DEVELOPMENT AND CHARACTERIZATION OF METHOTREXATE LOADED POLY(L-LACTIC ACID) MICROSPHERES FOR THE TREATMENT OF RHEUMATOID ARTHRITIS

by

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B.Sc. (Pharm), University of British Columbia, 1999

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Pharmaceutical Sciences)

THE UNIVERSITY OF BRITISH COLUMBIA

November 2005

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Methotrexate (MTX) has shown anti-inflammatory effects in the treatment of rheumatoid arthritis. Attempts by other groups have been made to improve the efficacy and reduce toxicity by administering the drug intra-articularly, but the outcomes were not successful due to rapid clearance of the drug from the joint cavity. MTX loaded polymeric microspheres may provide a controlled release drug delivery system to maintain an effective concentration of MTX in the joint cavity. The goals were to develop MTX loaded microspheres and to determine the in vivo biodistribution and efficacy following intra-articular injection in rabbit joints. MTX loaded poly(L-lactic acid) microspheres (size range 33-110 µm) manufactured from poly(L-lactic acid) with an average molecular weight of 2000 g/mole (PLLA2k) showed good tolerability in rabbit joints. The in vitro drug release profiles of MTX loaded PLLA2k microspheres demonstrated a rapid burst phase with more than 50% of drug being released within 5 days followed by a slow release phase.

Pharmacokinetics of MTX following intra-articular injection of both 1.5 mg and 10 mg doses of either MTX solution or MTX loaded microspheres (33-110 µm) were investigated in healthy rabbits. Plasma concentration peaked at 15 min (t_{max}) following intra-articular injection, and the maximum plasma concentration (C_{max}) for rabbits injected with MTX solution was 5 fold higher than for rabbits injected with MTX.
microspheres. Approximately 70% of injected MTX dose was excreted in the urine of the rabbits injected with MTX solution while only 12% of the dose was excreted in the urine of the rabbits injected with MTX microspheres 24 h following intra-articular injection.

*In vivo* efficacy of intra-articular MTX loaded PLLA2k microspheres (33-110 μm) or MTX solution was evaluated using an antigen-induced arthritis rabbit model. Arthritis was successfully induced in the joints of rabbits with the observation of histopathological features resembling rheumatoid arthritis. Based on the degree of swelling of the knee joints and a system of scoring the pathological features of the disease, there was no significant difference between MTX solution and microspheres treated groups compared to phosphate buffered saline (control) animals. The lack of therapeutic responses to MTX loaded microspheres treatment was likely due to the severity of the disease induced and insufficient length of the observation period.

MTX loaded PLLA2k microspheres were shown to be well tolerated in the rabbit knee joints and provide a controlled, localized delivery of MTX into the joint cavity following intra-articular injection.
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<td>$\eta_0$</td>
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<td>$\eta_{\text{rel}}$</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartamine transferase</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>AUMC</td>
<td>Area under the moment curve</td>
</tr>
<tr>
<td>C</td>
<td>Concentration</td>
</tr>
<tr>
<td>D</td>
<td>Diffusion coefficient</td>
</tr>
<tr>
<td>$D_0$</td>
<td>Dose administered</td>
</tr>
<tr>
<td>DMARD</td>
<td>Disease-modifying anti-rheumatic drug</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
</tr>
<tr>
<td>GPC</td>
<td>Gel permeation chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>J</td>
<td>Flux</td>
</tr>
<tr>
<td>Mn</td>
<td>Number-average molecular weight</td>
</tr>
<tr>
<td>Mv</td>
<td>Viscosity-average molecular weight</td>
</tr>
<tr>
<td>Mw</td>
<td>Weight-average molecular weight</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MRT</td>
<td>Mean residence time</td>
</tr>
<tr>
<td>MTX</td>
<td>Methotrexate</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDLLA</td>
<td>Poly(D,L-lactic acid)</td>
</tr>
<tr>
<td>PGA</td>
<td>Poly(glycolic acid)</td>
</tr>
<tr>
<td>PLA</td>
<td>Poly(lactic acid)</td>
</tr>
<tr>
<td>PLLA</td>
<td>Poly(L-lactic acid)</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear leukocytes</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>k</td>
<td>1st order rate constant for non-compartmental analysis</td>
</tr>
<tr>
<td>$k_a$</td>
<td>Absorption constant for one-compartmental model</td>
</tr>
<tr>
<td>$k_e$</td>
<td>Elimination rate constant for one-compartmental model</td>
</tr>
<tr>
<td>t</td>
<td>Time</td>
</tr>
<tr>
<td>$t_{1/2}$</td>
<td>Elimination half-life</td>
</tr>
<tr>
<td>$t_{1/2(abs)}$</td>
<td>Absorption half-life</td>
</tr>
<tr>
<td>Tc</td>
<td>Recrystallization temperature</td>
</tr>
<tr>
<td>Tg</td>
<td>Glass transition temperature</td>
</tr>
<tr>
<td>Tm</td>
<td>Melting point</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor-necrosis factor</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>Volume of distribution</td>
</tr>
<tr>
<td>Xc</td>
<td>Degree of crystallinity</td>
</tr>
<tr>
<td>XRD</td>
<td>X-ray diffraction</td>
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ACKNOWLEDGEMENTS

This thesis could not be complete without the help of many people. First, I would like to thank my supervisor, Dr. Helen Burt, for her high academic standards, guidance and support over the course of my Ph.D. years. I would also like to thank my committee members for their advice and direction on my research project: Drs. John McNeill, Don Brooks, Kishor Wasan, and Don Lyster.

Thank you to Mr. John Jackson for always being there to solve any problems in the lab and to provide support when I was frustrated with my research. Thank you to all my colleagues for their friendship and help: Karen, Kevin, Jason, Ruiwen, Chris, Tobi, Wes, and Chiming.

I would like to thank Mr. Michael Boyd and Ms. Vera Risovic for helping me with the pharmacokinetic study. I would also like to thank Drs. David Hart and Paul Salo and Ms. Carol Reno and Ms. Ruth Seerattan from the University of Calgary for their great help and valuable discussions in the in vivo efficacy study.

Financially support from the Canadian Arthritis Network, the Canadian Institutes of Health Research, and University Graduate Fellowship is gratefully acknowledged.

I would like to dedicate this work to my parents. Thank you for your unconditional love.
Chapter 1

PROJECT OVERVIEW AND BACKGROUND

1.1 PROJECT OVERVIEW

Rheumatoid arthritis is an autoimmune disease characterized by progressive and irreversible damage of the synovial-lined joints, resulting in the loss of joint space, a decrease in joint function and ultimately, deformity. Despite an increased understanding of the pathophysiology of rheumatoid arthritis, there are no treatments that cure rheumatoid arthritis. Therefore, the therapeutic goals are a remission of symptoms involving the joints, a return of full function, and the maintenance of remission with disease-modifying anti-rheumatic drug (DMARD) therapy (O'Dell et al., 2004).

Methotrexate (MTX) is one of the most frequently used DMARDs. Although the exact mechanism of action is still not fully understood, the efficacy of MTX is related to its cytotoxic and anti-inflammatory effects (Furst et al., 1990). When administered in low weekly oral doses, MTX effectively suppresses inflammation in rheumatoid arthritis. However, oral use of the drug is limited by its systemic toxic effects. Therefore, attempts have been made to improve the efficacy and reduce toxicity by administering the drug intra-articularly. These outcomes were not achieved due to rapid removal of the drug from the joint cavity (Wigginton et al., 1980).
Intra-articular injection of a controlled release drug delivery system has been proposed in order to maintain an effective concentration of the drug in the joint cavity. Several studies (Nishide et al., 1999; Ramesh et al., 1999; Horisawa et al., 2002a; Liggins et al., 2004) have shown that intra-articular microsphere formulations composed of biodegradable and biocompatible polymers are well tolerated in rabbit joints. Inflammatory responses were not significant, although a temporary increase in cell infiltration into the synovial fluid has been reported. However, very few studies have been carried out to study the pharmacokinetics and efficacy of a DMARD encapsulated controlled release system for intra-articular treatment of rheumatoid arthritis.

The aim of this work was to develop a MTX loaded polymeric microspheres formulation suitable for the potential intra-articular treatment of rheumatoid arthritis. The first part of the thesis focuses on the formulation and characterization of MTX encapsulated poly(L-lactic acid) microspheres. The second and major part of the thesis evaluates the pharmacokinetics of MTX in healthy rabbits and the last part describes an in vivo efficacy study of MTX encapsulated microspheres following intra-articular delivery in a rabbit model of antigen induced arthritis.
1.2 SYNOVIAL JOINT STRUCTURE

1.2.1 Synovial joint anatomy

Synovial joints are freely movable joints that have a joint cavity, and ligaments help to support the articulating bones. The functions of synovial joints are to provide a wide range of precise, smooth movements, at the same time maintaining stability, strength and, in certain aspects, rigidity in the body (van den Graaff, 1995).

The structure of a synovial joint is shown in Figure 1.1. Synovial joints are enclosed by a fibroelastic joint capsule that is filled with lubricating synovial fluid. Synovial fluid is confined within the cavity by a thin sheet of tissue called synovium (also known as synovial membrane or synovial lining). Synovium generates the synovial fluid and supplies it with oxygen and nutrients for transmission to the cartilage. It achieves this by virtue of its rich microcirculation and high blood flow. Around the margins of the joint cavity, small folds of synovium with or without blood vessels sometimes project into the cavity and these folds are called “villi” (Levick, 1995). The bones that articulate in a synovial joint are capped with a smooth articular cartilage. The avascular articular cartilage depends on the alternating compression and decompression during joint activity for the exchange of nutrients and waste products with the synovial
fluid. Tough, fibrous cartilaginous pads called menisci are located within the capsule of certain synovial joints and serve to cushion, as well as to guide, the articulating bones.
Figure 1.1  A synovial joint is represented by a lateral view of the human knee joint. Adapted from Human Anatomy (Adapted from van den Graaff, 1995)
1.2.2 Synovium

The synovium itself is very thin, typically around 15-20 \( \mu \)m thick in the rabbit knee (Knight and Levick, 1983; Levick and McDonald, 1989) and 60 \( \mu \)m thick in the human knee (Stevens et al., 1991). Synovium backs onto a broader zone of loose connective tissue and fat cells called subsynovium, where terminal arteries, veins, and a network of terminal lymphatic plexus drains away fluid and macromolecules that have seeped out through the synovial lining (Figure 1.2) (Simkin and Benedict, 1990; Jensen et al., 1993; Levick, 1995).

Two main types of cells are found in the synovial lining. Type A cells contain a prominent Golgi complex and many vesicles, but little rough endoplasmic reticulum. They are also more endowed with cell processes, mitochondria, intracytoplasmic filament, and lysosomes (Ghadially, 1983). Type B cells are well endowed with rough endoplasmic reticulum but with little Golgi complex, vesicles and vacuoles (Ghadially, 1983). Type A cells are often called macrophage-like cells because of the paucity of rough endoplasmic reticulum. The main functions of type A cells are to phagocytose and to secrete hyaluronic acid from Golgi complexes (Ghadially, 1983; Brown et al., 1991). Type B cells are often called fibroblast-like cells. The main function of type B cells is to produce a protein-rich secretion. The secretion may include procollagen,
collagenase, and lubricin, a lubricating glycoprotein found in the synovial fluid (Fraser et al., 1977). Type B cells also have phagocytotic ability when stimulated (Ghadially et al., 1982). Senda and coworkers (1999) showed that both type A and B synovial cells possessed the ability to phagocytose latex particles with a diameter of 240 nm, and that such activity was more intense in type A cells.

Normal synovium possesses a row of capillaries at about 5 µm (rabbit knee) or about 30 µm (human knee) beneath the surface. Many of the capillaries bear fenestrations which are often on the side facing the joint cavity (Levick and McDonald, 1995). The high capillary density, superficial location, and orientation of the fenestrations are well adapted for synovial fluid formation and nutrient supply.

1.2.3 Articular cartilage

Articular cartilage is an avascular, alymphatic, aneural tissue which covers the articular ends of bone. It is composed of cells (chondrocytes) set in an abundant matrix. The chondrocytes occupy only about 0.01 to 0.1% of the volume of the tissue (Ghadially et al., 1982). The matrix contains collagen fibrils, proteoglycans and a number of organic and inorganic solutes. The functions of articular cartilage include transmission and distribution of high loads to underlying bone, maintenance of contact stresses at
acceptably low levels, movement with little friction, and shock absorption (Ghadially et al., 1982).

The margin of articular cartilage blends gradually with the synovial membrane and periosteum. The thickness of articular cartilage varies from joint to joint, from one area of the joint to another in the same joint, and from species to species. The thicknesses of articular cartilage in the human hip and knee range from about 2 to 4 mm (Meachim and Stockwell, 1979) and the cartilage covering the femoral condyle of the rabbit is a little under 0.5 mm thick (Davies et al., 1962; Ghadially et al., 1982).

1.2.4 Synovial fluid

Synovial fluid is a slippery, viscoelastic liquid that fills joint cavity. It provides freedom of movement, lubricates the joint, and acts as a diffusional bridge and convective transport medium for nutrients and waste products (Levick, 1995). Synovial fluid formation begins with the passive ultrafiltration of plasma across the superficial, surface oriented fenestrations of the synovial capillaries. The ultrafiltrate flows through the interstitium, between the synovial lining cells to enter the joint cavity and becomes synovial fluid as lubricating macromolecules, hyaluronic acid and lubricin, are actively secreted into it by the synovial cells (Knox et al., 1988). Synovial fluid is not a static pool, but is continually being absorbed and replenished by the synovial lining of the joint.
cavity. As shown in Figure 1.2, synovial fluid and macromolecules drain from the joint cavity via the interstitial path between the lining cells to reach the lymphatic capillary network at the border between synovium and subsynovium (Levick, 1995; Levick and McDonald, 1995). The macromolecules are returned to the circulation by the lymphatic system (Simkin and Benedict, 1990; Jensen, et al., 1993). The synovial fluid volume in normal human knees is less than 2 mL (Netter et al., 1989) while the synovial fluid volume aspirated in rheumatoid arthritis and osteoarthritis patients is between 20-40 mL (Hunter and Blyth, 1999). The rate of volume turnover of synovial fluid is estimated to be of the order of 3-4 μL/h/cm² synovium in rabbit and human knees and this corresponds to a turnover time of roughly 1 h (Levick, 1987). In rheumatoid arthritis, the rate of turnover of synovial fluid increases 4 fold, while the fluid volume increases more than 8 fold, so the turnover time (i.e., volume/turnover rate) actually lengthens (Simkin, 1979; Levick, 1995)
Figure 1.2  Schematic cross section of a synovial joint (top). Inset: enlargement of synovial lining. Arrows indicate ultrafiltration of fluid from fenestrated capillaries into joint cavity and drainage of fluid from cavity through synovial interstitium into subsynovial space and lymphatics. (Adapted from Levick, 1995)
1.3 RHEUMATOID ARTHRITIS

Rheumatoid arthritis is a chronic inflammatory disease of unknown etiology and complex multifactorial pathogenesis, affecting joints and other tissues. It affects up to 1-3% of the population and is two to three times more frequent in women than in men (Grassi et al., 1998; Howe, 1998). The disease can start at any age, with a peak incidence between the fourth and the sixth decade of life (Smolen and Steiner, 2003).

1.3.1 Pathogenesis

Although the factors that initiate the inflammatory process of rheumatoid arthritis are unknown, many pathways involved in the generation of the disease have been recognized. The pathogenesis of rheumatoid arthritis has been reviewed by Hasunuma et al (1998), van den Berg and Bresnihan (1999), Bingham (2002), and Smolen and Steiner (2003). Rheumatoid arthritis is generally considered to be an autoimmune disease, with primary or secondary involvement of T lymphocytes (van den Berg and Bresnihan, 1999). It has been speculated that rheumatoid arthritis could be triggered by infectious agents, but proof of this is still lacking (Smolen and Steiner, 2003).

Inflammation and tissue destruction of the joint in rheumatoid arthritis results from a T lymphocyte response, triggered by the binding of foreign antigens to receptors on T lymphocytes (Schuna et al., 1996). T lymphocytes can undergo polarization into either
Th1 cells or Th2 cells which can be mutually inhibitory (Smolen and Steiner, 2003).

Th1 cells mainly secrete pro-inflammatory cytokines such as interferon-γ (IFN-γ) and tumor-necrosis factor-β (TNF-β); whereas Th2 cells mainly secrete anti-inflammatory cytokines such as interleukin-4 (IL-4), IL-5, IL-13, and IL-10 (Romagnani, et al., 2003).

The T lymphocytes infiltrating the synovial membrane in rheumatoid arthritis are primarily CD4+ memory cells that clearly have a Th1 bias (Smolen and Steiner, 2003). These T cells, by cell-cell contacts, or by different cytokines, such as IFN-γ, TNF-α and IL-17, activate monocytes, macrophages and synovial fibroblasts (Stout, 1993; Smolen and Steiner, 2003). These cells then overproduce pro-inflammatory cytokines, mainly TNF-α, IL-1, and IL-6 which seem to be the pivotal cytokines that lead to chronic inflammation (Firestein et al., 1990; Smolen and Steiner, 2003). These pro-inflammatory cytokines, once engaged to their receptors on specific cell surfaces, trigger various signal transduction cascades which lead to the induction of genes whose products mediate inflammation and tissue degradation (Bingham, 2002). These products include various cytokines, chemokines, and tissue-degrading enzymes, such as the matrix metalloproteinases (Bingham, 2002; Smolen and Steiner, 2003). The endogenous anti-inflammatory agents, such as soluble cytokine receptors and receptor antagonists, anti-inflammatory cytokines, or regulatory T lymphocytes, are insufficient to
counterbalance the cascade of pro-inflammatory events in chronic inflammation 
(Bingham, 2002; Smolen and Steiner, 2003).

B lymphocytes are also activated by T lymphocytes. Activated B lymphocytes produce plasma cells, which form antibodies. These antibodies, in combination with complement, result in the accumulation of polymorphonuclear leukocytes (PMNs). These PMNs release cytotoxins, free oxygen radicals, and hydroxyl radicals, which promote cellular damage to synovium and bone (Schuna et al., 1996; Howe, 1998; van den Berg and Bresnihan, 1999).

Overall, many cell populations are involved in rheumatoid arthritis pathogenesis. As a result of inflammatory infiltration, T lymphocytes, B lymphocytes, and plasma cells are found in greatly increased numbers in the subsynovial layer (Bingham, 2002). The synovial membrane contains type A and type B synovial cells which proliferate resulting in an aggressive tissue called pannus at the cartilage-bone junction (Bingham, 2002; Smolen and Steiner, 2003). Pannus contains matrix metalloproteinases and osteoclasts that invade the cartilage and eventually the bone surface, producing erosions of bone and cartilage, leading to destruction of the joint (Smolen and Steiner, 2003). A simplified schematic representation of the pathogenesis of rheumatoid arthritis is shown in Figure 1.3.
Figure 1.3 Schematic representation of pathogenesis of rheumatoid arthritis. Phase 1: Antigen-presenting cell phagocytoses antigen. Phase 2: Antigen is presented to T lymphocyte. Phase 3: Activated T cell stimulates Th1 and B lymphocyte production, promoting inflammation. Phase 4: Activated T cells and macrophages release factors that promote tissue destruction, increase blood flow, and result in cellular invasion of synovial tissue. Ag, antigen; PMN, polymorphonuclear leukocyte; TNF-α, tumor necrosis factor-α; IFN-γ, interferonγ; IL, interleukin, MMP, matrix metalloproteinases. (Adapted from Schuna et al., 1996)
1.3.2 Clinical presentation

The clinical features of rheumatoid arthritis can be classified as articular and extra-articular. Rheumatoid arthritis is a polyarthritis. It involves many joints (six or more), although in the early stages of the disease, only one or a few joints might be afflicted. Virtually all peripheral joints can be affected by the disease; however, the most commonly involved joints are those of the hands, feet and knees (Smolen et al., 1995), and distal interphalangeal joints are usually spared. Pain, early morning stiffness and functional limitation are typical of active disease. The onset is usually insidious and the joints become involved in an additive and progressive manner. Extra-articular symptoms include constitutional symptoms and involvement of other systems that range from rheumatoid nodules to life-threatening vasculitis (Schuna, et al., 1996).

1.3.3 Management of the disease

Medications that are used to treat rheumatoid arthritis are divided into three main classes: nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids and disease modifying anti-rheumatic drugs (DMARDs). According to the guidelines for the management of rheumatoid arthritis published by the American College of Rheumatology in 2002, DMARD therapy should be started within 3 month of the diagnosis of rheumatoid arthritis and corticosteroid and NSAIDs may be used for pain
relief and anti-inflammatory purposes (Figure 1.4). There are no treatments that cure rheumatoid arthritis; therefore, the therapeutic goals are a remission of symptoms involving the joints, a return of full function, and the maintenance of remission with DMARD therapy (O'Dell, 2004).

1.3.3.1 Nonsteroidal anti-inflammatory drugs

NSAIDs are particularly helpful during the first few weeks in which a patient has symptoms, because the drugs provide partial relief of pain and stiffness until a definitive diagnosis of rheumatoid arthritis can be established. NSAIDs have not been shown to slow the progression of the disease; therefore, in long-term care, NSAIDs should be used together with DMARDs (American College of Rheumatology, 2002; O'Dell, 2004). Long-term administration of NSAIDs may result in gastrointestinal ulcers, perforation, and hemorrhage (Howe, 1998). Cyclooxygenase-2 (COX-2) inhibitors, which decrease the incidence of gastric and duodenal ulcers by approximately 50 percent as compared with traditional NSAIDs, have been introduced (Bombardier et al., 2000; Silverstein, et al., 2000; Fitzgerald and Patrono, 2001). However, the efficacy of the COX-2 inhibitors is no better than that of the older and less expensive NSAIDs (Fitzgerald and Patrono 2001). It has been found that when used in high doses, COX-2 inhibitors may increase the risk of cardiovascular diseases in patients (Maxwell and Webb, 2005).
1.3.3.2 **Corticosteroids**

Corticosteroids are potent suppressors of the inflammatory response in rheumatoid arthritis and in many other diseases. Studies have shown that corticosteroids decrease the progression of rheumatoid arthritis, detected using radiographic techniques (Kirwan, 1995; Hickling *et al.*, 1998; van Everdingen *et al.*, 2002). Oral corticosteroids can be used in “bridging” therapy, continuous low-dose therapy, or short-term high-dose therapy to control flares of rheumatoid arthritis. In terms of bridge therapy, oral corticosteroids can be used to control pain and synovitis while DMARDs are taking effect (O’Dell, 2004). Adverse effects are the major limitations to the use of corticosteroids. They include hypothalamic-pituitary axis suppression, Cushing’s syndrome, osteoporosis, myopathies, glaucoma, cataracts, gastritis, hypertension, hirsuitism, electrolyte imbalance, glucose intolerance, skin atrophy, and increased susceptibility to infections (Schuna *et al.*, 1996).

1.3.3.3 **Disease modifying anti-rheumatic drugs**

Optimal management of rheumatoid arthritis requires rapid and sustained suppression of inflammation with DMARDs, which are defined as medications that retard or halt the progression of disease. Disease modification is most convincingly demonstrated by the ability of the medications to decrease radiographic progression. A meta-analysis of blinded clinical trials has suggested that the relative efficacies of
methotrexate (MTX), sulfasalazine, intramuscular gold, and penicillamine are similar (Felson et al., 1990; O’Dell, 2004). Antimalarial drugs (e.g., chloroquine and hydroxychloroquine) are less effective. Penicillamine, because of concern about its toxicity, and oral gold, because of its marginal efficacy (Felson et al., 1990), are rarely used today (Mikuls and O’Dell, 2000). However, all presently used DMARDs have limited efficacy, toxicity problems, or both. Drug retention rates in clinical practice are relatively low. Within 1-2 years, the majority of patients have to stop a given DMARD course, with the exception of MTX, which has a median retention rate of 3-4 years (Smolen and Steiner, 2003). MTX is most often selected for initial therapy (Mikuls and O’Dell, 2000). It has demonstrated efficacy and durability, a long-term track record of acceptable toxicity, and low cost (Ortendahl et al., 2002).

1.3.3.4 Biologic disease modifying anti-rheumatic drugs

Cytokines play important roles in the progression of rheumatoid arthritis. Currently, there are three biologic products available to treat rheumatoid arthritis. Infliximab, etanercept, and dalimimab inhibit the actions of TNF-\(\alpha\), and anakinra inhibits the action of IL-1 (Olsen and Stein, 2004). These biologic DMARDs have significantly improved the clinical response and radiographic progression of patients when used alone or in combination with MTX, compared to either placebo or MTX plus
placebo therapies (O'Dell, 2004). Currently, biologic DMARDs have been approved for use alone, or in combination with MTX, when MTX itself provides suboptimal therapeutic effects. The adverse effects of these agents include injection-site and infusion reactions, autoimmune responses and increase in infections (Olsen and Stein, 2004).
1. Establish Diagnosis of Rheumatoid Arthritis Early
2. Document Baseline Disease Activity and Damage
3. Estimate Prognosis

Initiate Therapy
- Patient Education
- Start DMARD(s) Within 3 Months
- Consider NSAID
- Consider Local or Low-Dose Systemic Steroids
- Physical Therapy/Occupational Therapy

Periodically Assess Disease Activity

Adequate Response with Decreased Disease Activity

Inadequate Response (i.e., ongoing active disease after 3 months of maximal therapy)

Change/Add DMARDs

MTX Naive
- MTX
- Other
- Combination

Suboptimal MTX Response
- Combination Rx
- Other Mono Rx
- Biologics

Multiple DMARD Failure

Symptomatic and/or Structural Joint Damage

Surgery

Figure 1.4 Outline of the management of rheumatoid arthritis. DMARD, disease-modifying antirheumatic drug; NSAID, nonsteroidal antiinflammatory drug; mono Rx, monotherapy; combination Rx, combination therapy. (Adapted from American College of Rheumatology guidelines, 2002)
1.4 METHOTREXATE

1.4.1 Chemistry

Methotrexate (MTX) (Figure 1.5) is

N-[4-{{(2,4-diamino-6-pteridinyl)-methyl}methyl-amino}benzoyl]glutamic acid.

MTX is also known as 4-amino-10-methylfolic acid and amethopterin. The chemical formula of MTX is C$_{20}$H$_{22}$N$_{8}$O$_{5}$ and the molecular weight of anhydrous MTX is 454.46 g/mole (Budavari, 1996). MTX is a folic acid analog. There are three main regions in its structure as follows (1) pteridine ring, (2) p-aminobenzoic acid (the bridge region), and (3) glutamic acid (Figure 1.5) (Rahman and Chhabra, 1988).

MTX is a bright yellow-orange, odorless crystalline powder. Melting of MTX is accompanied by decomposition in the temperature range 185-204°C (Budavari, 1996). The molecule is negatively charged at neutral pH with pKa values of 2.15 and 3.8 and therefore its aqueous solubility is pH dependent (Hansen et al., 1983). The solubility of MTX in double distilled water at 20°C was reported be 50 µg/mL (Chan and Gonda, 1991). The aqueous solubility of MTX is the lowest at pH 3 (Hansen et al., 1983). At pH below 2.5, a slight increase in solubility is observed due to the increased ionization of the MTX molecule in this region (Fort et al., 1990). MTX is practically insoluble in alcohol, chloroform, ether and dichloromethane (Gennaro et al., 1995).
At a constant pH and temperature, the degradation of MTX solutions display a first-order kinetic behavior (Chatterji, et al., 1978; Hansen et al., 1983). The degradation of MTX solution is pH dependent and subject to general acid-base catalysis by most of the buffer substances. Acetate, phosphate, and borate catalysis increases linearly with increasing catalyst concentration, while a non-linear variation of degradation rate with increasing carbonate buffer concentration has been observed (Hansen et al., 1983). The maximum stability of MTX is between pH 6.6-8.2 (Hansen, et al., 1983). For an isotonic buffer-free MTX solution at pH 8.5 and 25°C, the shelf-life for 10% degradation of the MTX is estimated to be 54 months. At pH above 6.5, N^{10}-methyl-folic acid is the only degradation production while in acidic solutions several compounds are formed (Hansen et al., 1983).

MTX undergoes photodegradation when stored in diluted solutions under light (Chatterji and Gallelli, 1978). Photodegradation can take place under normal lighting, but is more rapid in direct sunlight, with about 11% drug loss from a 1 mg/mL solution after 7 h. Storage under normal lighting resulted in little change in drug concentration over 24 h and a decrease of up to 12% by 48 h (McElnay, 1988). The major degradation products following photolysis are p-aminobenzoylglutamic acid and 2,4-diamino-6-pteridincarbaldehyde (Dyvik, et al., 1986).
Figure 1.5 Chemical structures of A) methotrexate, B) folic acid, C) folic acid (reduced form), D) 7-hydroxy methotrexate (7-OH-MTX).
1.4.2 Formulations

MTX is currently formulated as tablets for oral administration at strengths of 5, 7.5, 10 and 15 mg. Methotrexate Sodium Injection is formulated as 25mg/mL and 10mg/mL solutions. Nonmedicinal ingredients include sodium chloride and water for injection. Sodium hydroxide and/or hydrochloric acid may be used for pH adjustment. Benzyl alcohol may be used as a preservative.

1.4.3 Pharmacology and indications

MTX is a useful drug in the management of acute lymphoblastic leukemia in children and in choriocarcinoma and related trophoblastic tumors of women. Beneficial effects also are observed in patients with osteosarcoma and mycosis fungoides and when MTX is used as part of the combination therapy in Burkitt’s and other non-Hodgkin’s lymphomas and carcinomas of the breast, head and neck, ovary, and bladder (Chabner et al., 1996). MTX has also been used in the treatment of severe, disabling psoriasis (Chabner et al., 1996). The use of MTX in the treatment of rheumatoid arthritis was approved by the Food and Drug Administration in 1988 (van Ede et al., 1998).

The dose of MTX in the treatment of psoriasis in adults is 2.5 mg orally for 5 days following a rest of period of at least 2 weeks (Chabner et al., 1996). The use of MTX in the treatment of rheumatoid arthritis in adults is 10 to 15 mg/week and the maximum
dose is 25 to 30 mg/week (van Ede et al., 1998). The doses used for the treatment of cancers are much higher. For example, in the combination therapy of leukemias and non-Hodgkin’s lymphomas, 6 to 72-hour infusions of 250 mg to 7.5 g/m² of MTX may be employed (Chabner et al., 1996).

MTX is a folic acid (shown in Figure 1.5 B) analog in which the groups bonded to the C₄ carbon and N₁₀ hydrogen are NH₂ and CH₃, respectively. Reduced folic acids (tetrahydrofolates) are essential cofactors that provide single carbon groups in several reactions involved in the synthetic pathways for purines, pyrimidines, formation of polyamines, and transmethylation of phospholipids and proteins (Hillson and Furst, 1997). The enzyme dihydrofolate reductase is responsible for reducing partially oxidized folates (dihydrofolates) to tetrahydrofolates. MTX inhibits dihydrofolate reductase with high affinity, resulting in depletion of tetrahydrofolates that are required for the synthesis of purines and thymidylate (van Ede et al., 1998). A portion of intracellular MTX is metabolized to polyglutamates and these polyglutamates of MTX also inhibit the folate-dependent enzymes of purine and thymidylate metabolism. Subsequently, the synthesis of DNA and RNA, as well as other vital metabolic reactions are interrupted (Chabner et al., 1996; Cronstein, 1997; Hillson and Furst, 1997).
When MTX was first introduced in the treatment of rheumatoid arthritis in the 1980's, the rationale was that it inhibited proliferation of the lymphocytes or other cells responsible for inflammation in the joint. However, over the past 15 years, several lines of evidence have clearly suggested that MTX does not act simply as an antiproliferative agent for the cells responsible for the joint inflammation in rheumatoid arthritis (Cronstein and Merrill, 1996; Cronstein, 1997). The rapid clinical remission and the short term effect on the acute phase reactants, as seen with low dose MTX administration in most patients with rheumatoid arthritis, as well as the rapid flare of disease after drug discontinuation, suggest that the mechanism of the action of low dose MTX might be more anti-inflammatory than antiproliferative (Kremer, 1994; Cutolo et al., 2001).

