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# CONTROLLED RELEASE OF OSTEOTROPIC MOLECULES STIMULATES IN VITRO CELLULAR ACTIVITY AND IN VIVO LOCAL BONE REGENERATION

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ABSTRACT OF DISSERTATION

Ju Hyeong Jeon

The Graduate School  
University of Kentucky  
2007

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*IN VIVO* LOCAL BONE REGENERATION

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ABSTRACT OF DISSERTATION

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A dissertation submitted in partial fulfillment of the requirements  
for the degree of Doctor of Philosophy in Biomedical Engineering  
in the Graduate school at the University of Kentucky

By

Ju Hyeong Jeon

Lexington, Kentucky

Director: Dr. David A. Puleo

Professor of Biomedical Engineering

Lexington, Kentucky

2007

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## ABSTRACT OF DISSERTATION

### CONTROLLED RELEASE OF OSTEOTROPIC MOLECULES STIMULATES *IN VITRO* CELLULAR ACTIVITY AND *IN VIVO* LOCAL BONE REGENERATION

Bone defects treatment and reconstructive surgery continues to increase at a significant rate. Current bone defect treatments are autotransplantation, allograft, and xenografts create many problems such as, inflammation, infection and chronic pain. Moreover, allografts and xenografts arouse immune rejection. These problems have led to development of controlled release system for use as alternatives to autografts, allografts and xenografts in bone repair. There have been many approaches for sustained drug delivery in local bone regeneration using biodegradable polymers and osteotropic biomolecules. This dissertation presents new approaches that apply intermittent drug delivery for local bone regeneration. In the first, the osteotropic molecules simvastatin (Sim) or parathyroid hormone (PTH) were released with intermittent profiles. In the second, alternating delivery of Sim and PTH as well as alternating release of the antimicrobial agent cecropin B (CB) with Sim or PTH. An association polymer system of cellulose acetate phthalate (CAP) and Pluronic F-127 (PF-127) was used for the delivery vehicle. Each device showed discrete peaks in release profiles and lasted more than 10 days. Release profiles could be controlled by altering surface area exposed to aqueous environment, number of layers, loading, and blending ratios. Cells were cultured with sustained or intermittent exposure to Sim or PTH at various concentrations, and alternating exposure to CB and Sim or PTH and to Sim and PTH at different concentrations. Low dose Sim and PTH treatments stimulated higher osteoblastic activity than observed in control cultures. Furthermore, intermittent delivery was more effective than sustained exposure. *In vivo*, newly formed bone was found in animals implanted with both blank Sim-loaded devices. However, a greater anabolic effect was seen for Sim release devices. Further, intermittent release devices stimulated the greatest woven bone thickness, total bone area, and lamellar bone area. These results suggest that intermittent release devices containing a single molecule, Sim or PTH, and alternating release devices containing multiple molecules, CB with Sim

or PTH, possess promising potential as a treatment for local bone regeneration.

**KEYWORDS:** Controlled Release, Intermittent Release, Osteotropic Molecules, Association Polymer, Local Bone Formation,

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## **INTRODUCTION**

Bone loss is an important health care problem worldwide and current bone defect treatments have been mainly focused on replacing the lost bone with allogeneic, xenogeneic or synthetic materials. The use of allogeneic or xenogeneic tissue for bone repair involves immune rejection, and disease transmission. Although autogenic bone grafts are the most successful, they also face on additional surgery, and limitation of amount available. One approach to the success of bone regeneration via tissue engineering strategies is the proper design of the osteogenic molecule delivery devices. The requirements of devices for bone regeneration should include controlled release, biodegradability, mechanical integrity, and osteogenesis.

Controlled release technology has been studied for delivering a variety of biomolecules at the proper time, with the proper amount, and to the proper site. Most commonly, investigators seek to release one drug at constant concentrations for extended periods. However, non zero-order kinetics can be more desirable than zero-order kinetics for delivery of biomolecules for local bone regeneration. Two osteotropic biomolecules of interest are simvastatin (Sim), which is a widely used cholesterol-lowering drug, and parathyroid hormone (PTH), which is well known for its role in calcium homeostasis. At the systemic level, injections of Sim stimulate bone formation [1-6]. Intermittent or pulsatile administration of PTH also has an anabolic effect [7-12], but sustained treatment with PTH has catabolic effect on bone [13-15]. In this study, the objective was to mimic the intermittent concentrations resulting from daily

injections or oral dosing using controlled release methods.

An association polymer system based on blends of CAP and PF-127 was previously investigated for providing pulsatile release of biomolecules [16]. It was this CAP and PF-127 association polymer system that was used to develop devices for intermittent release of osteotropic molecules. Because certain bone defects can be contaminated with microorganisms, devices for delivery of osteotropic molecules with an antimicrobial peptide was also examined. This dissertation reports characterization of the release systems, *in vitro* bioactivity, and the bioactivity for select devices.

## **BACKGROUND**

The need to treat bone defects resulting from degenerative diseases, trauma, and reconstructive surgery continues to increase at a significant rate. Currently, bone graft procedures exceed 500,000 in the US and approximately 2.2 million worldwide [17]. Harvesting autogenous tissues needs an additional surgery at the donor site that can result in its own complications, such as inflammation, infection, and chronic pain. Also, the total amount of bone that can be harvested is limited and creates a supply problem. Although allograft tissue is treated by freezing, freeze-drying, gamma irradiation, electron beam radiation, or ethylene oxide, because it is obtained from a donor, a risk of disease transmission from donor to recipient exists [18]. Xenografts are an alternative approach to helping patients, but they still raise concerns with disease transmission and immune rejection. These problems have led to development of drug delivery devices, synthetic

materials, and tissue engineered constructs for use as alternatives to autografts and allografts in bone repair.

**i. Bone Formation**

**a. Intramembranous ossification**

Intramembraneous ossification is the formation of bone on fibrous connective tissue and involves making flat bones such as the mandible, skull, and some irregular bones. The bone is formed from mesenchymal cells and is first formed as connective tissue membranes. Osteoblasts proliferate and migrate to the membranes and then differentiate and deposit bone matrix. They finally turn into osteocytes and form Woven bone then produce osteons [19,20].

**b. Endochondral Ossification**

Endochondral ossification happens in long bone formation such as the femur and humerus. In this process, bone is firstly formed as hyaline cartilage. The perichondrium which surrounds the hyaline cartilage is infiltrated with artery and osteoblasts and changes into a periosteum. The osteoblasts form a collar of compact bone around the diaphysis. Then, the cartilage in the center of the diaphysis begins to disintegrate. Osteoblasts penetrate the disintegrating cartilage and replace it with trabecular bone. This forms a primary ossification center. Ossification continues from this center toward the ends of the bones. After trabecular bone is formed in the diaphysis, osteoclasts break down the newly formed bone to open up the medullary cavity. In epiphysis periosteal buds recruit mesenchymal cells and blood vessels and the process is similar that in a primary ossification center. This is secondary ossification center. Cartilage

between the primary and secondary ossification centers is the epiphyseal plate, and this epiphyseal plate keeps on forming new cartilage. New cartilage, then, is replaced by trabecular bone, and this process contribute to increase length of the bone [19,20].

## ii. **Controlled Release**

The original goal and direction of controlled release technology is to maintain appropriate concentrations of a single drug for extended periods. Current drug delivery devices primarily utilize a variety of polymeric biomaterials, such as polylactides (PLA), polyglycolides (PGA), poly(lactide-co-glycolides) (PLGA), polyanhydrides, and polyorthoesters. Mainly, micro- or nanospheres and porous scaffolds have been developed for delivery devices and [21-32]. In drug incorporating method, drug can be directly incorporated with delivery devices physically or it can be bind by chemically-electrostatic [32], ionic bond [33], or covalent bond [34] to devices.

Many approaches to intermittent biomolecules delivery have been investigated, such as injections, oral dosing, pulmonary inhalation and, transdermal delivery, [35-41]. In injections, patients may suffer from acute pain, and compliance can be problematic for prolonged administration. Oral doing is inefficient, because certain many parts of taken drugs can be destroyed in the gastrointestinal tract before they reach plasma or they may be excreted. Pulmonary inhalation requires continuous inhalation. As a result, a programmable device that does not need additional actions is required.

### iii. Drug release Kinetics

In most drug release, the rate of drug release changes when the release continues. Initially the rate of release relatively large, while at very long times the rate of release reaches to zero. In order to characterize the kinetic behavior of drug release, it is required to determine how the rate of release varies as the reaction progresses [42,43].

Rate law is differential equation because the rate of a drug release depends on the rate of change of the drug release with time. The differential rate law relates to the rate of drug release from the device to the system. Usually, differential rate laws have many different forms, however, most drug release kinetics belong to one of three differential rate laws [42,43].

#### a. **Zero-Order Release**

For a zero-order release, the rate of drug release is a constant. When the drug is completely consumed, the release abruptly stops.

Differential Rate Law:  $r = k$  Eq. 1

where  $k$  is rate constant, ( $L \text{ mole}^{-1} \text{ sec}^{-1}$ ).

#### b. **First-Order Release**

For a first-order release, the rate of drug release is directly proportional to the concentration of the drug ( $[A]$ ). When the drug is consumed during the release, the concentration and the rate of reaction reduces in the system.

Differential Rate Law:  $r = k a$  Eq. 2

where  $k$  is rate constant, ( $L \text{ mole}^{-1} \text{ sec}^{-1}$ ).

### c. **Second-Order Release**

For a second-order release, the rate of drug release is directly proportional to the square of the concentration of the drug ( $[A]$ ). For this type of release, the rate of release decreases rapidly (faster than linearly) as the concentration of the drug decreases.

Differential Rate Law:  $r = k [A]^2$  Eq. 3

where  $k$  is rate constant, ( $L \text{ mole}^{-1} \text{ sec}^{-1}$ ).

### iv. **CAP and PF-127 Polymer System**

Cellulose acetate phthalate (CAP) is a cellulose derivative that has been used in the pharmaceutical industry for enteric coating of oral tablets and capsules. While the regulatory status is clear for oral applications (*i.e.*, in FDA Inactive Ingredients Guide), it remains unknown with respect to parenteral devices. However, Heller's group has previously used CAP in formulations for treating heroin addiction [44,45]. Pluronic F-127 (PF-127) is a triblock copolymer of polyethylene oxide and polypropylene oxide. The numerous ether sites within PF-127 allow for hydrogen bonding with carboxylic acid groups in CAP. Blends of CAP and PF-127 form an association polymer that undergoes surface erosion following deprotonation at physiological pH and consequently shows zero-order release. In contrast to the commonly used biodegradable polyesters, such as poly(lactide-co-glycolide), which undergo bulk hydrolysis, the CAP/PF-127 system degrades by surface erosion, in which the material degrades from the outermost surface toward the inside [16]. The CAP/PF-127 polymer system can be used for programmed drug delivery, with the rate, duration, and amount of

released drug controlled by blending ratio, number of layers, and thickness [16,46,47]

v. **Osteotropic Molecules**

a. **Simvastatin**

Sim is a well-known member of the statin family. Statins are potent pro-drugs of hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors that block conversion of HMG-CoA to mevalonic acid, which is needed for cholesterol biosynthesis [48]. Sim inhibits mevalonate, farnesyl pyrophosphate, and geranylgeranyl pyrophosphate pathways [49], which are responsible for increasing bone mineral density by reducing osteoclastic activity [50]. Statins induce expression of bone morphogenetic protein 2 (BMP-2) *in vitro*, and they stimulate bone formation on the calvaria of mice following daily subcutaneous injections [1]. Oral dosing with Sim increases cancellous bone volume in rats [51], and it also increases transverse area of fracture callus as well as mechanical properties compared to controls [2]. Statins offer additional benefits, such as promotion of new blood vessel growth [52] and anti-inflammatory effects [53].

Some literature show skeptical result of Sim on bone formation [54,55]. They investigated oral dosing and systemic effect of Sim. However, many studies which carried local delivery of simvastatin showed positive effects on bone formation not only *in vitro* but also *in vivo*. The main difference between systemic and local delivery of simvastatin is effective concentration on the wound site. Former is absorbed by gastro-intestine system and mainly works in the liver and the latter works directly on the wound site. With 60 mg dosage forms, serum



concentrations of Sim and simvastatin acid are 18.7 +/- 4.7 ng/ml and 3.5+/-0.5 ng/ml, respectively. Further, the half-life of simvastatin acid is 5.9 +/- 0.3 hr [56]. In local delivery of Sim, we can keep the Sim concentration as high as we can. From the result of our study about *in vitro* simvastatin release [46], we can keep the local Sim concentration around 100 ng/ml-210ng/ml.

Sim has a very low aqueous solubility, approximately 1.4 µg/mL [57], because it has a rigid and hydrophobic section that is covalently linked to the HMG-like moiety. It has an inactive lactone ring in HMG-like portion. *In vivo*, this inactive lactone ring is enzymatically hydrolyzed to its active hydroxyacid form [58]. To increase local concentration of Sim, it is hydrolyzed to cleave the lactone ring and convert the molecule to its β-hydroxyacid form [59].

