DEVELOPMENT OF CO-LOADED FUSIDIC ACID AND RIFAMPICIN POLYMERIC MICROSPHERES AND NANOFIBERS: PHASE CHARACTERIZATION AND IN VIVO EVALUATION IN A RODENT MODEL OF ORTHOPAEDIC INFECTION

by

Samuel Edward Gilchrist

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Abstract

The overall goal of this project was to develop and characterize a biodegradable, polymeric formulation using poly (D,L-lactic acid-co-glycolic acid) (PLGA) for the controlled delivery of fusidic acid (FA) and rifampicin (RIF) to orthopaedic surgical sites to achieve high localized concentrations above the MIC of potential microorganisms to prevent implant-associated infections.

Our primary formulation strategy using PLGA microspheres was inappropriate for the controlled delivery of either agent due to a phase separation phenomena of the solid drugs from PLGA. The phase separation, lead us to investigate the solid state properties of FA and the phase behavior of FA and RIF in microspheres. Four distinct solid forms of FA (Form I-IV), and an amorphate were identified. Form IV and amorphous FA had significantly greater intrinsic dissolution rates, and the interconversion of both Form IV and amorphous FA to Form III in aqueous milieu suggests a risk of interconversion upon exposure to moisture if FA is formulated in the solid state.

The phase separation of FA and RIF from PLGA microspheres was characterized by drug microdomains localized on the microsphere surface (for FA), or a dimpled microsphere surface (for RIF). Novel micromanipulation techniques allowed the visualization of the phase separation events for FA and RIF, which was correlated with the compatibility between each drug and PLGA. When co-loaded, FA and RIF phase separate in a single event, intermediate to each drug alone. Surface distribution of drug microdomains, and drug release, was dependent on the weight fraction of FA.

A more suitable controlled release formulation was PLGA nanofibers prepared using electrospinning. The drug-loaded formulations were defect-free and had a biphasic drug release profile. All dual-loaded formulations showed direct antimicrobial activity in vitro against 4 Gram
positive microorganisms. Furthermore, lead formulations containing 10% (w/w) FA/SF and 5% (w/w) RIF were able to prevent the adherence of methicillin resistant *S. aureus* to a titanium implant in an *in vivo* rodent model of implant-associated infection.

The data in this thesis contributes to the understanding of drug phase separation from PLGA, and drug-loaded electrospun nanofibers provide a preclinical proof-of-principle for the prevention of implant-associated infections.
Preface

This thesis is comprised of the following 3 manuscripts, of which I am the principal author:


Chapter 2 is based on manuscript 1. I was the primary individual responsible for the design and conduct of the research experiments and preparation of the manuscript. Dr. Kevin Letchford helped with experimental design, conduct of experiments, and preparation of the manuscript. Dr. Brian Patrick and Anita Lam collected the XRPD data for all FA solid forms. The contribution of all co-authors was through the provision of intellectual discussion and editorial assistance.

Chapter 3 is based on manuscript 2. I was the primary individual responsible for the design and conduct of the research experiments and preparation of the manuscript. Dr. Deborah Rickard was responsible for the micromanipulation experiments, and Dr. Kevin Letchford performed compatibility parameter calculation and assisted with manuscript preparation. The contribution of all co-authors was through the provision of intellectual discussion and editorial assistance.
Chapter 4 is based on manuscript 3. I was the primary individual responsible for the design and conduct of the research experiments and preparation of the manuscript. Dr. Dirk Lange performed the animal surgeries and subsequent CFU counting. Dr. Horacio Bach assisted with the *in vitro* antimicrobial work. Dr. Ladan Fazli performed the immunohistochemistry and histopathological evaluation. All co-authors contributed through intellectual discussion and editorial assistance.

All animal work was carried out in accordance with the Canadian Council on Animal Care (CCAC) guidelines, and the animal care protocol was approved by the Animal Care Committee from the University of British Columbia (Animal Care Certificate A09-0519).
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## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>ACE</td>
<td>Acetone</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ALBC</td>
<td>Antibiotic-loaded bone cement</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
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<tr>
<td>CHCl₃</td>
<td>Chloroform</td>
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<tr>
<td>Da</td>
<td>Daltons</td>
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<tr>
<td>DCM</td>
<td>Dichloromethane</td>
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<tr>
<td>DMF</td>
<td>N,N,-dimethyl formamide</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
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<tr>
<td>EA</td>
<td>Ethyl acetate</td>
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<tr>
<td>EE</td>
<td>Encapsulation efficiency</td>
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<tr>
<td>FA</td>
<td>Fusidic Acid</td>
</tr>
<tr>
<td>H₂O</td>
<td>Water</td>
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<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
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<tr>
<td>HSM</td>
<td>Hot stage microscopy</td>
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<tr>
<td>IDR</td>
<td>Intrinsic dissolution rate</td>
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<tr>
<td>kV</td>
<td>Kilovolt</td>
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<tr>
<td>MBC</td>
<td>Minimum bactericidal concentration</td>
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<tr>
<td>MeOH</td>
<td>Methanol</td>
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<tr>
<td>Symbol</td>
<td>Name</td>
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<td>--------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>MH</td>
<td>Mueller-Hinton broth</td>
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<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
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<tr>
<td>mL</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mmHg</td>
<td>Millimeters of mercury</td>
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<tr>
<td>$\bar{M}_n$</td>
<td>Number-averaged molecular weight</td>
</tr>
<tr>
<td>$\bar{M}_w$</td>
<td>Weight-averaged molecular weight</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin-resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>O/W</td>
<td>Oil-in-water</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDI</td>
<td>Polydispersity index</td>
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<tr>
<td>PLGA</td>
<td>poly(D,L-lactic acid-co-glycolic acid)</td>
</tr>
<tr>
<td>PLLA</td>
<td>poly(D,L-lactic acid)</td>
</tr>
<tr>
<td>PMMA</td>
<td>poly(methyl methacrylate)</td>
</tr>
<tr>
<td>PVA</td>
<td>poly(vinyl alcohol)</td>
</tr>
<tr>
<td>R</td>
<td>Gas constant</td>
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<tr>
<td>RIF</td>
<td>Rifampicin</td>
</tr>
<tr>
<td>rpm</td>
<td>Rotations per minute</td>
</tr>
<tr>
<td>S.D.</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SF</td>
<td>Sodium fusidate</td>
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<tr>
<td>T</td>
<td>Absolute temperature</td>
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</table>
\( T_g \)  Glass transition temperature
TGA  Thermogravimetric analysis
THF  Tetrahydrofuran
\( T_m \)  Melting transition temperature
\( T_r \)  Polymer relaxation temperature
UV  Ultraviolet
\( v/v \)  Percent by volume
VB  Viscous boundary
VRE  Vancomycin resistant enterococcus
\( w/v \)  Percent weight in volume
\( w/w \)  Percent by weight
XRD  X-ray diffraction
XRPD  X-ray powder diffraction
\( \delta \)  Hildebrand solubility parameter
\( \delta_d \)  Hildebrand solubility parameter (dispersion components)
\( \delta_h \)  Hildebrand solubility parameter (hydrogen bonding components)
\( \Delta H_f \)  Enthalpy of fusion
\( \Delta H_M \)  Enthalpy of mixing
\( \Delta H_r \)  Enthalpy of recrystallization
\( \delta_p \)  Hildebrand solubility parameter (polar components)
\( \delta_t \)  Total Hildebrand solubility parameter
µg  Microgram
µL  Microlitre
µm  Micrometer
\( \chi_{sp} \)  
Flory-Huggins interaction parameter
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Finally, a very special thankyou to Erin for your unconditional love and support. Thanks for sticking this thing out with me; this is for you.
For Erin
Chapter 1: Background

1.1 Project overview

Prosthetic joint infections following total joint arthroplasty are a relatively uncommon occurrence in orthopaedics. However when infections do occur, they are the primary reason for joint failure. The management of these implant-associated infections is through perioperative administration of antibiotics. However, evidence shows that the rates of infection remain unchanged with this perioperative routine, suggesting that there is restricted access of circulating antimicrobials to the post-surgical tissue-implant compartment. Thus, most implant-associated infections are managed through two primary treatment options, in addition to systemic antibiotics: either joint debridement, or single and multiple exchange surgical procedures, where the infected implant is removed and replaced with a sterile implant. In some multiple exchange procedures, the sterile implant is not replaced immediately, but after 4-8 weeks. During this time, a temporary antibiotic-loaded acrylic bone cement spacer, created from poly(methylmethacrylate) (PMMA), is inserted into the bone space with the aims of avoiding both soft tissue shortening and scar formation, keeping a correct limb position, and sterilizing the infected areas (Steinbrink, 1990; Magnan et al., 2001). In the case of revision total hip and knee arthroplasty, these spacers can also withstand light load bearing (Hsieh et al., 2004), and allow for some joint articulation (Haddad et al., 2000), which has been shown to produce more favorable clinical outcomes than non-load bearing and non-articulating spacer devices. The evidence that treatment of implant-associated infections is linked to increased patient mobidity and mortality, and along with the co-morbidity of high doses of systemic antimicrobials, suggests that there is a need for an alternative treatment strategy to eradicate implant-associated pathogens.
A common surgical practice aimed at increasing local antimicrobial concentrations at the implant site is the mixing of a known amount of sterile antibiotic solid with PMMA bone cement as it polymerizes *in situ*. The antibiotic loaded bone cement (ALBC) is used as a structural solid to anchor implants, as a spacer device in two-stage revisions, or is formed into small diameter beads and placed in the surgical pocket (Kanellakopoulou and Giamarellos-Bourboulis, 2000). However, there is limited evidence for the efficacy of ALBCs in preventing, or treating established orthopaedic implant-associated infections. Furthermore, there are a number of additional disadvantages associated with the PMMA carriers as follows: 1) PMMA is not biodegradable and must be surgically removed from the implantation site, 2) the elution of antibiotics from ALBCs occurs primarily from the surface and large quantities of the antibiotics (>90%) are retained within the matrix leading to a very poor antibiotic elution profile (Levin, 1975; Baker and Greenham, 1988; Mader *et al.*, 1997; van de Belt *et al.*, 2000), 3) in spite of large antibiotic loads, ALBCs have been shown to encourage the growth and adherence of virulent pathogens (Kendall *et al.*, 1996), 4) the polymerization of PMMA carriers is exothermic, and has been associated with thermal necrosis and premature joint loosening (Berman *et al.*, 1984).

Although the use of biodegradable and bioresorbable polymers loaded with antibiotics would provide greatly improved drug elution profiles, these polymers typically cannot provide structural integrity and thus the required mechanical properties for implant fixation purposes. However, our group has proposed that antibiotic-loaded biodegradable polymeric delivery systems based on poly (D,L-lactic acid-co-glycolic acid) (PLGA) could be developed as an adjunctive means to deliver drugs without the need to provide structural characteristics. We have selected the fusidanes (fusidic acid, FA, and sodium fusidate, SF) and rifampicin (RIF) for co-loading into PLGA. These agents are receiving a great deal of attention for the treatment of
implant-associated infections due to activity against a broad range of microorganisms found to colonize implanted biomaterials, including *S. aureus, S. epidermidis*, coagulase-negative staphylococci, including strains that are methicillin-resistant (MRSA).

The work described in this thesis takes two main approaches to drug delivery system development. Firstly, the development of microsphere systems that could potentially be co-located at the implant site to release the loaded antibiotics and would not require surgical removal. Secondly, the development of very thin, flexible films that could conform to the surface of implants or spacer devices and release the loaded antibiotics at the implant/spacer site. Therefore, the primary objectives of this work were to develop and characterize controlled-release drug delivery systems based on PLGA and co-loaded with FA/SF and RIF and evaluate the effectiveness of a lead formulation in preventing implant-associated infection in a rodent model.

Previous studies in our laboratory in which FA was loaded as a single agent into PLGA microspheres, showed that a phase separation phenomenon resulted in the exclusion of solid amorphous FA-rich microdomains at the surface of the microsphere (Yang et al., 2009). Since the solid-state properties of FA have not been reported in the peer-reviewed literature, Chapter 2 of this thesis discusses our findings of new polymorphic forms of FA and an amorphous form. The solid-state characteristics and interconversions of the different forms are described.

Building on the earlier work in our laboratory (Yang et al., 2009), Chapter 3 of this thesis describes the characterization of PLGA microspheres co-loaded with FA and RIF. Phase separation behavior of FA and RIF, either loaded as single agents, or in combination in the forming microspheres was examined using real-time video recordings of single microsphere formation via the solvent-evaporation method. Phase diagrams were constructed illustrating the
phase changes taking place from the liquid through to the final glassy states as microspheres form.

Attempts to develop thin film delivery systems of co-loaded FA and RIF in PLGA are described in Chapter 4. Conventional solvent evaporation methods using a polymer/drug solution poured into a mold resulted in complete phase separation of a drug-rich viscous liquid phase beneath a solidifying PLGA film and were not investigated further. Electrospinning is a unique process for producing polymeric nanofiber membranes and has been investigated as a means of loading drugs into the nanofibers for controlled release purposes (Katti et al., 2004). The electrospinning fabrication process relies on acceleration of a jet of polymer/drug solution, via a high electrical field potential, towards a collector plate where a non-woven, thin film of nanofibers is produced (Rutledge and Fridrikh, 2007). Extremely rapid solvent evaporation leads to the formation of the solid nanofiber (Reneker et al., 2000). Electrospinning and formulation parameters were optimized to produce homogeneous, co-loaded FA/SF and RIF PLGA nanofiber membranes, and a lead formulation was evaluated in a rat model of implant-associated infection.

1.2 Joint replacement procedures and infection rates

Arthroplastic procedures are the surgical reconstructions or replacement procedures of malformed or degenerated joints. These surgeries are commonly performed to restore form and function, and alleviate pain in the knee, hip, shoulder, elbow, wrist, ankle, temporomandibular, metacarpophalangeal, and interphalangeal joints to improve quality of life (del Pozo and Patel, 2009). In 2006, nearly 800,000 total hip, and total knee primary arthroplasties were performed in the United States (Kurtz et al., 2008). During that same period, the rates of infection for both these procedures increased by 3-fold and 6-fold, respectively, accounting for nearly 2% of all restorative procedures that year (Kurtz et al., 2008). It is projected that primary restoration
procedures of the hip and knee will increase by ~175% and ~673% over the next 15 years, and the rates of revision surgeries are expected to increase by ~137% and ~601% over the same time (Kurtz et al., 2007). Therefore, with the rates of infection for joint replacements of the knee, hip, shoulder, and elbow, ranging between 0.5%-2% (Peersman et al., 2001), 1%-2% (Phillips et al., 2006), 1%-2.5% (Sperling et al., 2001), and 7%-9% (Morrey and Bryan, 1983; Gill and Morrey, 1998), respectively, and secondary joint revision surgeries shown to display infection rates between 5-40% (Widmer, 2001), the patient and economic burden of treating prosthetic joint infections will be extensive. The presentation of a prosthetic infection has been shown to incur significant health care costs, where the cost-of-care per case is estimated to be ~$68,000, compared to ~$39,000 for non-infected counterparts (Kurtz et al., 2008). However, the cost of an infected prosthetic implant significantly increases by ~1.5× if the infecting organism is resistant (~$107,000) (Parvizi et al., 2010).

Prosthetic joint infections have an unfavorable impact on patient’s subsequent quality of life, and result in prolonged hospital stays, revision surgeries, long-term intravenous antibiotic treatment, and increased morbidity and mortality rates (Kirkland et al., 1999; Whitehouse et al., 2002).

1.2.1 Pathogenesis of implant-associated infections

In this thesis, implant-associated infections will be used in the context of orthopaedic devices, however the term can be extrapolated to a range of other surgical implants that are prone to bacterial colonization such as heart valves, penile implants, vascular and urinary catheters, CNS shunts, pacemakers, and left ventricular assist devices (Darouiche, 2003).

Infections of surgical implants may occur perioperatively through bacterial contamination during surgery, immediately after surgery, or either hematogenously or contiguously by...
microbial spread from a distant infection through circulation or lymphatic transit, respectively (Waldman et al., 1997; Trampuz and Widmer, 2006). Implant-associated infections are classified as early (≤3 months), delayed (3-24 months), or late (≥24 months), according to the timeframe in which they are acquired (Trampuz and Widmer, 2006). Early and late infections are usually caused by highly virulent microorganisms such as *S. aureus* or Gram-negative bacilli, and delayed infections usually caused by less virulent microorganisms such as coagulase negative staphylococci (Trampuz and Zimmerli, 2006).

Risk factors for developing an implant-associated infection can be categorized into patient and surgery-related, both operatively and post-operatively. Patient-related risk factors include previous revision arthroplasty or previous infection associated with a prosthetic joint, tobacco use, obesity, rheumatoid arthritis, immunosuppression, and diabetes. Surgical risk factors include simultaneous bilateral arthroplasty, a long operative time (≥2.5 hours), and blood transfusion. Postoperative risk factors include atrial fibrillation, myocardial infarction, urinary tract infection, a prolonged hospital stay, and wound healing complications such as, superficial infection, hematoma, delayed healing, and wound necrosis (Bongartz et al., 2008; Dowsey and Choong, 2008; Sendi et al., 2008; Jamsen et al., 2009).

The pathogenesis of implant-associated infection is considerably different from all other post-surgical infections due to physiological phenomena related to the presence of the implanted biomaterial. The local immune depression in the environment surrounding the implant, the so-called *immune-incompetent fibro-inflammatory zone*, facilitates the colonization and growth of pathogens and promotes septic or aseptic failure of structural implants (Gristina, 1994). In fact, the number of microorganisms required to sustain an infection drops precipitously (~100,000-fold) when a foreign material is present (Elek and Conen, 1957).
The microorganisms responsible for implant-associated infections are opportunists, taking advantage of the weakened immune responses in the peri-implant milieu, and are typically of the Staphylococcal genus (Figure 1.1) (Campoccia et al., 2006). The initial seeding of bacteria on an implant can occur from exogenous or endogenous sources, and is reversible, non-specific and includes mainly hydrophobic interactions. However, the bacterial compliment of membrane autolysin/adhesion proteins mediates irreversible anchorage to the implant surface. In addition, these adhesion proteins have also been shown to anchor bacteria to host extracellular matrix proteins such as albumin, collagen, fibrinogen, fibronectin, and elastin, which are present as part of the body’s natural response to foreign materials (Patti et al., 1994). Adhesion proteins then promote the excretion of a complex polysaccharidic “slime”, called a glycocalix or biofilm. Proliferation of persister cells in the biofilm proceeds as the biofilm matures, and occurs as a response to changing environmental stimuli (Kussell et al., 2005). Mature biofilm communities exist in complex 3-dimensional mushroom-like structures separated by fluid-filled channels, which create a top-to-bottom gradient of oxygen and nutrients through the biofilm covering the implant. Bacteria at the top are able to disperse to colonize other surfaces, completing the biofilm lifecycle (Sauer et al., 2002). The distinct heterogeneous microenvironments within the biofilm have been shown to alter protein expression in some bacteria by up to 50% (Sauer et al., 2002), producing a number of bacterial phenotypes. Interestingly, it is the phenotypic alterations in bacteria located in these nutrient-depleted niches that constitute the primary mechanism of resistance of biofilm bacteria to antibiotics, and not a reduced antibiotic penetration (Anderl et al., 2000; Walters et al., 2003).
1.2.2 Treatment of implant-associated infection

The treatment options for established orthopaedic implant infections are based on a few key variables: interval after implantation (early, delayed, late), the type of infection, either exogenous from external contamination or hematogenous from a preexisting infection, the condition of the implant, and the patient’s comorbidity. Giulieri et al. have generated an algorithm for the treatment of established infections based on these key factors, and have shown that adherence to the algorithm resulted in significantly better outcomes compared to non-adherence (88% vs. 62%) (Giulieri et al., 2004). According to Giulieri’s model (Figure 1.2), the treatment of an established infection proceeds essentially one of three ways: debridement of necrotic tissue and retention of device, exchange of infected implant for sterile implant (one-stage exchange), or removal of infected implant, replacement with an antibiotic-loaded spacer
device for 2-6 weeks, followed by a secondary surgery to replace the spacer with a new implant (two-stage), with all cases receiving long duration, high-dose parenteral antibiotics.

The recommended antibiotic therapy for prosthetic joint infections is an early generation cephalosporin (e.g. cefazolin), lincosamide (e.g. clindamycin), or fluoroquinolone (e.g. levofloxacin) plus a second antimicrobial agent, typically RIF (Zimmerli and Ochsner, 2003; Trampuz and Widmer, 2006). However, if the microorganism is shown to be resistant (e.g. MRSA), the treatment is always RIF plus vancomycin for 2 weeks, followed by RIF combination therapy with one of ciprofloxacin, levofloxacin, teicoplanin, FA, cotrimoxazole, or minocycline (Zimmerli et al., 2004; Trampuz and Zimmerli, 2006; Garcia-Lechuz and Bouza, 2008).

With the epidemiology of nosocomial infections rapidly changing to include a number of multidrug resistant microorganisms other than MRSA, including vancomycin resistant Enterococcus (VRE), multidrug resistant Pseudomonas aeruginosa, and Acinetobacter species (Maviglia et al., 2009), and the rapid spread of resistance mediated by extended-spectrum β-lactamases, which hydrolyze the β-lactam ring of the penicillins and cephalosporins (Gold and Moellering, 1996), the list of antibiotics currently considered effective will have a limited therapeutic lifetime.
**Figure 1.2 Algorithm for the surgical and antibiotic management of a prosthetic joint infection.** Adapted from Giulieri et al. (Giulieri et al., 2004)
1.3 Clinical use of old generation antibiotics

The widespread use of antibiotics, each with a broad spectrum of activity has increased the incidence of drug resistant microorganisms, which now account for >70% of all nosocomial infections (Maviglia et al., 2009). This rapid rise in resistance has grossly outpaced the development of novel antimicrobials (Talbot et al., 2006), and has forced the reintroduction of a number of old generation antibiotics, such as polymyxin, fosfomycin, isepamicin, chloramphenicol (Falagas and Kopterides, 2007; Maviglia et al., 2009), and second-generation cephalosporins such as cefamandole and cefuroxime (Trampuz and Zimmerli, 2006). However two antibiotics that are receiving a great deal of attention for the treatment of implant-associated infections due to MRSA, are fusidic acid (FA) and rifampicin (RIF) (Trampuz and Zimmerli, 2005; Trampuz and Zimmerli, 2006; Aboltins et al., 2007; Forrest and Tamura, 2010). In addition, the spectrum of coverage appears to go beyond MRSA. RIF has been shown to act synergistically with daptomycin and is effective against VRE (Rand and Houck, 2004), and in an evaluation of 35 clinical VRE strains, none were resistant to FA and only a small percentage (~10%) resistant to RIF (Hoeffler and Zimmermann, 1997). Therefore, both FA and RIF may be extremely useful in treating nosocomial, and prosthetic joint infections.

1.3.1 Fusidic acid

1.3.1.1 Chemistry

FA is a tetracyclic triterpenoid derived from the fermentation broth of the fungus Fusidium coccineum. FA exists in two chemical forms: free acid, and a sodium salt (Figure 1.3). FA/SF are chemically related to the “super cephalosporins”, helvolic acid and viridominic acid through the addition of a few acetyl groups, and SF closely resembles the bile salts sodium cholate and taurocholate (Godtfredsen et al., 1962; Turnidge, 1999). Due to the similarity to bile
salts, SF self-aggregates in aqueous solution, and forms micelles at 1.44-4.56 mM (Coello et al., 1994). FA is commercially available as a crystalline free acid ($C_{31}H_{48}O_6$), with a molecular weight of 516.72 Da (anhydrous) and a pKa of 5.35 (Merck and Co, 1983). FA possesses very low aqueous solubility, however, its sodium salt (sodium fusidate; SF) is readily water-soluble.

![Chemical structure of (A) fusidic acid (FA) and (B) sodium fusidate (SF)](image)

**Figure 1.3 Chemical structure of (A) fusidic acid (FA) and (B) sodium fusidate (SF)**

There is very little information available on the chemical stability of FA, however it is generally shown to be very chemically stable, showing no degradation in aqueous media over 6 months, and with multiple freeze-thaw cycles in IV preparations (Sewell and Palmer, 1991). When formulated into plaster of Paris beads at 37°C, the degradation of FA was apparent after 3 weeks, with 60% of initial dose remaining (Mousset et al., 1995).

FA and its salts are formulated as an oral tablet, oral suspension, IV solution, and in various topical preparations (2011). The oral tablet is formulated using the sodium salt (SF). Initially available as an enteric-coated tablet, it was reformulated as a film-coated tablet, which displayed marked improvements in oral bioavailability (Taburet et al., 1990). The oral oily suspension of the diethanolamine salt, was reformulated as a flavored aqueous suspension of the
hemihydrate to improve tolerability and bioavailability. The IV formulation also underwent a reformulation from the diethanolamine salt to the sodium salt. There are four topical formulations of FA: A cream (2% FA in an oil-in-water cream base; Fucidin H®); an ointment (2% SF; Fucidin®); a gel (2% SF); and sterile gauze squares impregnated with Fucidin® (Fucidin® Intertulle). In addition, Leo® Pharma has also marketed an ophthalmic preparation (Fucithalmic®), a microcrystalline aqueous suspension of FA, for the treatment of superficial eye infections (Turnidge, 1999).

1.3.1.2 Pharmacokinetics and pharmacology

FA is exclusively active against Gram-positive organisms, in particular, *S. aureus*, *S. epidermidis*, and coagulase-negative staphylococci including strains that are methicillin-resistant (Coombs and Menday, 1985; Coombs, 1990). FA acts by inhibiting bacterial protein biosynthesis. FA binds to elongation factor G (EF-G) in the bacterial cytoplasm, an essential protein encoded by *fusA* that promotes the translocation of peptides along the ribosome. FA binds to the EF-G ribosome complex in combination with either guanosine di- or triphosphate (GDP or GTP) and stabilizes EF-G-GDP/GTP complex on the ribosome, thus preventing further elongation of the polypeptide by inhibiting the GTPase function of EF-G (Tanaka et al., 1968; Bodley et al., 1970; Collignon and Turnidge, 1999), halting protein synthesis.

Bacterial resistance to FA is due to two primary mechanisms: point mutation in the *fusA* gene, and acquisition of FA-resistant genes. Point mutations in *fusA* occurs in approximately 1 in every 10⁶-10⁸ colony forming unit (CFU) in *S. aureus* (Besier et al., 2003), and results in a decreased affinity of FA for EF-G (Turnidge and Collignon, 1999). A more frequent and major cause of resistance is the acquisition of plasmid or chromosomal-mediated resistance genes, namely *fusB*, *fusC*, and *fusD* (O'Neill et al., 2007; Castanheira et al., 2010). Both *fusB* and *fusC*
have been shown to prevent the binding of FA to EF-G, and fusD has been shown to be an intrinsic factor for resistance in *S. saprophyticus* (O'Neill *et al.*, 2007). Other mechanisms such as sequestering, deacetylation, and efflux of FA have also been reported (Turnidge and Collignon, 1999; O'Neill *et al.*, 2002).

The oral bioavailability of FA (given as a 500 mg dose in adults) is moderate-to-low for the capsule (SF) and suspension (FA), with bioavailable fractions of 0.69, and 0.46, respectively (Wise *et al.*, 1977). However, the reformulated film-coated tablet has a bioavailable fraction of 0.91 (Taburet *et al.*, 1990). FA exhibits dose-dependent pharmacokinetics with peak plasma concentrations (C<sub>max</sub>) of ~30 mg/L reached within 2-3 h (T<sub>max</sub>) for a 500 mg oral dose (Turnidge, 1999). FA is almost entirely bound to serum albumin (~99%) (Guttler *et al.*, 1971; Brodersen, 1985), and eliminated exclusively by non-renal mechanisms, with an elimination half-life (t<sub>1/2.β</sub>) ranging between 8-16 h (Barrett and Watt, 1979). The apparent volume of distribution (V<sub>d.β</sub>) and steady state volume of distribution (V<sub>d.ss</sub>) are 0.52 L/kg and 0.42 L/kg, respectively (Munkholm *et al.*, 1994; Turnidge, 1999), indicating that the drug is primarily located in the central compartment, comparable with other hydrophilic antibiotics (i.e. β-lactams) that are highly protein bound (Reeves, 1987). Systemic clearance (Cl<sub>s</sub>) values for a single dose of FA range from 33-43 mL/min and decrease (up to 50%) with multiple dosing (Munkholm *et al.*, 1994; Turnidge, 1999). Accumulation of FA is well documented with repeated dosing and is likely due to the ability of the free fraction of FA to penetrate into deep tissue compartments (Vaillant *et al.*, 2000), and due to saturable clearance mechanisms (Macgowan *et al.*, 1989; Taburet *et al.*, 1990).

The hepatic uptake and subsequent metabolism of FA is poorly understood. It is suggested that FA is taken into the hepatocyte via a bile salt efflux pump (Bode *et al.*, 2002), and metabolized via Phase I and II hepatic enzymes, with the major metabolites being a dicarboxylic
derivative, and a glucuronide conjugate (Godtfredsen and Vangedal, 1966). FA has also been shown to induce/inhibit various Phase I hepatic enzymes, which may play a role in the clearance of co-administered drugs, such as simvastatin (Burtenshaw et al., 2008), and L-methadone (Reimann et al., 1999), although the mechanism underlying the alteration of the Phase I system remains unclear.

Parenteral administration of FA has shown serious side effects and toxicities such as hepatotoxicity, hyperbilirubinemia, thrombophlebitis, and venospasm (ElKassar et al., 1996; Turnidge, 1999). In addition, oral dosing shows adverse gastrointestinal effects such as epigastric pain, anorexia, vomiting and diarrhea (Macgowan et al., 1989; Mandell, 2000). However, local administration of FA has been shown to be effective through joint irrigation with an aqueous FA solution with no adverse reactions (Mackechnie-Jarvis, 1985).

1.3.2 Rifampicin

1.3.2.1 Chemistry

RIF is a semi-synthetic derivative of rifamycin B (Maggi et al., 1966), a member of the ansamycin family of antibiotics, so called due to their 3-dimensional basket-like architecture (Figure 1.4).
The rifamycins were first isolated in 1957 from a complex mixture of related chemicals found in the fermentation broth of *Amycolatopsis mediterranei*, an Actinobacteria genus isolated from a pine forest soil sample on the French Riviera (Margalith and Pagani, 1961). RIF is commercially available as a crystalline, red/orange powder ($C_{43}H_{58}N_{4}O_{12}$) with a molecular weight of 822.96 Da (Merck and Co, 1983). RIF is zwitterionic, and has a pKa of 1.7 related to 4-hydroxy, and a pKa 7.9 related to 3-piperazine nitrogen. RIF is very slightly water soluble, at ~1g/762mL (pH <6), with increased solubility at lower pH values (Merck and Co, 1983).

The degradation of RIF occurs in aqueous media (Woo *et al.*, 1987), and is pH dependent (Gallo and Radaelli, 1976). In alkaline media, RIF has been shown to undergo oxidation to an inactive quinone derivative (Gallo and Radaelli, 1976), and hydrolyzes to a formyl rifamycin derivative, with subsequent precipitation in acidic media (Maggi *et al.*, 1966). RIF has been shown to be unstable in human plasma, losing up to 50% of initial concentration by 8h (Le Guellec *et al.*, 1997). Due to these instabilities, ascorbic acid has been used to prevent the oxidation of RIF in plasma, and aqueous samples (Weber *et al.*, 1983).
RIF is available as an oral capsule (Rifadin®) and fixed-dose combination tablet of RIF-isoniazid-pyrazinamide (Rifater®) through Aventis Pharma. An IV formulation (Rifampin) is also available as part of Health Canada’s Special Access Programme (2011).

1.3.2.2 Pharmacokinetics and pharmacology

RIF has a very broad and potent spectrum of activity. RIF is active against mycobacterial organisms, including Mycobacterium tuberculosis and M. leprae. It is highly active against S. aureus, coagulase-negative staphylococi, Listeria monocytogenes, Neisseria meningitidis, Haemophilus influenzae, Brucella, some strains of E. coli, Proteus mirabilis, anaerobic cocci, Clostridium spp., and Bacteroides (DiPalma and DiGregorio, 1990).

RIF acts by inhibiting RNA synthesis through a non-covalent interaction with DNA-dependent RNA polymerase. RIF binds the β subunit of the polymerase, which is encoded by the rpoB gene (Naryshkina et al., 2001), and blocks the exit of the transcribing RNA strand from the polymerase. RIF does not bind to the isolated β subunit, but binds very tightly to the holo enzyme (α2ββ′σ), or a partially reconstituted enzyme where the β subunit is associated with two α subunits (α2β) (Lill and Hartmann, 1977; Naryshkina et al., 2001).

Resistance to RIF occurs, albeit at a low frequency relative to other antituberculosis drugs (David, 1970), via mutation in a small region (~100 base-pairs) of the rpoB gene (Heep et al., 2000). However, some reports have indicated that a decreased sensitivity of the RNA-polymerase (Floss and Yu, 2005), presence of plasmid-mediated efflux proteins (Chandrasekaran and Lalithakumari, 1998), or chemical inactivation of RIF (Dabbs et al., 1995), may also play a role.

RIF is typically administered via the oral route (600 mg dose for adults; 10 mg/kg for children), however IV dosing has also been reported. RIF is well absorbed from the
gastrointestinal tract, with an oral bioavailable fraction of ~0.95 (Loos et al., 1985), and peak plasma concentrations of ~10 µg/mL reached within 2-3 h (Boman, 1974; Acocella, 1978; Acocella, 1983). RIF is highly protein bound (~85%) (Boman and Ringberger, 1974), and is metabolized primarily through hepatic mechanisms, with small contributions of renal filtration (~10-20%). The $t_{1/2}$ of RIF is ~ 3 h, and total systemic clearance ($Cl_s$) of ~5 L/h. The apparent volume of distribution ($V_{d,area}$) for RIF was ~25 L, suggesting that RIF is well-distributed beyond the central compartment (Loos et al., 1985).

RIF enters the hepatocytes via an organic anion transporter (Tirona et al., 2003), and is metabolized to a deacetylated form, which is also active, by the phase I drug metabolizing enzyme cytochrome P450 3A4, with subsequent elimination as a phase II glucuronide. The parent drug is also seen in biliary excretions but has been shown to undergo enterohepatic recycling, and thus the elimination of parent drug contributes very little to the total clearance of RIF (Acocella, 1983). RIF is a potent inducer of hepatic drug metabolizing enzymes, and efflux transporters, such as P-glycoprotein (P-gp), and is responsible for its self-regulating (autoinduction) metabolism with chronic administration (DiPalma, 1994; Bolt, 2004). Autoinduction from chronic administration (22 d) has been shown to significantly decrease bioavailable fraction, $t_{1/2}$, and total body exposure (as measured by the AUC) by ~30%, ~58%, and ~45%, respectively, while total systemic clearance increased by ~150% (Loos et al., 1985). The induction of phase I enzymes and P-gp has major clinical significance on the rate of metabolism of a number of co-administered psychotropic agents (sertraline hydrochloride, clozapine, midazolam), cardiovascular drugs (simvastatin, digoxin), antiretrovirals (ritonavir, indinavir), antibiotics (azithromycin, clarithromycin, itraconazole), immunosuppressants (tacrolimus, cyclosporine), opiates (morphine, codeine), and a range of other drugs (Finch et al., 2002).
RIF is well tolerated, with rare cases of toxicity. The most common side effects are transient increases in hepatic enzymes such as the transaminases, bilirubin and alkaline phosphatase, gastrointestinal distress causing nausea, abdominal discomfort, and diarrhea, and a red-orange appearance of the urine, feces, saliva, sweat, and tears (Scholar et al., 2000).

1.3.3 Fusidic acid and rifampicin co-administration

The broad spectrum of activity of both FA and RIF confers protection against ≥ 70% of organisms that commonly cause prosthetic joint infections (Figure 1.1) (Darley and MacGowan, 2004; Campoccia et al., 2006). When FA and RIF are evaluated in combination, the potency as determined by the minimum inhibitory concentration (MIC) has been shown to be additive or synergistic using in vitro screening methods (Zinner et al., 1981; Howden et al., 2004; Saginur et al., 2006). Clinically, there is also considerable evidence to support the use of this double therapy against surgical infections, where a number of clinical studies have shown favorable outcomes using oral dosing of FA and RIF following joint debridement, with no evidence for the development of bacterial resistance to either FA or RIF (Drancourt et al., 1997; Aboltins et al., 2007). In addition, there are no documented cases of drug-drug interactions with co-administration of RIF and FA.