Although the exact mechanisms are still not fully understood, the anti-inflammatory effects of MTX are speculated to be related to adenosine induced immunosuppression. MTX inhibits both dihydrofolate reductase and other folate dependent enzymes and leads to adenosine overproduction (Furst, 1997; Hillson and Furst, 1997; Cutolo et al., 2000). Extracellular adenosine can bind to adenosine surface receptors types A1, A2α, A2β, A3, which have been found on many different cell types such as fibroblasts and endothelial cells (van Ede et al., 1998). Low dose MTX exerts its anti-inflammatory effect by inducing extracellular adenosine, which acts predominantly through A2 receptors and
inhibits phagocytosis and secretion of TNF-α, IFN-γ, IL-2, IL-12 and many other proinflammatory cytokines (Cutolo et al., 2001). Through adenosine mediated pathways, MTX treatment in rheumatoid arthritis seems to decrease the monocytic and macrophagic cytokines IL-1, IL-6 and TNFα secretion and increase IL-1 receptor antagonist production (Cronstein, 1997). At the same time, MTX increases anti-inflammatory IL-4 and IL-10 gene expressions and decreases gene expression of proinflammatory Th1 cytokines such as IL-2 and IFN-γ, with resulting anti-inflammatory effects (Seitz et al., 1995; Seitz et al., 1996; Cutolo et al., 2001).

As for anti-proliferative effects, intermediate MTX concentrations (50 μg/mL), as obtained in serum after low dose treatment, can induce both a significant cell growth inhibition and apoptosis in immature Th1 monocytic cells but has no significant effect on synovial macrophage proliferation (Cutolo et al., 2000). This finding suggests that MTX might inhibit recruitment of immature and inflammatory monocytes into inflammatory sites and could reduce the survival of these cells in the inflamed synovial tissue (Cutolo et al., 2000).

Low dose MTX in rheumatoid arthritis treatment seems to exert anti-inflammatory effects by acting at different levels of the pathophysiological cascade. The direct inhibitory effects on proliferation and the induction of apoptosis in cells involved in the
immune/inflammatory reaction represent the first step of the intervention while the inhibition of both monocytic/lymphocytic proinflammatory cytokines involved in rheumatoid synovitis, seems to be the key role in the sustained anti-inflammatory actions exerted by low dose MTX (Cutolo et al., 2001).

1.4.4 Toxicity

When used in low doses for the treatment of rheumatoid arthritis, MTX has proved to be a very effective, fast working disease modifying agent with an excellent efficacy-toxicity ratio. Nevertheless, the main reason for discontinuation of MTX is not the lack of efficacy, but toxicity. In approximately 30% of rheumatoid arthritis patients, toxicity leads to discontinuation of MTX therapy (van Ede et al., 1998).

Table 1.1 outlines the range of toxicities of methotrexate found in the medical literature (Furst, 1997). Gastrointestinal toxicities including stomatitis, nausea and abdominal distress are most common. Although the precise mechanisms of toxicity are still not clear, some side effects have been directly related to MTX involvement in the previously mentioned metabolic pathways. Side effects of MTX such as GI distress and bone marrow suppression seem to be directly related to folate antagonism and its cytotoxic effects, especially in tissues with a high cell turnover such as bone marrow and the gastrointestinal tract, which have a high requirement for purines, thymidine and
pyrimidine (O'Dell, 1997; van Ede et al., 1998). Adenosine release in the central nervous system and its activation of A1 receptors in the brain may be responsible for induction of fatigue and lethargy (Grim et al., 2003). A1 receptors are also present in endothelial cells and their activation provokes vasodilatation and therefore explains the headache that appears in many patients a few hours after administration of low dose MTX (Grim et al., 2003). Other side effects like pneumonitis and progressive subcutaneous nodules probably have a more complex origin (O'Dell, 1997; Salaffi et al., 1997). MTX can induce acute parenchymal damage and fibrosis in the liver. In a clinical setting, serum transaminases, aspartamine transferase (AST) and alanine transferase (ALT) are often monitored (Kirchain and Gill, 1996).
Table 1.1  Adverse effects when using methotrexate for the treatment of rheumatoid arthritis (Furst, 1997).

<table>
<thead>
<tr>
<th>Methotrexate dose (mg/week)</th>
<th>7.5-48</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of use (months)</td>
<td>4-183</td>
</tr>
<tr>
<td>% of incidence</td>
<td></td>
</tr>
</tbody>
</table>

**Adverse events**

| Central nervous system      | 13-35  |
| (headache, fatigue, “fuzziness”, malaise) |
| Gastrointestinal (GI)       | 19-65  |
| Nausea, GI distress         | 19-65  |
| Stomatitis                  | 2-55   |
| Hematological               |        |
| Anaemia                     | 1-2    |
| Leucopenia                   | 2-21   |
| Thrombocytopenia             | 1-5    |
| Infection                   | Rare   |
| Integument                  |        |
| Alopecia                    | 1-6    |
| Rash                        | 2-15   |
| Liver                       |        |
| Cirrhosis                   | Rare   |
| Elevated transaminases      | 8-30   |
| Osteopathy                  | Rare   |
| Pulmonary                   | Rare   |
| Pneumonitis (hypersensitivity) | 1-7   |
| Pseudolymphoma              | Rare   |
| Teratogenicity              | Definite |
1.4.5 Pharmacokinetics

In the treatment of rheumatoid arthritis, MTX is usually given orally. In adult patients, after oral administration, active absorption of the drug occurred in the proximal jejunum (Hillson and Furst, 1997; Grim et al., 2003). When given intramuscularly or subcutaneously, the drug was absorbed more rapidly and reached higher serum concentrations compared with the oral route (Grim et al., 2003). Nevertheless, the mean absolute bioavailability was about 70-80% regardless of the route of administration and a large inter-individual variation between 30-90% has been observed (Grim et al., 2003).

In blood, 30-70% of MTX was bound to proteins, almost exclusively to albumin (Hillson and Furst, 1997; Grim et al., 2003). The volume of distribution at steady state ranged from 0.33-1 L/kg in 10 patients receiving a single 7.5 mg dose of MTX (Sinnett et al., 1989). Enterohepatic cycling of the drug has been suggested since a significant increase in drug plasma concentration was observed 8 h following MTX administration (Edno et al., 1996). The concentrations of MTX in the synovial fluid were approximately equal to plasma concentrations at 4 and 24 h after oral or intramuscular administration (Herman et al., 1989).
MTX is transported into cells by passive transmembrane diffusion and by a folate surface receptor mediated active transport system (Bannwarth et al., 1996). Once inside the cell, up to six glutamate residues may be added to the drug molecule. MTX polyglutamates cannot be transported extracellularly unless they are hydrolysed back to the monoglutamate (Hillson and Furst, 1997; Grim et al., 2003). MTX has been found to accumulate in erythrocytes (Kremer et al., 1986), peripheral blood T-lymphocytes, fibroblasts, myeloid precursors in bone marrow and keratinocytes (Grim, et al., 2003).

Three metabolic pathways for MTX have been described in humans. First, the drug may be metabolized by intestinal bacteria to 4-amino-4-dexoy-N\textsuperscript{10}-methyl-pterolic acid and this metabolite usually accounts for less than 5% of administrated dose (Grim et al., 2003). Secondly, 3-11% of a MTX dose is converted by an aldehyde oxidase in the liver to 7-hydroxy-methotrexate (7-OH-MTX) (Figure 1.5) (Hillson and Furst, 1997). 7-OH-MTX is poorly water soluble and a 10 fold less potent inhibitor of dihydrofolate reductase as compared to MTX (Furst, 1997). Due to its slower rate of urinary excretion, plasma concentrations of 7-OH-MTX usually exceed those of MTX within 8-10 h after drug administration and thus 7-OH-MTX is the major circulating metabolite of MTX (Bannwarth et al., 1996; Grim et al., 2003). Thirdly, MTX is converted to
polyglutamates intracellularly. MTX polyglutamates have 7 fold higher inhibitory effects for dihydrofolate reductase than MTX (Furst, 1990).

Elimination of MTX from plasma was shown to be biphasic or triphasic depending on the length of sample collection period. The elimination half-lives of the β phase ($t_{1/2}\beta$) for MTX and its 7-OH-MTX were 5 to 8 h and 8 to 11 h, respectively in rheumatoid arthritis patients (Edno et al., 1996; Grim et al., 2003). The longer the sampling, the longer the reported $t_{1/2}\beta$ of the drug, probably due to intracellular MTX storage, polyglutamylation and slow release back to plasma (Shen and Azarnoff, 1978; Grim et al., 2003). Renal excretion is the major elimination route for MTX (Grim et al., 2003). This route of elimination accounts for 60-90% of the MTX excretion, while biliary elimination is responsible for up to 10 to 30% of MTX excretion (Bannwarth et al., 1996). In the kidneys, MTX is filtered in the glomeruli and undergoes active secretion and active reabsorption (Edno et al., 1996). Both secretion and reabsorption in renal tubules can be saturated even at low MTX plasma concentrations within the range of 0.1-1 μmole/L. Thus non-linear elimination may result following the administration of 7.5-30 mg of MTX and contribute to the interindividual variability in MTX concentrations (Hendel and Nyfors, 1984).
1.5 INTRA-ARTICULAR DRUG DELIVERY

1.5.1 Synovial permeability and effects of inflammation

The synovium has a complex physiology and can be likened to a double barrier consisting of a capillary endothelium (first barrier), which controls the transit of large molecules such as proteins, and the synovial interstitium (second barrier) which limits the transit of small hydrophilic solutes (Netter et al., 1989).

Synovial inflammation induces anatomical changes such as synovial cell proliferation, cellular infiltration, villous hypertrophy, and angiogenesis (Smolen and Steiner, 2003). Levick (1995) showed that in normal human synovium, the capillaries are close to the joint cavity with a modal depth of 36 μm, while in chronic rheumatoid arthritis, the capillaries become buried under the hypertrophied synovial lining and the mean distance from the surface is more than twice as great. Stevens et al (1991) showed that the synovial thickening and cellular infiltration in chronic rheumatoid arthritis caused a greater than two fold increase in average transport distance to the joint cavity and a reduction in capillary density to one-third of normal.

Simkin and Pizzorno (1974) have shown that most small molecules cross the synovium in both directions by passive diffusion, and are limited primarily by the relatively long, narrow diffusion path between synovial lining cells and not by any part of
the microvascular barrier. Comparing synovial permeabilities in normal subjects and patients with rheumatoid arthritis, Simkin et al (1979) have shown that the synovium in joints of arthritic patients was less permeable to small molecules such as tritiated water, glucose or urate than that of normal individuals. On the other hand, total proteins moved across arthritic synovium faster than normal synovium. It was suggested that small molecules left the rheumatoid joint space slowly because the synovial proliferation characteristics of the disease decreased the diffusion path between synovial cells. The permeability to proteins was increased due to increased protein permeability of the microvascular barrier (Simkin, 1979). Vascular wall changes secondary to disease did not increase the delivery of small molecules because the vessel wall is generally not a significant barrier to small molecules (Simkin, 1979; Simkin and Nilson, 1981).

1.5.2 Intra-articular drug therapy

Intra-articular therapy has been used for patients in whom the arthritis manifests in only a limited number of joints. The rationale for intra-articular therapy is to target the drug to the site of action and to minimize systemic toxic effects of the drug (Itoh, 1992).

The intra-articular injection of corticosteroids has played an important role in the management of inflammatory arthritis at an early stage. Injections of corticosteroids into joints have been shown to be effective in easing the pain and stiffness and may even
improve advanced destructive arthritis (Derendorf et al., 1986). Microcrystalline steroids such as triamcinolone, methylprednisolone, and rimexolone are commonly injected intra-articularly as suspensions of poorly water soluble crystals, and the duration of benefits can be up to several months depending on the pharmacokinetic properties of the particular steroid (Hunter and Blyth, 1999). On the other hand, the response to a soluble hydrocortisone succinate injection was very brief due to the rapid clearance of hydrocortisone solution from the joint cavity (Hunter and Blyth, 1999).

Pharmacokinetic studies of intra-articular steroid suspensions have shown that the rate-limiting step for steroid disposition in the joint was the dissolution rate in the synovial joint (Kahn et al., 1970; Derendorf et al., 1986). In the plasma concentration-time profiles, the terminal part of the curve represented systemic absorption from the joint rather than elimination, while the early phase of the curve represented the elimination ("flip-flop-case") (Derendorf et al., 1990). Despite their effectiveness in suppressing pain, swelling and redness, intra-articular steroids produced an acute flare following injection due to their crystalline nature (Kahn et al., 1970). They have also been shown to produce damage to the articular cartilage and increase the risk of infection in the joint (Hunter and Blyth, 1999).
Intra-articular injection of hyaluronic acid has been approved as a viscosupplement in the management of osteoarthritis (Gossec and Dougados, 2004). Hyaluronic acid may help restore the viscoelasticity of the synovial fluid and promote the endogenous synthesis of a high molecular weight, and possibly more functional hyaluronan, thereby improving mobility and articular function and reducing pain (Gossec and Dougados, 2004). Studies have been conducted to investigate the efficacy of hyaluronic acid in controlling the symptoms of rheumatoid arthritis (Goto et al., 2001; Tanaka et al., 2002). The results showed that following 5 consecutive injections of 25 mg per week of high molecular weight sodium hyaluronate, patients had significant improvement in pain symptoms, and inflammation. The elimination of radio-labeled hyaluronic acid from the knee joints of healthy men has been demonstrated to be triphasic with half-lives of 1.5 h, 1.5 days and 4 weeks. An increase in intra-articular fluid volume at 24 h post injection was also observed (Lindqvist et al., 2002).

MTX has been administered as an intra-articular injection but the results have not been very encouraging (Marks et al., 1976; Bird et al., 1977). Intra-articular injection of 5 mg MTX solution and intra-articular 20 mg triamcinolone hexacetonide were compared in arthritic patients with persistent bilateral knee effusions and showed that MTX had no immediate anti-inflammatory effect, nor did it give the relief of intra-articular steroid
Intra-articular combination therapy of 20 mg triamcinolone and 50 mg MTX in arthritic patients reduced joint effusion and knee circumference compared to triamcinolone alone (Blyth et al., 1998).

Wigginton and coworkers (1980) studied MTX pharmacokinetics after intra-articular injections in patients with rheumatoid arthritis. Patients were administered 5 mg of MTX every 24 h into the same knee joint up to 48 h. The synovial fluid and serum MTX concentration profiles showed that the elimination of MTX from the joint was biphasic over 24 h, with half-lives of 0.54 and 2.90 h. The intra-articular apparent volume of distribution was calculated to be 69 mL and the intra-articular MTX clearance was 0.28 mL/min. The authors concluded that intra-articular MTX was clinically ineffective, primarily because the intra-articular half-life of MTX was too short relative to the probable synovial cell cycle generation time (Wigginton et al., 1980).

Repeated intra-articular MTX doses have produced better results (Gao et al., 1998; Iagnocco et al., 2005). Rheumatoid arthritis patients were treated with up to 6 intra-articular injections of 10 mg MTX every 3 to 7 days and their synovial fluids were analysed for leukocyte counts and cytokine levels (Gao et al., 1998). The granulocyte counts and IL-8 levels decreased in all MTX treated patients, whereas IL-6 and IL-10 showed only minor changes. IL-8 has been reported to be related to the activity of the
inflammatory process (Verburgh et al., 1993). Iagnocco and coworkers (2005) have demonstrated that multiple intra-articular injections of 10 mg MTX every week for 8 weeks in rheumatoid arthritis and psoriatic arthritis patients resulted in a decrease in local and systemic inflammation. There was a significant reduction in synovial thickness and joint effusion and an improvement in maximum knee joint flexion following treatment (Iagnocco et al., 2005).

Liposomes encapsulating MTX have been formulated for intra-articular delivery (Foong and Green, 1993; Williams et al., 1996). Increased retention of MTX in inflamed rabbit knee joints was observed following intra-articular injection of liposomal MTX (Foong and Green, 1988). This formulation reduced joint swelling, synovial fluid production, and synovium proliferation after intra-articular injection in rabbits with established antigen-induced arthritis (Foong and Green, 1993). Williams et al (1996) showed that a single intra-articular injection of MTX encapsulated in multilamellar liposomes was retained within the joint and produced a rapid reduction in knee swelling of rats within 24 hours, and a progressive reduction in joint swelling over the next 20 days.
1.6 POLYMERIC DRUG DELIVERY SYSTEMS

1.6.1 Controlled release drug delivery systems

Controlled release drug delivery occurs when a polymer, whether natural or synthetic, is combined with a drug or other active agent in such a way that the active agent is released from the material in a designed manner (Robinson, 1997). Goals of controlled release drug delivery systems include, maintaining the drug in the desired therapeutic range with just a single dose, localizing delivery of the drug to a particular body compartment, reducing the need for follow-up care, preserving medications that are rapidly destroyed by the body, and increasing patient comfort and improving compliance (Langer, 1990).

The ideal drug delivery system should be inert, biocompatible, possess appropriate mechanical properties, comfortable for the patient, capable of achieving high drug loading, safe from accidental drug release, simple to administer and remove, and easy to fabricate and sterilize (Robinson, 1997).

1.6.2 Biodegradable polymers for drug delivery

A range of polymers have been employed to control the release of drugs and other active agents. To be successfully used in controlled drug delivery formulations, the polymer must be chemically inert and free of leachable impurities (Piskin, 1994). It
must also have an appropriate physical structure, with minimal undesired aging, and be readily processable. More and more polymers are designed to degrade within the body so that they do not require retrieving following administration (Robinson, 1997). Most commonly used biodegradable polymers include poly(lactic acid)(PLA), poly(glycolic acid) (PGA) and the copolymers of PLA and PGA, poly(lactic-co-glycolic acid) (PLGA) (Figure 1.6).
Figure 1.6 The chemical structures of A) poly(lactic acid) (PLA), B) poly(glycolic acid) (PGA), and C) poly(lactic-co-glycolic acid) (PLGA). * indicates the chiral center.
1.6.2.1 **Structure and molecular weight of polymers**

Polymers are large molecules built up by the linking together of large numbers of much smaller molecules, which are termed monomers (Odian, 1991). The structure of the polymer depends on the monomers used in its preparation. Typically, polymers have hundreds or more repeating units. If the polymer is prepared from a single monomer the product is referred to as a homopolymer. For example, PLA and PGA are both homopolymers with the repeating units being lactic acid and glycolic acid, respectively (Figure 1.6). If more than one monomer is employed, the product is a copolymer such as PLGA (Figure 1.6).

With only a few exceptions, a synthetic polymer is always a mixture of molecules with different molecular weights in different amounts and distributions. Therefore, there is no well-defined molecular weight and only averaged values can be given (Allcock and Lampe, 1981). The processing behavior and many end-use properties of polymers are influenced not only by the average molecular weight but also by the molecular weight distribution. Some properties, including tensile and impact strength, are strongly influenced by the short molecules; for other properties, such as solution viscosity and low shear melt flow, the influence of the middle length of the chains is
predominant; yet other properties, such as melt elasticity, are highly dependent on the amount of the longest chains present (van Krevelen, 1997).

Several characteristic molecular weight averages exist. Most important are the number and the weight averages of the molecular weight, \( M_n \) and \( M_w \), respectively.

The number average molecular weight (\( M_n \)) is defined as the total weight of all the molecules in a polymer sample divided by the total number of moles present, as follows:

\[
M_n = \frac{W}{N} = \frac{\sum n_i M_i}{\sum n_i} \quad \text{Equation 1.1}
\]

Where \( W \) is the total weight of polymer sample, \( N \) is the total number of moles and \( n_i \) is the number of moles of molecular weight \( M_i \).

The weight average molecular weight (\( M_w \)) is defined as

\[
M_w = \frac{\sum w_i M_i}{W} = \frac{\sum n_i M_i^2}{\sum n_i M_i} \quad \text{Equation 1.2}
\]

Where \( w_i \) is the weight of molecules of molecular weight \( M_i \). \( M_w \) is an average which is weighted so that the contribution of each chain length depends on its proportion by weight in the total sample. Thus, a small chains would have the same contribution as one chain ten times its length.

The molecular weight of a polymer is also expressed by its intrinsic viscosity \([\eta]\). For a linear, unbranched, and non cross-linked polymer of the same chemical constitution, the viscosity of a dilute polymer solution is directly related to the molecular
weight average. By determining the relative viscosity using a viscometer, several viscosity numbers can be calculated (Allcock and Lampe 1981). The relative viscosity, \( \eta_{\text{rel}} \) is the ratio of the viscosity of a diluted polymer solution (\( \eta \)) to the viscosity of the pure solvent (\( \eta_0 \)) as shown in Equation 1.3. The specific viscosity, \( \eta_{\text{sp}} \), is the fractional increase in viscosity caused by the presence of the dissolved polymer in the solvent as shown in Equation 1.4. The specific viscosity and the relative viscosity clearly depend on the concentration of the polymer in solution. The quantity \( \eta_{\text{sp}}/C \), where C is the concentration of polymer in g/cm\(^3\), is sometimes called the reduced viscosity. The intrinsic viscosity, \([\eta]\), is defined as the limit of the reduced viscosity as the concentration approaches zero (Equation 1.5) (Allcock and Lampe, 1981).

\[
\eta_{\text{rel}} = \frac{\eta}{\eta_0} \quad \text{Equation 1.3}
\]

\[
\eta_{\text{sp}} = \frac{\eta - \eta_0}{\eta_0} = \eta_{\text{rel}} - 1 \quad \text{Equation 1.4}
\]

\[
[\eta] = \lim_{c \to 0} \left( \frac{\eta_{\text{sp}}}{c} \right) \quad \text{Equation 1.5}
\]

Two methods have been used to determine \([\eta]\): the extrapolation method and the single point method. The extrapolation method involves measuring \( \eta_{\text{rel}} \) for a polymer solution at a series of concentrations (Collins et al., 1973). The single point method uses Equation 1.6 as shown below. This equation is only valid for solutions of polymer in a
good solvent, and the concentration should be chosen such that \( \eta_{sp} \leq 0.2 \) (Solomon and Ciuta, 1962).

\[
[\eta] = \frac{\sqrt{2(\eta_{sp} - \ln \eta_{rel})}}{c} \quad \text{Equation 1.6}
\]

Intrinsic viscosity can be used to calculate a molecular weight average using the Mark-Houwink equation:

\[
[\eta] = KM_v^\alpha \quad \text{Equation 1.7}
\]

Where K and \( \alpha \) are constants and \( M_v \) is the viscosity average molecular weight. The constants K and \( \alpha \) are specific for a given polymer/solvent system at a particular temperature. If these values can be obtained from the literature, then \( M_v \) can be calculated (Allcock and Lampe, 1981).

Gel permeation chromatography (GPC) is a process for the separation of polymers according to their molecular size. Using this method, the rate of permeation of polymer chains in solution through a gel packed with microporous beads of fixed particle and pore sizes, expressed as retention time, is related to the hydrodynamic volume of each chain. The time for passage of the polymer molecules through the gel column decreases with increasing molecular weight. A calibration curve for a given column may be constructed by measuring the retention time of a series of monodisperse polymer standards of known molecular weight (MW) and plotting \( \log(MW) \) versus retention time.
After calibrating the column, the molecular weight (both Mn and Mw) and the polydispersity of polymer samples can be determined (Collins et al., 1973).

1.6.2.2 Polymer morphology

Polymer morphology describes the arrangement in three dimensions of polymer chains with respect to long range order (Rosen, 1993). Most polymers show, simultaneously, the characteristics of both crystalline solids and highly viscous liquids. The known polymers range from those that are completely amorphous, to semicrystalline polymers with crystallinities from low to high.

Two models of crystallinity have been used to describe the nature of semicrystalline polymers (Rosen, 1993). The “fringed-micelle model” assumes that polymers consist of small-sized, ordered, crystalline regions embedded in a disordered, amorphous polymer matrix. Crystallites are formed when chain segments from different polymer chains are precisely aligned together and undergo crystallization. Each chain can pass through several different crystalline regions and contribute ordered segments to several crystallites. The segments of the chain in the regions between the crystallites make up the disordered amorphous matrix (van Krevelen, 1997) (Figure 1.7A). In the “chain folded model” of polymer crystallinity, a single polymer chain folds upon itself to form lamellar crystallites interspersed in an amorphous phase. Several chains are involved in
each crystallite, and extend into the amorphous regions of the matrix (Rosen, 1993) (Figure 1.7B).
Figure 1.7  Schematic representation of A) fringed micelle model B) the chain folded model of polymer crystallinity.
1.6.2.3 *Thermal transitions*

Polymeric materials are characterized by two major types of thermal transitions, glass transitions and crystalline melting transitions. Whether a polymer sample exhibits both transitions, or only one, depends on its morphology. Completely amorphous polymers show only a glass transition. A theoretically 100% crystalline polymer would show only a melting transition. Semicrystalline polymers exhibit both the crystalline melting and glass transitions.

In an amorphous polymer, at temperatures below the glass transition temperature (Tg), the polymer chain segments are frozen in fixed positions. Some molecular movements of chain segments take place in the form of vibration about a fixed position. The polymer is rigid and glassy. With an increase in temperature, the amplitude of segmental vibrations increases. At the glass transition temperature, the chain segments have sufficient energy to overcome the secondary intermolecular bonding forces resulting in a transition from the glass to the rubber stage. In the rubbery state, the segmental motions are very rapid whereas the motion of the entire molecule is restricted by chain entanglement (Allcock and Lampe, 1981).

Many physical properties change profoundly at the glass transition temperature, including heat capacity, refractive index, mechanical damping, and electrical properties.
Nicholson, 1997). All of these are dependent on the relative degree of freedom for molecular motion within a given polymeric material and each can be used to monitor the point at which the glass transition occurs.

The melting of a semicrystalline polymer takes place over a wider temperature range than that observed for small organic compounds, due primarily to the presence of crystallites of different sizes and the more complex process for melting of large molecules (Allcock and Lampe, 1981). The crystalline melting temperature, \( T_m \), is generally reported as the temperature of the onset of melting. The effect of polymer structure on \( T_m \) is similar to that on \( T_g \). Polymers with low \( T_g \) values usually have low \( T_m \) values. The two thermal transitions are generally affected in the same manner by the molecular weight, the molecular symmetry, structural rigidity, and secondary bonding forces of polymer chains. For linear polymers, the \( T_g \) and \( T_m \) values are increasing functions of the molecular weight (Billmayer, 1984; Mark et al., 1984).

Among commonly used synthetic biodegradable polymers, poly(glycolic acid) (PGA) is a semicrystalline polymer with a \( T_m \) of 224-226°C and a \( T_g \) of 36°C (Piskin, 1994). Poly(lactic acid) (PLA) is a chiral molecule and can exist as two optically active stereoregular polymers, poly(D-lactic acid) and poly(L-lactic acid) (PLLA) and the racemic form, poly(D,L-lactic acid). For biomedical applications, PLLA and poly
(D,L-lactic acid) have been the most widely studied (Piskin, 1994). PLLA polymers are semicrystalline polymers with a Tm of 184°C and a Tg of 57-60°C. Poly(D,L-lactic acid) is amorphous and has a Tg of 54-59°C (Holland and Tighe, 1992; Piskin, 1994). For PLGA, the copolymers of lactic acid and glycolic acid, the degree of crystallinity and Tg varies with the ratio of glycolic acid and lactic acid (Holland and Tighe, 1992; Piskin, 1994).

1.6.2.4 Biodegradation

Biodegradation is a broad term that refers to hydrolytic, enzymatic or bacteriological degradation processes occurring in a polymer matrix (Holland and Tighe, 1992).

The most common degradation mechanism for biodegradable polymeric drug delivery systems is hydrolytic degradation. Hydrolytic biodegradation is dependent on the ability of water to insert itself into a susceptible functional group in the polymer backbone, which results in cleavage of the bond. Factors affecting the rate of hydrolysis of the functional group include hydrophobicity of the polymer, steric hindrance of the polymer side chains near the functional group, the crystallinity of the polymer and environmental factors such as temperature, pH and ionic strength (Kakino et al., 1986; Holland and Tighe, 1992; Pistner et al., 1993).
For PLA, hydrolytic degradation is considered to be the major biodegradation mechanism. The cleavage of an ester bond yields carboxyl and hydroxyl end groups. The carboxyl end groups are capable of catalyzing hydrolysis of other ester bonds, a phenomenon called autocatalysis (Li, 1999). The hydrolytic degradation starts with a random chain scission process as the ester bonds are hydrolyzed by the water molecules. The molecular weight of the polymer decreases significantly, but there is initially no appreciable weight loss and no soluble monomer products are formed. When the molecular weight drops sufficiently, the degradation products become soluble or separate from the matrix, and loss of material will occur. Soluble monomer products are then formed from soluble oligomeric fragments (Jalil and Nixon, 1990). Loss of mass from an implanted material is called bioerosion. Bioerosion may occur as a result of degradation but it may also occur with non-degradable materials, which dissolve or disintegrate in the body (Jalil and Nixon, 1990).

The final elimination phase in which dissolved monomers and oligomers of polymer are absorbed is called bioresorption (Vert et al., 1992). Small particles of PLA and dissolved oligomers are taken up into macrophages for subsequent digestion by lysozomal enzymes (Woodward et al., 1985; Bos et al, 1991). The final degradation product of PLA is lactic acid, which enters the tricarboxylic acid cycle and is metabolized.
and subsequently eliminated from the body as carbon dioxide and water (Jain et al., 1998). The effects of biodegradation and subsequent bioerosion of biodegradable drug delivery systems are seen as changes in inherent viscosity and molecular weight of the polymer and in mechanical properties such as tensile strength and drug release kinetics (Holland and Tighe, 1992).

### 1.6.2.5 Biocompatibility

All biomaterials are evaluated in terms of biocompatibility depending on the intended medical application (Park and Park, 1995). Biocompatibility can be defined as the acceptance of an artificial material by the surrounding tissues and by the body as a whole (Wang et al., 2004). The term biocompatibility encompasses many different properties of the materials, including toxicity, blood compatibility, and tissue compatibility (Wang et al., 2004).

When a biomaterial is exposed to blood, certain blood proteins adsorb rapidly to the biomaterial, and protein adsorption, depending on the type of adsorbed proteins, is followed by platelet adhesion. The activation of adherent platelets leads to the formation of thrombi on the surface, which may result in blockage of blood supply or may affect the drug release profiles of the drug delivery systems (Park and Park, 1995).
Tissue damage created by the implantation procedure usually results in inflammation (Park and Park, 1995). The inflammatory process is accompanied by a series of defensive reactions by neutrophils, eosinophils, macrophages and foreign body giant cells. Macrophages initiate the repair of damaged tissue by forming the scaffold for repair, which is called granulation tissue. If the implant is not phagocytosed by the cells, the body tends to completely isolate the foreign implant by forming a sheath-like fibrous membrane capsule around the implants, which is termed scar tissue (Park and Park, 1995). The fibrous capsule often contracts and causes pain in patients and deformation of the implant. For a drug delivery implant, the fibrous capsule may alter the drug release kinetics (Park and Park, 1995).

The biocompatibility of PLA implants was first noted by Kulkarni and coworkers (1971). In vivo evaluation of PLA drug delivery systems, such as microspheres, was carried out by several groups of workers (Ratcliffe et al., 1984; Jain, 2000; Johansen et al., 2000; Liggins et al., 2004). In general, tissue responses were mild with no abnormal reactions leading to the rapid clinical acceptance of lactic acid polymers. In vivo evaluation of PLLA bone fixation plates and screws three years after implantation, showed a foreign body reaction at the site of the implant although the presence of inflammation was considered minimal (Bergsma et al., 1993). PLLA microspheres in
the size range of 1-80 μm injected subcutaneously in mice, caused a mild inflammatory response initially and the response disappeared 6 months following injection. Histology showed a fine capsule around the implant at all time points but no remnants of scar tissues were discovered, illustrating excellent biocompatibility of PLLA (Lemperle et al., 2004). Hooper et al (1998) investigated the biocompatibility of PLLA implants as a function of molecular weight of PLLA. The results showed that the tissue response to the implant fluctuated as a function of the degree of degradation, exhibiting an increase in the intensity of inflammation as the implant began to lose mass. A thick capsule containing fibroblasts and macrophages was observed surrounding the implant. There was no ingrowth of tissue into the implant.

1.6.3 Mechanisms of drug release from polymeric drug delivery systems

Polymeric controlled release drug delivery systems can be generally divided into two types; reservoir-type and matrix type. In the reservoir-type system, the drug is encapsulated inside a system enclosed by a polymeric membrane and the rate of drug release is controlled by its permeation through the membrane wall. In the matrix-type drug delivery system, the active agent is homogeneously dispersed throughout a polymeric matrix (Chien, 1982). In this thesis, the discussion is focused on the matrix-type drug delivery system.
Three different mechanisms of drug release can be identified and are referred to as diffusion controlled, chemical controlled and solvent controlled release. These classifications represent theoretical situations where the rate of drug release is controlled predominantly by the diffusion of drug through a polymeric matrix or a membrane, the chemical processes such as polymer degradation, erosion or the cleavage of a drug from a polymeric carrier, and solvent interactions such as swelling of the polymer, respectively (Sinkon and Kohn, 1993).

