Controlled Release of Alendronate from Polymeric Films

by

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ABSTRACT

Bisphosphonate drugs are used for bone disorders such as osteoporosis, hypercalcaemia of malignancy and Paget's disease. Bisphosphonates have been shown to reduce bone resorption and increase bone mass, but have low oral bioavailability. Localized delivery of bisphosphonates has the potential to improve efficacy and decrease side effects common to oral bisphosphonate therapy.

A sensitive high performance liquid chromatography method for the determination of the bisphosphonate, alendronate, was developed and validated. Novel alendronate-loaded biodegradable polymeric films were prepared, which could be used as a targeted drug delivery device in areas where predictable bone ingrowth was desired. The drug release profiles of various formulations of alendronate in films composed of poly(lactic-co-glycolic acid) (PLGA) and polyethylene glycol or poly(DL-lactic acid)-*block*-methoxy poly(ethylene glycol) (termed "diblock") were determined. Increasing the proportion of diblock copolymer in the formulation decreased the glass transition temperature of PLGA, as measured by differential scanning calorimetry, and a miscible blend was formed. Controlled release over a 3 week period was observed for films composed of 10% diblock in PLGA.

Scanning electron microscopy and X-ray powder diffraction showed that grinding the alendronate prior to film preparation did not adversely affect the crystal structure. In addition, evidence from thermogravimetric analysis and powder diffraction patterns showed that preparation of the films in dichloromethane did not result in the formation of solvates.

Alendronate-loaded films were sterilized by glow discharge in preparation for in vitro studies with osteoblast cells. The drug release profiles of films that had and had not been glow discharged were the same and no difference in the mean molecular weight of the diblock was observed by gel permeation chromatography after 7 days at 37°C in phosphate buffered saline.

The effect of various concentrations (10^{-4} to 10^{-8} M) of alendronate on the proliferation of osteoblast cells in vitro was determined. Drug-loaded polymeric films containing 0.25% alendronate increased osteoblast proliferation after 4 days compared to polymer alone. However, films containing 0.5% alendronate appeared to inhibit proliferation. When these experiments were repeated using the MTS assay

rather than manual counting, the results showed no significant effect of alendronate on osteoblast proliferation.

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2 θ	2 theta, X-ray diffraction angle
α -MEM	Minimal essential medium
AU	Absorbance units
$^{\circ}\text{C}$	Degrees Celsius
C ₁₈	Octadecylsilane
CO ₂	Carbon dioxide
CPS	Counts per second
DAPI	4',6-diamidino-2-phenylindole
DCM	Dichloromethane, or methylene chloride
DSC	Differential scanning calorimetry
EDTA	Ethylenediamine tetraacetic acid
GPC	Gel permeation chromatography
HPLC	High performance liquid chromatography
Hz	Hertz
ICH	International Conference on Harmonization
kV	Kilovolt
λ_{em}	Emission wavelength
λ_{ex}	Excitation wavelength
MePEG	Methoxy polyethylene glycol
Mn	Number average molecular weight
mPA	MilliPascals
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt)
Mw	Weight average molecular weight
M.W.	Molecular weight
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PLGA	Poly (lactic-co-glycolic acid)
PDLLA	Poly(DL-lactic acid)
RF	Radiofrequency

Rhod	Rhodamine
RSD	Relative standard deviation (standard deviation/mean x 100)
SEM	Scanning electron microscope
Tg	Glass transition temperature
TGA	Thermogravimetric analysis
TRIS	Tris(hydroxymethyl)methylamine buffer
UV-Vis	Ultraviolet-visible
w/w	Weight to weight
XRD	X-ray powder diffraction

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1 PROJECT OVERVIEW

Bisphosphonate drugs are used for bone disorders such as osteoporosis, hypercalcaemia of malignancy and Paget's disease. Bisphosphonate drugs are potent inhibitors of osteoclastic bone resorption and there is evidence that they may trigger the proliferation of bone-building osteoblast cells (Fleisch 1998). This alters the balance of bone resorption and formation, leading to a net increase in bone density.

The oral bioavailability of the bisphosphonates is low (1-6%) and decreases dramatically in the presence of food because they form insoluble complexes with calcium or iron. Oral bisphosphonates must be taken after an overnight fast, and then the patient must sit upright for at least 30 minutes to prevent irritation to the esophagus (Suri *et al.* 2001), and continue fasting for 30 minutes to 2 hours, depending on the bisphosphonate drug. Localized and controlled delivery of bisphosphonates has the potential to improve efficacy by targeting the site of action. Local delivery would also avoid the inconvenience of fasting and the potential complication of gastrointestinal disturbance.

Alendronate was selected from the class of bisphosphonates for this work because it is readily available and has been used in other studies in film coating formulations for porous tantalum implants for orthopaedic applications. Of the different bisphosphonates, alendronate has one of the lowest oral bioavailabilities (0.7%) and highest incidence of gastrointestinal side effects.

Recent studies have shown that local delivery of bisphosphonates can improve bone growth around dental (Yaffe *et al.* 1995; Yaffe *et al.* 1997; Meraw *et al.* 1999; Meraw and Reeve 1999; Binderman *et al.* 2000; Yoshinari *et al.* 2002) and orthopedic implants (Astrand and Aspenberg 2004). Most of these studies have applied the bisphosphonate either topically into the implant cavity or as a drug coating to the implant itself; but neither of these approaches affords controlled drug release. In a periodontal procedure called guided tissue regeneration, a barrier film is inserted under the gum and over damaged bone in order to guide bone regeneration. A potential application for bisphosphonate-loaded polymer films might be as biodegradable barrier films in guided tissue regeneration (Tengvall *et al.* 2004; Skoglund *et al.* 2004). (Kajiwara *et al.* 2005; Eberhardt *et al.* 2005; Peter *et al.* 2005a; Peter *et al.* 2006). There are a very limited number of reports of the development and characterization of polymer-based, controlled release delivery systems for bisphosphonates. Although polymeric microsphere preparations of other bisphosphonates, clodronate (Perugini *et al.* 2001) and pamidronate (Weidenauer *et al.* 2003; Weidenauer *et al.* 2004) have been reported, there has been no

work on the development of biodegradable films for the controlled and localized delivery of alendronate for periodontal applications. This would represent a potentially important advancement in therapy.

In this work, poly(lactic-co-glycolic acid) (PLGA) was selected as the polymer vehicle because it is biodegradable, biocompatible and commercially available in a series of different molecular weights and copolymer ratios that would allow for modulating the drug release characteristics. In addition, previous work in this laboratory has shown that PLGA forms suitable film formulations for drugs such as paclitaxel (Jackson *et al.* 2004).

The goals of this project were to develop and characterize a biodegradable, controlled-release film formulation for alendronate that would release alendronate over a period of 2-4 weeks and to determine the effects of alendronate release from the formulations on osteoblast proliferation.

Since alendronate is a very hydrophilic drug and PLGA is a hydrophobic polymer, our experimental approach to formulation development was to reduce the solid alendronate particle size and employ polymer blends to achieve homogenous dispersions of the drug in the matrix and modulate drug release rates. Lead formulations were selected for in-depth characterization and for determination of osteoblast proliferation effects.

2 BACKGROUND

2.1 Bisphosphonates

2.1.1 Pharmacology

The bisphosphonates are a group of compounds analogous to pyrophosphate, but characterized by a stable phosphate-carbon-phosphate (P-C-P) bond. They are used to treat bone disorders such as osteoporosis, osteolytic bone metastases, and Paget's disease (Fleisch 1998). Bisphosphonates bind avidly to calcium as shown in Figure 1.

Figure 1 Chemical structure of the bisphosphonate alendronate bound to calcium. Carbon atoms are shown in grey, phosphorus in pink, oxygen in red, nitrogen in blue and hydrogen in white.

Bone is the major site of action of bisphosphonates. Bone tissue consists of ~65% mineral layers (hydroxyapatite, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$), and ~35% protein layers (primarily collagen) (Fleisch 1997). At any time, approximately 10% of bone surface in the adult skeleton is undergoing active remodelling, whereas the remaining 90% is quiescent (Hughes *et al.* 1989;Fleisch 1997). Remodelling can be divided into four phases: resorption, reversal, formation and quiescence, as shown in Figure 2. Activated osteoclasts migrate to the bone where they secrete acid that dissolves a shallow trench in the hydroxyapatite (resorption). Then there is a reversal phase in which there may be degradation of the collagen, and the release of growth factors to initiate the formation phase. The resorption cavity is filled in by successive layers of osteoblast cells that also deposit a mineralizable matrix (Raisz 1999). The balance of bone resorption and

formation may be disrupted by factors such as age, immobilization, inflammation or disease (Raisz 1999).

Figure 2 Bone remodelling cycle (Ralston 2005), used with permission.

The principal effect of bisphosphonates is the inhibition of osteoclast-mediated bone resorption. Bisphosphonates have a high affinity for calcium in hydroxyapatite (Fleisch 1985). Calcium-bound drug is dissolved under the acidic conditions created by the osteoclasts during resorption. The solubilised bisphosphonate is then taken up by the osteoclast where it triggers various biochemical effects. At the molecular level, nitrogen-containing bisphosphonates inhibit the mevalonate pathway, which perturbs cell activity and can induce apoptosis (Reszka and Rodan 2004). At the cellular level, there is a decrease in osteoclast recruitment and adhesion (Cecchini *et al.* 1987). The loss of the ruffled border on the osteoclast renders it inactive for further resorption, resulting in shallower resorption sites (Tenenbaum *et al.* 2002). Other effects include: prevention of

the development of osteoclast precursors (Hughes *et al.* 1989); stimulation of the production of osteoclast inhibitory factor (Vitte *et al.* 1996), and ultimately, induction of osteoclastic apoptosis (Hughes *et al.* 1995). In addition to direct effects on the osteoclasts, bisphosphonates also promote osteoblast proliferation and maturation (Im *et al.* 2004; Von Knoch *et al.* 2005). The net effect is a reduction in bone resorption leading to a net gain in bone density.

2.1.2 Structure-Activity Relationship

The administration of bisphosphonates varies with compound and indication. The Canadian Pharmacists Association *Compendium of Pharmaceuticals and Specialties* (2005) reports that the drug is taken orally either daily, weekly or monthly. For hypercalcemia of malignancy and Paget's disease, it is given as an intravenous infusion (Hillner *et al.* 2003).

There are several bisphosphonate drugs with various substitution groups, relative potencies, routes of administration, and indications, as shown in Table 1. Bone affinity and antiresorptive abilities appear to depend on separate properties of the bisphosphonate structure. Where the R₁ side group is -OH, the affinity for calcium ions increases due to tridentate rather than bidentate chelation to the calcium atom. The R₂ side chain determines antiresorptive potency. The addition of an amino alkyl group increases the potency 10 fold and increases selectivity for inhibiting bone resorption over reducing mineralization (Rodan and Fleisch 1996). Compounds containing a tertiary nitrogen group or a nitrogen-containing heterocyclic ring are even more potent (Fleisch 1998).

Table 1 **Commercially Available Bisphosphonate Drugs**

Name (trade name)	R ₁ ^a	R ₂ ^a	Relative Potency ^b	Route	Indication(s)
Etidronate (Didrocal, Didronel)	-OH	-CH ₃	1	oral or iv	Paget's disease, heterotopic ossification
Clodronate (Bonefos, Ostac)	-Cl	-Cl	10X	oral daily or iv	hypercalcaemia of malignancy
Tiludronate (Skelid)	-H		10X	oral daily	Paget's disease
Pamidronate (Aredia)	-OH	-(CH ₂) ₂ NH ₂	100X	iv	Paget's disease, hypercalcemia of malignancy, breast cancer, multiple myeloma
Alendronate (Fosamax)	-OH	-(CH ₂) ₃ NH ₂	>100-1000X	oral weekly	osteoporosis, Paget's disease
Ibandronate (Boniva, Bondronat)	-OH		>1000- 10,000X	oral monthly	Paget's disease, osteoporosis, hypercalcaemia of malignancy
Risedronate (Actonel)	-OH		5000- 10,000X	oral weekly	Paget's disease, osteoporosis
Zoledronic acid (Zometa)	-OH		>10,000X	iv infusion	hypercalcaemia of malignancy

^a R₁ and R₂ represent substituent groups on the following backbone:

^b Relative potencies established by Fleisch (Fleisch 1997)

2.1.3 Toxicity

Reported toxicities include mild gastrointestinal disturbances and diarrhoea (Russell *et al.* 1999). Severe esophagitis and upper gastrointestinal events have been also reported, particularly for alendronate (Greenspan *et al.* 2000;Suri *et al.* 2001).

Recently, rare reports of osteonecrosis of the jaw have been reported in patients treated with bisphosphonates (>95% of cases were attributed to pamidronate and zoledronate); most cases have been in cancer patients undergoing dental procedures (Cheng *et al.*

2005;Marx *et al.* 2005). The labelling of all bisphosphonates was recently updated to reflect this.

The incidence of gastrointestinal events in bisphosphonate clinical trials was lower than that observed post-marketing (Marshall 2002). The number of upper gastrointestinal disorders observed in the randomized comparative trials for alendronate in 2027 women over 3 years was 8 (2.0%) patients receiving placebo and 9 (4.6%) patients taking alendronate, 10 mg. None of these events was defined as serious (Greenspan *et al.* 2000). The New England Journal of Medicine reported that as of March 5, 1996, alendronate had been prescribed for ~475,000 patients worldwide, and a total of 199 patients had adverse effects related to the esophagus (51 categorized as serious or severe). Thirty-two patients were hospitalized, and two were temporarily disabled. Endoscopic findings generally indicated chemical esophagitis, with erosions or ulcerations and exudative inflammation accompanied by thickening of the esophageal wall. Esophagitis was associated with swallowing alendronate with little or no water, lying down during or after ingestion of the tablet, continuing to take alendronate after the onset of symptoms, and having pre-existing esophageal disorders (Suri *et al.* 2001).

The cause of this gastrointestinal damage may be linked to the mechanism of action for this class of drugs. Bisphosphonates can cause apoptosis in a variety of cell types in vitro, such as macrophage-like cells (Rogers *et al.* 1996), osteoclasts (Hughes *et al.* 1995), and myeloma cells (Shipman *et al.* 1998). Nitrogen-containing bisphosphonates can cause apoptosis and inhibit growth of gut epithelial cells due to inhibition of the mevalonate pathway, following loss of prenylated proteins and sterols (Suri *et al.* 2001). In a Caco-2 cell model in the presence of bisphosphonates, proliferating cells were more susceptible to apoptosis than confluent cells (Suri *et al.* 2001). In addition, nitrogen-containing bisphosphonates may compromise the surface hydrophobicity barrier of the gastrointestinal lining, which could trigger mucosal injury or ulceration (Lichtenberger *et al.* 2000).

The toxicity of bisphosphonates in a local delivery application, therefore, is dependent on the concentration of drug and the type of cells. Cells such as osteoclasts or gastric epithelial cells, which depend on the products of the mevalonate pathway to function, would be more susceptible to toxicity from nitrogen-containing bisphosphonates than other cells surrounding an orthopaedic or dental implant.

2.1.4 Physicochemical Characteristics

Bisphosphonates are negatively charged at the normal intestinal pH range. For example, alendronate has 5 pKa values (0.8, 2.2, 6.3, 10.9 and 12.2). At the pH of the small intestine (pH = 6 to 8), it has a net negative charge (-2, including the positively charged amino group). Etidronate has 4 pKa values (1.7, 3.1, 7.5 and 11.5) and also carries a negative charge (-3) in the intestine (Ezra and Golomb 2000).

The poor intestinal absorption is likely due to the low lipophilicity and negative charge of bisphosphonates. Alendronate has an octanol/buffer partition coefficient of 0.0017 (independent of pH values ranging from 2 to 11) (Boulenc *et al.* 1993; Lin *et al.* 1994).

Bisphosphonates are generally supplied as the sodium salt; the acid form has been found to be irritating, and the calcium salts (particularly of alendronate and pamidronate) are highly insoluble and more toxic than their free acid forms (Twiss *et al.* 1999). There was one report in the literature on the development of a gel formulation of calcium alendronate for subcutaneous or intramuscular administration (Ostovic and Brenner 1995); but it was not pursued. The various crystalline forms of alendronate have different solubilities. Alendronic acid monohydrate, alendronate monosodium salt trihydrate and alendronate monosodium salt anhydrous have solubilities in water of 8, 40 and 300 mg/mL, respectively (Ezra and Golomb 2000). The marketed compound is alendronate monosodium trihydrate. It is insoluble in liquids other than water.

2.1.5 Pharmacokinetics

Oral absorption of bisphosphonates is low (1-6%) under fasting conditions and negligible in the presence of food. Bisphosphonates are transported across the intestinal lining by passive diffusion. They are highly polar and charged at physiological pH, so absorption across the gastrointestinal mucosa is primarily by the paracellular, rather than the transcellular route (Lin 1996). Bisphosphonates can sequester calcium ions from the tight junctions of the gastrointestinal epithelium, resulting in increased paracellular transport (Suri *et al.* 2001).

Upon absorption, 70-80% of the drug is bound to albumin in the plasma, and then rapidly distributed throughout the body. It is then either taken up and sequestered into bone or eliminated by renal excretion (Lin *et al.* 1991). Uptake into the bone is greatest in areas of high physiological activity i.e., in active sites of bone resorption (Sato *et al.* 1991; Barrett *et al.* 2004). Approximately 65% of alendronate is present in non-calcified tissues within 5 minutes of administration of the drug. This is reduced to 5% within an hour and <1% within 6-24 hours (Lin *et al.* 1991).