1.4 Solid state properties of drugs

Solid drug substances can display a wide variety of solid state properties (Byrn et al., 1995; Brittain, 1999). The crystalline nature, or lack thereof in amorphous solids, of a drug substance can influence drug stability, solubility, dissolution, and bioavailability. Therefore, the solid state properties of drugs must be completely understood, as they can affect both the production and performance characteristics of a pharmaceutical product (Byrn et al., 1995).
1.4.1 Crystal structure

Crystals are highly ordered, three-dimensional arrays of molecules held together by non-covalent bonds, and possess long-range order extending many molecule diameters. The simplest repeat unit in the crystal lattice is the unit cell. Each unit cell in a lattice is the same size, and contains the same number of molecules or atoms arranged the same way. The size and shape of the unit cell is described by 3 crystallographic axes, whose lengths are $a$, $b$, and $c$, and the angles between them are $\alpha$ (between sides $b$ and $c$), $\beta$ (between sides $a$ and $c$), and $\gamma$ (between sides $a$ and $b$) (Figure 1.5).

According to the relative position of molecules, or atoms, the unit cell can be categorized into one of seven crystal systems based on primitive centering, where each apex of the unit cell represents an atom or molecule: triclinic, monoclinic, orthorhombic, tetragonal, rhombohedral, hexagonal or cubic. However, in addition to primitive centering (P), atoms/molecules can exist at the center of the top/bottom faces (base-centered; C), at the center of every face (face-centered; F), or at the center of the unit cell (body-centered; I). Combining the 7 crystal systems with various possible lattice centerings ($P$, $C$, $F$, $I$), Bravais illustrated that there are 14 possible point lattices that can describe all possible unit cells (Bravais and Shaler, 1949). The combinations are called the Bravais lattices (Figure 1.5). Drugs most commonly form triclinic, monoclinic, or orthorhombic unit cells (Florence and Attwood, 2006).
Figure 1.5 The 14 Bravais lattices categorized by the seven crystal systems based on (P) primitive, (C) base-centered, (I) body-centered, and (F) face-centered spacing. Dots indicate the location of atoms/molecules around the unit cell (shaded area). The sides and angles listed describe the geometry of each unit cell.
The external morphology of a crystal is referred to as the crystal habit. The habit of any crystal is dependent on the crystallization environment, and affects the external shape of the crystal without changing its internal structure. There are numerous descriptions of crystal shapes, including, acicular (needle-like), prismatic, pyramidal, tabular, equant, columnar, and lamellar (Florence and Attwood, 2006). The size and shape can vary depending on the conditions of the crystallization process, such as solvent used, temperature, agitation, concentration of drug, and the presence of impurities (Florence and Attwood, 2006). Tiwary (Tiwary, 2001), and Halebian (Halebian, 1975), provide excellent reviews on the influence of crystal habit on a number of important physical properties of drug powders, such as tableting behavior, syringeability, dissolution, and powder flow. For example, ibuprofen crystallizes in various crystal habits depending on the solvent used and recrystallization method (Khan and Jiabi, 1998; Garekani et al., 2001). The polyhedral habit is formed from crystallization out of methanol or ethanol, and has much better flow and compression characteristics compared to ibuprofen crystallized from isopropanol or hexane, which is needle-like (Garekani et al., 2001). The plate-like crystals of ibuprofen, formed from crystallization out of acetone, have a much higher dissolution rate than the rod-like crystals formed from crystallization out of 2-propanol (Khan and Jiabi, 1998).

1.4.2 Amorphous solids

In contrast to crystalline solids, amorphous solids possess no long-range order within the solid, and therefore have no defined molecular structure (Brittain, 1999). Amorphous solids have excess thermodynamic properties of entropy, enthalpy, and free energy relative to crystalline solids, and risk converting to a more stable crystalline form (Carstensen, 2000). In addition to the risk of conversion, amorphous forms of a particular compound possess a number of different physical properties from crystalline forms of the same compound, such as increased solubility
and vapor pressure, greater chemical reactivity, enhanced dissolution and bioavailability, due to
the higher internal energy and specific volume of the amorphous state (Hancock and Zografi,
1997; Craig et al., 1999).

Figure 1.6 illustrates a theoretical plot of the enthalpy ($H$) or specific volume ($V$) of a
solid in both the amorphous and crystalline forms, as a function of temperature. As the
temperature is increased for the crystalline form, a small increase in $H$ and $V$ is observed,
characteristic of the heat capacity ($C_p$), and thermal expansion coefficient ($\alpha$), respectively
(Brittain, 1999). At the melting point of the crystalline material ($T_m$), there is a discontinuity in
the slope, representing the first-order phase transition from a solid to a liquid. If the melt is
rapidly cooled or quenched, such that crystallization does not occur, the liquid enters the
supercooled liquid region, also known as the “rubbery state”. Upon further cooling, there is a
point at which the rubber becomes “frozen” in the glassy state. At this temperature, called the
glass transition temperature ($T_g$), the molecular structure of the liquid form is retained but the
translational and rotational motion of molecules is halted, leaving only vibrational motion to take
place below the $T_g$ (Brittain, 1999; Carstensen, 2000). The change in slope of $H$ or $V$ at the $T_g$ is
characteristic of a secondary thermodynamic transition with no latent heat transfer. Thus the $T_g$ is
dependent on, and characterized by, the onset of molecular mobility within the solid.
Figure 1.6 Thermodynamic plot of the enthalpy (H) and volume (V) change of a crystalline and amorphous solid upon heating. $T_m$ is the melting temperature and $T_g$ is the glass transition temperature.

The glassy material formed below the $T_g$ deviates from the equilibrium state with markedly higher $H$ and $V$, which is responsible for the higher internal energy and differing thermodynamic and physical properties of amorphous solids compared to crystalline solids (Brittain, 1999).

Amorphous solids can be produced through solidification from the melt (quenching) (Yoshioka et al., 1994; Hancock and Zografi, 1997), reducing particle size by milling or grinding, spray-drying, and lyophilization (Otsuka and Kaneniwa, 1988).

The degree of crystallinity of any solid may be calculated using data obtained from X-ray powder diffraction (XRPD) peak intensities, and heats of recrystallization/fusion. However a number of other techniques have also been used, including solution calorimetry, density, and infrared absorption measurements (Saleki-Gerhardt et al., 1994; Buckton and Darcy, 1999). Degree of crystallinity determinations were not carried out in this thesis, and will not be described further.
1.4.3 Polymorphism

Polymorphism is characterized as the ability of a drug substance to exist as two or more crystalline phases that have different arrangements and/or conformations of the molecules in the crystal lattice (i.e. a different unit cell) but exhibit identical liquid and vapor states (Haleblian, 1975).

Due to differences in the dimensions, shape, symmetry, number of molecules, and void volumes of the different unit cells, polymorphs of a given substance may demonstrate marked differences in a number of physical properties (Brittain, 1999; Grant, 1999), such as: packing properties, which includes molar volume, density, refractive index, and hygroscopicity; thermodynamic properties, which include melting temperature, internal energy, enthalpy, heat capacity, entropy, and solubility; kinetic properties, which include dissolution rate, solubility, and rates of reaction; surface properties, which include surface free energy and interfacial tension; and mechanical properties, which include hardness, tensile strength, compactibility, tableting, handling, and flow (Brittain, 1999; Grant, 1999). Of particular importance in pharmaceutical systems are the thermodynamic differences, which generally exist between any set of polymorphic pairs. Differences in internal energy, enthalpy, heat capacity, entropy, and free energy have significant impact on a number of physical properties of drug dosage forms, including solubility and dissolution, stability, and bioavailability (Singhal and Curatolo, 2004). For example, the recall of the antiviral drug ritonavir, was due to the precipitation of a new polymorphic solid form (Form II) upon storage, which had significantly lower solubility (Bauer et al., 2001); the polymorph-dependent cleavage and subsequent absorption of chloramphenicol from chloramphenicol palmitate (CAPP), resulted in overdosing and toxicity (Aguiar and Zelmer, 1969); and the photodecay of carbamazepine Form II, was significantly faster than Form I and III, resulting in degradation when exposed to light (Matsuda et al., 1994). Therefore,
evaluating the polymorphic tendency of any drug, and choosing the most stable polymorph is very important in producing a safe, stable, and effective drug product (Haleblian and McCrone, 1969).

The processes governing polymorphic transformation or interconversion can be mediated through 4 primary mechanisms: solid-solid transformations, melt transformations, solution transformations, and solution-mediated mechanisms (Brittain, 1999; Grant, 1999).

1.4.3.1 Solid-solid transformations

In solid-solid transformations, a polymorphic conversion occurs without passing through a liquid phase, and is influenced by environmental variables such as temperature, pressure, and relative humidity, and by the presence of crystalline defects, particle size, and distribution of impurities (Grant, 1999). Solid-solid transformations have been shown to occur in carbamazepine (Rustichelli et al., 2000), and tripalmitin polymorphs on slow heating (Sato and Kuroda, 1987), where rates of transformation increase with increasing temperature.

1.4.3.2 Melt transformations

Melt transformations occur when the liquid form of a drug is allowed to cool. Rates of nucleation, crystal growth, and cooling influence the formation of a solid phase, and have been well characterized for tripalmitin and lithium polymorphs (Sato and Kuroda, 1987; Chryssikos et al., 1990).

1.4.3.3 Solution transformations

Solution transformations occur when a drug is dissolved in a solvent, which is subsequently allowed to evaporate. In solution transformations, only the fraction of drug that
initially underwent dissolution in the solvent is capable of undergoing transformation (Grant, 1999). This method for generating polymorphs is the most common, and has been used in the generation of a number of polymorphs, including FA (discussed in Chapter 2 of this thesis) (Gilchrist et al., 2012), famotidine (Lu et al., 2007), and fluconazole (Caira et al., 2004). However, according to Ostwald’s rule of stages, any system moves to equilibrium from an initial high-energy state through minimal changes in free energy, and therefore a metastable form may often be generated during this process (Ostwald, 1897). This phenomenon has been well documented for indomethacin, and required the use of silane substrates to suppress the growth of the metastable form (Cox et al., 2007).

1.4.3.4 Solution-mediated transformations

A more efficient way to generate stable polymorphs is solution-mediated transformation. In solution-mediated transformation, the less stable polymorph is suspended in a saturated solution (Grant, 1999). This solution is then super-saturated with respect to the most stable form, and over time the most stable form will crystallize at the expense of the dissolution of the less stable form according to Scheme 1.1:

\[
\text{Metastable Form} \xrightarrow{\text{Dissolution}} \text{Solution} \xrightarrow{\text{Nucleation, Crystal Growth}} \text{Stable Form}
\]

Scheme 1.1 Process of polymorphic conversion via solution-mediated phase transformation

Solution-mediated transformation has been shown in this work for FA (Gilchrist et al., 2012), and is very well documented for a number of other drugs such as sulfamerazine (Gu et al., 2001), progesterone (Wang et al., 2000), and erythromycin (Wang et al., 2007).
1.4.3.5 Polymorphic phase diagrams

For any polymorphic pair, only one solid form is stable at any one temperature and pressure; the other polymorph is considered metastable. The two principle types of polymorphic pairs are described as monotropic or enantiotropic (Brittain, 1999). In a monotropic system, only one polymorph is stable at all temperatures and pressures below the melting point, with all other polymorphs being metastable. The conversion of a metastable form to the stable form is irreversible. Conversely, in an enantiotropic system, one polymorph is stable over a certain temperature/pressure range and the other polymorph is stable across a different range of temperatures/pressures (Burger and Ramberger, 1979; Brittain, 1999; Grant, 1999). In these systems the conversion of one polymorph to another is reversible. Polymorphic pairs of solid 1 ($S_1$) and solid 2 ($S_2$) representing monotropic polymorphism and enantiotropic polymorphism are illustrated in Figure 1.7 as a pressure-temperature phase diagram. In this diagram, solid lines represent equilibrium phase boundaries, and dotted lines represent metastable phase boundaries, with transitions denoted by parentheses representing transitions that involve a metastable phase. For both polymorphic pairs, $S_1$-V is the vapor pressure vs. temperature curve for solid 1, while $S_2$-V is the curve of solid 2. $S_1$-L is the melting point curve of solid 1, while $S_2$-L is that of solid 2. The curve L-V is the vapor pressure vs. temperature curve of the liquid. This transition is always an equilibrium transition as differences between polymorphs disappear in vapor and liquid phases. That is, polymorphs have identical liquid and vapor states (Haleblian, 1975).

In monotropic polymorphism, the transition point between $S_1$ and $S_2$ (line FG) occurs at a temperature beyond the melting point of both $S_1$ (line BG) and $S_2$ (line EG); that is, it is a virtual transition point. Furthermore, the vapor pressure vs. temperature curve for $S_1$ (line AB) is lower than that of $S_2$ (line DE) over all temperature ranges. Thus, in a monotropic system, $S_2$ is
metastable with respect to $S_1$ at all temperatures, and the conversion between polymorphs is irreversible.

In enantiotropic polymorphism, the transition point between $S_1$ and $S_2$ (Line BG) occurs at a temperature lower than the melting point of both $S_1$ (line FG) and $S_2$ (line CG); that is, it is an observable transition point. In addition, the vapor pressure vs. temperature curve for $S_1$ (line AB) intersects with that of $S_2$ (line BC) at the transition temperature (line BG). Thus, in an enantiotropic system, $S_1$ is stable below the transition point, and $S_2$ is stable above the transition, and the conversion between polymorphs is reversible (Burger and Ramberger, 1979; Brittain, 1999; Grant, 1999).

[A] Monotropic Polymorphism

[B] Enantiotropic Polymorphism

Figure 1.7 Phase diagrams of pressure versus temperature for a theoretical (A) monotropic or (B) enantiotropic polymorphic pair. $S$, $L$, $V$, refers to solid, liquid, and vapor phase, respectively. Solid lines represent equilibrium phase boundaries and dotted lines represent metastable phase boundaries. Parentheses are used to represent processes that involve a metastable phase.
The assignment of any given polymorphic pair to enantiotropy or monotropy is possible using 4 thermodynamic rules defined by Burger and Ramberger (Burger and Ramberger, 1979), which are outlined in Table 1.1. Of these rules, the two that are the most useful for appropriately assigning polymorphic pairs are the heat of transition rule, and the heat of fusion rule. The heat of transition rule states that if an endothermic solid-solid polymorphic transition is observed, that is the conversion between polymorphs is a real transition, the pair are enantiotropes. Conversely, if an exothermic solid-solid polymorphic transition is observed, the two are monotropes. The heat of fusion rule states that if the higher melting polymorph has a lower heat of fusion, the two are enantiotropes, and if the higher melting polymorph has a higher heat of fusion, the two forms are monotropes (Figure 1.7) (Burger and Ramberger, 1979).

Table 1.1 Thermodynamic rules for classification of polymorphic pairs, S₁ and S₂, based on polymorphic transitions according to Burger and Ramberger (Burger and Ramberger, 1979).

<table>
<thead>
<tr>
<th>Monotropy</th>
<th>Enantiotropy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transition (S₁-S₂) &gt; melting S₁ (line BG)</td>
<td>Transition (S₁-S₂) &lt; melting S₁ (line FG)</td>
</tr>
<tr>
<td>S₁ always stable</td>
<td>S₁ Stable &lt; transition (line BG)</td>
</tr>
<tr>
<td>—</td>
<td>S₂ Stable &gt; transition (line BG)</td>
</tr>
<tr>
<td>Transition irreversible</td>
<td>Transition reversible</td>
</tr>
<tr>
<td>Solubility S₁ always lower than S₂</td>
<td>Solubility S₁ higher &lt; transition</td>
</tr>
<tr>
<td>—</td>
<td>Solubility S₁ lower &gt; transition</td>
</tr>
<tr>
<td>Transition S₂ → S₁ is exothermic</td>
<td>Transition S₂ → S₁ is endothermic</td>
</tr>
<tr>
<td>( \Delta H_{f,S1} &gt; \Delta H_{f,S2} )</td>
<td>( \Delta H_{f,S1} &lt; \Delta H_{f,S2} )</td>
</tr>
<tr>
<td>IR peak S₁ after S₂</td>
<td>IR peak S₁ before S₂</td>
</tr>
<tr>
<td>Density S₁ &gt; density S₂</td>
<td>Density S₁ &lt; density S₂</td>
</tr>
</tbody>
</table>

The differences between enantiotropic and monotropic pairs can be characterized and interpreted from understanding the thermal characteristics of the polymorphic drug. Differential
scanning calorimetry (DSC) is a technique most commonly used, and measures the change of the difference in heat flow to a sample and a reference while they are subjected to a controlled temperature program (Hohne et al., 2003). A number of transitions can be visualized including, solid-solid transitions, melting endotherms, and recrystallization exotherms, and there are several possible scenarios associated with the transformations that either a monotropic or enantiotropic system may undergo, depending on thermodynamic and kinetic parameters. Figure 1.8 shows theoretical DSC traces for a monotropic or enantiotropic pair of polymorphs (Craig, 2006).

Figure 1.8 Schematic DSC traces showing the thermal behavior of (A) monotropic and (B) enantiotropic systems under different sets of conditions. For monotropic pairs: (a) melting of S₁; (b) solid-state transformation of S₂ to S₁ followed by melting of S₁; and (c) melting of S₂ followed by recrystallization and then melting of S₁. For enantiotropic pairs: (a) persistence of metastable S₂ followed by melting; (b) solid state transformation of S₁ to S₂ followed by melting of S₂; (c) solid state transformation from S₂ to S₁, with subsequent solid-state transformation from S₁ back to S₂ with melting of S₂; (d) melting of metastable S₁ followed by recrystallization and melting of S₂.
Data generated by DSC can be influenced by a number of key variables including heating rate (McGregor et al., 2004), sample purity (Giron and Goldbronn, 1995), and the presence of moisture (Yoshinari et al., 2002), and therefore, the DSC thermograms must be interpreted carefully, and in conjunction with other characterization techniques.

Haleblian outlines a scheme for the naming of polymorphic forms, which follows convention where each form is designated with Roman numerals (I, II, III, etc.) (Haleblian and McCrone, 1969). However, contrary to Ostwald (Ostwald, 1897), who suggested that the least stable form should be Form I, current conventions hold that Form I should generally be the most stable form at room temperature, with forms II, III, etc. following by the order in which they are discovered, and ideally in order of stability. However, with the discovery of new polymorphic forms, the nomenclature may not always follow the convention in order of stability (Desiraju, 2004).

The existence of polymorphic forms can be elucidated using a number of solid-state characterization techniques, such as crystallography (powder, or single crystal X-ray diffraction), microscopy (optical or thermal), thermal analysis (differential scanning calorimetry, thermogravimetric), vibrational patterns (infrared and Raman spectroscopy), and solid-state nuclear magnetic resonance (NMR) (Brittain, 1999). In Chapter 2 of this thesis we investigate the solid state properties of FA using crystallography, microscopic, and thermal analyses, and demonstrate significant differences in the solid state characteristics and dissolution behavior of a number of FA polymorphs generated from solvent evaporation and solution-mediated phase transformation.
1.5 Polymeric drug delivery

Polymers are long chain macromolecules of very high molecular weight composed of a series of smaller molecular weight repeat units (Sperling, 2005). Polymers have been used extensively as “carriers” to deliver a wide range of therapeutics, ranging from small molecules to proteins (Langer and Folkman, 1976). These delivery systems can be fabricated in many forms, including drug-polymer conjugates (Duncan, 1992), nano- and micro-particulates (Ravi Kumar, 2000), implants (Sershen and West, 2002), coatings (Gollwitzer et al., 2003), films/membranes (Jackson et al., 2004), or sutures (Blaker et al., 2004), and can be of natural or synthetic origin, and be biodegradable or nondegradable. The loading of drugs into biodegradable polymers and their application as controlled release drug delivery systems is discussed in section 1.6.

The advantages of using polymers to achieve a controlled release drug delivery system include: 1) the continuous maintenance of systemic or local drug levels within the therapeutic window; 2) reduced quantity of drug, and dosing frequency required to achieve therapeutic levels; 3) reduction in local or systemic side effects (Langer, 1998).

1.5.1 Polymer constitution and conformation

Polymers can take on a range of architectures depending on the types and orientation of monomers used. Polymer constitution is the atomic structure that makes up a single repeat unit. Stereoregular repeat units are those with an asymmetric carbon atom, and can take on a D or L form based on the orientation of groups around the chiral carbon atom; that is, they are enantiomers (Rosen, 1982). Based on the relationship of the chiral center of each repeat unit along the polymer backbone with respect to its neighbouring repeat units, polymers can take on one of three conformations: 1) Isotactic, where the polymer is composed of only one stereoisomer and all pendent groups are in the same orientation; 2) syndiotactic, where both
enantiomers are incorporated and alternate between adjacent monomers; or 3) atactic, where both enantiomers are incorporated and are randomly distributed along the polymer backbone (Figure 1.9) (Rosen, 1982).

The properties of each type of polymer are quite different to one another. The order imparted by isotactic and syndiotactic conformations produces a polymer with the ability to crystallize over small molecular ranges producing a semi-crystalline polymer. However, the irregular conformation of atactic chains does not allow for crystallization, and produces an amorphous polymer (Rosen, 1982). The characteristics of the repeat units creating a polymer chain can influence many of the properties of a drug delivery system, including mechanical, thermal, degradation, and drug release.

Figure 1.9 Tacticity in polymer chains containing stereoregular repeat units. Isotactic polymers are formed from one enantiomer (D or L); syndiotactic polymers are formed from both enantiomers and the polymerization reaction allows for the alternate addition of D or L; atactic polymers are formed from both enantiomers and polymerization has no preference for either enantiomer and proceeds in a random fashion.
In addition to the orientation of repeat units with respect to one another, the polymer can take on a number of different architectures depending on the type of monomers used. If the repeat units are all identical, the resulting polymer is called a homopolymer, and can take on any one of the three conformations shown in Figure 1.9. Examples of homopolymers are poly(lactic acid) and poly(glycolic acid). However, if the polymer is made of two or more different monomers (A and B), the polymer is referred to as a copolymer. Copolymers may be subdivided into four main classifications (Figure 1.10):

1) Random copolymers, where the A and B repeat units repeat in a random order.

2) Alternating copolymers, with a perfectly alternating arrangement of A and B repeat units along the polymer chain.

3) Block copolymers, which contain large sequences, called blocks, of each repeat unit.

4) Graft copolymers, which contain one main chain composed of a single repeat unit (A) with a series of side chains made up of a different repeat unit (B).

The properties of a polymer differ greatly depending on its classification. For example, a theoretical block copolymer composed of monomer A and monomer B, will have properties of both homopolymer A and homopolymer B. However, if A and B are randomly distributed, or alternating along the polymer backbone, the polymer will have properties intermediate between homopolymer A and homopolymer B, and dependent on the relative contributions of monomer A and monomer B in the final polymer. In this thesis, we utilize poly(D,L-lactic acid-co-glycolic acid) (PLGA), with a LA:GA ratio of 1:1, which is a random copolymer composed of equal amounts of lactic acid and glycolic acid. The physical properties of PLGA are discussed in section 1.6.2.
Figure 1.10 Polymer classifications based on composition of repeat units A (cube; ■) and repeat units B (polyhedron; ●).
1.5.2 Polymer morphology

Polymer morphology refers to the arrangement of a polymer chains in three dimensions with respect to the long-range order (Rosen, 1982). Since polymers do not achieve equilibrium in the solid state, the partial ordered or disordered arrangement between chains gives rise to semi-crystalline and amorphous material, respectively (Rosen, 1982). Semi-crystalline polymers contain regions of crystallinity, or crystallites, distributed throughout amorphous polymer chains (Seymour and Carraher, 1996). This concept is best illustrated using the “fringed micelle” model (Figure 1.11). In this model, polymer crystallites, or fringed micelles, are composed of regularly ordered areas of aligned segments of polymer chains interspersed in an amorphous matrix. Since the polymer backbones are many times longer than the crystallites, the polymer chains may pass from one crystallite to another through amorphous regions of randomly arranged polymer chains (Seymour and Carraher, 1996).

![Figure 1.11 Schematic representation of the fringed micelle model of polymer morphology](image)

Figure 1.11 Schematic representation of the fringed micelle model of polymer morphology
Several factors contribute to the ability of a polymer to form polymer crystallites, including a lack of pendent side groups or branching, rigidity of the backbone, regular repeat unit conformation (i.e. tacticity) and the ability to form hydrogen bonds or dipole interactions (Rosen, 1982).

1.5.3 Polymer molecular weight

One of the primary characteristics of polymers is the lack of a single, defined molecular weight. Due to the random nature of the polymerization process, no two polymer chains grow equally fast or for the same duration of time, and therefore, there exists a distribution of molecular weights in any polymer material (Cowie, 1973). The molecular weight of a polymer is expressed as the average molecular weight of the entire molecular weight distribution. The molecular weight distribution of polymers is characterized by different average molecular weights, including the number averaged molecular weight ($\bar{M}_n$), and the weight averaged molecular weight ($\bar{M}_w$), depending on the analytical method used (Figure 1.12) (Rosen, 1982).

The number averaged molecular weight ($\bar{M}_n$) uses the numerical fraction of molecules in a class as a weighting factor, and may be determined by any method that calculates the number of molecules in each weight class, such as end-group titration (Cowie, 1973; Rosen, 1982). This average is sensitive to the presence of very small chains since all molecules contribute equally to the average value (Rosen, 1982). A small fraction of the polymer sample, by weight, may exist as small polymer chains or low molecular weight impurities, yet this fraction may represent a large proportion of the total number of molecules present in the polymer, skewing $\bar{M}_n$ to a lower value.

The weight averaged molecular weight ($\bar{M}_w$) uses the mass fraction of molecules in a class as the weighting factor. This average is less sensitive to the presence of short chains in a
polymer sample. $M_w$ is weighted such that the contribution of each chain length fraction depends on its contribution by weight to the total sample (Rosen, 1982). For instance, for $M_w$, ten small polymer chains would have the same weighted importance as a single polymer chain ten times their mass. The lower sensitivity of $M_w$ to low molecular weight chains means that $M_w$ values are always greater than $M_n$ values.

![Molecular Weight Distribution Curve](image)

**Figure 1.12** Representation of a molecular weight distribution curve with the number-averaged molecular weight ($M_n$), and weight-average molecular weight ($M_w$) indicated.

$M_n$ and $M_w$ are used to describe the shape of the molecular weight distribution by using the ratio of $M_w$ to $M_n$, which is called the polydispersity index; a number closer to unity indicates a narrower molecular weight distribution (Rosen, 1982).
1.5.3.1 Influence of polymer molecular weight on polymer properties

The properties of polymers are often more dependent on the size, or weight of polymer chains rather than the number of polymer chains. Therefore, molecular weight is an important determinant of a number of physical properties including glass transition temperature and melt temperature.

The glass transition temperature \( (T_g) \) of a polymer is defined as the onset of large-scale cooperative motion, due to rotational and translational movement, of polymer chain segments (in the order of 20-50 consecutive carbon atoms) upon heating (Ebewele, 2000). The \( T_g \) is affected by the polymer molecular weight according to the following equation, derived by Fox and Flory (Fox and Flory, 1950):

\[
T_g = T_g^\infty - \frac{k}{M_n}
\]

(Equation 1.1)

where \( T_g \) and \( T_g^\infty \) are the glass transition temperature of the polymer with molecular weight \( M_n \), and a polymer of an infinite molecular weight, respectively, and \( k \) is a constant that is proportional to the free chain ends, and thus magnitude of free volume in the polymer (Fox and Flory, 1950). Therefore, an increase in polymer molecular weight results in a proportional increase in polymer \( T_g \).

Semi-crystalline polymers contain small crystallites dispersed throughout an amorphous matrix. The melting of these crystallites is considerably different from the melting behavior of small molecules, as they usually melt over a broad range, and are subject to melting point depression due to the presence of small molecule diluents (termed plasticizers), such as solvent molecules, which may originate due to the method of crystallization (Mandelkern, 2004). The
melting temperature observed in polymers is dependent on polymer molecular weight as described in the following equation (Mandelkern, 2004):

\[
\frac{1}{T_m} - \frac{1}{T_m^\infty} = \frac{2R}{\Delta h} \times \frac{1}{M_n}
\]

(Equation 1.2)

where \(T_m\) is the observed melting point, \(T_m^\infty\) is the equilibrium melting point, \(\Delta h\) is the enthalpy of fusion per repeat unit, and \(R\) is the gas constant.

As previously mentioned, polymers melt over a broad range of temperatures, reflecting both a range in the nature of crystallites within the matrix and in the molecular weights of polymer chains in polydisperse materials (Mandelkern, 2004).

1.6 Controlled release drug delivery systems

Controlled release drug delivery systems may be designed and intended to achieve both a temporal and local or systemic drug delivery. A number of controlled release systems based on polymers have been developed to treat a wide range of disease states such as drug-eluting stents for the treatment of restenosis (Regar et al., 2001), in situ forming drug depots (Ravivarapu et al., 2000), and nanoparticles (Kim et al., 2004) for the delivery of a range of cancer therapeutics, ocular implants (Mansoor et al., 2009), thin polymer films for the treatment of restenosis (Jackson et al., 2004), periodontal infections (Kalachandra et al., 2002), and tissue adhesions (Jackson et al., 2002). In addition, a myriad of drugs have been incorporated into polymeric microspheres (see section 1.9.3) and nanofibers (see section 1.9.4), which are the localized delivery systems developed in this thesis work, for a range of therapeutic applications.
Two fundamental types of controlled release systems exist: the reservoir system and matrix system. In the reservoir system, a drug phase (with or without polymer) is surrounded by a rate-limiting polymer phase, and in a matrix system, the drug is uniformly dispersed within a polymer (Figure 1.13). In this thesis we focus our efforts in the development of matrix-type drug delivery systems for the treatment of implant-associated infections.

![Reservoir System and Matrix System](image)

Figure 1.13 Schematic illustration of reservoir and matrix controlled release drug delivery systems

1.6.1 Biodegradable drug delivery systems

Biodegradable materials degrade by the cleavage of hydrolytically or enzymatically-sensitive bonds, ultimately leading to polymer erosion. Natural polymers, such as polysaccharides and proteins, belong to the latter class of enzymatically-sensitive polymers, whereas synthetic polymers, such as the aliphatic polyesters, polyanhydrides, poly(ortho esters), belong to the former. The selection of synthetic polymers over natural polymers for drug delivery purposes is due to a number of factors such as, being more biologically inert, and the
ability to specifically tailor the polymer properties to suit the application (Nair and Laurencin, 2007).

In general, the most widely used synthetic class of polymers is the aliphatic polyesters, which includes poly(glycolic acid), poly(lactic acid), poly(D,L-lactic acid-co-glycolic acid) and poly(ε-caprolactone). These polyesters offer the attractiveness of low cost, their reproducibility in manufacturing, their biocompatibility and lack of toxicity with prolonged use (Vert, 2005).

### 1.6.2 PLGA structure and properties

Poly(D,L-lactic acid-co-glycolic acid) (PLGA; Figure 1.14) is an aliphatic polyester, which is synthesized via ring-opening polymerization reactions of corresponding cyclic dimers of lactic acid diastereomers (D and L) and glycolic acid, yielding a random copolymer which is identified by the ratio of monomers used (Vert et al., 1992).

![Chemical structure of poly(D,L-lactic acid-co-glycolic acid) (PLGA) copolymer. The subscripts, x and y represent the number of repeat units of lactic acid and glycolic acid, respectively.](image)

PLGA synthesized from D,L-lactide is amorphous and a glass at room temperature, with a glass transition temperature ($T_g$) in the range of ~55-60°C, which is dependent on the molecular
weight and monomer composition. Decreasing the LA content from 85 mol% to 50 mol% causes a decrease in the $T_g$ from between 50-55°C to 45-50°C (Park, 1995). If PLGA is synthesized with stereoregular L-LA, the resulting copolymer displays some degree of crystallinity, but only when lactic acid is present over 70 mol%. Annealing a copolymer containing ≥70 mol% L-LA at temperatures well above the glass transition temperature, has been shown to further increase the overall degree of crystallinity to a maximum of ~20% (Chye Joachim Loo et al., 2005). Although the degree of crystallinity is limited, it can be controlled to some extent through the inclusion of stereoregular monomers, and annealing.

1.6.2.1 Biodegradation of PLGA

The degradation of PLGA is via hydrolysis and bulk erosion. Upon exposure to aqueous milieu, the ester carbon in PLGA is subject to nucleophilic attack by water molecules according to Scheme 1.2 (Houchin and Topp, 2008).

Overall, the hydrolytic degradation of a PLGA matrix occurs in 5 stages: 1) Uptake of water from aqueous/tissue fluids into polymer; 2) Random cleavage of polymer ester bonds through nucleophilic attack by water molecules; 3) Cleavage of long polymer chains into shorter chains or oligomers; 4) Dissolution of small molecular weight oligomers in aqueous media; 5) Erosion and mass loss of polymer (bulk erosion). In addition, the carboxylic acid end groups produced from the hydrolytic degradation of PLGA ester bonds are able to catalyze the hydrolysis of other ester groups, a process called autocatalysis (Gopferich, 1996). Therefore, the degradation of PLGA occurs heterogeneously throughout the matrix, with rates increasing towards the center of the device (von Burkersroda et al., 2002).
The degradation of PLGA is affected by several factors, including molar ratio of LA to GA, polymer crystallinity, and polymer molecular weight (Alexis, 2005). Zhou et al. have shown that changing the molar ratio of LA and GA monomers from 85:15 to 75:25, 65:35, and 50:50 resulted in an increase in rate of hydrolysis and mass loss from PLGA microspheres (Zhou et al., 2004). Increasing the GA ratio of the copolymer has been shown to influence water uptake, resulting in faster degradation rates due to preferential degradation of GA units (Li, 1999; Wu and Wang, 2001). In addition, increasing the molar content of stereoregular LA can increase the overall matrix crystallinity (Chye Joachim Loo et al., 2005), resulting in longer degradation time. However, since PLGA is primarily amorphous, matrix crystallinity is not expected to contribute to overall degradation rate. The molecular weight of PLGA has also been shown to be a significant factor in the rate of degradation and mass loss. Wu and Wang, have shown that decreasing the $M_w$ of PLGA (75:25) from ~241,000 Da, to ~166,000 Da, ~66,000 Da, ~31,000 Da, and ~10,000 Da, resulted in a proportional increase in the rate of degradation (Wu and Wang, 2001). That is, the rate constants for biodegradation increase with increasing molecular weight.
weight. The longer polymer chains in polymers with higher $\bar{M}_w$ may have more chance to be attacked by water molecules, which is required for polymer chain hydrolysis or biodegradation. However, higher molecular weight polymers do not exhibit any obvious morphology change until their molecular weight is lowered enough to lose mechanical integrity because of their high molecular weight (Wu and Wang, 2001). Although the degradation rates are faster, the erosion of matrices composed of higher molecular weight polymers is slower. In this thesis, we use PLGA (50:50) with a molecular weight of 49,100 Da, which shows significant mass loss (i.e. matrix erosion resulting in $\geq 50\%$ mass loss) at $\sim40$ d when evaluated in vitro (Wu and Wang, 2001).

The breakdown of PLGA to oligomers or constitutive monomers allows it to be cleared by endogenous metabolic pathways: LA is oxidized to pyruvate or converted to glucose via gluconeogenesis where it is subsequently cleared from the body through the Krebs or Cori cycles, respectively, and GA is excreted unchanged in the urine (Vert et al., 1994).

### 1.6.3 Mechanisms of drug release from biodegradable polymeric matrices

The rate of drug release from biodegradable controlled-release systems is governed by drug diffusion and the rate of polymer degradation. The diffusional transport of drug from a polymer matrix is described by Fick’s first law of diffusion, and defined as:

$$J = -D \frac{dC}{dx}$$

(Equation 1.3)

where $J$ is the flux (rate of diffusion per unit area), $D$ is the diffusion coefficient, and $dC/dx$ is the concentration gradient (Fick, 1855). In drug delivery systems where a drug is dissolved and molecularly dispersed within the matrix, it is called a monolithic solution. In monolithic
solutions there initially exists no concentration gradient to drive drug transport. However, as surface drug is depleted a concentration gradient is produced allowing drug to diffuse down the concentration gradient, through the polymer to the surface and is gradually released at the surface (Baker, 1987).

Where drug is not molecularly dispersed but present as solid drug particles throughout the polymer matrix due to drug insolubility in the polymer matrix or a drug loading that exceeds the drugs solubility in the polymer, it is defined as a monolithic dispersion (Baker, 1987). The dispersion can be classified as simple, complex, or as a monolithic matrix system and is based on the amount of drug loaded into the polymer matrix.

Simple monolithic dispersions are formed with drug loadings which occupy 0-5% of polymer volume. In these systems, solid drug particles are well dispersed and separated and release involves dissolution of drug particles in the polymer free volume followed by diffusion through polymer chains to the surface of the matrix. Release from simple monolithic devices can be described by the simple Higuchi model as follows (Higuchi, 1963):

\[
Q = \left[ D (2A - C_s)Cs \right]^\frac{1}{2} \tag{Equation 1.4}
\]

where \( D \) is the diffusion coefficient of the drug in the matrix, \( A \) is total amount of drug in unit volume of matrix, \( C_s \) is the solubility of the drug in the polymer, and \( t \) is time. Release from a simple monolithic device will be linear when plotted as a function of the square root of time.