1.6.3.1 **Diffusion controlled drug release**

In the diffusion controlled system, if the drug to be released is dispersed uniformly throughout the polymer, the system is called a monolithic diffusion system (Baker, 1987). A monolithic system can be either a monolithic solution in which the drug is dissolved in the polymer or a monolithic dispersion in which only a portion of the drug is dissolved in the matrix and the remainder is dispersed as particles throughout the matrix (Chien, 1982; Baker, 1987). The release of solutes from matrix systems is based upon Fick’s laws of diffusion. Fick’s first law states that the flux \( J \) or the rate of solute transfer across a plane of unit area is given by:

\[
J = -D \frac{dC}{dx}
\]

Equation 1.8
where $\frac{dC}{dx}$ is the concentration gradient or the change in concentration ($C$) with respect to distance ($x$), and $D$ is the diffusion coefficient. In the case of a monolithic solution, no concentration gradient exists in the matrix prior to the onset of drug release. As the drug begins to be released from the surface of the matrix, a concentration gradient is established. Drug begins to diffuse down the concentration gradient from the interior of the matrix towards the surface and is gradually released at the surface (Baker, 1987).

In monolithic dispersion systems, the amount of initial drug loading has an effect on the release mechanism (Baker, 1987). At low drug loading levels (less than 5% by volume), the release of the drug involves dissolution of the drug in the polymer followed by diffusion to the surface of the device. At slightly higher loading levels (5-10% by volume), the release mechanism becomes more complex, since the cavities remaining from the loss of the drug near the surface are filled with fluid from the external medium, and these cavities provide preferred pathways for the escape of material remaining within the device. At these loading levels, the cavities are not connected to form continuous pathways to the surface, but they may increase the overall permeability of the agent in the device (Baker, 1987). When loading of dispersed agent exceeds 20% by volume, the cavities left by the loss of material are sufficiently numerous to form a continuous channel to the surface of the matrix. The majority or the entire active agent is released
by diffusion through these channels. The solubility and diffusivity of the dispersed agent in the fluid filling the channels determines its rate of release (Baker, 1987).

**1.6.3.2 Diffusion and degradation controlled drug release**

The mechanisms of drug release from biodegradable polymeric devices are more complicated than from non-degradable devices. The kinetics of drug release are not only governed by the diffusion of drug from the device but also by the degradation rate of the polymer. The influences of these mechanisms may also vary at different time points of drug release. Attempts to match release profiles to kinetic equations are usually not possible because too many variables exist (Chien, 1982).

If the drug diffuses from the device rapidly relative to the degradation of the polymer, the drug release from the polymer is mainly controlled by simple diffusion during the initial stages. However, subsequent degradation of the polymer can increase the permeability of the polymer matrix significantly and hence increase drug release rates (Gopferich, 1996a).

In degradation-controlled monolithic systems, degradation of the polymer leads to erosion, which is the process of material loss from the polymer bulk (Gopferich, 1996b). Depending on the rate of polymer degradation and water diffusion into the polymer, either surface or bulk degradation and erosion may occur. In the first case, polymer
degradation is faster than water diffusion. Thus degradation and erosion are surface phenomena. In the case of bulk degradation and erosion, water ingress is rapid and degradation and erosion occurs throughout the polymer matrix (Gopferich, 1996b).

1.6.3.3 *Swelling controlled drug release*

A swelling-controlled system consists of a dispersion of a drug in a hydrophilic polymer matrix. In a swelling-controlled system, penetration of water from the environment changes the dimensions as well as the physical properties of the polymer matrix and thus the drug release kinetics. Drug release from such systems is a function of the rate of uptake of water from the surrounding media and the rate of drug diffusion (Baker, 1987).

1.6.4 *Factors affecting drug release from biodegradable polymers*

A number of factors can affect the kinetics of drug release from a polymer matrix. These include the properties of the drug and the properties of the polymer.

In diffusion controlled systems, the molecular weight and solubility of the drug in the polymer and release medium influence the drug release kinetics. The diffusion coefficient of the drug in the polymer decreases as the molecular weight of the drug increases (Pitt and Schindler, 1980). The solubility of the drug in the polymer matrix and in the release medium can influence the diffusion of the drug in the polymer matrix.
and the partition coefficient of the drug between the polymer matrix and release medium.

An increase in the drug solubility in both the polymer matrix and the release medium generally leads to an increase in the drug release rate (Chien, 1982).

For degradation controlled systems, acidic or basic drugs can affect polymer degradation through pH changes (Gopferich, 1996a). For example, amine drugs such as meperidine and methadone have been shown to increase the rate of polyester hydrolysis (Cha and Pitt, 1989).

The degree of crystallinity of a polymer can affect the permeability of the drug molecules in the polymeric matrix. In a semicrystalline polymer, drug diffusion usually occurs in the amorphous regions since the ordered alignment of polymer chains in the crystalline regions lowers the free volume, thus preventing the diffusion of drug and water molecules (Chien, 1982). Therefore, lowering the crystallinity of a polymer will increase its permeability to drugs and water and thus increase the release rate of the drug (Pitt et al., 1979ab). The flexibility of polymer chains in the amorphous regions also plays an important role. High flexibility, as indicated by low Tg values, allows the polymer chains to move more readily and thus increase the permeability to drug and water (Pitt et al., 1979ab). Biodegradable polymers that have low Tg's exhibit faster biodegradation rates due to the individual polymer molecules possessing more mobility,
leading to greater chemical and possible enzymatic attack (Pitt et al., 1979ab). The molecular weight of a polymer also affects drug diffusion and polymer degradation. As the molecular weight of a polymer decreases, the permeability of the polymer increases due to increased free volume caused by a greater number of polymer chain ends (Cha and Pitt, 1989). For polyesters such as PLA, hydrolysis increases as molecular weight decreases due to a higher percentage of hydrophilic end groups in the lower molecular weight polymer. The increased hydrophilicity permits a more rapid influx of water into the polymer matrix and leads to a higher water content in the matrix (Pitt et al., 1979ab).

Other factors such as drug loading and device geometry can influence drug release. In both monolithic solution and dispersion systems, an increase in drug loading results in an increased concentration gradient, thus increasing release rate (Chien, 1982). In a monolithic dispersion system, the levels of drug loading also influence the formation of cavities and channels for diffusion. The geometry of the drug delivery system can affect the diffusion path and surface area of the device. Increasing surface area results in a higher drug release rate (Chien, 1982; Baker, 1987).
1.7 INTRA-ARTICULAR MICROSPHERES AS A DRUG DELIVERY SYSTEM

1.7.1 Microspheres composition and size

Microspheres manufactured using different polymers and in different size ranges have been investigated for their tolerability in joints following intra-articular administration and their suitability for delivering anti-inflammatory drugs to the joints. Using empty microparticles, sized 1-10 μm prepared from poly(lactic acid), poly(butylcyanoacrylate), gelatin, and albumin, Ratcliffe et al (1984) observed various degrees of inflammatory response following intra-articular injection of all polymeric microparticles except albumin. However, this study failed to investigate the effect of particle size on the degree of inflammation induced by the different polymers.

Greis et al (1994) investigated the interactions of particles with synovial fibroblast cell cultures by measuring the collagenase synthesis induced by particles. Standard sized latex beads (0.4, 15, 45, 90 μm) and particles from prosthetic anterior cruciate ligament materials, Dacron and carbon particles, were selected for the study. The ability of latex beads to induce collagenase was strongly size dependent. Particles that were 0.4 μm in diameter were readily phagocytosed by the cells and induced collagenase production, while 15 μm latex beads were not readily internalized. Nevertheless, the synthesis of collagenase was induced if the beads were internalized. Latex particles
with diameters of 45 and 90 μm were too large for uptake by synovial fibroblast cells and
did not induce the synthesis of collagenase. Both the size and physical properties of
Dacron and carbon influenced their ability to activate synoviocytes. Internalized
Dacron and carbon particles all induced collagenase secretion. Certain cells that
contained no ingested particles also produced collagenase when in co-culture with cells
with ingested particles, indicating that phagocytosis, in addition to inducing collagenase,
also induced the release of cell-activating factors which then activated additional cells in
the culture (Greis et al., 1994).

Nishide et al (1999) investigated the biodegradation and tissue responses to
intra-articular poly(D,L-lactic acid) microspheres in the knee joints of rabbits. Different
sized (< 20 μm – 200 μm) microspheres containing a fluorescent dye were prepared from
poly(D,L-lactic acid) polymers with different molecular weights (3000-7000 g/mole).
Although there was a temporary increase in the number of white blood cells in the joint,
irrespective of the microsphere size, no inflammatory responses to the microspheres by
the synovial tissue were observed within 3 days after injection. It was found that
following intra-articular injection, the microspheres were localized in the adipose tissues
of the popliteal region of the knee cavity, irrespective of the microspheres size and
molecular weight (Nishide et al., 1999).
Horisawa and coworkers (2002a) investigated the size-dependency of intra-articular nanospheres and microspheres of poly(lactic-co-glycolic acid) (PLGA) on tolerability in rat knee joints. Histological analysis showed that fluoresceinamine bound PLGA nanospheres with a mean diameter of 265 nm were extensively phagocytosed in the synovium by the macrophages infiltrated through the synovial tissues. The phagocytosed nanospheres were delivered to the deep underlying tissues and the synovium was fairly proliferated, 3 days following the injection. On the other hand, microspheres with a mean diameter of 26.5 µm were not phagocytosed in the macrophages. A mild proliferation was observed in the epithelial lining synovial cells and the microspheres were covered with a granulation of multinucleated giant cells. The number of inflammatory leukocytes in the synovial tissue slightly increased one day after the injection of either nanospheres or microspheres, but no further inflammatory responses were detected. The authors concluded that both nanospheres and microspheres were well tolerated in the joint (Horisawa et al., 2002a).

Using an in vivo isolated horse joint model, Bragdon and coworkers (2001) assessed initial biocompatibility and early (within 3 h) vascular and transsynovial fluid alterations in horse knee joints, following intra-articular injection of paclitaxel loaded PLGA microspheres with an average size of 50 µm. Compared with control (non-injected)
joints, intra-articular injection of paclitaxel loaded PLGA microspheres did not affect joint blood flow and blood pressure during this short term study, and early joint reaction was minimal. Gross and histological morphology of synovium and articular cartilage were normal in all isolated joints (Bragdon et al., 2001).

In our laboratory, experiments have been conducted to investigate the in vivo biocompatibility of microspheres made from different biomaterials and with different particle sizes. Poly(L-lactic acid), poly(lactic-co-glycolic acid) and poly(caprolactone) microspheres with different size ranges (1-20 μm, and 35-105 μm) were injected into joints of healthy rabbits. The swelling and inflammatory responses were scored and graded. There was no apparent difference in responses caused by different biomaterials. The microspheres in the size ranges of 35-105 μm appeared to be well tolerated in the joint while smaller microspheres in the size range of 1-20 μm induced a higher degree of cellular infiltration and proteoglycan loss (Liggins et al., 2004).

In summary, previously published works using microspheres made of biodegradable polymers such as PLGA or PLA in size ranges above 20 μm have shown that the microspheres were not phagocytosed by macrophages following intra-articular injections and that there were minimal inflammatory responses (Bragdon et al., 2001; Horisawa et al., 2002a; Liggins et al., 2004). On the other hand, microspheres or nanospheres in
sizes less than 10 μm injected intra-articularly, were generally likely to be phagocytosed and in some studies, produced inflammatory reactions such as synovial proliferation, cellular infiltration, and collagenase production (Ratcliffe et al., 1984; Greis et al., 1994; Horisawa et al., 2002a).

1.7.2 Pharmacokinetics and efficacy studies

There are only a few studies that have investigated the pharmacokinetics and in vivo efficacy of drug encapsulated microspheres following intra-articular administration.

Ramesh and coworkers (1999) investigated plasma levels of dexamethasone following intra-articular injection of either dexamethasone loaded poly(D,L-lactic acid) microspheres (size 40 – 110 μm) or dexamethasone solution. The results showed that approximately 30% of the dose of the dexamethasone solution was detected in the serum 30 min following injection, and thereafter the drug concentration in the serum rapidly decreased over 4 h. No drug was detected in the rabbit serum over 24 h when the rabbit was injected with dexamethasone encapsulated microspheres. When the microspheres were collected from the synovial cavity, drug was detected in the microspheres up to 7 days following injection. The authors concluded that most of the drug incorporated in the microspheres was localized in the synovial cavity (Ramesh et al., 1999).
Horisawa and coworkers (2002b) investigated the efficacy of intra-articular delivery of betamethasone encapsulated within PLGA nanospheres (300-490 nm) in an antigen-induced arthritis rabbit model. Monoarthritis was induced in the left knee joint of the rabbits by the injection of ovalbumin. Then, betamethasone encapsulated nanospheres, betamethasone solution or saline were injected immediately after the antigen challenge. The progression of the disease was monitored for 42 days. Compared with betamethasone solution, and saline, the nanospheres formulation showed improved effectiveness in reducing joint swelling and cell infiltration over 21 days. However, the levels of proteoglycan and hydroxyproline in the cartilage slightly decreased in the nanospheres treated group compared to the betamethasone solution and saline groups, suggesting possible cartilage damage caused by the nanospheres (Horisawa et al., 2002b).

Liggins et al (2004) investigated the efficacy of paclitaxel encapsulated PLA microspheres (35-105 µm) in antigen-induced arthritis in rabbits. Forty milligrams of control microspheres or 20% paclitaxel loaded microspheres were injected into rabbit knee joints. Compared with control microspheres, 20% paclitaxel loaded microspheres produced significantly less joint swelling, cellular infiltration, and cartilage degradation as measured by proteoglycan loss and chondrocyte necrosis over 29 days (Liggins et al., 2004).
It was suggested that paclitaxel loaded PLA microspheres in the size range of 35-105 μm may be a potential formulation for the intra-articular treatment of inflammation in arthritis (Liggins et al., 2004).

1.8 THESIS GOALS AND OBJECTIVES

Intra-articular drug delivery can be very useful for treating rheumatoid arthritis disease flare-ups, synovitis and pain when a small number of joints are affected or for those joints that do not respond to systemic treatments. Surprisingly, little work has been done in terms of developing suitable site directed and controlled release intra-articular drug delivery systems to treat inflammatory arthritic conditions. To our knowledge, MTX has not been previously formulated in polymeric microspheres for intra-articular injection.

We therefore test the following hypothesis: MTX loaded polymeric microsphere formulations based on biodegradable polymers may be developed for intra-articular injection, which are well tolerated, retained in the synovial joint and provide a controlled, localized delivery of MTX into the joint cavity to produce a therapeutic response. The goals were to develop and characterize controlled release polymeric microsphere formulations of MTX and to investigate the in vivo biodistribution and efficacy of
microsphere formulations following intra-articular injection in an established arthritis rabbit model.

The development of a controlled release formulation of MTX for use in selected joints would represent a significant advance in rheumatoid arthritis therapy. A knowledge of the in vivo efficacy and biodistribution of MTX encapsulated microspheres is fundamental to future work in which this formulation may be optimized for intra-articular controlled release drug delivery in human subjects.

The research objectives were:

1) To develop and characterize MTX loaded PLLA microsphere formulations;

2) To determine the in vitro degradation of MTX loaded PLLA microspheres;

3) To evaluate the biocompatibility of MTX loaded PLLA microspheres in healthy rabbit joints;

4) To determine the pharmacokinetics and biodistribution of MTX following intra-articular administration of MTX encapsulated PLLA microspheres in healthy rabbits;

5) To evaluate the in vivo efficacy of MTX loaded PLLA microspheres following intra-articular administration in the antigen-induced arthritis rabbit model.
Chapter 2

FORMULATION AND CHARACTERIZATION OF METHOTREXATE ENCAPSULATED POLY (L-LACTIC ACID) MICROSPHERES

2.1 INTRODUCTION

Poly(L-lactic acid) (PLLA) was selected as a suitable biodegradable polymer for the development of MTX loaded microspheres due to its biodegradability, biocompatibility, mechanical strength and ability to achieve prolonged drug release (Watt et al., 1990). PLLA is commercially available over a wide range of molecular weights from 2k to 100k g/mole. The ability to prepare microspheres using different molecular weights of polymers was considered important, so that drug loaded microsphere formulations with significantly different release rate profiles could be developed. It is well established that molecular weights of the polymer greatly influences drug release rates (Chien, 1982; Okada, 1997; Siparsky et al., 1998; Tracy, 1998).

Other biodegradable and biocompatible polymers, poly(lactic-co-glycolic acid) (PLGA) and poly(D,L-lactic acid) (PDLLA) polymers, along with PLLA, have been used extensively in the formulation of microspheres loaded with a wide range of different drugs (for reviews, see Okada, 1997; Jain et al., 1998; Jain et al., 2000; Tracy et al., 2000

and Kakinoki et al., 2003) Previous work in this laboratory has shown that drug loaded PLLA microspheres can be prepared with very low molecular weight polymer (around 2k g/mole) and still retain good mechanical integrity (Liggins and Burt, 2001). For this reason, PLLA polymers only were selected for formulation development with MTX.

One of the methods commonly employed to manufacture microspheres is the solvent evaporation method. Figure 2.1 is a schematic diagram showing microsphere formation by emulsification and solvent evaporation. The solvent evaporation method involves the emulsification of a polymer solution containing drug (either dissolved or in suspension) into a second, immiscible liquid phase containing an emulsifier to form a dispersion of drug/polymer/solvent droplets. The solvent is then removed from the dispersed droplets by application of heat, vacuum, or by allowing evaporation at room temperature, producing a suspension of drug loaded polymer microspheres that can then be separated by filtration or centrifugation, washed, and dried (Watts et al., 1990). The properties of microspheres can change upon even slight variations of the manufacturing process, resulting in different microstructures (Donbrow, 1992; Gopferich, 1996a). The main variables that influence the microencapsulation process and the final microsphere product, include a) the nature and solubility of the drug being encapsulated; b) the polymer concentration, composition, and molecular weight; c) the drug/polymer ratio; d)
the organic solvent used; e) the concentration and nature of the emulsifier used; f) the
temperature and stirring/agitation speed of the emulsification process; and g) the
viscosity and volume ratios of the dispersed and continuous phase (Jain et al., 1998).

The biocompatibility of intra-articular PLLA microspheres has been investigated in
healthy rabbit joints (Liggins et al., 2004). Intra-articular injections of control (no
drug) PLLA (100k g/mole) microspheres in the size range of 35-105 µm, induced mild
swelling and cell infiltration (Liggins et al., 2004). However, PLLA microparticles in
the size range of 1-10 µm induced marked synovial hyperplasia, cellular infiltration and
fibrosis in rabbit joints (Ratcliffe et al., 1984). In this study, we evaluated the
biocompatibility of control and MTX loaded low molecular weight PLLA microspheres
in the size range of 33-110µm. Rabbits were chosen as a suitable animal model for this
work because the larger joints of rabbits are easier to inject compared to the joints of rats
or mice, and rabbits were used in the pharmacokinetic studies in the following chapter.

In this chapter, we report the results of development and characterization of poly
(L-lactic acid) (PLLA) microspheres loaded with MTX. The objectives of this study
were:

1. To prepare MTX loaded microspheres using PLLA with three different molecular
weights;
2. To characterize the solid state and degradation properties of MTX loaded microspheres;

3. To determine the *in vitro* MTX release profiles from microspheres;

4. To determine the biocompatibility of MTX loaded microspheres following intra-articular injection in healthy rabbits.
1. Polymer dissolved in organic solvent. Drug added to form a solution or suspension.

3. Emulsifier dissolved in second liquid immiscible with drug/polymer solution.

4. Polymer/drug solution mixed into emulsifier solution to form a dispersion of drug/polymer/solvent droplets.

5. Solvent removed by:
   a) Stirring at room temp.
   b) Application of heat.
   c) Application of vacuum.

Microspheres recovered by filtration or centrifugation, then washed and dried.

**Figure 2.1** Schematic diagram of microspheres formation by the solvent evaporation method. Adapted from Watts *et al* (1990).
2.2 EXPERIMENTAL

2.2.1 Materials

Methotrexate (MTX) was purchased from Hande Tech Development Co. (U.S.A). Poly (vinyl alcohol) (PVA) (98% hydrated, MW: 13,000-23,000) was obtained from Aldrich Chemical Company Inc. Poly (L-lactic acid) (MW: 2k g/mole, 50k g/mole and 100k g/mole) was obtained from Polysciences (Warrington, PA). Poly (ethylene glycol) standards were purchased from Polymer Laboratories Inc. Sodium dihydrogen orthophosphate, sodium phosphate, sodium chloride (NaCl) were purchased from Fisher Scientific (Napean, Ontario, Canada). All solvents used were High Performance Liquid Chromatography (HPLC) grade. Nitrogen and prepurified helium gases were supplied by Praxair (Burnaby, BC, Canada). Double distilled water was used throughout the studies. Phosphate buffered saline (PBS, pH 7.4) was prepared by dissolving 0.32g sodium dihydrogen orthophosphate, 2.15 g sodium phosphate, 8.22 g NaCl in one liter of distilled water. The pH of the buffer was in the range of 7.2-7.4.

2.2.2 General equipment and supplies

General equipment and supplies used in this study are listed in Table 2.1.
Table 2.1  List of general equipment and supplies used in the study.

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<th>Equipment and Supplies</th>
<th>Supplier</th>
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<td>Fisher Scientific, (Fairlawn, NJ)</td>
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<td>Olympus BH-2 microscope</td>
<td>Olympus Optical Company Limited, (Japan)</td>
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<td>Corning hot plate/stirrer, model PC-351</td>
<td>Corning Glass Works, (Corning, NY)</td>
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<td>GS-6 centrifuge and eppendorf Centrifuge model 5415 C</td>
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<td>Eppendorf tubes</td>
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2.2.3 Validation of UV-vis spectrophotometric assay

The UV-vis assays for MTX in PBS were validated by measuring UV absorbance at a wavelength of 304 nm of four sets of standards on three separate days. The linearity, limit of detection, limit of quantitation, inter-day precision, intra-day precision, and accuracy of the calibration curves were measured. Linearity was expressed as the coefficient of determination ($R^2$) for each of the twelve calibration curves assembled over three days and for the standard curve consisting of each of the twelve standards’ data points for each concentration. According to the “Reviewer Guidance” published by the Center for Drug Evaluation and Research, Food and Drug Administration (1994), the limit of detection was calculated from the equation

$$\text{Limit of detection} = \frac{3.3\sigma}{s} \quad \text{Equation 2.1}$$

Where $\sigma$ is the standard deviation of the y-intercepts of the twelve calibration curves (four a day for three days) and $s$ is the mean slope of the twelve calibration curves. The limit of quantitation was calculated from the equation

$$\text{Limit of quantitation} = 10\sigma/s \quad \text{Equation 2.2}$$

Inter-day precision was expressed in terms of the coefficient of variation (CV) of the mean of the twelve standards’ data points collected over three days for each concentration. Intra-day precision was expressed in terms of the CV of the mean of the
four standards’ data points on each day for each concentration. Accuracy was
determined by assembling a calibration curve and comparing three different standards at
each concentration against the calibration curve each day for three days. Accuracy was
expressed in terms of the percent deviation (bias) of each of the three standards and their
mean bias, compared to the daily calibration curve.

2.2.4 Solubility of methotrexate

Three 10 mg samples of methotrexate were placed in 2 mL Eppendorf vials. The
powder was dispersed in 1mL of pre-warmed double distilled water, 10 mM pH 7.4,
phosphate buffered saline (PBS) and 10% poly(vinyl alcohol) solution by vortexing for
10 seconds. The Eppendorf vials were then placed in a 37°C oven with circular shaking
at a rate of 60 rpm and samples were taken at 24 h. Eppendorf vials were centrifuged at
325 x g for 10 minutes. The supernatants were diluted with PBS and the absorbances
measured using a UV-vis spectrophotometer (Hewlett Packard Diode Array 8452A) at a
wavelength of 304 nm.

2.2.5 Stability of MTX in PBS at 37°C

Three sets of MTX standards in PBS were prepared in the concentration range of 0.4
µg/mL to 50 µg/mL. The UV-vis absorbances were determined by a UV-Vis
spectrophotometer at a wavelength of 304 nm. The standard MTX solutions were stored
in a 37°C oven and the absorbances were measured at time intervals of 5 h, 24 h, and 7 days.

2.2.6 Preparation of MTX loaded microspheres

The microspheres were prepared by the solvent evaporation method. Five hundred milligrams of poly(L-lactic acid) with different molecular weights (2k g/mole, 50k g/mole or 100k g/mole) were dissolved in 2.5 to 5 mL methylene chloride. MTX was suspended in the PLLA/ methylene chloride solution by vortex mixing. The drug suspension was then slowly dispersed into 100 mL of a 2.5% PVA solution and stirred at 1000 rpm using an overhead stirrer (BDC2002 Caframo, Ont, Canada). The resultant emulsion was continuously stirred for 2.5 hours at room temperature under ambient pressure until all the methylene chloride had evaporated. The solidified microspheres were recovered by centrifugation. The microspheres were sieved through 33 and 110 μm sieves and washed with distilled water. The washed microspheres were then air-dried overnight and stored in a desiccator at room temperature for further drying. Either control or MTX loaded microspheres batches intended for injection into rabbit joints were sterilized by gamma irradiation from a Co-60 source at a dose of 25 kGy (Nordion International Inc.).
2.2.7 Encapsulatio efficiency

To determine the MTX content in microspheres, 5 mg of microspheres were dissolved in 1 mL of methylene chloride in test tubes. MTX was then extracted by adding 10 mL of 10 mM, pH 7.4 PBS to the test tube and the tubes were shaken vigorously for 30 seconds. The drug concentration in the aqueous phase was measured using a UV-Vis spectrophotometer at a wavelength of 304 nm. The encapsulation efficiency was expressed as: (the amount of MTX in the microspheres / the theoretical amount of MTX in the microspheres) × 100%.

2.2.7.1 Validation of encapsulation efficiency studies

To validate the encapsulation efficiency test method, 20 μL, 50 μL or 100 μL of a 10 mg/mL MTX in a dimethylsulfoxide (DMSO) stock solution were mixed with 100 μL of PLLA (2k g/mole) in a methylene chloride stock solution in a 12 mL glass test tube to give 4%, 10% and 20% (w/w) theoretical loadings. The mixture was dried under a stream of nitrogen gas. The dried mixture was then dissolved with 1 mL of methylene chloride. MTX was extracted by adding 10 mL of 10 mM, pH 7.4 PBS to the test tube and the tubes were shaken vigorously for 30 seconds. The drug concentration in the aqueous phase was measured using a UV-Vis spectrophotometer as previously described.
2.2.8 Microspheres characterization

2.2.8.1 Particle size determination

The PLLA microspheres (20 mg) were homogeneously dispersed in distilled water (70 mL) with a few drops of 1% polysorbate 80 solution. Particle size distributions of microspheres were determined using a Malvern Hydro 2000SM laser diffraction particle size analyzer. Three measurements were taken from each batch and the particle size was expressed as the volume weighted mean.

2.2.8.2 X-ray powder diffraction of microspheres

The X-ray powder diffraction patterns of microspheres and MTX were determined using a Geigerflex X-ray powder diffractometer (Rigaku Inc., Tokyo, Japan). The sample size was approximately 100 mg. The X-ray tube was operated at a potential of 35kV and 18mA. The range of scans was from 5 to 35 degrees(2θ), and the scan speed was 1 degree per minute.

2.2.8.3 Scanning electron microscopy

Microspheres were mounted on aluminum disks with double sided adhesive tape impregnated with carbon. The mounted samples were coated with 100 Å of gold-palladium using a Hummer sputter coater and analyzed by scanning electron microscopy with an electron voltage of 5-10 kV.
2.2.8.4 Thermal properties of microspheres

Differential scanning calorimetry (DSC) of microspheres was carried out using a Pyris 1 DSC, cooled with liquid nitrogen using a Perkin Elmer Cryofill. The purge gas was prepurified helium at a pressure of 20 psi. Microspheres weighing 3-5 mg were placed in crimped, but not hermetically sealed, aluminium pans using an empty pan as a reference. The samples were heated from -20°C to 200°C at 10°C / min.

The degree of crystallinity (Xc) of PLLA was calculated using the equation:

\[ Xc = \left( \frac{AH_f - AH_c}{93.7 \text{ J/g}} \right) \times 100\% \]  
Equation 2.3

Where \( AH_f \) and \( AH_c \) are the enthalpies of fusion and recrystallisation, respectively, calculated from the area under the curve for both PLLA melting and recrystallisation peaks. The enthalpy of fusion for 100% crystalline PLLA polymer has been reported as 93.7 J/g (Celli et al., 1992).

2.2.9 In vitro release of MTX from PLLA microspheres

The in vitro release studies were carried out in PBS at 37°C. Into 12 mL glass, screw capped tubes were placed 10 mg of MTX loaded microspheres and 10mL PBS. The tubes were tumbled end-over-end at 30 rpm in a thermostatically controlled oven. At given intervals, the tubes were centrifuged at 325 x g for 10 min and 5 mL of the supernatant were saved for analysis. The remainder of the supernatant was removed and
the microsphere pellets were resuspended in fresh PBS (10 mL). The buffer was
replaced at each sampling interval in order to maintain sink conditions. The
concentration of MTX in the release medium was measured by UV-Vis
spectrophotometry.

2.2.10 Degradation study of microspheres

Into 12 mL glass, screw capped tubes were placed 15 mg of control or drug
loaded PLLA microspheres with 10 mL PBS and tumbled at 30 rpm in a 37°C oven.
The buffer solution was replaced every day. At predetermined intervals, the
microspheres were collected and washed with distilled water three times, dried under
vacuum and stored in a desiccator at room temperature. The weights of microspheres
samples were then measured to determine the weight loss of microspheres due to
degradation.

2.2.10.1 Gel permeation chromatography (GPC)

The molecular weights of degraded PLLA polymer samples were determined
using gel permeation chromatography (GPC). The system consisted of a Waters 515
HPLC pump, a Waters 717 plus autosampler, and a Waters 2410 refractive index detector
with a detection cell temperature of 40°C. The analytical columns were Styrogel® HR3
connected with HR0.5 (Waters Inc.). Assay conditions for the analysis of 0.5 % w/v
polymer in tetrahydrofuran were an injection volume of 20 µL, and a mobile phase of tetrahydrofuran flowing at 1 mL/min. A calibration curve of refractive index versus log molecular weight was generated by the Millennium software program using poly (ethylene glycol) standards (Polymer Laboratories Inc.). The number average molecular weights and weight average molecular weights of the polymer were calculated using the Millennium software program.

2.2.11 In vivo tolerability of MTX loaded microspheres in rabbit joints

All animal experiments were conducted according to the animal care guidelines of the University of Toronto. The studies were carried out by our collaborators, Drs. Weixian Ming and Tony Cruz, at Mount Sinai Hospital, Toronto, Ontario.