Bisphosphonates are not metabolized. The P-C-P bond is completely resistant to hydrolysis by enzymes such alkaline phosphatase and pyrophosphatase (Lin *et al.* 1991).

The elimination of alendronate by urinary excretion has several phases. In the first phase, more than 90% of the drug (that has not been retained on the bone surface) is excreted within the first 24 hours; in the second elimination phase, alendronate has a half life of ~10 days (Reszka and Rodan 2004). Once the bisphosphonate has localized on the bone resorption surface, it becomes embedded in the bone until it is eventually released back into circulation as part of normal bone turnover. The average terminal elimination half life of alendronate from the skeleton is ~10 years (Lin *et al.* 1991).

2.2 Therapeutic Applications of Delivery of Bisphosphonates to Bone Tissue

The inconvenience of fasting and the risk of esophagitis may reduce patient compliance with oral delivery of bisphosphonates. Localized or targeted bisphosphonate delivery at the site of orthopaedic or dental implants offers several key benefits:

- higher bioavailability
- decreased toxicities
- convenience

Development of localized delivery systems for therapeutic applications would represent an important advancement in therapy.

Previous research on local delivery of bisphosphonates has taken a variety of forms. Several groups have explored topical or subcutaneous application of the drug prior to inserting an implant device. Other groups have used bisphosphonates as a coating on an orthopaedic implant, in order to improve the rate and extent of bone fixation. Bisphosphonates have also been used in periodontal implant applications and finally, bisphosphonates might be of use in conjunction with bone void fillers. A summary of the literature accounts of each type of local bisphosphonate delivery—including the drugs, doses and delivery methods—is shown in Table 2, and discussed in detail in the following sections.

Table 2 Local Bisphosphonate Use with Medical Implants

(Page 1 of 2)

Drug (Reference)	Device	Concentration	Model	Effect
Topical or Subcutaneous Delivery				
Alendronate (Astrand and Aspenberg 2004)	titanium bone screws	20 µl of 1 mg/ml solution applied for 1 minute	rat tibia implant	Reduced soft tissue damage and bone resorption following 5 days oscillating fluid pressure
Ibandronate (Skoglund <i>et al.</i> 2004)	stainless steel bone screws	0.1 mg applied locally or daily 3 µg subcutaneous injections	rat tibia implant	after 2 weeks, pullout force increased 15% (local application) or 30% (IM injection)
Ibandronate (Eberhardt <i>et al.</i> 2005)	uncoated and hydroxyapatite coated titanium implants	saline, 1 µg/kg or 25 µg/kg subcutaneous injection	rat femur implant	high dose significantly enhanced extent of osseointegration
Implant Coatings				
Alendronate (Meraw and Reeve 1999; Meraw <i>et al.</i> 1999)	hydroxyapatite coated or titanium dental implants	soak in 0.1 mM, 1 week at 37°C or 2.8 µg	dog dental implant	increased amount of bone around implants
Pamidronate Ibandronate (Tengvall <i>et al.</i> 2004)	cross-linked fibrinogen coated stainless steel screws	0.12 µg/cm ² or 10 Å thick	rat tibia implant	increased pullout force after 2 weeks
Pamidronate (Kajiwara <i>et al.</i> 2005)	titanium implants	Soak in 3 mg/mL, 24 hr, 37°C	rat tibia implant	significantly accelerates formation of new bone
Zoledronic acid (Peter <i>et al.</i> 2005a; Peter <i>et al.</i> 2006)	hydroxyapatite coated titanium implants	0, 0.2, 2.1, 8.5, and 16 µg/implant	rat femoral implants	Mechanical fixation and bone density increased with dose (up to 8.4 µg/implant)

Table 3 Local Bisphosphonate Use with Medical Implants (Continued)

(Page 2 of 2)

Drug (Reference)	Device	Concentration	Model	Effect
Periodontal Applications				
Alendronate (Yaffe <i>et al.</i> 1995; Yaffe <i>et al.</i> 1997)	surgical pellet	0.15, 0.75, and 1.5 mg/ml	rat dental surgery	reduces bone resorption activated by dental surgery
Alendronate (Binderman <i>et al.</i> 2000)	insert drug/gelatin sponge after periodontal surgery	10, 50, 200, 400 µg	rat dental surgery	reduced bone loss (esp. 400 µg)
Pamidronate (Yoshinari <i>et al.</i> 2002)	titanium + Calcium phosphate implants	Soak in 0.01 M, pH 6.2, 24 hr, 37°C	dog dental implant	increased bone implant contact after 12 w

Several groups have explored the use of oral or intravenous bisphosphonates in conjunction with implant surgery (Millett *et al.* 2002; Jeppsson *et al.* 2003; Zou *et al.* 2003; Skoglund *et al.* 2004; Wise *et al.* 2005; Bobyn *et al.* 2005; Altundal and Gursoy 2005). This approach has been effective, but it does not represent a novel drug delivery system and is therefore outside of the scope of this review.

2.2.1 Topical or Subcutaneous Bisphosphonates with Implant Devices

The immediate goal after inserting a surgical implant is to achieve osseointegration, or the direct attachment of bone tissue to the surface the implant, without intervening connective tissue (Meraw *et al.* 1999). Bisphosphonates can be applied topically at the site of an implant device to aid in bone regrowth.

Immediately following insertion, implants are surrounded by traumatized bone and blood vessels. Osteoclasts will begin the bone remodelling process; but fluid pressure, mechanical instability and wear particles can cause excessive bone resorption around the implant which can lead to aseptic prosthetic loosening. To demonstrate that bisphosphonates could reduce peri-prosthetic bone resorption Åstrand *et al.* applied

alendronate topically (20 μ l of 1 mg/ml for 1 minute, then rinsed 3 times with saline solution) to the site of a titanium bone screw in a rat tibia model (Astrand and Aspenberg 2004). After 5 days to allow fibrous tissue to form between the implant and the bone, 20 cycles of pressure (0.6 MPa, 0.17 Hz) was applied twice a day for 5 days to stimulate bone resorption. Rats given topical alendronate had half the soft tissue damage at the interface, compared to saline treated controls.

To demonstrate that bisphosphonates can enhance early screw fixation, Skoglund compared local (0.1 mg) and systemic (0.6 ml of 3 μ g subcutaneous injections, daily) administration of ibandronate in rats with stainless steel screws implanted in their tibiae (Skoglund *et al.* 2004). After 14 days, the tibiae were harvested and the biomechanical pull-out force and stiffness were evaluated. Pull-out force increased by 15% over control for local ibandronate and 30% for systemic ibandronate. Stiffness was 28% greater than control with local treatment; no difference was observed for systemic administration.

Topical administration demonstrated a rapid effect on implant fixation and osseointegration, which may be important in the prevention of aseptic loosening. The effects were evaluated after only 2 weeks of healing. However, a sustained dose of bisphosphonate may be important in applications where the bone surface is larger and healing is slower.

A recent study (Eberhardt *et al.* 2005) attempted to address the important issue of optimal dose for local delivery. Rats received uncoated or hydroxyapatite-coated titanium implants in the femur, and were randomly assigned to daily subcutaneous administration of low or high concentrations of ibandronate (1 μ g/kg, osteoporosis dose or 25 μ g/kg, hypercalcaemia of malignancy dose) or saline control. Histomorphometric evaluation after 28 days showed significant enhancement in osseointegrated implant surfaces in the high dose group compared to the low dose or control. No significant differences were observed between the 2 types of implant.

2.2.2 Bone Regeneration in Orthopaedic Procedures

Hip implant prostheses are composed of a long stem that is inserted in the femur and an acetabular cup which is inserted in the cup-shaped socket of the hip. Topical application of a bisphosphonate solution at the site of the implant would not be convenient over such a large surface area and it would be difficult to ensure that the drug concentration would be consistent. This suggests that an implant coating might be a more efficient drug delivery system. Dr. R. Wang in the UBC Department of Materials Engineering has

developed a method to coat porous tantalum hip prostheses with calcium phosphate, collagen and bisphosphonate (Duan *et al.* 2005; Fan *et al.* 2005).

Incorporating a bisphosphonate drug around an implant is intended to enhance bone integration and inhibit aseptic loosening. Aseptic loosening can occur when particles of wear debris from the prosthetic cup enter the joint space around the implant and trigger an inflammatory immune response. This can activate the osteoclasts leading to increased bone resorption at the implant interface (Al Saffar and Revell 1999).

Several groups have observed enhanced peri-implant bone growth when bisphosphonates are immobilized on the implant surface. Locally applied alendronate significantly increased the amount of bone peripheral to both hydroxyapatite and titanium implants in a dog dental model (Meraw and Reeve 1999). After 28 days, the rate of bone formation was faster and the amount of bone-to-implant contact increased for both types of implants in the presence of alendronate (Meraw *et al.* 1999).

Tengvall *et al.* coated stainless steel screws with cross-linked fibrinogen, then immobilized pamidronate on the fibrinogen and finally adsorbed ibandronate on the surface. These screws were inserted in the tibiae of male rats. After 2 weeks, the bisphosphonate-coated screws required 28% greater force to pull out, supporting the idea that bone fixation is increased in the presence of bisphosphonates (Tengvall *et al.* 2004).

A similar effect was observed using pamidronate immobilized on titanium implants that had calcium ions embedded using an ion beam dynamic mixing apparatus (Kajiwara *et al.* 2005). The implants were soaked in 3 mg/ml pamidronate solution for 24 hours at 37°C. After 4 weeks, significantly more new bone had formed around the pamidronate-coated implant than controls.

Peter *et al.* have developed a calcium phosphate/bisphosphonate delivery system (Peter *et al.* 2005a). Titanium alloy cylinders were plasma coated with hydroxyapatite then soaked in zoledronate solution of various concentrations in ultrapure water. The amount of adsorbed zoledronate was quantified by determining the remaining drug in the supernatant. Implants coated with 0, 0.2, 2.1, 8.5 and 16 µg zoledronate were obtained. The drug-coated cylinders were implanted in rat femurs. After 3 weeks, mechanical fixation increased with increasing drug concentration, except at the 2 highest concentrations. The same group repeated the experiment on osteoporotic rats (Peter *et al.* 2006). In this study, concentrations up to 8.5 µg/implant had positive effects on mechanical fixation.

2.2.3 Bone Regeneration in Periodontal Surgery

Inflammation due to periodontitis can infiltrate the periodontal ligament and the marrow spaces of the jawbone. This can lead to osteoclastic bone resorption around the tooth and a loss of tooth attachment (Kinane and Lindhe 2000). Treatment usually consists of tooth scaling, root planing and removal of the inflamed tissue. However, tissue regeneration following surgery can be erratic; rapid proliferation of the epithelial cells into the wound space can discourage bone growth (Laurell *et al.* 1998).

In a technique called guided tissue regeneration, a barrier membrane is placed under the gum and over the remaining bone (Trombelli *et al.* 2002). The purpose of the procedure is to preserve the ridge and allow for the regeneration of new bone where it has been lost. It is sometimes done in combination with bone void fillers. Some of the barrier membranes are biodegradable and others (i.e., polytetrafluoroethylene, Gore-Tex®) require a second surgery to be removed (Hammerle and Jung 2003).

Including a bisphosphonate in the barrier membrane could have a potentially important role in promoting bone regrowth in periodontal applications (Tenenbaum *et al.* 2002).

In addition, dental surgeons have observed that a burst of bone resorption follows periodontal surgery. Administration of topical alendronate inhibits active bone resorption without interfering with bone formation. In an initial experiment, alendronate solution was applied locally in a rat dental model but no reduction in bone resorption was observed (Yaffe *et al.* 1995). However, when the investigators applied an alendronate-soaked surgical pellet to the exposed bone surface during mucoperiosteal flap surgery in rats, a significant reduction in bone resorption was observed (Yaffe *et al.* 1997). They also found that an alendronate-soaked gelatin sponge reduced bone loss both at the site of dental surgery and also had a systemic effect (Binderman *et al.* 2000).

The effect of immobilizing pamidronate in calcium phosphate coated onto titanium dental implants was observed in a dog model. After 12 weeks, greater bone contact was observed compared to calcium phosphate coated and pure titanium implants (Yoshinari *et al.* 2001)

2.2.4 Bone Void Fillers

Another potential application for local delivery of bisphosphonates is in conjunction with bone void fillers. Where there has been extensive local bone loss, bone void fillers are placed into the cavity to guide bone regeneration. Autogenous bone grafts are the most effective bone graft material. They contain osteoclastic precursors but there is limited

availability, significant morbidity due to blood loss and the procedure can lead to chronic pain at the harvest site (usually the iliac crest) for up to 3 months. Allografts are demineralized, freeze-dried human cadaver bone. Allografts contain the osteoinductive properties of autografts, but can be expensive and can carry the potential for infection (hepatitis, HIV, etc.) (Moore *et al.* 2001). Other frequently used materials include bovine bone, hydroxyapatite, and bioglass (Nasr *et al.* 1999). It has been difficult to find a synthetic material that is osteoconductive and consistently stays in place for the duration of healing (Cornell 1999). The addition of a bisphosphonate, either as a coating or in controlled release microspheres, to synthetic bone void filler might improve bone ingrowth and speed healing.

The effective dose for local administration of bisphosphonates around implants is unclear. Early studies indicated that if the local concentrations were too high, (alendronate 3.0 mg/kg) bone growth would be inhibited (Tenenbaum *et al.* 2002). Although the methods for coating the implants are described, only one group (Peter *et al.* 2005a; Peter *et al.* 2006) determined the final drug content on the implant. In addition, it is evident that a controlled release delivery system for bisphosphonates would be beneficial in situations where the area is large or the healing period is long. Characterizing the loading and release profiles is a critical component of the development of local controlled release drug delivery system for bisphosphonates.

2.3 Polymeric Drug Delivery Systems

2.3.1 Biodegradable Polymers as Biomaterials

There are four major classes of biomaterials: polymers, metals, ceramics, and plant- or animal-derived natural materials (Hoffman 2004). Polymers represent the largest class of biomaterials, ranging from natural materials such as cellulose, sodium alginate, natural rubber, collagen, heparin, hyaluronic acid and deoxyribonucleic acid to synthetic materials such as silicone rubber, polyethylene, poly propylene, poly(ethylene terephthalate), poly tetrafluoroethylene, and poly(methyl methacrylate). Another large class of synthetic polymers are the more polar compounds such as poly(vinyl chloride), the nylons, poly(ethylene glycol) and poly(lactic-co-glycolic acid). Some of these compounds are hydrolytically unstable in the presence of water and degrade in the body (Cooper *et al.* 2004). Biodegradable polymers have been widely used in such varied applications as: wound closures, orthopaedic pins, drug delivery systems, vascular grafts, vascular stents, to augment bone defects, and as ligament/tendon prosthesis (Chu 2000).

Biodegradable polymers have been used extensively in the development of controlled release drug delivery systems for a broad range of drugs and in many different applications, including anticancer agents, hormones, growth factors, vaccines, local anaesthetics, antibiotics, anti-inflammatory agents, anti-psychotics (for reviews, see (Bala *et al.* 2004; Jones 2004; Park *et al.* 2005)).

The goals of controlled release drug delivery include: maintaining the drug at the desired therapeutic range (for days to years) with a single dose, localizing delivery of the drug to the site of action, reducing need for follow up care, increasing patient comfort and improving compliance (Langer 1990). A biomaterial should have suitable biomechanical properties (depending on the proposed application), biocompatibility, ease of purification and sterilization and suitable surface properties (Piskin 1995).

2.3.2 Polymer Structure and Morphology

The structure and morphology of a biodegradable polymer will impact its biomaterial characteristics.

Polymers are composed of a linked series of monomers, often dozens or hundreds of units long. Normally, a polymer sample will be composed of molecules with a distribution of different chain lengths. There are two statistically useful definitions of average molecular weight: the number-average molar mass (M_n) or the weight-average molar mass (M_w).

M_n is defined as:

$$M_n = W/N = \sum N_i M_i / \sum N_i \quad \text{Equation 1}$$

Where W is the total weight of a polymer sample, N is the number of moles of polymer. N_i is the number of moles species i , and M_i is the molecular weight of species i .

In contrast, the weight-average molar mass (M_w) is calculated by replacing the number of polymers of molecular weight i (N_i) in the above equation, with the molecular weight of the polymer of particular weight i ($N_i M_i$). This gives:

$$M_w = \sum N_i M_i^2 / \sum N_i M_i \quad \text{Equation 2}$$

Mn is sensitive to the number of molecules of lower molecular weight, and Mw is sensitive to the contribution of longer polymer chains. Mw is always larger than Mn. The ratio of the number average (Mn) to the weight average (Mw) is called the polydispersity index. A polymer with a narrow distribution of chain lengths would have a polydispersity index closer to 1. The molecular weights and polydispersity index can be determined by gel permeation chromatography, which separates polymer chains by molecular weight.

Polymer morphology describes the extent to which a polymer sample can be described as amorphous or crystalline. The term “crystallinity” in polymers refers to small areas within a disordered amorphous polymer which are able to form areas of parallel alignment. Either segments from different polymer chains align and become crystalline (fringed micelle model), or a single polymer chain can fold upon itself to form lamellar crystallites (chain folded model) (Rosen S.L. 1993). Polymer morphology can impact mechanical properties, thermal behaviour, permeability, and degradation rates.

2.3.3 Drug Release Mechanisms

A drug delivery device in which the drug is evenly distributed throughout the polymer is called a monolithic device. The rate of drug release from a monolithic device can be controlled either by the rate of diffusion of the drug out of the polymer, or by the rate of degradation of the polymer itself.