If drug loading is increased such that drug particles occupy 5-10% of polymer volume, it is called a complex monolithic dispersion (Baker, 1987). Release of drugs from complex monolithic dispersions is still proportional to the square root of time, however have a higher rate than predicted from simple Higuchi kinetics. The increase in release is due to cavities formed on
the surface of the device from drug loss, which increase polymer permeability and provide a preferred route of drug release (Baker, 1987).

If drug loading is further increased to occupy \( \geq 20\% \) of polymer volume, nearly all drug particles dispersed in the polymer matrix are in contact with one another, forming a monolithic matrix (Baker, 1987). Release from monolithic matrices can be described by the percolation theory, where drug release occurs by dissolution of the active ingredient and diffusion through water-filled capillaries created from the dissolution of interconnecting drug particle clusters, creating a porous network (Holman and Leuenberger, 1988).

In these systems drug must first partition into the water-filled pores, and thus the diffusion coefficient of the drug through the polymer no longer describes the release profile. The geometry of the drug channels is described by a term called tortuosity \( (\tau) \). The presence of pores, and porosity of the matrix \( (\varepsilon) \) significantly increases the release of drug from monolithic matrices, and is defined by the second form of the Higuchi equation (Higuchi, 1963), which incorporates both \( \varepsilon \) and \( \tau \):

\[
Q = \left[ \frac{D\varepsilon}{\tau} (2A - \varepsilon C_0) C_{s,t} \right]^{1/2}
\]

(Equation 1.5)

The Higuchi equation is a pseudo-steady-state model describing Fickian release, where the amount of drug dissolved in the matrix remains constant due to the presence of excess, undissolved drug.

In many polymeric, controlled-release formulations, an initial large bolus of drug is released when introduced to the release medium, prior to the onset of diffusion controlled and polymer degradation and erosion mediated drug release. This is referred to as the “burst phase”
of drug release, and is an inherent property of diffusion-controlled devices (Allison, 2008). The burst phase is diffusion rate limited, and is the release of drug that is closely associated with the surface of the device. There are many factors that contribute to the burst phase of drug release, including drug-polymer compatibility (see section 1.8), physicochemical properties of the drug, processing, and polymer/drug crystallinity. However, the primary factors influencing the burst phase are drug loading, surface area, and polymer molecular weight.

Figure 1.15 is a schematic illustration of drug release curves from both diffusion and polymer degradation/erosion controlled systems. Curve A represents drug release from a device with a significant burst phase of drug release (Allison, 2008); curve B represents drug release from monolithic matrices where no burst phase is observed (Baker, 1987); curve C represents drug release from granular matrices (Higuchi, 1963); and curve D represents drug release from matrices undergoing bulk erosion over the course of drug release, where the onset of polymer erosion causes significant drug release from the polymeric (Baker, 1987).
Figure 1.15 Schematic diagram of drug releases profiles showing the effects of different drug release mechanisms. (A) Significant burst phase of drug release (Allison, 2008); (B) Release from monolithic matrices (Baker, 1987); (C) Release from granular matrices (Higuchi, 1963); (D) Release from matrices undergoing bulk erosion over the course of drug release (Baker, 1987).

In addition to diffusional transport, polymer degradation rate also influences drug release, as shown in Figure 1.15. In bulk degradation, as seen in PLGA matrices, water influx into the polymer matrix is faster than the breakdown of the matrix into smaller, water soluble components (Sinko and Kohn, 1993). Therefore, hydrolysis of susceptible polymer bonds (i.e. esters) proceeds throughout the whole matrix (Gopferich, 1996). The pores and channels formed from the hydrolysis of polymer chains into soluble oligomers and monomers leads to erosion and subsequent mass loss of the polymeric device (Figure 1.16).
Polymer degradation proceeds much faster than matrix hydration, and release is dependent on diffusional drug transport and the rate of polymer degradation.

1.6.4 Factors influencing drug release from PLGA matrices

There are a number of factors that influence the release of drug from a biodegradable matrix. These factors can be categorized into polymer properties, including molecular weight, monomer ratios, end-group capping, and crystallinity; drug properties, including physicochemical characteristics, extent of loading, and distribution throughout the polymer.
matrix; and device characteristics, including size, porosity, density, and shape (Fredenberg et al., 2011). This section will focus on the major factors influencing drug release from PLGA matrices. Since these matrices are primarily amorphous, factors such as crystallinity will not be discussed further.

The influences of monomer ratio and molecular weight have been shown to be strong regulators of matrix erosion, and drug release from biodegradable matrices (Witt et al., 2000). For PLGA, increasing the lactide content has been associated with a decrease in drug release rate for a number of drugs, including nalbuphine (Sung et al., 1998), nafarelin (Sanders et al., 1986), vapreotide (Rothen-Weinhold et al., 1997), and RIF (Makino et al., 2004). The increased lactide content in PLGA reduces matrix hydrophilicity, slows matrix hydration and delays erosion (Zhou et al., 2004).

The physicochemical properties of the drug can also play a role in regulating drug release. Miyajima et al. performed release experiments using a range of acidic, and neutral drugs in PLGA matrices and showed that the release is controlled by the solubility limit of the drug in the release media (Miyajima et al., 1998). In addition to solubility, the compatibility between drug and polymer (see section 1.8) has also been shown to play a major role in governing drug solubilization and release.

The geometry of the drug delivery system is another predictor of drug release. Crank (Crank, 1956), and Cobby (Cobby et al., 1974), developed mathematical models for the release of drug from a matrix of various geometries, including slab, sphere, cylinder, and tablet. In each case, drug release is dependent on device thickness/diameter, with thinner or smaller geometries predicted to have faster drug release. This phenomenon has been supported through experimental observations of drug release from microspheres (see section 1.9.3.2), nanofibers (see section 1.9.4.3), and thin films (Klose et al., 2008) composed of PLGA, suggesting that the surface area
of the device is a strong predictor of drug release rate. In fact, an alteration in processing to produce a porous device has been shown to markedly improve drug release. Klose et al. have shown that the release of lidocaine was significantly increased with an increase in matrix porosity (Klose et al., 2006).

The influence of a number of these factors on the drug release from PLGA microspheres and nanofibers will be reviewed in sections 1.9.3.2 and 1.9.4.3, respectively.

1.7 Polymer-drug dispersions

In solid polymeric (matrix type) drug delivery systems, the encapsulated drug can be dispersed throughout the polymer matrix as a molecular level dispersion, suspended in discrete solid drug particles or drug-rich domains, or a combination of the two. The nature of the polymer-drug dispersion has been shown to play a significant role in the performance of the drug delivery system (Leuner and Dressman, 2000).

When drug is present as a molecular level dispersion within a carrier, it is also termed a solid solution. Solid solutions are comparable to liquid solutions, consisting of a single phase irrespective of the number of molecules or components (Leuner and Dressman, 2000). Solid solutions can be classified according to miscibility, and may be described as a continuous or discontinuous solid solution (Leuner and Dressman, 2000).

1.7.1 Solid solutions

1.7.1.1 Continuous solid solutions

Continuous solid solutions occur when the two components are miscible in all proportions (Chiou and Riegelman, 1971; Leuner and Dressman, 2000). This suggests that the bonding strength between the individual components is stronger than the bonding strength
between molecules of their own kind. No continuous solid solutions have been reported in the peer-reviewed drug delivery literature (Leuner and Dressman, 2000).

1.7.1.2 Discontinuous solid solutions

Discontinuous solid solutions arise when the solubility between the two components is not zero but is limited (Chiou and Riegelman, 1971; Leuner and Dressman, 2000). A typical phase diagram of a discontinuous solid solution is shown in Figure 1.17. Alpha (α) and beta (β) show the regions of a true solid solution, where one of the components is molecularly dispersed in the other solid component. Increasing the amount of one of these components above its solubility limit produces a system characterized by 2 phases. Solid solutions of sulfathiazole and urea are classic examples of a discontinuous solid solution (Chiou and Niazi, 1971).

Figure 1.17 Phase diagram for a discontinuous solid solution. Alpha (α) and beta (β) show the regions of a true solid solution. Increasing the content of either component beyond the limits of α and β yields a 2-phase system.
1.7.1.3 Amorphous solid solutions

Polymeric drug delivery systems are likely to form an amorphous solid solution, as the polymers are often present in an amorphous, or semi-crystalline form. In amorphous solid solutions, the drug molecules are dispersed on a molecular level throughout the amorphous polymer matrix. Amorphous solid solutions are a single phase, characterized by a reduction in polymer $T_g$ due to the plasticizing effects of the drug molecules. In semi-crystalline polymers, where some degree of crystallinity exists, drug may also partition into spaces between polymer chains in crystallites, and be characterized by a lower $T_g$ and a melting point depression.

Amorphous solid solutions were first described by Chiou, who used citric acid to increase the solubility of griseofulvin (Chiou and Riegelman, 1969). Other examples have been illustrated by van Drooge who used thermal analysis to illustrate the interactions of diazepam with PVP. Using melt quenching, diazepam formed amorphous solid solutions in PVP up to 80% (w/w), however using spray-drying or freeze-drying, diazepam formed amorphous solid suspensions above 35% (w/w) drug loading (van Drooge et al., 2006).

1.7.2 Amorphous solid suspensions

If the compatibility between the drug and the polymer is low or negligible, drug may solidify into amorphous domains throughout the matrix, producing an amorphous suspension (Chiou and Riegelman, 1971). Amorphous suspensions contain two distinct phases: one characteristic of the polymer, and one characteristic of the drug. Solid suspensions occur when there is a reduced solubility of the drug in the polymer (Chiou and Riegelman, 1971). For example, Yang et al. have shown that the solubility of FA in PLGA is <1% (w/w) (Yang et al., 2009). When formulated as a PLGA microsphere using solvent evaporation techniques (see section 1.9.3.1.1), FA was dispersed into phase-separated domains suggesting an amorphous
solid suspension (Yang et al., 2009). In chapter 3 of this thesis, we expand these previous observations made by our group, and characterize a PLGA microsphere formulation containing FA and RIF as an amorphous solid suspension of drug rich domains dispersed throughout a polymer matrix.

1.7.3 Preparation and characterization of polymer-drug dispersions

Drug/polymer solutions/suspensions can be prepared using a number of techniques, including hot-melt extrusion (Naima et al., 2001), freeze-drying (Guyot et al., 1995), spray-drying (Chauhan et al., 2005), supercritical fluid processes (Van Nijlen et al., 2003), and electrospinning (Verreck et al., 2003). Characterization of drug-polymer solutions/suspensions can be done using X-ray diffraction to discriminate between crystalline and amorphous solutions based on the presence of characteristic diffraction peaks. However, XRD is unable to discriminate between amorphous solid solutions and amorphous solid suspensions. Additional techniques, such as Raman spectroscopy, and thermal analysis allow for the collection of more useful information, allowing for proper categorization of the dispersed system (Chiou and Riegelman, 1971; van Drooge et al., 2006).

The dispersion of a drug in a polymeric carrier has been shown to markedly improve the dissolution and release profile of many hydrophobic drugs including, griseofulvin (Chiou and Riegelman, 1969), itraconazole (Jung et al., 1999), nilvadepine (Okimoto et al., 1997), phenytoin (Muhrer et al., 2006) and fusidic acid (Yang et al., 2009). Therefore, the nature of drug-polymer dispersions is important when designing and evaluating drug delivery devices. Whether a polymeric drug delivery device will form a solution or suspension (i.e. one phase or two) depends on the compatibility between the drug and polymer, and thus the solubility of the drug in the polymer matrix.
1.8 Polymer-drug compatibility

The compatibility between a drug and polymer is a critical determinant of drug stability (Greenhalgh et al., 1999; Six et al., 2002), drug encapsulation (Letchford et al., 2008), and drug release kinetics (Liu et al., 2004).

In order for a solute to be solubilized by a solvent (in this case a drug solute molecule solubilized in the polymer solvent matrix), the intermolecular forces between the solute and solvent must be greater than solvent-solvent and solute-solute interactions. The amount of energy necessary to overcome the solute-solute, and solvent-solvent interactions must be greater than the cohesive energy between molecules in each of the two phases. In other words, the cohesive energy \( E_{coh} \) is the amount of energy required to remove a unit volume (per mole) of molecules from their neighboring molecules into infinite separation (Bicerano, 2002). The amount of energy required to do so in a unit volume of material \( V \) is defined as the cohesive energy density \( e_{coh} \), such that:

\[
e_{coh} = \frac{E_{coh}}{V}
\]

(Equation 1.6)

Direct methods for calculating \( E_{coh} \) are done by determining the heat of evaporation, which is simple for low molecular weight solvents, but does not apply to material that does not evaporate (i.e. polymers and drugs). Therefore, indirect methods, such as comparative swelling, or dissolution in liquids of known \( E_{coh} \), may yield values of \( E_{coh} \) for non-evaporating material (Bicerano, 2002). However, in 1928, Dunkel showed that the \( E_{coh} \) for low molecular weight organic compounds is additive, and can be predicted by summing the contributions of each molecular group constituting the overall compound (Dunkel, 1928). This idea was later expanded
by Hoftyzer and van Krevelen to include polymers, and they published a series of tables which can be used for the calculation of $E_{coh}$ from functional groups in a polymer repeat unit (Van Krevelen, 1997).

The cohesive forces between materials are a result of three intermolecular forces: dispersion forces (atomic), polar forces (molecular), and hydrogen bonding (electron exchange) (Van Krevelen, 1997). Dispersion forces are one of the Van der Waal’s intermolecular forces, and arise from instantaneous dipole moments induced in a molecule as a result of charge oscillation. This induced polarization (dipole), attracts temporary dipoles in neighboring molecules, resulting in cohesion. In contrast to dipole moments, polar cohesive forces are due to permanent dipoles in neighboring molecules, which arise as a result of electrostatic interactions between electronegative and electropositive atoms between molecules. Hydrogen bonding is similar to polar cohesive forces, and is the interaction between an electronegative atom of one molecule and an electropositive hydrogen (bonded to a nitrogen, oxygen, or fluorine atom) on a neighboring molecule. It is described as a strong electrostatic dipole-dipole interaction, but has features of covalent bonding. Therefore the $E_{coh}$ of any molecule must be the sum of the individual energies that make it up, and is defined by van Krevelen as:

$$E_{coh} = E_d + E_p + E_h$$  \hspace{1cm} (Equation 1.7)

where $E_d$, $E_p$, and $E_h$ are the dispersion, polar, and hydrogen forces, respectively.

The calculation of $E_{coh}$, thus far, has been used in the context of vaporization. That is the energy required to overcome cohesive forces between like molecules. However, using the idea of “like seeks like”, Hildebrand and Scatchard suggested that the $E_{coh}$ can be used to predict solvency behavior between solvent and solute (Hildebrand and Scott, 1951). Thus the
Hildebrand-Scatchard solubility parameter ($\delta$) is a numerical estimate of the degree of interaction between materials, and is related to the $E_{coh}$ by:

$$\delta = \sqrt{e_{coh}} = \sqrt{\frac{E_{coh}}{V}}$$  \hspace{1cm} (Equation 1.8)

In polar solvents, however, the Hildebrand-Scatchard equation was expanded by Hansen (Hansen, 2007), such that the total solubility parameter ($\delta_t$; the Hansen solubility parameter) is the sum of squares of the dispersion ($\delta_d$), polar ($\delta_p$), and hydrogen bonding ($\delta_h$) forces:

$$\delta_t^2 = \delta_d^2 + \delta_p^2 + \delta_h^2$$  \hspace{1cm} (Equation 1.9)

Where each component is calculated using the molar dispersion constant ($F_{di}$), the polar attraction constant ($F_{pi}$), and the hydrogen bonding energy ($E_{hi}$) according to the following equations:

$$\delta_d = \sum \frac{F_{di}}{V}$$  \hspace{1cm} (Equation 1.10)

$$\delta_p = \left( \sum \frac{F_{pi}^2}{V} \right)^{1/2}$$  \hspace{1cm} (Equation 1.11)

$$\delta_h = \left( \sum \frac{E_{hi}}{V} \right)^{1/2}$$  \hspace{1cm} (Equation 1.12)

The Hansen solubility parameter can then be used to estimate whether two components will be miscible by similarities in their respective solubility parameters, as is shown from the
thermodynamics of mixing. Thermodynamic considerations require the free energy of mixing ($\Delta G_M$) be zero, or negative, for the solution process to take place spontaneously. The free energy change for the solution process is given by:

$$\Delta G_M = \Delta H_M - T \Delta S_M$$  \hfill (Equation 1.13)

where $\Delta H_M$ is the heat of mixing, $T$ is the absolute temperature, and $\Delta S_M$ is the entropy change that occurs on mixing. According to Hildebrand and Scott (Hildebrand and Scott, 1951), $\Delta H_M$ can be calculated using the following equation:

$$\Delta H_M = \phi_1 \phi_2 (\delta_1 - \delta_2)^2$$  \hfill (Equation 1.14)

Which can be expanded to the following equation to account for all specific interactions:

$$\Delta H_M = \phi_1 \phi_2 \left[ (\delta_{d,1} - \delta_{d,2})^2 + (\delta_{p,1} - \delta_{p,2})^2 + (\delta_{h,1} - \delta_{h,2})^2 \right]$$  \hfill (Equation 1.15)

where $\phi_1$ and $\phi_2$ are the volume fractions of the two components. Therefore, as the solubility parameters approach each other, $\Delta H_M$ approaches zero, favoring solubility.

In the case of polymer solutions, Flory (Flory, 1941) and Huggins (Huggins, 1941) expanded on observations from Fried, who experimentally determined that predictions for ideal solutions were not always appropriate when dealing with molecules with larger molecular sizes (such as polymers) (Fried, 2004). The Flory-Huggins interaction parameter ($\chi_{sp}$) was developed
to explain the thermodynamics of polymer solutions, which has since been able to correctly predict the solubility of drugs in polymers and is calculated by:

\[ \chi_{sp} = \left( \delta_{\text{solute}} - \delta_{\text{polymer}} \right)^2 \frac{V_S}{RT} \]  

(Equation 1.16)

Where \( R \) is the gas constant; \( T \) is the absolute temperature; and \( V_S \) is the molar volume of the added drug as calculated by the group contribution method according to van Krevelen (Van Krevelen, 1997). The Flory-Huggins solution theory states that the \( \chi_{sp} \) is always positive, and is proportional to the enthalpy of mixing. A lower \( \chi_{sp} \) predicts favorable mixing of two components, whereas a large positive \( \chi_{sp} \) indicates a tendency for phase separation to occur between drug and polymer, as the components would prefer to be surrounded by molecules of their own kind (insoluble>0.5>soluble) (Li et al., 1995a; Bicerano, 2002).

### 1.8.1.1 Influence of drug-polymer compatibility on the glass transition temperature

Compatibility between a given drug and polymer pair means that in the final drug-loaded formulation, the drug will be dispersed at a molecular level within the free volume between polymer chains, forming a solid solution. The molecular-level drug effectively plasticizes the polymer by increasing the free volume and subsequently lowers the polymer \( T_g \). Therefore, a miscible blend of two amorphous components where one component is solubilized by the other, in this case a compatible drug-polymer pair, will exhibit a single \( T_g \) that will vary with the composition. The composition-dependent shift in \( T_g \) for a miscible blend \( (T_{g,BLEND}) \) has been defined by a number of equations including the Couchman-Karasz (Couchman, 1978), Gordon–Taylor (Gordon and Taylor, 1952), Jenckel–Heusch (Jenckel and Heusch, 1953), and Utracki...
(Ajji and Utracki, 1996) equations. However, the most commonly employed equation is the Fox equation (Fox, 1956), which is defined as:

\[
\frac{1}{T_{g,\text{BLEND}}} = \frac{\phi_1}{T_{g,1}} + \frac{\phi_2}{T_{g,2}}
\]

(Equation 1.17)

where \(\phi_1\) and \(\phi_2\), and \(T_{g,1}\) and \(T_{g,2}\), are the weight fractions and the glass transition temperatures of component 1 and 2, respectively.

Where a drug-polymer combination is incompatible and immiscible and the drug forms an amorphous solid, there will be two \(T_g\)’s, characteristic of each component, and the \(T_g\)’s will be unaffected by the overall composition. However the \(T_g\)’s may shift closer to one another and become broadened.

1.9 **Localized drug delivery approaches to orthopaedic surgical site infections**

Localizing drug release to the surgical site provides a number of advantages over traditional systemic administration. The critical tenet of systemic antibiotics is that there exists a relationship between plasma drug concentrations and drug concentrations at the surgical site. In fact, for infections of extracellular compartments, this has been evaluated for a number of antibiotic classes. In these studies, tissue penetration has been related with plasma protein binding (Bergan *et al*., 1987; Nix *et al*., 1991), making predictions of local concentrations possible. However, these predictions only represent the diffusional transport of drug into extracellular tissue fluid. Antibiotic penetration into abscesses and sub-cutaneous fibrin clots has been shown to be considerably slower than into extracellular fluid compartments (Barza and Weinstein, 1974). Thus, it has been suggested that serum/plasma concentrations may not be the
ideal marker of efficacy for targets in more isolated compartments where chronic inflammation, as seen in fibrin-encapsulation and deep trauma sites, presents a range of physiological barriers to antibiotic penetration (Ryan, 1993). Therefore, the limitation to successful antimicrobial therapy is an inability to reach adequate tissue levels due to the threshold of systemic toxicity. The delivery of the drug locally, to the infection site, may circumvent this limitation.

There are a number of proposed techniques for the local administration of antibiotics to prevent and treat infections associated with surgical implants. These include, low pressure irrigation with an antimicrobial solution (Bhandari et al., 2001), dipping of the surgical implant in an antimicrobial solution (Actis Dato et al., 1992), antimicrobial coating of the implant (Kazemzadeh-Narbat et al., 2010), mixing of sterile antibiotic with polymerizing acrylic bone cement prior to fixation (Jaeblon, 2010), and the placement of a biodegradable antibiotic carrier (Stemberger et al., 1997).

1.9.1 Bone cement and spacer devices

The first efforts to control infection in total joint arthroplasties were proposed in the 1970’s. Buchholz and Engelbrecht, proposed that poly(methyl methacrylate)(PMMA) resins, which had been used since the 1930’s in medical applications, would serve as an appropriate matrix for the delivery of gentamicin sulphate and would provide long-term protection against bacteriological seeding (Buchholz and Engelbrecht, 1970).

PMMA is a nondegradable polymer (Figure 1.18) that exists in two stereoisomer forms. PMMA possesses an atactic conformation and is a semicrystalline polymer with a $T_g$ of ~100°C (Migliaresi et al., 1994), which has been shown to be dependent on its molecular weight (Beevers and White, 1960).
The contents of commercial packages of PMMA for bone cement applications include an ampule of liquid components and a package of powder components (Webb and Spencer, 2007; Jaeblon, 2010). The major liquid components are methylmethacrylate monomer, benzoyl peroxide initiator, hydroquinone as a stabilizer to prevent premature polymerization, and a green chlorophyll dye, and the principle powder components are high molecular weight PMMA microbeads, barium sulphate or zirconium dioxide as radio-opacifiers, and a green chlorophyll dye, which helps to distinguish between cement and bone in the final mixture (Webb and Spencer, 2007; Jaeblon, 2010). The liquid and powder components are combined and mixed to form a malleable grout, which is then used to fix implantation devices. In a two-stage revision surgery, where the primary implant fails, PMMA has been manually formed into spacers of similar shape to the prosthetic, which are used to occupy boney voids to promote tissue healing prior to the implantation of a new sterile prosthetic. It is during the mixing stage that antibiotics are introduced into the bone cement for use as an antibiotic eluting device.

There has been widespread adoption of antibiotic-loaded bone cements (ALBCs) among surgeons in the United States for a number of years (Heck et al., 1995). In Canada, there is limited information on the use of ALBC in clinical practice, however the Calgary Health Region

![Figure 1.18 The chemical structure of poly(methyl methacrylate) (PMMA)](image)
reports that 79% of practicing orthopaedic surgeons utilized premixed ALBC in primary knee and hip arthroplasty (Sabuda et al., 2009). Until recently, the widespread adoption of ALBC in North America has been done off-label, through the mixing of sterile antibiotic in polymerizing PMMA bone cements in the operating theatre, as no current guidelines exist for the use of ALBCs as a prophylactic. Since 2004, the FDA has cleared 6 ALBC products containing gentamicin, including: Cobalt™ G-HV (Biomet Orthopedics), Palacos® R+G (Heraeus Medical), SmartSet® GHV (DePuy Inc.), Cemex® Genta (Exactech), VersaBond® AB (Smith and Nephew Inc.), and Simplex® P (Stryker). These ALBC products are only approved for use in the second stage, of a two-stage revision surgery where initial infection was cleared (see section 1.2.2). The proposed algorithm for the use of ALBCs or spacer devices is shown in Figure 1.19.
Figure 1.19 The proposed clinical guidelines for the use of antibiotic-loaded bone cements (ALBCs) and spacer devices in surgical prophylaxis or treatment of established orthopaedic infections. These guidelines are proposed as off-label instructions for the incorporation of sterile antibiotic powder into polymerizing PMMA bone cement. PROSTALAC is a pre-fabricated functional spacer made of antibiotic-loaded acrylic cement with a small metal-on-polythene articular surface (Haddad et al., 2000).

Although there are a number of studies suggesting that there is sufficient evidence for the use of ALBCs as a method for prophylaxis or treatment of infection, there exists much debate over its widespread use (Bourne, 2002; Bourne, 2004; Hanssen, 2004). In fact, Espehaug and Engesæter are the only two investigators to perform very large retrospective studies comparing ALBCs plus systemic antibiotics with systemic antibiotics alone. They showed no difference in infection rates and revision surgeries with the use of ALBCs in Norwegian hospitals (Espehaug...
et al., 1997; Engesæter et al., 2003). The major concerns regarding the use of ALBCs are due to the possible promotion of antibiotic resistance through drug release profiles that lead to sub-MIC at the surgical site.

Due to the impermeability of PMMA, antibiotic release from acrylic bone cements is a surface phenomenon, and is almost entirely dependent on the exposed surface area (van de Belt et al., 2000), with approximately 10% of the total antibiotic load eluting throughout the lifetime of the cement (DiCicco et al., 2003). The sub-therapeutic elution has been a concern for the development of antibiotic resistance. In fact, in 1989, Hope et al. illustrated that ALBCs promoted the development of microorganisms resistant to gentamicin in nearly all cases (88% in ALBC group versus 16% in bone cement only) (Hope et al., 1989). Furthermore, Dunne et al. showed that increasing the amount of gentamicin to a level that would result in mechanical failure, did not prevent the colonization of bone cement by S. epidermidis, S. aureus, and S. capitas (Dunne et al., 2007). These data suggest that the resistance of clinical isolates to gentamicin should preclude the use of ALBCs containing gentamicin. In fact, due to the emerging resistance of bacteria to aminoglycoside antibiotics, a number of groups have suggested the incorporation of a secondary antibiotic, including fusidic acid (Neut et al., 2006), clindamycin (Anagnostakos et al., 2008), and vancomycin (Streuli et al., 2006), into the ALBCs. However, Anagnostakos et al. showed that neither gentamicin nor gentamicin-vancomycin loaded spacer devices (beads) were able to eradicate clinical infections, and the bead surface became a growth plane for a number of Staphylococcal microorganisms (Anagnostakos et al., 2008).

Investigations of the tensile strength, hardness and flexion of ALBCs has been thoroughly investigated. Failure of the bone cement matrix can lead to aseptic loosening and implant failure via macrophage-mediated inflammatory response, which recruits osteoclasts to
the cement-bone interface, resulting in bone resorption (Horowitz and Purdon, 1995). In almost all cases, ALBCs had reductions in mechanical strength, which was dependent on the type and amount of antibiotic used (De Palma et al., 1982; DeLuise and Scott, 2004; Postak and Greenwald, 2006), and in the case of RIF, the polymerization of PMMA was completely prevented (De Palma et al., 1982).

In addition to bacterial growth, the thermal curing of PMMA in situ may also lead to the degradation of antibiotics and local tissue damage. The curing of PMMA has been shown to be in excess of 122°C (Jefferiss et al., 1975). Thermal necrosis of bone tissue has been shown to occur when exposed to temperatures of 50°C for 1min (Eriksson et al., 1984), and damage to sensory nerves has been shown to occur at temperatures of 45°C with longer exposures (De Vrind et al., 1992). Thermally induced necrosis of bone tissue through the curing of PMMA has been associated with early joint loosening, and thus exposure should be limited to temperatures ≤70°C (Berman et al., 1984).

1.9.2 Delivery systems based on biodegradable polymers

Many biodegradable materials have been evaluated as adjuncts to ALBCs, including protein-based materials (collagen, fibrin, thrombin, clotted blood), bone-graft, bone-graft substitutes and extenders (hydroxyapatite, beta-tricalcium phosphate, calcium sulfate, bioglass), and synthetic polymers (polyanhydrides, poly(lactic acid), poly(glycolic acid), PLGA, poly(hydroxybutyrate-co-hydroxyvalerate), polyhydroxyalkanoates).

The collagen sponge is the most widely used product of the protein-based materials, and has been evaluated for the localized delivery of gentamicin, cefotaxime, fusidic acid, clindamycin and vancomycin (Wachol-Drewek et al., 1996). In all cases, the drugs eluted from collagen sponge over 4d, with the majority of drug released within the first 24 h. Gentamicin-
collagen sheets have been shown to be a clinically effective treatment in intramedullary nailing (Hettfleisch and Schottle, 1993), periprosthetic breast implant infections (Lapid, 2011), and in the treatment of osteomyelitis (Ipsen et al., 1991), with no adverse effects. However, some physicians suggest that further refinement is needed before it should be used clinically, which is likely one of the reasons why it is not approved for use in the US (McLaren, 2004).

Polymeric implants have been used in many forms, including thin films for periodontal applications (Agarwal et al., 1993; Owen et al., 2010), and the treatment of osteomyelitis (Ramchandani and Robinson, 1998), and have been formulated as drug-eluting biodegradable orthopaedic screws (Makinen et al., 2005), and microspheres for the delivery of a number of antibiotics, including ofloxacin (Habib, 1999), cefazolin, gentamicin (Wang et al., 2004), fusidic acid (Yang et al., 2009), rifampicin (Suarez et al., 2001), cephalexin (Chaisri et al., 2009), and vancomycin (Atkins et al., 1998).

1.9.3 Application of microspheres in orthopaedics

Polymeric microspheres may be defined as particles with a diameter ranging from 1-1000 µm in which drugs are homogenously dissolved or dispersed in a polymeric matrix (Kissel et al., 2006). Microspheres loaded with cefazolin have been particularly successful in treating infections associated with tibial fractures in animal models when placed in the surgical pocket immediately following surgery, prior to wound closure (Jacob et al., 1993; Jacob et al., 1993; Jacob et al., 1997; Jacob et al., 1997). Thus, our group has proposed that a biodegradable polymer-based microsphere system could potentially be co-located at the implant site to release the loaded antibiotics, would not require surgical removal, and may be an appropriate delivery system to combat prosthetic joint infections.
1.9.3.1 Fabrication of microspheres and drug loading

There are a number of different techniques used to fabricate polymeric microspheres, and these include, polymerization of monomers in solution (emulsion, suspension, dispersion, or sedimentation) (Piirma, 1986), solvent-evaporation, and spray-drying techniques (Freiberg and Zhu, 2004). The most commonly employed method of drug-loaded microsphere preparation is the solvent evaporation technique, where the diffusion and evaporation of organic solvent from dispersed drug/polymer/organic solvent droplets dispersed in an aqueous phase results in solid drug-loaded microspheres. This is also referred to as the oil-in-water (O/W) single emulsion method (Figure 1.20).

1.9.3.1.1 Solvent evaporation

For poorly water-soluble drugs, an oil-in-water (O/W) single emulsion technique is frequently utilized to create polymeric microspheres (Figure 1.20). This method consists of four major steps: 1) drug solubilization in an organic solvent containing polymer, creating the dispersed phase; 2) dispersion of the organic phase as droplets in the continuous phase (an aqueous phase containing a small amount of surfactant) using high sheer stresses imparted by an overhead propeller stirring at high rpm; 3) the diffusion of solvent from dispersed phase into continuous phase, and subsequent evaporation; 4) washing, recovery, and drying of microspheres.
Figure 1.20 Schematic illustration of the formation of microspheres using the oil-in-water (O/W) single emulsion and solvent evaporation technique.

1.9.3.2 Factors influencing drug release from PLGA microspheres

The properties of microspheres formed using solvent evaporation techniques are strongly dependent on the material properties and processing methods used during microsphere manufacturing. A great deal of work by Bodmeier and coworkers in the 1980’s (Bodmeier and McGinity, 1987; Bodmeier and McGinity, 1988; Bodmeier et al., 1989), and Liggins and Burt in the late 1990’s, highlighted the importance of a number of material variables on the properties of poly(ester) microspheres (Liggins and Burt, 2001; Liggins and Burt, 2004; Liggins and Burt, 2004). The major determinants of drug release from biodegradable microspheres include, polymer molecular weight and crystallinity, microsphere diameter, and drug loading.

The release of drug from polymeric microspheres can be controlled by the molecular weight of the polymer. Makino et al. demonstrated that the release of β-estradiol from PLGA was slower with increasing PLGA molecular weight (Makino et al., 2000). The release of drug
from lower molecular weight PLGA (19,000 and 44,000 Da) was biphasic, where the higher molecular weight (74,000 Da) was triphasic. Furthermore, the release from high molecular weight polymers was incomplete until the onset of erosion and mass loss, and therefore it was suggested that the higher molecular weight may have caused a decrease in drug diffusion through the matrix due to decreased matrix hydration.

Microsphere diameter has a pronounced influence on drug release rates (Siepmann et al., 2004). Smaller microspheres have been shown to release their contents more rapidly, due to an increased surface area/volume ratio (Herrero-Vanrell et al., 2000; Fu et al., 2005). The smaller microspheres will have a larger proportion of drug on, or near the microsphere surface, and thus release is usually characterized by a larger burst phase of drug release.

Drug loading has also been shown to significantly increase the rate of drug release from PLGA microspheres for a number of drugs including paclitaxel (Mu and Feng, 2003), fentanyl (Choi et al., 2002), ketoprofen (Gabor et al., 1999), RIF (O'Hara and Hickey, 2000), and FA (Yang et al., 2009). As shown in section 1.6.3, the release of drug from polymeric matrices is influenced by the initial drug concentration in the device (i.e. $C_0$). Increasing the drug loading increases the initial concentration in the device leading to a more rapid rate of release down the concentration gradient. Increasing drug loading can also influence the release of drug due to an alteration of the nature of the drug dispersion (e.g. solid solution or monolithic dispersion). As discussed in section 1.6.3, increasing the drug loading from $\leq 5\%$ to $\geq 20\%$ of polymer volume is associated with the creation of interconnected drug channels. Thus, the increased drug release observed with an increase in drug loading may also be due to the increased porosity of the polymer matrix through the formation of water-filled pores and channels created from the dissolution and loss of drug from the polymer matrix. A report from Faisant et al. showed that the loading of 5-Fluorouracil (5-FU) in PLGA microspheres formed a monolithic matrix with an
interconnected 5-FU drug network that serves to increase the porosity and release rate from monolithic matrices (Faisant et al., 2002). Monolithic matrices have also been observed in leuprolide-loaded PLGA microspheres (Okada et al., 1991).

1.9.4 Application of nanofibers in orthopaedics

The development of nanofibers produced through electrically charged polymer solutions, in a process called electrospinning, has garnered much attention as a potential biomaterial in orthopaedic applications. Electrospun polymeric nanofibers are ultra-thin, and ultra-flexible, and ultra-light fibers, which have high porosity (50%-90% of total volume) (Burger et al., 2006), high surface/volume ratios (~100-1000 m²/g) (Megelski et al., 2002), and very small diameters (≤1000nm). These properties have been shown to be favorable in orthopaedic applications, as nanofibers have been used in the coating of medical implants to create a nanoporous matrix in which cells would preferentially adhere and enhance biointegration (Badami et al., 2006; Schnell et al., 2007). They have been used as a hemostatic due to high surface area and absorptive capacities (Zhang et al., 2005), and have also been shown to be highly gas permeable allowing the passage of oxygen (Gibson et al., 2001), which may help alleviate any tissue viability issues. Furthermore, nanofiber membranes are flexible and can adhere to complex contours, which are often present in operative surfaces.

These electrospun matrices can also serve as drug delivery vehicles through direct incorporation of active therapeutics in the polymer fiber structure, either within a matrix fiber, or within a fiber core (see section 1.9.4.3). Accordingly, nanofibers have been used as drug delivery vehicles to deliver a number of low molecular weight antibiotics including cephazolin (Katti et al., 2004), RIF (Zeng et al., 2003), mefoxin (Zong et al., 2002), tetracycline (Kenawy el al., 2002), gentamicin (Torres-Giner et al., 2011), and FA in Chapter 4 of this work, along with
others (Said et al., 2011; Said et al., 2012). The high porosity and surface area/volume ratios facilitate diffusional transport of drugs from the fiber matrix, and therefore release characteristics may be modified by changes in fiber morphology.