#### **b. Parathyroid Hormone**

PTH 1-34 fragment is well known for its role in calcium homeostasis, and PTH (1–34) delivery has either an anabolic or catabolic effect on bone, depending on mode of delivery. It has been reported that daily injections of low doses of PTH (1–34) stimulate bone formation and increase bone mass in humans and animals [7,12,14,60-63], whereas continuous administration of PTH (1–34) has a catabolic effect [8,9,15,64]. Many *in vivo* and *in vitro* studies have been conducted to explain the anabolic effect of PTH (1–34) on bone formation, and it is clear that PTH (1–34) enhances proliferation of primary osteoblastic cells from humans and animals *in vitro* [65,66]. In addition, PTH (1–34) exerts diverse effects on osteoblast differentiation depending on differentiation stage [67,68], and as a result, PTH (1–34) stimulates alkaline phosphatase (AP) activity in the

mouse osteoblastic cell line MC3T3-E1 [68].

Intermittent administration of PTH shows anabolic effect on bone formation by increasing number of osteoblasts [69-72], but the entire cellular mechanisms involving this effect are still not fully understood. Many literatures have shown that PTH activates survival signaling on osteoblasts and that result delays of osteoblastic apoptosis. This is a main way to increasing osteoblast number. PTH stimulates multiple intracellular signal pathways, mediated by cAMP, and activates both protein kinase A (PKA) and C (PKC) [73]. PTH activates  $\beta$ -arrestin and  $\beta$ -arrestin activates extracellular regulated kinase (ERK) signaling [74,75].  $\beta$ -arrestin is involved in desensitization of cAMP signaling by PTHr.

There are significantly fewer PTH receptors on osteoclast cells compared to osteoblast cells. Consequently, osteoclast cells are indirectly affected by PTH [76]. PTH binds to osteoblast cells and stimulates expression of receptor activator of nuclear factor kappa B ligand (RANKL). RANKL binds to osteoclast precursors with receptor activator of nuclear factor kappa B (RANK), and stimulates these precursors fusing and forming new osteoclasts cells [77].

The plasma half life of intact PTH (1–34) is less than 11 min [36]. The fast metabolic degradation of PTH requires multiple administrations of PTH to keep the effective concentration in the plasma [78]. For example, plasma levels of PTH in rats treated by pulmonary inhalation were elevated for less than 120 min [36,79]. However, local application of relatively small amount of biomolecules will have different requirements.

vi. **Antimicrobial Agents**

Regeneration of bone defects is complex and involves many sequential cascades of events [80]. Furthermore, they may be faced with the threat of microbial infection. Once an infection is established, it is difficult to solve the problem. Treatment of deep infections requires multiple surgeries, prolonged antibiotic treatment, and long-term problems [81]. Therefore, every effort is required to prevent this problem with early treatment.

Continuous use of antibiotics results in increasing resistance to antimicrobial drugs, including the occurrence of bacterial strains which are resistant to antibacterial agents and has created a public health problem of potentially crisis proportions (58). Antibiotic resistance is an evolution via natural selection. The antibiotic action is a kind of environmental pressure and those bacteria have a mutation allowing them to survive and reproduce, resulting in a fully resistant generation [82,83]

Cecropin B (CB) is a naturally occurring cationic amphipathic insect peptide [84,85] that can notably act not only against certain gram-positive bacteria but also against gram-negative bacteria, fungi, and parasites [86-88]. The mechanism of action of cecropin B involves channel formation in membranes and subsequent lysis [89]. CB has a broad spectrum of  $EC_{50}$  (half maximal effective concentration; the concentration of a drug which responses half from the baseline to maximum) from  $1\mu\text{M}$  to  $20\mu\text{M}$ , depending on microorganism and fungi [86,90,91].

**vii. Delivery of Multiple Molecules**

Because multiple cascades are required to direct the processes involved in bone repair to completion, the success of most current research focusing on delivery of a single biomolecule may be limited [92]. A method to overcome limitation of single molecule delivery is to release multiple molecules with distinct kinetics to trigger the tissue regeneration at the proper site of action [92,93].

One of the polymeric multiple biomolecules delivery systems is the use of scaffolds and drug loading methods into the scaffolds is that one molecule is mixed in scaffold and another drug was encapsulated into microspheres. In this method Mooney's group investigated dual growth factors delivery using PLGA scaffold for 40 days. Another approach was made by Mikos' group, they encapsulated dual growth factors and crosslinked them each other [94]. They seek for the target of traditional controlled release system-simultaneous sustained release system, whereas our devices aimed at short term intermittent delivery.

**viii. Significance and Objectives**

Sim has an anabolic effect on bone formation, especially when intermittently administered. Similarly, intermittent/pulsatile delivery of PTH can promote bone formation, whereas it is destructive if constantly administered. There are few studies about local delivery of Sim or PTH with biodegradable polymeric devices. Furthermore, there has been no study related to devices combining release of Sim and PTH or such devices combined with an antimicrobial agent, such as CB. Developing devices for intermittent delivery of

single or multiple osteotropic and antimicrobial molecules for placement in bone defects may lead to better treatments for local bone regeneration.

The objectives of these studies were to develop devices that intermittently or sustained release of single molecule (Sim or PTH) and multiple releases of molecules (CB/Sim, CB/PTH, or Sim/PTH). The hypotheses were that the mode of local Sim release affects *in vitro* osteoblastic activity and *in vivo* bone formation and that the mode of local PTH(1-34) release affects osteoblastic activity. To test these hypotheses, devices for intermittent delivery of Sim or PTH(1-34) were developed, and they were demonstrated that the cellular effects of Sim or PTH(1-34) on osteoblastic proliferation and differentiation were dependent on mode of administration and degree of new bone formation relied on mode of release of Sim.

## **Chapter One**

# **BIOERODIBLE DEVICE FOR INTERMITTENT RELEASE OF SIMVASTATIN ACID**

### **1.1 INTRODUCTION**

The need to treat bone defects resulting from degenerative diseases, trauma, and reconstructive surgery continues to increase at a significant rate. More than 500,000 bone grafting procedures are performed annually in the United States [95]. Harvesting autogenous tissues requires an additional surgery at the donor site that can result in its own complications, such as inflammation, infection, and chronic pain. Also, the total amount of bone that can be harvested is limited and creates a supply problem. Although allograft tissue is treated by freezing, freeze-drying, gamma irradiation, electron beam radiation, or ethylene oxide, because it is obtained from a donor, a risk of disease transmission from donor to recipient exists [18]. These problems have led to development of drug delivery devices, synthetic materials, and tissue engineered constructs for use as alternatives to autografts and allografts in bone repair.

Simvastatin is a well-known member of the statin family. Statins are potent pro-drugs of hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors that block conversion of HMG-CoA to mevalonic acid, which is needed for cholesterol biosynthesis [48]. Simvastatin occupies a portion of the binding site for HMG-CoA, thus blocking access of substrate to the active site [96].

Mevalonic acid is a precursor not only of cholesterol but also of isoprenoids, such as geranyl pyrophosphate, which is important in the control of osteoclast-mediated bone resorption [97]. Statins offer additional benefits, such as promotion of new blood vessel growth (Kureishi et al., 2000) and anti-inflammatory effects [53]. Most relevant to the present work, Mundy's group originally demonstrated that statins induce expression of bone morphogenetic protein 2 (BMP-2) and that they stimulate bone formation on the calvaria of mice following daily subcutaneous injections [1]. Subsequent studies have shown that oral dosing with simvastatin increases cancellous bone volume in rats [51], and it also increases transverse area of fracture callus as well as mechanical properties compared to controls [2].

For local delivery of simvastatin, typical controlled release devices that attempt to achieve zero-order kinetics may not be the most effective. To mimic alternating concentrations resulting from daily injection or oral dosing, devices providing intermittent release profiles would be useful. We previously investigated an association polymer system based on blends of cellulose acetate phthalate (CAP) and Pluronic F127 (PF-127) for providing pulsatile release of macromolecules [16].

The objectives of this study were to develop devices that intermittently release simvastatin acid and demonstrate that exposure of osteoblastic cells to alternating concentrations of the drug enhances bioactivity.

## **1.2 MATERIALS AND METHODS**

### **1.2.1 Hydrolysis of Simvastatin**

To decrease hydrophobicity of simvastatin (Aldrich, Milwaukee, WI), 42 mg were dissolved in 1 ml of 95% ethanol, and 1.5 ml of 0.1 M NaOH was added. The solution was heated at 50°C for 2 hours. Then the final solution was neutralized to pH 7.2 with 0.1 M HCl, and the volume was brought to 10 ml with deionized water [59]. Simvastatin acid (10 mM) was stored frozen at -20°C.

### **1.2.2 CAP/PF-127 Microspheres**

Microspheres containing different weight fractions of CAP (Fluka, Buchs, Switzerland) and PF-127 (Sigma, St Louis, MO) were prepared by a water-acetone-oil-water (W/A/O/W) triple emulsion process. Two g of different polymer blends of CAP and PF-127 (7:3, 6:4 and 5:5 by weight of CAP:PF-127) were dissolved in acetone. One ml of phosphate-buffered saline (PBS), pH 7.4, with or without 100 µM simvastatin acid was added to the acetone solution and mixed into rapidly stirring corn oil and sonicated. The CAP/PF-127 suspension and 5% Triton X-100 were added to deionized water and then stirred to harden the microspheres for 5 minutes. Following collection, microspheres were washed three times with deionized water, filtered, and dried in vacuum up to seven days.

Release devices were made using a pressure-sintering process. Microspheres were first treated by UV radiation in a laminar flow hood for 30 minutes. Ten to 15 mg of UV-treated microspheres containing simvastatin acid were placed in the wells of a Delrin mold (diameter, 6.2 mm; depth, 20 mm) and consolidated by applying 20 MPa pressure for 5 sec. Next, blank microspheres



were added on top of the first layer, and pressure was reapplied. By repeating this process, six-, eight-, and ten-layer devices were prepared. To provide directional control of drug release, the bottom and sides of the devices were coated three times with 10% poly(lactic-co-glycolic acid) (75:25,  $M_w \sim 75$  kDa; Alkermes, Cincinnati, OH) solution in methylene chloride.

### **1.2.3. *In Vitro* Release**

Samples were immersed in 5 ml of 150 mM PBS, pH 7.4, and incubated at 37°C under either static or dynamic (shaking at 80 rpm) conditions using a MaxQ Mini 4450 shaker (Barnstead/Lab-Line, Dubuque, IA). Supernatant was collected and replaced daily to maintain a constant volume. To allow quantification of released simvastatin acid, CAP was precipitated from the 1 ml of supernatant samples by treatment with 0.1 ml of 0.1 M hydrochloric acid. Acid-treated supernatants were then centrifuged at 1000 rpm for 5 minutes. Solutions were placed into 96-well assay plates, along with simvastatin acid standards made by serial diluting from a 1 mM stock solution. The concentration of simvastatin acid was determined fluorometrically using a SpectraMAX Gemini XS ( $\lambda_{ex}=390$  nm,  $\lambda_{em}=413$  nm).

Profiles were predicted using the approach of [47], who proposed the use of nonuniform initial concentration distributions as a means to regulate the release of biomolecules from drug delivery devices. For erosion-controlled systems, Eq. 1 describes the fractional release from a planar sheet with initial biomolecule distribution  $f(x)$ , half-thickness  $a$ , and erosion rate constant  $B$ .

$$\frac{M}{M_{\infty}} = \frac{\int_{a-Bt}^a f(x)xdx}{\int_0^a f(x)xdx} \quad \text{Eq. 1}$$

#### 1.2.4 Cell Culture

MC3T3-E1 preosteoblastic cells (CRL-2593; ATCC, Manasas, VA) were seeded at a density of 15,000/cm<sup>2</sup> into 24-well tissue culture plates in  $\alpha$ -Minimum Essential Medium (MEM) containing 10% fetal bovine serum (GIBCO/Invitrogen, Carlsbad, CA), 50  $\mu$ g/ml ascorbic acid (Sigma), 5 mM  $\beta$ -glycerophosphate (Sigma), and 0-1  $\mu$ M simvastatin acid. In a deviation from previous simvastatin treatment studies in the literature, medium was exchanged every day. In sustained release cultures, cells were constantly exposed to simvastatin acid at a fixed concentration, *i.e.*, the medium always contained the same concentration of drug. In intermittent release cultures, cells were exposed to alternating concentrations, *i.e.*, the medium varied between simvastatin-containing and drug-free medium. Multiple dilutions of simvastatin acid were selected based on preliminary cytotoxicity studies. Medium in control cultures was changed on the same schedule, but it did not contain simvastatin acid at any time.