2.3.3.1 Diffusion Controlled Release

In diffusion controlled release, the polymer matrix is assumed to be inert, and the rate of diffusion depends on the solubility of the drug in the polymer.

The Higuchi model is often used to describe diffusion-controlled drug release from a monolithic dispersion (Higuchi 1961). Assuming that the drug is dispersed evenly throughout the polymer, the drug on the surface layer will diffuse out of the polymer first. When the surface layer is depleted, the next layer will then diffuse through the drug-depleted layer to the surface based on the permeability of the polymer and the diffusivity of the drug. This can lead to a burst effect at the beginning of drug release.

The equations describing the theoretical drug release profiles for polymer matrix systems in a variety of geometries are derived in detail by Baker (Baker 1987). He showed that the release rate from a simple monolithic dispersion in slab geometry is:

$$\frac{dM_t}{dt} \approx \frac{A}{2} \left(\frac{2DC_{s(m)}C_o}{t} \right)^{1/2} \quad \text{for } C_o \gg C_{s(m)} \quad \text{Equation 3}$$

Where:

dM_t/dt is the steady state drug release rate at time t

A is the total area of the slab (both sides)

D is the diffusion coefficient of the drug in the polymer matrix

$C_{s(m)}$ is the solubility of the drug in the polymer matrix

C_o is the total concentration of drug initially present

t is time

This equation predicts that drug release will be directly proportional to surface area of the slab, and inversely proportional to the square root of time.

2.3.3.2 Degradation Controlled Release

The second drug release mechanism is degradation controlled release. This mechanism is significant for polymers that are susceptible to hydrolysis, such as polyesters and polyanhydrides. For example, in the degradation of poly(lactic-co-glycolic acid), the ester linkages are hydrolyzed in water to produce lower molecular weight oligomers and monomers of lactic and glycolic acids (Anderson and Shive 1997; Freiberg and Zhu 2004). Lactic and glycolic acid resulting from the degradation of PLGA polymers can decrease the local pH values which can then accelerate polymer degradation due to autocatalysis (Shenderova *et al.* 1999). There is a rapid loss of mass in the polymer as the lower molecular weight oligomers and monomers become soluble and disperse (Jain 2000).

The influence of diffusion and degradation controlled drug release mechanisms varies as a function of different time points in the drug release profile. It is common for both diffusion and degradation mechanisms to be occurring simultaneously.

2.3.4 Factors Influencing Drug Release

Numerous factors can impact the rate of degradation and drug release from biodegradable polymers, such as polymer composition and structure (morphology, molecular weight, glass transition temperature), nature of the drug, extent of drug loading and the presence of a second polymer or plasticizer (Alexis 2005). Of these, the extent of drug loading, the composition and molecular weight of the polymer and the type of polymer blend were of particular interest in this work.

In a biodegradable monolithic system, the drug is evenly distributed in the polymer and is released by a combination of diffusion and polymer erosion (Langer 1998). At low drug loading levels (0-5%) the release of the compound involves diffusion of water into the polymer followed by diffusion of the drug to the surface. At higher loading levels (5-10%), diffusion of drug near the surface leaves cavities in the polymer matrix that provide preferred pathways for drug diffusion. These pathways may not be continuous, but they will increase the apparent permeability of the device. When the drug loading exceeds 20% by volume, cavities left by drug diffusing out the polymer will leave a continuous channel to the surface of the matrix (Baker 1987).

The composition of the polymer is an important factor influencing the degradation rate. An increase in hydrophobicity will decrease the rate of water penetration and subsequent polymer hydrolysis and degradation. An increase in crystallinity will also impact the rate of water penetration, as will an increase in molecular weight. As the molecular weight increases, the rate of degradation and drug release decreases (Alexis 2005).

The addition of another polymer or plasticizer to the matrix can also alter the rate of drug release and polymer degradation. If the polymer or plasticizer is more water-soluble than the pure polymer, it may leach out of the matrix, leaving pores and channels for greater water penetration. This could lead to increased hydrolysis and degradation (Alexis 2005).

2.3.5 Polymers Used in Film Manufacture

Three polymers were used to manufacture films for this project: PLGA, and blends of PLGA and poly(ethylene glycol), or PLGA and diblock copolymer. Each of these polymers is described in the following sections.

2.3.5.1 Poly(DL-lactic-co-glycolic acid) (PLGA)

PLGA is a copolymer of lactic acid and glycolic acid. The general structure is shown in Figure 3.

Figure 3 Structure of poly(lactic-co-glycolic acid)

There are many different types of PLGA depending on the ratio of lactic to glycolic acid repeat units, the length of the polymer chains, and whether the monomer units are distributed randomly or in an ordered fashion. Poly(glycolic acid) is more crystalline than poly(lactic acid) because it lacks the methyl side group. Lactic acid is also more hydrophobic than glycolic acid, so PLGA copolymers that contain a greater proportion of lactic acid are more hydrophobic, absorb less water and subsequently degrade more slowly. Upon degradation, both monomers are readily metabolized through the Krebs cycle as carbon dioxide and water (Jain 2000).

PLGA is the most widely investigated and most commonly used synthetic, biodegradable polymer. PLGA devices are commercially available and approved in the form of sutures, grafts, bone pins, implants and prosthetic devices (Kohn *et al.* 2004). PLGA can also be used to manufacture microspheres, strands and injectable implants capable of controlled drug release over 1 to 4 months (Jiang *et al.* 2005). Commercially available drug delivery systems based on PLGA copolymers include: goserelin (Zoladex[®]), leuprolide acetate (Eligard[®], Lupron[®]), and recombinant human growth hormone (Nutropin[®]).

The PLGA selected for use in this study was an amorphous, random copolymer of lactide and glycolide units in the weight ratio of 85:15, respectively. The glass transition temperature has been previously reported to be 47°C (Jackson *et al.* 2004), 51°C (Wang and Wang 2003), or in the range of 50-55°C (Park and Jonnalagadda 2006).

Dr. Burt's lab has extensive experience using polyesters such as PLGA and poly(L-lactic acid) in combination with release-modifying agents to formulate drug-loaded micelles (Zhang *et al.* 1996), microspheres (Liggins and Burt 2001; Liggins and Burt 2004; Liggins and Burt 2004) and films (Jackson *et al.* 2002; Jackson *et al.* 2004; Owen *et al.* 2005).

2.3.5.2 Polyethylene glycol (PEG)

In this work, two different polymers were blended with PLGA for forming films. Bisphosphonates are ionic salts, so the addition of a hydrophilic polymer was intended to improve drug encapsulation efficiency and modify the drug release characteristics. Polyethylene glycol (PEG) is a hydrophilic polyether available in a variety of chain lengths, ranging from viscous liquids at low molecular weights to waxy, crystalline solids at higher molecular weights (Fruijtier-Polloth 2005). The general structure is shown in Figure 4.

Figure 4 Structure of polyethylene glycol (PEG). PEG 600 is also known as PEG 12.

PEGs are used extensively as biomaterials, and also in cosmetic and drug delivery applications due to their broad chemical compatibility, water solubility, and biocompatibility (Sedlak 2005). PEG 300 and 400 are commercially available as solubilising excipients in methocarbamil and lorazepam intramuscular injections, and as a cosolvent in numerous soft gelatin capsule oral formulations such as nifedipine, etoposide and digoxin (Strickley 2004). The end groups can be conjugated to other polymers, proteins or drugs to make PEG-conjugates that have increased circulation times and are resistant to recognition by the immune system (Bhadra *et al.* 2002; Veronese and Pasut 2005).

2.3.5.3 Amphiphilic Diblock Copolymer

The second polymer blended with PLGA was an amphiphilic diblock copolymer (referred to in this work as “diblock”). The structure of poly(DL-lactic acid)-*block*-methoxy poly(ethylene glycol) is shown in Figure 5.

Figure 5 Structure of poly(DL-lactic acid)-*block*-methoxy poly(ethylene glycol), or “diblock”

The diblock polymer was composed of a block of poly(DL-lactic acid) approximately 30 repeat units long, coupled to a block of methoxy poly(ethylene glycol), approximately 28 repeat units long. It was synthesized from D,L-lactide and MePEG by a ring opening bulk polymerization in the presence of stannous octoate (Burt *et al.* 1999). The ratio of the two polymer components was 40% PLLA to 60% MePEG by weight, using MePEG with a molecular weight of 2000. The theoretical molecular weight is therefore 3333 g/mol, but

the observed molecular weight was reported to be $M_{GPC} = 5240$ g/mol (Zhang *et al.* 1996).

PDLLA-MePEG polymer chains will self-assemble into micelles in aqueous solution, if they are above the critical micelle concentration. The hydrophobic block of the polymer will congregate in the centre of the micelle, while the hydrophilic block forms a corona on the outer aqueous side. These micelles have been used to solubilise paclitaxel within their hydrophobic core (Zhang *et al.* 1996; Liggins and Burt 2002). Similar micelles made of amphiphilic diblock copolymers have been used to encapsulate adriamycin (Piskin *et al.* 1995), indomethacin (Kim *et al.* 1998) and testosterone (Allen *et al.* 2000).

2.3.6 Controlled Release of Bisphosphonates from Polymers

The use of biodegradable polymers as a local delivery system for bisphosphonates has been previously reported for clodronate and pamidronate.

Perugini *et al.* encapsulated clodronate into microspheres intended for parenteral administration for bone diseases (Perugini *et al.* 2001). Several batches of microspheres were prepared by the water-in-oil-in-water double emulsion method from PLGA copolymers of various molecular weights and molar compositions and one PDLLA homopolymer. Clodronate is a low molecular weight, hydrophilic compound, and it was released from the PLGA copolymer microspheres within 48 hours. The drug release rate was prolonged to 20 and 45 days respectively when the additives carboxymethylcellulose or sorbitan monolaurate 20 (Span 20) were added. The authors attributed this prolonged release behaviour to better drug dispersion in the polymer matrix and a more rigid barrier against water penetration and drug diffusion. The PDLLA homopolymer also had a 20-day release profile. PDLLA is more hydrophobic than the copolymers.

Khang *et al.* prepared implantable biodegradable wafers of PLGA loaded with 10-40% pamidronate by direct compression. The 10 and 20% loaded wafers released the drug within 30-50 days. Reduced osteoclast and increased osteoblast activity was observed after implanting the wafers on the mastoid (behind the ear) of Mongolian gerbils with experimental aural cholesteatoma (Khang *et al.* 2003).

Another group prepared pamidronate-loaded microspheres using a star-branched polymer poly(DL-lactide-co-glycolide-D-glucose) (Weidenauer *et al.* 2003). They found that if they increased the drug loading beyond 15%, there was an unfavourable initial burst of drug release (>99% within 24 hours). An investigation of the internal structure of the microspheres showed many vacuoles, pores and channels which allowed the drug to

dissolve out quickly. The same group also prepared a lyophilized pamidronate/chitosan complex which was embedded in the same star-branched polymer (Weidenauer *et al.* 2004). This system offered controlled release of pamidronate over 36 days. They also found that micronizing the drug particles allowed for better dispersion throughout the polymer matrix and a more continuous release profile over 48 days with drug loadings up to 30%.

Enhancing bone regeneration by embedding a drug in a biodegradable polymer membrane has also been explored (Park *et al.* 2000). In this paper they used tetracycline rather than a bisphosphonate, because it has both antibacterial properties as well as tissue regenerating activity. The barrier membranes were composed of porous poly(L-lactic acid) cast on poly(glycolic acid) mesh. Controlled release of tetracycline over a 4 week period was observed.

2.4 Thesis Goals and Objectives

The overall objective of this work was to develop and characterize a controlled-release alendronate-loaded drug polymeric film that might be suitable to promote bone growth in a periodontal application. Desirable characteristics for a controlled-release bisphosphonate delivery system include: a biocompatible and biodegradable polymer matrix, controlled release of the bisphosphonate over 2-4 weeks, good encapsulation efficiency, and suitable physical properties (i.e., will adhere to the desired surface, has convenient shape and flexibility).

The specific aims of the project were to:

- Develop and validate a sensitive HPLC method for the determination of alendronate in drug release studies.
- Prepare and characterize various formulations of controlled-release alendronate-loaded polymeric films.
- Determine the effect of alendronate-loaded polymeric films on osteoblast proliferation.

3 EXPERIMENTAL

3.1 Materials

3.1.1 Sodium Alendronate

Sodium alendronate trihydrate (M.W. 325.12) was provided by Dr. Rizhi Wang, from the Department of Metals and Materials Engineering at the University of British Columbia. Standard sodium alendronate trihydrate (lot 105K4707) was obtained from Sigma-Aldrich (St. Louis, MO).

3.1.2 Polymers

Polymer films were manufactured from 85:15 poly (DL-lactic-co-glycolic acid) (PLGA) with an intrinsic viscosity of 0.61 dl/g in chloroform at 30°C (lots d96051 and d96056, Birmingham Polymers Inc.). Carbowax Sentry[®] polyethylene glycol (PEG) (NF, FCC grade) with a molecular weight of 600 was purchased from Union Carbide (Danbury, CT). Diblock copolymer consisting of poly(DL-lactic acid)-*block*-methoxy poly(ethylene glycol) (PDLLA-MePEG) was generously provided by Angiotech Pharmaceuticals. The PDLLA/MePEG weight ratio is 40:60, using MePEG with a molecular weight of 2000.

3.1.3 Chemicals and Solvents

HPLC-grade acetonitrile, dichloromethane and methanol, and ACS-grade sodium dihydrogen orthophosphate, sodium phosphate, sodium chloride, sodium hydroxide, cupric nitrate, nitric acid and (ethylenedinitrilo)-tetraacetic acid disodium salt (EDTA) were obtained from Fisher Scientific (Fairlawn, NJ). Ceric ammonium nitrate and fluorescamine were obtained from Sigma (St. Louis, MO).

3.1.4 General Equipment

General equipment and supplies used during the course of these experiments are listed in Table 3.

Table 3 General Equipment and Supplies

Equipment and Supplies	Supplier
Mettler model AE 163 or AJ100 balance	Mettler Instruments (Zurich, Switzerland)
Accumet® Model 230 pH meter	Fisher Scientific (Fairlawn, NJ)
Model 370 magnetic stirrer/heater	VWR Scientific (Mississauga, ON)
Branson ultrasonic cleaner	Branson Ultrasonics Corp. (Danbury, CT)
Vanlab vortex mixer	VWR Scientific (Mississauga, ON)
15 x 45 mm glass vials with rubber closures	Ampolletas, S.A. (Mexico City, Mexico)
Eppendorf 5415D centrifuge	Brinkmann (Mississauga, ON)
Beckman GS-6 centrifuge	Beckman Coulter (Mississauga, ON)
Eppendorf series 2100 adjustable volume pipettes	Brinkmann (Mississauga, ON)
Pipetman, variable volume pipettes	Gilson Company (Middleton, WI)

3.1.5 Stock Solutions for Drug Release Studies

Drug release experiments were performed in phosphate buffered saline (PBS). One litre of PBS contains 2.3 g sodium phosphate dibasic, 0.3 g sodium dihydrogen orthophosphate and 8.22 g sodium chloride in deionised water adjusted to pH 7.35.

The fluorescence HPLC mobile phase was prepared as a 1 mM solution of EDTA (e.g., 0.372 g in 1 litre deionised water) adjusted to pH=6.5 with saturated sodium hydroxide solution. The solution was filtered through a 0.20- μ m white nylon Millipore filter (Fisher GNWP, 47 mm), and then 3% was replaced with methanol.

Fluorescamine solution was prepared by dissolving 2 mg/ml in acetonitrile.

The 0.13 M EDTA buffer solution was prepared by dissolving 4.84 g EDTA in ~80 ml deionised water, adjusting the pH to 10 with saturated sodium hydroxide solution, and quantitatively diluting to 100 ml with deionised water.

3.1.6 Cell Culture

Osteogenic cells from newborn Sprague-Dawley rat calvaria were isolated by Dr. Brunette's laboratory. Primary cultures were subcultured by trypsinization (0.25% trypsin (Sigma, St. Louis, MO), 0.1% glucose (Sigma), citrate/saline buffer pH 7.8 (BDH Chemical Ltd., Poole, England)) and maintained in minimal essential medium (α -MEM, Stem Cell Technologies Inc., Vancouver, BC) containing 15% fetal calf serum (Cansera International Inc., Rexdale, ON) and antibiotics, 100 μ g/mL penicillin G (Sigma); 50 μ g/mL gentamicin (Sigma); 3 μ g/mL amphotericin B (Fungizone[®], Gibco, Grand Island, NY) in a humidified atmosphere with 5% CO₂ at 37°C (Forma Scientific Water-jacketed incubator, Caltec Scientific, Richmond BC).

Cultures were maintained in BD Falcon[™] tissue-culture-treated flasks, with 75 cm² growth area and 0.2 μ m vented blue caps (BD Bioscience, Mississauga, ON). Osteoblast experiments were performed in 12- or 24-well Falcon[™] tissue-culture-treated plates (BD Bioscience). Thermanox[™] (Nunc) sterile coverslips were used as positive controls in cell culture experiments. Coverslips were 0.2 mm thick and 13-mm in diameter. They were made of a flexible transparent proprietary polymer (of the polyolefin family) that has been culture treated on one side for enhanced cell attachment and growth.