Therefore, our group has suggested that highly flexible nanofiber membranes loaded with antimicrobials may possess favorable characteristics appropriate for use as drug delivery device to prevent, or treat infections associated with orthopaedic implants. We have proposed several target performance properties of the drug-loaded membranes in Table 1.2.

Table 1.2 Target properties of an electrospun nanofibrous membrane for applications in preventing and/or treating infections associated with orthopaedic implants

<table>
<thead>
<tr>
<th>Feature of nanofiber membrane</th>
<th>Target properties and characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placement</td>
<td>▪ Conform to surface of implant and/or biological tissues (e.g. bone, and soft tissues)</td>
</tr>
<tr>
<td>Tissue-membrane interface</td>
<td>▪ Biocompatible for tissue integration</td>
</tr>
<tr>
<td>Lifetime of nanofiber membrane</td>
<td>▪ Biodegradable to avoid surgical removal</td>
</tr>
<tr>
<td></td>
<td>▪ Biodegradation lifetime of $\geq 4-6$ weeks to allow coverage in secondary revision, and spacer device surgeries</td>
</tr>
<tr>
<td>Mechanical properties</td>
<td>▪ Flexibility to conform to biological and implant surfaces</td>
</tr>
<tr>
<td>Drug loading</td>
<td>▪ Loading of one or more antibiotic which shows efficacy against common Gram positive bacteria associated with orthopaedic infections</td>
</tr>
<tr>
<td>Drug release</td>
<td>▪ Initial burst release to eradicate bacterial contamination at time of surgery</td>
</tr>
<tr>
<td></td>
<td>▪ Controlled release above MIC over membrane lifetime to prevent hematological seeding of implant surface</td>
</tr>
<tr>
<td>Other</td>
<td>▪ Chemical stability of drugs maintained</td>
</tr>
</tbody>
</table>
1.9.4.1 Fabrication of nanofibers using electrospinning

Polymeric nanofibers can be fabricated by a variety of methods including wet spinning (Lee et al., 2007), pseudo-dry spinning (Notin et al., 2006), melt spinning (Wan et al., 2005), gel spinning (Notin et al., 2006), and self-assembly (Sargeant et al., 2008). However, electrospinning is the most straight-forward, versatile, and cost-effective technique for the production of uniform, non-woven polymeric nanofibers (Reneker and Chun, 1996; Frenot and Chronakis, 2003; Nair and Laurencin, 2008).

The basic electrospinning apparatus consists of three components: a suitable vessel to deliver the polymer/drug solution (“spin dope”), a high voltage power supply, a charging and grounded electrode, and a suitable collector (Figure 1.21).
Figure 1.21 (A) Illustration of the basic electrospinning apparatus consisting of a high voltage generator, positive and grounded electrodes, syringe pump, and grounded collector. (B) Blueprint drawing of the electrospinning apparatus highlighting the operational and solution conditions used in this thesis to form continuous, defect free PLGA nanofibers (see section 4.3.2). Note: This figure is not to scale.
During electrospinning, a dissolved polymer solution is pumped out of a syringe in the presence of a very high electrical field potential. The electric field potential which deforms the fluid meniscus at the capillary tip, ejects the polymer solution into an axial stable jet, which accelerates and may splay repeatedly within the radiant electrical field. As the fiber accelerates, instabilities force it to whip towards the ground collector as it stretches, thins, and solidifies into a uniform polymer fiber. Fiber formation can be broken down into five distinct processes: 1) charging of the polymer fluid; 2) formation of the cone-jet; 3) thinning of the jet in the electrical field; 4) whipping instability and drying of the jet in the radiant electrical field; and 5) collection of the solidified fiber on a grounded target.

1) Induction charging in the electrospinning process arises from direct contact of solution, or flow of solution across an electrode held at a very high potential (Rutledge and Fridrikh, 2007). Charged ions, generated within the spin dope rapidly move in response to the charge, acting as a charge carrier to the bulk and in turn transfer a force vector to the bulk polymer solution pushing molecules and radiant charge towards the open capillary tip (Reneker and Chun, 1996).

2) The spherical droplet formed at the end of the open capillary becomes distorted in the presence of an electric field. As the field strength is increased, the spherical droplet begins to show signs of convexity as repulsion between like-charges at the free surface of the polymer solution work against surface tension. At a critical electrical potential, slightly greater than the potential for conical equilibrium by overcoming surface tension, the distorted droplet becomes unstable and an axial jet is projected at the apex of the conical meniscus at the point of maximum field strength. The initiation of the axial jet represents the beginning of fiber formation (Taylor, 1964; Rutledge and Fridrikh, 2007).
3) During the initial formation of the cone-jet, electrical forces dominate to eject the axial jet. Upon ejection, the jet is accelerated and thins rapidly due to the viscoelastic properties of the spin dope. The rapid thinning of the jet decreases bulk solution conductivity and results in a rapid increase in surface charge density. Surface charge density increases until a near asymptotic value, at which point the jet thins more slowly as a result of only electrical potential (Hohman et al., 2001; Hohman et al., 2001; Feng, 2002; Feng, 2003). In addition, as the distance between the jet lead and the spinneret increases, the electric potential of the jet decays exponentially. At sufficient distance from the spinneret, electrostatic attraction forces oppose fluid inertial forces and cause the jet acceleration to slow down (Ganan-Calvo, 2000; He et al., 2005).

4) As the acceleration of the ejected liquid jet slows, it becomes very sensitive to small fluid instabilities. Instabilities can result in jet splaying or splitting to form multiple jets. However, the most common instability associated with electrospun jets is bending (or whipping) instability. The mechanism of whipping instability is strong charge repulsion at the surface of the thinning jet, where strong repulsion between neighboring charges forces an area of the jet to deviate (perpendicular) from the axial path. Surface tension, inertia, and jet viscosity serve to counteract this instability by reducing surface area, velocity, and shear, respectively, resulting in relatively small perturbations that amplify as they travel down the jet path producing wave-like, or helix-like perturbations (Figure 1.22). These perturbations allow for further jet thinning and solidification into fibers (Reneker et al., 2000; Hohman et al., 2001).
Figure 1.22 Schematic illustration showing the onset of 3 cycles of instability in an electrified jet produced by the electrospinning process as it accelerates towards the grounded collector. Each cycle of instability results in a reduction in fiber diameter, and is responsible for the high draw ratio seen in electrospun nanofibers.

5) The process of electrospinning constitutes an electrical circuit. Electrified jets, and subsequent solidified fibers remain charged as they traverse the electrical field until they impact
on a grounded, or lower potential electrode where they are collected. The electrospinning setup is axial symmetric, and consequently, impact on the collector electrode with no preferred orientation results in a non-woven fiber membrane (Rutledge and Fridrikh, 2007).

1.9.4.2 Factors influencing nanofiber fabrication

In general, the ‘spinnability’ of the polymer solution refers to the conditions under which continuous, and uniform fibers are obtained (Ziabicki, 1976; Shenoy et al., 2005). Outside of these limiting conditions, weak charge repulsion within the hydrodynamic jet (Lin et al., 2004), or capillary breakup due to surface tension effects (Fong et al., 1999), results in altered morphology, producing beaded nanofibers, or jet breakup, a process called electrospraying, which creates discrete nanospheres.

Reneker’s group, have published an extensive model of the electrospinning process (Reneker et al., 2000; Yarin et al., 2001), and provide an excellent review on the major parameters impacting the formation, and diameter of electrospun nanofibers (Thompson et al., 2007). Using their mathematical models of nanofiber formation, the authors divide operational and solution parameters into 3 categories based on their influence on the forming nanofiber. Strongest effects come from jet radius, charge density, nozzle-to-collector distance, and viscosity, with moderate effects from polymer concentration, solvent vapor pressure, and electric potential, and minor effects from surface tension. A more simplified review from Rutledge and Fridrikh, evaluated the primary variables using an empirical scaling relationship, which defined jet diameter in terms of a number of the fluid and operational parameters in controlling nanofiber production (Rutledge and Fridrikh, 2007):
where $d$ is the jet diameter, $Q$ is the solution flow rate, $\rho$ is the fluid density, $E_{\text{app}}$ is the applied field strength (a function of applied voltage and inter-electrode distance), $I$ is the current, and $z$ is the axial coordinate. Accordingly, since the rate of diameter change governs the spinnability of a polymer solution, Rutledge suggests that there are a number of relevant fluid properties, including viscosity, density, surface tension, conductivity, and dielectric constant, and operational parameters, including flow rate, electric current, and applied field, that influence nanofiber diameter, and can be manipulated to alter the behavior of the electrospinning jet.

### 1.9.4.2.1 Fluid properties

Solution viscosity and density can be varied through simple alterations to polymer concentration or polymer molecular weight. In each case, an increase in viscosity or density results in greater polymer chain interaction and entanglement, which play a major role in determining fiber morphology. Shenoy et al. (Shenoy et al., 2005), suggest a relationship between chain entanglements and spinnability, where chain entanglements in solution ($n_e$), is a function of polymer molecular weight ($M_W$), volume fraction ($\phi_p$), chain entanglement in the molten state ($M_e$; a function of viscosity), and defined as:

\[
(n_e)_{\text{solution}} = \frac{\phi_p M_W}{M_e}
\]  

(Equation 1.19)
Using this relationship, the authors produced a phase diagram illustrating the conditions under which nanofibers could be produced using a number of polymeric systems, including polystyrene (PS), PLA, PEG, and PVP (Shenoy et al., 2005). Using a modified approach, Gupta et al., derived a similar equation to evaluate the critical chain overlap concentration \( c^* \), that is the point when the concentration inside a single macromolecular chain equals the solution concentration, as a function of chain dimensions:

\[
\frac{3M}{4\pi R_g^3 N_{av}}
\]

\[ (\text{Equation 1.20}) \]

where \( M \) is the polymer molecular weight, \( R_g \) is radius of gyration, and \( N_{av} \) is Avogadro’s number. Normalizing the solution concentration to the critical concentration, Gupta et al. evaluated a range of molecular weight PMMA polymers for their ability to form continuous, defect free nanofibers and found that there exists a critical polymer concentration, which they termed semidilute entangled, where there is sufficient polymer chain overlap in solution to produce nanofibers. According to Gupta, an increase in molecular weight, or polymer concentration, can significantly impact the critical concentration required for chain overlap (Gupta et al., 2005). Accordingly, similar analyses of entanglements have been evaluated using the Berry number \( [\eta][C] \) which is a function of intrinsic viscosity and concentration (Hager and Berry, 1982), and has been shown to be a determining factor in the production of nanofibers from PVA (Dai and Shivkumar, 2007; Tao and Shivkumar, 2007), PMMA (Gupta et al., 2005), and PVP (Shui and Li, 2009).

The surface tension and charge density in electrospinning are two other critical factors, and have been shown to significantly influence nanofiber morphology. The work of Reneker’s
group (Shkadov and Shutov, 2001), suggests that the two factors are related as a function of jet diameter, as calculated by a stability factor ($S$) in the empirical relationship described by:

\[
\frac{1}{S} = \left( \frac{\pi (2 \ln \chi - 3)}{2 \gamma k} \right)^{\frac{1}{3}} \left( \frac{I}{Q} \right)^{\frac{2}{3}} d
\]

(Equation 1.21)

According to the relationship, as jet diameter decreases, the forces of instability, due to charge density and repulsion, are balanced by surface tension and viscoelasticity. The longer the electrospun jet stays in a state of instability, the more the jet thins. Therefore, the addition of charging agents (i.e. organic salts), has been associated with smaller and more uniform nanofibers (Reneker and Chun, 1996). Similarly, Son et al. (Son et al., 2004), and Yang et al. (Yang et al., 2004), investigated the influence of solvent dielectric constant on the spinnability of PEG and PVP solutions, respectively. In both cases, these authors suggested that increasing the dielectric constant resulted in smaller nanofiber diameters due to higher solution charge density. Yang and coworkers compared individual solvents with binary solvent mixtures, suggesting that nanofiber morphology is a property better controlled through small manipulations in binary solvent mixtures (Yang et al., 2004).

1.9.4.2.2 Operational parameters

A number of the above empirical relationships governing nanofiber properties can be extrapolated from solution phenomena, and applied to the mechanics of nanofiber formation. According to Reneker’s model, the operational parameters categorized to produce the strongest effects are initial jet diameter (i.e. needle orifice), volumetric charge density (i.e. applied voltage), and jet-to-collector distance (Thompson et al., 2007). Katti et al. (Katti et al., 2004),
illustrate the influence of operational parameters on PLGA nanofibers, and show that decreasing the orifice diameter, increasing voltage, and increasing jet-to-collector distance all result in a decrease in nanofiber diameter. Katti et al. also illustrate the limits of these parameters in influencing overall nanofiber morphology, and suggest that any change in operational parameter will reach an asymptotic range where further changes do not reflect similar changes in nanofiber morphology.

1.9.4.3 Drug loading and release characteristics of electrospun nanofibers

Drugs are typically loaded into polymeric nanofibers by solubilizing drugs in an organic solvent phase containing polymer, prior to electrospinning. The resulting nanofiber displays matrix-type morphology and the loading and subsequent release characteristics of any encapsulated drug will influenced by a number of variables previously discussed, including drug loading, drug-polymer compatibility, and polymer degradation rate (see section 1.6.3).

The release mechanism of drugs from non-porous electrospun nanofibers is dominated by diffusional transport (Xie and Buschle-Diller, 2010). The release of tetracycline and paclitaxel from polyester nanofibers was shown to follow Fickian diffusional release using swellable device models and when assuming release via a mono-dispersed cylinder system (Xie and Wang, 2006; Xie and Buschle-Diller, 2010). However, if the nanofibers contain a nanoporous surface, a desorption-limited mechanism of release has been suggested, where interconnected nanopores serve to significantly increase the overall surface area of the nanofiber membrane (Srikar et al., 2008).

Physicochemical properties of the encapsulated drugs can also play an important role in regulating drug release. Drugs with favorable solubility in the polymer have been shown to disperse throughout the fiber forming a solid solution (see section 1.7), with release being a
function of the solubility of the drug in the release media and the diffusivity of the drug through the hydrated polymer (Zeng et al., 2005). Incompatible drugs, are found localized at the fiber surface, and display an uncontrolled burst phase of release (Zeng et al., 2005). Similarly, the amount of drug loaded into the nanofiber membrane can also influence drug release. An increase in drug loading has been associated with a greater burst phase of release (Kim et al., 2004; Peng et al., 2008; Xie and Buschle-Diller, 2010).

Although polymer properties, such as molecular weight (Xie and Buschle-Diller, 2010), can influence drug release, the degradation of nanofibers over the typical time courses studied, does not have a major influence on release rate. In an interesting experiment, Puppi et al. compared the drug release, and degradation behavior of PLGA nanofibers and films loaded with retinoic acid, and showed that the release of retinoic acid from PLGA nanofibers was diffusion controlled, whereas the release from films was a function of diffusion and matrix erosion (Puppi et al., 2010). Therefore, it was suggested that the influence of matrix erosion in ultra-fine nanofibers might not play an important role in drug release over moderate incubation times (Xie and Buschle-Diller, 2010).
1.10 Thesis goal and research objectives

The overall goal of this project was to develop and characterize a biodegradable, polymeric formulation for the delivery of two potent antimicrobial agents, FA and RIF, which could be used for the controlled delivery of these two agents to orthopaedic implant surgical sites to achieve high localized concentrations above the MIC of potential microorganisms to prevent implant associated infections. In order to achieve this goal, co-loading of FA and RIF in PLGA microspheres was first explored. Previous studies in our laboratory in which FA was loaded as a single agent into PLGA microspheres, showed that a phase separation phenomenon resulted in the exclusion of solid amorphous FA-rich microdomains at the surface of the microsphere (Yang et al., 2009). Since the solid-state properties of FA have not been reported in the peer-reviewed literature, Chapter 2 of this thesis discusses our findings of new polymorphic forms of FA and an amorphous form. Chapter 3 subsequently describes the characterization of PLGA microspheres co-loaded with FA and RIF. Phase separation behavior of FA and RIF was examined using real-time video recordings of single microsphere formation via the solvent-evaporation method. Phase diagrams were constructed illustrating the phase changes taking place from the liquid through to the final glassy states as microspheres form. A more suitable controlled release formulation for FA and RIF was subsequently determined to be co-loading in PLGA nanofibers prepared using electrospinning (Chapter 4). Electrospinning and formulation parameters were optimized to produce homogeneous, co-loaded FA/SF and RIF PLGA nanofiber membranes, and a lead formulation was evaluated in a rat model of implant-associated infection.

Accordingly, the following were the specific research objectives:

1) To characterize the solid state properties of FA (Chapter 2).
2) To investigate and characterize the phase separation behavior of FA and RIF in PLGA using a microsphere formulation (Chapter 3).

3) To develop and characterize a PLGA nanofiber formulation, prepared by electrospinning, for the co-loading of FA and RIF (Chapter 4).

4) To determine the in vitro bactericidal susceptibility of the Gram positive microorganisms S. aureus, S. epidermidis, and two strains of MRSA, and the Gram negative microorganism A. baumannii to the combination of FA and RIF and evaluate the in vitro efficacy of co-loaded FA and RIF nanofiber formulation against these microorganisms using direct time-kill assays (Chapter 4).

5) To evaluate the efficacy of lead FA and RIF co-loaded nanofiber formulations to prevent the colonization of a titanium implant by a highly virulent clinical isolate of MRSA in an in vivo rat model of subcutaneous implant infection (Chapter 4).
Chapter 2: The solid state characterization of fusidic acid

2.1 Introduction

With the increased average age of the population, the number of total joint arthroplasties has steadily increased over the past few decades as the population ages. However, a disproportionate increase in implant-associated infections has prevented the success of numerous restoration procedures. Between 1990 and 2004, the incidence of infection following revision total knee and total hip arthroplasty increased 300% and 700% respectively, despite only a doubling in the number of surgical procedures (Kurtz et al., 2008). The persistence of SSI following invasive surgery is due, at least in part, to the emergence of multi-drug resistant microorganisms. For instance, in Staphylococcus aureus alone, there are reports of resistance to β-lactams (Ayliffe, 1997), macrolides (Schmitz et al., 2000), vancomycin (Khatib et al., 2011), daptomycin (Yang et al., 2010), and linezolid (Toh et al., 2007). In addition, clinical isolates from orthopaedic surgical sites have shown that methicillin-resistant S. aureus (MRSA) is present in >24% of infections, and >70% of those MRSA isolates display multi-drug resistance (Sisirak et al., 2010). Therefore, there is a clinical need for alternative treatment strategies. Currently, there is much interest in the use of old generation antibiotics in surgical prophylaxis and treatment due to the decreased incidence of antibiotic resistance to these drugs (Maviglia et al., 2009). One class of antibiotics that has garnered attention is the steroidal antibiotics, specifically fusidic acid (FA). FA is a tetracyclic triterpenoid derived from the fermentation broth of the fungus Fusidium coccineum (Figure 1.3). It has been available since the 1960’s as either the sodium salt or free acid and is commercially available in Canada, Australia, and the UK as oral, topical, ophthalmic and intravenous dosage forms, and is most often prescribed for

1 A version of this chapter has been published. Gilchrist, S.E. et al. (2012). The solid-state characterization of fusidic acid. Int J Pharm. 422 (1-2): 245-53.
skin and eye infections (Spelman, 1999). However, it has yet to be approved for use in the United States (Howden and Grayson, 2006). FA has high antimicrobial activity against Gram-positive bacteria, in particular *S. aureus*, *S. epidermidis*, and coagulase-negative *staphylococci* including strains that are methicillin-resistant (Coombs and Menday, 1985; Coombs, 1990), and strains that display multi-drug resistance (Sisirak *et al.*, 2010). It is due to this spectrum of activity that FA has become increasingly popular in the treatment of bone and prosthetic joint infections as Gram-positive organisms are commonly the culprits of orthopaedic infections.

Current guidelines state that FA is indicated for the treatment of prosthetic joints infections when MRSA is the infecting microorganism, and is dosed orally at 500 mg every 8 h for a minimum of 3 months (Zimmerli and Ochsner, 2003; Mastrokalos *et al.*, 2006; Trampuz and Zimmerli, 2006). However, despite this dose being equivalent to intravenous dosing, maintaining minimum inhibitory concentrations at the site of infection as well as systemic side effects are often of concern. Therefore, a primary focus of our lab is the development of a locally applied drug delivery system of FA for the treatment of orthopaedic infections. The local administration of antibiotics to the site of infection is an attractive option for the treatment of bone infections due to the ability to maintain high tissue levels for prolonged periods of time, while simultaneously avoiding systemic side effects. To date, several locally applied antibiotic delivery systems have been investigated including ciprofloxacin, gentamicin and vancomycin loaded poly(methylmethacrylate) bone cement beads (Mader *et al.*, 1997), gentamicin loaded collagen sponges (Mendel *et al.*, 2005), vancomycin and tobramycin loaded allograft bone (Winkler *et al.*, 2008), and ciprofloxacin loaded poly(lactic acid) pellets (Koort *et al.*, 2005). In many cases, these systems consist of solid drug particles dispersed throughout the device matrix, such as in the case of ciprofloxacin and fosfomycin loaded polyurethane films (Schierholz *et al.*, 1997), cefazolin and ciprofloxacin loaded glycerol monostearate implants (Allababidi and Shah,
and gentamicin loaded poly(L-lactic acid) films (Aviv et al., 2007). Recently, our group has demonstrated that when FA is formulated in poly(D,L-lactic acid-co-glycolic acid) microspheres and films cast from dichloromethane (DCM), the drug phase separates into drug-rich microdomains, suggesting that FA is present in the solid-state within the polymer (Yang et al., 2009).

Even though FA has been in use for several decades and there is renewed interest in its use in implant associated infections, its solid-state properties have not been reported in the peer-reviewed literature. FA is commercially available as a crystalline free acid (C₃₁H₄₈O₆), with a molecular weight of 516.72 Da (anhydrous) and a pKa of 5.35 (Figure 1.3) (O'Neil, 2001). The patent literature (Jensen and Andersen, 2006), and one monograph (Reeves, 1987), claim that FA free acid exists as a hemihydrate. In this work, we describe the preparation and characterization of the commercially available solid, 2 new polymorphic forms, and an amorphous form of FA.

2.2 Materials and methods

2.2.1 Chemicals

Commercially available FA was kindly supplied by Ercros, Pharmaceuticals Division (Madrid, ES). The solvents dichloromethane (DCM), acetonitrile (ACN), ethyl acetate (EA), acetone (ACE), chloroform (CHCl₃), methanol (MeOH), and phosphoric acid were of HPLC grade and purchased from Fisher Scientific (Ottawa, ON).

2.2.2 Generation of FA solid forms

2.2.2.1 Solvent-mediated polymorphic transformation

Commercially sourced FA was slurried in anhydrous ACN, or MeOH:H₂O (50:50) at 50 mg/mL in 1mL chromatography vials with plastic cap. The vials were tumbled end-over-end at
room temperature and 10 rpm using a benchtop rotor (Labquake®; Barnstead Thermolyne). At predetermined time points, the suspension was filtered using a 0.45 µm white nylon filter (Millipore Corp.; Billerica, MA) and the solid was subsequently dried under 635 mmHg vacuum at room temperature in the presence of a desiccant until analysis (24 h minimum).

2.2.2.2 Recrystallization via solvent evaporation

Fusidic acid was recrystallized from the following solvents: DCM, EA, ACE, CHCl₃, and MeOH. Recrystallization of FA was achieved by dissolving the drug in 2 mL of solvent in 20 mL glass scintillation vial at a concentration of 50-100 mg/mL followed by evaporation of the solvent at either room temperature or -20°C. Amorphous FA was prepared by dissolving 250 mg of FA in 1 mL of DCM in a 20 mL glass vial followed by rapid removal of the solvent at 100°C in an oil bath under a stream of nitrogen. All recrystallized forms of FA were stored under 635 mmHg vacuum at ambient temperature until analysis.

2.2.3 Characterization of FA polymorphs

2.2.3.1 Single crystal x-ray diffraction

A FA crystal, obtained through the evaporation of solvent from a FA/ACN solution, was mounted on a glass fiber. Measurements were made on a Bruker APEX DUO diffractometer with cross-coupled multilayer optics Cu-Kα radiation. The data were collected at a temperature of -183.0 ± 0.1°C to a maximum 2θ value of 132.0°. Data were collected in a series of φ and ω scans in 1° oscillations using 3.0 sec exposures. The crystal-to-detector distance was set to 59.65 mm.
2.2.3.2 X-ray powder diffraction

X-ray powder diffraction (XRPD) patterns of FA solid forms were obtained at 25°C with a Bruker (Milton, ON) APEX DUO diffractometer with cross-coupled multilayer optics Cu-Kα radiation. Samples were packed into a thin-walled capillary tubes (special glass; Charles Supper Company, Natick, MA) and sealed using a small amount of capillary wax. Samples were scanned from 4-60° 2θ using a step size of 0.02° and a step time of 3 sec/step. Data was collected and analyzed with Bruker Diffrac Plus XRD Commander version 2.3 software.

2.2.3.3 Scanning electron microscopy

The crystal habits of FA polymorphs were evaluated using SEM. Samples were scattered onto a SEM stub and sputter-coated with a layer of 60:40 gold:palladium alloy using a Denton Vacuum Desk II sputter-coater (Moorestown, NJ) at 50 mTorr. SEM images were captured using a Hitachi S-3000N system (Tokyo, Japan) scanning at 5–20 keV.

2.2.3.4 Thermal gravimetric analysis

Samples were analyzed for weight loss during heating using a TA Instruments (New Castle, DE) Q50 thermal gravimetric analyzer. Approximately 5 mg of sample was weighed into an open pan and heated from 40°C to 250°C at 10°C/min under nitrogen gas purge flowing at 20 mL/min.

2.2.3.5 Differential scanning calorimetry

Differential scanning calorimetry (DSC) thermograms of FA samples were recorded using a TA Instruments Q100 differential calorimeter with a refrigerated cooling system. Approximately 5 mg of sample was weighed into a hermetically sealed pan with a pinhole.
Samples were equilibrated at 40°C for one minute followed by heating to 250°C at a rate of 10°C/min. The heat flow, and heat capacity of the instrument was calibrated using a high purity indium standard.

2.2.3.6 Hot stage microscopy

Thermal events were visually monitored by hot stage microscopy. Samples were placed on a glass microscope slide, equilibrated at 100°C and then heated at 10°C/min to 250°C using a Metler Toledo (Columbus, OH) FP900 Thermosystem hot stage with temperature controller. Samples were viewed using an Olympus CX41 transmission light microscope with magnification of 10x and a polarizing filter over the light source. The thermal events were digitally recorded using Olympus Stream Basic imaging and documentation software (v. 1.6).

2.2.3.7 Polymorphic stability in aqueous slurry

FA as Form I, Form III, Form IV, and amorphous solids were slurried in H$_2$O at 50 mg/mL as described above. At pre-determined time points, the FA suspensions were filtered, dried, and analyzed using XRPD as described above.

2.2.3.8 Intrinsic dissolution rate

Solid forms of FA were evaluated for intrinsic dissolution rate using a Wood’s intrinsic dissolution apparatus (Hanson Research; Chatsworth, CA) and a Mandel SR8 Plus Dissolution Test Station (Guelph, ON). A 50 mg sample of FA was compressed at 1500 psi for 60 sec in a 8mm electropolished die using an Enerpac P142 hydraulic press (Butler, WI). The die housing the drug compact was attached to a shaft and holder and submerged in 500 mL degassed phosphate buffered saline (PBS; pH 7.4) at 37°C with the rotation speed set to 50 rpm. At pre-
determined time points, 2 mL of the dissolution media were withdrawn and analyzed for FA content using an HPLC (Waters® Millennium System) assay utilizing a mobile phase of 50/30/20 (v/v/v) ACN/MeOH/0.01M phosphoric acid solution, flowing at 1 mL-min\(^{-1}\) through a C18 reverse phase Vovapak column (Waters®), with a 20 µL sample injection volume and detection \(\lambda\) at 235nm. FA was quantified against a standard curve prepared by dissolving FA in ACN over a range of 0.1 to 500 µg-mL\(^{-1}\). Data were analyzed using Waters Millennium 32 software.

### 2.2.4 Statistical analysis

IDR values were calculated by a linear regression analysis of the amount of FA dissolved (mg) per unit surface area of the compressed disk, over 30-minutes in the IDR experiments. Differences between the IDR of each of the FA polymorphs were evaluated using a one-way ANOVA and a Newman-Keuls multiple comparison \textit{post-hoc} test with significance level set a \(p < 0.05\). All analysis was done in GraphPad Prism v. 5 for Mac OS X (GraphPad Software, San Diego CA, USA).

### 2.3 Results

#### 2.3.1 X-ray diffraction

##### 2.3.1.1 Powder XRD

XRPD patterns for the 4 solid samples were different, indicative of the presence of 4 different solid forms of FA (Figure 2.1). Based on single crystal XRD and thermal analysis findings, given below, we found no evidence of nydrate of solvate forms of FA. Hence the different XRPD patterns were indicative of 4 FA polymorphic forms, which have been designated Forms I-IV. Major diffraction peaks were present at 7.23°, 12.67°, 13.12°, 18.18°,
and 22.82° 2θ for Form I, 11.18°, 13.7°, 15.82°, 17.4°, and 18.19° 2θ for Form II, 7.18°, 13.7°, 15.99°, 16.42°, and 17.91° 2θ for Form III, and 11.78°, 13.1°, 15.12°, 16.44°, and 18.11° 2θ for Form IV (Figure 2.1). Amorphous FA lacked any diffraction peaks and was characterized by a characteristic broad halo.

![XRPD patterns of FA, Forms I-IV and amorphous FA.](image)

Figure 2.1 XRPD patterns of FA, Forms I-IV and amorphous FA.

Slurrying Form I or Form III in water for up to 28 days did not result in a change in the XRPD pattern (Figure 2.2). However, the same treatment of either Form IV or amorphous FA
resulted in a significant time dependent change in the diffraction pattern of these forms until both forms had identical XRPD patterns as Form III. The identification of the various polymorphs with respect to their source, and crystallization conditions are summarized in Table 2.1.
Figure 2.2 XRPD patterns of (A) Form I, (B) Form III, (C) Form IV, and (D) Amorphous FA, slurried in H₂O for 1, 7, 14, and 28d.
Table 2.1 Summary of thermal events for FA solid forms.

<table>
<thead>
<tr>
<th>Polymorph</th>
<th>Preparation</th>
<th>Transition&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Transition Temperature (°C)</th>
<th>ΔH (J/g)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Degradation Temperature (°C)&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Form IV</td>
<td>Slurry Form III in MeOH/ H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>( T_{m,1} )</td>
<td>128.2 ± 0.2</td>
<td>11.0 ± 1.7 (( \Delta H_f ))</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>( T_c )</td>
<td>153.4 ± 1.0</td>
<td>40.0 ± 1.4 (( \Delta H_r ))</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>( T_{m,2} )</td>
<td>175.1 ± 0.3</td>
<td>42.7 ± 4.8 (( \Delta H_f ))</td>
<td>178.4 ± 5.4</td>
</tr>
<tr>
<td>Form I</td>
<td>Slurry any form in ACN</td>
<td>( T_m )</td>
<td>190.0 ± 0.1</td>
<td>50.3 ± 2.5 (( \Delta H_f ))</td>
<td>188.9 ± 0.5</td>
</tr>
<tr>
<td>Form II</td>
<td>Recrystallized any form from DCM at -20°C</td>
<td>( T_m )</td>
<td>179.2 ± 0.6</td>
<td>58.5 ± 5.4 (( \Delta H_f ))</td>
<td>179.9 ± 1.0</td>
</tr>
<tr>
<td>Form III</td>
<td>As received from Ercros or Slurry Form IV or amorphous in H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>( T_m )</td>
<td>148.7 ± 1.2</td>
<td>18.9 ± 3.7 (( \Delta H_f ))</td>
<td>174.5 ± 0.5</td>
</tr>
<tr>
<td>Amorphous</td>
<td>Rapid evaporation of any form in DCM</td>
<td>( T_g )</td>
<td>116.9 ± 2.1</td>
<td>—</td>
<td>171.4 ± 0.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> \( T_m \), melting temperature, the peak temperature of an endothermic transition; \( T_r \), recrystallization temperature, the peak temperature of an exothermic transition \( T_g \), glass transition temperature, the midpoint of the transition as determined by differential scanning calorimetry (DSC).

<sup>b</sup> Where endothermic transitions were observed, enthalpy values represent the heat of fusion (\( \Delta H_f \)), whereas exothermic transitions are expressed as the heat of recrystallization (\( \Delta H_r \)).

<sup>c</sup> Onset temperature of mass loss as determined by thermogravimetric analysis (TGA).

Results are expressed as the mean ± S.D. \( n = 3 \).
2.3.1.2 Single crystal XRD

Only FA recrystallized from ACN generated a single crystal large enough to perform single crystal XRD analysis. To investigate the packing orientation of FA in this polymorph, a crystal with approximate dimensions of 0.12 x 0.18 x 0.46 mm was mounted on a glass fiber. Figure 3 shows the crystal structure of FA, highlighting the oxygen atoms (O1-O6) involved in inter-molecular hydrogen bonding between FA molecules in the unit cell. The unit cell of Form I is a primitive monoclinic lattice system containing two FA molecules (Figure 2.3), with lattice parameters as follows: \( a = 12.23 \ \text{Å} \); \( b = 8.0 \ \text{Å} \); \( c = 13.9 \ \text{Å} \); \( \alpha = 90^\circ \); \( \beta = 94.3^\circ \); \( \gamma = 90^\circ \); \( V = 1357.7 \ \text{Å}^3 \).
Figure 2.3. (A) Single crystal structure of FA Form I; (B) Packing diagram for FA Form I. View is looking down the B-axis and illustrates the orientation of fusidic acid molecules and hydrogen bonding between molecules in 3 consecutive unit cells.
2.3.2 Scanning electron microscopy

SEM micrographs of the solid forms of FA are shown in Figure 2.4. Form I, produced by solution-mediated polymorphic transformation of Form III in ACN, produced needle- or rod-like crystals. Form II, formed by recrystallizing Form III from DCM was comprised of single- and multi-layered plates. Form III, either the commercially available form or through H$_2$O-mediated polymorphic transformation of Form IV or amorphous FA, was composed of very small plates, with some scattered irregular particles. Form IV, produced by solution-mediated polymorphic transformation of Form III in MeOH:H$_2$O (50:50), yielded hexagonal plates. Amorphous FA was a mixture of particles, with no distinct crystal habit. The FA solid forms produced from the evaporation of ACE, CHCl$_3$, and MeOH, are not shown, as XRPD showed no crystalline characteristics.
Figure 2.4 Scanning electron micrographs showing the morphologies and crystal habits of (A) Form I, (B) Form II, (C) Form III, (D) Form IV, and (E) amorphous FA.
2.3.3 Thermal gravimetric analysis

Upon heating, all FA polymorphs, except Form II, displayed a gradual weight loss between 40°C and 150°C of 0.7%, 2.5%, 1.6%, and 1.2%, for Form IV, Form I, Form III, and the amorphous form, respectively. After this small initial weight loss, the mass remained constant until approximately 178°C for Form IV, Form II, Form III, and amorphous FA. Rapid mass loss did not occur until approximately 190°C for Form I. Thermal degradation events observed by TGA are summarized in Table 2.1.

2.3.4 Differential scanning calorimetry

The thermal behavior of the FA polymorphs differed as demonstrated by the thermograms in Figure 2.5. Form I, Form II, and Form III were characterized by endothermic thermal events at 190°C, 179°C, and 148°C, respectively. Form IV displayed three thermal events with an endothermic transition with a peak at 128°C, an exotherm at 153°C, followed by an endotherm at 175°C. The thermogram of the amorphous FA showed a baseline shift, indicative of a glass transition temperature, at approximately 117°C. All solid forms showed a broad endotherm around 193°C, corresponding to significant weight loss and thermal degradation. The thermal events observed by DSC are summarized in Table 2.1.
2.3.5 Hot stage microscopy

The thermal transitions of each of the solid-state forms of FA were observed using hot stage microscopy (Figure 2.6). Polarized light was used to distinguish crystalline material, which appeared as bright birefringent areas, from non-crystalline material, which appeared as dark grey regions. Form I and II remained birefringent throughout the heating cycle until the samples melted at 178°C (Form II), or 190°C (Form I). Form III initially showed only small areas of birefringence, but upon melting at 130°C the birefringent areas became more visible. Form IV melted at 130°C. This thermal event was followed by recrystallization at 153°C as indicated by the reappearance of birefringence, and a final melt at 178°C. The amorphous form of FA was devoid of birefringence throughout the entire heating cycle.
Figure 2.6: Hot stage micrographs of the solid forms of FA at 100°C, 130°C, 153°C, 178°C, and 190°C. The scale bar (white rectangle) is 100px across, and represents 50µm.
2.3.6 Intrinsic dissolution rate

In Figure 2.7, the IDR of Form IV was found to be 0.092 mg/min/cm², and was statistically different ($p<0.05$) from the IDR of forms I, II, and III, which had IDR values of 0.053, 0.043, and 0.045 mg/min/cm², respectively (Figure 2.7). The amorphous FA had an IDR of 0.125 mg/min/cm², and was significantly higher ($p<0.05$) than any other solid form. There was no statistical difference in the IDR of form I, II, or III.