Twelve female New Zealand white rabbits (approximately 2.5 kg) were divided into two groups of 6 animals. The right knee joint of each rabbit was injected intra-articularly either with 25mg control PLLA (2k g/mole) microspheres or 10% MTX loaded PLLA (2k g/mole) microspheres in 200 µL PBS. After the injections, the animals were observed daily and scored for swelling of joints during the first week. All animals were sacrificed on day 14 after injection. The joints were fixed in formalin and then decalcified in 10% formic acid with repeated changes. The decalcified joints were paraffin-embedded and joint sections containing synovium, cartilage and bone were
prepared. The sections were stained for cellularity with hematoxylin and eosin or for proteoglycan content with Saffranin “o”. The swelling in joints and the loss of proteoglycans in the cartilage of the joints was evaluated according to the following standard reported previously by Liggins et al (2004):

**Scoring standard for swelling in joints**

Grade 0: no swelling

Grade 1: little swelling

Grade 2: moderate swelling

Grade 3: heavy swelling

Grade 4: severe damage and pain (animal cannot walk on the limb)

**Scoring standard for loss of proteoglycans in the cartilage by histological analysis**

Grade 0: no loss

Grade 1: small loss (less than 1/3 of total)

Grade 2: moderate loss (around 1/3 of total)

Grade 3: heavy loss (1/3 to 2/3 of total)

Grade 4: total loss
2.3 RESULTS

2.3.1 Validation of UV-vis spectrophotometry assay for MTX in PBS

The precision data are given in Table 2.2. Acceptable assay precision was taken to be < 20% for the coefficient of variation value at the lowest concentration measured and < 15% at all other concentrations. These criteria were met using the UV-vis spectrophotometry assay at a concentration range of 0.156 to 40 µg/mL. A linear relationship between MTX concentrations in PBS and the UV absorbance at a wavelength of 304 nm in the concentration range of 0.156 µg/mL to 40 µg/mL ($r^2 > 0.97$) was established. The overall inter-day and intra-day coefficient of variation was about 6%. The limit of detection of MTX in PBS was 0.1753 µg/mL, and the limit of quantitation was 0.5313 µg/mL for MTX. The accuracy data are shown in Table 2.3.
Table 2.2  Intra-day and inter-day precision of UV-vis spectrophotometry assay of MTX in phosphate buffer saline (pH 7.4)

<table>
<thead>
<tr>
<th>MTX concentration (µg/mL)</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>All days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absorbance at 304 nm</td>
<td>%CV</td>
<td>Absorbance at 304 nm</td>
<td>%CV</td>
</tr>
<tr>
<td>0.156</td>
<td>0.008</td>
<td>16.5</td>
<td>0.001</td>
<td>4.5</td>
</tr>
<tr>
<td>0.312</td>
<td>0.014</td>
<td>1.89</td>
<td>0.015</td>
<td>4.4</td>
</tr>
<tr>
<td>0.625</td>
<td>0.033</td>
<td>3.1</td>
<td>0.031</td>
<td>9.6</td>
</tr>
<tr>
<td>1.25</td>
<td>0.065</td>
<td>2.5</td>
<td>0.064</td>
<td>5.8</td>
</tr>
<tr>
<td>2.5</td>
<td>0.134</td>
<td>0.4</td>
<td>0.129</td>
<td>1.8</td>
</tr>
<tr>
<td>5</td>
<td>0.261</td>
<td>1.2</td>
<td>0.257</td>
<td>4.0</td>
</tr>
<tr>
<td>10</td>
<td>0.514</td>
<td>1.6</td>
<td>0.510</td>
<td>5.0</td>
</tr>
<tr>
<td>20</td>
<td>1.020</td>
<td>2.2</td>
<td>1.005</td>
<td>5.7</td>
</tr>
<tr>
<td>40</td>
<td>1.971</td>
<td>3.0</td>
<td>1.962</td>
<td>4.3</td>
</tr>
</tbody>
</table>

*Absorbance at 304 nm is the average calculated from four standard curves at each concentration for each day (n=16 for all days).

%CV is the coefficient of variation, which is the ratio of the standard deviation to the average, expressed as a percentage. A value less than 20% at the lowest concentration and less than 15% at all other concentrations was taken to indicate sufficient precision.
Table 2.3  Accuracy of UV-vis spectrophotometry assay of MTX in phosphate buffer saline solution (pH 7.4)

<table>
<thead>
<tr>
<th>MTX concentration (µg/mL)</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Measured value</td>
<td>%CV</td>
<td>Bias (%)</td>
</tr>
<tr>
<td>0.156</td>
<td>0.11</td>
<td>27.2</td>
<td>-28.8</td>
</tr>
<tr>
<td>0.312</td>
<td>0.36</td>
<td>4.8</td>
<td>14.8</td>
</tr>
<tr>
<td>0.625</td>
<td>0.42</td>
<td>7.0</td>
<td>-32.2</td>
</tr>
<tr>
<td>1.25</td>
<td>1.13</td>
<td>3.4</td>
<td>-9.9</td>
</tr>
<tr>
<td>2.5</td>
<td>2.58</td>
<td>0.5</td>
<td>3.2</td>
</tr>
<tr>
<td>5</td>
<td>5.30</td>
<td>1.8</td>
<td>6.0</td>
</tr>
<tr>
<td>10</td>
<td>10.76</td>
<td>1.8</td>
<td>7.5</td>
</tr>
<tr>
<td>20</td>
<td>21.74</td>
<td>0.9</td>
<td>8.7</td>
</tr>
<tr>
<td>40</td>
<td>42.47</td>
<td>0.2</td>
<td>6.1</td>
</tr>
</tbody>
</table>

*a* Measured value is the average (n=3 for each day) of values of concentration calculated from one standard curve on that day.

*b* %CV is the ratio of the standard deviation to the average, expressed as a percentage. A value less than 15% was taken to indicate sufficient accuracy at each concentration.

*c* Bias is the ratio of the deviation of measured value from the actual concentration measured, expressed as a percentage.
2.3.2 Solubility and chemical stability of MTX

The solubilities of MTX at 37°C in double distilled water, PBS (pH 7.4) and 2.5% PVA were 0.15, 6.71 and 0.47 mg/mL, respectively.

The chemical stability of MTX in PBS at 37°C over a concentration range from 0.156 to 40 μg/mL over a week is summarized in Table 2.4. The percentage degradation of MTX was determined by comparing the absorbance at 304 nm of the same sample measured by the UV-vis spectrometer on the day of sample preparation and after 7 days at 37°C. The results showed that at low MTX concentrations (0.156 – 0.625 μg/mL) the percentage MTX degraded was more than 20%, while the degradation for higher concentrations (1.25 – 40μg/mL) was less than 10%.

Table 2.4 Percentage degradation of MTX in phosphate buffered saline (pH. 7.4) following 7 days of storage at 37°C. The values are mean ± standard deviation (n=4).

<table>
<thead>
<tr>
<th>MTX concentration (μg/mL)</th>
<th>Mean degradation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.156</td>
<td>27.0 ± 7.1</td>
</tr>
<tr>
<td>0.312</td>
<td>28.6 ± 2.1</td>
</tr>
<tr>
<td>0.625</td>
<td>25.5 ± 0.5</td>
</tr>
<tr>
<td>1.25</td>
<td>9.4 ± 0.4</td>
</tr>
<tr>
<td>2.5</td>
<td>9.1 ± 0.6</td>
</tr>
<tr>
<td>5</td>
<td>11.4 ± 0.6</td>
</tr>
<tr>
<td>10</td>
<td>5.4 ± 1.3</td>
</tr>
<tr>
<td>20</td>
<td>9.2 ± 0.4</td>
</tr>
<tr>
<td>40</td>
<td>5.7 ± 0.2</td>
</tr>
</tbody>
</table>
2.3.3 Optimization of manufacturing of MTX loaded microspheres

Poly (L-lactic acid) of three different molecular weights (2k, 50k and 100k g/mole) (PLLA2k, PLLA50k, and PLLA100k) were used to manufacture MTX loaded microspheres. When stirring rate and the concentration of emulsifying agent remained constant, the effects of changing polymer concentration and MTX loading on the encapsulation efficiency and particle size of the microspheres were evaluated and are shown in Table 2.5. For PLLA2k microspheres, when the polymer concentration was increased from 10% to 20%, the encapsulation efficiency increased from 35% to 68% and the mean particle diameter increased from 28 μm to 83 μm. Therefore, for further characterization, all PLLA2k microspheres were manufactured using 20% polymer concentrations. The microspheres exhibited unimodal particle size distributions as illustrated in Figure 2.2. The microspheres prepared from PLLA 2k had smaller mean particle sizes (62-83 μm) while the microspheres prepared from high molecular weights (PLLA 50k and 100k) showed larger mean particles sizes (greater than 100 μm).

The manufacturing process allowed for high levels of drug encapsulation with all three polymers. PLLA2k microspheres produced between 64-89% MTX encapsulation efficiency while PLLA50k and PLLA100k microspheres showed more than 80% MTX encapsulation efficiency. To ensure that the encapsulation efficiency test could
correctly determine the amount of MTX encapsulated in the microspheres, PLLA2k and MTX solutions of known concentrations were dispensed into a test tube and dried out using nitrogen gas. The dried PLLA-MTX suspension was processed the same way as for the encapsulation efficiency test and the amount of MTX in the suspension was determined. The results showed that when the theoretical loading was 5% and 10%, the recovery of MTX was approximately 96%. The recovery of MTX dropped to 88% when the theoretical loading was increased to 20%.

All microspheres for further characterization studies were in the size range of 33 μm-110 μm. The size range was controlled by sieving microspheres through sieves with mesh opening of 33 μm and 110 μm. The percentage yields of microspheres following sieving are given in Table 2.6. For PLLA 2k microspheres, approximately 60% of microspheres manufactured were in the size range of 33-110 μm while for PLLA50k and 100k, 70-80% of total microspheres manufactured were in this size range.
Table 2.5  Manufacturing conditions and properties of MTX loaded microspheres manufactured from 2k, 50k and 100k g/mole PLLA polymer.

<table>
<thead>
<tr>
<th>Polymer molecular weight (g/mole)</th>
<th>Polymer concentration (w/v %)</th>
<th>Theoretical loading (w/w %)</th>
<th>Encapsulation efficiency (%)</th>
<th>Mean diameter (μm)(^a)</th>
<th>Diameter at 90th percentile (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2k</td>
<td>20</td>
<td>0</td>
<td>n/a</td>
<td>83.7±0.5</td>
<td>128.5±0.5</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>2.5</td>
<td>63.6</td>
<td>79.5±0.8</td>
<td>128.1±1.2</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>5</td>
<td>65.6</td>
<td>61.6±0.4</td>
<td>101.3±0.1</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>10</td>
<td>68.0</td>
<td>76.9±0.7</td>
<td>130.5±1.9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10</td>
<td>35.3</td>
<td>28.7±0.4</td>
<td>70.5±0.4</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>20</td>
<td>89.5</td>
<td>77.5±0.5</td>
<td>127.8±0.4</td>
</tr>
<tr>
<td>50k</td>
<td>10</td>
<td>10</td>
<td>89.6</td>
<td>104.3±0.4</td>
<td>179.0±0.5</td>
</tr>
<tr>
<td>100k</td>
<td>10</td>
<td>10</td>
<td>82.5</td>
<td>187.6±0.6</td>
<td>325.3±1.7</td>
</tr>
</tbody>
</table>

\(^a\)The volume weighted mean is reported as the mean diameter values. The values are shown as the mean of 3 batches of microspheres ± one standard deviation (n=3).
Table 2.6 Percentage yield of microspheres manufactured from PLLA2k, 50k and 100k following sieving between 33 μm, and 110 μm sieves.

<table>
<thead>
<tr>
<th>PLLA molecular weight (g/mole)</th>
<th>Theoretical loading of MTX (w/w %)</th>
<th>% yield of 33 -110 μm microspheresa</th>
</tr>
</thead>
<tbody>
<tr>
<td>2k</td>
<td>10</td>
<td>57.33 ± 2.35</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>59.71 ± 5.95</td>
</tr>
<tr>
<td>50k</td>
<td>10</td>
<td>67.64 ± 1.93</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>68.68 ± 2.88</td>
</tr>
<tr>
<td>100k</td>
<td>10</td>
<td>80.09 ± 0.66</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>77.27 ± 2.97</td>
</tr>
</tbody>
</table>

*a% yield of 33 -110 μm microspheres was determined as the percentage weight of microspheres in the size range of 33 -110 μm divided by the theoretical weight of microspheres which is the weight of MTX plus the weight of polymer.
Figure 2.2  Particle size distributions of 10% MTX loaded PLLA microspheres manufactured from 2k, 50k, and 100k g/mole PLLA polymer using the solvent evaporation method.
2.3.4  *In vitro* drug release profiles

2.3.4.1  The effect of polymer molecular weight on MTX release

Figure 2.3 shows the *in vitro* release profiles of MTX loaded microspheres in the size range of 33-110 μm as a function of molecular weight. Microspheres prepared from 2k, 50k, and 100k PLLA all demonstrated a rapid burst phase of release of MTX followed by a slower release period. More than 50% of the drug was released from PLLA 2k microspheres within the first day followed by a slow release period in which about 10% of the drug was released in the remaining 14 days. The burst phase of release for PLLA50k and PLLA100k microspheres produced less than 20% of the total drug being released over the first day. Following the burst phase, only about 4% of the drug was released over the remaining 35 days. Approximately 95% of the total MTX was released over 20 days from PLLA2k microspheres, while 27% and 24% of total MTX was released from PLLA50k and PLLA100k microspheres, respectively, over 40 days.

MTX loaded microspheres prepared using PLLA2k were selected as the lead formulation for future characterization work, based on the faster and more complete release of MTX from the PLLA 2k microspheres over a period of 3-4 weeks, compared to the PLLA50k and PLLA100k microspheres.
2.3.4.2 The effect of MTX loading on MTX release

The effects of drug loading on MTX release was further studied in PLLA2k microspheres. Figure 2.4 shows the in vitro release profiles for MTX from microspheres prepared from PLLA2k loaded with 4 different amounts of MTX. PLLA2k microspheres loaded with 2.5, 5, 10 and 20% (w/w) MTX all showed a burst phase of release followed by a much slower phase of release over 14 days. The detailed release profile within 24 h is also shown (Figure 2.4B). The amount of drug released was dependent on the amount of MTX loaded in the microspheres. Approximately 60% of the loaded amount of MTX was released in the burst phase followed by a phase giving approximately zero order release at rates of 1.34, 2.36, 2, and 4.5 μg/day for 2.5, 5, 10 and 20% (w/w) loaded microspheres, respectively, over the remaining 20 days.

2.3.5.3 The effect of gamma irradiation on drug release

Cumulative MTX release profiles for non-irradiated and γ-irradiated PLLA2k microspheres loaded with MTX are shown in Figure 2.5. It showed that irradiated microspheres have release profiles almost identical to non-irradiated microspheres.
Figure 2.3 Cumulative *in vitro* release profiles of MTX in 0.1M PBS at 37°C from microspheres loaded with 10% (w/w) MTX manufactured from PLLA polymers with molecular weights of 2k, 50k and 100k g/mole. Microspheres were in the size range of 33-110 μm. Values are mean ± one standard deviation, (n=4). (×) PLLA2k, (▲) PLLA50k, (■) PLLA100k.
Figure 2.4 Cumulative *in vitro* release profiles of MTX in 0.1 M PBS at 37°C from A) microspheres loaded with various amounts of MTX manufactured from PLLA 2k g/mole. Microspheres were in the size range of 33-110 μm. The detailed release profile within 24 h is shown in B). Values are mean ± one standard deviation, (n=4). MTX loadings were (×) 20% (w/w), (▲) 10% (w/w), (■) 5% (w/w), (♦) 2.5% (w/w).
Figure 2.5  Cumulative *in vitro* release profiles of MTX in 0.1 M PBS at 37°C from γ-irradiated (25 kGy from Co-60 source) and non-irradiated PLLA2k microspheres loaded with 10% MTX (w/w). Microspheres were in the size range of 33-110 μm. Values are mean ± one standard deviation, (n=4). (■) non-irradiated, (♦) irradiated microspheres samples.
2.3.5 Characterization of microspheres

2.3.4.2 Surface morphology

The scanning electron microscopic surface morphologies of both control (no drug) and 10% MTX loaded PLLA2k microspheres are shown in Figure 2.6. Both control (A) and 10% loaded microspheres (B) appeared to be smooth and spherical. There were no MTX crystals precipitated on the surface of MTX loaded microspheres.

2.3.4.3 X-ray powder diffraction patterns of MTX and PLLA microspheres

Figure 2.7 shows the X-ray powder diffraction patterns of crystalline MTX (A) and PLLA2k microspheres (B and C). Both control (B) and 10% MTX loaded PLLA2k microspheres (C) had similar X-ray powder diffraction patterns, with the most intense peak located between 16.4 and 16.8°2θ and two less intense diffraction peaks at 15 and 19°2θ. No peaks could be attributed to crystalline MTX in the microsphere matrix from the X-ray powder diffraction pattern of 10% MTX loaded microspheres.

2.3.4.4 Thermal properties

Figure 2.8 (A and B) shows DSC thermograms of control and 10% (w/w) MTX loaded PLLA2k microspheres and Table 2.6 provides a summary of the thermal data. The solidification of PLLA2k in the microspheres resulted in a semicrystalline polymer matrix with a degree of crystallinity of approximately 5% (Table 2.7). The amorphous component of the PLLA matrix showed a Tg of 52°C, which was taken as the peak
temperature of enthalpy relaxation (Figure 2.8A). Above the Tg, an exotherm was observed at 95°C and was taken as the crystallization temperature (Tc). Further heating of the sample resulted in melting of the crystalline regions of the polymer matrices, with some evidence of double melting endothermic peaks (Figure 2.8A). The peak of the second melting peak was taken as the melting point, which was about 145°C for both control and 10% MTX loaded PLLA 2k microspheres. The Tg and Tm of the PLLA2k microspheres were not significantly affected by the addition of 10% MTX (Figure 2.8B).
Figure 2.6 The surface morphology of A) control and B) 10% (w/w) MTX loaded PLLA2k microspheres.
Figure 2.7 X-ray powder diffraction patterns of A) crystalline MTX (from Handetec), B) control PLLA2k microspheres in the size range of 33-110 μm, C) 10% MTX loaded PLLA2k microspheres in the size range of 33-110 μm.
Figure 2.8  DSC thermograms of control and 10% MTX loaded PLLA (2 kg/mole) microspheres following degradation in 0.1M PBS at 37°C. A) Control microspheres, B) 10% (w/w) MTX loaded microspheres before degradation, C) 10% MTX loaded microspheres 1 day, D) 10% MTX loaded microspheres 28 days following degradation. Samples (3-5mg) were heated from -20°C to 200°C at 10°C /min. (Endotherms down)
Table 2.7  Thermal properties of control and 10% MTX loaded PLLA2k microspheres following *in vitro* degradation in 0.1m PBS (pH 7.4) at 37°C. Microspheres are in the size range of 33-110 μm.

<table>
<thead>
<tr>
<th>Degradation time (days)</th>
<th>Control microspheres</th>
<th>10% MTX loaded microspheres</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tg</td>
<td>Tc</td>
</tr>
<tr>
<td></td>
<td>(°C)</td>
<td>(°C)</td>
</tr>
<tr>
<td>0</td>
<td>52</td>
<td>97</td>
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<tr>
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<td>54</td>
<td>100*</td>
</tr>
<tr>
<td>3</td>
<td>54±1</td>
<td>101*</td>
</tr>
<tr>
<td>7</td>
<td>55±1</td>
<td>101*</td>
</tr>
<tr>
<td>14</td>
<td>53±1</td>
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<tr>
<td>21</td>
<td>51</td>
<td>100*</td>
</tr>
<tr>
<td>28</td>
<td>57±1</td>
<td>100*</td>
</tr>
</tbody>
</table>

Tg: glass transition temperature, Tc: crystallization temperature, Tm: melting temperature, Xc: degree of crystallinity

*The standard deviation of the mean temperatures is not shown if less than 1°C.

bThe standard deviation of the mean degree of crystallinity is not shown if less than 1%.

c Significantly different by one-way ANOVA test (F<0.0001) among Tc values for microspheres after days of degradation.

* Significantly different from day 0 by Tukey-Kramer test.

Values are mean values ± standard deviation, (n=3).
2.3.6 Degradation of microspheres

An *in vitro* degradation study in PBS at 37°C was conducted for PLLA2k microspheres using GPC determination of molecular weight changes over time. A calibration curve of log molecular weight versus retention time using poly(ethylene glycol) standards is shown in Figure 2.9. Figure 2.10 shows the number average molecular weight and weight average molecular weight changes for the PLLA2k microspheres incubated in PBS at 37°C. There was no difference between the molecular weights of drug loaded and control microspheres over the degradation time period.

The results showed that there was an apparent increase in the molecular weights of the polymer for both number average molecular weight and weight average molecular weight after one day of degradation. Despite the initial increase in molecular weight, by 30 days of degradation, PLLA 2k microspheres had lost approximately 20% of the original molecular weight (Figure 2.10).

The mass loss profile (Figure 2.11) shows that approximately 13% of total mass of 10% drug loaded microspheres was lost after one day of degradation while 9% of total mass of control microspheres was lost after one day of degradation. The mass of the microspheres decreased in a linear fashion with the time of degradation. After 35 days of incubation, approximately 22% of the total mass was lost due to degradation.
Figure 2.9  Molecular weight calibration curve using poly(ethylene glycol) standards. Chromatographic conditions: Styrogel® HR3 and HR0.5 columns in series, mobile phase was THF at 1 mL/min, and the detector was a differential refractive index detector.
Figure 2.10  A) Number average molecular weight and B) weight average molecular weight profiles of control and 10% (w/w) MTX loaded PLLA2k microspheres in 0.1M PBS at 37°C. Microspheres were in the size range of 33-110 μm. Values are mean ± one standard deviation, (n=3). (■) 10% MTX loaded, (♦) control microspheres.
**Figure 2.11** Cumulative weight loss profile of control and 10% MTX loaded PLLA2k microspheres incubated in 0.1M PBS at 37°C. Microspheres were in the size range of 33-110 μm. Values are mean ± one standard deviation, (n=3). (■) 10% MTX loaded, (♦) control microspheres.
Figure 2.12 Scanning electron micrographs of control and 10\% (w/w) MTX loaded PLLA2k microspheres following degradation in 0.1 M PBS at 37°C. Microspheres were in the size range of 33-110 \mu m. A) Day 3, control, B) day 3, 10\% MTX, C) day 7, control, D) day 7, 10\% MTX.
2.3.6.1 Thermal analysis of degraded microspheres

Representative DSC thermograms of degraded microspheres (day 3 and day 25) are shown in Figure 2.8 (C and D). The thermograms showed the disappearance of the enthalpy relaxation endotherm and an elevation of Tc by 5°C (Figure 2.8 C and D). The Tg values of the degraded microspheres were taken as the mid-point in the change in heat flow. The changes in Tg, Tc, and Tm of the microspheres following degradation are summarized in Table 2.7. Both control and 10% MTX loaded microspheres showed a significant elevation in the values of Tc following degradation while the Tg and Tm were not affected by degradation. The crystallinity was observed to increase from 5% to 11% after one day of degradation although the change was not statistically significant.

2.3.6.2 Surface morphology of degraded microspheres

The surface morphology of degraded microspheres is shown in Figure 2.12. On day 3, both control and drug loaded microspheres exhibited some evidence of degradation (Figure 2.12, A and B) with the appearance of some pores and holes in the surface. On day 7, the MTX loaded microspheres showed greater degradation and loss of integrity (Fig 2.12, D) compared to control microspheres (Fig 2.12, C).
2.3.7 Biocompatibility of MTX loaded and control microspheres

Healthy rabbits were injected intra-articularly with 25 mg of either control or 10% MTX loaded PLLA2k microspheres. After the injection, animals were observed daily and scored for swelling of joints during the first week. The results are given in Table 2.8. In the first 3 days, little to moderate swelling was observed in both groups. The swelling reaction decreased three days after the injection. There was no swelling observed in the remaining 4 days.

Fourteen days after the injection, the animals were sacrificed and the joints were taken for histological study. Both safranin-O stain and toluidine blue stain showed that the animals from both groups had a small degree to no loss of proteoglycans from the cartilage (Table 2.8). The results indicated that the 10% MTX loaded and control PLLA2k microspheres in the size range of 33-110 μm were well tolerated in the joints of the rabbits.
Table 2.8  Joint swelling and histological analysis of proteoglycan loss in cartilage of rabbit joints injected intra-articularly with 25 mg control or MTX loaded PLLA2k microspheres in the size range of 33-110 μm. Joint swelling and proteoglycan loss were scored on a 0 to 4 point scoring system.

<table>
<thead>
<tr>
<th>Treatment /Animal Number</th>
<th>Scores for swelling of joint</th>
<th>Loss of proteoglycan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day1</td>
<td>Day2</td>
</tr>
<tr>
<td>Control Microspheres</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>MTX Microspheres</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
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</tr>
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<td>5</td>
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<td>1</td>
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<tr>
<td>6</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

“Control microspheres” animals were treated with 25 mg PLLA2k microspheres and “MTX microspheres” animals were treated with 25 mg 10% MTX loaded PLLA2k microspheres.
2.4 DISCUSSION

The solvent evaporation method was used to encapsulate MTX in PLLA microspheres. The data showed that MTX may be encapsulated at relatively high efficiency (between about 64-90%) in microspheres manufactured from PLLA of various molecular weights (Table 2.5). The lower MTX encapsulation efficiency achieved for the low molecular weight polymer (2k g/mole) compared to the high molecular weight polymer (50k and 100k g/mole) was likely due to the difference in the precipitation rates of the different polymers during the microsphere formation process. To determine relative rate of polymer precipitation and hardening during microspheres formulation, samples of polymer droplets were taken every 5 min and observed using optical microscopy. It was noted that high molecular weight PLLA precipitated and hardened as microspheres more rapidly than PLLA2k. Within 5 to 10 min, PLLA50k, and 100k polymers precipitated and hardened as microspheres, while PLLA2k still appeared as emulsion droplets in the water phase up to 30 min. Given that the solubility of MTX in 2.5% PVA solution was found to be approximately 0.5mg/mL (Section 2.2), the slower precipitation rate of PLLA2k and longer hardening time would provide a longer time for MTX suspended in the polymer solution droplets to partition into the aqueous phase resulting in a lower encapsulation efficiency.
Particle size data shown in Table 2.5 showed that particle size distributions were reproducible between batches made with a given stirring rate and PVA concentration. The increase in mean particle sizes of the microspheres observed with increasing polymer molecular weight (Table 2.5 and Figure 2.2) may be explained by the difference in the viscosities of solutions of polymers with different molecular weights in methylene chloride. It has been reported that the intrinsic viscosities of 100k g/mole PLLA, 50k g/mole PLLA and 2k g/mole PLLA are 1.067 ± 0.016, 0.855 ± 0.003, and 0.153 ± 0.011 dL/g, respectively (Liggins, 1998). For a given rate of stirring, higher viscosities of polymer in the organic phase generally result in an increased resistance of the organic phase droplets to shear stress and break up, resulting in larger microspheres (Jail and Nixon, 1990; Freiberg and Zhu, 2004). Particle size of microspheres is known to be a factor influencing the drug release rates and solid state properties of microspheres (Jali and Nixon, 1989; Jalil and Nixon, 1990) and, therefore, only microspheres in the size range (33-110 μm) intended for intra-articular injection were selected for release study and further characterization.

The in vitro MTX release studies of MTX loaded microspheres composed of PLLA with three different molecular weights were conducted in order to select a potential lead formulation for further characterization work. In vitro MTX release profiles from MTX...
loaded microspheres manufactured from PLLA2k, PLLA50k, and PLLA100k showed that the rate and extent of release were dependent on polymer molecular weight (Figure 2.3). The release rate and the extent of release was highest for PLLA2k microspheres followed by PLLA50k and PLLA100k microspheres. There was an initial burst phase of MTX release followed by a slower and controlled release of the drug for all microspheres. The high molecular weight polymer microspheres released MTX very slowly after the burst phase and only 20% of the loaded drug had been released in 40 days. There was no evidence of matrix erosion on day 7 following drug release from PLLA50k and PLLA100k microspheres indicating that the major factor contributing to MTX release from high molecular weight PLLA microspheres was diffusion rather than erosion (data not shown). It has been reported that PLLA with molecular weights of 103k g/mole and 153k g/mole showed negligible weight loss during the first 270 days of incubation in Ringer’s solution at 37°C (Migliaresi et al., 1994). The high molecular weight PLLA microsphere formulations were not felt to be optimal formulation for future in vivo work because the very low release rates of MTX could result in sub-therapeutic tissue concentrations of drug particularly in the acute antigen-induced arthritis model. The long degradation lifetime of high molecular weight PLLA microspheres could be of potential concern for long-term tolerability and biocompatibility in the joint. Hence,
MTX loaded PLLA2k microspheres were selected as the lead formulation for subsequent studies.

The thermal analysis of PLLA2k microspheres showed that the solidification of PLLA (2k g/mole) resulted in a semicrystalline polymer matrix with a degree of crystallinity around 5% for both 10% MTX loaded and control microspheres (Table 2.7). Glass transitions (Tg) observed in control or 10% MTX loaded microspheres exhibited concurrent enthalpy relaxation (Figure 2.8 A and B). Enthalpy relaxation may occur upon heating a polymer through its Tg. Below Tg the polymer chains may relax by undergoing short range motion and becoming ordered over the range of a few monomer units of polymer chains. The region of order is not large enough to be considered crystalline and has been termed “microstructure” in the amorphous phase (Bodmeier et al., 1989). Upon heating through Tg, an enthalpy of relaxation is observed because energy is required to overcome the short-range order established through relaxation (Bodmeier et al., 1989; Liggins and Burt, 2001). The peak attributed to enthalpy relaxation at the Tg has been reported for other PLLA polymers (Migliaresi et al., 1994; Gonzalez et al., 1999; Liggins and Burt, 2001). Above Tg, a crystallisation exotherm was observed as the polymer chains gained more mobility and were able to align into a more stable configuration by releasing energy. The peak of the
crystallization exotherm was taken to be the Tc. Further heating resulted in melting of the crystalline regions of the polymer matrices with an evidence of double melting endotherms (Figure 2.8 A and B). Based on the melting and recrystallization model of multiple melting of polymers, as the initial crystalline lamellae in the polymer matrix melt and give rise to the low temperature endotherm, the molten material can undergo a recrystallization process during the DSC scan and form thicker lamellae (Wang et al., 1999). The recrystallized lamellae melt at a higher temperature and result in the endotherm at a higher temperature (Wang et al., 1999). The double melting phenomenon has been observed in some PLLA polymers before and following degradation (Gonzalez et al., 1999; Li, 1999; Martin et al., 1999).

The Tg and Tm of 10% loaded PLLA 2k microspheres were not significantly affected by the addition of MTX (Table 2.7) likely due to the fact that drug had negligible solubility in the PLLA matrix. In general, when a drug is soluble or miscible in a polymer matrix, the polymer chains are either stiffened by interactions with the drug molecules resulting in a elevation of Tg or they are plasticized by interruption of polymer-polymer interactions by the presence of drug molecules resulting in a depression of Tg (Mumper et al., 1992; Liggins 1998). Thus, MTX was likely dispersed as solid particles in the polymer matrix. MTX has a low solubility in methylene chloride of
approximately 30 µg/ml (preliminary study). During microspheres preparation, the organic phase was a suspension of MTX in a PLLA/methylene chloride solution. This observation and DSC evidence suggests that the dispersion of MTX in the PLLA microspheres matrix was likely a particulate dispersion.

The X-ray powder diffraction pattern of MTX, as received, indicates that the drug was crystalline in nature (Figure 2.7). However, no peaks could be attributed to crystalline MTX in the microsphere matrix from the X-ray powder diffraction pattern of 10% MTX loaded microspheres (Figure 2.7) likely due to the low sensitivity of the X-ray powder diffraction technique and inability to detect crystalline MTX at low loadings. The solid state characterization of crystalline MTX conducted by Chan and Gonda (1991) has shown that crystalline MTX was hydrated with 4% of water. The dehydration of MTX took place over a temperature range of 40°C to 120°C and the melting of MTX occurred in a temperature range of 175°C to 200°C accompanied by decomposition of MTX (Chan and Gonda, 1991). We confirmed these observation by DSC analysis of MTX, as received (data not shown). Thermal analysis of 10% MTX loaded PLLA2k microspheres did not detect any peak for MTX melting. We speculate that particulate MTX in the polymeric matrix could have gradually dissolved in the polymeric matrix.
during DSC analysis at temperatures above Tm (145°C) of the PLLA resulting in disappearance of the melting peak of MTX.

The effects of MTX loading on drug release were investigated in PLLA2k microspheres. The *in vitro* drug release profiles of PLLA2k microspheres loaded with 2.5%, 5%, 10% and 20% MTX all demonstrated a burst phase of release followed by a much slower phase of release over 14 days (Figure 2.4). MTX loading levels affected the rate and extent of MTX release. The rate of MTX release was increased with higher loading levels of MTX. According to the kinetics described for diffusion controlled release from a sphere by Baker (1987), the amount of drug released at any given time increases directly proportionally to the total drug loading. The initial burst phase of MTX release was likely due to dissolution of MTX near the surface of the microspheres. Since MTX in PLLA microspheres matrix was likely dispersed as solid particles, as the release medium penetrated the microsphere matrix, it would dissolve particulate MTX, leaving cavities and pores that were filled with release medium and served as a preferred pathway for drug diffusion, contributing to a rapid burst release phase. The initial stages of formation of cavities and pores were demonstrated by the scanning electron micrographs of MTX loaded microspheres following 3 days of drug release (Figure 2.12B). The slower release phase following the burst phase was likely controlled by a
combination of drug diffusion and polymer degradation. The scanning electron micrographs of MTX loaded and control PLLA2k microspheres by day seven of incubation in PBS showed substantial evidence of polymer erosion (Figure 2.12 D).