For MTS cell proliferation assays, the osteoblast media was exchanged with PC-3 media and samples were transferred into Corning[®] Inc. 96-well tissue-culture-treated plates (Corning, NY). One litre of PC-3 media contains 1 packet of F-12 nutrient media, 111 ml fetal bovine serum, 11 ml penicillin/streptomycin (all from Invitrogen, Carlsbad, CA) and 1.176 g sodium bicarbonate (Sigma, St. Louis, MO) diluted to volume in distilled, deionised water, and sterile filtered through a Nalgene[®] 0.2 μ m bottle-top filter (VWR, Mississauga, ON).

3.2 Equipment

3.2.1 UV-Vis Spectrophotometer

Spectroscopic analysis of alendronate was performed on an HP8452A diode array spectrophotometer equipped with 1 cm² quartz cuvettes and Agilent UV-Visible Chem Station software.

3.2.2 HPLC

Ion chromatography was performed on a Waters Alliance system consisting of a 1525 binary HPLC pump, 717+ autosampler, 2410 refractive index detector and 2487 dual wavelength absorbance detector, with Waters Breeze software (Waters Corporation, Mississauga ON). Separation was effected with an IC-Pak™ Anion-HR column (4.6 x 15 mm).

Analysis of alendronate by fluorescence chromatography was performed on a Waters HPLC (Milford, MA) system, consisting of a model 717+ autosampler, 600 Controller Pump, and a 470 Scanning Fluorescence detector with a bus SAT/IN module interface and Waters Millenium v4.0 software. The column was a Nova-pak® C18 4 µm, 3.9 x 150 mm cartridge. Halfway through the project, the detector was replaced with a Waters 474 Scanning Fluorescence detector and the column was replaced with an Alltech Nucleosil 100 (C18) 10 µm, 4.6 x 250 mm column.

3.2.3 Liquid Chromatography/Mass Spectrometry

LC/MS experiments were performed on a Waters Alliance HT 2795 Separations Module with an IC-Pak™ Anion-HR column (4.6 x 15 mm) and a Waters Micromass Quattro *micro*™ ESCi™ multimode ionization mass spectrometer with MassLynx 4.0 software.

3.2.4 Scanning Electron Microscope

Surface morphology studies were performed with a Hitachi S-3000N scanning electron microscope (Tokyo, Japan). Samples were coated with a ~10 nm thick layer of 60:40 alloy of gold:palladium using a Denton Vacuum Desk II sputtercoater (Moorestown, NJ) at 50 millitorr.

3.2.5 X-Ray Powder Diffractometer

The crystal structures of sodium alendronate samples were determined using a Bruker D8 Advance (Madison, WI) X-ray powder diffractometer.

3.2.6 Thermal Analysis

Thermal analysis was performed at Angiotech Pharmaceuticals using a Mettler Toledo MX5 microbalance and either a TA Instruments DSC Q1000 with a refrigerated cooling system for differential scanning calorimetry, or a TA Instruments TGA Q500 for thermogravimetric analysis.

3.2.7 Gel Permeation Chromatography

The gel permeation chromatography system consisted of a Waters 515 HPLC pump, 717plus autosampler, and a 2410 refractive index detector at 40°C with Waters Millenium v4.0 software. Styragel[®] 7.8 x 300 mm HR3 and HR1 (THF) columns (Waters, Mississauga ON) were connected in series. The mobile phase was chloroform at a flow rate of 1 ml/minute.

3.2.8 Cell Culture

After trypsinization, osteoblast density was determined on a Z1[™] Coulter Counter[®] cell and particle counter. A 0.5 ml aliquot of cells was diluted to 10.0 ml with Isoton[®] II diluent (Beckman Coulter Inc., Fullerton, CA) in a 35 ml Accuvette[®] II vial (Beckman Coulter). The number of cells greater than 5 μ m was determined in triplicate and averaged.

Polymer films were sterilized with a Harrick PDC-32G Plasma Cleaner/Sterilizer connected to a SPX Robinair CoolTech high performance vacuum pump (SPX Corp, Owatonna MN).

Fluorescent microscope images were obtained on a Zeiss Axioplan-2 imaging microscope (Carl Zeiss, Toronto, ON) using the DAPI and Rhod filters. Images were recorded using Northern Eclipse (Empix, Mississauga ON) imaging software.

Cell proliferation was measured using CellTiter 96[®] AQueous One Solution Cell Proliferation Assay (Promega, Madison WI). The change in absorbance at 492 nm (minus the blank reading at 600 nm) was recorded on a Labsystems Multiskan Ascent photometric plate reader using Ascent Software version 2.4.2 (Labsystems, Helsinki, Finland).

3.3 Preparation of Polymer Films

Teflon squares measuring 1 cm by 1 cm were applied to glass slides as film templates. They were cleaned with 70% isopropyl alcohol and dried prior to film manufacture.

Film casting solutions were prepared by dissolving PLGA containing PEG 600 or diblock copolymer (and ground alendronate if applicable) in a small quantity of dichloromethane (DCM). The mixtures were vigorously mixed by vortexing and ultra-sonication until the polymers had dissolved and the alendronate was evenly suspended. The mixtures were cooled in an ice bath to increase viscosity and slow evaporation after casting. A 50 μ l aliquot was cast over the template using an Eppendorf pipettor fitted with a disposable tip

with 2 mm of the end snapped off. The glass slides were placed in a loosely closed plastic container and placed in the refrigerator for 3-5 days to let the solvent evaporate slowly.

The proportions used to manufacture the lead formulation at various concentrations of alendronate are shown in Table 4.

Table 4 Theoretical loadings of 0, 0.1, 0.25, 0.5% alendronate into 10% diblock in PLGA films

Theoretical Alendronate Concentration (%)	Weight of Diblock (mg)	Weight of Alendronate (mg)	Weight of PLGA (mg)
0	30	—	270
0.1	30	0.3	270
0.25	30	0.8	270
0.5	30	1.5	270

3.4 Physicochemical Characterization

3.4.1 Solubility of Alendronate

Samples (0.5 g) of alendronate were placed in 10 mL volumetric flasks and dispersed in either distilled, deionised water (pH 5.74) or PBS (pH 7.47) previously warmed to 37°C. The flasks were placed in a 37°C oven with circular shaking at a rate of 50 rpm and 0.5 ml samples were taken at 1, 2, 5, 24, 48 and 72 hours. The samples were filtered through a Millex[®] HV syringe-driven filter unit and diluted by half in either water or PBS. Samples were further diluted 0.4 ml into 10.0 ml prior to analysis by fluorescence HPLC. The reported solubilities are the mean of the concentrations determined at 24, 48 and 72 hours for the samples dissolved in water, and the mean of the concentrations determined at 48 and 72 hours for the samples dissolved in PBS.

3.4.2 Alendronate Analysis by Spectrophotometry

A 1.2 mg/ml stock solution of alendronate in water was prepared. Aliquots were transferred to a series of 10 ml volumetric flasks to give final alendronate concentrations of 0, 2.4, 4.8, 9.6, 14.4, 24.0, and 36.0 µg/ml. A 1.5 ml aliquot of 0.1% ceric nitrate solution (0.025 g ceric ammonium nitrate in 25.0 ml 0.5 M H₂SO₄) was added and the solutions were allowed to stand at room temperature for 1 hour. The samples were quantitatively diluted to 10.0 ml with 0.5 M sulphuric acid. The absorbance at ~ 320 nm was recorded and subtracted from the blank. Typical spectra are shown in Figure 6.

Figure 6 UV-Vis spectra of alendronate standards in ceric nitrate solution containing 0, 2.4, 9.6 and 28.8 µg/ml alendronate. Loss of signal at 320 nm is proportional to alendronate concentration.

3.4.3 Alendronate Analysis by Ion Chromatography

Anion exchange chromatography was used for samples dissolved in buffers containing amine groups (e.g., HEPES) that would interfere with the fluorescence assay. Samples did not require pre-treatment. The mobile phase was composed of 0.5 mM cupric nitrate in 1.0 mM nitric acid (e.g., 116.3 mg $\text{Cu}(\text{NO}_3)_2 \cdot 5\text{H}_2\text{O}$ and 0.063 H_2NO_3 in 1 l deionised water, pH ~2.9) at a flow rate of 1.0 ml/minute. An IC-PAK™ anion exchange column (Waters, Mississauga ON) was used for separation. Alendronate can be detected indirectly by UV-Vis spectroscopy at 245 nm. Alendronate elutes at 1.5 minutes, well separated from the injection front. Copper elutes as a negative peak at approximately 5 minutes. A representative chromatogram is shown in Figure 7.

Figure 7 Representative chromatogram of 25 $\mu\text{g/ml}$ alendronate standard by ion chromatography. Mobile phase: 0.5 mM cupric nitrate, 1.0 mM nitric acid, 1 ml/min. UV-Visible detection at 245 nm.

3.4.4 Alendronate Analysis by Fluorescence HPLC

Samples were prepared for HPLC analysis by mixing 100 μl of sample with 900 μl of 0.13 M EDTA buffer (pH 10) and 500 μl of 2 mg/ml fluorescamine in acetonitrile in a 4 ml glass vial. Samples were gently agitated until the solution was clear and a yellow colour developed, approximately 10 seconds. One ml of dichloromethane was added to extract the acetonitrile, and the vials were capped and shaken vigorously. The layers were allowed to separate and the yellow upper layer was withdrawn for HPLC analysis. The sample-processing scheme is shown in Figure 8.

Figure 8 Sample preparation flow chart for the determination of alendronate by fluorescence HPLC

Separation of the fluorescamine-derivatized alendronate peak was achieved by reverse-phase HPLC. The mobile phase was composed of a 97:3 solution of 1 mM EDTA:methanol at a flow rate of 1 ml/minute through a 15- or 25-cm C18 column, with fluorescence detection ($\lambda_{ex} = 395 \text{ nm}$, $\lambda_{em} = 480 \text{ nm}$).

The derivatized alendronate peak eluted at approximately 3.5 minutes. Unreacted fluorescamine was well separated from the peak of interest, as shown in Figure 9.

Figure 9 Fluorescence HPLC chromatogram of alendronate standard, concentration 0.01 mg/ml. Mobile phase: 97:3 (1 mM EDTA (pH 6.5):methanol), 1 ml/min. Excitation wavelength, 395 nm, emission wavelength 480 nm.

3.4.5 Processing of Alendronate

A portion of the original alendronate powder, termed “ground”, was ground to a fine powder with a mortar and pestle for 10 minutes. Another sample, termed “lyophilized”, was dissolved in water and lyophilized at -40°C under vacuum for 3 days using a FreeZone 4.5-liter Benchtop Freeze Dry System (Labconco Corporation, Kansas City, MO).

3.4.6 In vitro Release Studies

Drug release from various polymer film formulations was determined by weighing individual films into 4 ml glass vials. One ml of PBS was added and the films were capped and incubated at 37°C with agitation. At defined intervals, the PBS was withdrawn for analysis and replaced with fresh PBS and returned to the chamber. Since alendronate is very soluble in water, sink conditions were maintained at all times. Details of specific studies are provided below.

To determine the amount of residual alendronate left in the films at the end of the in vitro release study, the films were dried under vacuum (27 mm Hg) for 90 minutes then dissolved in 1 ml dichloromethane. One ml water was then added and the alendronate was extracted into the aqueous layer with vigorous shaking. The concentration of alendronate in the aqueous layer was determined by fluorescence HPLC.

Loading efficiency was determined by weighing individual films into 4 ml glass vials. One ml dichloromethane was added to each film to dissolve the polymer. One ml water was then added and the alendronate was extracted into the aqueous layer with vigorous shaking. The concentration of alendronate in the aqueous layer was determined by fluorescence HPLC.

Release studies to determine the effects of alendronate processing on drug release from 85:15 PLGA were conducted as follows: individual films (n=5, ~10 mg/film) composed of 10% (w/w) lyophilized, as received or ground alendronate in PLGA were weighed into 4 ml glass vials, and 1 ml of phosphate buffered saline (PBS, pH 7.5) was added. The vials were capped and incubated at 37°C with gentle agitation. At 1 hour, and 6 hours, and then daily for 10 days, and every second day until day 18, the entire volume of buffer was removed and replaced with fresh PBS buffer. The concentration of alendronate in the withdrawn buffer was determined by the fluorescence HPLC method. Buffer samples that could not be tested immediately were sealed and stored at 2-8°C.

Release studies to determine the effects of alendronate processing on drug release from 20% PEG 600 in 85:15 PLGA were conducted in the same manner, except that the films were composed of 10% (w/w) lyophilized, as received or ground alendronate in 20% PEG 600 in 85:15 PLGA, and the study ended after 9 days because full release of the drug from the films had been achieved.

Release studies to determine the effects of increasing concentrations of diblock copolymer were performed in the same manner using films (n=5) composed of 0%, 10%, 20% and 30% diblock copolymer (w/w) in PLGA containing 5% alendronate by weight. Samples were taken 1 hour, 6 hours, daily for 8 days, every 3-4 days until day 29 and weekly thereafter. Studies on samples containing 20% and 30% diblock were ended after 18 and 7 days respectively, when full drug release from the films had been achieved.

Release studies to determine the effect of low drug concentration films were performed using 3 different batches of films loaded with 0.10%, 0.25% and 0.50% (w/w) alendronate in 10% diblock in PLGA films. The PBS buffer was replaced on days 1, 2, 4 and 7 and weekly thereafter until day 28. Three films from each batch at each concentration were tested, and the plotted values are the mean of the 3 batches (n = 9 for each data point).

Release studies to determine the effect of glow discharging on drug release were performed in the same manner as the low drug concentration films. Films from the same

3 manufacturing batches were glow discharged for 4 minutes as described in 3.5.1 and run in parallel with the low drug concentration studies.

3.4.7 Surface Morphology

Surface morphology was determined using a scanning electron microscope (SEM). Powder samples of alendronate were dusted on the adhesive surface of the sample holder. A ~10 nm thick layer of 60:40 alloy of gold:palladium was sputtered on to the samples under vacuum. After coating, samples were placed in the SEM and observed at 20 kV.

The mean particle size was estimated by measuring the longest diameter of each particle clearly visible in the micrograph.

Figure 10 Scanning electron micrograph of sodium alendronate trihydrate as received from Dr. Wang. Mean particle size ~ 30 μm (range 9-97 μm).

3.4.8 X-Ray Powder Diffraction

X-ray powder diffraction patterns of alendronate and alendronate in polymer films were obtained using a Bruker D8 Advance diffractometer in Bragg-Brentano configuration with a Cu source at 25°C. Samples were scanned from 5.000 - 45.000° 2 θ , using a step size of 0.020°, and a step time of 1 second per step. Samples of approximately 300 mg

were packed into standard Bruker sample holders. The polymer films were adhered to a glass slide and the slides were mounted, with plasticine, flush to the diffracting plane (top) of the sample holders. To improve particle statistics, all samples were rotated during data collection.

The theoretical powder diffraction data were created by converting the fractional atomic coordinates of the published structure from the Cambridge Structural Database, from the .cif to the .res format using Mercury Crystal Structure Visualization software (CSD, Cambridge, UK). Then Powdercell 2.4 (Federal Institute for Materials Research and Testing, Berlin, Germany) was used to open the .res file and calculate the theoretical diffraction pattern.

3.4.9 Thermal Analysis

Polymer samples weighing 2-5 mg were accurately weighed on a microbalance into aluminum pans and an aluminum lid was crimped in place. Samples were heated to 200°C at a rate of 10°C/min. The initial heat was followed by a cooling scan from 200°C to -50°C and a second heating scan from -50°C to 200°C under the same conditions. Diblock samples followed the same protocol, except they were cooled to -100°C. The glass transition temperatures were taken as the midpoint of the heat capacity change in the second heating.

Thermogravimetric analysis was performed by weighing ~50 mg alendronate samples directly onto the pan. The temperature was increased from 20–500°C at a rate of 20.0°C/minute, or isothermal if the change in percent weight was greater than 1.00%.

3.4.10 In vitro Degradation Studies

The short-term degradation of diblock from the diblock in PLGA films was monitored by gel permeation chromatography. Duplicate samples (~10 mg) from three batches of 10% diblock in PLGA films with and without glow discharging were placed in glass tubes with 1 ml PBS in a 37°C chamber with agitation. After 3 or 7 days, the PBS was removed and the films were dried under vacuum at room temperature.

Gel permeation chromatography was used to determine any change in the polymer ratios or diblock molecular weight (Jackson *et al.* 2004). HPLC grade chloroform (Fisher) was used as the mobile phase at a flow rate of 1 ml/min. Samples were prepared at a concentration of approximately 1.0% (w/v) i.e. a 10 mg piece of film dissolved in 1 ml of chloroform. The injection volume was 20 µl.

The experiments were performed on films that did not contain alendronate. Alendronate is not soluble in chloroform and the particles could create an obstruction in the chromatography system.

A typical gel permeation chromatogram is shown in Figure 11. PLGA elutes at 10 minutes; the diblock peak appears as a shoulder at 12 minutes.

Figure 11 GPC elution profile of a 10% diblock in 85:15 PLGA film (not glow discharged) after 3 days in PBS at 37°C. Solvent: chloroform, 1 ml/minute; injection volume 20 μ l.

Polystyrene standards were used to construct a calibration curve to detect any changes in the diblock molecular weight. A calibration graph of log molecular weight versus retention time (Figure 12) was established for the column using polystyrene standards with molecular weights of 300, 1000, 4000, 9000, 20,000, and 30,000 g/mol (PolySciences Inc., Warrington PA). A linear relationship between log molecular weight and retention time was observed ($R^2 = 0.993$).

Figure 12 GPC standard curve for polystyrene standards with molecular weights from 300 to 30,000 g/mol. (Mobile phase: chloroform at 1.0 ml/min. through two Styragel[®] columns (HR3 and HR1) connected in series with refractive index detection at 40°C, injection volume: 20 µl). Equation: $y = -0.3103x + 7.6411$, $R^2 = 0.993$.