![Intrinsic dissolution profiles](image)

**Figure 2.7** Intrinsic dissolution profiles Form I (●), Form II (▼), Form III (◆), Form IV (■), and amorphous FA (★). $n=3$; Mean ± S.D.

2.4 Discussion

The generation of FA solid forms was achieved through solvent-mediated polymorphic transformation (for solvents with low FA solubility; <50 mg/mL) or recrystallization from solvents (for solvents with high FA solubility; >50 mg/mL). During solvent-mediated polymorphic transition, the solution is saturated with respect to the metastable polymorph, and
supersaturated with respect to the most stable polymorphic form. Thus, with time, the most stable form will nucleate and precipitate out to maintain thermodynamic equilibrium (Miller et al., 2005). In order to perform these studies, we slurried FA in H₂O, ACN, and MeOH:H₂O (50:50). In ACN, Form III, Form IV and amorphous FA, all generated Form I within 24h (Figure 2.8; Pathway A). However, using H₂O, only Form IV and amorphous forms of FA transformed to generate Form III over 28d (Figure 2.8; Pathway B), as determined by XRPD (Figure 2.2). Recent reports highlight the importance of proper solvent selection in screening for stable polymorphic forms (Gu et al., 2001; Miller et al., 2005). These authors suggest that the hydrogen-bonding propensity (HBP) of the solvent and the overall solubility of the drug are the critical determinants for producing the most stable polymorph. Solvents with high HBP but very poor drug solubility will retard polymorphic transformation favoring a metastable form. Conversely, solvents with weak HBP and higher solubility will favor a more stable polymorph (Gu et al., 2001; Miller et al., 2005). Therefore, since ACN is best represented by the former relationship, and H₂O by the latter, it is likely that Form I generated from ACN is a more thermodynamically stable form, and Form III generated from slurry experiments in H₂O, is metastable. In order to determine if Form III is metastable, we slurried excess Form III in ACN for 7d and found that Form III converts to Form I by 24h, confirming that Form III is indeed metastable (Figure 2.2). However, despite the metastable nature of Form III (also the “as received” commercial sample), its stability in H₂O over 28d suggests that this form is suitable for its commercial use in topical/ocular aqueous suspensions.
Figure 2.8 Conversion of FA polymorphic forms by solvent-mediated transformation. Note: Form II is generated by recrystallizing any form through the evaporation of DCM at -20°C, and thus is absent from this figure as it does not occur through solvent-mediated transformation.

Single crystal XRD analysis of Form I yielded a primitive monoclinic lattice system containing two FA molecules, with no evidence of the presence of H$_2$O or solvent within the lattice (Figure 2.3). We were unable to generate crystals large enough for single crystal XRD analysis from the other polymorphic forms, and therefore it is unclear how the molecular packing arrangements may differ between the solid forms. Additionally, there is no reference pattern published in peer-reviewed literature, or in any online database (i.e. the Cambridge Structural Database (CSD)) (Allen, 2002). Therefore, this report represents the first published single crystal XRD analysis of FA in the peer-reviewed literature.
DSC and HSM of the bulk FA samples were performed to confirm the presence of the proposed polymorphs. Heating Form IV resulted in several thermal events. The first was a small endotherm with an onset of 120°C and peak of 128°C, which was indicative of melting of crystalline drug. When this thermal event was visualized by hot stage microscopy it was confirmed that the crystalline starting material, which displayed a high degree of birefringence, completely melted at 130°C, resulting in a sample void of any birefringence, thus, confirming that the endotherm at 128°C was due to melting of crystalline FA. Upon further heating, a large exotherm in the DSC thermogram appeared with a peak at 153°C, indicative of recrystallization of another polymorph. This recrystallization was confirmed by hot stage microscopy in which crystal growth was observed at 153°C resulting in the reappearance of birefringence in the sample. Upon further heating, the newly recrystallized FA finally melted at 175°C characterized by an endotherm in the DSC thermogram and loss of birefringence when visualized by hot stage microscopy. To investigate the nature of the newly recrystallized form at 153°C, Form IV was evaluated using variable-temperature XRPD. Form IV was heated from to 153°C at a rate of 5°C/min, and rapidly cooled to 25°C using liquid nitrogen. The resulting XRPD pattern of Form IV after heating was different from all other polymorphs (data not shown). However, due to thermal degradation, as evidenced by sample discoloration and degradation peaks upon HPLC analysis, we were unable to characterize this mixture further.

The DSC thermogram of Form I, II, and III all displayed single melt endotherms at 190°C, 178°C, and 145°C, respectively (Figure 2.5). The presence of each thermal event was confirmed by HSM, which displayed the loss of birefringence/melting at the observed endothermic temperatures. Amorphous FA was characterized by a single glass transition at 117°C. All solid forms displayed an endothermic event at 193°C, which was due to a thermal
degradation event, as it occurred in the temperature range during which rapid mass loss was observed by TGA, and occurred concurrently with the melting of each crystal form (Table 2.1). Through TGA analysis, we observed that all FA polymorphs (except Form II) gradually lost a small amount of weight over the broad temperature range of 40-150°C, suggesting the presence of surface adsorbed water. The patent literature claims that FA exists as a hemihydrate (Jensen and Andersen, 2006), which would be characterized as weight loss in a stepwise fashion in a TGA analysis. This was not observed in our experiments, and we found no evidence that the polymorphic forms of FA investigated in this work were hydrates. All samples, regardless of their method of preparation, were characterized by a rapid weight loss at elevated temperatures starting at approximately at 172°C. This weight loss was attributed to degradation and vaporization of the samples and was further supported by the discolored appearance of the samples upon completion of the heating cycle.

To determine if there were any solubility differences between the solid forms of FA, the intrinsic dissolution rates of the solid forms of FA were compared using the rotating disk method using a Wood’s apparatus. Given the pKa of 5.35 for FA, we investigated the IDR at pH 3 and pH 7.4 (~ pKa ± 2 pH units) to compare between unionized and ionized drug. However, the concentration of FA in the dissolution media at pH 3 was below the level of detection, and therefore we were unable to quantify the IDR. Thus, for comparison between all forms, the IDR was evaluated at a physiologically relevant pH of 7.4. It was found that Form IV and the amorphous form of FA had greater IDR values (0.092 and 0.125 mg/min/cm², respectively) than Form I, II or III (IDR of 0.053, 0.043, and 0.045 mg/min/cm², respectively). It is well known that polymorphism can have a dramatic impact on the solubility of a drug, with more stable polymorphs leading to lower dissolution rates as compared to their metastable counterparts (Grant, 1999). Therefore, the lower solubility of Form I, II and III suggest much lower lattice
energies and an increased stability of these polymorphs compared to Form IV and the amorphous FA. Despite the fact that we were unable to demonstrate any statistical difference between the IDR of Form I, II, or III, even though we would expect the higher melting polymorph (Form I) to have the lowest IDR of all solid forms, our data suggests that there are only minor differences in the lattice energies between Form I, II, and III. This phenomenon is supported by a literature survey of 55 polymorphic compounds by Pudipeddi and Serajuddin who demonstrated that the vast majority of polymorphic forms exhibit limited spread in solubility ratios (solubility of any polymorphic form, normalized to the solubility of the most stable form) or dissolution rates, and is likely due to undetectable differences in the entropy between polymorphs (Pudipeddi and Serajuddin, 2005).

2.5 Conclusions

In this work, we demonstrated that FA undergoes a polymorphic transformation from amorphous or Form IV into Form I, or Form III when slurried in ACN or H₂O, respectively. In addition, we have shown that recrystallization of FA, through the evaporation of DCM, yields Form II. Our experiments confirm the existence of Form I and III, which have been previously reported in the patent literature. Furthermore, we have demonstrated that the commercial form of FA (Form III) is metastable, and converts to Form I in the presence of ACN, but remains stable in aqueous solution. We have also shown the existence of two unreported polymorphic forms of FA (Form II and IV). Forms I-III had similar IDR values, but were significantly lower than that of Form IV or amorphous. This similarity between IDR values of Form I-III is likely due to only small lattice energy differences between these forms. Differences in dissolution rates between the polymorphs could conceivably have an impact on the release rate of FA from local delivery devices if the drug was present in the solid-state as Form IV or amorphous FA. However, it is
cautioned that formulation of FA as one of theses forms may lead to interconversion to Form III in the presence of an aqueous milieu potentially resulting in a reduction in the release rate of the drug.
Chapter 3: The phase separation behavior of fusidic acid and rifampicin in PLGA microspheres

3.1 Introduction

Polymeric microspheres composed of the polyesters poly(lactic acid) and poly(lactic acid-co-glycolic acid) (PLGA) copolymers, loaded with a wide range of drugs, have been the subject of extensive studies over the past three or four decades (Edlund and Albertsson, 2002). Drug loaded microspheres injected subcutaneously, intramuscularly or locally at target sites can provide controlled release of bioactive agents over days, weeks or months to treat a variety of disease states (Sinha and Trehan, 2003; Menei et al., 2005; Patil and Sawant, 2008; Reisacher and Liotta, 2011; Tran et al., 2011). Accordingly, there are several successful marketed PLGA microsphere formulations loaded with drugs, such as leuprolide acetate, triptorelin pamoate, octreotide acetate, lanreotide, risperidone, naltrexone and exenatide, for the treatment of a range of conditions, including alcohol dependence, prostate cancer, acromegaly, endometriosis, and type II diabetes (Letchford et al., 2011).

Recently, our group examined the loading of fusidic acid (FA), an antimicrobial agent active against a number of Gram positive microorganisms including *S. aureus*, *S. epidermidis*, and coagulase negative staphylococci, including strains that are methicillin resistant, into PLGA microspheres for potential application for the controlled and localized delivery of FA to sites of orthopedic infection (Yang et al., 2009). In these studies, very distinctive microsphere morphology was observed as a result of phase separation of FA from PLGA as FA-rich, amorphous microdomains producing uniform and spherical protrusions on the microsphere.

surface. During microsphere formation PLGA and FA were co-dissolved in the organic solvent dichloromethane (DCM). Upon formation of the initial oil in water emulsion, DCM rapidly leaves the dispersed phase, diffusing into the surrounding aqueous, continuous phase. Consequently, the polymer and FA solution concentrates, and just before solidification of the polymer, a FA-rich DCM phase appears to separate. Using a variety of techniques, including real-time recording of single-microsphere formation, it was shown that coalescence of these liquid FA-rich microdroplets throughout the forming microsphere and at the interface, resulted in the appearance and growth of rounded protrusions at the surface (Yang et al., 2009). This same phenomenon has also been observed by two other groups studying the encapsulation of cyclosporine A in PLGA microspheres (Passerini and Craig, 2002; Malaekhe-Nikouei et al., 2006), and was described as “islands” of drug surrounded by the polymer matrix. It was suggested by both groups that the “islands” were composed of amorphous phase-separated drug, based on the fact that the “island” diameters increased with increasing drug loading. We agree with this conclusion, and our single microsphere studies directly demonstrated a similar phase separation process for FA (Yang et al., 2009).

Phase separation of drug within the polymer matrix and at the surface of microspheres can occur during fabrication, most commonly using the solvent evaporation method, and has been extensively reported (O'Donnell and McGinity, 1997; Li et al., 2008). It is well established that drug may precipitate in the polymer in a crystalline form or as an amorphous form within the carrier (Bodmeier and McGinity, 1987; Rosilio et al., 1991; Shenderova et al., 1997; Liggins and Burt, 2004). Interestingly, phase separation may be accompanied by changes in microsphere morphology from the completely smooth surfaces observed for control (no drug) microspheres to a concave dimpled appearance, as in, for example, paclitaxel loaded poly(L-lactic acid) microspheres (Liggins and Burt, 2004) and progesterone loaded PLGA microspheres (Rosilio et
al., 1991), or “rough” or “porous” surfaces, as in for example, quinidine loaded poly(D,L-lactic acid) microspheres (Bodmeier and McGinity, 1987) and peptide loaded PLGA microspheres (Blanco-Prieto et al., 1996). Phase separation of drug in a number of binary and ternary solvent-cast films has also been reported (Lu and Zografi, 1998; Panyam et al., 2004; Taepaiboon et al., 2007; Janssens et al., 2008).

Several reports have shown surface-associated drug phase separation from the polymer matrix, after the microspheres have been formed. However, there are only a few reports attempting to explain the underlying mechanisms of formation behind these phenomena. DeLuca and co-workers have carried out detailed kinetic and thermodynamic modeling of the formation of polymeric microspheres using the solvent evaporation method, and the processes underlying the formation of porous microspheres (Li et al., 1995a; Li et al., 1995b). In these investigations, porous microspheres were formed when salmon calcitonin peptide was loaded into PLGA microspheres using dichloromethane (DCM) as solvent and methanol as a cosolvent (to dissolve the loaded peptide). The authors explain that as solvent is transported out of the liquid phase droplet, the liquid droplet becomes increasingly viscous and eventually forms a gel at what is termed the viscous boundary (VB). Loss of more solvent results in crossing the glassy boundary, eventually leading to a glassy state composed of solid polymer and drug (Li et al., 1995a). Using a ternary phase diagram of DCM, methanol and PLGA, Li et al. (Li et al., 1995b) described the droplet phase composition changing with solvent loss and two phases being produced as the viscous boundary is approached, referred to as a polymer-rich phase, containing primarily polymer and solvent with small amounts of cosolvent and drug, and a polymer-poor phase, containing the bulk of the drug. Pores within the resulting microspheres were postulated to be due to the peptide, either trapping absorbed cosolvent (methanol) and/or imbibed continuous phase (water) in the bulk matrix or dissolved peptide in water adsorbing onto PLGA via
hydrophobic bonding (Li et al., 1995b). Using the micropipette techniques discussed in this investigation, it is possible to view, in real time, the events of phase separation to confirm or refute any of these postulated mechanisms.

An understanding of the phase behavior of drugs, and methods to study these phenomena in polymeric carriers are critical for the optimization of drug-delivery formulations, as phase separation can impact drug release profiles and drug stability. We have expanded our investigations of the phase separation phenomena of FA from PLGA microspheres to include dual FA and rifampicin (RIF) loaded microspheres. RIF is active against S. aureus, S. epidermidis, but also shows activity against Streptococcal organisms (including Streptococcus pneumoniae), Clostridium welchii, Neisseria meningitidis, and Pasteurella multocida (McCabe and Lorian, 1968; Kunin et al., 1969). The broad spectrum of activity of both FA and RIF confers protection against ≥ 70% of organisms that commonly cause prosthetic joint infections (Campoccia et al., 2006), and thus there is considerable clinical use of combinations of FA and RIF in implant associated orthopedic infections (Drancourt et al., 1997; Trampuz and Zimmerli, 2006). Therefore, a combination product that provides controlled release of FA and RIF at the site of infection may be of clinical benefit. In this work, we explore the ternary phase separation behavior and miscibility characteristics of FA and RIF in PLGA microspheres prepared by solvent evaporation from DCM, and bring a new mechanistic understanding to these complex, and thus far unseen, phase separation behaviors using real-time video recording of forming microspheres.
3.2 Materials and methods

3.2.1 Chemicals

Fusidic acid (FA), rifampicin (RIF), and poly(vinyl alcohol) (PVA; 98% hydrolyzed; MW 13-23g/mol) were purchased from Sigma Aldrich (Oakville, ON, CA). Poly(d,l-lactic acid-co-glycolic acid) (PLGA; 50:50) with a number-averaged molecular weight (Mn) of 49,100Da was purchased from Lactel® Absorbable Polymers (Pelham, AL, USA). The solvents dichloromethane (DCM), acetonitrile (ACN), methanol (MeOH), phosphoric acid, were of HPLC grade and purchased from Sigma Aldrich (Oakville, ON, CA).

3.2.2 Fabrication of antibiotic-loaded PLGA microspheres

Drug loaded PLGA microspheres were prepared by an oil-in-water (O/W) single emulsion and solvent evaporation method. Briefly, PLGA (50:50), FA and/or RIF, were co-dissolved at various concentrations in 5 mL of DCM to achieve a final combined drug-polymer concentration of 10% (w/v). Ratios were: 20% FA with 0%, 5%, 10%, 20%, and 30% RIF; and 20% RIF with 30%, 20% 10%, 5%, and 0% FA. The drug/PLGA solution was then added drop-wise into a flask containing 100 mL of 2.5% (w/v) aqueous PVA solution with overhead propeller stirring at 600 rpm (BDC 2002; Caframo, Wiarton, ON, CA) to form the O/W emulsion. The resulting emulsion was stirred continuously for 2.5 h at room temperature to evaporate the organic solvent. The microspheres were allowed to sediment in the flask on the bench top for 5 min and subsequently washed three times with distilled water with bench top sedimentation for 5 minutes to remove unencapsulated drug, residual DCM and PVA. The microspheres were then dried under 635 mmHg vacuum at room temperature for 24 h using a bench top vacuum oven (Napco No. 5831) and stored in a desiccator until further analysis.
3.2.3 Microsphere particle size distribution

The mean particle size, and size distributions, of FA, RIF, and co-loaded PLGA microspheres were determined using a Malvern Mastersizer 2000 laser diffraction particle size analyzer (Malvern Inc., Malvern, Worcestershire, UK). Briefly, approximately 5 mg of microspheres were carefully weighed out and suspended in 5 mL of distilled water with two drops of 1% polysorbate 80 (Tween 80) and sonicated for 2 min to prevent aggregation of microspheres. The particle size distribution of microspheres was expressed as mean diameter along with the span, where span is calculated according to the following equation:

\[
Span = \frac{\text{[Diameter at 90% cumulative size]}}{\text{[Diameter at 10% cumulative size]}} - \frac{\text{[Diameter at 50% cumulative size]}}{\text{[Diameter at 10% cumulative size]}}
\]  
(Equation 3.1)

3.2.4 Drug encapsulation efficiency

To determine the encapsulation efficiency of the FA and RIF antibiotics, ~2.5 mg of antibiotic-loaded microspheres were weighed and dissolved in 1 mL ACN in a screw-top test tube with a Teflon®-coated cap. 5 mL of phosphate buffered saline (PBS; pH 7.4) were added and vortexed for 60s to precipitate the polymer. The solution was centrifuged at 18,000 \( \times \) g (Microfuge® 18; Beckman Coulter, Mississauga, ON, CA) for 5 min to pellet any precipitate, and the supernatant was sampled for FA and RIF content.

FA content of the dissolved PLGA microspheres was quantified using a HPLC (Waters® Millennium System) assay utilizing a mobile phase of 50/30/20 (v/v/v) ACN/MeOH/0.01M phosphoric acid solution, flowing at 1 mL/min through a C18 reverse phase Nova-Pak® column (Waters®), with a 20 \( \mu \)L sample injection volume and UV vis detection at 235nm. FA was
quantified against a standard curve prepared by dissolving FA in ACN over a range of 0.1 to 500 µg/mL.

RIF content was evaluated using a UV-spectrophotometer (Varian Cary 50-BIO) assay with a medium scan speed and detection at 475nm. RIF was quantified against a standard curve prepared by dissolving RIF in DMSO:PBS over a range of 0.2 to 25 µg/mL.

3.2.5 **In vitro drug release from PLGA microspheres**

Antibiotic-loaded microspheres were accurately weighed (~5 mg), and placed into 15 mL PBS (pH 7.4; 0.1 mg/mL ascorbic acid) in screw-top test tubes with a Teflon®-coated cap. Ascorbic acid was added to the PBS release media as an antioxidant, since it has been shown to stabilize RIF and prevent oxidative degradation (Weber *et al.*, 1983). Each tube was tumbled end-over-end at 10 rpm in a thermostatically controlled oven at 37°C. At pre-determined time intervals, release tubes were removed and centrifuged at 1,400 × g (GS-6 Centrifuge; Beckman Coulter, Mississauga, ON, CA) to sediment the microspheres and 1 mL of the supernatant was withdrawn for HPLC or UV analysis as described above. The remaining media was removed and replaced with fresh PBS (pH 7.4; 0.1 mg/mL ascorbic acid) to maintain sink conditions. The solubility of FA is $5.2 \times 10^{-3}$ g/L, and that of RIF is $4.13 \times 10^{-2}$ g/L, and at no point did the concentration of either drug reach these limits during this measurement of drug release from the PLGA microspheres.

3.2.6 **Laser confocal microscopy**

The detailed surface morphologies of the FA, RIF, and co-loaded PLGA microsphere formulations were characterized using confocal laser scanning microscopy. Samples were placed
on double-sided tape adhered to a glass microscope slide, and images were captured using a LEXT OLS3100 confocal laser scanning microscope (Olympus; Markham, ON Canada).

3.2.7 Raman spectroscopy

High spatial resolution Raman spectroscopy surface mapping analyses of FA-RIF co-loaded PLGA microsphere formulations were kindly performed by Dr. Tim Smith of Renishaw, plc (Wotton-under-Edge, UK). Specifically, Raman spectra were obtained on a Renishaw RM100 confocal Raman Microscope (Renishaw, plc), recorded at a spatial resolution of 1 µm. Images were subsequently created using component method (using FA, RIF, and PLGA reference spectra) and images were generated from StreamLineTM images of Anadin Extra tablet as reference with argon ion laser excitation at λ = 785nm.

3.2.8 X-ray powder diffraction

X-ray powder diffraction (XRPD) patterns of FA, RIF, and FA-RIF co-loaded microspheres, and physical drug-polymer mixtures were obtained at 25°C with a Bruker APEX DUO diffractometer equipped with an area detector, cross-coupled multilayer optics, and Cu-Kα radiation. Samples were packed into thin-walled capillary tubes (special glass; Charles Supper Company, Natick, MA, USA) and sealed using a small amount of capillary wax. For each sample, three frames of data were collected at different 2θ angles and the frames were merged together producing a scan range of 4-60° 2θ. The sample to detector distance was 150 mm and each sample was rotated 360° about the phi axis with a 5 min data collection time per frame. The data were merged and integrated using Bruker XRD² EVAL software and analyzed using the Bruker EVA program.
3.2.9 Differential scanning calorimetry

DSC analysis of FA, RIF and FA-RIF co-loaded PLGA microspheres, and physical drug-polymer mixtures was carried out using a TA Instruments DSC Q100 (New Castle, DE, USA) with liquid nitrogen cooling system. The heat flow and heat capacity of the instrument was calibrated routinely using a high purity indium standard. Briefly, accurately weighed samples between 4–6 mg were hermetically sealed in aluminum pans with a pinhole, and heated from 25°C to 300°C at a rate of 10°C/min under nitrogen flow. The initial heat scan was followed by a rapid quench cooling scan from 300°C to −50°C at a rate of 35°C/min and a second heating scan from −50°C to 250°C at a rate of 10°C/min. All DSC thermograms were analyzed using TA Instruments Universal Analysis (v. 4.7A).

DSC was used to determine the ability of FA and RIF to form a miscible blend. FA and RIF were co-solidified from DCM at room temperature in the absence of PLGA. Approximately 5 mg of co-solidified drug was hermetically sealed in aluminum pans with a pinhole, and heated from 25°C to 300°C at a rate of 10°C/min under nitrogen flow. Using published $T_g$ values for FA (117°C) (Yang et al., 2009) and RIF (160°C) (Agrawal et al., 2004), the Fox equation, which defines the weight fraction-dependent shift in glass transition temperature of any blend ($T_{g,BLEND}$), was used to generate a reciprocal plot over a range of FA:RIF weight fractions and was plotted along with the experimental $T_g$ values observed in the drug-loaded microspheres, and as co-solidified drug. The Fox equation is expressed as:

\[
\frac{1}{T_{g,BLEND}} = \frac{W_{FA}}{T_{g,FA}} + \frac{W_{RIF}}{T_{g,RIF}}
\]

(Equation 3.2)
where, \( w_{FA} \) and \( w_{RIF} \) are the weight fractions of FA and RIF, and \( T_{g,FA} \) and \( T_{g,RIF} \) are the glass transition temperatures for FA and RIF, respectively.

### 3.2.10 Micromanipulation and video imaging of single microsphere formation

Real-time recordings of the formation of single FA, RIF, and FA-RIF co-loaded PLGA microspheres were captured using a micropipette manipulation system as described in detail elsewhere. (Rickard et al., 2010) Briefly, single droplets of FA/RIF/PLGA solution in DCM were formed at the tip of a 5 µm diameter borosilicate glass micropipette into a solution of 0.025 M SDS. Once the emerging droplet reached the desired diameter (~100 µm), the micropipette manipulator was gently tapped to release the solution droplet, which was either allowed to fall to the bottom of the chamber or immediately recaptured on the end of the micropipette. The droplet was observed and the image digitally recorded as the DCM diffused into the surrounding aqueous phase, resulting in a solidified microsphere. The experimental setup includes an inverted optical microscope (Diaphot 200; Nikon, Melville, NY, USA) with a 40× or an oil-immersion 100× objective lens, micropipette manipulation system, and video capturing equipment. The camera (Pike F-100B; Allied Vision Technologies, Stadtroda, DE) was controlled via a computer with StreamPix software (Norpix, Montreal, QC, CA) and the captured video analyzed using ImageJ software (National Institutes of Health). The chamber used with the 40× objective lens was a standard glass cuvette (2-mm path length) (Duncan and Needham, 2006; Rickard et al., 2010). For experiments with the oil-immersion objective, the chamber was formed from coverslips (22 × 30 mm) and cut glass microscope slides (Duncan and Needham, 2004). Coverslips were affixed to the top and bottom of the glass slide pieces with vacuum grease to create a chamber with final dimensions of approximately 22 × 25 × 2 mm, which was open on two sides.
3.2.11 Mass composition calculations for single microdroplets

The solute concentration during phase separation was calculated as a function of time and was based on the volume change of the single droplet. The initial solution density, $\rho_i$, and total solute concentration (i.e. drug + PLGA), $c_i$, were calculated from the weight fraction ($w$) of each component assuming no volume change on mixing, where:

\[
\frac{1}{\rho_i} = \frac{w_{DCM}}{\rho_{DCM}} + \frac{w_{PLGA}}{\rho_{PLGA}} + \frac{w_{DRUG}}{\rho_{DRUG}} \quad \text{(Equation 3.3)}
\]

and

\[
c_i = \left( w_{PLGA} + w_{DRUG} \right) \rho_i \quad \text{(Equation 3.4)}
\]

In all calculations, it was assumed that neither drug nor polymer dissolved into the surrounding aqueous phase, and therefore, the solute mass in the droplet remained constant. The solute concentration was then calculated as a function of time based on the volume change of the droplet according to $c = c_i v_i / v$, where $v_i$ is the initial volume of the droplet and $v$ is the volume of the droplet as a function of time. The solute concentration at the time of phase separation was calculated according to the diameter of the droplet when the first signs of phase separation were visible (as indicated in Figure 3.7A). To calculate this in terms of the solute mass fraction, the mass of DCM in the droplet was also calculated as a function of time by subtracting the mass lost from the initial DCM mass, $m_{DCM} = m_{i,DCM} - m_{lost,DCM}$. The initial mass is given by $m_{i,DCM} = w_{i,DCM} v_i \rho_i$, and the mass lost is given by $m_{lost,DCM} = (v_i - v) \rho_{DCM}$. The mass fraction of total solute, $w_{PLGA+DRUG}$, can then be calculated according to:
Solute mass fractions were calculated assuming the following densities: \( \rho_{PLGA} = 1.34 \text{ g/ml}; \rho_{DCM} = 1.33 \text{ g/ml}; \rho_{RIF} = 1.34 \text{ g/ml}; \rho_{FA} = 1.16 \text{ g/ml} \).

3.2.12 FA, RIF, and PLGA compatibility calculations

The compatibility between FA and RIF and PLGA was calculated by the Hildebrand-Scatchard equation and the enthalpy of mixing (\( \Delta H_M \)) as described in section 1.8.

3.3 Results

3.3.1 FA, RIF and FA-RIF co-loaded PLGA microspheres

As shown in Figure 3.1 A, pure FA loaded PLGA microspheres (at 20% loading) showed regular spherical protrusions. Microspheres containing 20% RIF were characterized by small concave surface dimples Figure 3.1F. For the mixed systems, microspheres containing 20% (w/w) FA with increasing RIF co-loading (Figure 3.1B-E) displayed uniform microdomains that appeared as spherical surface protrusions with distinct boundaries. The interstitial surface space between these spherical protrusions showed a dimpled appearance characteristic of pure RIF. For RIF-loaded microspheres (20% w/w) with increasing amounts of FA, the dimpled morphology became more pronounced with FA addition up to 10% FA (w/w), but once the FA loading reached or exceeded 20% (w/w), spherical protrusions appeared, reminiscent of the 20% FA system with added RIF. Detailed surface morphologies for both initial and drug-released released microspheres are shown in Figure 3.1.
Figure 3.1 Laser confocal micrographs illustrating the detailed surface morphology of microspheres containing FA and RIF before (A-I) and after (J-R) 7d release in PBS (pH 7.4; containing 0.1 mg/mL ascorbic acid) at 37°C. The scale bar represents 30 µm.

FA-loaded microspheres showed an increase in mean diameter with the co-loading of RIF in a dose dependent manner with mean diameters of 71.5 µm for FA mono-loaded microspheres increasing to 93.4 µm, 87.4 µm, 99.9 µm, and 101.6 µm, for RIF co-loadings of 5, 10, 20, and 30% (w/w), respectively. In contrast, RIF-loaded microspheres demonstrated a decrease in mean diameter with FA co-loading, with mean diameters of 131.6 µm for RIF mono-loaded microspheres decreasing to 112.3 µm, 121.1 µm, and 94.2 µm, for FA co-loadings of 5, 10, and
30% (w/w), respectively. Formulations were characterized by high FA encapsulation efficiency ranging from approximately 82% to 97%, with no apparent dependence on drug loading. RIF encapsulation was slightly lower than FA, ranging from approximately 75% to 87%, with higher RIF loadings achieved once FA theoretical loadings were ≥ 20% (w/w). The physical characteristics of the microspheres are summarized in Table 3.1.
<table>
<thead>
<tr>
<th>Microsphere Formulation</th>
<th>Thermal Characteristics</th>
<th>Physical Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$T_r$ (°C)$^a$</td>
<td>$\Delta H_r$ (J/g)$^b$</td>
</tr>
<tr>
<td>20% FA; 0% RIF</td>
<td>56.8 ± 0.17</td>
<td>7.9 ± 1.1</td>
</tr>
<tr>
<td>20% FA; 5% RIF</td>
<td>57.0 ± 0.15</td>
<td>6.7 ± 1.0</td>
</tr>
<tr>
<td>20% FA; 10% RIF</td>
<td>57.1 ± 0.32</td>
<td>6.2 ± 1.2</td>
</tr>
<tr>
<td>20% FA; 20% RIF</td>
<td>57.4 ± 0.18</td>
<td>6.5 ± 0.5</td>
</tr>
<tr>
<td>20% FA; 30% RIF</td>
<td>57.9 ± 0.33</td>
<td>4.7 ± 0.5</td>
</tr>
<tr>
<td>30% FA; 20% RIF</td>
<td>57.5 ± 0.11</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td>10% FA; 20% RIF</td>
<td>57.2 ± 0.46</td>
<td>6.4 ± 0.9</td>
</tr>
<tr>
<td>5% FA; 20% RIF</td>
<td>56.2 ± 0.16</td>
<td>6.6 ± 0.2</td>
</tr>
<tr>
<td>0% FA; 20% RIF</td>
<td>55.2 ± 0.83</td>
<td>6.5 ± 0.6</td>
</tr>
</tbody>
</table>

$^aT_r$ - Enthalpy of relaxation temperature. The peak temperature of the first endothermic transition in the first heating cycle.

$^b\Delta H_r$ = Enthalpy of relaxation. Integration of the first endothermic transition in the first heating cycle.

$^cT_g$ = Glass transition temperature for the mono- or co-loaded drugs. The midpoint of the heat capacity change in the first heating cycle.

$^d$PDI = Polydispersity index. Measured by the span, according to Equation 3.1. A smaller span indicates a narrow size distribution.

$^e$EE = Encapsulation efficiency. Recovered drug expressed as a percentage of the theoretical drug loading.

Results are expressed as the mean ± S.D. $n = 3$. 
3.3.2 Raman spectroscopy analysis of FA and RIF co-loaded PLGA microspheres

In order to determine the nature of the various surface microdomains shown in Figure 3.1, Raman surface mapping of FA and RIF co-loaded PLGA microspheres was performed using Raman shift data between 1800 cm\(^{-1}\) and 700 cm\(^{-1}\). Unfortunately, the high scattering intensity of RIF precluded the ability to map the microsphere surface for actual drug localization; however, we were able to map the surface of the co-loaded microsphere for PLGA distribution. Figure 3.2 shows that the PLGA signal is limited to the areas around the surface microdomains, suggesting that the drugs are localized within the protruding and dimpled microdomains.

![Raman confocal images of 20% (w/w) FA and 20% (w/w) RIF co-loaded into PLGA microspheres.](image)

(A) Confocal image illustrating the microsphere morphology; (B) Chemical map showing the variation in distribution of PLGA. Light areas show the presence of PLGA and dark areas the absence.
3.3.3 XRPD analysis of FA, RIF, and FA-RIF co-loaded PLGA microspheres

Representative XRPD patterns of the drug-loaded microspheres, and physical drug-polymer mixtures are shown in Figure 3.3.

Figure 3.3 Representative XRD patterns of physical mixtures of FA, RIF, and PLGA and microspheres containing FA, RIF, and FA:RIF (1:1). There were no diffraction peaks present in any mono- or co-loaded formulations.
There was no evidence of crystallinity in any of the microsphere formulations, as illustrated by a characteristic amorphous halo in the XRPD pattern.

3.3.4 Thermal characterization of FA, RIF, and FA-RIF co-loaded PLGA microspheres and co-solidified drugs

Representative DSC scans of FA, RIF, and FA-RIF co-loaded PLGA microspheres are shown in Figure 3.4 and summarized in Table 3.1. All formulations displayed an enthalpy of relaxation endotherm ($T_r$) at approximately 57°C, which was associated with the $T_g$ of PLGA; the second heating cycle, after quench cooling, was also characterized by a single $T_g$ between 40-43°C, characteristic of PLGA (Table 3.1). A glass transition ($T_g$) between 116-150°C was also observed on the first heating cycle, and this was characteristic of a thermal transition attributed to the drug(s). For the 20% FA in PLGA, the $T_g$ was at 117.9°C ± 0.06°C, and for 20% RIF in PLGA, the $T_g$ was at 159.22 ± 1.7°C. As expected from a reciprocal combination of the mixtures, the measured $T_g$ for the first heating cycle was dependent on the FA and RIF content of the co-loaded microspheres. That is, the inclusion of RIF in 20% FA loaded microspheres resulting in a concentration-dependent increase in $T_g$ (Figure 3.4A). Conversely, the inclusion of FA content in 20% RIF microspheres resulted in a concentration-dependent decrease in the $T_g$ (Figure 3.4B).
Figure 3.4 DSC thermograms of drug-loaded PLGA microspheres showing the first heating cycle of (A) 20% FA-loaded PLGA (50:50) microspheres containing 0-30% RIF, and (B) 20% RIF-loaded PLGA (50:50) microspheres containing 0-30% FA. The shaded areas represent the enthalpy of relaxation ($T_r$), at ~57°C, and a glass transition temperature ($T_g$) between 117-150°C, dependent on RIF or FA loading.
Thermal analysis of FA and RIF and FA-RIF mixtures co-solidified by solvent evaporation from DCM in the absence of PLGA showed single observable $T_g$s covering the range between the two pure materials. The $T_g$ for co-solidified drug and microspheres is illustrated in Figure 3.5, plotted along with the calculated $T_g$ of a miscible blend of FA-RIF, determined by the Fox equation.

![Figure 3.5](image)

Figure 3.5 Effect of blending increasing amounts of RIF with FA on the glass transition temperature ($T_g$) of the blend from (○) 20% (w/w) FA-loaded PLGA microspheres prepared by single O/W emulsion and solvent evaporation, and (▲) the antibiotics co-solidified from DCM with no polymer present. The solid reciprocal line represents theoretical values based on the Fox equation. Each data point represents the mean ± S.D. of $n = 3$ scans.