The effect of gamma irradiation on drug release from MTX loaded PLLA2k microspheres was investigated. The results showed that the release profiles of MTX loaded microspheres for 14 days were not altered upon exposure to gamma irradiation at a dose of 25 Gy (Figure 2.5). Gamma irradiation has generally been employed for sterilization of biodegradable polymer devices although the dramatic decrease in polymer molecular weight on treatment is a well-known concern (Scholes et al., 1997). The effects of gamma irradiation on the properties of polymeric microspheres have been widely investigated (Montanari et al., 1998; Blanco et al., 1999; Montanari et al., 2001; Wang et al., 2003; Faisant et al., 2003). In general, gamma irradiation has a dose dependent effect on the molecular weight and thermal properties of the polymer (Calis et al., 2002; Martinez-Sancho et al., 2004). It has been found that the drug release rate from microspheres increased as the dosage of gamma irradiation increased, but seemed to be less sensitive to the effect of gamma irradiation compared to polymer molecular weight (Calis et al., 2002; Faisant et al., 2003). Other studies have reported that the drug release profiles were not significantly influenced by gamma irradiation even though
the molecular weight of the polymer was decreased by chain scission of the polymer chains by gamma irradiation (Martinez-Sancho et al., 2004).

The molecular weights of control and 10% MTX loaded microspheres following degradation were monitored by GPC. The number average molecular weight of PLLA2k polymer before degradation was determined to be 3500 g/mole (Fig 2.10A), which is higher than that reported by the manufacturer. This difference is likely due to the different methods used to measure the molecular weight of the polymer. The manufacturer used an end group titration method, while GPC was used to evaluate the molecular weight in this work. Since no PLLA standards were available, poly(ethylene glycol) (PEG) standards were used for GPC calibration. GPC separates according to the hydrodynamic volumes of the polymer coils in solution and not according to the absolute molecular weights, and hence the GPC values should only be seen as providing relative molecular weights for comparative purposes (Hakkarainen et al., 1996).

One day following the degradation, there was an increase in the molecular weight of the polymer (Fig 2.10A and B), which was likely due to the rapid leaching of a very low molecular weight fraction of the polymer matrix, resulting in an increase in the average molecular weight of the polymer. The mass loss study also showed that approximately 9% and 14% of total mass was lost following one day of degradation for control and 10%
MTX loaded microspheres, respectively (Figure 2.11). Liggins and Burt (2001) reported that approximately one-third of 1k g/mole PLLA was water soluble and GPC analysis of the water soluble fraction of low molecular weight PDLLA showed that the highest molecular weight that dissolved in water was approximately 650 g/mol (Gradfils et al., 1996). It has also been shown that the average molecular weight of poly (L-lactic acid) films increased from 22k g/mole to 30k g/mole during the first week of degradation in a mineral medium at 37°C due to the extraction of low molecular weight products that became soluble in the mineral medium (Hakkarainen et al., 2000). Starting on day 3, the molecular weight of PLLA2k microspheres decreased continuously (Figure 2.10 A and B) and approximately 20% of the total weight of microspheres was lost following 40 days of degradation (Figure 2.11) suggesting that the degradation of microspheres was accompanied by erosion.

Thermal analysis of degraded PLLA2k microspheres showed that Tc was elevated and crystallinity of the microspheres was increased one day following degradation (Table 2.7). The degradation process starts with a random chain scission process as the ester bonds are hydrolyzed by the water molecules. When the molecular weight drops sufficiently by hydrolysis, the degradation products become soluble or separate from the matrix, and loss of material will occur (Jalil and Nixon 1990). Hydrolysis usually takes
place in the amorphous regions of the polymer matrix due a higher permeability of the aqueous medium to the matrix and the susceptible functional groups in the polymer backbone. Therefore, degree of crystallinity of polymers generally is increased during the initial hydrolysis of the polymer due to the preferential hydrolysis of the amorphous regions (Hakkarainen et al., 2000). It has been shown that following 110 weeks of degradation in phosphate buffer (pH 7.4) at 37°C, the crystallinity of high molecular weight PLLA (130k g/mole) increased by 50% and the Tc of the polymer was elevated by 2°C (Li, 1999; Vert et al., 1994). Thermal analysis of degraded microspheres did not show significant changes in Tg and Tm values of the microspheres even though the molecular weight was decreased 25% after 28 days of degradation (Table 2.7 and Figure 2.11). Tm and Tg values have been reported to be less sensitive to chain cleavage and molecular weight reduction than the degree of crystallinity (Migliaresi et al., 1994).

Biocompatibility or tolerability studies in healthy rabbit joints were carried out using control PLLA2k and 10% MTX loaded PLLA2k microspheres in the size range of 33-110 μm. This size range was chosen based on previous studies which showed that PLLA microspheres in this size range were well tolerated in rabbit joints, while microspheres in the smaller size range frequently induced a greater inflammatory response (Ratcliffe et al., 1984; Liggins et al., 2004). Intra-articular injections of 25 mg
of microspheres (control or MTX loaded) produced a mild inflammatory response over
the first three days and subsided thereafter without any significant loss of proteoglycans
from cartilage 14 days following injection (Table 2.8). These findings were consistent
with our previous work (Liggins et al., 2004) which showed that intra-articular
microspheres in the size range of 33-105 μm only induced mild knee joint swelling and
cellular infiltration seven days following administration of a 40 mg dose. Nishide and
coworkers (1999) investigated the biodegradation and tissue response of D,L-lactic acid
oligomer microspheres of sizes less than 20 μm, 20-100 μm and 100-200 μm injected
into the knee joints of rabbits. Cell infiltration into the joint increased 4 fold for all
microsphere size ranges compared to saline control animals, up to 9 h following
intra-articular injection and then decreased to basal levels at 3 days. Decreasing the
amount of microspheres injected into the joint from 20 mg to 5 mg per knee decreased the
inflammatory response significantly (Nishide et al., 1999). The injected microspheres
were found to be located primarily in the popliteal region of the knee joint. Horisawa et
al (2002a) showed that both PLGA microspheres (26 μm) and PLGA nanospheres (265
nm) were compatible and well tolerated in the joints of rats following intra-articular
injections. The microspheres were not phagocytosed and were observed to cause the
formation of granulation tissues surrounded by multinuclear giant-cells in the synovial
membrane. The nanospheres, on the other hand, were extensively phagocytosed and infiltrated through the synovial tissues inducing the proliferation of the synovium.

In summary, 10% MTX loaded PLLA2k microspheres in the size range of 33-110 μm were developed and characterized as the lead formulation for further \textit{in vivo} evaluation. The lifetime of MTX release and degradation/erosion of the microspheres was approximately 3-4 weeks.
Chapter 3

PHARMACOKINETICS OF METHOTREXATE FOLLOWING INTRA-ARTICULAR INJECTION OF METHOTREXATE LOADED MICROSPHERES

3.1 INTRODUCTION

MTX for the treatment of rheumatoid arthritis was introduced in the 1980's, and numerous studies have been conducted to evaluate the pharmacokinetics and pharmacodynamics of oral low dose MTX in patients with arthritis (Bannwarth et al., 1996; Grim et al., 2003). In human subjects, MTX is absorbed through the proximal jejunum, and about 5-7% of the administered MTX is metabolized by hepatic methotrexate 7-hydroxylase to 7-hydroxymethotrexate (7-OH-MTX) (Grim et al, 2003). Renal excretion accounts for between 60-90% of the MTX and 7-OH-MTX eliminated (Bannwarth et al., 1996). The antifolate activity of 7-OH-MTX is significantly less than MTX, but it is slowly excreted and may crystallize in the kidneys resulting in nephrotoxicity (Chen and Chiou, 1983). The hepatic methotrexate 7-hydroxylase activity in animals and humans has been shown to be quite variable, with highest activity

\[ \text{\textsuperscript{2} A version of this chapter has been published. Liang LS et al., (2005) Pharmacokinetic study of methotrexate following intra-articular injection of methotrexate loaded poly(L-lactic acid) microspheres in rabbits. J Pharm Sci. 94(6):1204-15.} \]
in rabbits, followed by rats, hamsters, and monkeys and undetectable in dogs (Chen and Chiou, 1983; Iven et al., 1985; Kitamura et al., 1999).

For the evaluation of the pharmacokinetics of various drug delivery systems of MTX, rabbits are often chosen as the animal model for the reason that they have similar metabolic pathways to MTX to human subjects (Chen and Chiou 1982; Foong and Green, 1988; Wang et al., 1995). The pharmacokinetics of MTX following intravenous dosing ranging from 1 to 12 mg/kg in rabbits could be fitted to a linear three-compartment model with a terminal half-life between 2.4 to 3.6 h. For 8 h post-dosing, 50% of the dose of MTX was excreted into urine in the unchanged form and 15% as the metabolite 7-OH-MTX and these fractions were not influenced by changes in dose (Iven et al., 1985). The renal clearance of 7-OH-MTX was similar to MTX (Chen and Chiou, 1983). Renal clearance decreased with the increasing plasma levels, suggesting active tubular secretion as one of the excretion mechanisms (Iven et al., 1985; Chen and Chiou, 1983). Infusion studies of MTX and 7-OH-MTX in rabbits revealed that the metabolite has a longer residence time and a larger volume of distribution compared to MTX (Chen and Chiou, 1983).

The pharmacokinetics of MTX following intra-articular injection of MTX solution and MTX encapsulated liposomal formulations have been investigated in rabbits (Foong
and Green, 1988). The data showed that MTX solution was rapidly cleared from the joint cavity and was detectable in plasma within 1 h of injection and 79% of the injected MTX dose was excreted in the urine within 24 h of injection. The MTX encapsulated liposomes, on the other hand, slowed down the clearance of MTX from joint cavity and 45% of the injected dose was recovered from the joint 24 h following injection (Foong and Green, 1988). MTX has not been previously formulated in polymeric microspheres for intra-articular injection and very few studies have been carried out to study the pharmacokinetics of a controlled release microspheres drug delivery system for intra-articular drug delivery. A knowledge of the pharmacokinetics and biodistribution of MTX following intra-articular injection of MTX loaded microspheres is considered fundamental to future in vivo efficacy studies.

In this chapter, we report the results of two pharmacokinetic studies of MTX following intra-articular injection of MTX loaded PLLA2k microspheres (33-110 μm) into the knee joints of healthy rabbits. The size range of 33-110 μm was selected based on previous data in Chapter 1 showing good tolerability and biocompatibility in rabbit joints. This size range of microspheres would likely remain trapped within the synovial fluid since they are too large to be phagocytosed (Greis et al., 1994). Thus, MTX released from microspheres should be available for delivery to synovial tissues. MTX
release studies from MTX loaded PLLA2k microspheres showed that release of MTX was 95% complete in 2 weeks. Thus MTX loaded PLLA2k microspheres were felt to be an appropriate formulation for pharmacokinetic evaluation. The first study was a pilot study in which MTX solution or MTX loaded microspheres (dose of 1.5mg MTX) was injected into the knee joints of the rabbits. The concentrations of MTX in plasma, urine and synovial tissues were determined. The study was repeated with a higher dose of MTX (10mg) and histological analysis was also conducted in some of the rabbits. The objectives of these studies were:

1. To determine the plasma and urine concentrations of MTX and 7-OH-MTX following intra-articular injection of MTX loaded microspheres or MTX solution;

2. To investigate the biodistribution of MTX following intra-articular injection of MTX loaded microspheres or MTX solution;

3. To determine the tissue localization of MTX microspheres following intra-articular injection of MTX loaded microspheres.
3.2 EXPERIMENTAL

3.2.1 Materials

Methotrexate (MTX) (MW: 454.4g/mole) was purchased from Hande Tech Development Co. (U.S.A). Aminopterin was purchased from Sigma Chemical Co. Poly (vinyl alcohol) (PVA) (98% hydrated, MW: 13,000-23,000) was obtained from Aldrich Chemical Company Inc. 7-hydroxy-methotrexate (7-OH-MTX) was purchased from Schircks Laboratories (Jona, Switzerland). Poly(L-lactic acid) (MW: 1600-2400 g/mol, intrinsic viscosity: 0.1-0.2, polydispersity: 2-3) was obtained from Polysciences (Warrington, PA). All solvents used were High Performance Liquid Chromatography (HPLC) grade. Phosphate buffered saline (PBS, pH 7.4) was prepared by dissolving 0.32 g sodium dihydrogen orthophosphate, 2.15 g sodium phosphate, 8.22 g NaCl in one liter of distilled water. Phosphate buffer (pH 6.5) was prepared by dissolving 0.936 g sodium dihydrogen orthophosphate, 0.644 g sodium phosphate in one liter of distilled water and filtered through 0.2 mm filter paper.

3.2.2 Preparation of MTX loaded microspheres

The microspheres were prepared by the solvent evaporation method as described in Chapter 2. Briefly, MTX was suspended in 20% PLLA2k polymer in methylene chloride. The drug suspension was then slowly dispersed into 100 mL of a 2.5% PVA
solution and stirred at 1000 rpm using an overhead stirrer (BDC2002 Caframo, Ont, Canada). The resulting emulsion was continuously stirred for 2.5 hours at room temperature under ambient pressure until all the methylene chloride had evaporated. The solidified microspheres were recovered by centrifugation. The microspheres were sieved through 33 and 110 μm sieves and washed with distilled water. The washed microspheres were then air-dried overnight and stored in a desiccator at room temperature for further drying. Either control or MTX loaded microspheres batches intended for injection into rabbit joints were sterilized by gamma irradiation from a Co-60 source at a dose of 25kGy (Nordion International Inc.).

3.2.3 MTX and 7-OH-MTX assays

3.2.3.1 MTX and 7-OH-MTX extraction

MTX and the metabolite 7-OH-MTX in the plasma and urine samples were extracted according to the method reported by Cociglio et al (1995) with modifications. One mL acetonitrile was added to the plasma or urine sample (0.5 mL), vortex mixed and centrifuged at 16000 x g for 5 min. The supernatant was transferred to a 12 mL glass tube and 2 mL of methylene chloride were added to extract the aqueous phase. The aqueous phase (200 μL) was dried under a stream of nitrogen gas at 45°C. The dried sample was reconstituted with 200 μL phosphate buffer pH 6.5.
Five gram samples of organs and the whole joint tissues were homogenized in 5 mL PBS (pH 7.4) (Polytron, Brinkman Instruments, Mississauga, ON. Canada) and the homogenates were centrifuged at 16000 x g for 30 min. The supernatants were removed and processed in the same manner as the plasma and urine samples to extract MTX and 7-OH-MTX.

To determine whether MTX plasma samples were stable during storage at -20°C, blank plasma and tissues samples (n=3) were spiked with 0.1 µg and 1 µg/mL MTX and stored at -20°C for 18 days. The samples were then processed and analysed for MTX as previously described.

3.2.3.2 HPLC assay for plasma and tissue samples

MTX and 7-OH-MTX in plasma and tissue samples were assayed by HPLC with fluorescence detection using the method developed by Albertioni et al (1995) with modifications. The HPLC system consisted of a Shimadzu LC-10AD pump (Shimadzu Corporation, Japan) and a Shimadzu SIL-9A automatic injector. A Waters 470 scanning fluorescence detector was used (Ex: 370nm, Em: 417 nm). A photochemical reactor unit with a 254 nm UV lamp and a 10 m x 0.25mm reaction coil (Aura Industries Inc, NY, USA) was used post-column (before the detector). The analytical column was a Nov-Pak C18 column with dimensions of 3.9 x 150 mm. The mobile phase consisted of
10 mM phosphate buffer pH 6.5, with 3.2% acetonitrile and 6% hydrogen peroxide (Sigma Chemical Co.) at a flow rate of 1mL/min under ambient temperature. The injection volume was 50 μL. A folic acid antagonist, aminopterin (Figure 3.1), at a concentration of 0.2 μg/mL was used as the internal standard. Blank plasma or tissue supernatants were spiked with known concentrations of MTX and 7-OH-MTX and the internal standard. Calibration curves were constructed by plotting the ratio of peak areas of MTX/internal standard and 7-OH-MTX/internal standard versus concentrations of MTX and 7-OH-MTX, respectively.

3.2.3.3 HPLC assay for urine samples

MTX and 7-OH-MTX in the urine samples were assayed by HPLC with a UV detector. The HPLC system consisted of a Waters 600S controller pump, a Waters 717 plus autosampler, and a Waters 486 absorbance detector (304 nm). The analytical column was a Novo-Pak C18 column with dimensions of 3.9 x 150mm. The mobile phase consisted of phosphate buffer pH 6.5, with 3.7% acetonitrile. The injection volume was 50 μL. Standard curves were constructed by plotting the peak area of MTX or 7-OH-MTX versus concentrations of MTX and 7-OH-MTX. No internal standard was used in this assay.
3.2.3.4 Recovery of MTX and 7-OH-MTX from tissues

Two New Zealand White rabbits were sacrificed and their livers, lungs, spleens, kidneys and synovial tissues were removed and stored at -20°C until analysis. One gram samples of each tissue were spiked with 0.2 μg, 0.4 μg, and 4 μg MTX. Four mL of PBS (pH 7.4) were added to the tissues and the tissues were processed the same way as described in section 3.2.3.1. The MTX recovery was measured by comparing the peak areas of MTX obtained from the tissue samples and the peak areas of MTX obtained from direct injections of 0.05, 0.1 and 1 μg/mL in phosphate buffer pH 6.5.

3.2.3.5 Validation of the assays

The assays for MTX in plasma and urine were validated by measuring four sets of standards on three separate days. The retention time and specificity of the MTX peak were characterized. The linearity, limit of detection, limit of quantitation, inter-day precision, intra-day precision, and accuracy of the calibration curve were measured. The specificity of the assay was determined by observing blank replicates for lack of peaks at the retention time of MTX. Linearity was expressed as the coefficient of determination (R²) for each of the twelve calibration curves assembled over three days and for the standard curve consisting of each of the twelve standards’ data points for each concentration. The limit of detection was calculated from Equation 2.1 and the limit of quantitation was calculated from Equation 2.2 (given in Chapter 2).
Inter-day precision was expressed in terms of the coefficient of variation (CV) of the mean of the twelve standards’ data points collected over three days for each concentration. Intra-day precision was expressed in terms of the CV of the mean of the four standards’ data points on each day for each concentration. Accuracy was determined by assembling a calibration curve and comparing three different standards at each concentration against the calibration curve each day for three days. Accuracy was expressed in terms of the percent deviation (bias) of each of the three standards and their mean bias compared to the daily calibration curve.

3.2.4 Microspheres recovery from joint fluids

Two New Zealand White rabbits were injected with 56mg of 18% loaded microspheres in the right hind knee joint while under inhalation anesthesia. The rabbits were immediately sacrificed. One mL of heparinized (10 units/mL) saline was injected into the right knee joint of each rabbit following sacrifice of the rabbits. The right knee joints of the rabbits were flexed and massaged and then the fluid in the joints was aspirated with a syringe with a 23 gauge needle. The washing and aspiration of the right knee joint was repeated with another 1mL of heparinized saline. The aspirated fluids were pooled and centrifuged at 325 x g. The supernatant was removed and analysed for MTX by UV-vis spectrophotometry. The sedimented microspheres were dissolved in
1mL of methylene chloride and MTX encapsulated in the microspheres was extracted with 1mL of PBS (pH 7.4) and analysed by UV-vis spectrophotometry.

3.2.5 Pharmacokinetic studies in rabbits

Both pharmacokinetics studies were conducted according to the protocol # A02-0066 approved by the UBC animal care committee. The experiments were conducted at the Animal Care Facility of the Vancouver Hospital and Health Sciences Center. The animals were housed in metal cages and monitored by Mr. Michael Boyd. The room was maintained at a temperature of approximately 20°C with 30-70% relative humidity and a light/dark cycle of 12 hours/12 hours. Rabbit chow and tap water were provided ad libitum to the animals for the duration of the study.

3.2.5.1 Low dose pharmacokinetic study

Fourteen female New Zealand White Rabbits (weight 2.5-3.0 kg) were surgically implanted with catheters (Access Technologies Inc.) into the jugular veins. The animals were randomized to receive an intra-articular injection into the right knee joint cavity of either 1.5 mg MTX solution in 200 µL PBS (MTX solution) or 25 mg of 6% MTX loaded PLLA2k microspheres (sized 33-110 µm) in 200 µL PBS. The MTX solution was prepared by dissolving 15mg MTX in 500 µL sodium hydroxide (0.4N) followed by dilution with PBS to 2mL. The pH remained at 7.0. Total dose of MTX in
the microspheres was equivalent to 1.5 mg. Serial blood samples (1.5 mL) were obtained from the jugular vein immediately before intra-articular injection and at 5, 15, and 30 minutes, 1, 2, 4, 6, 8, 10, 24, and 48 hours after the intra-articular injection. An equivalent volume of 0.9% sodium chloride injection was administered into the animal after each blood draw. The blood samples were centrifuged for 10 minutes at 16000 x g, and the plasma harvested and stored at -20°C before drug analysis. Urine samples of the rabbits were collected from the trays below the cage of the rabbits at 0-8 h, 8-24 h, and 24-48 h intervals. The animals were sacrificed 48 hours following the intra-articular injection and major organs (livers, kidneys, lungs, heart, and spleen) and the joint tissues were removed from the rabbits for further analysis.

3.2.5.2 High dose pharmacokinetic study

Sixteen female New Zealand White Rabbits (weight 2.5-3.0 kg) were used for the study. The animals were randomized to receive an intra-articular injection into the right knee joint cavity of either 10 mg MTX solution in 400 μL PBS or 56 mg of 18% MTX loaded PLLA2k microspheres (sized 33-110 μm) in 400 μL PBS. The MTX stock solution was prepared by dissolving 100 mg MTX in 500 μL sodium hydroxide (0.4 N) followed by dilution with PBS to 4 mL. The pH remained at pH 7.0. Total dose of MTX in the microspheres was equivalent to 10 mg. Immediately before (0 h) and following
the intra-articular injection, serial blood samples (1.5 mL) were obtained via the jugular vein at 5, 15, and 30 min, 1, 2, 3, 4, 5, 6, 8, 24 h after the intra-articular injection. An equivalent volume of normal saline was administered into the animal after each blood draw. The blood samples were centrifuged for 10 min at 16000 x g, and the plasma harvested and stored at -20°C for drug analysis. To ensure complete collection of urine samples, the animals were kept under light inhalation halothane (1.5%) anesthesia and the urine samples were collected from a catheter inserted into the urethra of the rabbits in the first 8 h following intra-articular injection. Between 8 to 24 h, the urine samples were collected from the trays below the cage of the rabbits. Eight rabbits (four injected with MTX solution, four injected with MTX microspheres) were sacrificed 6 h following intra-articular injection, and the rest of the rabbits were sacrificed 24 h following intra-articular injection. The synovial joint was flushed with 2 mL of heparinized saline and the fluid was aspirated for drug analysis. Major organs (livers, kidneys, lungs, heart and spleen) and the joint tissues were removed from the rabbits for further analysis.

3.2.6 Pharmacokinetic calculations

The plasma curves were resolved with the nonlinear regression computer program WinNONLIN (Scientific Consulting Inc., Standard Edition, and Version 1.1). The terminal rate constant, and mean residence time were calculated using the
non-compartmental analysis with extravascular dose input. The 1st order rate constant (k) associated with the terminal portion of the MTX plasma curve was estimated via linear regression of time versus log concentration. The elimination half-life was determined by:

\[ t_{1/2} = \frac{0.693}{k} \]  
Equation 3.1

The area under the concentration-time curve (AUC\(_{0,\infty}\)) and area under the moment curve was calculated by the trapezoidal rule for the observed values and then extrapolated to infinity as shown in Equations 3.2 and 3.3.

\[
AUC_{0,\infty} = AUC_{0,t} + AUC_{t,\infty} = \sum_{n=1}^{N} \frac{C_n + C_{n+1}}{2} (t_{n+1} - t_n) + \frac{C_t}{k}  
\]  
Equation 3.2

\[
AUMC_{0,\infty} = \sum_{n=1}^{N} \frac{C_n + C_{n+1}}{2} (t_{n+1} - t_n) \left(\frac{t_n + t_{n+1}}{2}\right) + \frac{C_t \cdot t_{last}}{k} + \frac{C_t}{k^2}  
\]  
Equation 3.3

where the summation is calculated over N trapezoids formed by n + 1 data points. C and t represent the concentration and the time after administration, respectively. C\(_t\) is the plasma concentration of MTX at the last data point.

The mean residence time (MRT) was calculated by

\[
MRT = \frac{AUMC_{0,\infty}}{AUC_{0,\infty}}  
\]  
Equation 3.4

The plasma data were also fitted with a one-compartment and a two-compartment model with first order dose input to determine the absorption constant and terminal
elimination constant. The Akaike’s information criterion suggested that the one-compartmental model (AIC: -57) was more efficient in describing the plasma data compared to the two-compartmental model (AIC: -23). Therefore, the plasma data were fitted to a one-compartment model and the absorption rate constant was determined from equation 3.5.

$$C(t) = \frac{D_0 k_a}{V(k_a - k_e)} (e^{-k_e t} - e^{-k_a t})$$  
Equation 3.5

where $D_0$ and $V$ represent dose administered and volume of distribution, respectively. $k_a$ and $k_e$ are absorption and elimination rate constants, respectively. The absorption half life $t_{1/2}(\text{abs})$ was calculated by:

$$t_{1/2}(\text{abs}) = \frac{0.693}{k_a}$$  
Equation 3.6

3.2.7 Histological analysis

In the high dose study (10 mg MTX), one of the knee joints from both treatment groups, sacrificed at 6 h and 24 h following intra-articular injection, were used for histological analysis. The knee joints were removed in toto and fixed in 10% formalin for 7 days. The muscles were trimmed off and the knee joints were fixed in decalcification fluid, which consisted of 10% formic acid in 4% formaldehyde, for 4 weeks. Sections were cut in the 8 sagittal planes and stained with haematoxylin/eosin.
3.2.8 AST analysis of rabbit plasma samples

The asparate aminotransferase (AST) activities in the rabbit plasma samples from the high dose pharmacokinetic study were analysed using an AST transaminase analysis kit (Sigma Diagnostics). The principle of AST analysis is based on the colorimetric method proposed by Reitman and Franke (1957). The reaction of aspartic acid and α-ketoglutaric acid is catalyzed by AST to form oxalacetic acid and glutamic acid. The oxalacetic acid is then reacted with a color reagent, 2,4-dinitrophenylhydrazine to form phenylhydrazones. The color intensity of phenylhydrazones is proportional to the transaminase activity.

A set of standards was first prepared using the transaminase calibration standard solution (sodium pyruvate) and substrate solution which contains DL-asparate, and α-ketoglutaric acid. A calibration curve of the UV absorbance values at the wavelength of 505 nm versus the corresponding units of AST (0-216 SF units/mL) was created.

To analyse the activity of AST in plasma, 20 μL of plasma sample was added to the substrate solution (100 μL) and kept at 37°C for an hour to allow the reaction between the substrate solution and AST in the plasma. Color reagent containing 2,4-dinitrophenylhydrazine (100 μL) was then added and the whole mixture was shaken gently and left at room temperature for 20 min to allow the reaction between oxalacetic
acid and 2,4-dinitrophenylhydrazine. One mL of 0.40 N Sodium Hydroxide Solution was then added to the mixture to terminate the reaction. The UV absorbance of the mixture was then read and recorded at 505 nm using a UV-Vis spectrophotometer with water as the reference. AST activity was determined in Sigma-Frankel (SF) units/mL from the calibration curve.

3.2.9 Statistical analysis

Student’s $t$ test ($p<0.05$) was used to compare the concentrations of MTX in various samples and pharmacokinetic parameters between two treatments.
Figure 3.1 Chemical structure of aminopterin.
3.3 RESULTS

3.3.1 MTX and 7-OH-MTX assay validation

3.3.1.1 Specificity, stability, and recovery

MTX and 7-OH-MTX in plasma samples and tissue samples were analyzed by HPLC using a post column UV reactor and a fluorescence detector. The assay was able to separate internal standard aminopterin, MTX and 7-OH-MTX into three distinctive peaks with retention times of 7.3, 19.8 and 25.1, minutes respectively. Blank rabbit tissue samples gave chromatograms that did not exhibit any peaks at these positions.

Attempts were made to analyze MTX in urine using the HPLC assay with the fluorescence detection. The substances in the urine produced significant peaks around the peak positions of MTX and 7-OH-MTX. Therefore, an HPLC assay with a UV detector was developed. The assay was able to separate MTX and 7-OH-MTX into two distinct peaks with retention times of 11.9 and 15.2 minutes, respectively. Blank urine samples showed no peaks at positions for MTX and 7-OH-MTX but peaks were present in the position for aminopterin. Therefore, no internal standard was used in this assay.

The rabbit plasma and tissue samples were spiked with 0.05, 0.1 and 1 µg /mL MTX. A sample of plasma and each tissue was also spiked with 0.1 µg and 1 µg /mL MTX and stored at -20°C for 18 days. The percent recovery of MTX from these tissues
was calculated by extraction and HPLC analysis of these samples and comparing the MTX peak area obtained with those from control assays, in which MTX was present but no tissue was used. Table 3.1 shows the percent recovery data for each tissue, plasma and urine tested at different MTX concentrations. The percent recovery of MTX from liver, kidneys and synovial tissues was approximately 50% at 3 different concentrations. The recoveries of MTX from plasma and urine were over 90% at 1 and 0.1 μg/mL, but dropped to 70% and 85% for plasma and urine samples, respectively, when spiked with 0.05μg/mL of MTX. Recovery of MTX from plasma and tissue samples spiked at 0.1μg and 1μg and stored at -20°C over 18 days did not show any significant differences when compared with the recovery of MTX from tissues processed immediately, suggesting that MTX was stable in the rabbit tissues stored at -20°C over this period of time.

3.3.1.2 Precision, accuracy, linearity, range, and limit of detection

The assays for MTX and 7-OH-MTX in plasma and urine were validated over three days. A linear relationship between MTX or 7-OH-MTX concentrations in plasma and ratios of peak areas of MTX or 7-OH-MTX to the internal standard were obtained in the concentration range of 0.01 to 1μg/mL ($R^2$>0.99) by the HPLC assay.

Representative standard curves in the concentration range of 0.01 to 1 μg/mL MTX and 7-OH-MTX in plasma are shown in Figure 3.2. The 3-day inter and intra-day precision
data are given in Table 3.2. The overall inter-day and intra-day coefficient of variation was about 14%. Acceptable assay precision is generally considered to be < 20% at the lowest concentration measured and < 15% at all other concentrations for the % CV values. These criteria were met using this HPLC assay at concentration range of 0.01 μg/mL to 1 μg/mL for MTX. The limit of detection of MTX and 7-OH-MTX in plasma were 0.038 μg/mL and 0.046 μg/mL respectively, and the limit of quantitation was 0.13 μg/mL for MTX and 0.14 μg/mL for MTX and 7-OH-MTX, respectively. The accuracy data are summarized in Table 3.3. A CV value less than 15% at concentration range from 0.05 to 1 μg/mL indicates sufficient accuracy at this range.

For analysis of MTX and 7-OH-MTX in urine samples, the overall inter-day and intra-day coefficient of variation was less than 15% in the concentration range of 0.5 to 20 μg/mL (Table 3.4). A linear relationship between MTX concentrations in urine and peak areas of MTX was obtained in the concentration range of 0.1 to 20 μg/mL (R^2 > 0.99) by the HPLC assay with UV detection (Figure 3.3). The limit of detection of MTX and 7-OH-MTX in plasma were 0.13 μg/mL and 0.14 μg/mL respectively, and the limit of quantitation was 0.4 μg/mL for MTX and 0.43 μg/mL for MTX and 7-OH-MTX, respectively.
Table 3.1  Percentage of MTX recovered from tissues, plasma and urine samples spiked with known concentrations of MTX following extraction and HPLC analysis. The values are shown as the mean of 3 samples ± one standard deviation (n=3).

<table>
<thead>
<tr>
<th>Spiked MTX concentration (µg/mL)</th>
<th>MTX recovered (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Synovial tissues</td>
</tr>
<tr>
<td>0.05</td>
<td>57.5±1.5</td>
</tr>
<tr>
<td>0.1</td>
<td>49.8±0.8</td>
</tr>
<tr>
<td>1</td>
<td>50.1±2.9</td>
</tr>
</tbody>
</table>

<sup>a</sup>MTX recovered was measured by dividing the peak areas of MTX obtained from the tissue, plasma and urine samples by the peak areas of MTX obtained from direct injections of 0.05, 0.1 and 1 µg/mL in phosphate buffer pH 6.5 and expressed as a percentage.
**Figure 3.2** Representative standard curves of A) MTX and B) 7-OH-MTX extracted from rabbit plasma and analysed by HPLC. Peak area ratio is the ratio of peak area of MTX or 7-OH-MTX to the peak area of aminopterin measured from HPLC chromatographs. $R^2$ is the coefficient of determination.
Figure 3.3  Representative standard curves of A) MTX and B) 7-OH-MTX extracted from rabbit urine and analysed by HPLC. Peak area is the area under the peak of either MTX or 7-OH-MTX measured from HPLC chromatographs. \( R^2 \) is coefficient of the determination.
Table 3.2 Inter- and intra-day precision for the HPLC assay with fluorescence detection for A) MTX, B) 7-OH-MTX extracted from rabbit plasma.