#### 1.2.5 Assays

After 3, 7, 10, and 14 days of culture, cells were rinsed twice with PBS and then lysed by sonication in a high salt solution (0.05M NaH<sub>2</sub>PO<sub>4</sub>, 2M NaCl, and 2mM EDTA). DNA standards were prepared by serial diluting calf thymus DNA in the high salt solution. Hoechst 33258 (final concentration, 0.5  $\mu$ g/ml; Sigma) was added to DNA standards and samples and allowed to react in the dark for 10

minutes [16,98]. The amount of DNA in the samples was determined by measuring fluorescence ( $\lambda_{\text{ex}}=356$  nm,  $\lambda_{\text{em}}=458$  nm).

To measure alkaline phosphatase (AP) activity, cell lysate was incubated with substrate solution prepared by dissolving 10 mM of p-nitrophenyl phosphate (Sigma) in 0.6 M 2-amino-2-methyl-1-propanol buffer, pH 10 [16]. After 30 minutes, 0.25 N NaOH was added to each well to immediately stop enzyme activity. Absorbance at 410 nm was measured with an MR5000 microplate reader (Dynatech Laboratories, Chantilly, VA), and the amount of substrate cleaved was determined using  $\epsilon=1.7 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ . Activity was expressed as nmol of substrate cleaved per minute and then normalized by DNA content.

For measuring osteocalcin (OCN) secretion, conditioned medium was assayed using an EIA kit (Biomedical Technologies, Stoughton, MA) following the manufacturer's protocol. Concentrations were determined from a standard curve constructed using mouse OCN.

### **1.2.6 Statistical Analysis**

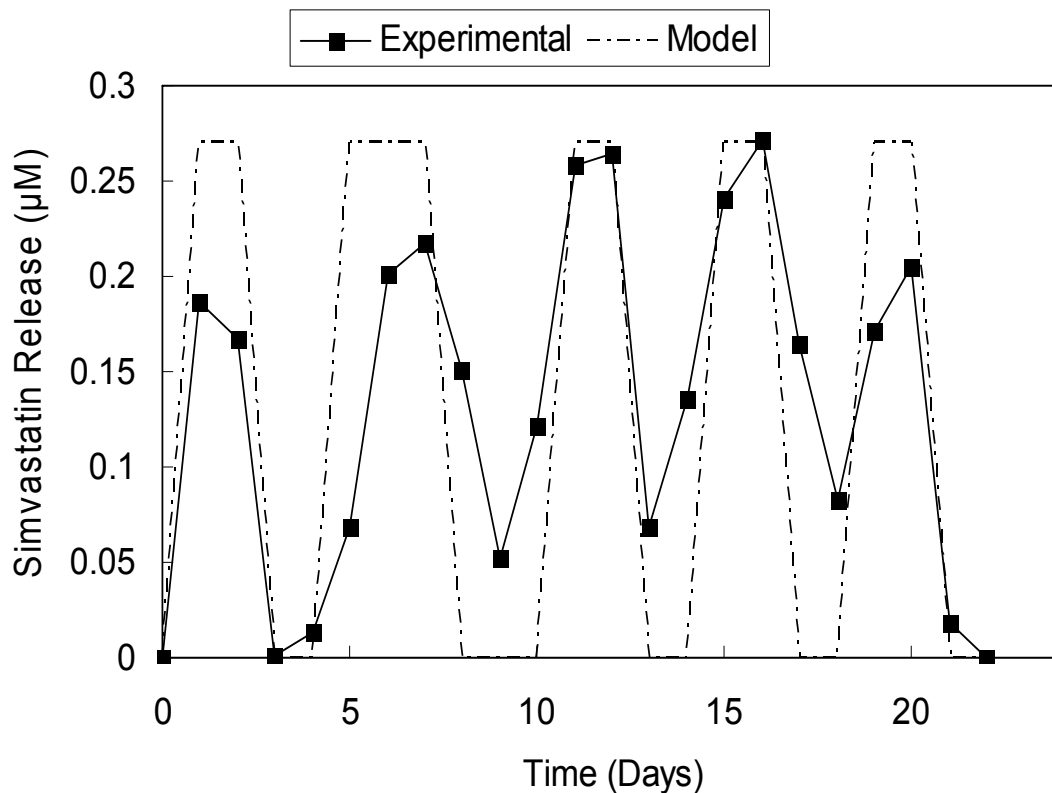
Results (mean and standard deviation) were calculated from at least six replicate samples. One-way analysis of variance (ANOVA) was conducted using the computer application InStat (Graphpad Software, San Diego, CA). Post-hoc comparisons were made using the Tukey-Kramer test when the p-value was significant ( $p < 0.05$ ).

## 1.3 RESULTS

### 1.3.1 Release Profiles

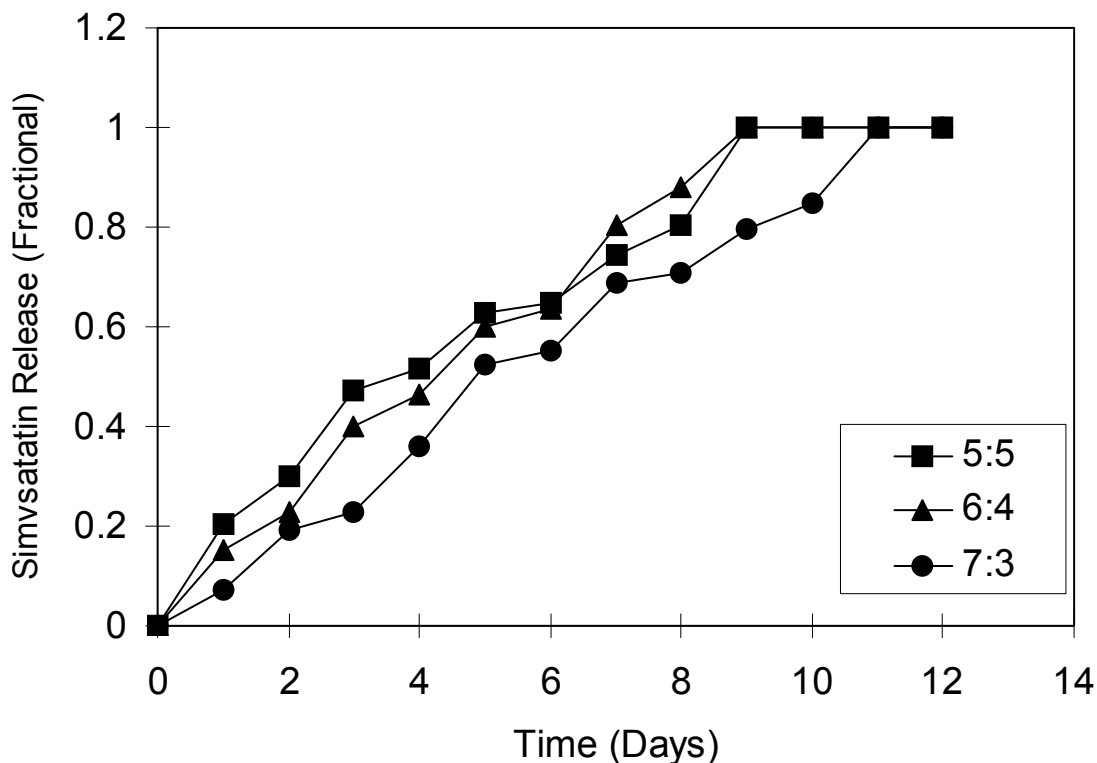
To create intermittent release profiles, drug-loaded microspheres were alternately layered with blank (unloaded) microspheres. Microspheres were 50-150  $\mu\text{m}$  in diameter and had a loading of 68-73 ng simvastatin acid per mg of polymer. By multilayering blank and loaded microspheres, six-, eight-, and ten-layer devices had approximate totals of 3, 4, and 5  $\mu\text{g}$  simvastatin acid, respectively. Figure 1.1 shows both the designed/predicted and experimentally determined release profiles for 10-layer devices. In general, the shape and duration of the experimental profile were comparable to those of the desired/predicted release profile. Two differences were noted, however. Whereas the designed profile had zero release between the peaks, small amounts of simvastatin acid were measured. In addition, the peak release values were not identical to those predicted in the model. Regardless, the desired intermittent release profiles were obtained.

Figure 1.2 shows cumulative release of simvastatin acid for the three different blend ratios under dynamic release conditions. All three profiles were linear. The polymer with the highest ratio of CAP to PF-127 (7:3) had a slightly, but significantly ( $p < 0.05$ ), slower release rate. This blend also lasted for a longer time, approximately two days longer. Behavior of the 5:5 and 6:4 blends was statistically similar. And although differences were observed in the rate and duration of release, statistically similar total amounts of simvastatin acid were delivered for all blends.



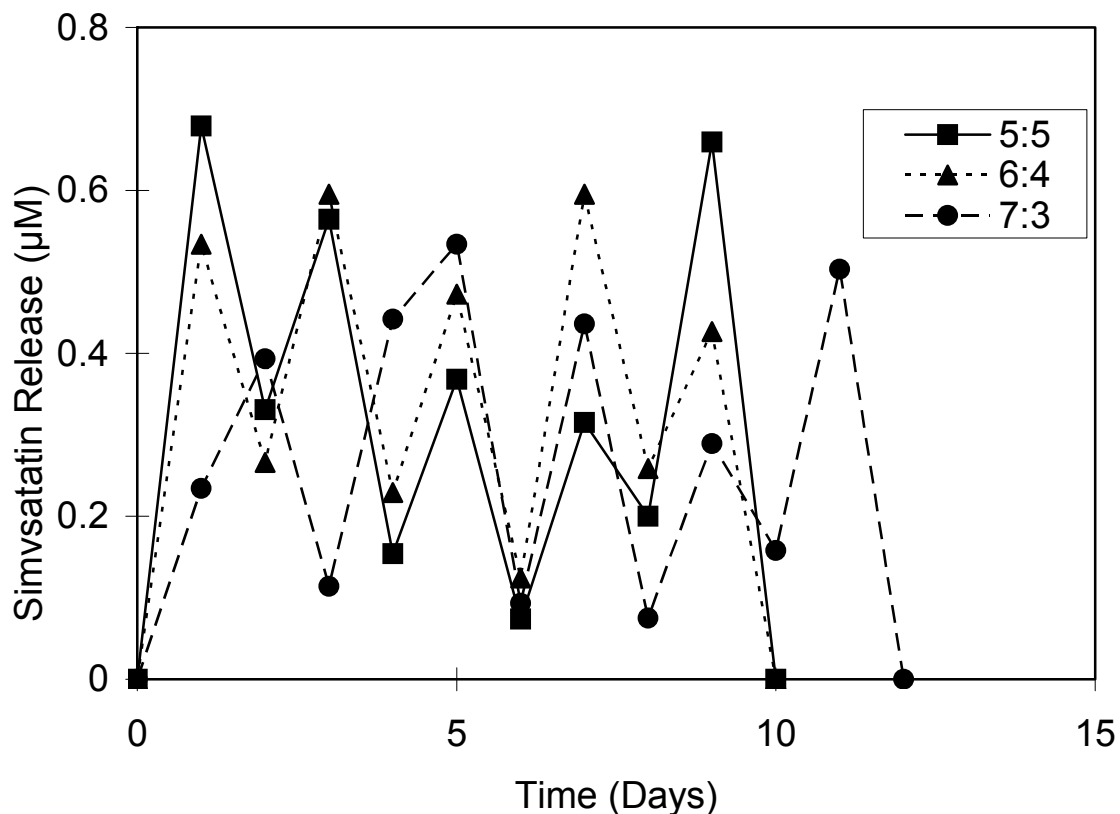
**Figure 1.1. Comparison between the desired/predicted and the experimental release profiles for 10-layer devices (7:3 CAP:PF-127).**

Figure 1.3 shows the effect of CAP to PF-127 blend ratio on intermittent release of simvastatin acid under dynamic conditions. All three devices showed exactly five discrete peaks, with each layer degrading over two to three days. As expected from Figure 1.2, 7:3 blends lasted slightly longer than did 5:5 and 6:4 devices.