### 3.3.5 Micromanipulation and analysis of single microsphere formation

Using a micropipette technique, PLGA microsphere formation was observed in real time for solutions of 20, 40, and 60% (w/w) drug/PLGA content for FA, RIF, and co-loaded (FA:RIF;
1:1) microspheres. All solutions had a starting composition of ~10% (w/v) of total solids (drug and polymer) in DCM. Representative videomicrographs of 20% (w/w) FA and RIF mono-loaded, and 20% (w/w) FA:RIF (1:1) co-loaded PLGA microspheres are shown in Figure 3.6. Note that, in order to see the details of microstructure and phase separations, a higher magnification oil immersion lens was used to obtain the images in Figure 3.6. This necessitated studying smaller microspheres than those that provided the data in Figure 3.7, and thus, the time lines for Figure 3.6 and Figure 3.7 do not match, as the relatively small microspheres of Figure 3.6 lose solvent and reach the phase precipitation faster than the larger population shown in Figure 3.7.

All formulations were characterized by FA or RIF phase-separation events. For 20% (w/w) FA-loaded microspheres (Figure 3.6A), the first phase separation of FA-rich droplets, occurred very rapidly (see Figure 3.6A; 3s and 8s). Since water is not very soluble in DCM, it would seem that this is a FA-DCM phase in the PLGA:DCM droplet. In the absence of FA, there are no water inclusions, but the aqueous solubility of FA is $5.2 \times 10^{-3}$ g/L, and so we cannot completely rule out that this FA-rich phase does not contain any water. With further loss of DCM solvent and shrinking of the microdroplet volume, these FA-rich microdroplets within the forming microsphere coalesced to form larger microdroplets. As the microsphere approached the viscous boundary, the viscosity of the forming microsphere increased (Brownian motion of internal particles slowed dramatically) and some of the FA-rich droplets in the interior of the solidifying microsphere became kinetically trapped within the bulk of the microsphere, while ones closest to the surface condensed out at the surface (see Figure 3.6A; ~15s). Thus, what appeared to be the FA-rich phase was excluded from the PLGA matrix and localized as microdomains at the surface of the solidifying microsphere (Figure 3.6A; 20s). These data are
very similar to, and confirm, our previous observations for FA:PLGA microsphere formation (Yang et al., 2009).

For the 20% (w/w) RIF-loaded microspheres (Figure 3.6C), small water inclusions that can sometimes be incorporated into the droplet as it emerges from the micropipet (due to differential wetting and displacement of the water film on the glass). RIF phase separates from PLGA (Figure 3.6C) considerably later than FA does in a microsphere of comparable (60 µm) size. RIF is more water soluble in an aqueous phase (4.13 × 10⁻² g/L) and might be expected to imbibe more water than FA into the PLGA microsphere, and therefore we cannot rule out that this RIF-rich phase contains some water. As shown in Figure 3.6C, the RIF-rich microdroplets did not appear to coalesce very much, which might be explained by the fact that by the time the RIF phase separates as small droplets, the viscosity of the polymer droplet was high enough to prevent much Brownian motion and RIF-rich droplet motion, at a relative droplet volume of 0.194 ± 0.000, which corresponds to a solute mass percent of 39.7 ± 0.1%. Thus, the separation of RIF was much later in the solidification process than the separation of FA in the FA-loaded formulation. In this mono-loaded system, what appeared to be the phase-separation of drug-rich microdomains occurred much closer to the VB (relative droplet volume of 0.109 ± 0.001) as shown in Figure 3.6C, and the drug was more-well encapsulated throughout the microsphere.

In the co-loaded formulation (Figure 3.6B), the phase separation of both drugs occurred in a single event at a relative droplet volume of 0.251 ± 0.002, which corresponds to a solute mass percent of 31.1 ± 0.3%, midway between the two mono-loaded formulations. The phase separation was also consistent with each drug’s behavior in that some droplets were retained in the interior, similar to RIF, while, larger domains precipitated at the microsphere surface, characteristic of FA phase separation.
Figure 3.6 Time lapsed video images (FOV = 58 μm × 43 μm) illustrating the formation of PLGA (50:50) microspheres containing (A) 20\% (w/w) FA, (B) 20\% (w/w) FA:RIF (1:1 as 10\%:10\%), and (C) 20\% (w/w) RIF. All microspheres were created with an initial polymer and drug concentration of 10\% (w/v). The detailed surface morphologies of the corresponding microspheres by surface SEM are depicted in Figure 3.1.
The data from the micromanipulation experiments on larger microdroplets (95.9 ± 3.0 µm) were then used to plot the changes in relative microsphere volume during microsphere solidification for microspheres composed of FA, RIF, or FA-RIF (1:1), in PLGA over the course of solidification (Figure 3.7A). The mass composition of drug, PLGA, and DCM at the point of phase separation was quantified according to Equations (3.3) to (3.5). The viscosity boundary (VB), defined as the onset of polymer gelation (Li et al., 1995a), is indicated at a relative droplet volume of 0.095 ± 0.044, as shown by the inflection in Figure 3.7A. Consistent with the images in Figure 3.6, the slightly larger population of microspheres showed the same drug and drug mixture phase separation in relation to the viscous boundary (FA<FA-RIF<RIF).

The data from the micromanipulation experiments were then used to construct a ternary phase diagram to better illustrate the solute composition of the mono- and co-loaded microspheres at the point of phase separation (Figure 3.7B). The apex entitled “Drug” represents FA, RIF, or FA-RIF (1:1). Each formulation, FA, RIF, and FA-RIF (1:1), was evaluated at 20%, 40%, and 60% (w/w) relative to PLGA, to evaluate the role of drug load on the phase separation. This figure illustrates that the phase separation of FA occurred much earlier in the solidification process than RIF. Furthermore, when both FA and RIF were encapsulated (1:1), the phase boundary of the co-loaded formulation (represented as the “drug” apex on the diagram) was between the two mono-loaded formulations.
Figure 3.7 (A) Representative plot of the changes in relative microsphere volume during microsphere solidification for microspheres composed of FA (○), RIF (■), or FA-RIF (1:1) (▲), in PLGA as determined by micropipette experiments. The droplet volume at the point of phase separation is shown in red for FA (○), RIF (■), and FA-RIF (▲). (B) Ternary phase diagram illustrating the composition at the point of drug phase separation for microspheres containing FA (○), RIF (■), and FA-RIF (▲). All compositions were calculated using Equations 3-5 and are given in mass %. The dotted lines represent the microsphere composition at the viscous boundary for FA (⊙) or RIF (□), which is shown as the inflection point in (A).
3.3.6 Drug-polymer compatibility

The Hildebrand-Scatchard equation (Equation 1.15) was used to calculate the Flory-Huggins interaction parameter ($\chi_{sp}$) for the binary mixtures of FA or RIF and PLGA, using the group contribution method. The partial solubility parameters were also used to calculate the enthalpy of mixing ($\Delta H_M$) of the binary mixtures using Equation 1.14. The total solubility parameters ($\delta_{\text{drug}}$ or $\delta_{\text{PLGA}}$), molar volume ($V$), $\chi_{sp}$, and $\Delta H_M$ values are shown in Table 3.2.

Table 3.2 FA, RIF, and PLGA compatibility data.

<table>
<thead>
<tr>
<th>Microsphere component</th>
<th>$\delta_{\text{drug}}$ or $\delta_{\text{PLGA}}$ $^a$ (MPa$^{1/2}$)</th>
<th>$V$ $^b$ (cm$^3$/mol)</th>
<th>$\chi_{sp}$ $^c,d$</th>
<th>$\Delta H_M$ $^e$ (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA</td>
<td>21.1</td>
<td>443.4</td>
<td>0.72</td>
<td>9.47</td>
</tr>
<tr>
<td>RIF</td>
<td>24.6</td>
<td>591.2</td>
<td>0.55</td>
<td>3.57</td>
</tr>
<tr>
<td>PLGA (50:50)$^e$</td>
<td>23.1</td>
<td>47.7</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

$^a$ $\delta_{\text{drug}}$ or $\delta_{\text{PLGA}}$ – Total solubility parameter for the drugs and polymer as calculated from Equation 1.8.

$^b$ $V$ – Molar volume of the drug or polymer, calculated according to Fedors (Fedors, 1974).

$^c$$\chi_{sp}$ – Flory-Huggins interaction parameter calculated from Equation 1.15 (see section 1.8).

$^d$$\Delta H_M$ – Enthalpy of mixing calculated from Equation 1.14 (see section 1.8).

$^d$Critical solubility value – $\chi_{sp}$ value for solubility limits; Insoluble>0.5>Soluble (Schott, 1969).

$^e$The $\delta_{\text{PLGA}}$ and $V$ of PLGA (50:50) were taken from literature values (Schenderlein et al., 2004).
3.3.7 *In vitro* release profiles of FA and RIF from mono- and dual-loaded PLGA microspheres

The *in vitro* release profiles of FA and RIF are plotted in Figure 3.8. All formulations containing 20% (w/w) FA (± RIF) were characterized by an initial burst of FA (Figure 3.8A) and RIF (Figure 3.8B) over 1 hr, with no further FA release and a moderate release of RIF over 7 days, with the exception of microspheres containing 30% (w/w) RIF, which showed a moderate controlled-release over 7 days. In contrast, formulations containing 20% (w/w) RIF (± FA) did not release FA (Figure 3.8), and only released very low quantities of RIF (< 5%; Figure 3.8D). It was not until a 20% (w/w) co-loading of FA that any appreciable release of FA or RIF was observed (Figure 3.8A and B). Figure 3.1 (J-R) shows the detailed surface morphology of microspheres following 7 days release in PBS (pH 7.4) revealing the disappearance of the spherical protrusions on the microsphere surface.
Figure 3.8 *In vitro* release profiles of FA and RIF from mono- and co-loaded PLGA (50:50) microspheres performed in PBS (pH 7.4; containing 0.1mg/mL ascorbic acid) at 37°C. (A) FA release from 20% (w/w) FA-loaded PLGA containing (●) 0% RIF, (■) 5% RIF, (▲) 10% RIF, (▼) 20% RIF, and (◆) 30% RIF. (B) RIF release from 20% (w/w) FA-loaded PLGA microspheres containing (■) 5% RIF, (▲) 10% RIF, (▼) 20% RIF, and (◆) 30% RIF. (C) FA release from 20% (w/w) RIF-loaded PLGA microspheres containing (●) 0% FA, (■) 5% FA, (▲) 10% FA, and (◆) 30% FA. (D) RIF release from 20% (w/w) RIF-loaded PLGA microspheres containing (●) 0% FA, (■) 5% FA, (▲) 10% FA, and (◆) 30% FA. n = 3 ± S.D.

3.4 Discussion

In this investigation, we explored the ternary phase separation behavior and miscibility characteristics of FA and RIF loaded into PLGA microspheres. This exploration was mainly
based on observations of interesting morphological features in the mono- and co-loaded PLGA microspheres. Although a small number of reports have shown microspheres with similar features (Rosilio et al., 1991; Passerini and Craig, 2002; Liggins and Burt, 2004; Malaekeh-Nikouei et al., 2006; Yang et al., 2009), there have been no attempts to explain the phase phenomena underlying these observations. Of particular importance was the ability to visualize microscopic images of the whole process of microsphere formation from single PLGA-DCM-drug solution microdroplets using a micropipet technique.

Microsphere formation is a process of interface creation, mass transfer, and phase separation. It occurs in three distinct steps: 1) droplet formation into a second aqueous phase; 2) solvent removal into the aqueous solvent and solidification of the polymer and drug; 3) final drying to remove all solvent. Parameters that influence the diffusion of solvent from the dispersed phase and subsequent solidification of the polymer and the co-dissolved drugs are critical to the ultimate drug release profiles of the resulting microspheres. Understanding the mixing relations, mutual solubilities and insolubilities of the components is essential for the rational design of controlled release. This processes governing the solidification of microspheres can be divided into three temporal states: 1) the solution state, 2) the gel state, and 3) the glassy state. In the solution state, the mass transfer of solvent to the continuous phase results in the liquid-liquid phase separation of a polymer-rich outer layer and a polymer-poor inner core, which has been termed binodal decomposition (Li et al., 1995a). According to DeLuca, the polymer-rich liquid phase is composed of PLGA, organic solvent, and small amounts of encapsulated drug, while the polymer-poor liquid phase contains the majority of the encapsulated drug and solvent. This initial phase separation then is dependent on the relative polymer concentration in each phase, and is the equilibrium between a concentrated polymer swollen by solvent, and solvent with a small amount of polymer dissolved. In stage 2, as the mass transfer of
solvent continues from the polymer-drug dispersed phase, the droplet reaches a gelation point, where the polymer-drug solution becomes concentrated and more viscous. This point is termed the viscous boundary (VB) depicted earlier in Figure 3.7. The mass transfer of organic solvent out of the now gelatinous microsphere is slowed considerably at the VB, and the transport of molecular solvated drug or drug particles inside the microsphere is restrained. The drug-polymer droplets remain in the gel state until mass transfer is complete, when the droplets reach the third stage, the glassy state and form the final solid-state microsphere. Drug can associate with polymer as a molecular level dispersion of drug within the polymer interstitial space (solid solution), or it could precipitate as distinct separate domains/clusters (forming a solid suspension), in which the drug particles may be crystalline or amorphous (van Drooge et al., 2006). However if the outer shell of the microsphere is sufficiently viscous, drug that is not molecularly dispersed (i.e. drug that exists as phase separated domains/clusters) cannot be efficiently transported into the outer layer, trapping it in this more concentrated polymer phase.

As shown in Figure 3.1A, mono-loaded microspheres containing 20% FA displayed uniform, spherical surface protrusions with distinct boundaries on the microsphere surface. This morphology has been shown before by our group for FA-PLGA microspheres (Yang et al., 2009), and similar morphologies have been shown for PLLA microspheres loaded with Cyclosporine A (Passerini and Craig, 2002; Malaekhe-Nikouei et al., 2006), as well as cellulose acetate films containing Vitamin E (Taepaiboon et al., 2007). Similar spherical protrusion on the surface of polymer films and microspheres are therefore thought to be due to an insolubility of the drug in the polymer and the phase exclusion of drug-rich domains from the polymer matrix in PLGA, PLLA, and cellulose acetate. The fact that they are spherical indicates that they have a liquid origin, that is, an immiscible liquid phase with a significant interfacial tension.
Microspheres containing 20% (w/w) RIF were characterized by small concave surface dimples (Figure 3.1F). These concave dimples increased in size with FA loading until FA loading reached 20% (w/w) where spherical protrusions were once again visualized (Figure 3.1D). Similar concave dimpled morphologies have been shown for PLLA microspheres encapsulating the anti-cancer drug paclitaxel (Liggins and Burt, 2004), and were attributed to solid-solid phase separation of drug from the polymer. It was suggested that the encapsulated drug was a principal component of the initially formed surface layer during microsphere hardening (Liggins, 2001). Therefore, it is likely that the dimpled morphology in microspheres with higher RIF loadings began as numerous small spherical domains, similar to what is seen with FA, as a result of RIF being a component of the highly viscous surface layer. However, due to the higher aqueous solubility of RIF, these domains undergo dissolution in the continuous phase during the microsphere hardening process resulting in loss of phase-separated domains, forming concave dimples. Although we were unable to visually confirm this, the loss of surface associated drug may explain the lower encapsulation efficiency in the microspheres with higher RIF loadings. Nevertheless, the change from a dimpled morphology to one with spherical surface protrusions with increasing FA content suggests that at higher loadings (i.e. 20%), the FA incompatibility in PLGA begins to determine the phase separation, and is also promoted by the lower aqueous solubility of FA ($5.2 \times 10^{-3}$ g/L for FA compared to $4.13 \times 10^{-2}$ g/L for RIF).

To further investigate the nature and composition of the phase-excluded microdomains, each microsphere formulation was subjected to XRPD and thermal analysis by DSC. XRPD patterns of each formulation were compared to XRPD patterns of physical mixtures of each drug with PLGA. All microsphere formulations were characterized by an amorphous halo, with no evidence of crystallinity in any formulation, suggesting that the drugs were present in the solid microsphere in the amorphous form. Thermal analysis supported this observation as no melt
endotherms were observed upon heating. All FA, RIF, and FA-RIF co-loaded microspheres were characterized by an enthalpy of relaxation endotherm ($T_r$), attributed to PLGA, and a glass transition ($T_g$) at ~45°C on the second heating cycle after quench cooling (Figure 3.4; second heating cycle not shown). The enthalpy of relaxation occurs concurrently with the polymer $T_g$, and is a result of short range order within the glassy regions of the polymer that occurs during ageing or processing (Liggins, 2001). Quenching the sample (i.e. removing the thermal history) eliminates the enthalpy of relaxation, and a single $T_g$, characteristic of PLGA, is observed at ~45°C on the second heating cycle. Since there was no influence of drug loading on the $T_g$ of PLGA, this suggests that both drugs have extremely limited solubility in PLGA and completely phase separate from the polymer. Accompanying the polymer transition, there was only one other observable thermal event on the first heating cycle, a $T_g$ ranging from ~116°C-150°C (Figure 3.4). This single $T_g$, well above the $T_g$ of PLGA, suggests that the amorphous phase-separated microdomains contained both FA and RIF as a single miscible glass. To evaluate whether PLGA has an influence on the miscibility of FA and RIF, the drugs were co-solidified by solvent evaporation from DCM in the absence of PLGA. DSC analysis on the drug co-solidified from DCM showed a single $T_g$ spanning the same range observed in the PLGA microspheres, with a dependence on the weight contribution of RIF (Figure 3.4). All measurements from microspheres and co-solidification experiments closely matched the theoretical calculations predicted by the Fox equation. Thus, we suggest that FA and RIF are miscible in all proportions, and the single thermal event in microspheres and co-solidified drugs, indicated that both FA and RIF were co-localized as an amorphous solid in spherical microdomains on the surface and within the bulk of the PLGA microspheres.

Surface mapping of co-loaded microspheres using high spatial resolution Raman spectroscopy was employed to confirm that the phase separated microdomains on the surface of
the microsphere were composed of co-localized FA and RIF. Unfortunately, RIF produced very high fluorescence and scattered light at a magnitude of ~100× and ~30× that of FA and PLGA, respectively, which did not allow useful Raman information to be collected on the distribution of RIF. It is likely that a resonant effect is occurring to amplify the Raman signal, which typically happens when the virtual energy level of the input laser coincides with the electronic state of the molecule. However, we were able to map the surface for PLGA distribution. As shown in Figure 3.2, PLGA distribution is confined to the areas surrounding the spherical microdomains, and not within them (light areas show the presence, dark areas the absence of PLGA). Hence, this provides additional evidence that the microdomains observed in the PLGA microspheres were composed of both FA and RIF as a single, amorphous micro-phase-separated solid.

The molecular solubilization of a drug in a polymeric carrier is largely determined by the compatibility between these two components. That is, in order for the drug to remain dissolved in the polymer, the adhesion forces (i.e. dispersion, polar, and hydrogen bonding forces) must be greater between polymer and drug molecules than between drug molecules. In the case of microsphere formation, if the drug and polymer are not sufficiently compatible, the drug molecules will associate into localized regions in the solidifying microsphere until saturation is reached, at which time the drug will become insoluble in the polymer and phase separate (Shen et al., 2002). Thermodynamically, the ability of two components to form miscible blends requires favorable intermolecular interactions between the two components. Drug/polymer compatibility and miscibility can be quantified by determining the Flory Huggins interaction parameter, either experimentally by methods such as melting point depression or ellipsometric measurements, or through calculating the total Hildebrand solubility parameters (and subsequent $\chi_{sp}$ calculations) and the enthalpies of mixing ($\Delta H_m$). The calculated Flory-Huggins interaction parameters and enthalpies of mixing both predicted that neither FA nor RIF would have
complete solubility in PLGA ($\chi_{sp}$ value for solubility limits; Insoluble>0.5>Soluble (Schott, 1969)). However, our calculations do show that RIF is predicted to have higher compatibility with PLGA compared to FA (as demonstrated by the lower $\chi_{sp}$ and $\Delta H_M$ values) (Table 3.2). Therefore, we suggest that the formation of phase-separated, drug-rich microdomains and the time at which this phenomenon occurs is regulated by the absolute solubility of each drug in PLGA.

Micromanipulation techniques were used to visualize and characterize the phase separation of FA and RIF from PLGA microspheres. Real-time video images were temporally recorded from the solution phase of microsphere formation until they passed the viscous boundary and reached the gel phase. Representative videomicrographs in Figure 3.6 illustrate that both FA and RIF phase separate from PLGA in all formulations. Analysis of the real-time videos of microsphere formation allows for quantification of microdroplet composition at the point of phase separation. Figure 3.9 is a schematic representation of the stages of phase separation for mono- and co-loaded PLGA microspheres compared to solidification of control PLGA microspheres. The phase separation of FA occurs very early during the solution state (relative droplet volume = 0.311 ± 0.014 for 20%FA) when the microsphere is still fluid (Figure 3.9; Point A). The phase separation results in the coalescence of FA-rich microdroplets in the polymer-poor microsphere interior, where convective currents within the microsphere (from the mass transfer of solvent from the core) exclude them to the organic-aqueous phase boundary at the microsphere surface. Once the microsphere undergoes gelation (i.e. passes the VB), the microdroplets are no longer able to coalesce, and are seen as smaller microdomains localized to the microsphere bulk. On the other hand, the phase separation of RIF (20% loading) occurs much closer to the gelation point, at a relative droplet volume of 0.194 ± 0.000, and supports the higher compatibility in PLGA (as demonstrated by the lower $\chi_{sp}$ and $\Delta H_M$ values) (Figure 3.9; Point C).
Interestingly though, the microsphere has still not reached the VB and remains fluid, yet there is no visible convection within the forming microsphere, and the phase separated RIF-rich microdomains are not excluded to the surface of the forming microsphere. When co-loaded, the phase separation of FA and RIF occurs as a single event, rather than an early phase separation of FA followed by the later phase separation of RIF. This event is governed by the compatibility of the FA-RIF mixture in PLGA, and happens intermediate between FA and RIF phase separation (relative droplet volume = 0.251 ± 0.002) (Figure 3.9; Point B). During this phase separation, there is still sufficient fluidity in the microsphere to allow for some surface exclusion, however the majority of separated drug mixture remains within the microsphere bulk. It is likely that RIF is altering the FA partitioning through an increased miscibility of FA in the solvent-rich RIF phase. However, the incompatibility of RIF with PLGA precludes complete compatibility in the ternary mixture containing FA, RIF, and PLGA. Kang et al. demonstrated a similar phenomenon using a two-phase polymer system composed of binary mixtures of PLGA and PEG, to which paclitaxel (PTX) was incorporated (Kang et al., 2007). In this investigation, the authors show that PTX preferentially partitioned into the PEG domains in films containing up to 20% (w/w) PEG in PLGA. The formulation presented as a two-phase system, where the interactions between PTX and PEG were sufficient to prevent PEG crystallization, forming an amorphous solid solution of PTX in PEG, dispersed in an amorphous PLGA matrix (Kang et al., 2007). Therefore, we suggest that FA preferentially partitions into the solvent-rich RIF phase during microsphere formation. At the solubility limit of RIF in PLGA, both drugs phase separate as a single amorphous phase with a distribution dependent on the relative contribution of FA.
Figure 3.9 Schematic illustration of the phase separation of FA, RIF, and a combination of FA-RIF from PLGA in microspheres. Note: Images are to illustrate the morphologies involved and are not representative of the obvious volume changes, especially in the glassy state, where microspheres would be much smaller than illustrated. The pink shading represents the first signs of drug phase separation. As DCM diffuses out of the dispersed phase, (A) FA phase separates at a relative droplet volume of 0.311 ± 0.014. The early separation in the solution state at a relatively low solution viscosity allows for coalescence of FA-rich microdroplets within the microsphere interior. Convective currents drive the microdroplets to the microsphere interface prior to final polymer gelation. The resulting microsphere is characterized by FA-rich microdomains localized to the surface of the microsphere. (B) In FA-RIF co-loaded microspheres, the phase separation of both FA and RIF occurs at a relative droplet volume of 0.251 ± 0.002 due to the increased compatibility between RIF and PLGA. At this point, the solution still has a sufficiently low viscosity to allow for coalescence of microdroplets; the relative contribution of FA allows for surface exclusion of some drug-rich microdomains. The resulting morphology is characterized by larger, but fewer, surface domains. (C) RIF phase separates from PLGA at a relative droplet volume of 0.194 ± 0.000 due to increased RIF/PLGA compatibility. As this occurs closer to the VB, the solution viscosity is relatively large and there is limited coalescence of individual phase separated RIF droplets. RIF remains largely localized to the bulk of the microsphere. Surface exposed drug solubilizes in the continuous phase during gelation (higher RIF aqueous solubility) and the resulting morphology is characterized by small surface dimples.
In vitro release of FA and RIF from mono- and co-loaded microspheres was almost completely dependent on the surface phase separation of drug-rich spherical domains (Figure 3.8). Where release was achieved, it was characterized exclusively by a large initial burst phase of release, with minimal release thereafter. The burst release was found to be due to the dissolution of the surface microdomains, as seen in Figure 3.1 (J-R), where the microdomains are absent following 7d release in PBS. A similar effect has been observed with the encapsulation of Vitamin E in cellulose acetate films, where the release of Vitamin E was characterized exclusively by a burst release, and dissolution of phase-separated surface domains (Taepaiboon et al., 2007). These data indicate that the release of drug over 7d is limited to microspheres where there are surface microdomains, and that release is almost entirely attributed to the dissolution of these spherical drug-rich reservoirs. In addition, for microspheres with higher loadings of RIF, there was little surface deposition of drugs, resulting in minimal burst release of both FA and RIF. This inverse relationship suggested that the co-loading of RIF in the FA microspheres resulted in the drug remaining trapped in the interior so a limited drug exclusion to the microsphere surface and hence, a reduced burst drug release.

3.5 Conclusion

In a polymeric drug delivery system, the compatibility of the drug (or mixture, in this case) with the encapsulating polymer will determine the drug deposition in the resulting formulation. Drugs may exist as molecular dispersions in the polymer interstitial space, or phase separated into microdomains, which occupy large voids in the polymer matrix. Detailed surface analysis of FA, RIF, and FA-RIF co-loaded PLGA microspheres by confocal microscopy, Raman spectroscopy, real-time video imaging of microsphere formation, and thermal analysis by DSC confirm the phase exclusion of both FA and RIF from PLGA as a separate amorphous
phase, manifesting as surface protrusions or surface dimples respectively (due to rapid dissolution of RIF in the forming aqueous phase). This final morphology had ultimate influence on the \textit{in vitro} release of the drugs. Our 7d \textit{in vitro} release experiments indicated that the release of FA or RIF from PLGA was only achieved through a burst release, characterized by the dissolution of the surface-excluded microdomains, and was dependent on the drug(s) being excluded from the bulk of the microsphere during hardening. The release of FA and RIF was inversely related to the RIF loading in the microsphere. This was due to the observation that RIF limited the surface disposition of FA due to an increased compatibility in the PLGA matrix (i.e. a delayed phase separation). These results showed that the extent of FA or RIF burst from PLGA microspheres was controlled to a certain extent by variations in FA:RIF loadings, which govern the phase separation kinetics. These findings highlight the importance, and limitations, of polymer-drug miscibility and compatibility on the physical properties of drug-loaded polymeric microspheres.
Chapter 4: Novel Fusidic acid and Rifampicin Co-Loaded PLGA Nanofibers For the Treatment of Orthopaedic Implant Associated Infections

4.1 Introduction

Arthroplastic procedures are the surgical reconstructions or replacement procedures of malformed or degenerated joints. These surgeries are commonly performed to restore form and function, and alleviate pain in the knee, hip, shoulder, elbow, wrist, ankle, temporomandibular, metacarpophalangeal, and interphalangeal joints to improve quality of life (del Pozo and Patel, 2009). In spite of the rapid progression in orthopaedic technologies and surgical techniques, implant-associated infection rates following invasive orthopaedic surgery remain high and difficult to treat. This can be attributed to the fact that the number of microorganisms required to sustain an infection drops precipitously (~100,000-fold) when a foreign material is present (Elek and Conen, 1957), and consequently, infections due to foreign body implants are one of the primary reasons for joint failure following total joint arthroplasty (Rand, 1993; Segawa et al., 1999). The rates of infection for joint replacements of the knee, hip, shoulder, and elbow, range between 0.5%-2% (Peersman et al., 2001), 1%-2% (Phillips et al., 2006), 1%-2.5% (Sperling et al., 2001), and 7%-9% (Morrey and Bryan, 1983; Gill and Morrey, 1998), respectively, while those associated with secondary joint revision surgeries ranges between 5%-40% (Widmer, 2001).

The current method for the management of implant-associated infections is the use of perioperative systemic antibiotics, the distribution of which is limited by the physiological nature of the wound. The reduction in tissue transport, perfusion, and homeostasis, render the surgical site nearly inaccessible to drugs circulating within the central compartment (Mader et al., 1993; ________

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3 The data presented in this chapter have been submitted for publication. Gilchrist S.E. et al. (2012). Novel Fusidic acid and Rifampicin Co-Loaded PLGA Nanofibers For the Treatment of Orthopaedic Implant Associated Infections.
Giulieri et al., 2004). As a result the antibiotic concentration reaching the desired site may not be high enough to eradicate the infection, but instead, may result in the development of antibiotic-resistant microorganisms (Hanssen and Spangehl, 2004). Furthermore, the elevated systemic levels can lead to serious non-target toxicities (Ambrose et al., 2004). In addition to this, most patients have to undergo invasive procedures including joint debridement to sufficiently clean the implant, or undergo single and multiple exchange procedures to introduce a new sterile implant. As a result, the treatment of implant-associated infections is linked to increased patient morbidity and mortality, and along with the co-morbidity of high systemic levels of antimicrobials, underscores the need for an alternative treatment strategy to eradicate implant-associated pathogens.

A promising alternative approach to systemic antibiotic administration is a localized, controlled release drug delivery strategy, where the drug is targeted to the surgical site and released at high concentrations over a prolonged period of time. A common surgical practice aimed at increasing local antimicrobial concentrations is the mixing of a known amount of sterile antibiotic solid with poly(methylmethacrylate) (PMMA) bone cement as it polymerizes in situ. The antibiotic loaded bone cement (ALBC) is used as a structural solid to anchor implants, used as a spacer device in two-stage revisions, or formed into small diameter beads and placed in the surgical pocket (Kanellakopoulou and Giamarellos-Bourboulis, 2000). Despite conventional use, there is limited evidence for the efficacy of ALBCs in preventing, or treating established orthopaedic implant-associated infections. Furthermore, there are a number of additional disadvantages associated with the PMMA carriers, including: 1) PMMA is not biodegradable and must be surgically removed from the implantation site, 2) the elution of antibiotics from ALBCs occurs primarily from the surface and large quantities of the antibiotics (>90%) are retained within the matrix leading to a very poor antibiotic elution profile (Levin, 1975; Baker
and Greenham, 1988; Mader et al., 1997; van de Belt et al., 2000), 3) in spite of large antibiotic loads, ALBCs have been shown to encourage the growth and adherence of virulent pathogens (Kendall et al., 1996), 4) the polymerization of PMMA carriers is exothermic, and has been associated with thermal necrosis and premature joint loosening (Berman et al., 1984).

Given the need to preserve the mechanical strength of orthopaedic biomaterials, including bone cement, our group has proposed that ultra-thin, flexible antibiotic-loaded films that conform readily to the surface of implants or cement spacers/beads, may provide a novel and adjunctive means of controlled delivery of antibiotics to orthopaedic surgical sites without any compromise in implant/biomaterial structural integrity. In this study, we have evaluated the use of the biodegradable polymer poly(D,L-lactic acid-co-glycolic acid) (PLGA) as a suitable matrix for the delivery of the antibiotics, fusidic acid (FA), its sodium salt (SF), and rifampicin (RIF), and fabricated as either solvent-cast films or nanofibrous membranes.

The fusidanes (FA and SF) are active exclusively against Gram-positive organisms, in particular, *Staphylococcus aureus*, *S. epidermidis*, and coagulase-negative Staphylococci including strains that are methicillin-resistant (MRSA) (Coombs and Menday, 1985; Coombs, 1990). RIF is also active against *S. aureus*, *S. epidermidis*, but also shows activity against streptococcal microorganisms (including *Streptococcus pneumonia*) (McCabe and Lorian, 1968; Kunin et al., 1969). The broad spectrum activity of both FA and RIF offers protection against ≥ 70% of organisms that commonly cause prosthetic joint infections (Campoccia et al., 2006).

A serious confounding problem in implanted device infections is the formation of a bacterial biofilm on the device surface. The most common pathogens, *S. aureus* and *S. epidermidis*, produce a biofilm that is 100-1000 fold less susceptible to antibiotics than planktonic bacteria (Saginur et al., 2006). In a study of antimicrobial susceptibility of pathogenic strains of staphylococci, RIF, vancomycin, and FA were the antibiotics most commonly included
in combinations that are active against a staphylococcal biofilm, with RIF being the most active (Saginur et al., 2006). Only combinations containing RIF and FA, plus either ciprofloxacin or vancomycin were consistently bactericidal against MRSA biofilms.

Electrospinning is a process that produces ultrafine fibers with diameters in the nanometer size range and possessing very high surface area-to-volume ratios (Reneker and Chun, 1996). In electrospinning, dissolved polymer/drug solutions are pumped out of a syringe in the presence of a very high electrical field potential. This positive potential deforms the fluid meniscus at the tip of a capillary, and forces the ejection of an axial, electrified jet, which accelerates within the radiant electrical field towards a grounded collector (Reneker et al., 2000). The resulting drug-loaded non-woven membranes possess several unique properties, including flexibility, high porosity, varying morphologies, good cell adhesion, and controlled drug release, and have been explored as tissue-engineering scaffolds for vasculature (Boland et al., 2004), bone (Yoshimoto et al., 2003), neural (Yang et al., 2005), and tendon tissues (Sahoo et al., 2006), and delivery systems for a wide range of drugs including antibiotics (Zeng et al., 2003; Katti et al., 2004; Said et al., 2011; Torres-Giner et al., 2011; Said et al., 2012), and antineoplastics (Zeng et al., 2005; Xie and Wang, 2006; Xu et al., 2008). Recently, Said et al. prepared FA-loaded PLGA nanofibers as a potential wound dressing material and showed that the nanofibrous membranes retained bacteria on the surface and inhibited the proliferation of three test organisms (Said et al., 2011).

The objectives of this work were to develop and characterize ultrathin, flexible PLGA films and nanofibrous membranes suitable for the co-delivery of FA/SF and RIF, to determine the in vitro activity against a number of bacterial strains commonly associated with skin or orthopaedic infections, and validate lead formulations in a rat model of implant-associated infection.
4.2 Materials and methods

4.2.1 Chemicals

Fusidic acid (FA) and rifampicin (RIF) were purchased from Sigma Aldrich (Oakville, ON, CA). Sodium fusidate (SF) was purchased from Molekula (Shaftesbury, UK). Poly(D,L-lactic acid-co-glycolic acid) (PLGA) with a lactic acid:glycolic acid composition of 50:50 and molecular weights ($M_n$) ranging from 5,200-72,800 Da was purchased from Lactel® Absorbable Polymers (Pelham, AL, USA). The solvents tetrahydrofuran (THF), dimethylformamide (DMF), acetonitrile (ACN), methanol (MeOH), phosphoric acid, were of HPLC grade and purchased from Sigma Aldrich (Oakville, ON, CA). BBL™ Mueller-Hinton bacterial broth (MH) was purchased from BD Biosciences (Mississauga, ON, CA), and MH plates were prepared by the addition of 1.5% agar (BD Biosciences).

4.2.2 Preparation of antibiotic-loaded PLGA solvent-cast films

Films containing FA/SF and RIF were prepared by dissolving 10% (w/w) FA or SF plus 2, 3, or 5% RIF (w/w), and PLGA (50:50) in THF and solvent casting at a concentration of 10% (w/v) on 1 cm² Teflon® templates applied to glass slides. Films were retained for 24h in a fume hood for solvent evaporation, vacuum dried at 625 mm Hg at room temperature for an additional 24h using a bench top vacuum oven (Napco, No. 5831), and subsequently stored in a desiccator for further analysis.

4.2.3 Fabrication of non-woven PLGA nanofibers by electrospinning

PLGA with molecular weights ($M_n$) ranging from 5,200-72,800 Da was dissolved in THF:DMF (75:25) at concentrations ranging from 0.1 g/mL to 0.35 g/mL. The resulting solutions were electrospun under fixed conditions (syringe flow rate = 1 mL/h; needle gauge =
18G blunt end; needle-to-collector distance = 10 cm; applied voltage = 1.5 kV/cm) to determine the most appropriate solution parameters to achieve continuous, defect free nanofibers.

4.2.3.1 Preparation of antibiotic-loaded PLGA nanofiber membranes

Non-woven nanofiber mats containing FA/SF and RIF were prepared by electrospinning. Briefly, PLGA (50:50) was co-dissolved with FA/SF [10% (w/w)] and RIF [2, 3, 5% (w/w)] in THF:DMF (75:25) to achieve a final concentration for total solids of 0.25 g/mL. This solution was pumped out of a 10 mL glass syringe housing an 18G blunt-end needle at 1 mL/hr using a KDS 100 syringe pump (KD Scientific; Holliston, MA) in the presence of a 1.5 kV/cm electrical field differential and a syringe-to-collector distance of 10 cm. The resulting charged jet was collected on a grounded rotating drum, dried under 625 mmHg vacuum for 24h to remove residual solvent, and stored under desiccant until analysis.