A universal calibration curve for gel permeation chromatography can be used to calculate molecular weight from retention time. The product of the intrinsic viscosity $[\eta]$ and molecular weight is related to hydrodynamic volume, and once established for a given column, temperature and solvent, it can be used to determine the molecular weight of any random coil polymer (Grubisic *et al.* 1967). A plot of $\log [\eta]M.W.$ versus retention time will yield a linear curve for a wide range of polymers. Unfortunately, the intrinsic viscosity for the diblock was not determined, so the reported values for molecular weight are not absolute, but do show relative changes.

The retention time of the PLGA peak was not in the calibration range, so the molecular weight of PLGA was not determined. However, the ratio of the diblock to PLGA peak heights was determined in an attempt to determine a significant loss of diblock from the films over time.

3.4.11 Film Thickness

The thickness of the polymer films was determined by taking measurements in at least 3 places on each film with callipers capable of reading 0.001 mm (Mitutoyo, Kawasaki, Japan). Samples of various drug concentrations (0 to 0.5%) were taken from 4 batches of alendronate-loaded polymer films.

3.5 Osteoblast Culture Studies

3.5.1 Glow Discharge

PLGA films were treated with argon plasma glow discharge to provide sterility and a negatively charged surface to which the cells could adhere. The films, still on their Teflon[®] templates, were placed in the glow discharge chamber. The chamber was evacuated for 4 minutes, then flushed with argon and re-evacuated 3 times. An RF field was used to generate an argon plasma for 4 minutes.

3.5.2 Cell Proliferation by Staining

Polymer films composed of 10% diblock in PLGA and containing 0, 0.1, 0.25 or 0.5% ground alendronate were sterilized by glow discharge and placed in individual wells in a 12-well plate. Thermanox[™] tissue-culture treated cover slips were used as a control.

Two milliliters of osteoblast media and 50,000 osteoblast cells were added. Media was exchanged on day 1, 2, 3, 4 and 7. On days 1, 2, 4, and 7, films of each concentration were removed to a separate 12 well plate and fixed by replacing the cell culture medium with 4% formaldehyde solution (EM Grade, Canemco Int. St. Laurent) in PIPES buffer (Sigma, St. Louis, MO) for 10 minutes at room temperature. The sample films were then rinsed 3 times with 2 ml PBS buffer.

Osteoblasts on sample films were stained with sulforhodamine B acid chloride (Fluka Biochemika, Oakville, ON) for 30 minutes, and then rinsed with PBS until the solution ran clear. The cell nuclei were stained with Hoescht Stain Solution (Sigma, St. Louis, MO) for 1 minute then the films were rinsed 3 times with 2 ml PBS buffer.

Films were mounted between 2 glass slides and 8 representative images per film were recorded at 10X magnification using the DAPI filter to visualize entire osteoblasts or the Rhod filter for nuclei only. The numbers of cells per image were counted manually.

3.5.3 Cell Proliferation by MTS

The effect of alendronate in solution on osteoblast cells was determined by seeding 24-well plates with 1 ml of osteoblast media containing 20,000 cells/ml. A 100- μ l aliquot

containing 0, 0.01, 0.1, 1, 10, or 100 $\mu\text{g/ml}$ alendronate in water was added and the plates were incubated for 2 days in an incubator at 37°C in a humidified atmosphere and 5% CO_2 . Then the osteoblast media was replaced with 1 ml of PC-3 media and 0.1 ml of MTS solution was added (osteoblast media interferes with the MTS assay). The plates were incubated 2 hours, and 100 μl samples from each well were transferred to a 96-well plate for analysis. The absorbances at 492 and 620 nm were recorded. The difference between the absorbance at 492 nm and the blank absorbance at 620 nm was reported. Data were normalized with respect to the absorbance of the control wells (no alendronate). The experiment was repeated three times with different batches of films and different passages of osteoblasts, with $N=4$ replicates at each alendronate concentration.

The proliferation of osteoblast cells on polymer films was determined as follows. Polymer films containing 0, 0.1, 0.25 and 0.5% alendronate were sterilized by glow discharge and placed treated side up in individual wells in a 12-well plate. Two millilitres of osteoblast media containing 20,000 cells/ml was added to each well and the osteoblasts were allowed to proliferate over the films and along the sides of the well as shown in Figure 13. Control wells contained the same amounts of cells and osteoblast media, but no films or drug.

Figure 13 Schematic diagram of osteoblast cells proliferating over drug-loaded films and along walls of 12-well plate

In order to distinguish proliferation on the films from total osteoblast proliferation, at days 1, 2, 4 and 6 or 7, the films were removed to a second 12-well plate and incubated in 1 ml PC-3 media and 0.1 ml MTS solution for 2 hours. In the control wells and the now empty treatment wells, the osteoblast media was replaced with 1 ml PC-3 media and 0.1 ml MTS solution. After 2 hours incubation at 37°C , a 0.1 ml aliquot from each well was transferred to a 96-well plate and read at 492 and 620 nm. The absorbance at 620 nm was subtracted from the value at 492 nm as a blank, and the results were normalized with

respect to the absorbance in the control wells, which contained no films and no drug. Osteoblast proliferation on the alendronate-loaded films alone, as well as total osteoblast proliferation in the sum of the films and the wells were recorded. The experiment was repeated 3 times with different batches of films and different passages of osteoblasts, with N=3 replicates at each alendronate concentration. However, one set of experiments had to be discarded because the osteoblasts had reached confluence prior to the start of the experiment and failed to continue to proliferate.

3.6 Statistical Analysis

Data collected from multiple independent samples from the same experiment are presented as mean $\pm$ standard deviation, where the N number represents the number of samples. If the experimental design provided for multiple sample sets each composed of several replicates, the data are reported as mean $\pm$ standard error of the mean, with the N number representing the number of samples sets used in the analysis. Statistical analysis was performed using OriginPro version 7.5 (OriginLab Corporation, Northampton MA) with a significance level of $p < 0.05$. The two-tailed Student's *t*-test were used for comparisons between groups with a significance level of $\alpha < 0.05$ considered to be statistically significant and marked with an asterisk (*).

An analysis of similarity and difference (*f*₁, *f*₂) of the drug release curves was performed in accordance with the FDA Guidance for Industry: Dissolution Testing of Immediate Release Solid Oral Dosage Forms (Center for Drug Evaluation and Research 1997). The following description of the approach is quoted from this guidance document:

“This model-independent approach uses a difference factor (*f*₁) and a similarity factor (*f*₂) to compare dissolution profiles (Moore and Flanner 1996). The difference factor (*f*₁) calculates the percent (%) difference between the two curves at each time point and is a measurement of the relative error between the two curves:

$$f_1 = \{ [\sum_{t=1}^n |R_t - T_t|] / [\sum_{t=1}^n R_t] \} \cdot 100$$

Equation 4

where *n* is the number of time points, *R*_{*t*} is the dissolution value of the reference (prechange) batch at time *t*, and *T*_{*t*} is the dissolution value of the test (postchange) batch at time *t*.

The similarity factor (f2) is a logarithmic reciprocal square root transformation of the sum of squared error and is a measurement of the similarity in the percent (%) dissolution between the two curves.

$$f_2 = 50 \cdot \log \left\{ \left[1 + \left(\frac{1}{n} \right) \sum_{t=1}^n (R_t - T_t)^2 \right]^{-0.5} \cdot 100 \right\}$$

Equation 5

For curves to be considered similar, f1 values should be close to 0, and f2 values should be close to 100. Generally, f1 values up to 15 (0-15) and f2 values greater than 50 (50-100) ensure sameness or equivalence of the two curves and, thus, of the performance of the test (postchange) and reference (prechange) products.

This model independent method is most suitable for dissolution profile comparison when three to four or more dissolution time points are available. As further suggestions for the general approach, the following recommendations should also be considered:

- The dissolution measurements of the test and reference batches should be made under exactly the same conditions. The dissolution time points for both the profiles should be the same (e.g., 15, 30, 45, 60 minutes). The reference batch used should be the most recently manufactured prechange product.
- Only one measurement should be considered after 85% dissolution of both the products.
- To allow use of mean data, the percent coefficient of variation at the earlier time points (e.g., 15 minutes) should not be more than 20%, and at other time points should not be more than 10%."

4 RESULTS AND DISCUSSION

4.1 Assay Development

4.1.1 Sodium Alendronate Trihydrate

Sodium alendronate trihydrate has a molecular formula of $C_4H_{12}NaNO_7P_2 \cdot 3H_2O$ and a molecular weight of 325.12. The chemical structure is shown in Figure 14 in two representations.

Figure 14 Chemical structure and ball-and-stick model of alendronate. Carbon atoms are shown in grey, phosphorus in pink, oxygen in red, nitrogen in blue and hydrogen in white.

Alendronate has 5 pKa values: 0.8, 2.2, 6.3, 10.9 and 12.2. At physiological pH, alendronate is a zwitterion with an overall charge of -2 (3 negative charges on the phosphate groups and a protonated amine group). Alendronate is freely soluble in water (10 mg/ml), but insoluble in most other common solvents. The trihydrate has a monoclinic crystal structure (Vega *et al.* 1996) and there are no reported polymorphs.

The solubility of sodium alendronate trihydrate in deionized water and phosphate buffered saline (pH = 7.5) at 37°C was determined in order to establish that solution saturation would not be a limitation in the planned drug release studies (Figure 15).

Figure 15 Solubility of sodium alendronate trihydrate at 37°C in (×) phosphate buffered saline (pH 7.5) and (◇) distilled water (pH 5.7).

The solubility of alendronate in 37°C deionized water was determined to be 34 mg/ml. In PBS, the solution showed a small degree of supersaturation at 24 hours, which then re-equilibrated and the solubility was determined to be 47 mg/ml. The difference in solubility was attributed to the difference in pH between water (5.7) and PBS (7.5). Alendronate has a pKa value at 6.3, so at pH 7.5 the concentration of ionized species would be higher, thus increasing the solubility.

4.1.2 Analytical Method Literature Review

The first goal of the project was to develop, optimize and validate a suitably sensitive analytical method for the determination of alendronate in the drug release assays. The published analytical methods for bisphosphonates similar to alendronate (i.e., alendronate, etidronate and pamidronate) were reviewed. These compounds do not possess a chromophore, so detection must be performed indirectly or by derivatization. The literature on bisphosphonate analysis can be roughly divided into 4 approaches of increasing complexity, as shown in Table 5.

Table 5 Literature Review: Bisphosphonate Analytical Methods

Analytical Method	Observations	References
UV Spectroscopy	<ul style="list-style-type: none">- Suitable for samples of known composition and concentration (e.g., pharmaceutical preparations)- Fast- Cannot distinguish impurities or matrix effects- Limited linear range	(Ostovic <i>et al.</i> 1993; Kuljanin <i>et al.</i> 2002; Taha and Youssef 2003; Al Deeb <i>et al.</i> 2004; Tzanavaras <i>et al.</i> 2005)
Ion Chromatography	<ul style="list-style-type: none">- Suitable for samples of known composition (e.g., pharmaceutical preparations)- Detection by refractive index, indirect UV or conductivity measurement- Unsuitable for samples with a high concentrations of ions in the matrix	(Tsai <i>et al.</i> 1992; denHartigh <i>et al.</i> 1993; Quitasol and Krastins 1994; Tsai <i>et al.</i> 1994; Sparidans <i>et al.</i> 1995)
HPLC with On-line Derivatization	<ul style="list-style-type: none">- Suitable for samples of unknown composition- Significant sample preparation required- On-line reactors required for some methods- Wide linear range- Low limit of quantitation- Good reproducibility	(Daley-Yates <i>et al.</i> 1989; DeMarco J.D. <i>et al.</i> 1989; Flesch and Hauffe 1989; Kline <i>et al.</i> 1990; Flesch <i>et al.</i> 1991; Kline and Matuszewski 1992)
Co-precipitation, extraction and derivatization followed by fluorescence HPLC	<ul style="list-style-type: none">- Suitable for biological samples- Extensive sample preparation required- Low limit of quantitation- Internal standard required for reproducibility in co-precipitation/extraction steps	(King and Vieth 1996; Sparidans <i>et al.</i> 1997; Sparidans <i>et al.</i> 1997; Sparidans <i>et al.</i> 1998; Sparidans and den Hartigh 1999; Ptacek <i>et al.</i> 2002; Wong <i>et al.</i> 2004; Yun and Kwon 2006)

The suitability and feasibility of each of these analytical approaches was evaluated, as shown in the following sections. Methods for the determination of alendronate in biological fluids such as urine and plasma were not considered, because under the original scope of this project, biological samples were not expected. These methods generally involve an extensive sample preparation scheme including protein precipitation, calcium precipitation, solid phase extraction and derivatization to determine alendronate. A similar bisphosphonate such as pamidronate can be added as an internal standard to improve reproducibility.

4.1.3 Alendronate Analysis by UV-Vis

Spectrophotometric methods for the analysis of alendronate have been recommended as a rapid, inexpensive assay method for the analysis of pharmaceutical samples with predictable concentrations (Kuljanin *et al.* 2002;Taha and Youssef 2003;Taha and Youssef 2003;Al Deeb *et al.* 2004;Tzanavaras *et al.* 2005). Using the method reported by Taha (Taha and Youssef 2003) that measures the reduction in cerium ammonium nitrate signal intensity at 320 nm in the presence of alendronate, a linear response in the range 2 – 24 µg/mL was obtained. The published method suggests a 1 hour incubation period after the addition of the ceric ammonium nitrate solutions; however, it was found that the length of the incubation period could be reduced to as little as 10 minutes without loss of linearity. In addition, standard solutions were found to remain stable for at least 2 hours after the incubation period.

The linear range (2 – 24 µg/mL) was in agreement with the published value. However, this narrow linear range was not suitable for drug release studies, where low concentration samples were expected. An analytical method with broader range and lower limit of quantitation was required.

4.1.4 Alendronate Analysis by Ion Chromatography

The ion chromatography HPLC methods developed by Tsai (Tsai *et al.* 1992;Tsai *et al.* 1994) and by Han (Han and Qin 1996) were investigated. All bisphosphonates are strongly ionic at neutral pH, making ion chromatography a logical choice. The stationary phase of an anion exchange column contains positively charged resin particles, such as a quaternary ammonium group covalently bound to a polymeric resin. Negatively charged molecules in the test solution, such as alendronate, would replace the counter ion and bind to the positively charged group on the resin. The alendronate was then detected indirectly by loss of refractive index or UV-Vis (245 nm) signal compared to the mobile phase (nitric acid).

In the first method, the mobile phase was composed of 6 mM nitric acid at a flow rate of 0.5 ml/minute with a refractive index and UV-Visible detector (235 nm) connected in series, as shown in Figure 16. The linear ranges were determined to be 150-1000 µg/mL by refractive index detection or 50-1000 µg/mL by UV detection. UV detection was selected as the method of choice because of its lower limit of quantitation. The method was found to be rugged to changes in the flow rate from 0.45 to 0.55 ml/minute.

Figure 16 Chromatogram of 0.5 mg/ml alendronate in phosphate buffer by ion chromatography. Mobile phase: 6 mM nitric acid, flow rate: 0.5 ml/minute, injection volume 20 μ l, detection by A) refractive index or B) UV-Vis at 235 nm.

The specificity of the method with respect to other common ions was determined by injecting samples of various salt solutions and observing the peak positions as shown in Table 6. The negative peak at ~2.5 minutes (UV-Vis) was common to all chromatograms including the blank and was associated with the absence of nitrate in the sample. All of the common ion peaks were well separated from the alendronate peak.

Table 6 Specificity of Alendronate by Ion Chromatography: Retention Times of Common Ions

Ion Solution	Concentration	UV-Vis Retention Time (minutes)
Alendronate	0.15 mg/ml	Alendronate = 3.331
Potassium Chloride	0.2 mg/ml	K^+ = 1.535
Sodium Chloride	0.29 mg/ml	Na^+ = 1.540
Sodium Phosphate	0.35 mg/ml	Na^+ = 1.529 PO_4^- = 4.947
Calcium Phosphate	0.15 mg/ml	Ca^{2+} = 1.453 PO_4^- = 4.783

In an attempt to lower the limit of quantitation, the method of Sparidans *et al.* (Sparidans *et al.* 1995) was investigated. This group adapted the original ion chromatography method by adding copper (II) nitrate to the mobile phase and altering the UV detection wavelength to 245 nm to obtain the best signal-to-noise ratio. In aqueous solutions, bisphosphonates and copper form soluble UV-absorbing complexes. Sparidans *et al.* showed that altering the flow rate and the concentrations of nitric acid and copper (II) nitrate could optimize the method for a variety of bisphosphonates similar to alendronate.

This method was adapted by adding 0.5 mM copper (II) nitrate, decreasing the nitric acid concentration to 1 mM and increasing the flow rate to 1.0 ml/minute. This reduced the limit of quantitation to 5 µg/mL. A representative chromatogram is shown in Figure 7. Alendronate eluted at 1.5 minutes, well separated from the injection front (0.7 minutes). Copper eluted as a negative peak at 4-5 minutes. The specificity of the copper/ion chromatography method was confirmed by injecting samples containing low concentrations of other common ions. All chromatograms showed peaks for the injection front and copper. No additional peaks were observed for 10 mM sodium chloride or 0.2 mg/ml potassium chloride solutions. The peak positions and orientations for the following solutions are indicated in brackets: pH 6.5 phosphate buffer (small positive peak at 1.3, large positive peak at 3.5, large negative peak at 7.8 minutes), 5 mM TRIS buffer (positive peak at 3.6 min.), and $Ca_5(PO_4)_3OH$ (negative peak at 6.7 min.), 2.5 mg/ml collagen (large negative peak at 13 minutes), pH 7.4 phosphate buffered saline

(large positive peak at injection front, large positive peak at 4 minutes, very large negative peak at 15-22 minutes). Hence the method was specific for alendronate in the presence of low concentrations of other ions.