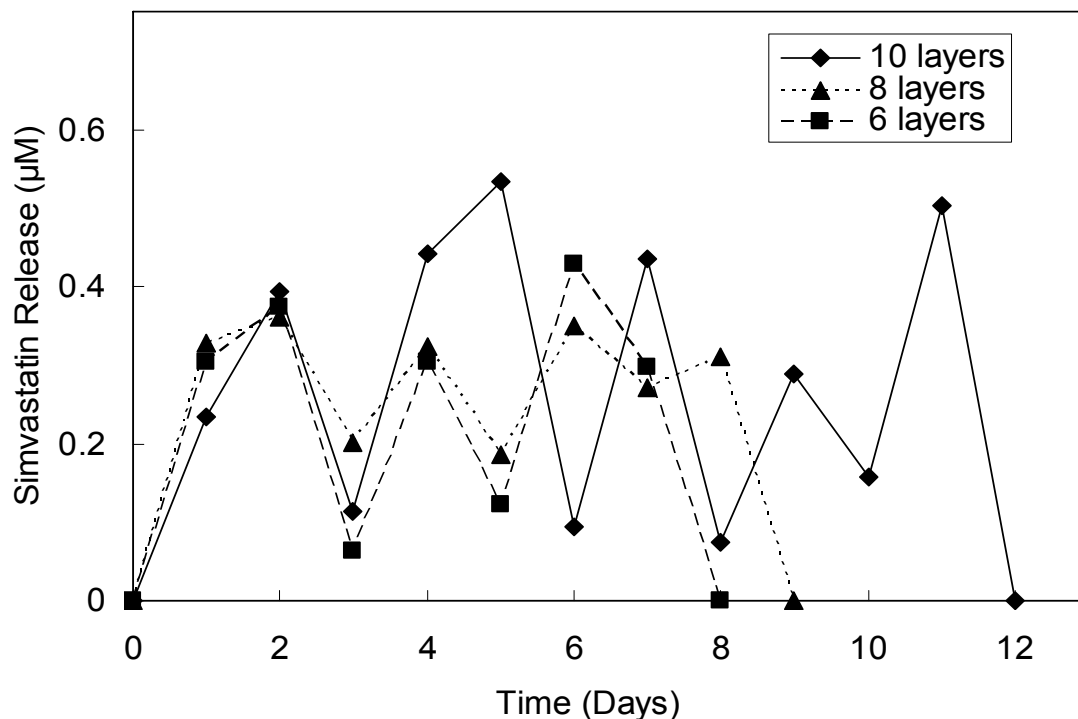
**Figure 1.2. Effect of blend ratio on cumulative release of simvastatin acid. Data are the mean of at least six replicates. Error bars, which ranged from 3-11% of the mean, are not shown to prevent obscuring the curves.**

The number of layers can be altered to modulate release (Figure 1.4). Six layer devices had exactly three discrete peaks, eight layer devices had four peaks, and ten layer devices had five peaks under dynamic release conditions. In these experiments, each layer lasted approximately two days, independent of the number of layers.



**Figure 1.3. Effect of blend ratio on intermittent release of simvastatin acid from 10-layer devices. Data are the mean of at least six replicates. Error bars, which ranged from 5-9% of the mean, are not shown to prevent obscuring the curves.**

The release conditions were also found to affect the kinetics of polymer erosion and subsequent release of simvastatin acid (Figure 1.5). Five discrete peaks were seen when samples were degraded under both static and dynamic conditions. Under static release conditions, however, 10-layer devices lasted for more than 20 days, compared to only 12 days under dynamic conditions.



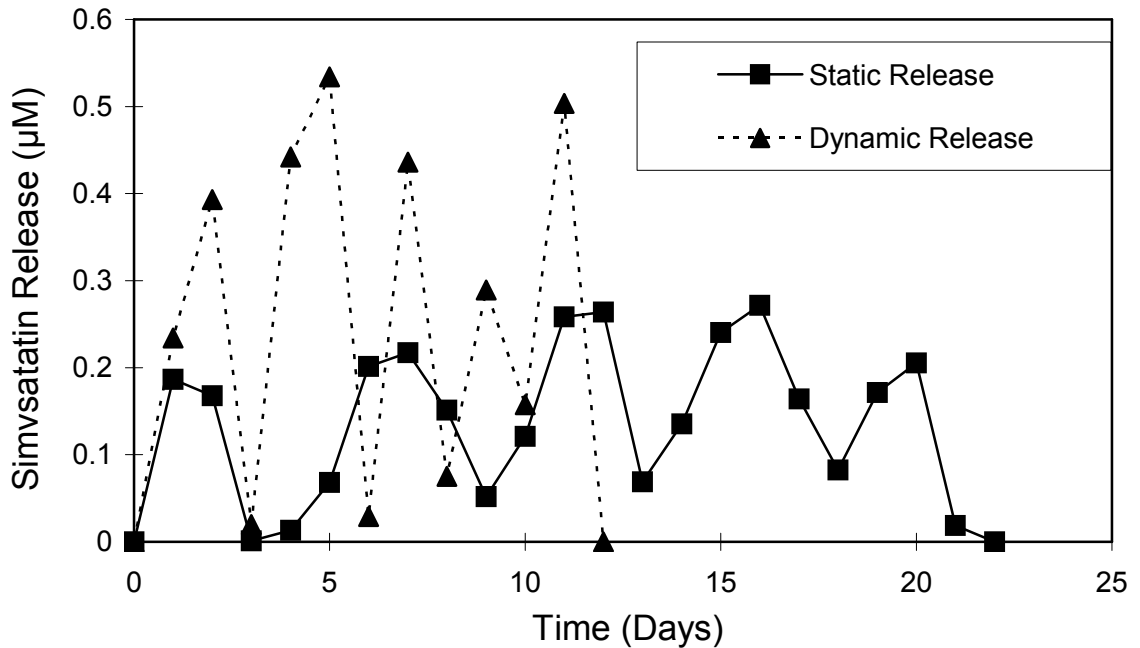
**Figure 1.4.** Effect of number of layers on intermittent release of simvastatin acid (blend ratio, 7:3). Data are the mean of at least six replicates. Error bars, which ranged from 3-14% of the mean, are not shown to prevent obscuring the curves.

### 1.3.2 Cytotoxicity

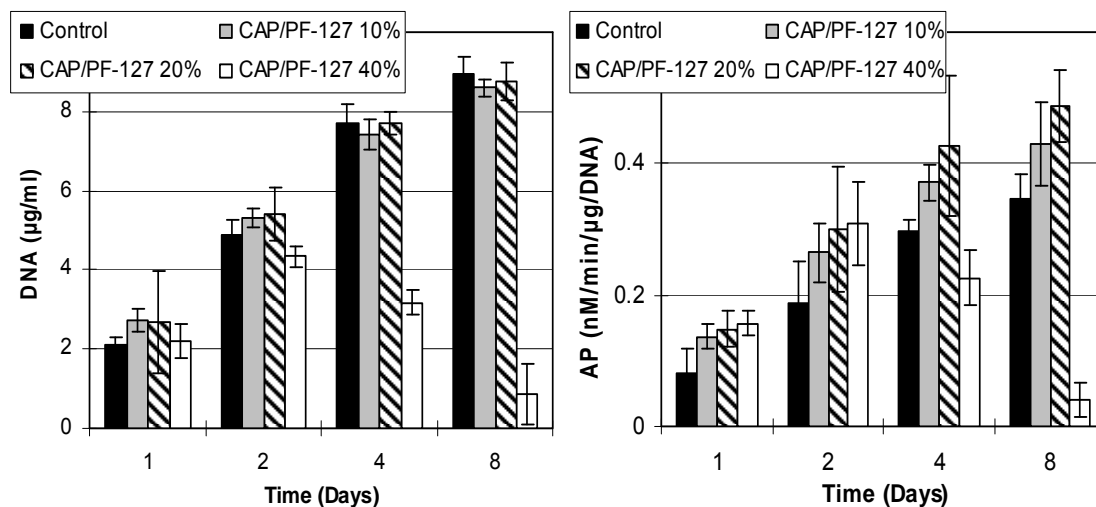
Degradation products from polymer erosion experiments (*i.e.*, dissolved CAP and PF-127 in the PBS) were diluted in cell culture medium and added to MC3T3-E1 osteoblastic cells (Figure 1.6). The undiluted release supernatant was considered 100%, and increasing dilutions into the cell culture medium were tested. High concentrations of CAP/PF-127 had a cytotoxic effect, causing cell



death. Even 40% degradation products had a time-dependent, adverse effect on the cells ( $p < 0.01$ ). While cell responses were similar within the first two days of exposure, cytotoxic effects became apparent by four days. At 20% and below, however, the cells were unaffected; both the DNA content (reflecting number of cells) and production of alkaline phosphatase (reflecting bioactivity) were statistically similar to levels in control cultures.



**Figure 1.5. Effect of incubation conditions on intermittent release of simvastatin acid from 10-layer devices (blend ratio, 7:3).**

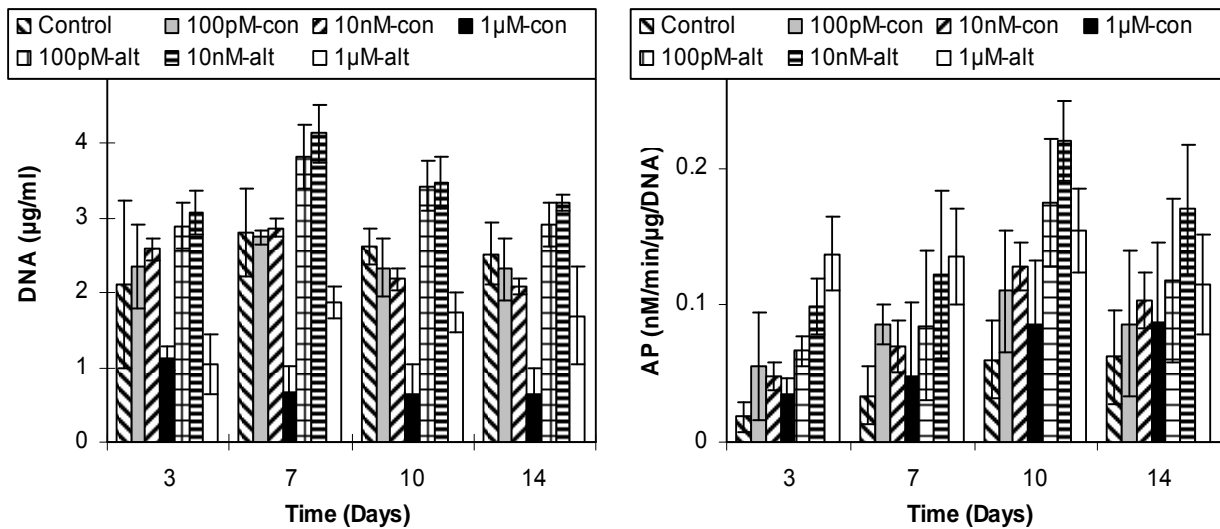


**Figure 1.6. Effect of CAP/PF-127 (7:3) degradation byproducts on osteoblastic cells.**

### 1.3.3 Cell Responses to Alternating or Constant Concentrations of Simvastatin Acid

Having determined acceptable levels of degradation products, effects of different profiles of exposure to simvastatin acid were investigated. DNA content and alkaline phosphatase (AP) activity in MC3T3-E1 cultures exposed to pulsatile/alternating and sustained/constant delivery of simvastatin acid are shown in Figure 1.7. In all groups, except 1 µM constant concentration, the amount of DNA increased until the seventh day, and the growth rates were somewhat diminished afterward. Intermittently exposing cells to 1 µM simvastatin acid reduced, but did not eliminate, the adverse effect of this higher concentration. Alternating exposure to lower concentrations of simvastatin acid increased DNA contents in the earlier stage of culture ( $p < 0.05$ ). AP was elevated

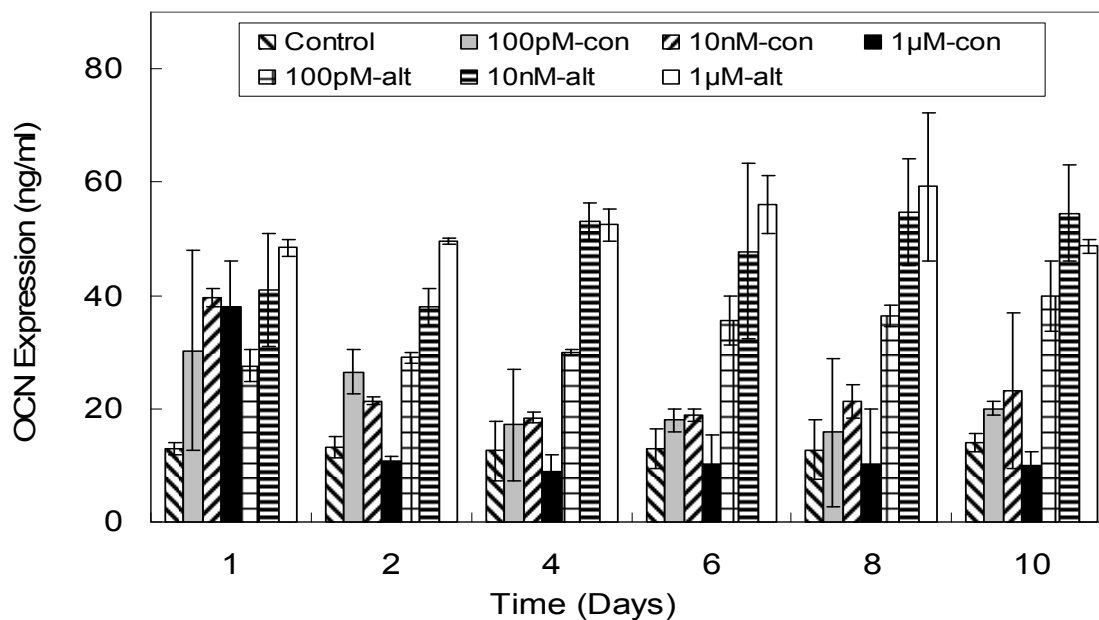
by intermittent exposure to simvastatin acid. Activity was significantly higher for the 100 pM and 10 nM alternating groups at 10 days compared to the other treatments ( $p < 0.05$ ).