A)

<table>
<thead>
<tr>
<th>MTX concentration in plasma (µg/mL)</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>All days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak area</td>
<td>%CV&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Peak area</td>
<td>%CV&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>ratio&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>ratio&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>0.005</td>
<td>0.0038</td>
<td>35.5</td>
<td>0.0033</td>
<td>42.5</td>
</tr>
<tr>
<td>0.01</td>
<td>0.0044</td>
<td>17.4</td>
<td>0.0042</td>
<td>19.5</td>
</tr>
<tr>
<td>0.05</td>
<td>0.0267</td>
<td>9.9</td>
<td>0.0336</td>
<td>6.6</td>
</tr>
<tr>
<td>0.1</td>
<td>0.0511</td>
<td>5.8</td>
<td>0.0571</td>
<td>14.1</td>
</tr>
<tr>
<td>0.5</td>
<td>0.2696</td>
<td>5.1</td>
<td>0.2627</td>
<td>6.5</td>
</tr>
<tr>
<td>1</td>
<td>0.5362</td>
<td>6.0</td>
<td>0.5738</td>
<td>4.5</td>
</tr>
</tbody>
</table>

B)

<table>
<thead>
<tr>
<th>7-OH-MTX concentration in plasma (µg/mL)</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>All days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak area</td>
<td>%CV&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Peak area</td>
<td>%CV&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>ratio&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>ratio&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
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<td>0.0043</td>
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<tr>
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<td>17.8</td>
<td>0.0048</td>
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<td>9.9</td>
<td>0.0352</td>
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<td>5.1</td>
<td>0.3172</td>
<td>6.3</td>
</tr>
<tr>
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<td>0.6520</td>
<td>8.2</td>
<td>0.6677</td>
<td>10.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> Peak area ratio is the ratio of peak area of MTX or 7-OH-MTX to the peak area of aminopterine. The value reported is the average calculated from four standard curves at each concentration for each day (n=16 for all days).

<sup>b</sup>%CV is the coefficient of variation, which is the ratio of the standard deviation to the mean area ratio values, expressed as a percentage. A value less than 20% at the lowest concentration and less than 15% at all other concentrations was taken to indicate sufficient precision.
Table 3.3  Accuracy for the HPLC assay with fluorescence detection for A) MTX, B) 7-OH-MTX extracted from rabbit plasma.

A)     

<table>
<thead>
<tr>
<th>MTX concentration (µg/mL)</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Measured value a</td>
<td>%CV b</td>
<td>Bias c</td>
</tr>
<tr>
<td></td>
<td>(%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>0.013</td>
<td>39.5</td>
<td>30.0</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>0.058</td>
<td>8.4</td>
<td>16.0</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>0.103</td>
<td>5.4</td>
<td>3.1</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0.51</td>
<td>6.5</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.98</td>
<td>8.3</td>
<td>-1.6</td>
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</table>

B)     

<table>
<thead>
<tr>
<th>7-OHMTX concentration (µg/mL)</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Measured value a</td>
<td>%CV b</td>
<td>Bias c</td>
</tr>
<tr>
<td></td>
<td>(%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>0.015</td>
<td>34.8</td>
<td>50.0</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>0.061</td>
<td>7.5</td>
<td>22.0</td>
</tr>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>0.101</td>
<td>5.5</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0.541</td>
<td>5.9</td>
<td>7.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.998</td>
<td>9.9</td>
<td>-1.5</td>
</tr>
</tbody>
</table>

aMeasured value is the average (n=3 for each day) of values of concentration calculated from one standard curve on that day.

b%CV is the ratio of the standard deviation to the average, expressed as a percentage. A value less than 15% indicates sufficient accuracy at each concentration.

Bias is the ratio of the deviation of measured value from the actual concentration measured, expressed as a percentage.
Table 3.4  Inter- and Intra-day precision of the HPLC assay with UV detection for A) MTX, B) 7-OH-MTX extracted from rabbit urine.

A)  MTX concentration (µg/mL)  |  Day 1  |  Day 2  |  Day 3  |  All days  
--- | --- | --- | --- | ---
| Peak Area | %CV\(^b\) | Peak Area | %CV | Peak Area | %CV | Peak Area | %CV |
| 0.1 | 35457 | 4.8 | 38132 | 20.5 | 301445 | 31.4 | 33785 | 31.6 |
| 0.5 | 72657 | 16.5 | 67887 | 13.4 | 61292 | 10.7 | 63945 | 17.4 |
| 1 | 154065 | 6.4 | 146987 | 8.7 | 145478 | 4.6 | 154785 | 6.4 |
| 5 | 687121 | 4.7 | 727697 | 11.6 | 702109 | 2.9 | 672309 | 5.4 |
| 10 | 1454386 | 8.5 | 1528885 | 10.4 | 1516005 | 9.8 | 1512974 | 10.4 |
| 20 | 2847956 | 9.4 | 2757286 | 4.7 | 2920613 | 5.7 | 2733949 | 8.8 |

B)  7-OHMTX concentration (µg/mL)  |  Day 1  |  Day 2  |  Day 3  |  All days  
--- | --- | --- | --- | ---
| Peak Area | %CV\(^b\) | Peak Area | %CV | Peak Area | %CV | Peak Area | %CV |
| 0.1 | 38233 | 20.4 | 29878 | 15.7 | 20457 | 25.4 | 59365 | 20.3 |
| 0.5 | 82767 | 13.9 | 98787 | 17.3 | 87898 | 16.8 | 97086 | 19.1 |
| 1 | 178329 | 5.8 | 180428 | 4.4 | 153483 | 10.8 | 162412 | 10.6 |
| 5 | 751731 | 9.3 | 853864 | 6.3 | 742016 | 5.4 | 852848 | 12.5 |
| 10 | 1726327 | 8.3 | 1901803 | 9.1 | 1801428 | 2.3 | 1727183 | 13.1 |
| 20 | 3548789 | 5.4 | 3372828 | 5.8 | 3617751 | 3.7 | 3521411 | 8.5 |

\(^a\) Peak area ratio is peak area of MTX or 7-OH-MTX measured from HPLC chromatographs. The value reported is the average calculated from four standard curves at each concentration for each day (n=16 for all days).

\(^b\)%CV is the coefficient of variation, which is the ratio of the standard deviation to the mean area ratio values, expressed as a percentage. A value less than 20% at the lowest concentration and less than 15% at all other concentrations indicated sufficient precision.
3.3.2 Microspheres recovery from rabbit joint

To investigate the feasibility of recovering MTX and MTX loaded microspheres from the synovial fluid of the rabbits, two rabbits were injected intra-articularly with 56 mg of 18% MTX loaded microspheres, and the joints were immediately flushed with 2 mL of heparinized saline and the fluids in the joints were aspirated for analysis. The pooled fluids from synovial joints were clear in appearance but there was evidence of microspheres retrieved from the joint space via the needle and syringe. The amounts of MTX in the aspirated fluids and microspheres were analysed and the results are shown in Table 3.5. About 10% of the injected dose was recovered from the joints of the rabbits with 3-4% of the dose found in the aspirated fluids and 7-8% of the dose was found in the retrieved microspheres.
Table 3.5 The amounts of MTX recovered from aspirated fluid from rabbit joints immediately following intra-articular injection of 56 mg of 18% MTX loaded PLLA2k microspheres.

<table>
<thead>
<tr>
<th>Rabbit #</th>
<th>Volume of fluid aspirated (mL)</th>
<th>Amt of MTX in the fluid (µg)</th>
<th>Amt of MTX in microspheres (µg)</th>
<th>MTX recovered$^a$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.5</td>
<td>437.6</td>
<td>856</td>
<td>12.9</td>
</tr>
<tr>
<td>2</td>
<td>1.2</td>
<td>304.6</td>
<td>725</td>
<td>10.3</td>
</tr>
</tbody>
</table>

$^a$MTX recovered was calculated by dividing the total amount of MTX determined in the joint (amount in the fluid + amount in the microspheres) by the dose of MTX injected (10mg), expressed as a percentage.
3.3.3 Pharmacokinetic studies in rabbits

3.3.3.1 Low dose pharmacokinetic study

The plasma MTX concentrations following a single intra-articular injection of either 25 mg of 6% (w/w) MTX loaded PLLA2k microspheres (33 -110 μm) or 1.5 mg MTX solution are shown in Figure 3.4. The plasma MTX concentration rose 5 min after the injection of MTX solution and plasma concentrations reached a maximum 15 min after the injection (t$_{max}$ of 15 min and C$_{max}$ of 0.6 μg/ml). The plasma concentrations gradually declined and were undetectable 8 h after the injection. For the rabbits injected with MTX loaded microspheres, the plasma MTX concentrations plateaued at about 0.06 μg/ml between 5 min and 3 h and were undetectable, 4 h following the injection. The concentrations of the major metabolite 7-OH-MTX in plasma were not analysed since the 7-OH-MTX standard was not available at the time this study was conducted.

The amounts of MTX excreted in the urine of rabbits following the intra-articular injections of microspheres and MTX solution are shown in Figure 3.5. Some samples of urine were missed during the time period of 0-3 h as the rabbits were recovering from anesthesia in a heated incubator following intra-articular injection. The results showed that in the period of 8 to 24 h post intra-articular injection, the amount of MTX excreted into the urine from rabbits injected with MTX solution was more than 6 fold higher than
that from rabbits injected with MTX loaded microspheres and the difference was statistically significant (p < 0.05). In the period of 24-48 h, similar amounts of MTX were excreted from both groups. The total amount (0-48 h) of MTX excreted into the urine from rabbits injected with MTX solution was also significantly higher (p < 0.05) than that from rabbits injected with MTX loaded microspheres.

There was no MTX detected in the major organs from either rabbits injected with MTX solution or MTX loaded microspheres. When analyzing synovial tissues, approximately 0.6 μg of MTX was detected in the joint tissues of two of the rabbits injected with MTX microspheres while no MTX was detected in the joints of rabbits injected with MTX solution.
Figure 3.4  MTX concentrations in rabbit plasma following a single intra-articular injection of either MTX solution or 25mg of MTX loaded PLLA2k microspheres (33-110 μm) in 200 μL PBS. Values are mean ± one standard deviation, (n=7). The dose of MTX injected was 1.5mg. *Indicates statistical difference between MTX plasma concentrations of rabbits injected with MTX solution and MTX loaded microspheres by paired t-test (p<0.05). (♦) MTX solution, (■) MTX microspheres.
Figure 3.5 The amount of MTX (μg) excreted at different time periods in the urine of rabbits following a single intra-articular injection of either MTX solution or 25mg of MTX loaded PLLA2k microspheres (33-110 μm) in 200μL PBS. * Indicates statistical difference between the amounts of MTX excreted from rabbits injected with MTX solution and MTX loaded microspheres by paired t-test (p<0.05). aPart of the urine sample was missed from both groups during this time period. Values are mean ± one standard deviation, (n=3). The dose of MTX injected was 1.5mg.
3.3.3.2 High dose pharmacokinetic study

The plasma MTX concentrations following a single intra-articular injection of either 10 mg MTX solution in 400 μL PBS or 56 mg of 18% MTX loaded PLLA2k microspheres (33-110 μm) in 400 μL PBS are shown in Figure 3.6. Plasma MTX concentrations rose 5 min following intra-articular injection of MTX solution, and peaked 15 min following injection (t_{max} of 15 min, C_{max} of 2.64 μg/mL). The concentration gradually declined and was still detectable 8 h following injection. For rabbits injected with 56 mg of 18% MTX loaded microspheres, the plasma MTX was detectable 5 min following injection and peaked 15 min following injection (t_{max} of 15 min, C_{max} of 0.4 μg/mL). The concentration gradually declined and was below the detection limit 6 h following injection.

The plasma concentrations of 7-OH-MTX, the major metabolite of MTX, following the injection of MTX solution are shown in Figure 3.7. The data for 2 animals (rabbit #5 and rabbit #15) are shown separately from the rest of the group due to significant inter-individual variability. The metabolite 7-OH-MTX was detectable in the plasma of rabbits injected with MTX solution 5 min following injection. With the exception of two rabbits (# 5 and #15), the plasma concentrations of 7-OH-MTX following the injection of MTX solution peaked at 1 h (Cmax 1.19 μg/mL) and gradually declined to
undetectable levels 6 h following the injection. The concentrations of 7-OH-MTX were higher than MTX in plasma from 2 h to 8 h post injection. The peak 7-OH-MTX concentrations in rabbit #5 and #15 occurred at 4 h following injection and were 3 to 5 fold higher than the peak concentration of the rest of the group. For rabbits injected with MTX microspheres, the concentration of 7-OH-MTX peaked at 1 h following injection (Cmax 0.2 μg/mL) except for two rabbits (rabbit # 4 and rabbit #14) (Figure 3.8). The peak 7-OH-MTX concentrations for rabbit #4 and rabbit #14 occurred at 2 h following injection and were 3 to 8 fold higher than the mean peak concentration for the rest of the group.

The amounts of MTX (μg) excreted at different time periods in the urine of rabbits after a single intra-articular injection of either MTX solution or MTX loaded microspheres are shown in Figure 3.9A. Between 0 to 3 h, about 1500 μg of MTX was excreted in to the urine from the rabbits injected with MTX solution and this accounted for 15% of the total dose of MTX injected. From 3 to 6 h and from 6 to 24 h, an additional 2.6% and 5.7% of the total dose were excreted, respectively. On the other hand, less than 2% of the total dose of MTX was excreted in the urine of those rabbits injected with 18% MTX loaded microspheres from 0 to 3 h and 3 to 6 h time periods and 2.48% of the total dose of MTX was excreted in the period of 6-24 h.
The amounts of 7-OH-MTX excreted in the urine following a single intra-articular injection of either MTX solution or MTX loaded microspheres are shown in Fig 3.9B. Between 0-3 h, about 1700 μg of 7-OH-MTX were excreted in the urine of the rabbits injected with MTX solution. Between 3-6 h and 6-24 h, an additional 800 and 1100 μg of 7-OH-MTX, respectively, were excreted in the urine. For rabbits injected with MTX microspheres, less than 200 μg of 7-OH-MTX were excreted in the period of 0-3 h and an additional 152 and 260 μg of 7-OH-MTX, respectively, were excreted in the urine between 3-6 h and 6-24 h, respectively. In terms of the percentage of total dose, approximately 37% of the total dose was excreted as 7-OH-MTX from rabbits injected with MTX solution while 5.7% of the total dose was excreted as 7-OH-MTX from rabbits injected with MTX microspheres.

To evaluate whether drug loaded microspheres were retained in the joint 6 h and 24 h following intra-articular injection of microspheres, synovial fluid and synovial tissues of the rabbits were removed for analysis. The joint was flushed with 2 mL heparinized saline and the fluid in the joint was aspirated and analyzed for MTX. The amounts of MTX in the aspirated fluids 6 h and 24 h following a single intra-articular injection of either MTX solution or MTX loaded microspheres are given in Figure 3.10A. At 6 h following the intra-articular injection, the amount of MTX in aspirated fluid of the rabbits
injected with microspheres was about 9 fold higher than that of the rabbits injected with MTX solution. At 24 h following the intra-articular injection, no MTX was detected in the aspirated fluid of the rabbits injected with MTX solution while the amount of MTX in the aspirated fluid in the rabbits injected with MTX microspheres decreased to 0.02 µg.

The synovial tissues of the rabbits were removed and analyzed for MTX after the animals were sacrificed. The amounts of MTX in the synovial tissues of the rabbits are given in Figure 3.10B. Since the recovery of MTX from the synovial tissue samples was 50% (Table 3.1), the results reported in Figure 3.10B were corrected to account for 100% recovery. At 6 h after intra-articular injection, the amount of MTX in the synovial tissues of the rabbits injected with MTX microspheres was about the same as in the synovial tissues of the rabbits injected with MTX solution. At 24 h after intra-articular injection, the amount of MTX in the synovial tissues of the rabbits injected with MTX microspheres was about three fold higher than that of the rabbits injected with MTX solution.

Samples of liver and kidneys were analyzed for the concentration of MTX. No MTX was detected in liver tissues from either group of rabbits. Analysis of kidney samples demonstrated that only rabbits administered MTX solution and sacrificed at 6 h following injection showed MTX present in the kidneys at about 0.1 µg/5g tissue.
Figure 3.6 MTX concentrations in rabbit plasma after a single intra-articular injection of either MTX solution or 56mg of 18% MTX loaded PLLA2k microspheres (33-110 μm) in 400 μL PBS. Values are mean ± one standard deviation, (n = 8 for 0-6 h period, n = 4 for 6-24 h period). The dose of MTX injected was 10mg. *Indicates statistical difference between MTX plasma concentrations of rabbits injected with MTX solution and MTX loaded microspheres by student’s t-test (p<0.05). (⊕) MTX solution, (■) MTX microspheres.
Figure 3.7 7-OH-MTX concentrations in rabbit plasma following a single intra-articular injection of 10mg MTX solution in 400 µL PBS. (♦) rabbit #15, (■) rabbit #5, (▲) the remaining animals in the group, values are mean ± one standard deviation (n = 6).
Figure 3.8 7-OH-MTX concentrations in rabbit plasma following a single intra-articular injection of 56 mg of 18% MTX loaded PLLA2k microspheres (33-110 μm) in 400 μL PBS. The injections provided a 10 mg MTX dose. (▲) rabbit #14, (●) rabbit #4, (♦) the remaining animals in the group, values are mean ± one standard deviation (n = 6)
Figure 3.9  The amount of A) MTX (µg) and B) 7-OH-MTX (µg) excreted at different time periods in the urine of rabbits following a single intra-articular injection of either 10 mg MTX solution in 400 µL PBS or 56mg of 18% MTX loaded PLLA2k microspheres (33-110 µm) in 400 µL PBS. Values are mean ± one standard deviation, (n = 8 for the period of 0-6 h, n= 4 for the period of 6-24 h). The dose of MTX injected was 10mg. * Indicates statistically different between two treatments by stduent's t-test (p<0.05).
Figure 3.10  The amount of MTX in A) pool fluid aspirated from synovial joints and B) the synovial tissues, 6 h and 24 h following a single intra-articular injection of either 10 mg MTX solution in 400 μL PBS or 56 mg of 18% MTX loaded microspheres in 400 μL PBS. The values reported in B) are corrected to account 100% MTX recovery from synovial tissues. Values are mean ± one standard deviation, (n = 4). The dose of MTX injected was 10 mg. * Indicates statistically different between two treatments by student’s t-test (p<0.05).
3.3.4 Pharmacokinetic analysis of plasma data

The plasma data of rabbits in both low dose and high dose pharmacokinetic studies were analysed using a non-compartmental and a one-compartmental model with extravascular dose input. The values of elimination half-life ($t_{1/2}$) determined using both the non-compartmental and the one-compartmental models were similar and therefore, only the values that were determined by the non-compartmental model are reported. The pharmacokinetic parameters for rabbit plasma data from both experiments are given in Table 3.6. At both doses, the area under the curve ($AUC_{0-\infty}$) of the group injected with MTX solution was about 7 fold higher than the $AUC_{0-\infty}$ of the group injected with MTX microspheres, while no significant difference was observed in elimination half-lives ($t_{1/2}$) and mean residence times (MRT) between the groups at either dose (Table 3.6). The absorption half-lives determined from one-compartmental analysis varied from 0.04 h to 0.13 h (Table 3.6) but were not significantly different at either dose.
Table 3.6  Pharmacokinetic parameters for MTX plasma data in rabbits following an intra-articular injection of either MTX solution or MTX loaded PLLA2k microspheres (33-110 μm). The dose injected was either 1.5mg or 10mg. The parameters are determined using WinNonlin computer program.

<table>
<thead>
<tr>
<th>MTX dose (mg)</th>
<th>Treatment</th>
<th>$t_{1/2(\text{abs})}$ (h)$^a$</th>
<th>$t_{1/2}$ (h)</th>
<th>$AUC_{0-\infty}$ (μg/mL.h)</th>
<th>MRT (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 MTX solution</td>
<td>0.13±0.08</td>
<td>1.57±0.90</td>
<td>0.98±0.65*</td>
<td>2.24±1.47</td>
<td></td>
</tr>
<tr>
<td>MTX microspheres</td>
<td>0.04±0.01</td>
<td>1.03±0.33</td>
<td>0.14±0.06</td>
<td>1.70±0.36</td>
<td></td>
</tr>
<tr>
<td>10 MTX solution</td>
<td>0.07±0.05</td>
<td>0.94±0.18</td>
<td>4.40±2.04*</td>
<td>1.28±0.30</td>
<td></td>
</tr>
<tr>
<td>MTX microspheres</td>
<td>0.06±0.03</td>
<td>0.92±0.18</td>
<td>0.65±0.23</td>
<td>1.34±0.23</td>
<td></td>
</tr>
</tbody>
</table>

*Statistical difference between two treatments by stduent's t-test (p<0.05). $^a$Absorption half life $t_{1/2(\text{abs})}$ was estimated by one-compartmental analysis.
3.3.5 Histological analysis

In an attempt to determine the sites of deposition of the microspheres within the joint, the synovial joint of one of the animals from each treatment group was processed for histological analysis. No synovial proliferation was observed in the joints either injected with either MTX solution or MTX microspheres at either time points (Figure 3.11). For rabbits injected with microspheres, circular empty spaces marked by dark color granules were observed close to the microvessels in the adipose layer of the synovium of the rabbit sacrificed at 6 h after injection (Figure 3.11 C and D). The circular spaces were in the size range of 50 μm and appeared to be surrounded by red blood cells in the synovial layer (Figure 3.11 C and D). These circular spaces were not observed in the synovium of rabbits sacrificed 24 h after injection.
Figure 3.11  Histological analysis of synovial tissues following intra-articular injection of 56 mg of 18% MTX loaded PLLA2k microspheres (33-110 μm) and sacrificed at 6 h following injection. (A) synovial tissue with dark color granules visible in the rectangular area (40x magnification), (B) higher magnification of the rectangular area (400x magnification).
Figure 3.12  Histological analysis of synovial tissues following intra-articular injection of A) 10 mg MTX solution in 400 μL PBS and B) 56 mg of 18% MTX loaded PLLA2k microspheres (33-110 μm) and sacrificed at 24 h following injection. (Magnification 100 x). Both graphs showed no evidence of synovial proliferation as indicated by the arrows.
3.3.6 AST analysis

MTX has been reported to induce acute hepatotoxicity. In a clinical setting, serum transaminases, aspartamine transferase (AST) and alanine transferase (ALT) are often monitored (Kirchain and Gill, 1996). The high concentration of these two enzymes and their ready release from the hepatocyte cytoplasm makes them good indicators of necrotic lesions within the liver. The AST activities in rabbit plasma at different times following intra-articular injection of 10mg MTX solution or MTX loaded microspheres were determined and the results are shown in Figure 3.13. There was no difference in the AST activity profiles for rabbits injected with either MTX solution or MTX loaded microspheres. The AST in the rabbit plasma increased slightly following the injection of MTX formulations and peaked at 6 h following the injection. The activity declined 24 h following injection. Although the AST activity slightly increased following the injection of MTX, the activities were within the normal range of AST (below 28 SF units/mL) (McLaughlin and Fish, 1994) indicating that the injection of MTX did not induce acute liver toxicity.
Figure 3.13  The AST activities in rabbit plasma after a single intra-articular injection of either 10 mg MTX solution in 400 μL PBS or 56 mg of 18% MTX loaded PLLA2k microspheres (33-110 μm) in 400 μL PBS. Values are mean ± one standard deviation, (n = 8 for 0-6 h period, n = 4 for 6-24 h period). The dose of MTX injected was 10 mg. (•) MTX solution, (■) MTX microspheres.
3.4 DISCUSSION

In both low dose and high dose studies, MTX appeared in the plasma of rabbits injected intra-articularly with either MTX solution or MTX microspheres about 5 min following injection, and peak concentrations occurred at 15 min, indicating that MTX was rapidly transferred from the synovial cavity into the systemic circulation (Figure 3.4 and Figure 3.6). This rapid transfer of drug was also confirmed by the short absorption half-life (0.04 to 0.13 h) observed in both groups at both doses (Table 3.6). A similar profile to the one obtained after injection of the MTX solution was observed by Foong and Green (1988) who administered a single intra-articular injection of $[^{3}\text{H}]$ MTX solution into normal or inflamed joints of rabbits. The maximum concentration was observed at 1 h following the intra-articular of 1mg $[^{3}\text{H}]$ MTX solution (Foong and Green, 1988).

The rapid appearance of MTX in the plasma of rabbit injected with MTX microspheres was likely due to the burst phase of MTX release from microspheres observed in previous in vitro drug release studies (Figure 2.4). In both the high and low dose studies, plasma levels of MTX attained following intra-articular injection of MTX microspheres were more than 5 fold lower than levels produced by MTX solution (Figure 3.4 and Figure 3.6). After about 2 h post intra-articular injection of microspheres,
plasma MTX concentrations were very low and below detection level 3-6 h following microspheres injection. The in vitro MTX release profiles of 10% and 20% (w/w) MTX loaded microspheres have demonstrated a rapid burst phase of release of MTX in the first 4 h followed by a slower release period (Figure 2.4). We speculate that the release of MTX from microspheres in the joint cavity slowed down 2 h following intra-articular injection due to the ending of the burst release phase and possible aggregation and adherence of microspheres to the synovial tissues resulting in low synovial fluid and plasma concentrations (Nishide et al., 1999).

When comparing the pharmacokinetic parameters for plasma MTX data from both studies, the AUC$_{0-\infty}$ of the groups injected with MTX solution was about 7 fold higher than the AUC$_{0-\infty}$ of the groups injected with MTX microspheres (Table 3.6) indicating that more drug entered the systemic circulation from the groups injected with the MTX solution. The Cmax (Figure 3.7) and AUC$_{0-\infty}$ values (Table 3.6) for the 10 mg MTX solution plasma concentration-time curve were between 5-7 fold greater than the Cmax (Figure 3.5) and AUC$_{0-\infty}$ (Table 3.6) values for the 1.5 mg MTX solution plasma concentration-time curve. This suggested that plasma levels following intra-articular injection were dose dependent and that the transfer of MTX solution from the synovial cavity to the systemic circulation did not reach saturation in the high dose study. The
mean residence times (MRT) and the elimination half-lives $t_{1/2}$ for both treatment groups at two doses were not significantly different (Table 3.6). Since MRT after an extravascular administration is a function of both drug absorption and elimination, the fact that the MRT values were similar in both treatment groups, confirms that the rates of absorption were similar.

The elimination half-life of MTX following systemic administration in rabbits, reported in the literature, varies from 2 to 47 h depending on the dose, the duration of the study and the detection limit of the assay (Sasaki et al., 1983; Chen and Chiou, 1983; Iven et al., 1985). The half-lives of MTX have been reported as 0.7 h ($\alpha_{t_{1/2}}$) and 47.7 h ($\beta_{t_{1/2}}$) following a 6-h 50mg/kg intravenous infusion (Sasaki et al., 1983). Chen and Chiou (1983) showed that following an intravenous bolus of a dose of 45 mg MTX in rabbits, MTX plasma levels decreased polyexponentially with an elimination half-life of 10 h. Iven et al (1985) found that following a short term infusion of 1.33 mg/kg MTX for 10 min in rabbits, the plasma concentrations decreased in a triexponential manner with $\alpha_{t_{1/2}}$, $\beta_{t_{1/2}}$ and $\gamma_{t_{1/2}}$ of 0.136 h, 0.632 h, and 2.402 h, respectively. In this study, plasma was sampled up to 48 h post injection and it is likely that the terminal phase had not been attained.
In the high dose study, 7-OH-MTX appeared in the plasma within 5 min and peaked between 1 to 2 h following intra-articular injection in both treatment groups (Figure 3.8A and Figure 3.8B) indicating the injected MTX was quickly converted to its metabolite 7-OH-MTX. In both treatment groups, there were two rabbits (#5 and #15 for MTX solution group) and (#4 and #14 for microspheres group) that possessed markedly different 7-OH-MTX plasma profiles than the rest of the rabbits (Figures 3.7 and 3.8). These differences in the plasma profiles of 7-OH-MTX could be due to individual variation in hepatic MTX-7-hydroxylase activities. A 48 fold range in MTX-7-hydroxylase activities in six human liver cytosol preparations have been shown by Kitamura et al (1999).

In the low dose study, during the period of 8-24 hours urine collection, 8% of the total drug was excreted in the urine following injection of MTX solution compared to 2% of the drug excreted from rabbits injected with MTX loaded microspheres (Figure 3.5). This result was consistent with the plasma data indicating that MTX from the solution formulation rapidly entered the systemic circulation and was eventually excreted in the urine, while the uptake of MTX from the joint cavity into the blood was slowed down in the microspheres formulation. In the period of 24-48 h, an additional 3% of MTX was excreted from rabbits injected with MTX loaded microspheres (Figure 3.5) suggesting
that MTX was still being released at a very slow rate from microspheres, despite the fact that the concentration of MTX in plasma was below detection level 4 hours after intra-articular injection. The in vitro release profile of 10% (w/w) loaded MTX microspheres have shown that following the burst phase of MTX release, the loaded MTX was released at an approximately zero order rate of 0.2% per day over 2 weeks (Figure 2.4). Urine data showed that 2.5 times more MTX was excreted in the urine from rabbits injected with MTX solution than rabbits injected with MTX loaded microspheres 48 h after intra-articular injection (Figure 3.5). For the low dose study, the total amount of MTX excreted in the urine at the end of the study accounted for 10% of total dose injected for rabbits injected with MTX solution and 5% for rabbits injected with MTX loaded microspheres (Figure 3.5). Due to the fact that we failed to collect urine samples in the first two hours following intra-articular injection, the urine data do not completely represent the total amount of MTX excreted in the urine.

In the low dose study, the synovial joints were not flushed with saline and the whole right joints of the rabbits were removed for MTX analysis. Approximately 0.60 \( \mu \text{g} \) of MTX was detected in the joint tissues of two of the rabbits injected with MTX microspheres while no MTX was detected in the joint of rabbits injected with MTX solution. The fact that we were not able to detect significant amounts of MTX in the
joints of rabbits injected with MTX microspheres was likely due to the large sample size of joint tissues (8-13 g) taken for analysis. The whole joint was homogenized, extracted and samples analyzed by HPLC. We speculate that the concentrations of MTX in the samples were below the quantitation limit of the assay.

In the high dose study, the rabbits were under light inhalation anesthesia and a catheter was inserted into the urethra of the rabbits to ensure the complete collection of urine samples. At 0-3 h following intra-articular injection, the amounts of MTX and 7-OH-MTX excreted in the urine of rabbits injected with solution were approximately 6 fold higher than in the urine of rabbits injected with MTX loaded microspheres (Figure 3.9A and B). Within 24 h following injection, 4-5 fold more MTX and 7-OH-MTX was excreted in the urine from rabbits injected with MTX solution than rabbits injected with MTX loaded microspheres (Figure 3.9A and Figure 3.9B). Both high and low dose studies showed that following intra-articular injection, MTX from MTX solution rapidly entered the systemic circulation and was excreted in the urine while MTX microspheres slowed down the uptake of MTX across synovium into blood circulation by releasing MTX in the joint in a slow and controlled manner.

In the high dose study, the synovial joints were flushed with heparinized saline in an attempt to recover the microspheres and MTX in the synovial fluid. At 6 h post
injection, the amount of MTX in aspirated fluid of rabbits injected with microspheres was significantly higher than that of the rabbits injected with MTX solution, suggesting that MTX microspheres were able to retain the drug in the joint (Figure 3.10A). At 24 h following intra-articular injection, no drug was detected in the aspirated fluid of rabbits administered MTX solution, while small amounts of MTX were detected in the aspirated fluid of the rabbits injected with microspheres (Figure 3.10A). We speculate that by 24 h following injection, some MTX microspheres may have became located in the joint in such a way that they were inaccessible to the joint flushing procedure. The significant amounts of MTX detected in synovial tissues at 24 h (Fig 3.10 B) may indicate that microspheres migrated to popliteal folds of the synovial cavity where they were not flushed out with saline but were extracted in the synovial tissue samples. In studies where rabbit joints were injected with microspheres (10 mg MTX dose) and then immediately flushed and fluids aspirated to recover the microspheres, only about 10-13% of the total injected dose was recovered (Table 3.5). This study illustrated the difficulty in retrieving the microspheres and synovial fluid from the joint.