However, when this method was used in drug release studies where the concentration of alendronate was low and the concentration of buffer ions, especially saline, was high, the anion exchange column was overwhelmed (Figure 17). The loss of resolution between the alendronate and chlorine peaks increased the limit of detection and decreased the precision to unacceptable levels.

Figure 17 Chromatogram of Alendronate in PBS by Ion Chromatography. Mobile phase: 0.5 mM copper (II) nitrate, 1.0 mM nitric acid, flow rate: 1.0 ml/minute, injection volume 20 μ l, detection by UV-Vis at 245 nm.

The ion chromatography method was useful for samples dissolved in simple matrices, such as the alendronate-coated tantalum samples prepared by Dr. Rizhi Wang's groups at the UBC Department of Metals and Materials Engineering. No sample preparation was required, the materials and equipment were commonly available, and the run time was only 6 minutes. However, the interference by PBS meant that another analytical method was required in order to perform drug release studies.

4.1.5 Other methods

In order to overcome the interference of PBS, the ion chromatography system was coupled to an electrospray mass detector in the negative ionization mode was attempted (Qin *et al.* 1994). The mobile phase was composed of 5% acetonitrile, 95% water, and 0.2% formic acid at a flow rate of 1.0 ml/minute with the same column from the previous ion chromatography experiments (IC-Pak™ Anion-HR column). The parent peak ($m/z = 248$, formula weight minus 3 waters of hydration, sodium and a proton) was observed at ~4.5 minutes. Low peak areas were resolved by increasing the injection volume from 10 to 200 μ l. However, a set of alendronate standards diluted in PBS had poor peak shapes indicating that a large fraction of the alendronate was not retained on the column. The mass spectrometry method was not pursued further.

The other published HPLC methods (DeMarco J.D. *et al.* 1989;Kwong *et al.* 1990;Kline and Matuszewski 1992;King and Vieth 1996;Sparidans *et al.* 1997;Sparidans *et al.* 1998;Ptacek *et al.* 2002) involved uncommon laboratory equipment such as on-line, pre-column or post-column derivatization apparatus or conductivity detectors. Automation of the derivatization process was essential for accuracy and reproducibility because some of these derivatized bisphosphonate compounds were unstable with respect to time. There were also reports of gas chromatographic methods (Sakiyama *et al.* 1996;Muntoni *et al.* 2004) or gas chromatography coupled to a mass spectrometer for detection (Leis *et al.* 2004;Endele *et al.* 2005). Finally, there have been 2 publications in which inductively coupled plasma was used to detect phosphorus in the bisphosphonate, either in solutions (Reed *et al.* 1995), or after separation on an ion exchange column (Kovacevic *et al.* 2004). A less elaborate method was considered optimal.

4.1.6 Alendronate Analysis by Fluorescence HPLC

The next analytical approach involved derivatizing the amino group on the drug with a fluorescent tag molecule which could then be analyzed by reverse-phase HPLC. A previously published method for the analysis of pamidronate in biological samples using fluorescamine as the fluorescent tag was adapted (Flesch and Hauffe 1989;Flesch *et al.* 1991;Wong *et al.* 2004;Weidenauer *et al.* 2004). Both pamidronate and alendronate have an amino group on the R_2 side group that can be readily derivatized with fluorescamine. The protein precipitation, calcium precipitation, and solid phase extraction steps common to many of the methods that were originally intended for plasma and urine samples were omitted. Briefly, the alendronate sample was diluted in an EDTA buffer at pH 10. A solution of fluorescamine in acetonitrile was added and reacted with the alendronate amino group within seconds, as shown in Figure 18.

Figure 18 Reaction of fluorescamine with an amino group creating a fluorescent fluorophore

The acetonitrile was extracted with dichloromethane and the aqueous layer was withdrawn for HPLC analysis with fluorescence detection. It was found that changes could be made from the published methods such as: altering the volumes and concentrations of EDTA buffer and fluorescamine, and omitting the centrifugation step separating the organic and aqueous layers, due to the comparative simplicity of the samples.

A summary of the validation results for the fluorescence method are shown in Table 7. The supporting data are discussed in detail in the following sections. The validation parameters have been taken from the ICH Harmonized Tripartite Guideline Q2B: Validation of Analytical Procedures: Methodology (International Conference on Harmonization 1996).

The linear range was very broad (1 – 2000 $\mu\text{g/mL}$), and the limit of quantitation was sufficiently low enough for drug release studies. More importantly, the method was specific for alendronate in the presence of PBS. It was therefore deemed suitable for determining alendronate in drug release studies.

Table 7 Method Validation Data for Fluorescence HPLC Analysis of Alendronate

Validation Characteristic	Result ^a
Specificity	Specific for alendronate in the presence of PBS, PLGA, diblock Not specific in the presence of TRIS and chitosan
Linearity	Typical equation: $y = 7 \times 10^6 x + 2000$, $R^2 = 0.999$
Range	0.5 – 2000 µg/mL
Accuracy	90-101%
Precision	
Repeatability	RSD = 0.9% (n=6, 10 µg/mL)
Inter-day precision	RSD = 5.0% (3 days, 10 µg/mL)
Detection limit	0.2 µg/mL (based on 3X signal to noise ratio)
Quantitation limit	0.5 µg/mL (RSD <2%)
Robustness	The following adjustments to the HPLC mobile phase did not change the size or position of the alendronate peak: 5 or 10% methanol 3% acetonitrile pH lowered to 5.3

^a RSD = Relative standard deviation (standard deviation/mean x 100)

4.1.6.1 Specificity

The International Conference on Harmonization defines specificity as “the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.” Fluorescence HPLC is an inherently specific method. The excitation wavelength of 395 nm and the emission wavelength of 480 nm exclude interference from most common solvents and organic impurities, which absorb below 300 nm. In addition, reverse-phase chromatography will separate an impurity that does have the same excitation/emission profile.

Typical chromatograms had only two peaks, as shown in Figure 9, the analyte eluting at ~3.5 minutes and the unreacted fluorescamine at ~10 minutes. Common buffers such as

phosphate buffered saline had no effect on the chromatographic profile. However, substances that contain an amino group (i.e., TRIS buffer, chitosan) that could be derivatized by fluorescamine are potential impurities. In small quantities, they would show up as additional peaks on the chromatogram. In large quantities, they will compete with alendronate to react with the fluorescamine, despite being well resolved from the alendronate peak. Therefore, the method was not specific for alendronate in the presence of TRIS or chitosan.

4.1.6.2 Linearity

A linear relationship between the concentration of standard alendronate and the alendronate peak area was demonstrated over the extended linear range of 0.5 to 2000 µg/ml, as shown in Table 8 and Figure 19. Under routine analysis conditions (0.5 to 10 µg/ml) the coefficient of determination (R^2) was generally >0.999, and a typical equation was $y = 7451565 + 1449x$.

Table 8 **Linearity of Alendronate Response by Fluorescence HPLC**

Standard Alendronate (µg/ml)	HPLC Response (Absorbance Units)			Mean HPLC Response	RSD ^a (%)
	Replicate 1	Replicate 2	Replicate 3		
0.1	3676	3466	3277	3473	5.7
0.2	3723	3603	3815	3714	2.9
0.5	5709	5687	5617	5671	0.8
1.0	9848	9866	9865	9860	0.1
5.0	33607	33674	33843	33708	0.4
10	66275	66627	66836	66579	0.4
50	418270	418269	419707	418749	0.2
100	838029	840921	838687	839212	0.2
500	4192424	4184897	4193570	4190297	0.1
1000	7956390	8006906	8023749	7995682	0.4
1500	11310551	11136838	11162489	11203293	0.8
2000	13744836	13486590	13422757	13551394	1.3

^a RSD = Relative standard deviation (standard deviation/mean x 100)

Figure 19 Linearity of fluorescence HPLC standard curve for sodium alendronate standard solutions with concentrations of A) 0.5 to 2000 µg/ml ($R^2 = 0.9936$) or B) 0 to 10 µg/ml ($R^2 = 0.9996$). Mobile phase 97:3 1 mM EDTA(pH 6.5):Methanol, 1 ml/min., 25 cm C18 column, $\lambda_{\text{ex}} = 395$ nm, $\lambda_{\text{em}} = 480$ nm.

4.1.6.3 Accuracy

Accuracy is the extent to which the test results represent the true value (International Conference on Harmonization 1996). The ICH recommends "Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals." The accuracy of the fluorescence method was assessed by comparing the result for a series of alendronate standards prepared from pure sodium alendronate trihydrate (Sigma, St. Louis, MO) with the results obtained from the working alendronate standards prepared from material donated by Dr. Wang, shown in Table 9. The concentration of the lower reference standards (0.5 to 100 µg/ml) was calculated using the working standards in the same (lower) concentration range, because the high standards distorted the intercept. A similar practice of using standards no greater than 10 times more concentrated than the samples was employed during subsequent drug release studies.

Table 9 Accuracy of Fluorescence HPLC Method for Determination of Alendronate

Expected Alendronate Concentration (µg/ml)	Observed Alendronate Concentration Mean (RSD ^a , n=3) (µg/ml)		% Recovery
0.5	0.5	(5.9%)	90%
1.0	0.9	(1.7%)	93%
10	9.5	(0.3%)	95%
100	98.9	(0.3%)	99%
1000	1008.9	(0.3%)	101%
2000	1976.6	(0.1%)	99%

^a RSD = Relative standard deviation (standard deviation/mean x 100)

The recovery data demonstrated that the method was accurate to within 10% of the expected value down to a concentration of 0.5 µg/ml. As expected, the accuracy decreased as the lower limit of quantitation was approached.

4.1.6.4 Precision

Precision is the measure of the degree of repeatability of an analytical method under normal operation and is normally expressed as the percent relative standard deviation for a statistically significant number of samples.

Repeatability or, inter-assay precision, should be determined from a minimum of nine determinations covering the specified range of the procedure or from a minimum of six determinations at 100% of the test or target concentration. At a concentration of 10 µg/ml, the RSD of 6 determinations was 0.9%. The repeatability results at other concentrations are reported in Table 8, and range from 0.1 to 5.7% RSD based on 3 determinations at each concentration. Adequate repeatability (<2.0%) was observed at all but the 2 lowest concentrations.

Intermediate precision is the variation in results from within the same laboratory due to random events such as different days, analysts, equipment, etc. The inter-day precision is shown in Table 10. Standard alendronate samples at 5 typical concentrations were analyzed in triplicate on 3 separate days. The relative standard deviations of the daily and overall mean peak area were at or below 5.0%.

Table 10 Inter-Day Precision of Fluorescence HPLC Method for Determination of Alendronate

Alendronate Conc. (µg/ml)	Day 1		Day 2		Day 3		All Days	
	Peak Area	RSD ^a (%)	Peak Area	RSD ^a (%)	Peak Area	RSD ^a (%)	Peak Area	RSD ^a (%)
5.2	44294	0.4	47158	1.2	48898	0.5	46783	5.0
10.4	82473	0.3	89843	0.3	82494	0.3	84937	5.0
20.8	177046	0.1	179602	0.2	164987	0.3	173878	4.5
52	431705	0.1	428098	0.3	418257	0.2	426020	1.6
104	849814	0.1	858425	0.2	844557	0.2	850932	0.8

^aRSD = Relative standard deviation (standard deviation/mean x 100)

During the course of the experiments, 3 different C₁₈ columns were used. No difference in the linear range was observed as a result of changing columns.

4.1.6.5 Detection Limit and Quantitation Limit

The limit of detection for alendronate was 0.2 µg/ml based on a signal-to-noise ratio of 3:1. The limit of quantitation was 0.5 µg/ml based on acceptable accuracy and linearity.

4.1.6.6 Range

The range of the assay, derived from the precision, accuracy and linearity experiments, was 0.5 to 2000 µg/ml.

4.1.6.7 Robustness

ICH defines robustness as "The reliability of the analysis with respect to deliberate variations in method parameters." The robustness of the method with respect to composition of the mobile phase, the sample vials and sample solution stability was examined.

The HPLC mobile phase normally contains 97% EDTA buffer (pH 6.5) and 3% methanol. The following adjustments to the composition of the mobile phase did not change the size or position of the alendronate peak: 5 or 10% methanol, 3% acetonitrile, 3% tetrahydrofuran, or pH lowered to 5.3. The position of the unreacted fluorescamine shifted ± 2 minutes.

Samples must be prepared in glass rather than plastic vials. The organic solvents in the sample preparation steps can extract impurities that increase noise of the baseline.

The sample solution stability was determined by observing the peak area of standard alendronate solutions analyzed after 24 hours. The recovery ranged from 99% to 139% at the lowest concentration. The concentrations tended to be higher than expected likely due to evaporation of the solvent.

Table 11 Sample Solution Stability of Fluorescence HPLC Method for Determination of Alendronate

Alendronate Conc. ($\mu\text{g/ml}$)	Initial		After 24 hours		Observed Concentration (mg/ml)	Recovery (%)
	Peak Area	RSD ^a (%)	Peak Area	RSD ^a (%)		
0.5	5279	0.7%	6374	6.4%	0.7	139%
1.04	9001	0.5%	10024	0.4%	1.2	111%
2.08	17753	0.2%	18739	1.2%	2.6	109%
5.2	41474	0.1%	41800	0.4%	5.2	99%
10.4	83337	0.3%	84497	0.6%	10.6	102%

^a RSD = Relative standard deviation (standard deviation/mean x 100)

The fluorescence HPLC assay for alendronate demonstrated sufficient specificity, linearity, accuracy, and robustness for drug release studies. It was used extensively in the next series of experiments to optimize the drug release rate from various polymer film formulations.

4.2 Formulation Development

Once the analytical method for alendronate was validated, the next objective of the project was to prepare controlled-release formulations of alendronate-loaded polymeric films.

4.2.1 Preparation and Characterization of Polymer Films

Three polymers were used in various combinations: PLGA, polyethylene glycol (PEG 600) and an amphipathic diblock copolymer of poly(DL-lactic acid-*block*-methoxy poly(ethylene glycol) (PDLLA-MePEG), referred to in this work as “diblock”. The films were cast from polymer solutions made by dissolving the appropriate amount of PLGA, PEG 600 or diblock in a small volume of dichloromethane. The finished films were clear and colorless; at high concentrations (5% or 10% w/w alendronate) the drug particles were visible in the films.

4.2.2 Effect of Processing on Alendronate Powder

The size of the alendronate particles dispersed in the polymer matrix had an impact on the drug release characteristics. Large particles were more difficult to disperse evenly and could protrude from the film, which would lead to low content uniformity among the films and could cause variability in drug release profiles.

4.2.2.1 Surface Morphology

Sodium alendronate trihydrate solid was obtained from Dr. R. Wang. The mean particle size was $\sim 30\ \mu\text{m}$ (range 9-97 μm). A second sample was ground to a fine powder with a mean particle size of 6 μm (range 2-18 μm). A third sample was lyophilized; it forms a fluffy white cake that was shown to be anhydrous by elemental analysis.

All three samples were examined by scanning electron microscopy (Figure 20). Grinding reduced the particle size and improved particle size uniformity. Lyophilized alendronate resembled fine glassy particles.

A)

B)

C)

Figure 20 Scanning electron micrographs of sodium alendronate A) as received (trihydrate), mean particle size 30 μm , B) ground (trihydrate), mean particle size 6 μm , C) lyophilized, glassy anhydrous particles.

4.2.2.2 X-Ray Powder Diffraction

The 3 samples were examined by X-ray powder diffraction (Figure 21) to determine if the sample processing had an effect on the crystal structure. The as received and ground samples possessed similar diffraction patterns with the exception that the 2θ peak positions of the as received sample were shifted by $\sim 0.1^\circ$. This was likely due to the displacement of the powdered sample above or below the diffraction plane of the sample holder. The peak positions and intensities were in good agreement with the published powder diffraction pattern for sodium alendronate trihydrate (Figure 22) (Vickers 2004). The lyophilized sample, however, had lost all evidence of reflecting planes. The featureless diffractogram indicated that lyophilization rendered alendronate amorphous.

Figure 21 X-Ray Powder Diffraction Patterns of Alendronate A) ground, B) as received and C) lyophilized.

Figure 22 Reference X-Ray Powder Diffraction Pattern of Sodium Alendronate Trihydrate, used with permission (Vickers 2004).

The crystal structure of a single crystal of sodium alendronate trihydrate has also been published (Vega *et al.* 1996). Using the published fractional atomic coordinates, the peak positions and relative peak intensities of the theoretical powder diffraction pattern were calculated. Table 12 compares the theoretical peak positions and intensities to those of the as received and ground samples. Peaks with relative intensities of less than 5% have been omitted for clarity.

The table shows excellent agreement between the theoretical peak positions and those of the ground sample. The as received sample also compared favourably, when the 0.1° shift due to sample position was taken into account. The differences in the relative peak intensities could be attributed to differences in the preferred particle orientations due to sample packing.