**Figure 1.7. DNA content and alkaline phosphatase activity for osteoblasts exposed to constant and alternating simvastatin acid.**

Results for OCN secretion by MC3T3-E1 cells exposed to alternating and constant delivery of simvastatin acid and the controls are shown in Figure 1. 8. In control cultures, OCN levels remained relatively constant at low concentration ( $p < 0.05$ ). At day 1, cells constantly exposed to simvastatin acid showed higher concentrations of OCN compared to controls and close to levels in cells exposed to alternating simvastatin acid. At days 2 and 4, cells constantly exposed to simvastatin acid showed decreased concentrations that then remained fairly constant ( $p < 0.05$ ). For 1 µM constant exposure, levels of secreted OCN were dramatically decreased compared to the 100 pM and 10 nM constant

concentrations. All cells intermittently exposed to simvastatin acid showed increased OCN concentrations in the earlier stage of culture ( $p < 0.05$ ), and levels remained high. Concentrations were significantly higher for the 10 nM and 1  $\mu$ M alternating groups after 2 days in comparison with the other groups ( $p < 0.05$ ).



**Figure 1.8. Osteocalcin secretion for osteoblasts exposed to constant and alternating simvastatin acid.**

#### 1.4 DISCUSSION

Simvastatin has an HMG-like moiety, which is present as an inactive lactone. *In vivo*, this prodrug is enzymatically hydrolyzed to its active hydroxyacid form [58]. Simvastatin also has a rigid, hydrophobic section that is covalently linked to the HMG-like portion. Consequently, the drug has a very low aqueous solubility, approximately 1.4  $\mu$ g/ml [57]. More hydrophilic molecules can

give a higher local concentration. Therefore, the approach used for enhancing simvastatin solubility was hydrolysis to cleave the lactone ring and convert the molecule to its  $\beta$ -hydroxyacid form [59]. The hydroxyacid form of simvastatin is approximately three orders of magnitude less lipophilic than the lactone form [99].

Cellulose acetate phthalate (CAP) is a cellulose derivative that has been used in the pharmaceutical industry for enteric coating of oral tablets and capsules. While the regulatory status is clear for oral applications (*i.e.*, in FDA Inactive Ingredients Guide), it remains unknown with respect to parenteral devices. However, Heller's group has previously used CAP in formulations for treating heroin addiction [44,45].

Pluronic F-127 (PF-127) is a triblock copolymer of polyethylene oxide and polypropylene oxide. The numerous ether sites within PF-127 allow for hydrogen bonding with carboxylic acid groups in CAP. Blends of CAP and PF-127 form an association polymer that undergoes surface erosion following deprotonation at physiological pH and consequently shows zero-order release.

In contrast to the commonly used biodegradable polyesters, such as poly(lactide-co-glycolide), which undergo bulk hydrolysis, the CAP/PF-127 system degrades by surface erosion, in which the material degrades from the outermost surface toward the inside [16,47]. Therefore, the overall shape of the release profiles was linear. When alternating loaded and unloaded layers, flatter regions reflecting erosion of the blank layers were also observed.

As a non-ionic surfactant, PF-127 is readily soluble in water, but CAP is swelled and then relatively slowly dissolved in neutral solutions. Consequently,

blends containing higher ratios of PF-127 had shorter duration delivery profiles. But the overall effect was small, in contrast to the work of Lee, in which larger differences in erosion were observed for the different blends[47,100]. This discrepancy may be related to structural differences resulting from the fabrication methods. Lee and associates made their devices by simply solvent casting, whereas in the present work, CAP/PF-127 microspheres were made first and then pressure-sintered into multilayered devices. Another reason for the different degradation rates may be the exposed surface area. Lee's samples were exposed on both top and bottom surfaces, so the degradation rate was relatively higher compared to the present devices, which were coated to protect all but one surface from the aqueous environment. Differences in the carboxylate content of CAP obtained from different sources may also have affected material degradation.

Directional control of release was obtained by coating with a more hydrophobic biodegradable polymer(PLGA). Poly Lactic acid(PLA) also can be considered as a coating materials, however the degradation rate of PLA is more than a year [101], consequently, PLA should be remained on the implant site for long period of time after CAP/PF-127 device degradation. It is not necessary that coating material remains on the implant site after delivery device degradation. Thus, only one surface was exposed to PBS, and as mentioned previously, the degradation rate was consequently reduced. The total duration of delivery depended directly on the number of layers and the release environment. For each additional set of blank and loaded layers, another release peak was

obtained. Even gentle mechanical agitation enhanced erosion of the polymer. These results indicate that, in addition to chemical dissolution effects, physical erosion also plays a role in degradation of CAP/PF-127. Therefore, even though intermittent release will be obtained, the duration of delivery will depend on the site of implantation. Consider, for example, low clearance of erosion byproducts following placement in a relatively confined bony defect compared to an implant more vigorously bathed in body fluids.

Comparison of the present doses to those used clinically is complicated by the significantly different uses of simvastatin: systemic lowering of cholesterol vs. stimulation of local bone formation. The clinically recommended therapeutic dose of simvastatin ranges from 5 to 80 mg/day. With 60 mg dosage forms, serum concentrations of simvastatin and simvastatin acid are 18.7 +/- 4.7 ng/ml and 3.5 +/- 0.5 ng/ml, respectively. Further, the half-life of simvastatin acid is 5.9 +/- 0.3 hr [56]. The devices described in the present work released simvastatin acid at a rate of approximately 36.5 ng/hr into 5 ml of PBS. To stimulate bone formation in animals, Mundy et al. [1] injected 1-10 mg/kg/day subcutaneously over the calvaria of mice for 5 days. Assuming 30 g animals, up to approximately 300 µg of simvastatin was administered each day. In contrast, the present devices contained up to only 5 µg of simvastatin acid.

As might be expected with many biodegradable/bioerodible materials, high levels of degradation byproducts can be cytotoxic. This has been well-documented for polymers and copolymers of lactic and glycolic acid [102-105]. When biodegradable materials are implanted, however, the concentration of toxic

byproducts is reduced via dilution in extracellular fluids and blood and by clearance via the circulatory and lymphatic systems. In the present work, although high concentrations of degradation products were cytotoxic, dilution of CAP/PF-127 byproducts to less than 40% prevented adverse effects. Preliminary results following implantation of CAP/PF-127 release devices in rats show no evidence of adverse effects (data not shown).

The results of DNA content, AP activity, and OCN secretion for preosteoblastic cells exposed to intermittent and sustained concentrations of simvastatin acid at lower concentrations (100pM - 10nM) indicate an anabolic effect. The continued inhibition of HMG-CoA reductase activity and therefore of cholesterol synthesis resulting from high, sustained concentrations of simvastatin acid appear to have adversely affected the cells and prevented growth and activity. After Mundy and colleagues [1] reported that simvastatin induced expression of BMP-2 in a model reporter system, Maeda et al. [51] showed that continuous low doses of simvastatin enhanced AP activity and mineralization and increased BMP-2 production in MC3T3E-1 cells.

In the present work comparing alternating exposure with constant treatment, the former stimulated higher osteoblastic activity. This result is similar to that for Mundy's delivery via subcutaneous injection and the resulting local pulsatile simvastatin profile that was found to enhance bone formation. Skoglund and associates delivered simvastatin by daily oral dosing and found that simvastatin-treated mice had a larger transverse area of fracture callus, and the force required to break the bone was greater than controls [2]. Many clinical



studies have suggested that statins are related to an increase in bone mineral density and significantly reduced fracture risk [106,107].

## 1.5 CONCLUSION

Intermittent release of simvastatin acid was achieved using the association polymer system of CAP and PF-127. The release profiles roughly mimic local exposure resulting from repeated oral dosing or subcutaneous injections. Furthermore, the release profiles can be controlled by varying polymer blending ratio, number of layers, and release conditions. Cell number, alkaline phosphatase activity, and osteocalcin secretion were enhanced in preosteoblastic cell cultures treated with alternating concentrations of simvastatin acid. Ongoing studies are directed at evaluating the bioactivity of intermittently released simvastatin acid *in vivo*. Overall, CAP/PF-127 devices can be designed to provide pulsatile release of simvastatin acid, and such alternating concentrations stimulate osteoblastic activity. These devices may be useful for promoting local bone formation.

## Chapter Two

### LOCALIZED INTERMITTENT DELIVERY OF SIMVASTATIN ACID STIMULATES BONE FORMATION *IN VIVO*

#### 2.1 INTRODUCTION

Stimulation of local bone formation using pharmacological compounds that upregulate synthesis of autogenous bone growth factors is a promising approach to treatment of bone defects. This method can lead to cost-effective devices and less complicated surgeries [3]. Application of growth factors, including bone morphogenetic protein-2 (BMP-2), can powerfully stimulate bone formation. However, such recombinant proteins are expensive and when exposed to a physiological environment, they can be degraded rapidly [108]. Furthermore the supraphysiological amounts needed may cause immune responses [109].

Simvastatin is a member of the statin family of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase competitive inhibitors, which are widely used as cholesterol-lowering drugs. Simvastatin promotes bone formation both *in vitro* and *in vivo* in animal models, associated with increased expression of BMP-2 [48,51,110]. In addition, simvastatin inhibits mevalonate, farnesyl pyrophosphate, and geranylgeranyl pyrophosphate pathways [49,111,112], which are responsible for bone formation by reducing osteoclastic activity [50]. This inhibition mechanism plays an important role in the stimulation of bone mass by

simvastatin.

Currently available simvastatin is chemically modified from naturally existing lovastatin. It has been developed for oral administration and to be hepatoselective to decrease cholesterol synthesis in the liver. When delivered via systemic circulation, simvastatin shows poor distribution to bone defect sites [110]. Consequently, developing a simvastatin delivery device that provides active amounts of drug with an appropriate time course to a desired site may be more biologically effective.

Unlike traditional controlled release devices, which often aim to achieve zero-order kinetics for constant concentration in the plasma, subcutaneous injection and oral administration show pulsatile drug concentration profiles. In a previous paper, we developed a device for intermittent release of simvastatin acid using an association polymer system of cellulose acetate phthalate (CAP) and Pluronic F-127 (PF-127). Pulsatile release profiles were demonstrated, and *in vitro* stimulation of preosteoblastic cell activity was shown [46].

Based on prior results, the objective of the present study was to investigate the ability of devices for controlled, localized delivery of simvastatin acid, either with intermittent or sustained release, to stimulate localized bone formation in a rat calvarial onlay model.

## **2.2 MATERIALS AND METHODS**

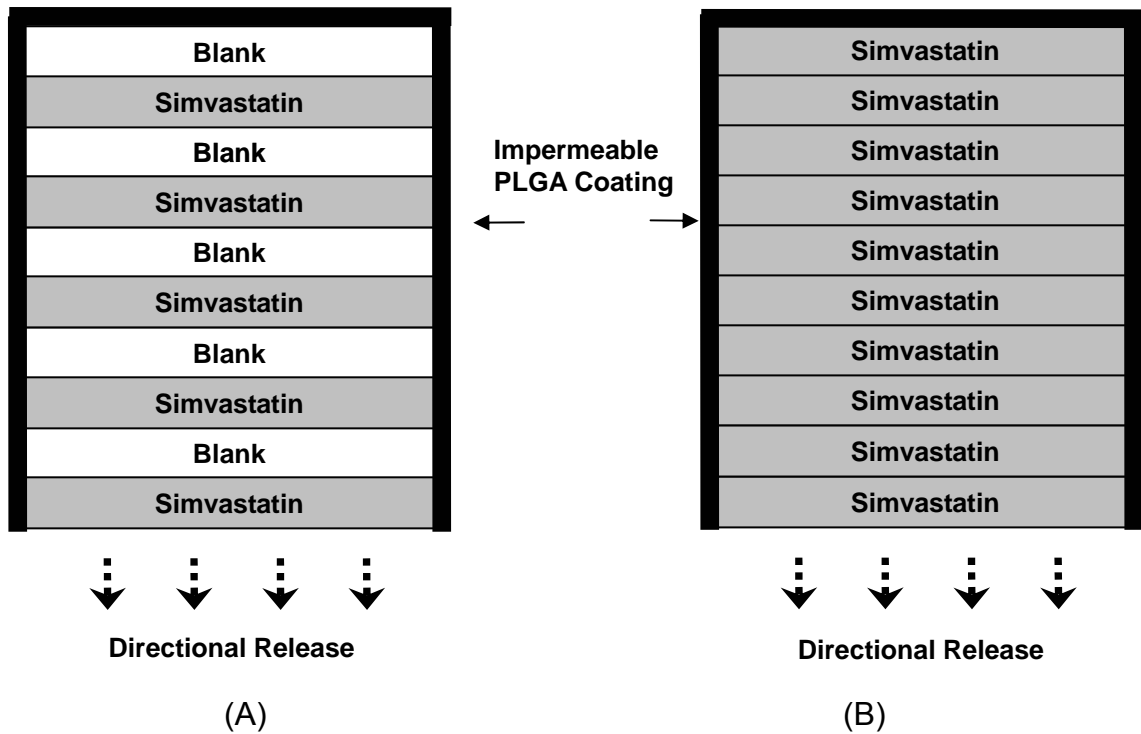
### **2.2.1 CAP/PF-127 Microspheres and Release Devices**

CAP/PF-127 microspheres and release devices were fabricated as reported previously [46] and are briefly described here. Drug-loaded

microspheres were made using a triple emulsion process to have a loading of 50 ng simvastatin acid per mg of polymer. Intermittent release devices were then made by alternating 20 mg layers of microspheres loaded with simvastatin acid and blank microspheres (without drug) (Figure 2.1). Sustained release devices were made in the same manner, but every 20 mg layer contained simvastatin acid. Control (blank) devices were made with the same amount of microspheres used in sustained and intermittent release devices, however no drug was present. To provide directional control of drug release, the bottom and sides of all devices were coated with 10% poly(lactic-co-glycolic acid) (75:25,  $M_w \sim 75$  kDa; Alkermes, Cincinnati, OH) solution in dichloromethane. To verify release profiles, devices were incubated in 150 mM phosphate-buffered saline at 37°C with gently shaking, and simvastatin concentrations were determined fluorometrically [46].