4.2.4 In vitro characterization of antibiotic-loaded PLGA nanofibers

4.2.4.1 Scanning electron microscopy

The morphologies of the FA/SF, RIF, and co-loaded PLGA nanofibers were characterized using scanning electron microscopy (SEM). Samples were spun directly onto a SEM stub covered with a conductive carbon tab, and subsequently sputter-coated with a layer of 60:40 alloy of gold:palladium using a Denton Vacuum Desk II sputter-coater (MooRESTOWN, NJ) at 50 mTorr. SEM images were captured using a Hitachi S-3000N system (Tokyo, Japan) scanning at 5–20 keV.
4.2.4.2 X-ray diffraction

X-ray diffraction (XRD) patterns of the FA/SF, and RIF co-loaded PLGA nanofibers were obtained with a Bruker D8 Advance (Madison, WI, USA) diffractometer in Bragg-Brentano configuration with a Cu source at 25°C. Samples were scanned from 2-50° 2θ using a step size of 0.02° and a step time of 1 sec/step. Approximately 2.5 cm² sections of each nanofiber mat were cut and adhered to a small glass slide and mounted inside a standard Bruker sample holder. Samples were rotated during data acquisition to avoid preferential orientation. Data was collected and analyzed with Bruker Diffrac Plus XRD Commander version 2.3 software.

4.2.4.3 Differential scanning calorimetry

Differential scanning calorimetry (DSC) analysis of the FA/SF, RIF, and co-loaded PLGA nanofiber formulations was performed on a TA Instruments DSC Q100 (New Castle, DE, USA) with liquid nitrogen cooling system. The heat flow and heat capacity of the instrument was calibrated routinely using a high purity indium standard. Briefly, nanofiber sections (~4–6 mg) were hermetically sealed in aluminum pans with a pinhole, and heated from 25°C to 300°C at a rate of 10°C/min under nitrogen flow. The initial heat scan was followed by a rapid quench cooling scan from 300°C to −50°C at a rate of 35°C/min and a second heating scan from −50°C to 250°C at a rate of 10°C/min. All DSC thermograms were analyzed using TA Instruments Universal Analysis (v. 4.7A).

4.2.4.4 Drug encapsulation efficiency

To determine the encapsulation efficiency of the loaded antibiotics, ~2.5 mg sections of antibiotic-loaded nanofiber were dissolved in 1 mL ACN in a screw-top test tube with a
Teflon®-coated cap. Phosphate buffered saline (PBS; pH 7.4) (5 mL) was added and vortexed for 60s to precipitate the polymer. The solution was centrifuged at 18,000 × g (Microfuge® 18; Beckman Coulter, Mississauga, ON, CA) for 5 min to pellet any precipitate, and the supernatant was sampled for FA and RIF content.

FA/SF content was quantified using a HPLC (Waters® Millennium System) assay utilizing a mobile phase of 50/30/20 (v/v/v) ACN/MeOH/0.01M phosphoric acid solution, flowing at 1 mL/min through a C18 reverse phase Nova-Pak® column (Waters®), with a 20 µL sample injection volume and UV/vis detection at 235nm. FA was quantified against a standard curve prepared by dissolving FA in ACN over a range of 0.1 to 500 µg/mL.

RIF content was evaluated using a UV-spectrophotometer (Varian Cary 50-BIO) assay with a medium scan speed and detection at 475nm. RIF was quantified against a standard curve prepared by dissolving RIF in DMSO at 1 mg/mL and diluting using PBS to cover a range of 0.2 to 25 µg/mL.

**4.2.4.5 In vitro drug release from antibiotic-loaded PLGA nanofibers**

The *in vitro* release of FA, SF, and RIF from antibiotic-loaded nanofibers, was carried out in PBS (pH 7.4) supplemented with 0.1 mg/mL ascorbic acid. Ascorbic acid was added to the PBS release media as an antioxidant to prevent the oxidative degradation of RIF (Weber *et al.*, 1983). Briefly, 2.5 cm² sections of nanofiber membrane were cut, weighed, and placed in 5 mL of PBS (pH 7.4; 0.1 mg/mL ascorbic acid) and incubated in a thermostatically controlled oven at 37°C under gentle rotation. At pre-determined time intervals, 1 mL samples were withdrawn for HPLC or UV analysis, and the remaining media was removed and replaced with fresh PBS (pH 7.4; 0.1 mg/mL ascorbic acid) to maintain sink conditions. Concentrations of FA, SF, and RIF in the release media were quantified using the HPLC or UV methods described above.
4.2.5 Antimicrobial efficacy

4.2.5.1 Microbial strains and culture media

Gram-positive organisms used in this study were *Staphylococcus aureus* (ATCC 25923), *S. epidermidis* (ATCC 14990), Methicillin-resistant *Staphylococcus aureus* (MRSA; ATCC 700698), and a clinical variant of MRSA (Newman strain), which was kindly provided by Dr. Julian Davies (Department of Microbiology and Immunology at The University of British Columbia, Vancouver BC, CA). As a representative Gram-negative bacterium, the strain *Acinetobacter baumannii* (ATCC BAA-747) was evaluated.

4.2.5.2 Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of FA, RIF, and FA-RIF (2:1) was determined using the micro-broth dilution technique as recommended by the Clinical and Laboratory Standards Institute (2009). Briefly, serial dilutions of antibiotics were prepared in Mueller-Hinton (MH) broth in 96-well microtitre plates. A bacterial suspension was then added to each well to achieve a final inoculum of ~10^8 bacteria/mL. Wells containing growth media alone or bacterial culture without antibiotic were used as negative and positive controls, respectively. The 96-well microtitre plates were inspected for bacterial growth after incubation at 37°C for 24h, and the MIC was taken as the lowest concentration of antibiotic where no growth was observed (i.e. no turbidity), in 3 consecutive experiments.

4.2.5.3 Bacterial survival curves

Cultures of each bacterial strain were grown at 37°C overnight in sterile MH broth. The inoculum was then adjusted to 0.5 McFarland Standard with sterile MH broth [equivalent to 10^7-10^8 colony forming units (CFU)/mL] (McFarland, 1907). Each stock was further diluted to a
final inoculum of $\sim 10^6$ CFU/mL and 1 mL of bacterial suspension was incubated with 0.5 cm diameter antibiotic-loaded nanofiber membrane sections in 24-well tissue culture plates. Controls consisted of bacterial cultures alone (no treatment) and control PLGA nanofiber membranes (no antibiotic). The plates were incubated at 37°C under gentle rocking in a thermostatically controlled incubator for 48h. At pre-determined time points (0, 24, 48h), 10 µL was withdrawn from each well, serial diluted with sterile MH broth, and cultured on sterile MH growth plates for 24 h at 37°C. Survival of bacteria was calculated by CFU counting.

4.2.5.4 *In vivo* evaluation of antimicrobial nanofibers

To test the ability of the antibiotic-loaded nanofibers to prevent the bacterial colonization of a titanium implant *in vivo*, the co-loaded nanofibers were implanted alongside a titanium disk into pockets made in the dorsum of rats, and challenged with $\sim 10^8$ CFU MRSA (Newman). The study protocol was approved by the University Animal Ethics Committee and carried out under the Canadian Council on Animal Care (CCAC) guidelines.

Female Sprague-Dawley rats ($n=13$) weighing between 195-225g were purchased from Charles River Canada (St. Constant, Quebec, Canada). Animals were maintained in a temperature and humidity controlled facility (22°C ± 2°C) on a 12-hour light:dark cycle (0700h – 1900h), fed standard rat chow *ad libitum* and had free access to water throughout the study period. Following a 7d acclimation period, each animal was deeply anesthetized using isofluorane. While in the prone position, the hair of the dorsum was shaved, and two small subcutaneous pockets were created in the upper left and right quadrants using blunt-end surgical scissors. The left pocket received a sterile titanium disk (0.5 cm $\times$ 0.1 mm) alongside a 0.5 cm diameter antibiotic-loaded nanofiber section. The right pocket received a sterile titanium disk (0.5 cm $\times$ 0.1 mm) alongside a 0.5 cm diameter blank PLGA nanofiber section (no drug).
Following implantation, ~10^8 CFU MRSA (Newman) were injected onto the surface of the titanium implant. The wound was subsequently closed using non-absorbable 5/0 vicryl sutures. Control animals received subcutaneous titanium implants with/without a bacterial challenge.

After 7d, the animals were sacrificed using an overdose of isoflurane with subsequent asphyxiation using CO_2. The wounds were opened to remove the implants, which were manually cleared of host tissue and washed in sterile PBS to remove non-adherent bacteria. To remove adherent bacteria, the implants were placed in 500µL fresh PBS and sonicated for 10min using a sonicating water bath (Aquasonic Model 50T). The solutions were serially diluted and the number of bacteria was calculated by CFU counting. The fibrous and inflammatory tissue surrounding each implant was excised and preserved in sterile 10% buffered formalin for immunohistochemistry and histopathological analysis

4.2.5.5 Immunohistochemistry and histopathological analysis

Representative sections of the excised inflammatory tissues were embedded in paraffin, and cut to a thickness of 5 µm using a tissue microtome. Each section was stained with hematoxylin and eosin using standard techniques, and for CD3+ T-Cell invasion using immunostaining (Abcam; Cambridge, MA, USA) on a Discovery XT platform (Ventana Medical System, Tuscan, Arizona) with enzyme labeled biotin streptavidin system and solvent resistant DAB Map kit.

Each slide was reviewed by a blinded pathologist (L.F.) using a Leica Microsystem digital microscope (DVM 2000; Leica Microsystems Inc, Concord, ON, CA) equipped with Digital Image Hub software (Leica Microsystems Inc, Concord, ON, CA). Samples were examined at 200× and 400× magnification and scored using a semi quantitative scoring system for inflammation. Each sample was scored for inflammatory cell infiltrate (1 – mild, 2 –
moderate, 3 – severe), necrosis (0 – none, 1 – mild, 2 – moderate, 3 – severe), and stage of healing process as determined by the presence of neovascularization and fibrosis (0 – none, 1 – late stage, 2 – intermediate stage, 3 – early stage). The total inflammatory reaction factor for each section was calculated as the sum of the numerical evaluation of the semi quantitative scores listed above, and the percentage of cells in the infiltrate that were positive for CD3.

4.2.6 Statistical analysis

Statistical analyses were carried out using one-way ANOVA and a Bonferroni multiple comparison post-hoc test with significance level set a $p < 0.05$. All analysis was done in GraphPad Prism v. 5 for Mac OS X (GraphPad Software, San Diego CA, USA).

4.3 Results

4.3.1 Antibiotic-loaded PLGA solvent-cast films

FA/SF, and RIF were co-loaded into solvent-cast PLGA films using THF. THF was chosen as a common solvent due to its ability to solubilize all three drugs used in this study. It was observed that, regardless of the drug combination, a drug-rich ultra-viscous liquid formed beneath the drying film (Figure 4.1). We further observed that this drug-rich phase remained as an ultra-viscous liquid, even after removal of the dried film layer.
4.3.2 Optimization of PLGA molecular weight and concentration for nanofiber fabrication

The effects of varying PLGA molecular weight and polymer concentration on the formation of PLGA fiber and particulate morphologies produced by electrospinning/spraying are shown in Figure 4.2, and termed a PLGA phase morphology map. Data points represent given PLGA morphologies determined by SEM. Lines have been drawn connecting data points possessing structures of similar morphology. The lowest molecular weight PLGA of 5,200 Da did not produce defined morphologies over the concentration range evaluated, and is described as “irregular polymer deposition”. Increasing the molecular weight to 17,100 Da, produced discrete PLGA nanobeads over a concentration range of 0.1-0.2 g/mL. Increasing solution concentration to 0.3 g/mL resulted in the formation of beaded nanofibers, and continuous nanofibers were formed at solution concentrations exceeding 0.3 g/mL. At a PLGA molecular weight of 30,100 Da, nanobeads were produced at 0.1 g/mL, beaded nanofibers formed at 0.15-
0.25 g/mL, and continuous nanofibers at any concentration exceeding 0.25 g/mL. A molecular weight of 49,100 Da, produced similar results with nanobeads being produced with solution concentrations of 0.1 g/mL, beaded nanofibers being produced with solution concentrations of 0.15 g/mL, and continuous nanofibers being formed between 0.15-0.3 g/mL. Beyond 49,100 Da, no discrete nanobeads were observed. PLGA with a molecular weight of 72,800 Da produced beaded nanofibers at low solution concentrations (0.1 g/mL), and produced continuous nanofibers between 0.1g/mL and 0.3g/mL. For PLGA with molecular weights of 49,100 Da, and 72,800 Da, the upper limit of nanofiber formation was observed at 0.35 g/mL and 0.3 g/mL, respectively, as the viscosity of the spinning solution did not allow for the production of nanofibers under the electrospinning conditions employed.

Based on the morphologies observed (irregular polymer deposition, beads, beaded-nanofiber, and continuous nanofibers), PLGA with a molecular weight of 49,100 Da and a solution concentration of 0.25 g/mL (highlighted by the red X in Figure 4.2) were selected as optimal solution parameters to produce continuous polymer nanofibers.
Figure 4.2 Electrospinning/electrospraying PLGA phase morphology map illustrating the influence of PLGA (50:50) molecular weight ($\bar{M}_n$) and solution concentration on the morphology of nanofibers formed via electrospinning. All samples were prepared using fixed electrospinning parameters (syringe flow rate = 1mL/hr; needle = 18G blunt end; needle-to-collector distance = 10cm; applied voltage = 1kV/cm).
4.3.3 Antibiotic-loaded PLGA nanofiber membranes

All antibiotic-loaded formulations produced continuous, defect-free fibers in the nanoscale range with narrow size distributions (Table 4.1). In addition, there was no visible evidence of drug phase separation from the polymer matrix (Figure 4.3).

Although the addition of antibiotics to the PLGA matrix had no influence over the surface morphology of the nanofibers as shown in Figure 4.3, the antibiotics did produce significant changes in the diameter of the resulting nanofibers. Both FA and RIF caused a significant decrease in nanofiber diameter from $993.9 \pm 178.7$nm (control PLGA) to $656.2 \pm 85.1$nm and $618.7 \pm 28.8$nm respectively, with no dependence on loading (FA data not shown). SF loading resulted in significantly smaller fibers with diameters of $285.8 \pm 30.6$nm. Nanofiber diameter was dependent on SF load with higher loadings resulting in a corresponding decrease in diameter (data not shown). The co-loading of FA/SF with RIF had no further influence on nanofiber diameter or morphology. All formulations showed high encapsulation efficiency (EE) of antibiotics (alone or in combination) ranging from ~75%-100%. A summary of the physical characteristics of FA, SF, and RIF-loaded PLGA nanofibers is given in Table 4.1.
Figure 4.3 Representative scanning electron micrographs of electrospun PLGA (50:50) nanofibers containing (A) no drug, (B) 10% (w/w) FA, (C) 10% (w/w) SF, (D) 5% (w/w) RIF, (E) 10% (w/w) FA; 5% (w/w) RIF, (F) 10% (w/w) SF; 5% (w/w) RIF. All formulations produced continuous, defect-free nanofibers, with no visible evidence of phase separation. The scale bar represents 10 µm.
Table 4.1 Summary of the thermal and physical characteristics of FA, SF, and RIF mono- and dual-loaded PLGA (50:50) nanofibers prepared by electrospinning.

<table>
<thead>
<tr>
<th>Nanofiber Formulation</th>
<th>Thermal Characteristics</th>
<th>Physical Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$T_r$ ($^\circ$C)$^a$</td>
<td>$\Delta H_r$ (J/g)$^b$</td>
</tr>
<tr>
<td>Control</td>
<td>53.7 ± 0.27</td>
<td>6.1 ± 1.18</td>
</tr>
<tr>
<td>10% FA</td>
<td>53.4 ± 1.03</td>
<td>2.7 ± 0.06</td>
</tr>
<tr>
<td>10% SF</td>
<td>47.3 ± 0.51</td>
<td>4.2 ± 0.89</td>
</tr>
<tr>
<td>2% RIF</td>
<td>54.8 ± 0.18</td>
<td>6.1 ± 0.65</td>
</tr>
<tr>
<td>3% RIF</td>
<td>55.1 ± 0.23</td>
<td>5.5 ± 0.12</td>
</tr>
<tr>
<td>5% RIF</td>
<td>55.6 ± 0.03</td>
<td>6.0 ± 0.20</td>
</tr>
<tr>
<td>10% FA; 2% RIF</td>
<td>54.9 ± 0.48</td>
<td>6.5 ± 0.89</td>
</tr>
<tr>
<td>10% FA; 3% RIF</td>
<td>54.5 ± 0.76</td>
<td>5.4 ± 0.23</td>
</tr>
<tr>
<td>10% FA; 5% RIF</td>
<td>53.9 ± 0.83</td>
<td>4.4 ± 0.41</td>
</tr>
<tr>
<td>10% SF; 2% RIF</td>
<td>55.5 ± 0.29</td>
<td>7.4 ± 0.57</td>
</tr>
<tr>
<td>10% SF; 3% RIF</td>
<td>56.0 ± 0.35</td>
<td>7.8 ± 0.80</td>
</tr>
<tr>
<td>10% SF; 5% RIF</td>
<td>55.8 ± 0.60</td>
<td>6.7 ± 0.92</td>
</tr>
</tbody>
</table>

$^a$ $T_r$ - Relaxation endotherm. The peak temperature of the first endothermic transition in the first heating cycle.

$^b$ $\Delta H_r$ = Enthalpy of relaxation. Integration of the first endothermic transition in the first heating cycle.

$^c$ $T_g$ = Glass transition temperature. The midpoint of the heat capacity change in the second heating cycle.

$^d$ $\text{EE}$ = Encapsulation efficiency. Recovered drug expressed as a percentage of theoretical drug loading.

Results are expressed as the mean ± S.D. $n = 3$. 
4.3.4 X-ray diffraction patterns of antibiotic-loaded PLGA nanofiber membranes

Representative XRPD patterns of the drug-loaded nanofibers are shown in Figure 4.4. There was no evidence of crystallinity in any of the nanofiber formulations, as illustrated by a characteristic amorphous halo in the XRPD pattern.

![XRD patterns](image)

**Figure 4.4** Representative XRD patterns of (A) control PLGA (50:50) nanofibers, (B) 10% (w/w) FA-loaded PLGA (50:50) nanofibers, (C) 10% (w/w) SF-loaded PLGA (50:50) nanofibers. There were no diffraction peaks present in any mono- or co-loaded formulations.
4.3.5 Thermal characterization of antibiotic-loaded PLGA nanofiber membranes

Thermal events observed from the DSC scans of FA, SF, and RIF mono- and dual-loaded PLGA nanofibers are summarized in Table 4.1. All formulations displayed a relaxation endotherm ($T_r$) at approximately 55°C, which was associated with the $T_g$ of PLGA, and the second heating cycle after quench cooling, was also characterized by a single $T_g$ at ~45°C, characteristic of PLGA (Table 4.1). There was no evidence of any transitions due to the presence of the antibiotics, and no influence of the drugs on any thermal transitions associated with the polymer.

4.3.6 In vitro drug release from mono- and co-loaded PLGA nanofibers

The in vitro release profiles of FA, SF, and RIF are given in Figure 4.5. All formulations were characterized by a biphasic release pattern with an extensive burst phase of release over 1-2d, followed by a slow, controlled release over 35d. The burst release accounted for ~20%, ~50%, and ~20% of total FA, SF, and RIF loading, respectively, in the mono-loaded formulations (Figure 4.5 C, D, and E, respectively). Co-loading of RIF and FA/SF slowed the release of SF, and increased the release of FA over 35d (Figure 4.5 A and B). The release of RIF during the initial burst phase of release was influenced by the presence of SF [10% (w/w)], which doubled the amount of RIF released over 2d (Figure 4.5 A and B).
Figure 4.5 *In vitro* release profiles of FA, SF, and RIF from mono- and dual-loaded PLGA (50:50) nanofibers performed in PBS (pH 7.4; containing 0.1 mg/mL ascorbic acid) at 37°C. (A) FA release from 10% (w/w) loaded PLGA nanofibers containing 0% (w/w) RIF (▼), 2% (w/w) RIF (■), 3% (w/w) RIF (◆), and 5% (w/w) RIF (●). (B) SF release from 10% (w/w) loaded PLGA nanofibers containing 0% (w/w) RIF (▼), 2% (w/w) RIF (■), 3% (w/w) RIF (◆), and 5% (w/w) RIF (●). (C) RIF release from 10% (w/w) FA-loaded PLGA nanofibers containing 2% (w/w) RIF (▼), 3% (w/w) RIF (■), and 5% (w/w) RIF (◆). (D) RIF release from 10% (w/w) SF-loaded PLGA nanofibers containing 2% (w/w) RIF (▼), 3% (w/w) RIF (■), and 5% (w/w) RIF (◆).
(◆). (E) RIF release from PLGA nanofibers containing 2% (w/w) RIF (▼), 3% (w/w) RIF (■), and 5% (w/w) RIF (◆).

4.3.7 In vitro antimicrobial efficacy of antibiotic-loaded PLGA nanofibers

The antimicrobial activity of FA, SF, and RIF mono- and co-loaded PLGA nanofibers was evaluated against five bacterial strains. Bactericidal activity was recorded after 48h when *S. epidermidis*, MRSA, and MRSA (Newman) were exposed to the co-loaded nanofiber formulations (Figure 4.6B, C, and D). However, none of the mono-loaded formulations were able to eradicate the bacterial strains, displaying only a bacteriostatic effect. In contrast, none of the mono- or co-loaded formulations show bactericidal activity against *S. aureus*, and only a bacteriostatic effect was observed during 48h (Figure 4.6A). Interestingly, *A. baumannii* was resistant to the mono-loaded FA, and SF formulations, however the co-loaded, and RIF-loaded formulations show a moderate bacteriostatic effect against *A. baumannii* in the first 24h; which subsequently recovered to control levels at 48h (Figure 4.6E).
Figure 4.6 Direct *in vitro* antimicrobial activity of FA, SF, and RIF mono- and co-loaded PLGA (50:50) nanofibers against (A) *S. aureus* (ATCC 25923), (B) *S. epidermidis* (ATCC 14990), (C) MRSA (ATCC 700698), (D) MRSA (Newman strain), (E) *A. baumannii* (ATCC BAA-747). Curve legend: Bacteria control (●); control PLGA nanofibers (no antibiotic; ■); 10% (w/w) FA loaded PLGA nanofibers containing 2% (w/w) RIF (▲), 3% (w/w) RIF (▼) 5% (w/w) RIF (♦); 10% (w/w) SF loaded PLGA nanofibers containing 2% (w/w) RIF (○), 3% (w/w) RIF (□), 5% (w/w) RIF (△); and mono-loaded formulations containing 10% (w/w) FA (▽); 10% (w/w) SF (◇); and 5% (w/w) RIF (★). * Represents mono-loaded formulations.
The MIC of FA, RIF, and FA-RIF (2:1) was also evaluated in susceptible bacterial strains (S. aureus, S. epidermidis, and MRSA), and is shown in Table 4.2. The MIC of FA was 0.156, 0.3125, 0.078, and 0.156 µg/mL and for RIF, 0.00078, 0.0031, 0.00078, and 0.00039 µg/mL against S. aureus, S. epidermidis, MRSA, and the clinical isolate of MRSA (Newman), respectively. When combined as a 2:1 ratio of FA:RIF, the MIC was lower than FA, but slightly higher than RIF alone, suggesting indifference of the combination against the pathogens investigated.

Table 4.2 Susceptibility of S. aureus, S. epidermidis, and MRSA to FA, RIF, and FA-RIF (2:1) as measured by the minimum inhibitory concentration (MIC).

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Minimum Inhibitory Concentration (µg/mL) a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FA</td>
</tr>
<tr>
<td>S. aureus (ATCC 25923)</td>
<td>0.156</td>
</tr>
<tr>
<td>S. epidermidis (ATCC 14990)</td>
<td>0.3125</td>
</tr>
<tr>
<td>MRSA (ATCC 700698)</td>
<td>0.078</td>
</tr>
<tr>
<td>MRSA (Newman)</td>
<td>0.156</td>
</tr>
</tbody>
</table>

a The minimum inhibitory concentration reported was taken as the concentration in which bacterial growth was not observed (i.e. no turbidity). n = 3.

4.3.8 *In vivo* antimicrobial efficacy of antibiotic-loaded PLGA nanofibers

The ability of the antibiotic-loaded PLGA nanofibers to inhibit the colonization of bacteria on the surface of an implant was evaluated for 2 lead formulations [10% (w/w) FA/SF co-loaded with 5% (w/w) RIF] in a rodent model of subcutaneous implant infection. Since all co-
loaded formulations were characterized by smooth and continuous morphology (Figure 4.3), and displayed similar release profiles (Figure 4.5) and similar in vitro activity against all pathogens evaluated (Figure 4.6), we chose the formulations containing 10% (w/w) FA/SF co-loaded with 5% (w/w) RIF due to their higher drug loadings. The elution profile of drugs from the nanofibers implanted alongside a titanium discs in rat subcutaneous tissue, significantly decreased the number of MRSA attached to the titanium surface by 99.9% compared to untreated, and bacterial controls ($p < 0.05$; Figure 4.7).

![Bar graph](image)

**Figure 4.7** Direct in vivo antimicrobial activity of FA/SF-RIF dual-loaded PLGA (50:50) nanofibers against MRSA (Newman). Titanium disks were implanted in the dorsum of rats, alongside an antibiotic-loaded nanofiber formulation, and inoculated with $\sim 10^8$ CFU MRSA (Newman) for 7d. Bacteria and blank nanofibers (no drug) were used as controls. The number of bacteria adhering to the titanium disk are represented as Mean CFU ± S.D. *, $p < 0.05$ compared to MRSA bacteria control.
The tissue surrounding the titanium implant was removed and sectioned to evaluate immune response. Figure 4.8 shows a representative cross section of the inflammatory tissue surrounding the titanium implant, and shows healthy epidermis (A) and dermis (B), evidence of cellular infiltration by CD3+ T-cells (C), evidence of healing via neovascularization and fibrosis (D), evidence of necrosis (E), and the presence of nanofibrous material (F). Immunopathological analysis revealed a significantly heightened immune response in all group, with an attenuated inflammatory score in the SF-RIF treated group ($p < 0.05$; Figure 4.8).
Figure 4.8 Representative immunopathological image of the inflammatory capsule encasing a subcutaneous titanium implant inoculated with ~10^8 CFU MRSA (Newman) for 7d. Tissue sections were stained for infiltrating CD3+ T-cells, and evaluated for evidence of necrosis, and markers of healing. The inflammatory reaction score for each group is expressed as Mean ± S.D. *, p < 0.05 compared to titanium control.
4.4 Discussion

Given the incidence of implant associated infections and the relative ineffectiveness of conventionally used management methods, new and more efficacious infection control options are badly needed. We propose that FA and RIF loaded ultra thin nanofiber membranes are a promising method for localized, high concentration drug delivery at an implant surface. Our initial attempts to generate drug loaded films using conventional solvent evaporation methods resulted in the formation of a drug-rich ultra-viscous liquid beneath the drying polymer film and the formation of non-homogeneous films that were unusable as a drug delivery device. Previous studies in our lab have shown the incompatibility of FA with PLGA in solvent-cast films using dichloromethane (DCM), which was demonstrated by spherical drug-rich solid microdomains on the surface of the PLGA film (Yang et al., 2009). This phase separation was attributed to a very low solubility of FA in PLGA (<1%). Switching the solvent from DCM to THF (due to the universal solubility of FA, SF, and RIF) changed the phase separation behavior from the formation of solid amorphous domains to the formation of an ultra-viscous liquid. THF has been shown to act as a hydrogen bond acceptor with cholesterol and cholesterol methyl ether (Góralski et al., 1990; Góralski, 1992). Since there are structural similarities between FA and cholesterol, it is possible that FA and THF will produce similar hydrogen bond interactions. Hence, after the solubility limit of FA and RIF in PLGA is reached, FA and THF molecules may interact strongly resulting in the viscous FA/THF/RIF-rich liquid. To investigate whether PLGA was influencing the separation of the drug-rich phase, we attempted to crystallize FA and RIF from THF in the absence of polymer. We observed the formation of the same ultra-viscous liquid that was found beneath the drying PLGA films, and similarly, it did not solidify under vacuum (data not shown). Therefore, we suggest that this phase separation seen in drying PLGA films is due to two factors: very low miscibility of the drugs in PLGA, and a high affinity of both drugs.
for THF. Thus, the ultra-viscous phase is likely a FA/THF/RIF-rich phase, containing some PLGA.

Given our inability to use conventional solvent-cast film systems, we sought an alternative processing method that allowed the creation of dual drug-loaded PLGA matrices. Electrostatic spinning (electrospinning) has recently reemerged as a novel and functional technology for biomedical applications (Reneker and Chun, 1996), and has been shown to provide superior control over size, morphology, and also to deliver superior drug-loading efficiencies when compared to traditional self-assembling polymer technologies (i.e. micelles, nanospheres, microspheres) (Chakraborty et al., 2009). In order to determine the optimal conditions to produce continuous, defect-free nanofibers, we screened a range of PLGA molecular weights over varying polymer solution concentrations with fixed operating parameters (applied voltage, diameter of the spinneret, spinneret-to-collector distance, and flow rate). SEM analysis showed that both PLGA molecular weight and solution concentration were critical determinants in the formation of continuous nanofibers (Figure 4.2). We constructed what we have termed, an “electrospinning/electrospraying PLGA phase morphology map” to show the conditions of polymer molecular weight and solution concentration that determine the formation of a particular PLGA morphology. Low viscosity solutions arising from use of low molecular weight polymer, and/or low solution concentration did not produce any specific morphology, such as fibers or beads. The irregular polymer deposition and bead formation may be explained by a combination of weak charge repulsion within the hydrodynamic jet due to dilute polymer solutions and poor polymer chain interactions due to low PLGA concentration and molecular weight (Lin et al., 2004). The lack of charge repulsion leads to jet instability and jet breakup due to surface tension effects, favoring the creation of beads; a process referred to as electrospraying (Fong et al., 1999; Katti et al., 2004). Increasing the polymer concentration or molecular weight
results in greater polymer chain interactions and the bead morphology changes from spherical to spindle-like and ultimately to continuous nanofibers (illustrated in Figure 4.2). We selected PLGA (50:50) of molecular weight 49,100 Da, at a concentration of 0.25 g/mL, since this was within the range (0.15-0.3 g/mL) over which continuous nanofibers were formed.

The addition of antibiotics to the spinning solution produced continuous and defect-free nanofibers in the nanoscale range with narrow size distributions (Figure 4.3 and Table 4.1). There was no visible evidence of phase separation from the polymer matrix in any formulation, indicating that the electrospinning technique was able to prevent the phase separation behavior seen in the solvent-cast films. Similar effects of electrospinning have been reported. Taepaiboon et al. completely eliminated the surface phase separation of vitamin E that was observed in cellulose acetate solvent-cast films by forming electrospun nanofibers (Taepaiboon et al., 2007). The ability to overcome phase separation is likely due to the very rapid solvent evaporation rate in the electrospinning process. During electrospinning, the forming fiber is drawn from a millimeter-sized jet to a nanometer-sized fiber over the course of a few milliseconds. This large draw ratio results in a very high surface area and extremely rapid evaporation of solvents (Reneker et al., 2000). This increased evaporation rate may not allow sufficient time for the formation of distinct drug-rich phases and thus preventing their ultimate exclusion from the polymer matrix. Thus, a metastable dispersion of the drugs in the PLGA nanofiber matrix was likely produced.

The nanofiber diameters were significantly influenced by the presence of drug. The addition of FA or RIF (alone or in combination) decreased the fiber diameter relative to control PLGA (no antibiotics) with no dependence on drug loading (FA data not shown). This decrease in diameter is likely due to a lowering of PLGA concentration in the spin-dope and the resulting fibers approach the diameters observed with lower PLGA concentrations seen in Figure 4.2.
Conversely, SF was able to decrease the nanofiber diameter more than the FA-loaded nanofibers at all concentrations, and was shown to be loading-dependent and not influenced by the degree of RIF loading. This may be explained by the presence of sodium ions in the spin-dope, which serve to increase the net solution charge. The addition of organic salts has not only been shown to favor smooth fibers over bead and beaded-fiber morphologies, but also to produce fibers with smaller diameters (Fong et al., 1999). However, it was noted that the limit for creating nanofibers containing SF was a loading percentage of 10% (w/w). Any further increase in SF content caused jet breakup and the formation of beads due to the large charge repulsion on the jet surface. Thus all formulations containing the fusidanes were limited to a 10% (w/w) loading for all experiments.

Both the morphology of the nanofibers, and the physicochemical properties of the encapsulated drugs influenced the profile of drug release. The release of FA, SF, and RIF from mono-loaded nanofibers was characterized by a biphasic release pattern involving an extensive initial burst over 2d followed by slower and controlled release. The release of FA was considerably slower than for SF in mono-loaded nanofibers (Figure 4.5 A and B), likely primarily due to a size effect, with smaller diameter fibers (higher specific surface area) releasing drug more rapidly (Cui et al., 2006; Srikar et al., 2008). In addition, since SF has been shown to exhibit surface active properties, the SF may be preferentially located at the surface of the nanofiber (Carey and Small, 1971). A similar phenomenon has been shown through the encapsulation of the surface active agent Span 80 in electrospun nanofibers (Li et al., 2008). For the dual-loaded formulations, the release of FA and SF was different in the presence of RIF. The addition of RIF to FA nanofibers caused an increase in the release of FA. The presence of RIF may have allowed for better water penetration into the nanofiber matrix (due to its slightly higher aqueous solubility) assisting in release. On the other hand, SF release was slower in the presence
of RIF, and could not be explained on the basis of fiber diameter, or hydrophilicity. Comparing Figure 4.5B with 7D, it is observed that the release of SF does appear to mirror the release of RIF from the co-loaded fibers. This may be indicative of an interaction between SF and RIF in the PLGA nanofiber, changing the surface distribution of SF. The burst release of RIF was influenced only by the presence of SF, which is likely due to a combination of the hydrophilic nature of SF, and the significantly smaller nanofibers. Our group has shown the ability of water-soluble excipients to enhance the release of a sparingly soluble drug from a polymer matrix in vitro by increasing water penetration and the opening of pores in the matrix created by the release of the hydrophilic agent (Owen et al., 2010). In addition, the amphiphilic properties of SF may play a role in the release of RIF through a localized-micellization mechanism, by which amphiphiles are able to increase the release of a hydrophobic drug from polymer matrices (Jackson et al., 2004; Owen et al., 2010).

To determine the nature of the drug dispersion in the nanofibers, each mono- and dual-loaded formulation was analyzed by X-ray diffraction and thermal analysis. The lack of diffraction peaks in any formulation indicates that the drug is dispersed either as solid amorphous particles or in molecular form (solid solution). All antibiotic-loaded nanofiber formulations were characterized by a relaxation endotherm \( (T_r) \) at \( \sim 55^\circ\text{C} \) on the first heating cycle, and a glass transition \( (T_g) \) at \( \sim 45^\circ\text{C} \) on the second heating cycle after quench cooling. Drug loading appeared to have no effect on either the \( T_r \) or the \( T_g \) of PLGA in any formulation (Table 4.1), and is likely that FA/SF and/or RIF formed solid amorphous particles and not a solid solution. However, we were not able to observe any thermal events related to solid drug in the polymer, probably due to lack of sensitivity of the measurement (Verreck et al., 2003). Ageing studies using nanofiber samples [20\% \( (\text{w/w}) \) FA with 5\% \( (\text{w/w}) \) RIF] at \( 37^\circ\text{C} \) over 50 days in a temperature-controlled oven, close to the \( T_g \) of the polymer, did not produce any additional thermal transitions,
suggesting that the metastable amorphous dispersions possessed sufficient stability over that time frame.

To determine the antimicrobial activity of our nanofibers, we determined the minimal inhibitory concentration (MIC) of FA, RIF, and FA:RIF (2:1). The ratio of FA:RIF used in the combined therapy matched the highest relative weight percentages of the two drugs in the co-loaded formulation. MIC values obtained in this work for each strain are consistent with literature values of activity of FA (Collignon and Turnidge, 1999), and RIF (Segreti et al., 1989), against *S. aureus*, *S. epidermidis*, and MRSA. The combination of FA:RIF was found to be indifferent, and not synergistic (Collignon and Turnidge, 1999), or antagonistic (Druegeon et al., 1994), as some reports have suggested.