Table 12 X-Ray Diffraction Peak Positions and Relative Intensities of Theoretical, As Received and Ground Sodium Alendronate Trihydrate

Theoretical		As Received		Ground	
Angle 2 θ °	Intensity %	Angle 2 θ °	Intensity %	Angle 2 θ °	Intensity %
9.22	100	9.35	100	9.22	82.7
12.38	41.7	12.50	47.9	12.38	100
13.48	80.6	13.60	98.0	13.48	88.8
13.98	6.0	14.10	8.8	13.96	9.0
17.00	7.8				
17.11	9.9	17.20	23.0	17.10	19.8
17.14	6.7				
18.50	16.0	18.61	19.0	18.50	15.2
19.71	16.6	19.81	22.4	19.72	21.4
20.25	15.8	20.34	27.5	20.26	19.9
20.98	19.5	21.09	22.5	20.98	17.1
21.80	8.2	21.89	11.1	21.80	11.8
23.34	20.0	23.43	24.5	23.34	21.9
24.26	4.2	24.35	12.6	24.25	9.5
24.88	26.0	24.97	40.3	24.88	42.6
24.91	7.7				
		25.35	7.0		
26.14	21.8	26.25	33.2	26.14	25.7
26.19	11.5				
26.41	2.5			26.41	6.5
28.00	2.9	28.10	8.2	28.00	5.1
28.41	11.4	28.49	18.7	28.41	13.4
29.96	35.1	30.05	46.2	29.96	39.1
30.61	5.6	30.70	8.6	30.61	6.4
31.21	7.8	31.36	12.3	31.23	8.4
31.37	6.1				
31.94	9.2	32.02	12.8	31.94	11.8
32.33	4.9	32.43	11.8	32.34	7.7
33.01	7.6	33.09	17.5	33.02	12.7
34.23	18.7	34.33	27.9	34.24	15.4
34.76	2.8	34.78	6.4	34.71	4.3
35.46	10.3	35.55	13.2	35.45	9.4
37.23	5.1	37.28	7.9	37.19	5.3
37.75	8.5	37.83	9.8	37.75	13.1
38.48	2.3	38.56	8.9	38.48	4.9
38.86	6.0	38.92	17.1	38.85	8.5
39.16	9.1	39.23	15.8	39.14	9.8
40.27	2.9	40.33	6.5	40.27	4.5
40.69	2.7	40.76	5.7	40.69	4.1
41.24	1.5	41.42	9.9	41.22	4.2
		41.58	7.8		
41.97	11.1	42.04	17.1	41.97	12.3
42.61	4.8	42.64	11.9	42.57	6.5

4.2.2.3 In Vitro Alendronate Release Profiles

To determine the comparative drug release profiles of the 3 types of alendronate (as received, ground and lyophilized), they were prepared as 10% (w/w) drug-loaded PLGA films. The lyophilized form was fluffy and difficult to weigh accurately. The cumulative release profiles are shown in Figure 23.

Figure 23 Cumulative Release of Alendronate from 10% (w/w) drug-loaded 85:15 PLGA films containing: (Δ) lyophilized, ($\diamond$) as received and ($\square$) ground alendronate in PBS at 37°C. 0.1 mg/mg film represents 100% theoretical loading. Error bars indicate standard deviation among $n=5$ films of each type.

The films containing lyophilized samples and larger alendronate crystals (as received) showed more rapid rates of drug release than films with ground alendronate. By Day 1, more than 50% of the theoretical alendronate content had released from the as received sample films; whereas less than 35% had been released from the ground sample films. Larger particles might be expected to leave larger pores as the drug dissolved then diffused from the films. The large particles may even have protruded from the film surface. The smaller particles were expected to be better dispersed throughout the polymer matrix, and thus released alendronate in a more controlled manner. In previous

reports of bisphosphonate-loaded polymeric drug delivery systems, reducing particle size led to more controlled drug release characteristics (Perugini *et al.* 2001; Weidenauer *et al.* 2004). The lyophilized and as received sample films also showed greater variability among films, as indicated by the size of the error bars (standard deviation).

The loading efficiency of the manufacturing process was determined by measuring the alendronate concentration in films ($n = 2$ to 5) prior to in vitro release studies. The loading efficiencies, expressed as percent of the theoretical drug loading (0.1 mg/mg film), were: as received 84%, ground 95% and lyophilized 91%. The different values for total alendronate content in the films was a function of the manufacturing process and the analytical method.

Based on the more consistent nature of the drug release profile and loading efficiency, ground alendronate was selected as the material of choice for future experiments.

4.2.2.4 Investigation of Potential Solvate Formation

The marketed pharmaceutical formulation is sodium alendronate trihydrate; however, both a monohydrate and an anhydrous form have been reported. Since, during the preparation of the polymeric films, alendronate was suspended in dichloromethane, it was possible that solvates might be formed. Hence, a sample of sodium alendronate trihydrate was agitated in dichloromethane for 3 days at 37°C and then dried at room temperature for two days. The resulting product was compared to untreated sodium alendronate trihydrate using thermogravimetric analysis and X-ray powder diffraction.

Thermogravimetric analysis (TGA, Figure 24) did not detect any events indicating a loss of solvated dichloromethane. Loss of 3 moles of water was observed at 123°C (expected: 16.6%, observed: 16.4%). The onset of decomposition was noted at 256°C.

Sample: Sodium Alendronate in DCM
Size: 55.1270 mg
Method: Stepwise isothermal

TGA

File: C:\...Sodium Alendronate.002
Operator: Troy
Run Date: 2006-01-26 13:06
Instrument: TGA Q500 V6.2 Build 187

Figure 24 Thermogravimetric analysis of sodium alendronate trihydrate after 3 days in dichloromethane at 37°C. The temperature ramp rate = 20°C/minute, unless a weight change of >1.00%/min. was detected, in which case the temperature was held isothermal.

The X-ray powder diffraction (XRD) patterns of as received and dichloromethane-treated alendronate are shown in Figure 25.

Figure 25 X-ray powder diffraction pattern of sodium alendronate trihydrate as received (black) and after 3 days in dichloromethane at 37°C (red).

The peak positions and intensities compared favourably with those observed in the first powder diffraction patterns (Figure 21) and with the published powder diffraction pattern (Figure 22). There were no new peaks or shifts in the diffraction pattern to indicate a difference in crystal structure that might be expected if a new solvate were formed.

The absence of a dichloromethane solvate was almost certainly related to the lack of lipophilic groups on alendronate.

4.2.3 Effect of Polyethylene Glycol

The rate of drug release from PLGA films can be modulated by incorporating a second, hydrophilic polymer to form a blend (Jackson *et al.* 2004; Alexis 2005). Polyethylene glycol (PEG) is a hydrophilic, biocompatible polyether that is widely used in biomedical and cosmetic applications (Fruijtier-Polloth 2005). PEG 600, is a viscous liquid at room temperature, and was selected for incorporation into PLGA.

In preliminary studies, a series of films containing 10% ground alendronate (w/w) and a polymer matrix composed of 0%, 10%, 20% or 30% PEG 600 (w/w) in PLGA were

manufactured. Incorporating PEG 600 into PLGA films had a dramatic effect on the drug release profiles. By Day 1, the PLGA only films had released approximately 40% of their theoretical loaded alendronate content. However, the films containing 10%, 20% and 30% PEG had released approximately 70%, 90% and 100% of the loaded alendronate, respectively.

The as received, ground and lyophilized samples of alendronate were manufactured into films composed of 20% PEG 600 in PLGA, and 10% alendronate (w/w). The results of the drug release study are shown in Figure 26. The three sample types showed similar trends to those observed in the PLGA-only films (Figure 23), however, there was a marked early burst phase of alendronate release. Almost all of the drug was released within the first 2 days. Ground alendronate showed a slower controlled release profile, probably due to improved dispersion of the alendronate particles throughout the polymer matrix.

Figure 26 Cumulative release of alendronate from 10%-drug loaded 20% PEG 600 in 85:15 PLGA films containing: (Δ) lyophilized, ($\diamond$) as received, and ($\square$) ground alendronate in PBS at 37°C. 0.1 mg/mg film represents 100% theoretical loading. Error bars indicate standard deviation among $n=5$ films of each type.

The loading efficiency in the 20% PEG 600 in PLGA films was determined by measuring the alendronate concentration in films ($n = 3$ to 5) prior to the drug release studies. The loading efficiencies, expressed as percent of the theoretical drug loading (0.1 mg/mg film), were: as received 76%, ground 83% and lyophilized 96%. It was likely that the lower loading efficiencies were due to loss of alendronate on the vials during manufacturing.

Incorporating a hydrophilic agent into the PLGA film matrix was intended to increase the solubility of alendronate and provide controlled release over a 2-4 week period. However, the use of PEG 600 led to a very rapid drug release. This was likely due to significant water uptake into the film matrix following rapid release of the water-soluble low molecular weight PEG from the film. This would cause the water-soluble alendronate to dissolve in water-filled pores or channels left by the loss of PEG and be released rapidly

from the film. Further investigation into other potential release-modulating agents was required.

4.2.4 Effect of Diblock Copolymer

Diblock copolymers have been previously used to improve the physical properties and drug-releasing characteristics of PLGA films (Jackson *et al.* 2004). The cumulative drug release profiles for films containing 5% (w/w) alendronate in a polymer matrix composed of 0%, 10%, 20% and 30% diblock copolymer (w/w) in PLGA are shown in Figure 27.

Figure 27 Cumulative release of alendronate from 5% (w/w) drug-loaded films composed of 85:15 PLGA and the following amounts of diblock: (Δ) 0%, ($\square$) 10%, ($\diamond$) 20% or ($\times$) 30% into PBS at 37°C. 0.05 mg/mg film represents 100% theoretical loading. Error bars indicate standard deviation among n=5 films of each type.

Increasing the diblock concentration increased the rate of alendronate release. Alendronate release from films composed of 20% and 30% diblock in PLGA was

complete within about 11 and 3 days, respectively. The 10% diblock in PLGA formulation demonstrated an initial burst of approximately 40% of the total drug loading in the first 3 days, followed by 2 weeks of controlled drug release. The PLGA-only samples showed a 3-day burst phase followed by slow controlled release.

The sample films were transparent at the beginning of the study, but became swollen and opaque after a day in PBS at 37°C. The blend of hydrophilic diblock with PLGA likely resulted in greater water penetration into the sample films, causing rapid dissolution of alendronate and subsequent diffusion out of the matrix. Increasing the proportion of diblock in the polymer matrix therefore increased the rate of drug release.

Given the controlled drug release profile and low variability among individual films, 10% diblock in PLGA was selected as the lead film formulation.

4.2.5 Mathematical Modeling

The cumulative drug release data from films composed of various concentrations of blends of diblock in PLGA (Figure 27) were plotted as a function of the square root of time to determine if the drug release kinetics could be described by the Higuchi model, as shown in Equation 3 in Section 2.3.3.1. This mathematical model applies to diffusion controlled drug release. The data are shown in Figure 28.

Figure 28 Cumulative release of alendronate as a function of the square root of time from 5% (w/w) drug-loaded films composed of 85:15 PLGA and the following amounts of diblock: (Δ) 0%, ($\square$) 10%, ($\diamond$) 20% or ($\times$) 30% into PBS at 37°C. 0.05 mg/mg film represents 100% theoretical loading. Error bars indicate standard deviation among $n=5$ films of each type. Lines shown are linear regressions of the given data points.

For PLGA films with and without diblock, the release of alendronate appeared to follow triphasic release kinetics. The initial burst phase of drug release was likely due to dissolution of the highly hydrophilic alendronate at or near the surface of the films into the medium. This was followed by a phase of drug release that was linear with respect to the square root of time for all films. However, there were differences in the duration of the linear phase for the different compositions. This phase was attributed to diffusion-controlled release, as predicted by the Higuchi equation. Films composed of PLGA only, showed a linear relationship for a duration of about 34 days ($R^2 = 0.995$). For films composed of 30% diblock, the period of the diffusion-controlled release was very short (less than 1 day, $R^2 = 0.958$). For the 10% and 20% diblock in PLGA films, the duration of the diffusion controlled phase of release was about 15 days and 6 days, respectively.

The third phase of release was much slower and clearly the Higuchi model no longer fits the data ($R^2 = 0.982$ and 0.984 , respectively). The Higuchi model was derived assuming a rate-limiting and nondegradable polymer matrix. For the PLGA-only films, this condition was probably maintained over the first 36 days of release since the PLGA (85:15) polymer has a long degradation lifetime (Alexis 2005). However, for films containing diblock, the GPC data (Figure 11 and Figure 12) showed that both the molecular weight of the diblock copolymer and the ratio of diblock to PLGA were decreasing. This indicated that the diblock was degrading and diffusing out of the matrix. At the diblock loadings of 10%, 20% and 30% in the films, it is possible that degradation and diffusion of diblock out of the films led to the formation of pores and channels leading to faster release rates of alendronate. Therefore, it is suggested that drug release from the diblock in PLGA blended films was determined by both molecular diffusion through the polymer matrix and diffusion of alendronate through water-filled pores and channels.

4.2.6 Effect of Alendronate Concentration

Once the lead formulation was selected, the drug release studies were repeated with 3 batches of films using a lower alendronate concentrations better suited to use with bone cells in culture. Figure 29 shows the drug release profiles from films loaded with 0.1%, 0.25% and 0.5% alendronate.

Figure 29 Cumulative release of alendronate from (Δ) 0.10%, ($\diamond$) 0.25% and ($\square$) 0.50% drug-loaded 10% (w/w) diblock in 85:15 PLGA films in PBS at 37°C. Data expressed as mean $\pm$ SEM (N=3).

The lower concentration films (0.1 to 0.5% alendronate) produced a burst phase that was shorter (1 versus 3 days) than that observed for 5% loaded films (Figure 27) and the controlled release phase was longer. After 4 weeks, the low concentration films still gave slow controlled drug release, while the 5% drug-loaded films had reached a plateau by week 3. The slower and more prolonged phase of release for the low loading films was likely due to a combination of the films being less porous (as drug particles dissolve) and possibly due to a difference in the concentration gradient (Baker 1987).

4.2.7 Effect of Glow Discharge

Prior to using the polymer films in cell culture work, they were sterilized by exposure to vacuum and argon gas glow discharge. A glow discharge cell contains the samples, 2 electrodes, and argon gas at low pressure. When a potential difference was applied across the electrodes, accelerated electrons formed a plasma of charged species that could collide and react with the samples. It is known that sterilization by γ -irradiation can cause

structural changes in polymers, by degradation, scission or cross-linking (Alexis 2005). These changes could in turn affect the rate of drug release from the polymer.

To determine whether the glow discharge process had an effect on drug release from 10% diblock in PLGA films, drug release studies were performed on three batches of films manufactured at 3 drug concentrations: 0.1%, 0.25% and 0.5%. Glow discharged film samples were compared to control films, as shown in Figure 30.

Figure 30 Cumulative release of alendronate from (Δ) 0.10%, ($\diamond$) 0.25% and ($\square$) 0.50% (w/w) drug-loaded 10% diblock in 85:15 PLGA films in PBS at 37°C. Glow discharged data are indicated by open symbols and dashed lines. Control data are indicated by filled symbols and solid lines. Data are expressed as mean $\pm$ SEM (N=3).

Glow discharging appeared to have a small inhibitory effect on the initial rate of alendronate release in the 0.1% and 0.5% alendronate loaded films, but a small increase was observed for the 0.25% loaded films. After the burst phase (day 2), the slopes of the release curves were similar.

An analysis of the difference and similarity (f1, f2) of the release curves was performed in accordance with the FDA Guidance for Industry: Dissolution Testing of Immediate Release Solid Oral Dosage Forms (Center for Drug Evaluation and Research 1997). This calculation is normally performed to compare dissolution profiles, for instance between a generic drug and the innovator product, or to compare batches made before and after a manufacturing change.

The difference factor, f1, is a measure of the relative error between two curves, and the similarity factor, f2, measures the similarity in the percent dissolution. For curves to be considered similar, f1 values should be close to 0, and f2 values should be close to 100. Generally, f1 values up to 15 (0-15) and f2 values greater than 50 (50-100) ensure sameness or equivalence of the two curves. The results of the calculation are tabulated in Table 13.

Table 13 Similarity and Difference Factors (f1, f2) for Drug Release Curves of Glow Discharged and Control 10% Diblock in PLGA Films Containing 0.1, 0.25 and 0.5% Alendronate

Drug Concentration (% w/w)	f1	f2
<i>Criteria for Sameness</i>	<i>< 15</i>	<i>> 50</i>
0.10	38	98.9
0.25	18	100
0.50	20	99.9

While all of the curves met the criteria for sameness (f2) in terms of amount of dissolution, the relative error (f1) did not meet the criteria. This was likely due to the size of the error bars relative to the amount of drug released.

It was possible that the differences in drug release between the films as a result of variability in manufacturing exceeded the variability introduced by the glow discharge process. But, since all films must be sterilized prior to use in in vitro experiments and the effect of glow discharge on drug release appeared to be minimal, it was concluded that glow discharging was an acceptable means to sterilize the films, so long as the process was performed in a repeatable manner with each batch.

4.2.8 Characterization of 10% Diblock in PLGA Blend Formulation

The physical properties of the 10% diblock in PLGA formulation were characterized by the following methods. The glass transition temperature was determined by differential scanning calorimetry. The extent of polymer degradation was determined by gel permeation chromatography, and the film thicknesses of various batches were determined with callipers. The results of each of these characterization tests are discussed in the following sections.