### **2.2.2 Animal Procedures**

Two month old male Sprague-Dawley rats were used in this study. A calvarial onlay model was used to determine effects of local delivery of simvastatin acid. Blank (no drug) devices (n=10), intermittent devices (n=10), and sustained devices (n=10) were tested. After anesthetization, a transverse incision was made toward the posterior end of the skull. The periosteum was reflected, and implants were placed directly on the calvarium, with the exposed drug-releasing side of the devices facing the bone. Incisions were closed with three sutures.



**Figure 2.1. Schematic representation of intermittent (A) and sustained release devices (B).**

### 2.2.3 Histological Preparation

After 9, 18, or 28 days, rats were euthanized, and the calvaria were harvested. Specimens were immediately stored in 10% buffered formalin phosphate (Fisher Scientific, Fair Lawn, NJ) solution for at least one week. Samples were decalcified overnight in 5% nitric acid, dehydrated in an alcohol series, and embedded in paraffin. Five  $\mu\text{m}$  thick coronal cross-sections were cut, before staining with hematoxylin for 15 minutes and eosin for 15 seconds to 2 minutes then dehydrate in 95% absolute alcohols for transmitted optical microscopy evaluation.

#### **2.2.4 Histological Analysis**

Representative sections from the control, sustained release, and intermittent release groups were examined at 40X and 100X magnification with an Olympus IX51 inverted microscope with Insight digital camera (Diagnostic Instruments, Sterling Heights, MI). In addition to qualitative assessments, the thickness and total area of newly formed woven bone, the thickness relative to implant center, and area percentage of lamellar bone islands within the woven bone layer were determined. Thickness measurements were made on at least six samples with 5 different regions in each group. Area calculations were based on examination of 3 different 1 mm<sup>2</sup> spots from at least six samples in each group. ImageJ software (National Institutes of Health, Bethesda, MD) was used for quantification.

#### **2.2.5 Statistical Analysis**

Data are presented as mean  $\pm$  standard deviation of up to nine measurements. All histological measurements were compared among groups by analysis of variance (ANOVA) using InStat (Graphpad Software, San Diego, CA). Post-hoc comparisons were made using the Tukey-Kramer Comparison Test when the p-value was significant ( $p < 0.05$ ).

### **2.3 RESULTS**

#### **2.3.1 Release Profiles**

The approximate loadings of simvastatin acid were 5  $\mu\text{g}$  for intermittent release devices (5 drug-loaded layers at 1  $\mu\text{g}/\text{layer}$ ) and 10  $\mu\text{g}$  for sustained

release devices (10 loaded layers at 1 µg/layer). Figure 2.2 shows profiles for release of simvastatin acid from the intermittent and sustained delivery devices. As expected from their design, intermittent release devices exhibited five discrete peaks separated by two to three days. In contrast, for sustained release devices, the concentration of simvastatin acid increased over the first 24 hours and remained relatively constant for the next ten days. Both devices achieved similar maximum concentrations and lasted for comparable durations.

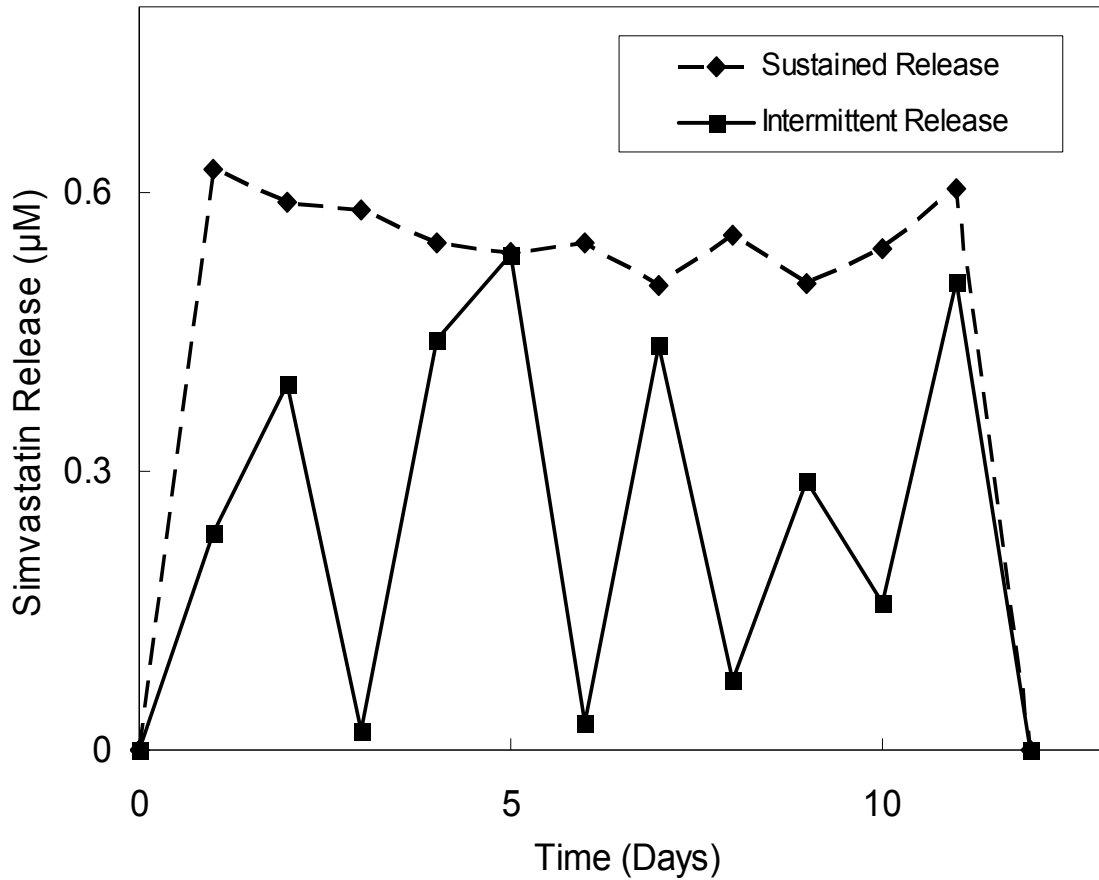
### **2.3.2 Clinical and Physical Examination**

During the postoperative healing period, wound dehiscence occurred in 3 rats (1 in each group), and infections resulting from bites from cage mates were observed in 3 rats (2 in blank group and 1 in sustained one). As a result, 7 control, 9 intermittent, and 8 sustained specimens were available for histological analysis. All animals gained weight (up to 50 g) during the postoperative healing period (9-28 days; data not shown).

### **2.3.3 Histological Analysis**

Figure 2.3(A) shows the gross morphology of the implant site. At 9 and 18 days, devices were surrounded by fibrous and inflammatory tissue. At 28 days, the amount of tissue was significantly reduced as the drug delivery component of the implants was degraded and the poly(lactic-co-glycolic acid) shell collapsed and began to disintegrate. In Figure 2.3(B), comparison of the three types of devices shows similar appearance after 28 days. In all groups, implants had

largely been degraded and some fibrous scar tissue was present at the surgical site.

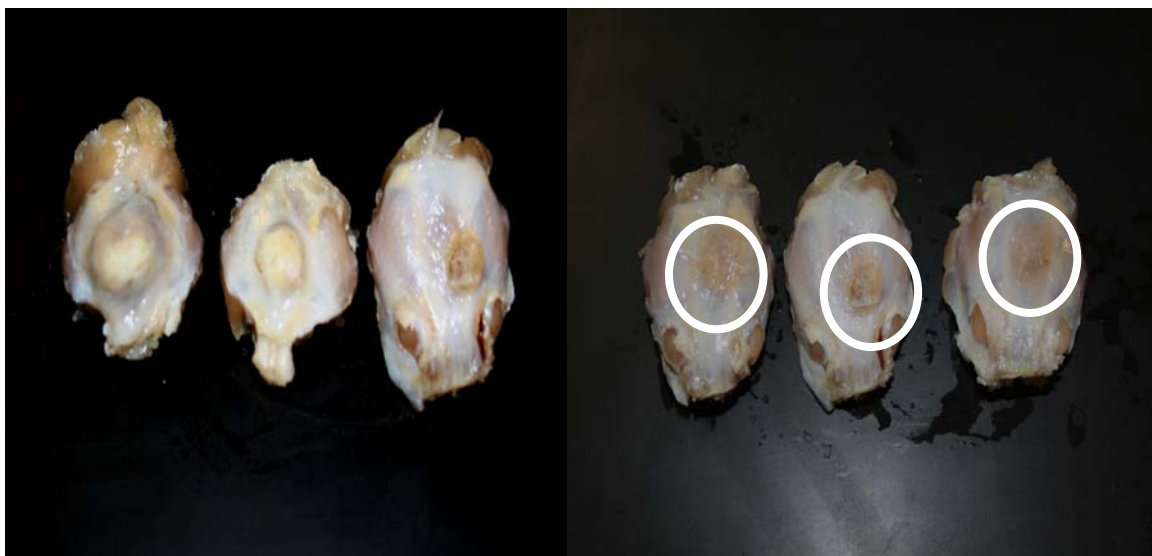


**Figure 2.2. Comparison between the intermittent and the sustained release profiles for 10-layer devices .**

Figure 2.4 shows the bone-forming ability of released simvastatin acid. Much newly formed woven bone with islands of matrix stained more intensely with eosin and osteocytes were found on the calvarium. Furthermore, many blood vessels were generated in and around the new bone area.



Newly formed woven bone thickness was significantly different between the control simvastatin acid release groups (Fig. 2.5 and 2.6). Fibrous and inflammatory tissue was found above the newly formed bone. Intermittent release devices stimulated 133% greater woven bone thickness ( $187\pm40\ \mu\text{m}$ ) compared to control devices ( $80\pm30\ \mu\text{m}$ ) ( $p<0.05$ ), and sustained release devices stimulated 77.5% greater woven bone thickness ( $142\pm56\ \mu\text{m}$ ) ( $p<0.05$ ) compared to controls. Comparing the two release profiles, intermittent devices stimulated a 32.3% greater response than did the sustained release ( $p<0.05$ ).