In the high dose study, for rabbits injected with MTX solution, 72% of the total dose was excreted in the urine (28% as MTX and 44% as 7-OH-MTX) and approximately 0.1% of the drug was detected in the synovial tissues and kidneys (Figure 3.10A and
MTX was not detected in other tissues removed for analysis. For rabbits injected with MTX loaded microspheres, 12% of total dose was excreted in the urine (5% as MTX and 7% as 7-OH-MTX) 24 h following intra-articular injection of MTX loaded microspheres and approximately 2% of the total MTX was recovered from synovial tissues and synovial fluid (Figure 3.10A and Figure 3.10B). No MTX was detected in other tissues. It is possible that the rest of the injected MTX that was not detected in the rabbits may have been metabolized into MTX polyglutamates intracellularly (Iven et al., 1985) which could not be analysed by the HPLC assay, and also distributed in the tissues and plasma at concentrations below the detection limit of the assay. It has been shown that MTX was rapidly converted to MTX polyglutamates and accumulated in the brain, liver, kidneys, and testes of rats as early as 3 h following an intra-peritoneal injection of 10mg/kg of MTX (Krakower and Kamen, 1983). The accumulation of MTX polyglutamates has also been demonstrated in erythrocytes, T-lymphocytes, fibroblasts, and hepatic tissues of patients with rheumatoid arthritis receiving long term methotrexate therapy (Bannwarth et al., 1996; Grim et al., 2003).

Renal excretion is the major route of elimination for MTX and its metabolite 7-hydroxy-methotrexate (7-OH-MTX) in rabbits (Chen and Chiou, 1983).
Chiou (1983) reported that 67% of drug was excreted as MTX and 33% as 7-OH-MTX in the urine of rabbits 48 h after an intravenous bolus injection of 45mg of MTX.

Administration of a 6 h 50mg/kg infusion in rabbits showed that 57% of the dose (45% as MTX and 10% as 7-OH-MTX) was excreted in the urine in 20 h with 10%, 1% and 1.8% of total dose in the tissues, GI tract, and bile, respectively (Sasaki et al., 1983). For the tissue samples, liver, kidney, and lungs possessed the highest concentrations (Sasaki et al., 1983). Foong and Green (1988) showed that following an intra-articular injection of 1mg [³H]MTX solution in rabbits, 79% of the total radioactivity was recovered in the urine, while 0.41% and 0.14% were recovered from liver and kidney, respectively, 24 h following injection.

The histological analysis showed that at 6 h post intra-articular injection of MTX loaded microspheres, there were empty spaces surrounded by dark color granules embedded in the adipose layer of the synovium (Figure 3.11A and B). Considering the similarity in sizes and shapes, these spaces were likely caused by tissue deposition of the microspheres that were subsequently removed by the processing conditions during the fixation procedures. There was no evidence of microsphere phagocytosis by the synovial cells. Horisawa et al (2002a) administered intra-articular injections of PLGA microspheres with a mean diameter of 26.5 μm in rat joints and demonstrated the
presence of a granulation tissue surrounded by multinucleated giant cells in the synovium. A possible explanation for the deposition of microspheres in the adipose layer observed in this work was the creation of turbulence brought about by intra-articular injection. Since the adipose layer is only 10-15 μm away from the synovial surface, the relatively high flow rate accompanying the intra-articular injection could have formed a channel in the tissue and carried the microspheres to the adipose layer. The accumulated red blood cells surrounding the spaces created by microspheres likely leaked from nearby microvessels due to the trauma. Despite the possible trauma caused by the initial intra-articular injection, no synovial proliferation was observed indicating the deposition of microspheres in the joint cavity did not induce acute injury (Figure 3.12 A and B). No evidence of microspheres deposition was found for the rabbit sacrificed at 24 h following injection. We speculate that since only one joint was taken from 4 treated animals and only 8 sagittal slides were cut across the synovial joint, that it is possible that microspheres migrated to the popliteal fold as suggested by Nishide et al (1999) and the section of the fold that contained the microspheres was missed.

In summary, MTX loaded PLLA2k microspheres were retained in the joint space and synovium and released MTX in a slow, controlled manner. MTX in solution was rapidly absorbed across the synovium into the plasma and systemic uptake of MTX was
considerably higher than for MTX microspheres. MTX levels in synovial tissues, 24 h after intra-articular injection were markedly higher for the microspheres formulation compared to the solution formulation.
4.1 INTRODUCTION

In the previous chapter, it was shown that intra-articular injections of MTX loaded microspheres into healthy rabbits resulted in low systemic levels of drug compared to MTX solutions. Urinary excretion data for MTX and 7-OH-MTX and synovial tissue levels of MTX suggested that MTX was released by the microspheres in the joint at low levels over at least 48 h.

Rabbits are generally considered to be a good animal model for studies of rheumatoid arthritis and for the evaluation of efficacy of anti-rheumatic drugs (Foong and Green, 1988). Dumonde and Glynn (1962) reported the induction of chronic synovitis in sensitized rabbits by the intra-articular injection of fibrin. Clinically and histologically acute, and later chronic, monoarthritis was produced, lasting for months and resembling rheumatoid arthritis synovitis. Lining cell hyperplasia, perivascular diffuse infiltration with lymphocytes, plasma cells, lymphoid follicles, pannus and cartilage erosions were the prominent features indistinguishable from those found in rheumatoid arthritic synovitis. Since then, antigen-induced arthritis has been one of the most intensively studied animal models of inflammatory arthritis (Magilavy, 1990).
Various antigens including bovine serum albumin, ovalbumin, and horseradish peroxidase are all capable of inducing infiltration of neutrophils, plasma cells, macrophages and lymphocytes (Magilavy, 1990). By injecting antigen locally into the joints of presensitized animals, monoarthritis induction is possible, as compared to polyarthritis in other animal models (Brahn, 1991). This model is particularly useful for the evaluation of intra-articular treatments in which the treatment is localized in one or two joints.

Evidence has shown that antigen-induced arthritis in rabbits and rats is responsive to both intra-articular and systemic MTX treatment. Foong and Green (1993) induced arthritis in ovalbumin-presensitized rabbits with 5mg of ovalbumin in both knee joints. MTX solution or a MTX liposomal formulation was injected into one of the knee joints of rabbits, either immediately after antigen challenge (day 0), day 7, day 21 or day 35 and the progression of the disease was monitored for 56 days. The results showed that the rabbits treated with 1mg MTX solution at day 0 and rabbits treated with the MTX liposomal formulation at day 0 and day 7 had less joint swelling, lower skin temperature, decreased synovial fluid production and synovium proliferation compared to the contra-lateral controls. The intra-articular injection of MTX solution or the MTX liposomal formulation administered several days after antigen challenge had no
significant effect on the established arthritis (Foong and Green, 1993). Williams et al (1996) evaluated the efficacy of intra-articular delivery of 200 µg MTX in an antigen-induced arthritis model in rats. MTX solution or a MTX liposomal formulation were given 7 days following the induction of arthritis and the progression was monitored for 21 days. No significant differences in the development of arthritis were observed in the MTX solution treated rats compared with saline treated control rats, while a rapid reduction in knee swelling within 24 h and a progressive reduction in joint swelling over the next 20 days was observed in the animals treated with the MTX liposomal formulation. The overall inflammatory scores in MTX liposomal formulation treated rats were significantly lower than those in saline-treated controls (Williams et al., 1996). Williams et al (2001) also evaluated the effects of a MTX liposomal formulation on cytokine mRNA expression following intra-articular injection. On the day of the arthritis challenge, rats were treated with a single intra-articular injection of MTX liposomal formulation, or MTX solution. On day 3 and day 7 after disease induction, animals were sacrificed. The results showed that the MTX liposomal formulation significantly reduced knee swelling by day 1 and inhibited the histological progression compared to MTX solution. The local proinflammatory cytokines IL-1β and IL-6 mRNA expression in synovial tissue extracts were also reduced (Williams et al., 1995;
Williams et al., 2001). Novaes et al (1996) showed that a low dose intramuscular treatment of MTX at 0.25mg/kg to rabbits 1 week before the induction of arthritis in the knee joint, significantly reduced the intensity of leukocyte influx, protein leakage, synovial membrane cell infiltrate, as well as the production of IL-1β cytokines.

The antigen-induced arthritis model has also been used to evaluate other intra-articular anti-rheumatic and anti-inflammatory drug formulations. Horisawa et al (2002b) have shown that betamethasone encapsulated nanospheres injected intra-articularly into the joints of antigen-induced arthritis rabbits produced a reduction in joint swelling and cell infiltration compared to betamethasone solution and saline controls. Liggins et al (2004) showed that paclitaxel loaded microspheres injected intra-articularly in an antigen-induced arthritis rabbit model, produced a greater reduction in joint swelling, cellular infiltration, and cartilage damage compared to control microspheres.

In this work, the in vivo efficacy of intra-articular MTX microspheres was evaluated in the antigen-induced arthritis rabbit model, using ovalbumin as the antigen.

The objectives of this work were:

1. To evaluate the efficacy of intra-articular MTX microspheres or MTX solution in rabbits with antigen-induced arthritis in hind knee joints.
2. To determine the pharmacokinetics of MTX in the antigen-induced arthritis rabbit model following intra-articular injection of MTX loaded microspheres or MTX solution.
4.2 EXPERIMENTAL

4.2.1 Materials

Methotrexate (MTX) (MW: 454.4g/mole) was purchased from Hande Tech Development Co. (U.S.A). Aminopterin was purchased from Sigma Chemical Co. Poly (vinyl alcohol) (PVA) (98% hydrated, MW: 13,000-23,000) was obtained from Aldrich Chemical Company Inc. 7-hydroxy-methotrexate (7-OH-MTX) was purchased from Schircks Laboratories (Jona, Switzerland). Poly(L-lactic acid) (MW: 1600-2400 g/mol, intrinsic viscosity: 0.1-0.2, polydispersity: 2-3) was obtained from Polysciences (Warrington, PA). All solvents used were High Performance Liquid Chromatography (HPLC) grade. Phosphate buffered saline (PBS, pH 7.4) was prepared by dissolving 0.32g sodium dihydrogen orthophosphate, 2.15 g sodium phosphate, 8.22 g NaCl in one liter of distilled water. Phosphate buffer (pH 6.5) was prepared by dissolving 0.936 g sodium dihydrogen orthophosphate, 0.644 g sodium phosphate in one liter of distilled water and filtered through 0.2 mm filter paper. 18% MTX loaded PLLA microspheres (33-110 μm) were from the same batch of microspheres used for the high dose pharmacokinetic study previously reported in Section 3.2.2.
4.2.2 Animals and housing

Twelve female New Zealand White rabbits (weight 4-5 kg) were obtained from Riemens farm (Calgary, Canada). The experiments were conducted at the Animal Resource Center of the University of Calgary and the animals were housed in metal cages. The room was maintained at a temperature of approximately 20°C with 30-70% relative humidity and a light/dark cycle of 12 hours/12 hours. Rabbit chow and tap water were provided ad libitum to the animals for the duration of the study.

4.2.3 Animal care and use committee approval

This study fell under the scope of protocol No. MO3147 approved by the Committee on Animal Care of the University of Calgary. The experiments were conducted in accordance with the principles contained in Care of Experimental Animals – A Guide for Canada, published by the Canadian Council on Animal Care.

4.2.4 Induction of rheumatoid arthritis

Arthritis was induced in the knee joints of rabbits using a procedure similar to that described by Foong and Green (1988). Twelve female New Zealand White rabbits were immunized by injecting subcutaneously a total of 1 mL of 10 mg/mL ovalbumin (Sigma, ON, Canada) in sterile PBS emulsified in Complete Freund’s Adjuvant (Difco, Fisher Scientific Inc.). Fourteen days later, a booster of 1 mL of 10 mg/mL ovalbumin in
sterile PBS and Incomplete Freund’s adjuvant (Difco, Fisher Scientific Inc.) were given subcutaneously. Twenty-eight days after the first immunization, arthritis was induced with a 0.5 mL intra-articular injection of 10 mg/mL ovalbumin in sterile PBS into the suprapatellar pouch of both left and right hind knee joints using the medial approach while animals were anaesthetized with 1.5%- 4% inhalation halothane.

4.2.5 **Intra-articular injection and blood collection from the rabbits**

Twenty-four hours following the induction of arthritis, the animals received treatments with MTX. The animals were under 1.5%- 4% inhalation halothane anesthesia throughout the process. The right knee joints of treated rabbits received an injection of either 10 mg MTX solution in 400 µL sterile PBS (pH 7.4) (n=5) or 56 mg of 18% MTX loaded PLLA microspheres (33-110 µm) in 400 µL sterile PBS (n=5). The MTX stock solution was prepared by dissolving 100 mg MTX in 1 mL Sodium Hydroxide and dilutes it with PBS to 4 mL. The left knee joints received an injection of 400 µL sterile PBS. Control animals received an injection of 400 µL sterile PBS in both knees (n=2). The detailed assignments of intra-articular treatments to individual rabbits are given in Table 4.1.

Due to the longer duration (up to 14 days) of the experiment, the use of a jugular catheter was not approved by the Animal Care Committee in the University of Calgary.
Depending upon the ease with which blood could be obtained from individual animals, blood samples were taken from either the ear vein or the ear artery of both ears of the rabbits using a needled catheter. Immediately before (0 h) and following the intra-articular injection, serial blood samples (0.5 mL) were obtained at 0.5, 1, 2, 4, 6, 8 h to study the pharmacokinetics of MTX following intra-articular injection.

Table 4.1  Intra-articular treatments to individual rabbits one day following the induction of arthritis by ovalbumin in both knee joints. PBS: 400 μL sterile PBS, MTX microspheres: 56 mg of 18% MTX loaded PLLA2k microspheres in 400 μL sterile PBS, MTX solution: 10 mg MTX solution in 400 μL sterile PBS. The dose of MTX injected was 10 mg.

<table>
<thead>
<tr>
<th>Rabbit #</th>
<th>Intra-articular Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Right knee</td>
</tr>
<tr>
<td>1</td>
<td>PBS</td>
</tr>
<tr>
<td>2</td>
<td>MTX microspheres</td>
</tr>
<tr>
<td>3</td>
<td>MTX solution</td>
</tr>
<tr>
<td>4</td>
<td>MTX microspheres</td>
</tr>
<tr>
<td>5</td>
<td>MTX solution</td>
</tr>
<tr>
<td>6</td>
<td>MTX microspheres</td>
</tr>
<tr>
<td>7</td>
<td>MTX solution</td>
</tr>
<tr>
<td>8</td>
<td>PBS</td>
</tr>
<tr>
<td>9</td>
<td>MTX solution</td>
</tr>
<tr>
<td>10</td>
<td>MTX microspheres</td>
</tr>
<tr>
<td>11</td>
<td>MTX solution</td>
</tr>
<tr>
<td>12</td>
<td>MTX microspheres</td>
</tr>
</tbody>
</table>
4.2.6 Analysis of MTX and 7-OH-MTX in rabbit plasma

MTX and the metabolite 7-OH-MTX in the plasma samples were extracted according to the method previously reported in Section 3.2.3.1. Briefly, 1 mL acetonitrile was added to the plasma sample (0.5 mL) vortex mixed and centrifuged at 16000 x g for 5 minutes. The supernatant was transferred to a 12mL glass tube and 2mL of methylene chloride were added to extract the aqueous phase. The aqueous phase (200 µL) was dried under a stream of nitrogen gas at 45°C. The dried sample was reconstituted with 200 µL phosphate buffer pH 6.5.

4.2.7 HPLC analysis of MTX and 7-OH-MTX

MTX and 7-OH-MTX in plasma were assayed by HPLC using the method reported previously in Section 3.2.3.2. The HPLC system consisted of a Shimadzu LC-10AD pump (Shimadzu Corporation, Japan) and a Shimadzu SIL-9A automatic injector. A Waters 470 scanning fluorescence detector was used (Ex: 370 nm, Em: 417 nm). A photochemical reactor unit with a 254 nm UV lamp and a 10m x 0.25mm reaction coil (Aura Industries Inc, NY, USA) was used post-column (before the detector). The analytical column was a Novo-Pak C18 column with dimensions of 3.9 x 150 mm. The mobile phase consisted of 0.01M phosphate buffer pH 6.5, with 3.2% acetonitrile and 0.2% of 30% hydrogen peroxide (Sigma Chemical Co.) at a flow rate of 1mL/min under
ambient temperature. The injection volume was 50 µL. A folic acid antagonist
aminopterin at the concentration of 0.2 µg/mL was used as the internal standard and
calibration curves were constructed by plotting the ratio of peak areas of MTX/internal
standard versus concentrations of MTX.

4.2.8 Pharmacokinetic calculations

The plasma curves were resolved with the nonlinear regression computer program
WinNONLIN (Scientific Consulting Inc., Standard Edition, Version 1.1) using the
non-compartmental and the one-compartmental model as described in Section 3.2.6.
The elimination half-life ($t_{1/2}$), the area under the curve ($AUC_{0-\infty}$) and the mean residence
time (MRT) were determined by using Equations 3.1, 3.2 and 3.4, respectively. The
absorption half-life $t_{1/2(abs)}$ was determined by Equations 3.5 and 3.6.

4.2.9 Statistical analysis

Student's $t$ test ($p<0.05$) was used to compare pharmacokinetic parameters between
two treatments.

4.2.10 Monitoring of arthritis development

The inflammatory response was monitored by measuring changes in the joint
diameter by a digital caliper (VWR, Canada). Measurements were taken when the hind
leg was at an extended angle and flexed at a 90° angle. The differences in these two
measurements were in the range of 0.1 mm and therefore, subsequent measurements were only taken at the 90° angle. The development of arthritis was monitored for 14 days by measuring the diameter of the joint daily for the first 7 days and then every other day.

Fourteen days post intra-articular treatment, all the rabbits were humanely sacrificed and the whole knee joints were taken for standard histological analysis.

4.2.11 Histological processing of knee joints

Histological processing was carried by technical staff at the Faculty of Medicine, University of Calgary. Rabbit knee joints were transected 3-4 cm above and below the knee joint. Muscle was stripped and the joint was fixed in 10% Neutral Buffered Formalin (Fisher Scientific Inc.) for 10 days at room temperature. Joints were transferred to Cal x II decalcifying solution, which consisted of 10% formic acid solution in 4% formaldehyde (Fisher Scientific) and kept at room temperature. The solution was changed daily for 14 days at which time the joint was bisected in the sagittal plane. Joints were left for a further 14 days with changes of decalcifying solution every 2 days. The end point was decided when bones could be cut smoothly with a blade without any grittiness. The gross appearance of a decalcified rabbit knee joint is shown in Figure 4.1. The tissues were then washed thoroughly under cool running tap water for 2 hours, before being transferred to an automatic paraffin processor (Leica TP 1020). Routine
processing for histological analysis including dehydration in ethanol, clearing in xylene
and infiltration in Paraplast Plus Wax (Fisher Scientific Inc.) was accomplished
overnight. The tissue was then transferred to a vacuum oven at 60°C for 2 hrs, to ensure
complete infiltration of paraffin into the tissue. Bones were embedded in paraffin
moulds and stored at room temperature until sectioned.

Serial sections were cut 12 μm in thickness using a Leica RM 2165 microtome.
Sections were adhered to Superfrost Plus slides (Fisher Scientific) and baked at 40 °C for
5 days. Alternate slides were then stained with haematoxylin, fast green and safranin O
(Fisher Scientific Inc.). Sections was cleared in xylene, cover slipped with Polymount
mounting media (Fisher Scientific Inc.) and allowed to dry at room temperature for
several days.
Figure 4.1  The gross appearance of a decalcified rabbit knee joint. A) Rabbit knee joint transected 3-4 cm above and below the knee joint. B) The joint was bisected in the sagittal plane. C) The gross appearance of the knee joint following bisection.
4.2.12 Grading histological slides

Histological grading was carried out by experienced histologists from the Faculty of Medicine, University of Calgary. All sections were coded prior to assessment and graded in a blinded fashion to eliminate observer bias. Since there are still no standard criteria for evaluating histopathology of rheumatoid arthritis, a list of criteria was compiled and modified from those previously reported (Kapila et al., 1995; Mould et al., 2003) and is provided in Table 4.2. First, all sections were viewed under an optical microscope (Leitz DMRB) and examined for typical defects of rheumatoid arthritis and a score was determined for each defect based on the criteria. Then every 10th section was examined and graded again to compare scores. Finally, 3 slides from both medial and lateral compartments of the joint were selected at similar locations on each joint and again graded by the same person. The scores were validated by a second independent observer. The total score was determined by adding the scores for the 6 histological criteria.
Table 4.2  Scoring criteria for histological analysis of rabbit joints induced with arthritis by ovalbumin and intra-articularly treated with MTX. The list of criteria was compiled and modified based on those previous reported (Kapila et al., 1995; Mould et al., 2003).

<table>
<thead>
<tr>
<th>Histopathological features</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Synovial lining hyperplasia</strong></td>
<td></td>
</tr>
<tr>
<td>1-3 layers</td>
<td>0</td>
</tr>
<tr>
<td>4-6 layers</td>
<td>1</td>
</tr>
<tr>
<td>&gt; 7 layers</td>
<td>2</td>
</tr>
<tr>
<td><strong>Villous hyperplasia</strong></td>
<td></td>
</tr>
<tr>
<td>Not present</td>
<td>0</td>
</tr>
<tr>
<td>Few scattered and short villi</td>
<td>1</td>
</tr>
<tr>
<td>Marked finger-like villi</td>
<td>2</td>
</tr>
<tr>
<td>Marked diffuse villi</td>
<td>3</td>
</tr>
<tr>
<td><strong>Inflammatory cell infiltrate</strong></td>
<td></td>
</tr>
<tr>
<td>Normal cellularity</td>
<td>0</td>
</tr>
<tr>
<td>Increased cellularity</td>
<td>1</td>
</tr>
<tr>
<td>Patchy discontinuous cellular infiltration</td>
<td>2</td>
</tr>
<tr>
<td>Diffuse continuous cellular infiltration</td>
<td>3</td>
</tr>
<tr>
<td><strong>Inflammatory exudates</strong></td>
<td></td>
</tr>
<tr>
<td>No exudates present</td>
<td>0</td>
</tr>
<tr>
<td>Mild exudates present intra-articularly</td>
<td>1</td>
</tr>
<tr>
<td><strong>Pannus formation</strong></td>
<td></td>
</tr>
<tr>
<td>No pannus formation</td>
<td>0</td>
</tr>
<tr>
<td>Pannus with superficial cartilage destruction</td>
<td>1</td>
</tr>
<tr>
<td>Pannus with destruction to depth of mid zone</td>
<td>2</td>
</tr>
<tr>
<td>Pannus with destruction to depth of tide mark</td>
<td>3</td>
</tr>
<tr>
<td><strong>Cartilage destruction</strong></td>
<td></td>
</tr>
<tr>
<td>Cartilage intact</td>
<td>0</td>
</tr>
<tr>
<td>Minimal abrasion with no obvious loss of chondrocytes or collagen disruption</td>
<td>1</td>
</tr>
<tr>
<td>Destruction with superficial loss of chondrocytes and/or collagen disruption</td>
<td>2</td>
</tr>
<tr>
<td>Moderate loss of chondrocytes and collagen disruption to mid zone</td>
<td>3</td>
</tr>
<tr>
<td>Destruction with severe loss of chondrocytes to tide mark</td>
<td>4</td>
</tr>
</tbody>
</table>
4.3 RESULTS

4.3.1 MTX and 7-OH-MTX plasma concentrations

An indwelling catheter was not approved for use in these long-term animal studies and blood samples were taken at each time point following intra-articular injection using a needled catheter from either the ear vein or artery. It was difficult to take blood samples via these routes and an average of 10 min was required to remove sufficient blood for a sample. Thus, samples could not be taken at either 5 min or 15 min, as for previous pharmacokinetic studies.

The MTX plasma profiles following intra-articular injection of either 10 mg MTX solution or 56 mg of 18% loaded microspheres are shown in Figure 4.2. Significant variability between different animals was observed in both treatment groups. Hence, individual MTX plasma profiles for the group treated with MTX solution and the group treated with MTX microspheres are given in Figure 4.3 and Figure 4.4, respectively. In the group treated with MTX solution, rabbits #7, #9 and #11 showed maximal MTX plasma concentrations within 2 h following intra-articular injection of the microspheres (Figure 4.3). MTX was still detectable 24 h following injection. Two rabbits #3 and #5, showed peak MTX concentrations at between 30 min to 1 h following intra-articular injection of MTX and peak plasma concentrations were about 10 fold higher than rabbits
Rabbit #5 showed a prolonged phase of elimination compared to all other rabbits. For rabbits injected with MTX microspheres, the inter-individual variability in MTX plasma profiles was not as large as in the group treated with MTX solution. The peak MTX concentrations in the group treated with MTX microspheres ranged from about 0.1 to 0.3 μg/mL and gradually declined to undetectable levels 8 h following the injection (Figure 4.4).

The 7-OH-MTX plasma profiles of rabbits injected with either MTX solution or MTX loaded microspheres are shown in Figure 4.5 and 4.6. For rabbits injected with MTX solution, the concentration of 7-OH-MTX peaked between 2 to 3 h and peak concentrations were between 0.5 and 1.3 μg/mL. Except in one rabbit (#5), the concentrations of 7-OH-MTX were higher than MTX in plasma from 2 to 8 h following injection (Figure 4.5). For rabbits injected with MTX microspheres, the concentrations of 7-OH-MTX peaked between 0.5 to 2 h following injection, and peak concentrations of 7-OH-MTX were between 0.1 and 0.3 μg/mL (Figure 4.6).

The pharmacokinetic analysis of the MTX plasma profiles following the intra-articular injections of MTX solution or MTX microspheres into inflamed joints are shown in Table 4.3. Data from normal joints previously reported in Table 3.6 are shown for ease of comparison. For rabbits with antigen induced arthritis, the area under the
curve (AUC \(0-\infty \)) of the MTX plasma profile of rabbits injected with MTX solution was significantly higher than the MTX plasma profile for rabbits injected with MTX microspheres. The elimination half-lives \( (t_{\frac{1}{2}}) \) were 6.7 h and 2.5 h for rabbits injected with MTX solution and MTX microspheres, respectively. The differences observed in elimination half-lives \( (t_{\frac{1}{2}}) \) and mean residence time (MRT) between two treatment groups were not statistically significant. Comparing diseased and normal animals, the MTX mean absorption half-lives \( (t_{\frac{1}{2}}_{\text{abs}}) \) and elimination half-lives \( (t_{\frac{1}{2}}) \) for diseased animals in both treatment groups were longer than those of the healthy animals. However, statistical analysis of the data did not show any significant difference due to the large standard deviations.
Figure 4.2 MTX concentrations in plasma of antigen (ovalbumin) induced arthritis rabbits following a single intra-articular injection of either 10 mg MTX solution in 400 μL PBS or 56 mg of 18% PLLA2k MTX loaded microspheres (33-110 μm) in 400 μL PBS into the right hind knee joint. Left hind knee joints were injected with 400 μL PBS. Values are mean ± one standard deviation, (n = 5). The dose of MTX injected was 10mg. (●) MTX solution, (■) MTX microspheres.
Figure 4.3  MTX plasma concentrations of individual antigen (ovalbumin) induced arthritis rabbits following a single intra-articular injection of 10 mg MTX solution in 400 μL PBS into the right hind knee joint. Left hind knee joints were injected with 400 μL PBS. (♦) rabbit #3, (■) rabbit #5, (▲) rabbit #7, (×) rabbit #9, (●) rabbit #11.
Figure 4.4  MTX plasma concentrations of individual antigen (ovalbumin) induced arthritis rabbits following a single intra-articular injection of 56 mg of 18% MTX loaded PLLA2k microspheres (33-110 \( \mu \)m) in 400 \( \mu \)L PBS into the right hind knee joint. Left hind knee joints were injected with 400 \( \mu \)L PBS. The dose of MTX injected was 10 mg MTX. (\( \bullet \)) rabbit #2, (■) rabbit #4, (▲) rabbit #6, (×) rabbit #10, (●) rabbit #12.
Figure 4.5 7-OH-MTX plasma concentrations of individual antigen (ovalbumin) induced arthritis rabbits following a single intra-articular injection of 10 mg MTX solution in 400 µL PBS into the right hind knee joint. Left hind knee joints were injected with 400 µL PBS. (♦) rabbit #3, (■) rabbit #5, (▲) rabbit #7, (×) rabbit #9, (●) rabbit #11.
**Figure 4.6** 7-OH-MTX plasma concentrations of individual antigen (ovalbumin) induced arthritis rabbits following a single intra-articular injection of 56 mg of 18% MTX loaded PLLA2k microspheres in 400 μL PBS to the right hind knee joint. Left hind knee joints were injected with 400 μL PBS. The dose of MTX injected was 10 mg MTX. (♦) rabbit #2, (■) rabbit #4, (▲) rabbit #6, (×) rabbit #10, (●) rabbit #12.
Table 4.3 Pharmacokinetic parameters for MTX plasma profiles of antigen (ovalbumin) induced arthritis rabbits following an intra-articular injection of either MTX solution or MTX loaded microspheres. The dose of MTX injected was 10mg. Data from normal rabbits injected with the same amount of MTX solution and MTX microspheres are shown here for ease of comparison (from Table 3.6). The parameters were determined using WinNonlin computer program.

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<th>Treatment</th>
<th>$t_{1/2(abs)}$ (h)$^a$</th>
<th>$t_{1/2}$ (h)</th>
<th>$AUC_{0-\infty}$ ($\mu g/mL.h$)</th>
<th>MRT (h)</th>
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<td>MTX solution</td>
<td>0.59 ± 0.46</td>
<td>6.72 ± 4.62</td>
<td>6.99 ± 7.20$^*$</td>
<td>8.81 ± 5.96</td>
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<td>MTX microspheres</td>
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<td>2.54 ± 1.79</td>
<td>0.47 ± 0.18</td>
<td>3.92 ± 2.68</td>
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<td>Normal joints</td>
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<td>MTX solution</td>
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<td>0.94 ± 0.18</td>
<td>4.40 ± 2.04$^*$</td>
<td>1.28 ± 0.30</td>
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<td>MTX microspheres</td>
<td>0.06 ± 0.03</td>
<td>0.92 ± 0.18</td>
<td>0.65 ± 0.23</td>
<td>1.34 ± 0.23</td>
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</table>

$^*$Statistical difference between two treatments by student’s t-test ($p<0.05$).

$^a$Absorption half life $t_{1/2(abs)}$ was estimated by one-compartmental analysis.
4.3.2 Joint swelling evaluation

The joint diameters of the rabbits were measured using a digital caliper. Except on day 0 and day 1, the rabbits were not under anesthesia when the knee joint diameter was measured. The results of knee swelling measurements (knee diameter on each day minus knee diameter on day 0) are provided in Figure 4.6 (A-C). The joint swelling consistently increased in the first three days following the injection of MTX solution or MTX microspheres and decreased on day 4. The swelling then peaked again at 7 days following the injection of MTX solution or microspheres and gradually declined to similar level as day 1. There were no significant differences between joints injected with MTX microspheres, MTX solution or PBS.
A) Days following induction of arthritis

B) Days following induction of arthritis

C) Days following induction of arthritis
Figure 4.7  Knee joint swelling following antigen (ovalbumin) induction of arthritis in rabbits. One day following the induction of arthritis, the right hind knees of the rabbits were injected with either 10 mg MTX solution in 400 μL PBS or 56 mg of 18% MTX loaded microspheres (33-110 μm) in 400 μL PBS. The left hind knee joints were injected with 400μL PBS. A) Comparison of knee joint swelling between MTX solution treated knees and 400μL PBS treated contralateral knees, B) comparison of knee joint swelling between MTX loaded microspheres treated and PBS treated contralateral knees, C) comparison of knee joint swelling between MTX solution treated knees and MTX microspheres treated knees. Values are mean ± one standard deviation, (n = 5). The dose of MTX injected was 10mg. (♦) MTX solution, (■) MTX microspheres. (×) PBS.
4.3.3 Histological Analysis

Fourteen days following the intra-articular treatment with MTX solution or MTX microspheres, the rabbits were sacrificed and the whole joints were isolated and processed for histological analysis.