The antibiotic-loaded nanofibers were evaluated for their ability to release FA/SF and RIF at concentrations exceeding the MIC using time-dependent killing assays *in vitro* against *S. aureus*, *S. epidermidis*, MRSA, and MRSA (Newman), and a relevant nosocomial Gram Negative bacterium, *A. baumannii*. All mono-loaded formulations were bacteriostatic against *S. aureus*, *S. epidermidis*, and both strains of MRSA over 48h, suggesting that the amount of drug released over 48h was only slightly above the MIC (Figure 4.6). Both FA and RIF have been shown to be bacteriostatic against a number of Gram Positive microorganisms *in vitro* (Grohs et al., 2003). However, incubation with FA/SF and RIF co-loaded nanofibers resulted in the complete eradication of bacteria after 48h incubation for both strains of MRSA and *S. epidermidis* (see Figure 4.6). No difference was observed between any co-loaded formulations indicating that the elution profiles of FA/SF-RIF combinations were all satisfactory in terms of exceeding the minimal bactericidal concentration of the microorganisms. Interestingly, the higher release of RIF when co-loaded with SF as opposed to FA, did not result in higher antimicrobial activity. The inoculum used in our studies may not have been high enough to observe a difference, and
thus the influence of SF on increasing RIF release may only be seen at higher inoculum. However, this has yet to be explored.

*S. aureus* was not susceptible to the combination of FA/SF and RIF, which may be a function of the indifference of the drug combination. Other reports suggest that this is related to the presence of RIF-resistant mutants. In some time-kill studies using RIF, the appearance of “skip-tubes” was observed at high inoculum concentrations (~$10^6$ CFU/mL) indicating the growth of a RIF-resistance mutant (McCabe and Lorian, 1968). In fact, the same authors have shown that ~20% of evaluated *S. aureus* strains were completely resistant to RIF (McCabe and Lorian, 1968). We suggest that since the mono-loaded RIF formulations displayed similar bactericidal activity as the co-loaded formulations, the existence of RIF-resistant mutants is unlikely. It has recently been shown that RIF is bacteriostatic against *S. aureus* isolated from clinical blood isolates at concentrations of 2-5 µg/mL over 24h (Soriano et al., 2005). Therefore, the release of FA/SF and RIF from mono- or dual-loaded nanofibers may simply not have been enough to exhibit anti-*S. aureus* activity.

The opportunistic, and multidrug resistant Gram negative *A. baumannii*, has recently emerged as a significant contributor to nosocomial infections. The resistance phenotype of this species is due to the horizontal acquisition of genes and the presence of five super families of efflux pumps on the cell surface (Coyne et al., 2011). The presence of *A. baumannii* in clinical infections (ranging from those of the respiratory tract, to bloodstream, soft tissue, and prosthetic devices) is a major clinical problem (Gordon and Wareham, 2010). There is considerable interest in the use of double, and even triple therapy to combat this microorganism, and currently the majority of multi-therapy approaches investigate RIF as a major component (Bergogne-Berezin and Towner, 1996). Unfortunately, none of the antibiotic-loaded formulations evaluated in this study were able to control the growth of this strain over 48h, but all formulations containing RIF
(either mono- or dual-loaded) were able to produce a bacteriostatic effect at least for the first 24h. Although *A. baumannii* may not be susceptible to FA/SF, the exchange of FA/SF for another antibiotic combination with RIF may lead to the development of new treatments to eradicate *A. baumannii*.

Given their effectiveness in the *in vitro* experiments, we evaluated the ability of the co-loaded nanofibers [10% (w/w) FA/SF co-loaded with 5% (w/w) RIF] to prevent the colonization of titanium implants by MRSA (Newman) in an *in vivo* rodent model of implant-associated infection. The elution of both FA-RIF and SF-RIF combinations from nanofibers were found to significantly decrease adherent bacteria by > 99.9% (Figure 4.7) by day 7 of the experiment. These results indicate that the concentration of each drug delivered *in vivo* is at least above the MIC range determined from *in vitro* experiments (Table 4.2), decreasing the initial ~10^8 inoculum to levels that were cleared by the immune system. Considering that the antimicrobial loaded nanofibers were able reduce a bacterial load that typically would far exceed the number of bacteria that would be introduced into the surgical site during implant procedures, the application of this technology might be expected to show potential in preventing implant associated infection in humans.

To establish whether decreased infection of the treated sides correlated with a decreased inflammatory state, we analyzed the tissue surrounding the implants for cellular infiltrates characteristic of chronic inflammation associated with bacterial infection. As shown in Figure 4.8, there was a significant increase in inflammatory reaction score with a bacterial inoculum in all groups, with the exception of the SF-RIF treatment group, which showed an increase in inflammation score that was not statistically different from the titanium control. This may suggest a decreased immune response in animals receiving SF-RIF nanofibers. The immune response to a bacterial invasion can be categorized, in part, by the appearance of CD3+ T-cells at
the infection site. In both positive and negative control animals, the immunohistochemistry showed that <1% and approximately 20% of the total foreign cell infiltrate were CD3+ lymphocytes respectively (data not shown). In a murine model of *S. aureus* implant-associated infection, the delayed and persistent appearance of CD3+ T-cells has been associated with a chronic infection, and the inability of host responses to clear the *S. aureus* biofilm (Prabhakara *et al.*, 2011). Interestingly, we observed a higher percentage of infiltrating T-cells in the tissues from the antibiotic-treated surgical pocket compared to the vehicle control (data not shown). Previous studies have shown the ability of *S. aureus* exotoxins (superantigens) to bind T-cells (Kotb, 1995), resulting in T-cell paralysis, or apoptosis in the presence of the excess proinflammatory cytokines tumor necrosis factor-alpha or interleukin-10 (Chau *et al.*, 2009), which are both upregulated in acute bacterial induced inflammation (Feezor *et al.*, 2003). The resulting homeostasis has been representative of an immunosuppressive state (Kotb, 1995), allowing the bacteria to evade host defenses and form a stable colony. Considering that similar exotoxins have been reported for MRSA, it is likely that this phenomenon explains the lower number of CD3+ lymphocytes in the untreated surgical pockets, where a persistent MRSA biofilm is attenuating the host immune response through T-cell apoptosis or quiescence. In addition, the presence of the nanofiber carrier may also have served to increase the initial T-cell upregulation, and cannot be disregarded as a confounding factor. In spite of the increased inflammatory response, and cellular infiltration of CD3+ T-cells in the antibiotic-treated animals, the ability of FA/SF-RIF co-loaded PLGA nanofibers to prevent the colonization of a highly virulent clinical strain of MRSA may be of clinical interest in preventing infections associated with orthopaedic implants.
4.5 Conclusion

The conditions of PLGA molecular weight and concentration required for producing continuous, homogeneous nanofibers were mapped out using an “electrospinning/electrospraying PLGA phase morphology map”. We developed a biodegradable, localized drug delivery system consisting of electrospun nanofibers co-loaded with FA/SF + RIF with good antibiotic encapsulation (~75%-100%), and a biphasic release pattern. All dual-loaded formulations showed antimicrobial activity \textit{in vitro} against \textit{S. epidermidis}, and two strains of MRSA, but were bacteriostatic against \textit{S. aureus} over 48h and \textit{A. baumannii} over 24h. Lead formulations containing 10% (w/w) FA/SF and 5% (w/w) RIF were able to reduce the number of adherent bacteria by >99.9%, in an \textit{in vivo} rodent model of implant-associated infection. We propose that these nanofiber membranes could be attached to any implant surface, and/or adhere to complex biological surfaces. Hence, electrospun biodegradable PLGA nanofibers loaded with a combination of FA/SF and RIF may be an effective approach for localized drug delivery and the prevention of orthopaedic implant associated infection.
Chapter 5: Summarizing discussions, conclusions, and suggestions for future work

5.1 Summarizing discussion

Complications due to infections of implants are the primary reason for implant failure in orthopaedics (Gristina, 1994). Contemporary practice of perioperative systemic antibiotics has been met with little clinical efficacy, due to an inability of circulating antibiotics to reach sufficient tissue concentrations at the implant site (Gristina, 1994). Furthermore, efficacy of all antibiotic therapy is challenged by the rapid increase in the prevalence of antibiotic-resistant microorganisms at the surgical site, including MRSA, for which traditional antibiotics are ineffective.

The prevalence of MRSA in US hospitals has sharply climbed from 2.4% in 1975, to nearly 60% in 2003 (Laxminarayan, 2007). In Canada, the incidence of MRSA infection is lower at ~20% (Laxminarayan, 2007), however the rate of acquisition is considerably greater with a ten-fold increase in MRSA prevalence between 1995 and 1999 (Simor et al., 2001). In spite of such a dramatic increase in the prevalence of resistant microbes, the development of new antibiotics has declined by 56% over the past 20 years (Spellberg et al., 2004). In fact, analysis of the drug pipelines from 15 major pharmaceutical companies in 2003 showed that only 1.6% of all pipeline drugs were novel antimicrobials (Spellberg et al., 2004). In addition, in an evaluation of all antimicrobial approvals by the FDA spanning the same 20-year range, only 2 had novel mechanisms of action (Bosso, 2005). Therefore, with the increasing prevalence of drug resistant microorganisms and the concomitant decline in the development of novel antimicrobials, older generation antibiotics that show efficacy against multidrug resistant microbes have been reintroduced into clinical practice (Maviglia et al., 2009). Two antibiotics that are receiving a great deal of attention for the treatment of implant-associated infections due to MRSA, and other
multi-drug resistant microbes such as VRE, are fusidic acid (FA) and rifampicin (RIF) (Trampuz and Zimmerli, 2005; Trampuz and Zimmerli, 2006), whose combination therapy has been shown in the literature to be additive or synergistic in vitro (Zinner et al., 1981; Howden et al., 2004; Saginur et al., 2006), and in vivo (Drancourt et al., 1997; Aboltins et al., 2007).

The delivery of drugs, like FA, using a localized delivery formulation for the treatment of infections has a number of advantages over traditional systemic delivery (Langer, 1998). This is particularly important in orthopaedic implant infections, where the infection is routinely localized to the bone-implant interface. The distribution of drugs to bone sites has been shown to be highly variable, and depends on the antibiotic used, with most penetrating at very low levels (Smilack et al., 1976; Bergan, 1981). Therefore, it has been suggested that the delivery of drugs to the orthopaedic surgical site, by direct application of antibiotic carriers, is an efficient way to overcome the poor distribution of systemic antibiotics (Diefenbeck et al., 2006). A common surgical practice aimed at increasing local antimicrobial concentrations at the implant site is the mixing of a known amount of sterile antibiotic solid with PMMA bone cement as it polymerizes in situ. The antibiotic-loaded bone cement (ALBC) is then formed into small diameter beads and placed at the surgical site, or used in implant fixation for primary or revision arthroplasty. However, these attempts to increase the localized antibiotic concentration using ALBC’s have not been able to improve patient outcomes due to an incomplete, and sub-inhibitory antibiotic release profile (Levin, 1975; Baker and Greenham, 1988; Mader et al., 1997; van de Belt et al., 2000). In addition, the need for surgical removal, may lead to increased patient morbidity. Therefore, the use of biodegradable carriers has been proposed to overcome the incomplete elution profile of ALBC’s and the required surgical removal of ALBC devices. For instance, Elliot Jacob’s group has shown that PLGA microspheres loaded with cefazolin were particularly successful in the treatment of infections associated with tibial fractures in animal models, when
placed in the surgical pocket at the end of the fracture fixation procedure prior to wound closure (Jacob et al., 1993; Jacob et al., 1993; Jacob et al., 1997; Jacob et al., 1997). In addition, these cefazolin-loaded PLGA microspheres have been shown to be significantly more effective than systemic drug administration at preventing infection in an established intramuscular infection model of MRSA when injected into the wound site (Fallon et al., 1999).

The goal of this work was to develop a PLGA-based formulation for the co-delivery of FA and RIF, to achieve localized controlled delivery (above the MIC of potential microorganisms) to surgical implant sites to prevent implant associated infections. In order to achieve this goal, our first approach was the co-loading of FA and RIF in PLGA microspheres. Earlier work by our group attempted to develop a PLGA microsphere formulation for the localized delivery of FA with potential application in orthopaedic infections (Yang et al., 2009). However, in these studies, very distinctive microsphere morphology was observed as a result of phase separation of FA from PLGA as FA-rich, amorphous solid microdomains producing uniform and spherical protrusions on the microsphere surface (Yang et al., 2009). We found no previous reports on the solid state characteristics of FA other than very limited evidence for a hemihydrate form (Reeves, 1987). Thus, we followed this up with a comprehensive study to screen and characterize different solid forms of FA (Chapter 2).

Our first strategy was recrystallizing commercial FA from a range of solvents with high FA solubility (>50 mg/mL) to generate different solid forms. Recrystallization from EA, ACE, CHCl₃, and MeOH, all yielded amorphous forms of FA, regardless of the rate of solvent evaporation as evidenced by an absence of either diffraction peaks (XRPD) or melt endotherms (DSC). However, the slow recrystallization of FA from DCM at -20°C yielded a crystalline solid form as evidenced by a unique X-ray diffraction pattern, which differed from the commercial FA. Our second strategy to generate FA solid forms was through solvent-mediated polymorphic
transformation (for solvents with low FA solubility; <50 mg/mL). Solvent-mediated polymorphic transformation involves the growth of the most stable form of a solid at the expense of the dissolution of the metastable form. Thus, solution-mediated transformation typically yields the most stable solid form (Grant, 1999), whereas recrystallization from solvent has been shown to proceed by the rule of stages, and occasionally a metastable form is produced (Ostwald, 1897).

We found that slurying excess FA in ACN or MeOH:H₂O generated two different polymorphic forms as evidenced by their sharp and unique diffraction patterns that differed from both the commercial FA, and FA recrystallized from DCM.

FA polymorphs were named according to their melting points as outlined by Haleblian (Haleblian, 1975), with the highest melting polymorph being Form I, and all others in decreasing order of their respective melting temperatures. The highest melting polymorph (Form I) was produced from the solution mediated transformation in ACN, giving needle-like crystals with a single melt transition at ~190°C. Form II was produced through slow solvent evaporation of FA from DCM at -20°C, giving plate-like crystals, which were stacked and irregular, and displayed a single melt endotherm at ~179°C. Form III was the commercial form of FA which was characterized by a single broad melting endotherm at ~148°C. Form IV was created through the solvent mediated transformation of FA in MeOH:H₂O, and formed hexagon plate-like crystals, characterized by three thermal events: an endothermic transition with a peak at ~128°C, an exotherm at ~153°C, followed by an endotherm at ~175°C. In addition, we were able to create an amorphous form of FA through the rapid solidification of FA from DCM at 100°C, with a single $T_g$ at ~117°C.

In section 1.4.3.5, polymorphism is discussed in terms of monotropic or enantiotropic relationships, based on the relative stabilities of a polymorphic pair. The heat of fusion rule for assigning polymorphs discussed in Chapter 1 (section 1.4.3.5) states that a polymorphic pair are
enantiotropically if the higher melting polymorph has a lower heat of fusion ($\Delta H_f$), and the pair is monotropic if the higher melting polymorph has a greater $\Delta H_f$. Using FA Form IV as an example, there is a polymorphic transition upon heating. Based on the heat of fusion rule, the $\Delta H_f$ of the highest melting polymorph, that is the polymorph that is created upon heating of Form IV, has a greater heat of fusion (Table 2.1), and therefore we suggest that Form IV is most likely to be monotypically related to the solid form created upon heating. However, due to the thermal degradation of FA above ~153°C, we were unable to determine whether the solid form generated upon heating of Form IV shared the diffraction pattern of any other FA solid forms generated in Chapter 2. Nevertheless, we suspect that the irreversible transformation to Form I through solution-mediated polymorphic transformation further supports the hypothesis that FA is monotypic, with Form I being stable at all temperatures and pressures, as illustrated in the theoretical monotypic phase diagram in Figure 1.7.

To investigate the stability of each polymorphic form of FA in aqueous media, we slurried Form I, III, IV, and amorphous FA in excess water for 28d. Form IV and amorphous FA both converted to Form III within 28d, suggesting that both Form IV and amorphous FA were metastable, and Form I and Form III are stable, as neither Form I nor Form III changed over this incubation time (Figure 2.2). We found that the metastable Form IV and amorphous FA have significantly higher dissolution rates than Form I-III. The conversion of metastable Form IV and amorphous FA to Form III in an aqueous environment may hold potential serious consequences if these metastable solid forms of FA transition to more stable states over time, due to possible effects on, for example, dissolution rate and solubility.

Chapter 3 of this thesis goes on to investigate the interesting phase separation phenomena of FA and RIF from PLGA during microsphere formation. When FA and/or RIF were mono- or co-loaded into PLGA microspheres using the solvent evaporation method, the resulting
microsphere surface morphologies were distinctly different, and characterized by uniform surface protrusions in FA-loaded microspheres, uniform surface dimples in RIF-loaded microspheres, and a transition from dimpled surface to spherical protrusions in RIF-loaded microspheres co-loaded with increasing FA loading beyond 20% (w/w) (Figure 3.1). Although a small number of reports have shown microspheres with similar features (Rosilio et al., 1991; Passerini and Craig, 2002; Liggins and Burt, 2004; Malaekh-Nikouei et al., 2006; Yang et al., 2009) there have been no attempts to explain the phase phenomena underlying these observations. There are an increasing number of commercial controlled release formulations based on drug-loaded microsphere technology and we believe that it is important to understand the phase behavior of drugs in polymeric matrices.

In collaboration with David Needham’s group at Duke University, we were able to use novel micromanipulation techniques to observe the phase separation events of FA and/or RIF from PLGA occurring in a single microsphere in real time. These micromanipulation techniques have been used for a number of investigations, including observation of protein glassification in aqueous phase protein droplets (Rickard et al., 2010), and measuring the diffusion of water molecules through organic phase droplets (Su et al., 2010), However, using this technique for observation of phase behavior in forming microspheres represents a new application of the method. We believe it may offer a powerful tool for understanding mechanisms of microsphere formation using solvent evaporation methods.

DeLuca’s group is one of the only groups to have attempted to model the mechanism of microsphere formation, and predict the kinetics of solvent mass transfer from the dispersed phase to the continuous phase during microsphere solidification (Li et al., 1995a; Li et al., 1995b). In DeLuca’s work, the forming microspheres are predicted to go through various phase changes as the organic solvent diffuses into the continuous, aqueous phase. The shrinking microdroplets first
enter a metastable period characterized by a polymer rich outer shell and a polymer poor inner core due to the rapid loss of solvent at the droplet surface. With further solvent loss, the microdroplet will pass the viscous boundary, which is the point where solvent loss from the dispersed droplet phase slows exponentially, and the microdroplet becomes a gel. With subsequent solvent loss, the viscous microdroplets will pass the glassy boundary, which is the point at which the microsphere is solid and the loss of solvent is complete. Using the micromanipulation techniques discussed in Chapter 3, we were able to identify the viscous and glassy phase boundaries and quantify the composition of the solidifying microsphere at the point of drug phase separation. We have shown that the phase separation of FA occurs very early in the solidification process, where coalescence of FA-DCM-rich droplets leads to surface phase exclusion due to the convective currents formed in the solidifying microsphere. On the other hand, RIF phase separates much later during microsphere solidification, and closer to the viscous boundary. At this point, there may be sufficient viscosity to prevent coalescence of RIF-DCM-rich microdroplets, as the RIF domains appear evenly distributed throughout the microsphere interior. Interestingly, when FA and RIF are combined, the phase separation occurs in a single event, intermediate to the separation of either FA or RIF alone. The majority of drug-rich domains appear throughout the bulk of the microsphere, however the relative contribution of FA to the drug mixture will determine the amount of drug present on the surface of the microsphere (Figure 3.6 and Figure 3.7).

In all of our microsphere formulations, the drug(s) were dispersed in the PLGA matrix in the amorphous form, based on evidence from X-ray diffraction and DSC studies. However, DSC analysis also showed that FA and RIF phase separated as a miscible glass of both drugs in PLGA. Raman spectroscopy supported this hypothesis that FA and RIF are co-localized in the drug-rich microdomains, as no PLGA was visualized in the drug-rich surface microdomains in
the co-loaded microspheres (Figure 3.2). The drug release profiles from the co-loaded microspheres were characterized, almost exclusively, by a large burst phase of release with limited diffusional transport of either drug after the initial burst, due in large part to the dissolution of the phase-separated surface microdomains of drug (Figure 3.8).

The observation that RIF phase separates later during microsphere formation suggested an increased compatibility with PLGA. Therefore, we calculated solubility parameters to predict the compatibility between FA or RIF and PLGA. Thermodynamically, the ability of two components to form miscible blends requires favorable intermolecular interactions between the two components, that is, the compatibility/solubility parameters between the components must be similar. If two components share similar solubility parameters, then the two components are more likely to be miscible. In this study we calculated the compatibility of FA, RIF, and PLGA using the group contribution method according to Van Krevelen (Van Krevelen, 1997), and the Flory Huggins interaction parameter ($\chi_{sp}$) (see section 1.8). The solubility parameter calculations support the observations that RIF has a greater compatibility with PLGA, and is likely the reason for the delayed phase separation seen in RIF-loaded PLGA microspheres. Therefore, the time at which each drug phase separates from PLGA appears to be a function of the calculated solubility and compatibility parameters. We suggest that these calculations play an important role in understanding phase separation phenomena in drug-loaded polymer systems. Our group previously used these solubility parameter calculations to show the correlation between the amount of drug solubilized and the compatibility between that drug and the hydrophobic polymer block in micellar systems (Letchford et al., 2008).

It was clear that although drug-loaded microspheres provided interesting phase separation phenomena for investigation, the formulation was not appropriate for further development. Preliminary studies in our lab had shown electrospinning to be a process capable of producing
FA and RIF co-loaded polymeric membranes that were ultra flexible and without any evidence of visible phase separation. Thus, we hypothesized that electrospun polymeric membranes loaded with FA and RIF could be spun directly onto the surface of a metal implant, such as a surgical screw, or fixation plate prior to implantation, or they could be spun as a membrane and used to conform to the complex contours of joint replacement implants, and surgical sites to increase localized concentrations of antibiotics. In Chapter 1, we outlined target properties for an electrospun membrane that could be used as a drug delivery device to prevent and/or treat infections associated with orthopaedic implants (Table 1.2).

During nanofiber fabrication via electrospinning, a number of operational and fluid parameters can be manipulated to create nanofibers or nanoparticles of varying architecture and size (see section 1.9.4.2). In order to determine the appropriate conditions under which PLGA nanofibers could be produced, we screened PLGA with a LA:GA ratio of 50:50, over a molecular weight range of 5,200 Da to 72,800 Da, and at solution concentrations ranging from 0.1 g/mL to 0.35 g/mL, using fixed electrospinning conditions. The resulting polymer nanostructure formed from each molecular weight and solution concentration was evaluated using SEM, and used to construct what we have termed a “PLGA phase morphology map” (Figure 4.2). Depending on the molecular weight and solution concentration, PLGA formed a range of nanostructure morphologies including random polymer deposition, discrete nanobeads, beaded nanofibers, and discrete nanofibers. The transition of morphology from beads to nanofibers occurred with an increasing PLGA molecular weight and solution concentration, which is supported by a number of other investigations (Hager and Berry, 1982; Katti et al., 2004; Gupta et al., 2005; Shenoy et al., 2005). Using the phase morphology map, we selected PLGA with molecular weight 49,100 Da, and solution concentration of 0.25 g/mL to evaluate the influence of drug loading on nanofiber production and morphology. The solution concentration
of 0.25 g/mL was chosen due to the large range over which discrete nanofibers could be produced (0.15-0.3 g/mL), and thus any morphological changes would be due to the presence of drug, and not due to dilution of PLGA beyond any of the phase boundaries depicted as dashed lines in Figure 4.2.

The loading of FA/SF and RIF, either alone or in combination, produced defect-free nanofibers, with narrow size distributions, and no visible evidence of phase separation of either FA/SF or RIF. XRD and thermal analysis of the antibiotic-loaded nanofiber formulations indicated the presence of only amorphous material. The presence of FA/SF or RIF, alone or in combination did not influence any of the thermal events associated with PLGA, suggesting a dispersion that was an amorphous solid suspension. However, we did not observe any thermal events characteristic of FA/SF or RIF, which we suggest was due to a lack of sensitivity in the DSC method. Similarly, Qi et al. performed studies to characterize the phase separation of felodipine in Eudragit® E PO solid dispersions prepared by hot-melt extrusion, and found that thermal analysis by DSC was unreliable in detecting phase separation, despite drug loadings which greatly exceeded the solubility of felodipine in Eudragit® E PO (Qi et al., 2010). However using a novel proton-NMR relaxation analysis techniques, the authors were able to quantify the domain size of phase separated amorphous felodipine, and showed that the thermally-undetectable nanodomains were between 5.6 nm and 22 nm in size (Qi et al., 2010). Ageing studies with drug-loaded nanofibers performed at 37°C over 50 days in a temperature-controlled oven, did not produce any additional thermal transitions, suggesting that the “metastable” amorphous suspensions were stable over that time frame. Solid dispersions of a number of other drugs including ketoconazole (Van den Mooter et al., 2001), carvedilol (Pokharkar et al., 2006), simvastatin (Ambike et al., 2005), etoricoxib (Shimpi et al., 2005), and indomethacin (Matsumoto and Zografi, 1999), have all been successfully produced and
stabilized using rapid solvent evaporation techniques, such as spray drying. Since electrospinning can be seen as a similar process as spray drying (Qi et al., 2006), it is likely that the kinetics of fiber formation also allows for stabilization of the amorphous drug domains. For example, both itraconazole and ketanserin have been shown to produce stable amorphous solid solutions/dispersions in polyurethane using electrospinning (Verreck et al., 2003).

The release of FA/SF and RIF was defined by a biphasic release pattern consisting of an initial drug burst phase of release over 2d followed by a slow controlled release of drug (Figure 4.5), characteristic of a diffusion-controlled polymeric device (Baker, 1987). Referring to our target drug release properties outlined in Table 1.2, we suggest that the large burst phase of release (ranging from 20%-40% of total drug loading) may be advantageous for the rapid killing of microorganisms contaminating the surgical site at the time of implantation. Furthermore, the longer period of controlled-release may be advantageous for preventing the adherence of microorganisms, which may be present at the implant site due to delayed hematological seeding from an existing infection.

To investigate whether the drugs remained biologically active, and were released at concentrations exceeding the MIC of potential microorganisms, we chose to perform time dependent kill curves against 4 Gram positive microorganisms from the staphylococcal genus: S. aureus, S. epidermidis, and two strains of MRSA. Combined, these staphylococcal microorganisms are responsible for > 65% of all implant-associated infections (Campoccia et al., 2006), and thus efficacy against members of the staphylococcal genus of microorganisms would represent a significant clinical advantage. In addition, we selected a representative Gram negative microorganism, A. baumannii, which has recently emerged as a significant contributor to nosocomial infections, and is extremely difficult to kill due, in part, to the presence of five super families of efflux pumps on the cell surface, which contribute to the multidrug resistant
phenotype (Coyne et al., 2011). Our choice to use time dependent killing assays contradicts the vast majority of published articles evaluating the antimicrobial activity of an electrospun wound-style dressing, which have been performed using a modified disk diffusion assay. In disk diffusion assays, drug-loaded nanofiber sections are incubated on top of agar pre-seeded with bacteria, and the diffusion of drug from the nanofiber membrane creates a spherical zone where bacteria do not grow into stable colonies. However, the Wound Healing Society and European Tissue Repair Society released a publication in 2005 showing that there exists no correlation between the zone of inhibition (ZOI) and inhibitory values required to kill bacteria, and thus are inappropriate assays for the evaluation of an eluting antimicrobial wound dressing (Gallant-Behm et al., 2005). Moreover, the diffusion of a hydrophobic compound throughout a hydrophilic medium, such as agar, will be slowed considerably, and thus direct assays such as kill-curves against planktonic bacteria are preferred (Gallant-Behm et al., 2005).

Our in vitro evaluation of the efficacy of FA/SF and RIF-loaded nanofibers showed that none of the drugs alone were able to eliminate any of the bacterial strains over 48h. However, the release of each drug from PLGA nanofibers was bacteriostatic against all Gram positive microorganisms tested, suggesting that the release of each drug alone was at, or slightly exceeding the MIC of S. aureus, S. epidermidis, and both strains of MRSA (Figure 4.6). In contrast, when co-loaded, all formulations showed bactericidal activity against the same microorganisms, suggesting that the release of both drugs exceeded the minimum bactericidal concentration, that is the lowest concentration of drug that prevented growth of more than one colony (Figure 4.6). However, caution must be taken in interpreting the in vitro MIC in relation to the cumulative drug release. Drug release experiments were performed in PBS of pH 7.4 (supplemented with 0.1 mg/mL ascorbic acid), and may not reflect the release of drug into bacterial broth during in vitro evaluation, which may represent a limitation to the study design.
Nevertheless, the ability of co-loaded nanofibers to eradicate planktonic bacteria in our time dependent killing assays is interesting, as the MIC experiments using a combination of free drugs, that is FA:RIF in a 2:1 ratio matching the highest ratio used in our nanofiber formulations, was shown to be indifferent (Table 4.2). However, in our MIC evaluation of free antibiotics, we did not evaluate the MBC, and it may be likely that the observed MIC was a complete killing of the isolates when FA and RIF were assessed in combination. More likely, however, is the method of evaluating MIC using microbroth dilution techniques may be misleading. Modified checkerboard assays, which are similar to microbroth dilution techniques, to evaluate the combinational effect of FA and RIF have largely shown indifference, whereas time-dependent killing assays have shown synergy in most Gram positive isolates (Farber et al., 1986). Many studies using RIF in combination with other antibiotics have shown very poor agreement (Zinner et al., 1981; Howden et al., 2004; Saginur et al., 2006; Perlroth et al., 2008; Maviglia et al., 2009), and the results have been suggested to be highly method-dependent, and largely unreliable at predicting in vivo outcomes (Perlroth et al., 2008).

In spite of the in vitro findings of antibiotic indifference in MIC evaluation using free drugs in microbroth dilution techniques, the positive results from in vitro time-dependent kill curves of the FA/SF and RIF co-loaded nanofibers against S. epidermidis and both strains of MRSA successfully met our target property of releasing drug at levels exceeding the MIC of potential microorganisms present in an infected surgical site in vitro. Therefore, the ability of FA/SF and RIF loaded nanofibers to eliminate infecting microorganisms warranted further investigation in vivo. To evaluate the efficacy of our nanofiber formulations in preventing the colonization of a titanium implant in vivo, we used a rodent model of subcutaneous implant infection. In this model, two subcutaneous pockets were created on the dorsum of a rat, with each pocket receiving sterile titanium disk implants alongside a blank (no drug) or drug-loaded
nanofiber, and inoculated with ~10^8 CFU MRSA. The animals are were allowed 7d recovery, at which time the disks were removed and evaluated for adherent bacteria. This model has been previously validated by Hancock’s group, and shown to be a good model for the evaluation of implant colonization by P. aeruginosa (Gao et al., 2011), and thus should be a good initial screening model to determine the efficacy of our nanofiber formulations at preventing the colonization of an implant by MRSA in vivo. We selected two lead formulations for in vivo evaluation, containing 10% (w/w) FA or SF plus 5% (w/w) RIF. These lead formulations were chosen since high drug loading could be achieved and with homogeneous fiber morphologies, good release profiles, and showing in vitro activity against all pathogens evaluated. These lead formulations were able to reduce to number of adherent bacteria by > 99.9% compared to bacterial control and unloaded nanofiber (no drugs) control, further supporting the in vitro data that the drug release exceeded the MIC of MRSA. Although a limitation to this study is not including a free drug control (either as local administration or systemic), which may have also shown efficacy against the bacterial challenge. In any case, the presence of FA and RIF above the MIC in the surgical site would likely have prevented the adherence and growth of MRSA on the titanium implants, and allowed for natural immune responses to clear the bacterial burden. In order to determine if the natural immune response was lower in tissue pockets receiving the FA/SF and RIF co-loaded nanofibers, tissue sections surrounding the implant were removed, fixed and stained for CD3+ T-cells. In addition, a blinded pathologist scored each tissue section for inflammation using a semi-quantitative scoring system based on CD3+ cellular infiltrate, necrosis, and stage of healing process as determined by the presence of neovascularization and fibrosis. Similar reaction scoring has been used to quantify chronic infections associated with subcutaneous sutures (Mehta et al., 1996), and vascular grafts (Schneider et al., 2008). We found a significantly enhanced inflammatory score in all animals, in spite of the reduction in adherent
bacterial counts with FA/SF and RIF co-loaded nanofibers. We suggest that this could be due to a few factors. First, the formulation has been shown to be inhibiting the adherence of MRSA to titanium, which may allow a strong inflammatory reaction, mediated by the ability of the adaptive immune response to scavenge the planktonic bacteria (Ainslie et al., 2009). Secondly, the presence of the nanofiber membrane may also be eliciting a high immune response through frustrated phagocytosis (Ratner, 2004), which causes an upregulation of macrophages and neutrophils in an attempt to clear and encapsulate the foreign materials (Ainslie et al., 2009). In spite of the high inflammatory reaction score in the treated tissue sections, the prevention of bacterial adherence to the surface of the titanium may be of significance in preventing infections associated with implanted devices.

Based on the data in Chapter 4, we suggest that we have developed a formulation that possesses the major target characteristics for the intended application of a device placed at orthopaedic implant surgical sites (Table 1.2). In addition, we believe that electrospinning is an exciting and unique way to circumvent the limitations associated with phase separation of incompatible or poorly miscible drugs and polymers, and may be a viable formulation approach for the localized application of antibiotics at the surgical site to prevent infection associated with implantable biomaterials.

5.2 Conclusions

Major findings of this work were:

1. Fusidic acid (FA) loaded PLGA microspheres fabricated using the solvent evaporation technique produced phase separated amorphous solid FA domains. Investigation of the solid state forms of FA produced 4 polymorphic forms (Form I, II, III, and IV), and an amorphate. Metastable Form IV and amorphous FA converted to Form III in aqueous
slurry. Each solid form differed in its solid-state properties; however only Form IV and the amorphous form showed significantly enhanced dissolution rates.

2. Co-loaded FA and RIF in PLGA microspheres phase separated as a single amorphous phase whose surface deposition was determined by the relative weight fractions of each drug. The phase separation events were monitored in real time using micromanipulation techniques and video recording of single forming microspheres. Ternary phase diagrams illustrated the microsphere composition at the point of drug phase separation for mono- and co-loaded microspheres and the phase separation events were governed by the compatibility of FA and RIF in PLGA.

3. A more suitable controlled-release formulation for FA and RIF was determined to be co-loading in PLGA nanofibers prepared by electrospinning. Highly flexible, homogeneous, co-loaded nanofiber membranes possessed biphasic release profiles that were effective against *S. aureus*, *S. epidermidis*, and two strains of MRSA in direct *in vitro* time-dependent killing assays.

4. Lead FA/SF-RIF co-loaded formulations composed of 10% (w/w) FA or SF and 5% (w/w) RIF in PLGA nanofiber membranes and implanted alongside titanium implants placed in subcutaneous tissue pockets made in the dorsum of rats, were able to completely prevent the *in vivo* colonization of the implant by a highly virulent clinical isolate of MRSA after 7 days.

5.3 Suggestions for future work

In Chapter 3, we investigated the phase separation of FA and RIF from PLGA microspheres using micromanipulation techniques, and video imaging of single microsphere formation. Micromanipulation represents a novel method for the investigation of drug and
polymer phase separation, and could be expanded to include a range of polymers and drugs to evaluate compatibility and miscibility.

In Chapter 4, we have shown the ability of FA and RIF co-loaded PLGA nanofibers to prevent the *in vivo* colonization of a titanium implant in a subcutaneous pocket in a rodent model of acute infection. It would be interesting to evaluate the ability of these nanofiber formulations to treat an established infection, where the implant is colonized with an established bacterial biofilm. A number of models have been developed in which the implant, either a stainless steel needle (Monzon *et al.*, 2001), or silicone tube (Christensen *et al.*, 2012), are pre-colonized *in vitro* using a highly adherent strain of microorganism. The biofilm-coated implant is then implanted *in vivo*, and the antibiotic efficacy determined using methods performed in Chapter 4 of this thesis. Even more interesting is a novel model of chronic orthopaedic infection, in which bioluminescent bacteria are added to the synovial joint space in a rodent model alongside a titanium wire implant (Pribaz *et al.*, 2012). *In vivo* bioluminescence imaging is then used to score infection burden, which can then be normalized prior to the initiation of treatment. These models might be more relevant for the evaluation of the efficacy of antibiotic loaded nanofibers to treat orthopaedic infections.
References


treatment with tobramycin and quorum-sensing inhibitors against pseudomonas aeruginosa in an intraperitoneal foreign-body infection mouse model. J Antimicrob Chemother.


Edlund, U. and A. Albertsson, 2002. Degradable polymer microspheres for controlled drug delivery

degradable aliphatic polyesters. 157: 67-112.


Muhrer, G., U. Meier, F. Fusaro, S. Albano and M. Mazzotti, 2006. Use of compressed gas precipitation to enhance the dissolution behavior of a poorly water-soluble drug: Generation of


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