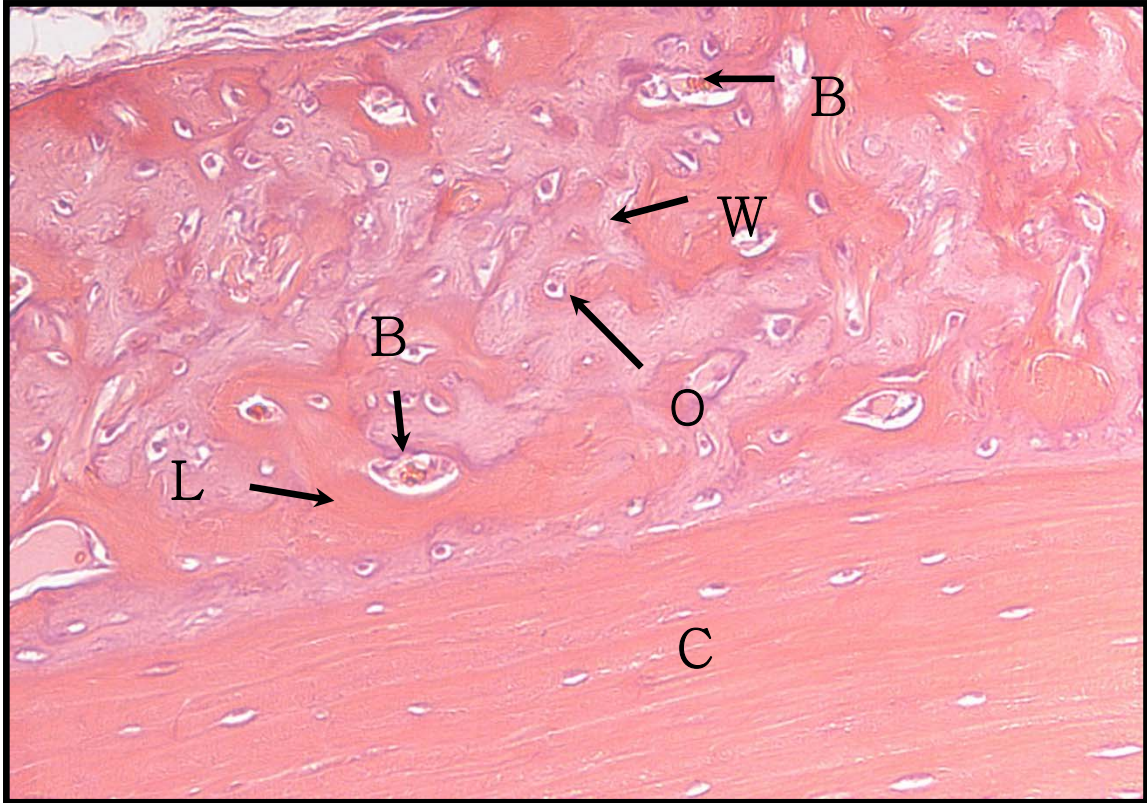
9 days 28 days 28 days

(A)

Control Sustained Intermittent

(B)

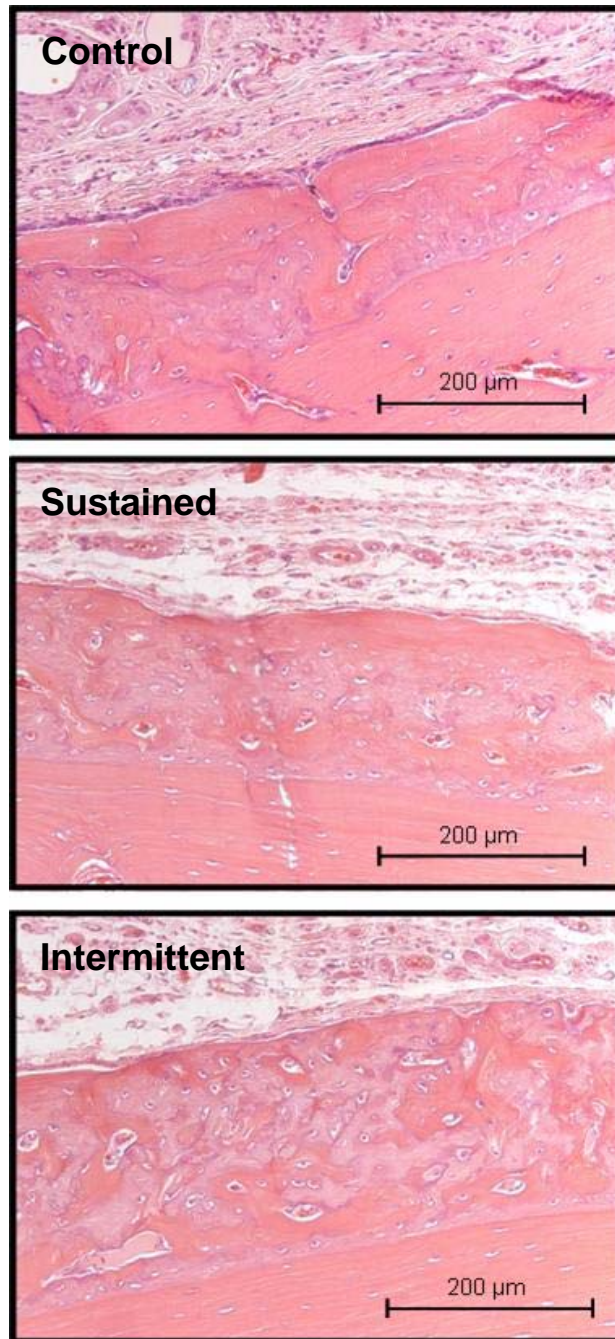
**Figure 2.3. Gross morphological state of tissues (A) around sustained release devices at different healing times (9, 18, and 28 days) and (B) around different devices after 28 days.**



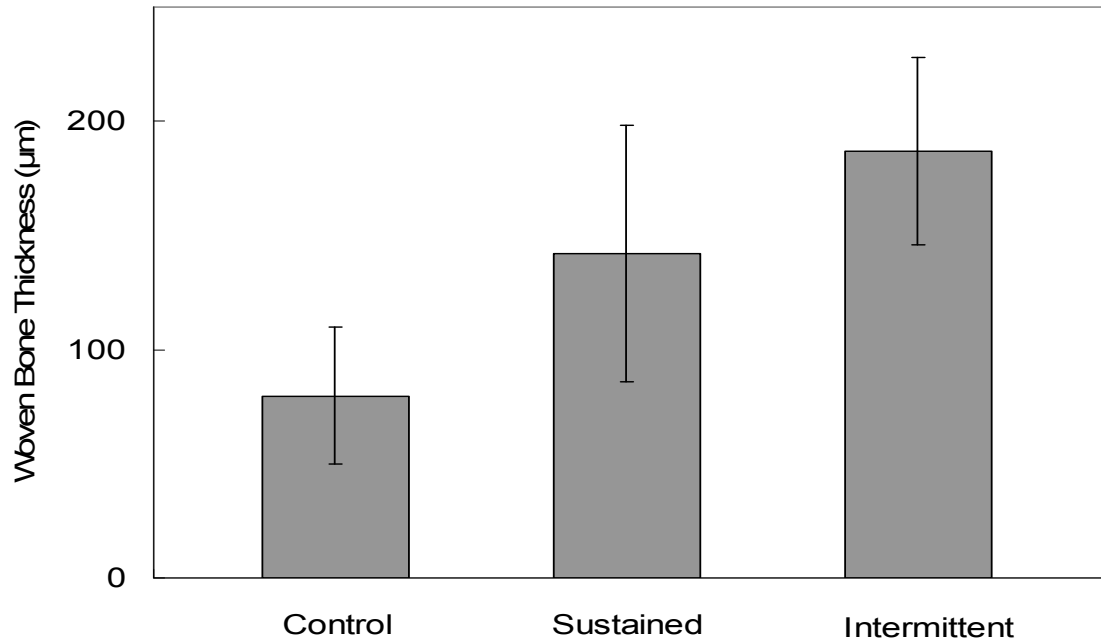
**Figure 2.4. Representative photomicrograph showing the formation of woven bone (W), lamellar bone (L), osteocytes (O), and blood vessels (B) above the calvarium (C).**

Woven bone thickness varied from implant center to periphery, as shown in Figure 2.7 The site directly below the device showed minimal woven thickness ( $18.8-36 \pm 7.6-40 \mu\text{m}$ ) compared to other locations ( $44-205.1 \pm 6.53-38.5 \mu\text{m}$ ) ( $p < 0.05$ ). Figure 2.8 shows the total area of new bone and the percentage of more intensely stained matrix relative to total bone. Intermittent release groups elicited 162.7% larger new bone area and sustained delivery stimulated 74.1% more new bone area than did control implants. The percentage of intensely-stained bone matrix for the intermittent (25.5%) and sustained groups (24.8%) was higher than that for the controls (21.3%) ( $p < 0.05$ ). Comparing alternating

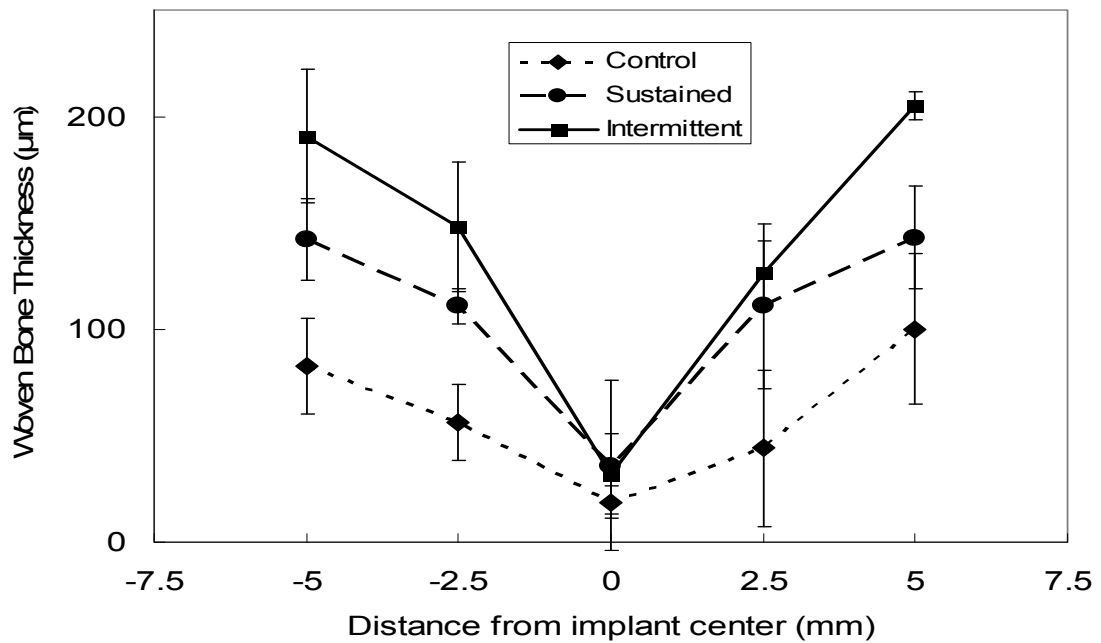
and sustained groups, although the alternating group was slightly higher, the difference was not statistically significant.



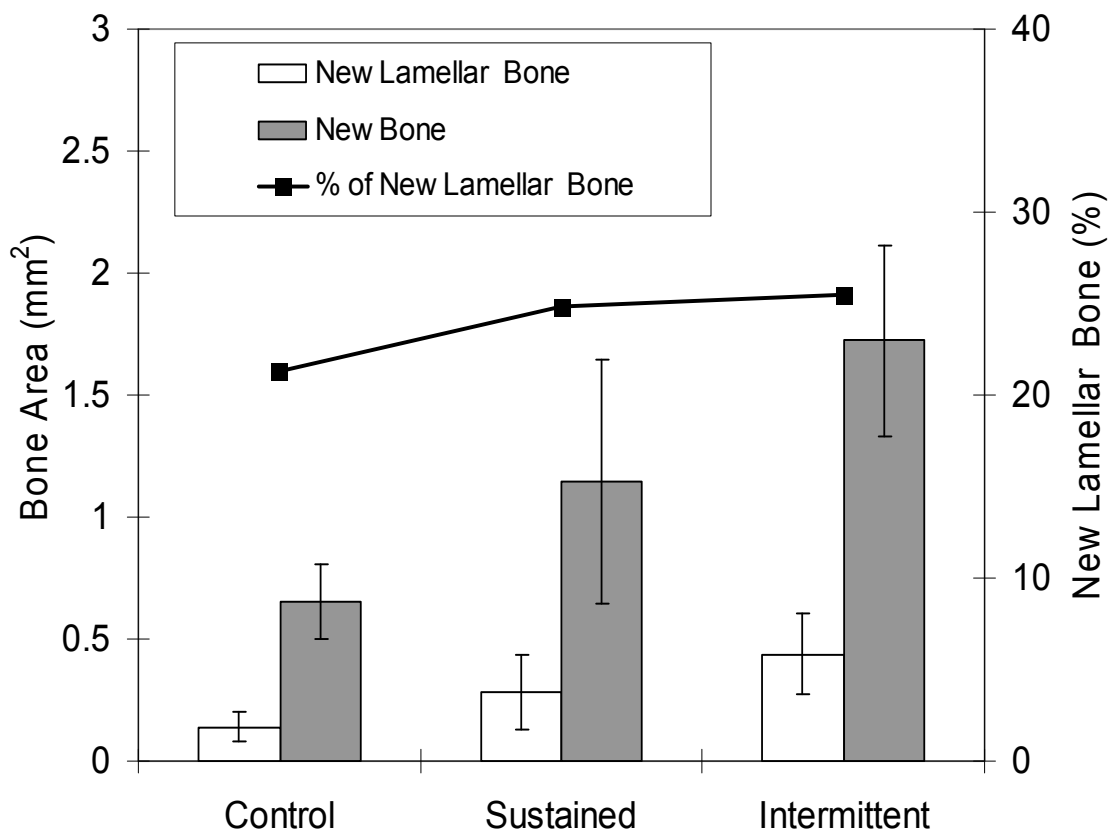
**Figure 2.5. Representative photomicrographs comparing new bone formation above the calvarium in all experimental groups.**



**Figure 2.6.** Thickness of newly formed woven bone over calvariae treated with control, sustained release, and intermittent release devices.