4.2.8.1 Thermal Analysis

Thermal analyses were performed at Angiotech Pharmaceuticals to determine the glass transition temperatures (T_g) for PLGA, diblock and their blends. Below the T_g, the amorphous component of a polymer is hard and brittle, above the T_g, it becomes more flexible and elastic. Thermograms of polymer films composed of PLGA, diblock and 10, 20 and 30% diblock in PLGA were obtained. Samples were heated to 200°C, cooled to -50°C (-100°C for diblock), and then reheated to 200°C. The glass transition temperatures observed on the second heating cycle are reported in Table 14.

Table 14 Observed Glass Transition Temperatures for PLGA and PLGA/Diblock Blends

Sample	Glass Transition Temperature (°C)				Standard Deviation
	Replicate 1	Replicate 2	Replicate 3	Mean	
85:15 PLGA	45.69	36.86	41.11	41.2	4.4
10% Diblock in PLGA	25.10	23.14	21.28	23.2	1.9
20% Diblock in PLGA	16.81	15.91	16.01	16.2	0.5
30% Diblock in PLGA	7.51	6.63	8.28	7.5	0.8
Diblock	-47.48	-47.54	-46.95	-47.3	0.3

The observed T_g for PLGA (41°C) and diblock (-47°C) were lower than values previously reported (47°C and -35°C, respectively) (Jackson *et al.* 2004). However, the data were collected on different batches of polymer, with different instruments and at different heating and cooling rates.

The cooling and second heating curve of a typical film (10% diblock in PLGA) is shown in Figure 31. The first heating curve has been omitted for clarity. The glass transition temperature of the polymer blend was observed at 21°C.

Sample: 10% Diblock in PLGA3
 Size: 2.6760 mg
 Method: Heat/Cool/Heat

DSC

File: C:\troy\Karen's Files\10%DB_PLGA3.001
 Operator: Troy/Karen
 Run Date: 2005-12-07 02:18
 Instrument: DSC Q1000 V6.2 Build 285

Figure 31 Differential scanning calorimetry cooling/reheating scan of 10% Diblock in 85:15 PLGA following one heating cycle at a heating rate 10°C/minute.

Miscible polymer blends exhibit a single glass transition; whereas immiscible blends will retain the glass transitions for each of the components, although they may become broadened and shift closer to each other. The shift in T_g in a miscible polymer blend depends on the composition of the blend. Increasing the proportion of diblock to PLGA in the polymer produced a concentration-dependent decrease in the glass transition temperature, Figure 32, as predicted by the Fox equation (Fox 1956):

$$\frac{1}{T_g(\text{blend})} = \frac{A}{T_g(\text{diblock})} + \frac{B}{T_g(\text{PLGA})} \quad \text{Equation 6}$$

Where: A is the weight fraction of diblock, B is the weight fraction of PLGA, and the glass transition temperature is in Kelvin.

Figure 32 Effect of blending increasing amounts of diblock into 85:15 PLGA films on glass transition temperature (Tg) of blend. Observed values ($\diamond$, $n=3$) were obtained by differential scanning calorimetry at a heating rate of 10°C/minute. Error bars indicate standard deviation. Line shown is the linear regression of the given data points.

The concentration-dependent reduction in Tg upon the addition of diblock to PLGA was in good agreement with the Fox equation. That, and the single intermediate Tg value for the polymer blends, indicated that the polymers formed a miscible blend.

The addition of 10% diblock to PLGA lowered the Tg from 41°C to 23°C. This is convenient for a dentistry application, because the films will be stiff enough to trim at room temperature, but become more pliable at body temperature. The diblock functioned as a plasticizer in the polymer blend.

4.2.8.2 Polymer Degradation

PLGA and diblock are both biodegradable, biocompatible polymers (Anderson and Shive 1997). Gel permeation chromatography was used to determine the extent of polymer degradation during the early stages of drug release. Since the degradation of 85:15 PLGA is generally of the order of weeks to months (Alexis 2005), it was felt to be more critical to measure changes in the molecular weight of the diblock. Hence, the molecular weight of the diblock was monitored for evidence of short-term polymer degradation. In addition, it was necessary to determine if the glow discharge process had an impact on the molecular weight—either increasing the molecular weight by cross-linking or decreasing it by scission. The changes in diblock molecular weights in 3 batches with and without glow discharging are shown in Figure 33.

Figure 33 Comparison of diblock molecular weights in glow discharged (solid) and not glow discharged (hatched) 10% diblock in 85:15 PLGA films agitated in PBS at 37°C for 3 or 7 days. Mean and SD for 3 batches determined by GPC, (*) statistically significant compared to time 0.

The GPC elution profiles of films that had or had not been glow discharged were indistinguishable. This indicates that the glow discharge process did not have a measurable effect on the molecular weight of the diblock. The small differences in the drug release profiles of glow discharged versus control films observed in Figure 30 were likely a result of film variation rather than the glow discharge process.

However, there was a change in the retention time of the diblock peak with time. By day 7, there was a significant reduction in the diblock molecular weight from day 0 in both the glow discharged and control samples, to 84% and 86% of original, respectively.

The initial diblock molecular weight determined in this study ($M_{GPC} \sim 9500$ g/mol) was considerably higher than either the theoretical molecular weight (3333 g/mol) or the previously reported molecular weight (5240 g/mol) (Zhang *et al.* 1996). However, the data in this work were collected on a different batch of polymer using polystyrene standards. As noted in the original paper, the Mark-Houwink parameters were not determined for the polymers, so the values were considered relative rather than absolute.

The retention time for PLGA was outside the range of the calibration curve, so the data were not reported. However, there was no apparent trend in the data and no evidence of a reduction in PLGA molecular weight in the 7-day period.

The PLGA and diblock peaks were not well resolved by GPC, so the peak heights rather than peak areas were used to monitor loss of diblock into the medium. Data from 3 batches of diblock in PLGA films incubated in PBS are shown in Figure 34. There was no statistical significance between films that had been glow discharged and control. A decline in peak height ratios was observed indicating a loss of lower molecular weight diblock from the films, but it was not statistically significant after 3 or 7 days. In a similar study using diblock in 50:50 PLGA, only 80% of the original diblock was left after one week (Jackson *et al.* 2004).

Figure 34 Comparison of diblock to PLGA peak height ratios in glow discharged (solid) and not glow discharged (hatched) 10% diblock in PLGA films agitated in PBS at 37°C for 3 or 7 days. Mean and SD for 3 batches determined by GPC.

4.2.8.3 Film Thickness

The areas of the polymer films were determined by the dimensions of the templates on which they were cast, but there could be some variability in the weight and thickness of the films. Weight and thickness data obtained from 4 batches are shown in Table 15. The mean weight was ~10 mg, and the mean thickness was ~0.1 mm. The films tended to be slightly thicker at the edges, but the relative standard deviation among 3 measurements of the same film was generally 5% or less. In the drug release studies, drug release was normalized to film weight.

Table 15 Film Thicknesses and Weights of 4 Batches of Alendronate Loaded 10% Diblock in PLGA Films

Batch	n	Mean Weight (SD) mg	n	Mean Thickness (SD) mm
2	18	9.9 (0.7)	54	0.106 (0.019)
3	18	10.8 (1.4)	54	0.095 (0.022)
5	24	9.6 (0.7)	72	0.133 (0.033)
6	24	9.0 (1.4)	72	0.104 (0.013)

4.3 Osteoblasts Cell Culture Data

With the lead formulation selected and characterized, the effect of alendronate-loaded polymer films on bone cells in vitro was evaluated.

4.3.1 Effect of Alendronate Concentration

The concentration of alendronate has been shown to be critical for both osteoclast and osteoblast cell culture (Moreira *et al.* 2005). At 2×10^{-9} mol/L, alendronate inhibited 50% of the resorptive capacity of osteoclasts (Sahni *et al.* 1993). Concentrations greater than 10^{-3} M have been shown to dramatically reduce cell viability within 48 hours in cultures of human normal osteoblasts (Garcia-Moreno *et al.* 1998). A similar effect was observed at concentrations $>10^{-4}$ M in MG-63 human osteoblasts (Im *et al.* 2004). Below the threshold alendronate concentration (10^{-5} to 10^{-12} M), however, an increase in osteoblast proliferation was observed. Proliferation and osteogenic differentiation of human bone marrow stromal cells was observed at a concentration of 10^{-8} M (Von Knoch *et al.* 2005).

Dr. Douglas Hamilton, from the laboratory of Dr. Brunette (Department of Oral Biological & Medical Sciences, UBC Faculty of Dentistry) kindly provided a line of rat osteoblast cells for the proliferation experiments. Unlike the human MG-63 line, these osteoblasts will only continue to proliferate and differentiate for ~10 passages.

Studies were carried out to determine the threshold alendronate concentration in the rat osteoblast line. The effect of various concentrations of alendronate in solution (i.e., not from polymer films) on the proliferation of rat osteoblasts was determined using an MTS assay. The data are shown in Figure 35.

Figure 35 Osteoblast proliferation in the presence of various concentrations of alendronate (2.8×10^{-4} to 2.8×10^{-8} M). Wells were seeded with 1 ml of media containing 20,000 osteoblasts and an aliquot of alendronate solution. The relative osteoblast proliferation after 2 days at 37°C, 5% CO₂ was measured by the MTS assay. Data expressed as mean $\pm$ SEM (N=3).

At 2.8×10^{-8} M alendronate there was an increase in osteoblast proliferation and at 2.8×10^{-4} M there was a decrease; however, these were not statistically significant from the control. The size of the effect was smaller than that reported in the literature (Garcia-Moreno *et al.* 1998; Im *et al.* 2004), and the correlation with alendronate concentration was not as pronounced.

4.3.2 Osteoblast Proliferation

The threshold alendronate concentration was particularly important for the controlled-release alendronate-loaded films. The drug release profiles showed a burst of drug release on the first day. But unlike an *in vivo* system, where excess drug is distributed by the circulatory system, the drug concentration in the cell culture plate can accumulate, possibly to toxic levels. Therefore low concentrations of alendronate (0.1, 0.25 and 0.5%)

were used in the preparation of films for cell culture studies, and the media was changed regularly throughout the study.

The concentration of alendronate in the cell culture media was not determined by HPLC because a suitable procedure for extracting alendronate from biological media had not been developed and validated. Furthermore, in cell culture alendronate would be expected to chelate to calcium and other components of the media, reducing its bioavailability. The effective amount of alendronate in solution would therefore be quite different to what could be determined by HPLC.

The initial cell culture experiments were designed to visually observe the proliferation of osteoblasts on the films to ensure that there were no local toxicities and that the cells would adhere to the films normally and proliferate.

As seen in Figure 36, the sulfurdhodamine B stain shows the morphology of the proliferating osteoblast cells under the rhodamine filter. The Hoechst stain labels only the nuclei, which were then visible under the DAPI filter. Counting the cells manually was found to be more accurate when only the nuclei were visible.

Figure 36 Fluorescence microscope images of rat osteoblasts on 0.25% alendronate-loaded 10% diblock in 85:15 PLGA Films, using the rhodamine (left) or DAPI (right) filters.

Figure 37 shows the number of osteoblast cells observed after 1, 2, and 4 days. Osteoblast cell number increased over a period of 4 days on control Thermanox™ cover slips, the polymer-only films and the 0.25% alendronate-loaded polymer films. Cell proliferation on the 0.5% films was consistently low, indicating a potentially toxic concentration of alendronate, at least initially. The large error bars are due to wide variations in osteoblast

populations on the films. Osteoblasts are known to prefer surfaces with variable topographies (Wieland *et al.* 2005;Owen *et al.* 2005), so the edges of the films, or any areas with bubbles or defects were frequently observed to be well populated; however, the smooth central areas of the films were often devoid of cells.

Figure 37 Osteoblast proliferation after 1, 2 or 4 days incubation with control Thermanox™ cover slips or 10% diblock in 85:15 PLGA films loaded with 0, 0.1, 0.25 or 0.5% alendronate. Proliferation is expressed as mean $\pm$ SEM (N=3 experiments) of stained osteoblasts visible per representative microscope image (10X magnification).

The cells were visually observed to adhere normally and proliferate on all surfaces except the 0.5% alendronate films. It was possible that the local alendronate concentrations reached toxic levels in the early time points due to the burst effect of drug release. In other studies that were followed out to 7 days, the osteoblast populations appeared to begin to recover after day 4.

Osteoblast proliferation on films loaded with 0.25% alendronate was greater than proliferation observed on either the Thermanox™ control slides or the alendronate-free

polymer controls. This effect might have been the result of optimal alendronate drug release, or possibly because the 0.25% alendronate films seemed to have a greater number of small contours and bubbles where the osteoblasts would congregate.

The manual counting method was lengthy and susceptible to bias in the choice of images. In addition, the duration of the proliferation experiments was limited, because the control cells became too numerous to count by day 7. A second method for cell quantitation was desired.

4.3.3 Cell Proliferation by MTS Assay

The next series of experiments were quantitated using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt) or MTS assay to determine the number of viable cells colorimetrically. The MTS tetrazolium compound can be reduced by viable cells within a few hours to a coloured formazan product that absorbs at 492 nm. The linear range of the assay was 0.2 to 1.1 absorbance units at 490 nm.

Figure 38 Chemical structure of MTS tetrazolium reagent being reduced to its formazan product

The proliferation experiments were repeated using the MTS assay. Using this assay, the quantities of proliferating cells on the films, as well as the quantities that had adhered to the sides of the wells were measured. This gave a measure of both the ability of cells to adhere to the polymeric films, as well as the effects of alendronate in solution. The data from 2 experiments are shown in Figure 39 and Figure 40.

Figure 39 Osteoblast proliferation data from experiment 1 on 0, 0.1, 0.25 and 0.5% alendronate-loaded 10% diblock in 85:15 PLGA films after incubation at 37°C for 1, 3, 4 or 6 days. Proliferation was measured by the MTS assay (n=3) and normalized to untreated control wells. A) Osteoblast proliferation on films only, B) osteoblast proliferation on films and wells.

Figure 40 Osteoblast proliferation data from experiment 2 on 0, 0.1, 0.25 and 0.5% alendronate-loaded 10% diblock in 85:15 PLGA films after incubation at 37°C for 1, 3, 4 or 7 days. Proliferation was measured by the MTS assay (n=3) and normalized to untreated control wells. A) Osteoblast proliferation on films only, B) osteoblast proliferation on films and wells.

The MTS cell proliferation experiments did not support the earlier observations using manual counting. The inhibitory effect of 0.50% alendronate-loaded films was not observed; nor was the increased proliferation in the presence of 0.25% alendronate-loaded films. The MTS experiments were performed on different batches of films, manufactured at lower temperatures to prevent small bubbles forming due to rapid

evaporation of the solvent. It is possible that increased proliferation on the 0.25% alendronate-loaded films observed in Figure 37 could be explained by the different and more textured topography of the films used in these studies.

The MTS results do not show the increase in osteoblast proliferation with time seen in Figure 37 because the data have been normalized with respect to a set of control wells that did not contain polymer films. While there appeared to be a trend of decreasing osteoblast proliferation as a function of time for measurements on films only (Figure 39A and Figure 40A), it is not known whether these differences were significant based on only 2 experiments. On the other hand, in studies evaluating the osteoblast proliferation in both the films and the wells (Figure 39B and Figure 40B), there appeared to be no effect with incubation time.

An interesting trend of decreased osteoblast proliferation was noted day 4 in all studies, and at all concentrations. This appeared to be followed by recovery of the cells on day 6 or 7. The media was changed on days 1, 2, 4 and 7, and the alendronate burst phase in low concentration films lasted one day (Figure 30), so a toxic accumulation of alendronate was not likely. It remains to be determined whether this is a reproducible feature of osteoblast proliferation in these types of studies and what causes this effect.

4.3.4 Future Experiments

The MTS data for osteoblasts were inconclusive with regard to determining an optimal alendronate concentration for polymeric films. In future experiments, the effects of alendronate release on osteoclasts in addition to osteoblasts should be determined, although osteoclasts can be difficult to obtain and culture. The optimal alendronate concentration for osteoblasts is likely different from that of osteoclasts. And since a potential application for the polymer films is to act as a barrier between the bone and epithelial cells in periodontal applications, the effect on proliferation on epithelial cells might also be of interest.

After osteoblasts have proliferated to the point of confluence, they will begin to differentiate and secrete bone matrix (Fleisch 1997). Other osteoblast studies typically include some experiments measuring the time to and extent of differentiation by measuring alkaline phosphatase activity and nodule formation. However, performing these experiments in the presence of bisphosphonates is complicated by the fact that the calcium ions essential for osteoblast differentiation will be chelated by the bisphosphonates in a concentration dependent manner (Peter *et al.* 2005b). While this effect is probably unimportant in vivo, it has been shown to be a factor in in vitro

experiments. Including an additional chelator, such as EDTA or excess Mg^{2+} and Zn^{2+} ions might overcome the inhibitory effect of alendronate on alkaline phosphatase (Vaisman *et al.* 2005).

Finally, the affinity of osteoblasts for surface topography might be exploited by casting the polymer films into ridged or dimpled templates. A comparison of smooth and uneven surfaces would make an interesting study.

5 SUMMARY AND CONCLUSIONS

A controlled-release drug delivery system for local delivery of alendronate has a number of potential applications, for which characterization of the quantity and rate of drug release was required. A sensitive fluorescence HPLC assay for alendronate was validated. The in vitro drug release profiles of several potential formulations of PLGA, alendronate and various release modulators and plasticizers were determined. The lead formulation of 0.25% alendronate in 85:15 PLGA containing 10% diblock copolymer gave controlled release of drug over the expected healing period after surgery (2-4 weeks). This lead formulation may increase osteoblast proliferation over polymer alone, but this effect could not be confirmed using the MTS assay.

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