Histological slides were scored in five categories: synovial lining hyperplasia, villous hyperplasia, inflammatory cell infiltrate, inflammatory exudates, pannus formation and cartilage destruction. These histopathological features were clearly observed in the rabbits induced with arthritis suggesting that arthritis was successfully induced in this animal model (Figure 4.10-Figure 4.16). Optical micrographs of the cartilage of a healthy rabbit knee joint obtained from another independent study are shown in Figure 4.8 for comparison. In the healthy joint, the cartilage surfaces covering femur and tibia are smooth with the different zones that are commonly used to describe the cartilage, clearly visible (Figure 4.8B). Zone I is the superficial zone of the cartilage that only comprises 1-3 layers of chondrocytes. Zone II is the transitional zone where oval or rounded chondrocytes are found. Zone III is the deep zone where large rounded chondrocytes are arranged in columns perpendicular to the surface. Zone IV is the calcified zone which rests on underlying subchondral bone. A line called the “tide mark” demarcates zone III from zone IV (Ghadially, 1983) (Figure 4.8B). The optical
micrographs of the synovial membrane from a healthy rabbit knee joint obtained from another independent study are shown in Figure 4.9. The synovial membrane is one to three layers in thickness lying on top of the adipose tissues.

All rabbits induced with arthritis showed cartilage destruction in both knee joints and the severity ranged from score 1 to 4 (Figure 4.10). Fissures or clefts were observed perpendicular to the surface of the cartilage and extended to the transitional zone (score 2) (Figure 4.10 B) or radial zone (score 3-4) (Figure 4.10 C-D) resulting in disorganization of the cellular rows and clustering of the cells in the regions. The disruption of the integrity of the tidemark due to the fissures and clefts (score 4) could be also observed in some rabbits (Figure 4.10 D). The destruction of the cartilage was also marked by the loss of proteoglycan as observed by the loss of matrix staining (Figure 4.11). There were regions in the cartilage showing interstitial loss of the matrix accompanied by the abnormality of the cells (Figure 4.11B and C). It appeared that these regions were more readily observable in the inflamed joints treated with PBS than in the joints treated with MTX microspheres. However, it was difficult to assign scores given each region had different sizes and different degree of loss of matrix. Therefore, we did not include this feature in evaluating cartilage destruction.
Pannus formation was observed in some animals from both treatment groups (Figure 4.12). Pannus is the region of proliferated synovial membrane that invades cartilage. It is usually enclosed with a membrane and attached to the cartilage. The destruction of cartilage by pannus ranged from superficial cartilage destruction (score 1) (Figure 4.12 A and B) to destruction to the depth of mid zone (score 2) (Figure 4.12 C and D).

Two fold to four fold increases in the thickness of the synovial lining were observed in the animals induced with arthritis (Figure 4.13). A few scattered and finger-like villi with hyperplasia were also observed close to the cartilage (Figure 4.14). Synovial hyperplasia was accompanied by increased cellularity in the subsynovial layer indicating inflammatory cellular infiltration. Local generation of fibrin due to inflammation could be observed as the pink staining (Figure 4.15).

Inflammatory exudates could be observed in the joints in some animals in the cavity between the synovial membrane and cartilage (Figure 4.16). Exudates make up the region of high protein content and cellular debris which have escaped from blood vessels and been deposited in tissues or on tissue surfaces (Ghadially, 1983). Sometimes the exudates may be confused with the pannus, because both exudates and pannus consist of hyperplastic synovial cells and are located in the joint cavity. However, the exudates are usually not enclosed with a membrane and the structure appears less dense than the
pannus. The exudates were generally located closer to the synovial membrane while the pannus was attached to the cartilage.

The histological evaluation and scoring of pathological features for rabbits treated with MTX microspheres, MTX solution and PBS controls, are shown in Tables 4.4 and 4.5. There were no significant differences in the total scores between the MTX treated (solution or microspheres) and the PBS treated joints. Although the total score was higher in the knee joints treated with MTX solution than in the knee joints treated with MTX loaded microspheres, the difference was not significant.

Additional micrographs demonstrating the pathological features of the synovial joints of individual animals from both treatment groups are shown in Appendix I.
Figure 4.8  Optical micrographs of the cartilage of a normal rabbit knee joint obtained from another independent study, provided by the Faculty of Medicine, University of Calgary.  A) Cartilage of femur, tibia and part of the meniscus of the knee joint. (25x), B).Cartilage of tibia at a higher magnification (100x). Zones of cartilage are marked. Round circular cells in dark purple color are the chondrocytes.
Figure 4.9  Optical micrographs of the synovial membrane of a normal rabbit knee joint obtained from another independent study, provided by the Faculty of Medicine, University of Calgary.  A) Synovial membrane (25x) (indicated by the arrows), B) Synovial membrane at a high magnification (100x). car: cartilage. adp: adipose cells. j cav: joint cavity.
Figure 4.10 Representative micrographs of cartilage destruction due to arthritis induced by ovalbumin in rabbit knee joints. One day following the induction of arthritis, the right hind knees of the rabbits were injected with either 10 mg MTX solution in 400 μL PBS or 56 mg of 18% MTX loaded PLLA2k microspheres (33-110 μm) in 400 μL PBS. The left hind knee joints were injected with 400 μL PBS. The rabbits were sacrificed 14 days following MTX treatment and knee joints were processed for histological analysis. A) Minimal abrasion as indicated by the arrow on the surface of the cartilage represents score 1. The joint was injected with 400 μL PBS. B) Destruction with superficial loss of chondrocytes and cartilage disruption indicated by the arrow (score 2). The joint was injected with MTX microspheres. C) Moderate loss of chondrocytes and cartilage disruption to mid zone (score 3). The joint was injected with PBS. D) Destruction with severe loss of chondrocyte to tide mark as indicated by the arrow (score 4). The joint was injected with PBS. All micrographs shown are at a magnification of 25x. men: meniscus, car: cartilage, j cav: joint cavity.
Figure 4.11 Representative micrographs of cartilage interstitial loss of matrix due to arthritis induced by ovalbumin in rabbit knee joints. One day following the induction of arthritis, the right hind knees of the rabbits were injected with either 10 mg MTX solution in 400 μL PBS or 56 mg of 18% MTX loaded PLLA2k microspheres (33-110 μm) in 400 μL PBS. The left hind knee joints were injected with 400 μL PBS. The rabbits were sacrificed 14 days following MTX treatment and knee joints were processed for histological analysis. No scores were given for this histopathological feature. A) interstitial matrix loss (pointed by arrows) but surface remained intact. The joint was injected with MTX solution. B) Interstitial matrix loss accompanied by abnormal cellularity indicated by the rectangle. The joint was injected with MTX microspheres. C) The rectangular section of B with a higher magnification (100 x). The interstitial matrix loss was evidenced by the decrease of intensity of the color stain compared to intensity of stained cartilage in Figure 4.8. men: meniscus, car: cartilage, j cav: joint cavity.
Figure 4.12  Representative micrographs of pannus formation due to arthritis induced by ovalbumin in rabbit knee joints. One day following the induction of arthritis, the right hind knees of the rabbits were injected with either 10 mg MTX solution in 400 µL PBS or 56 mg of 18% MTX loaded PLLA2k microspheres (33-110 µm) in 400 µL PBS. The left hind knee joints were injected with 400 µL PBS. The rabbits were sacrificed 14 days following MTX treatment and knee joints were processed for histological analysis.

A) Pannus with superficial cartilage destruction as indicated by the rectangle represents score 1. The joint was injected with PBS. A higher magnification (100x) of the rectangular section is shown in B). C) Pannus with destruction to the depth of mid zone as indicated by the rectangle (score 2). The joint was treated with MTX microspheres. The cut parallel to the surface of the cartilage as shown by the arrow is an artifact due to histological processing. A higher magnification (100x) of the rectangular section is shown in D). men: meniscus, j cav: joint cavity.
Figure 4.13  Representative micrographs of synovial tissue hyperplasia due to arthritis induced by ovalbumin in rabbit knee joints. One day following the induction of arthritis, the right hind knees of the rabbits were injected with either 10 mg MTX solution in 400 μL PBS or 56 mg of 18% MTX loaded PLLA2k microspheres (33-110 μm) in 400 μL PBS. The left hind knee joints were injected with 400 μL PBS. The rabbits were sacrificed 14 days following MTX treatment and knee joints were processed for histological analysis. A) 4-6 layer increase in synovial thickness as indicated by the arrow represents score 1. The joint was injected with PBS. B) >7 layer increase in synovial thickness as indicated by the double arrow (score 2). The joint was injected with PBS. Both A) and B) are at a magnification of 25x. car: cartilage.
Figure 4.14 Representative micrographs of villous hyperplasia in synovial tissues due to arthritis induced by ovalbumin in rabbit knee joints. One day following the induction of arthritis, the right hind knees of the rabbits were injected with either 10 mg MTX solution in 400 µL PBS or 56 mg of 18% MTX loaded PLLA2k microspheres (33-110 µm) in 400 µL PBS. The left hind knee joints were injected with 400 µL PBS. The rabbits were sacrificed 14 days following MTX treatment and knee joints were processed for histological analysis. A) A few scattered and short villous hyperplasia as indicated by the arrow represents score 1. The joint was injected with PBS. B) Marked finger like villi indicated by the arrow (score 2). The joint was injected with MTX microspheres. Both micrographs are at a magnification of 25 x. j cav: joint cavity.
Figure 4.15  Representative micrographs of inflammatory cellular infiltration in synovial tissues due to arthritis induced by ovalbumin in rabbit knee joints. One day following the induction of arthritis, the right hind knees of the rabbits were injected with either 10 mg MTX solution in 400 μL PBS or 56 mg of 18% MTX loaded microspheres in 400 μL PBS. The left hind knee joints were injected with 400 μL PBS. The rabbits were sacrificed 14 days following MTX treatment and knee joints were processed for histological analysis. A) Increased cellularity and fibrin (pink stain) in the subsynovial layer of the synovial tissue represents score 3. The joint was injected with PBS. Magnification 25x. A higher magnification (100x) of the region of increased cellularity is shown in B).
Figure 4.16  Representative micrographs of inflammatory exudates in the joint cavity due to arthritis induced by ovalbumin in rabbit knee joints. One day following the induction of arthritis, the right hind knees of the rabbits were injected with either 10 mg MTX solution in 400 µL PBS or 56 mg of 18% MTX loaded microspheres in 400 µL PBS. The left hind knee joints were injected with 400 µL PBS. The rabbits were sacrificed 14 days following MTX treatment and knee joints were processed for histological analysis. A) Exudates in the joint cavity represent score 1. The joint was treated with MTX solution. (Magnification 25 x). A higher magnification (100 x) of the exudates is shown in B).  exu: inflammatory exudates. j cav: joint cavity.
Table 4.4  Quantitative assessment of histopathological features of arthritis induced by ovalbumin in rabbit knees following MTX microspheres treatment. One day following the induction of arthritis, the right hind knees of the rabbits were injected with 56 mg of 18% MTX loaded PLLA2k microspheres (33-110 μm) in 400 μL PBS. The left hind knee joints were injected with 400 μL PBS. Control rabbits were injected with 400μL PBS in both knee joints. The rabbits were sacrificed 14 days following MTX treatment and knee joints were processed for histological analysis.

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<th>Synovial and Villous hyperplasia</th>
<th>Inflammatory cell infiltrate</th>
<th>Inflammatory exudates</th>
<th>Pannus formation</th>
<th>Cartilage destruction</th>
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Table 4.5  Quantitative assessment of histopathological features of arthritis induced by ovalbumin in rabbit knees following MTX solution treatment. One day following the induction of arthritis, the right hind knees of the rabbits were injected with 10 mg MTX solution in 400 µL PBS. The left hind knee joints were injected with 400 µL PBS. Control rabbits were injected with 400 µL PBS in both knee joints. The rabbits were sacrificed 14 days following MTX treatment and knee joints were processed for histological analysis.

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± SD
4.4 DISCUSSION

Antigen-induced arthritis in rabbits provides one of the best models of rheumatoid arthritis available (Foong and Green, 1988). The joint histopathology of antigen-induced arthritis closely resembles rheumatoid arthritis (Dumonde and Glynn, 1962; Foong and Green, 1993). It has been reported that from 1 to 4 h following intra-articular challenge in rabbits, acute inflammation associated with thick, purulent exudates, severe edema, and hemorrhagic synovitis developed and reached its peak between 24 and 48 h after antigen injection. The acute inflammatory response subsided slowly over a period of 7-10 days. By 2-4 weeks, histological examination showed a typical picture of chronic inflammation. The synovium appeared thickened and slightly congested and pannus tissue could be seen in some rabbits (Jasin, 1988). In this work, similar inflammatory responses were observed in the ovalbumin induced arthritis rabbit model. The knee joint swelling of all rabbits increased in the first three days following the induction of arthritis due to acute inflammation. The swelling gradually declined to similar levels as day 1 by 14 days following induction of arthritis. Histological slides of rabbit joints at 14 days following the induction of arthritis showed that the arthritis was well developed with distinct histopathological features such as cartilage destruction, synovial hyperplasia, pannus formation, and cellular infiltration (Figures 4.10 to 4.16).
In this study, MTX solution or MTX loaded microspheres were given one day following the induction of arthritis. Early use of MTX at the development of the arthritis would favor the suppression of the arthritis, based on several studies of the efficacy of intra-articular delivery of MTX in different animals with different onset of therapy (Foong and Green, 1993; Williams et al., 1996; Williams et al., 2001).

Significant variability was observed in the MTX plasma profiles of diseased rabbits following intra-articular injection of either MTX solution or MTX microspheres (Figures 4.5 and 4.6). The large variability observed in the plasma profiles was likely due to the arthritis developed in the joints. Within a day following the injection of antigen in both knee joints of the rabbits, the development of arthritis was quite variable. The degree of swelling ranged from 2.3 to 9.8 mm in the joints injected with MTX solution, and from 2.6 mm to 6.8 mm in the joints injected with MTX microspheres (Figure 4.7 C). For rabbits injected with MTX solution, the maximum MTX plasma concentrations were approximately 0.5 µg/mL for rabbits with a joint swelling greater than 8 mm (Rabbit # 7, 9, 11), while the maximum MTX plasma concentrations were approximately 5 fold higher for rabbits with a joint swelling less than 6 mm (Rabbit # 3 and #5). Similar relationship between the maximum MTX concentrations in plasma and the degree of joint swelling was observed in rabbit injected with MTX microspheres, such that rabbits
with a higher degree of joint swelling had lower maximum MTX plasma concentrations and rabbits with decreased joint swelling showed higher maximum MTX plasma concentrations. The maximum MTX plasma concentrations of rabbits with the highest swelling in both treatment groups also occurred at 0.5 to 1 h later than the rabbits that had smaller joint swelling. It has been shown that most small molecules cross the synovium in both directions by passive diffusion, and are limited primarily by the relatively long, narrow diffusion path between synovial lining cells (Simkin and Pizzorno, 1974). Joints with larger effusions as indicated by a higher degree of swelling would have lower MTX concentrations in the synovial fluid resulting in a smaller concentration gradient across the synovial membrane. Since an increase in joint inflammation also leads to increased synovial thickening, the rate of transport of MTX across the synovium into the circulation may be decreased. It has been shown that the synovial thickening and cellular infiltration in chronic rheumatoid arthritis can cause a greater than two fold increase in average transport distance from the capillary to the joint cavity (Stevens et al., 1991).

Although the development of arthritis in this study was not considered chronic at the time of the injection of MTX treatments, groups investigating similar arthritis models have shown that significant synovial proliferation took place 24 h following the induction of arthritis (Howson, et al., 1986). It is possible that slower transport of MTX across
inflamed synovium may have resulted in both lower maximum MTX plasma levels and increased mean absorption half-life in diseased animals (Table 4.3). However, statistical analysis of the absorption half-life data did not show any significant difference due to the large standard deviations. Similar to previous data in healthy joints, the AUC of the group treated with MTX solution was significantly higher than the group treated with microspheres (Table 4.3) indicating that more MTX entered systemic circulation following the injection of MTX solution than MTX microspheres.

7-OH-MTX appeared in the plasma within about 0.5 h and peaked between 1 and 2 h following intra-articular injection in both treatment groups (Figures 4.5 and 4.6) indicating the rapid conversion of MTX to its metabolite 7-OH-MTX. Significant individual variability was also observed in the 7-OH-MTX plasma profiles. Variable plasma MTX concentrations and variable hepatic MTX-7-hydroxylase activities among individual rabbits (Kitamura et al., 1999) likely contributed to the variability observed in the 7-OH-MTX plasma profiles.

Although the exact mechanism of action of MTX in the treatment of rheumatoid arthritis has not been fully elucidated, low dose MTX in rheumatoid arthritis treatment seems to exert anti-inflammatory effects by acting at different levels of the pathophysiological cascade. The direct anti-proliferative effects on monocytic cells
involved in the immune and inflammatory reactions represents the first step of intervention (Cutolo et al., 2001). However, inhibition of both monocytic and lymphocytic proinflammatory cytokines secreted by infiltrated macrophages and lymphocytes involved in rheumatoid synovitis seems to be the key role in the sustained anti-inflammatory actions exerted by low dose MTX (Cutolo et al., 2001). Evidence has shown that MTX reduced the production of proinflammatory monocytic and macrophagic cytokines such as IL-1, IL-6, and TNF-α, and increased, at least, gene expression of anti-inflammatory Th2 cytokines such as IL-4 and IL-10 with resulting anti-inflammatory effects (Cutolo et al., 2001). Williams et al (2001) have shown that the histological progression of antigen-induced arthritis in rats was significantly inhibited following an intra-articular injection of MTX liposomal formulation. The reduction in disease severity was accompanied by a reduction in local IL-6 and IL-1β mRNA expression in synovial tissue extracts from the rat knee joints.

Although we hypothesized that the delivery of MTX to joints via a controlled release formulation should exert an anti-inflammatory effect, the results of this in vivo efficacy study did not show that intra-articular delivery of MTX significantly improved the progression of arthritis in the antigen-induced arthritis model. Based on a system of scoring the pathological features of the disease including synovial lining and villous
hyperplasia, inflammatory cell infiltration and exudates, pannus formation, and cartilage
destruction, there was no significant difference between MTX solution and microspheres
treated groups compared to PBS (control) animals. One possible explanation for the
lack of efficacy in this animal model might be the severity of the disease induced.

Histological analysis of the cartilage showed that 14 days following the induction of
arthritis, fissures or clefts extended to the transitional zone or radial zone of the cartilage
resulting in cell death and loss of proteoglycans could be also observed (Figure 4.10).

These clinical features were considered rather severe in terms of cartilage destruction and
only are observed in patients with a late stage of rheumatoid arthritis development
(Howson et al., 1986). Howson et al (1986) have investigated the effects of the
challenge dose of ovalbumin on the joint tissues of rabbits. Arthritis was induced in
both knee joints of the rabbits either with 7.5 mg ovalbumin, which is considered a dose
at the upper end of the range used by most investigators or a 0.1 mg dose at the lower end
of the range. The animals were sacrificed from 1 day to 8 months post arthritis
induction in the high dose group and up to 11 months in the low dose group and
progression of the disease was evaluated by histological analysis. The gross and
histological changes in the knee joint in the high dose group was similar to acute cartilage
necrosis with profound inhibition of chondrocyte activity in the first 2 weeks resulting in
widespread cell death and some survival of chondrocytes. The low dose challenged animals on the other hand, developed a mild arthritis with a synovitis that persisted for 11 months. The cartilage showed irregular chondrocyte inhibition, death and degeneration of the matrix. The authors suggested that, depending on the challenge dose used, there is a tremendous variability in the degree of arthritis produced by the antigen induced arthritis model (Howson et al., 1986). In this study, 5 mg ovalbumin was used to induce arthritis in both knee joints of the rabbits. This dose was chosen because it is the most commonly used dose to induce arthritis in rabbits by other investigators (Foong and Green, 1988; Foong and Green, 1993; Horisawa et al., 2002b). However, given that the cartilage was severely damaged while other histopathological features were less prominent 14 days following the antigen challenge, the dose of ovalbumin may have been too high. The arthritis model developed in this study might not fully represent the disease features of rheumatoid arthritis. It is possible that the severity of the cartilage damage might have masked the efficacy of the drug. It would be beneficial to include a positive control treatment group, such as an intravenous injection of MTX to validate the therapeutic effects of MTX in controlling the progression of disease in this animal model.

In this work, the disease progression was monitored for 14 days. Foong and Green (1993) have shown that the effect of joint swelling and surface temperature suppression
was not observed until 14 days following treatment with an intra-articular injection of a 1 mg MTX liposomal formulation in inflamed rabbit joints. Gross and histological examination of the opened joints 56 days following arthritis induction showed a significant reduction in inflammatory changes and erosion of cartilage. Therefore, longer time periods for monitoring may be necessary for the observation of therapeutic effects.

In summary, a severe antigen-induced inflammatory arthritis was produced in rabbits who were subsequently treated with a 10 mg dose of MTX solution or MTX microspheres. Maximum plasma MTX concentrations seemed to correlate with the degree of inflammation such that lower maximum MTX concentrations were produced in animals with greater joint swelling. The data showed that there was no significant difference between MTX solution and MTX microspheres treated groups compared to PBS (control) animals.
Chapter 5

SUMMARIZING DISCUSSION, CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

5.1 SUMMARIZING DISCUSSION

MTX, an antifolate drug, has been shown to exert anti-inflammatory effects by inhibiting proliferation of cells involved in the inflammatory reaction and inhibiting both monocytic and lymphocytic proinflammatory cytokines involved in rheumatoid synovitis. Intra-articular delivery of MTX has been attempted in patients for the purpose of targeting the drug to the site of action and to minimize systemic toxic effects of the drug (Marks et al., 1976; Bird et al., 1977). However, intra-articular injections of a MTX solution were not therapeutically effective due to the rapid clearance of MTX from the synovial joint (Wigginton et al., 1980). Previous work has shown that following the intra-articular injection of liposomal formulations of MTX in both antigen induced arthritis rabbit and rat models, increased amounts of MTX were retained in the joints and joint swelling and synovium proliferation were decreased compared to the injection of MTX solution (Foong and Green, 1998; Williams et al., 1996). Thus, we have proposed that intra-articular injections of a controlled release MTX delivery system should maintain an effective concentration of the drug in the joint cavity. This would be
particularly beneficial for patients in whom the arthritis manifests in only a limited number of joints.

The goals of this work were to develop and characterize controlled release polymeric microspheres formulations of MTX and to investigate the *in vivo* biodistribution and efficacy of microspheres formulations following intra-articular injection in an established arthritis rabbit model. We have proposed the following ideal properties of the microspheres formulation for this purpose: the formulation should be composed of biocompatible and biodegradable polymeric materials; the formulation should possess an optimal and reproducible size distribution, and the formulation should be readily injected and sterilizable. Furthermore, based on the design of the *in vivo* pharmacokinetic and efficacy study, we also proposed an optimal MTX release lifetime of 2-3 weeks.

PLLA polymers were selected as a suitable polymeric biomaterial for the microspheres formulation. MTX loaded microspheres prepared using PLLA2k were selected as the lead formulation for characterization work, based on the faster and more complete release of MTX from the PLLA2k microspheres over a period of 2-3 weeks, compared to the PLLA50k and PLLA100k microspheres. Microspheres in the size range of 33-110 μm were selected for characterization based on previous work showing
that microspheres in this size range were well tolerated, while smaller size microspheres
induced higher levels of cellular infiltration and inflammation (Liggins et al., 2004).

Solid state characterization of MTX loaded microspheres showed that MTX was
likely dispersed in the microsphere matrix as a particulate dispersion. The drug release
profiles of MTX loaded PLLA2k microspheres in the 33-110 μm size range demonstrated
an extensive and rapid burst phase followed by a slow controlled release phase and
approximately 95% of loaded drug was released over 2 weeks. The initial burst phase of
MTX release was likely due to dissolution of MTX near the surface of the microspheres.
The cavities and pores remaining from the loss of particulate MTX following drug
release near the surface of microspheres served as a preferred pathway for drug diffusion,
contributing to the rapid burst release phase. The subsequent slower release phase was
controlled by a combination of drug diffusion and polymer degradation. In vitro
degradation studies of MTX loaded PLLA2k microspheres showed 20% of total mass
was lost in 40 days and the molecular weight of PLLA2k was decreased by 25 % in 28
days due to polymer degradation. Gamma irradiation sterilization produced no adverse
effects on the microspheres and had no influence on MTX release rates. Hence the
formulation could be readily sterilized prior to injection using this procedure.
The injection of control or MTX loaded PLLA2k microspheres in healthy rabbit joints produced a mild inflammatory response over the first three days and subsided thereafter without any significant loss of proteoglycans from cartilage. The completion of this characterization work demonstrated that the formulation possessed the properties felt to be ideal for the desired application, as previously discussed. Thus, PLLA2k microspheres in the 33-110 μm size range were selected for further pharmacokinetic and efficacy studies.

To understand the biodistribution and elimination of MTX following intra-articular injection, two studies were carried out in which either MTX solution or PLLA2k MTX loaded microspheres were injected into the joints of healthy rabbits with injected MTX doses of 1.5 mg and 10 mg. The plasma MTX profiles of both studies showed that MTX solution was rapidly transferred across the synovium and entered the systemic circulation while MTX microspheres released MTX slowly in the joint cavity resulting in lower plasma MTX concentrations. Urine data showed that during the period of study, higher amounts of drug were excreted in the urine following the injection of MTX solution than following the injection of MTX loaded microspheres. Synovial fluid and synovial tissues analysis at 6 h and 24 h following intra-articular injection showed that significantly higher amounts of MTX were retained in the joints injected with MTX.
microspheres than in the joints injected with MTX solution. Histological analysis of synovial tissues showed no evidence of phagocytosis of the microspheres and evidence suggested that microspheres were located in the adipose layer of the synovial tissues. The delivery of MTX in a controlled manner using microspheres, therefore achieved a greater retention of MTX in the joint compared to delivery of MTX as a solution.

It has been shown that for a typical plasma MTX concentration profile following an oral dose of 15 mg in patients with rheumatoid arthritis, the maximum plasma MTX concentration was about 0.7 μM and concentrations decreased in a triphasic manner. Constant levels of 1 nM were maintained for several days (Hillson et al., 1997). The concentration of MTX in synovial fluid has been reported to be roughly equal to the concentration of MTX in plasma at 4 and 24 h after an oral MTX dose of 10 mg/m² in human subjects (Herman et al., 1989). Therefore, we estimated that a target concentration for intra-articular delivery of MTX in the joint was likely to be in the range of 1 nM to 1 μM (0.5 ng/mL to 0.5 μg/mL). In the high dose study, the amounts of MTX detected in the joints ranged from 0.1 μg to 0.5 μg at 6 h and 24 h following intra-articular injection of MTX loaded microspheres. Based on these data, reaching effective concentrations of MTX in the joints seems to be achievable using MTX loaded microspheres at a dose of 10 mg MTX. The dose of MTX given to rabbits for the
treatment of arthritis has ranged from 1 mg total dose to 30 mg/kg given via different routes of administration (Foong and Green, 1988; Novaes et al., 1996; Neidel et al., 1998). A pretreatment in rabbits with a low dose of MTX at 0.25 mg/kg intramuscularly, one week before the induction of arthritis with bovine serum albumin, has been shown to reduce intra-articular production of proinflammatory cytokines, and the intensity of protein leakage, leukocyte afflux and synovial membrane polymorphonuclear cell infiltrate (Novaes et al., 1996). A treatment with MTX at a dose of 30 mg/kg per week intramuscularly for 12 weeks was shown to reduce cartilage damage in experimental osteoarthritis in rabbits and such a high dose did not cause major adverse effects on articular cartilage proteoglycan metabolism in normal rabbits (Neidel et al., 1998).

The in vivo efficacy study of intra-articular MTX loaded PLLA2k microspheres and MTX solution at a dose of 10 mg MTX was conducted using an antigen-induced arthritis rabbit model. The plasma levels of MTX at different time points following the injections were also evaluated. A higher inter-individual variability was observed in the MTX plasma levels of the diseased animals as compared to the healthy animals likely due to the differences in the degree of the joint swelling in diseased animals. The rabbits with greater joint swelling were found to have lower maximum MTX plasma
concentrations. The lower maximum MTX plasma concentrations observed could be due to a lower concentration of MTX in the synovial joint diluted by a larger volume of joint effusion leading to a smaller concentration gradient across the synovium membrane. The proliferated synovium following the induction of arthritis would also slow down the rate of transport of MTX across the synovium into the circulation.

Arthritis was successfully induced in rabbits with histopathological features of synovial lining hyperplasia, villous hyperplasia, inflammatory cell infiltrate, inflammatory exudates, pannus formation and cartilage destruction clearly observed. Severe cartilage destruction was observed in all rabbits regardless of the treatments received. Based on a system of scoring the pathological features of the disease, there was no significant difference between MTX solution and microspheres treated groups compared to PBS (control) animals, even though evidence has shown that more drug was retained in the joint cavity of the rabbits injected with MTX microspheres compared to the rabbits treated with MTX solution. We speculate that there may be several reasons that might explain the lack of efficacy observed in this animal study. The lack of the efficacy in the antigen induced arthritis rabbit model might be due to the severity of the disease induced. As discussed previously, a high level of joint inflammation including severe cartilage destruction was observed. Therefore, the levels of MTX achieved in the
joint tissues were likely not sufficient to inhibit disease progression. It is possible that the sustained levels of MTX released from microspheres after the burst phase was complete, were insufficient to maintain effective MTX tissue levels. There was a high degree of inter-individual variability observed among the animals in the same treatment group. It was apparent that the sample sizes in each treatment group were also not high enough to enable us to observe any statistical differences between treatment groups and controls. In a similar antigen-induced arthritis rabbit model to that used in this work, an intra-articular injection of MTX liposomal formulation at 1mg MTX dose was shown to reduce joint swelling and reduced inflammatory changes and erosion of cartilage. However, the reduction of joint swelling was not observed until 14 days following the intra-articular injection and the reduction in inflammatory changes and erosion was observed by histological analysis 56 days following arthritis induction (Foong and Green, 1993). It is possible that a lag time period exists before therapeutic effects may be observed following the injection of MTX treatments. Therefore, more than a 14 day time period may be necessary for monitoring the disease progression.

5.2 CONCLUSIONS

1. MTX was successfully encapsulated in PLLA microspheres manufactured from PLLA2k, 50k and 100k polymers.
2. MTX was released from PLLA microspheres (33-110 μm) with a rapid burst phase followed by a slow controlled released phase.

3. Control and MTX loaded PLLA2k (33-110 μm) microspheres were well tolerated in healthy rabbit knee joints for 7 days.

4. Following an intra-articular injection in the knee joints of healthy rabbits, MTX solution was rapidly absorbed across the synovium and entered the systemic circulation, while MTX loaded microspheres slowed down the uptake of MTX into the circulation due to the slow and controlled release of drug in the joint.

5. Significantly higher amounts of MTX were retained in the knee joints of healthy rabbits injected with MTX microspheres than in the joints injected with MTX solution.

6. Synovial inflammation was successfully induced in the knee joints of rabbits by ovalbumin with histopathological features resembling rheumatoid arthritis.

7. MTX loaded microspheres or MTX solution given intra-articularly did not significantly improve the progression of synovitis in rabbits compared to PBS treated controls.

5.3 SUGGESTIONS FOR FUTURE WORK

We believe it is worthwhile continuing to investigate the in vivo efficacy of MTX loaded microspheres following intra-articular injection. However, it is clear from this
preliminary efficacy study that future work should incorporate a number of revisions to the protocols, as follows. The number of animals in each treatment group should be increased to compensate for inter-individual variability. Different doses of antigen should be investigated to achieve an antigen induced arthritis model with less severe cartilage destruction. A dose escalation study should be conducted to determine the optimal dose of MTX treatment, and a longer time period may be necessary for the observation of the progression of the disease. For the evaluation of therapeutic effects of MTX, histological analysis and measuring joint swelling may not be sensitive enough to reflect the pharmacological effects of MTX in a short term study. A more quantitative analysis such as measuring the production of proinflammatory cytokines IL-1, IL-6, or TNF-α in the synovial joints, which are known to be modulated by the administration of MTX, should also be included for a better indication of the therapeutic effects of intra-articular MTX treatments. In the pharmacokinetic study, it was difficult to retrieve MTX and microspheres from synovial fluid resulting in an underestimation of the amounts of MTX in the joints. This problem may be alleviated by using radio-labeled MTX enabling the whole joint to be homogenized for measuring total radioactivity.

In this study, the slow controlled release phase following the burst phase of MTX release from microspheres may not provide sufficient amounts of MTX in the joint to
maintain effective MTX tissue levels. Future studies should investigate formulations of MTX loaded microspheres using other biomaterials to achieve an optimal drug release in which the rapid burst phase is reduced and MTX is released ideally at a constant rate.
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