**Figure 2.7.** Thickness of newly formed bone according to distance from implant/calvarium center (- Left/+ Right).



**Figure 2.8. Total area of new bone and percentage of lamellar bone.**

## 2.4 DISCUSSION

The present results demonstrate that locally released simvastatin acid stimulated supracalvarial bone growth. Osteogenesis was enhanced by both intermittent and sustained delivery.

All specimens showed signs of inflammatory responses up to 18 days. Newly formed woven bone was found in controls animals implanted with blank (drug-free) devices as well as in those in the sustained and intermittent release groups. Inflammatory responses on bone formation are related to cytokine mediators, which influence cells locally to change remodeling activities

associated with appositional bone growth [113-115]. Accordingly, inflammatory responses may be one of the main contributions to formation of new bone found in all groups, with localized release of simvastatin acid enhancing the effects.

Even though the studies were not intended to quantitatively assess angiogenesis, it is interesting to note that many newly formed blood vessels were found in around the woven bone in all groups. However the simvastatin treated groups had a higher degree of angiogenesis than control groups (data not shown). These blood vessels likely increased blood flow in and around the implantation site and expedited new bone formation. It was reported by Kureishi et al. that simvastatin acid administration promotes new blood vessel growth [52].

The newly formed bone was composed of large areas of woven bone intermixed with areas of lamellar bone. These islands were identified by their greater organization and differential staining, with the more mature bone staining more intensely. It is thought that an appositional growth occurred as layers of bone were formed over the existing calvarium, resulting in overall bone growth [116], similar to what is observed during intramembranous ossification. Even though new bone thicknesses stimulated by the intermittent release devices was highest and for the control devices was lowest, the morphology of newly formed bone and presence of islands of lamellar bone in all three groups were analogous. However, the slightly higher percentage lamellar bone stimulated by both types of drug-containing devices suggests that simvastatin acid delivery stimulated bone formation and bone maturation.

The present results are in accordance with previous reports of simvastatin delivered via injection in mice. Mundy's' group subcutaneously injected three different doses (1, 5, or 10 mg/kg/day, which would be 30, 150, or 300 µg/day for a 30 g mouse) of simvastatin for 5 days, and reported increases in total bone area of 2, 20, and 46%, respectively [1]. In other studies, treatment with low doses of simvastatin increased cancellous bone volume and the transverse area of fracture callus. Our release devices had alternating five or ten simvastatin-loaded layers and intermittently or continuously delivered 1 µg/layer for no more than 12 days, yet they promoted bone formation to either a 74% (sustained) to 163% (intermittent) increase in bone area. It is thought that the present devices would be a candidate for localized bone formation as well as or better than subcutaneous injections.

A limited number of groups have investigated materials for localized delivery of simvastatin [3-5,117]. Thylin et al. injected simvastatin mixed into methylcellulose gel [5]. They reported an optimal dose of 2.2 mg simvastatin yielding up to 170% increase in calvarial thickness. "Extensive" leakage of the gel from the injection site was noted for some animals. Simvastatin (crushed Zocor tablets) has also been mixed with absorbable collagen and implanted into rabbit calvarial defects [118]. A dose of 0.5 mg simvastatin per 0.2 g collagen sponge stimulated filling in the defects, but the kinetics of drug release were not studied. A reservoir system with methylcellulose gel containing 0.1-2.2 mg of simvastatin under a biodegradable poly(lactic acid) membrane enhanced bone formation by 45% on the rat mandible, but considerable inflammation occurred

[3]. Although not tested *in vivo*, other approaches to delivering simvastatin include incorporation in hydroxypropylmethyl cellulose, sodium carboxymethyl cellulose, and chitosan for short term (approximately 6 hr) release [117] and attachment to poly(lactic-co-glycolic acid) for extended retention of the drug [119,120]. The intermittent and sustained release devices described in this paper had approximate totals of 5  $\mu\text{g}$  and 10  $\mu\text{g}$  simvastatin acid, respectively, and they stimulated 78% (sustained) to 133% (intermittent) increased bone thickness compared to control groups.

In previous work, the drug delivery component of the implants was totally degraded within two weeks *in vitro*, but the PLGA membrane used for controlling directionality of simvastatin acid release remained in the sample solution [46]. Depending on the ratio of lactic acid to glycolic acid, PLGA degradation rates vary from 1 to 6 month [121]. Due to the large difference in degradation times for CAP/PF-127 and PLGA, even though the CAP/PF-127 drug release component was entirely degraded, some degree of fibrous and inflammatory tissue surrounded the remaining PLGA fragments after 28 days.

Diffusion of released simvastatin acid to peripheral tissues increased new bone thickness at the edges of the calvariae. Mechanical and/or chemical effects may have resulted in minimal bone formation directly under the devices. For example, relative motion between implant and bone may have occurred during daily activities of the animals, and locally concentrations of CAP/PF-127 erosion products would have been higher directly under the devices. To achieve a more even layer of new bone, additional space may be required directly under the



implant. Placement of the drug delivery device in conjunction with a biodegradable scaffold or other space-making implant could alleviate the nonuniformity. Alternatively or in addition, the implant may be protected from movement immediately following placement.

## 2.5 CONCLUSION

Using a multilayering method, devices for localized delivery of CAP/PF-127 were fabricated. Alternating release devices showed five discrete peaks, while sustained release devices showed zero-order release for approximately ten days. Based on our experimental results, local delivery of simvastatin acid with either sustained or intermittent release profiles stimulated formation of new bone *in vivo*. Furthermore, the intermittent devices were more effective than sustained devices, while requiring only one half the amount of drug.

## Chapter Three

### INTERMITTENT RELEASE OF PARATHYROID HORMONE (1-34) BIODEGRADABLE DEVICE ENHANCES OSTEOBLASTIC ACTIVITIES

#### 3.1 INTRODUCTION

Parathyroid hormone (PTH) is well known for its role in calcium homeostasis. The N-terminal fragment comprising the first 34 amino acids, PTH(1-34), has biological activity comparable to the whole molecule. It has been reported that daily injections of low doses of PTH or PTH(1-34) stimulate bone formation and increase bone mass in humans and animals [7,12,14,60-63]. Many *in vivo* studies have been conducted to explain the anabolic effect of PTH or PTH(1-34) on bone formation, and experiments with intermittent infusion and injection of PTH(1-34) have shown increased bone mass [26]. It is thought that PTH increases bone formation by decreasing the apoptosis of osteoblasts [11], whereas continuous administration of PTH or PTH(1-34) result in decreased bone mass [15,64,122]. These results imply that the effects of PTH on bone cells vary with cell type and experimental conditions, such as intermittent or continuous administration [10]. Many *in vitro* studies have been conducted to determine PTH effects on bone. From the results, it is clear that PTH enhances proliferation of primary osteoblastic cells from humans and animals [65,66]. In addition, PTH exerts diverse effects on osteoblast differentiation depending on differentiation stage [123,124].

To mimic the effect of injections, devices need to provide intermittent release profiles. We previously investigated an association polymer system based on blends of cellulose acetate phthalate (CAP), which is a well known enteric coating material in the pharmaceutical industry, and a nonionic surfactant, Pluronic F127 (PF-127), which is used for stabilizing proteins and peptides during lyophilization. The CAP/PF-127 polymer system is useful for programmed drug delivery, with the rate, duration, and amount of released drug controlled by thickness, blending ratio, and number of layers [16,47].

The objective was to develop devices for intermittent release of PTH(1-34) and to determine the effects of PTH(1-34) released with different profiles on osteoblastic cellular activity. Our hypothesis was that the mode of local PTH(1-34) release affects osteoblastic activity. To verify this hypothesis, in this study, devices for intermittent delivery of PTH(1-34) were developed, and it was demonstrated that the cellular effects of PTH(1-34) on osteoblastic proliferation and differentiation were dependent on mode of administration.

## **3.2 MATERIALS AND METHODS**

### **3.2.1 CAP/PF-127 Microspheres and Release Devices**

PTH(1-34) (Bachem, Torrance, CA) was labeled with Alexa Fluor 350 Carboxylic acid, succinimidyl ester (Invitrogen, Eugene, OR) to enable measurement of the released amount. CAP (Fluka, Buchs, Switzerland) and PF-127 (Sigma, St Louis, MO) microspheres were made by a water-acetone-oil-water (W/A/O/W) triple emulsion process. CAP and PF-127 polymer blend (7:3

weight fraction of CAP and PF-127) was dissolved in acetone. To this acetone solution, phosphate-buffered saline (PBS), pH 7.4, with 0 or 250 µg/ml of CB were added and then homogenized into corn oil and sonicated for 20 seconds. The CAP/PF-127 suspension and 5% Triton X-100 solution were added to deionized water and then stirred to harden the microspheres for 5 minutes. Following collection, microspheres were washed, filtered, and lyophilized overnight.

Release devices were made using a pressure-sintering process. Fifteen to 20 mg of UV-sterilized microspheres containing PTH (1-34) were placed in the wells of a Delrin mold (diameter, 6.2 mm; depth, 20 mm) and consolidated by applying 20 MPa pressure for 5 sec. Next, for intermittent devices, blank microspheres were added on top of the first layer, and pressure was reapplied. By repeating this process, ten-layer devices were prepared. For sustained release devices, all layers were composed of microspheres containing PTH(1-34). To provide directional control of drug release, the bottom and sides of the devices were coated three times with 10% poly(lactic-co-glycolic acid) (75:25,  $M_w \sim 75$  kDa; Alkermes, Cincinnati, OH) solution in methylene chloride.

### **3.2.2 *In Vitro* Release**

Devices were immersed in 5 ml PBS, pH 7.4, and incubated at 37°C. One ml of supernatant was collected and the remaining PBS replaced daily. To allow quantification of released PTH(1-34), CAP was precipitated from the 1 ml of supernatant by treatment with 50 µl of 0.1 M hydrochloric acid. Acid-treated supernatants were then centrifuged at 1000 rpm for 5 minutes. Solutions were

placed into 96-well assay plates, along with labeled PTH(1-34) standards made by serial diluting from a 100 ng/ml stock solution. The concentrations of labeled PTH(1-34) were determined fluorometrically using a SpectraMAX Gemini XS<sup>®</sup> ( $\lambda_{\text{ex}}=346$  nm,  $\lambda_{\text{em}}=442$  nm). In order to investigate pH changes, devices were immersed in 5 to 15 ml PBS at 37°C, and the pH was measured each day.

### **3.2.3 Cell Culture**

MC3T3-E1 preosteoblastic cells (CRL-2593; ATCC, Manassas, VA) were seeded at a density of 15,000/cm<sup>2</sup> into 24-well tissue culture plates in  $\alpha$ -Minimum Essential Medium (MEM) containing 10% fetal bovine serum (GIBCO/Invitrogen, Carlsbad, CA), 50  $\mu$ g/ml ascorbic acid (Sigma), 5 mM  $\beta$ -glycerophosphate (Sigma), and antibiotics (penicillin G and streptomycin GIBCO), and 0-5 ng/ml PTH(1-34). In sustained release cultures, one set of cells was constantly exposed to various PTH(1-34) at a fixed concentration. In intermittent release cultures, another set was exposed to alternating concentrations. In control cultures, medium was changed on the same schedule, but it did not contain PTH(1-34).

### **3.2.4 *In Vitro* Bioassays**

After 3, 7, 10, and 14 days of culture, cells were harvested and then lysed by sonication in high salt solution (0.05M NaH<sub>2</sub>PO<sub>4</sub>, 2M NaCl, and 2mM EDTA). DNA standards were prepared by serial diluting calf thymus DNA in the high salt solution. Hoechst 33258 (final concentration, 0.5  $\mu$ g/ml; Sigma) was added to DNA standards and samples and allowed to react in the dark for 10 minutes [125]. The amount of DNA in the samples was determined by measuring fluorescence

( $\lambda_{\text{ex}}=356$  nm,  $\lambda_{\text{em}}=458$  nm).

To measure alkaline phosphatase (AP) activity, lysed cell samples were incubated with substrate solution prepared by dissolving 10 mM of Sigma 104 phosphatase substrate in Sigma 221 alkaline buffer solution. After 30 minutes, 0.25 N NaOH was added to each well to immediately stop enzyme activity. Absorbance at 410 nm was measured with an MR5000 microplate reader (Dynatech Laboratories, Chantilly, VA), and the amount of substrate cleaved was determined using  $\epsilon=1.7 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ . Activity was expressed as nM of substrate cleaved per minute and then normalized by DNA content.

For measuring osteocalcin (OCN) secretion, conditioned medium collected from cultures samples was assayed using an EIA kit (Biomedical Technologies, Stoughton, MA) following the manufacturer's protocol. Concentrations were determined from a standard curve constructed using mouse OCN.

### **3.2.5 Statistical Analysis**

Results (mean and standard deviation) were calculated from at least six replicate samples. One-way analysis of variance (ANOVA) was conducted using the computer application InStat (Graphpad Software, San Diego, CA). Post-hoc comparisons were made using the Tukey-Kramer test when the p-value was significant ( $p < 0.05$ ).

## **3.3 RESULTS**

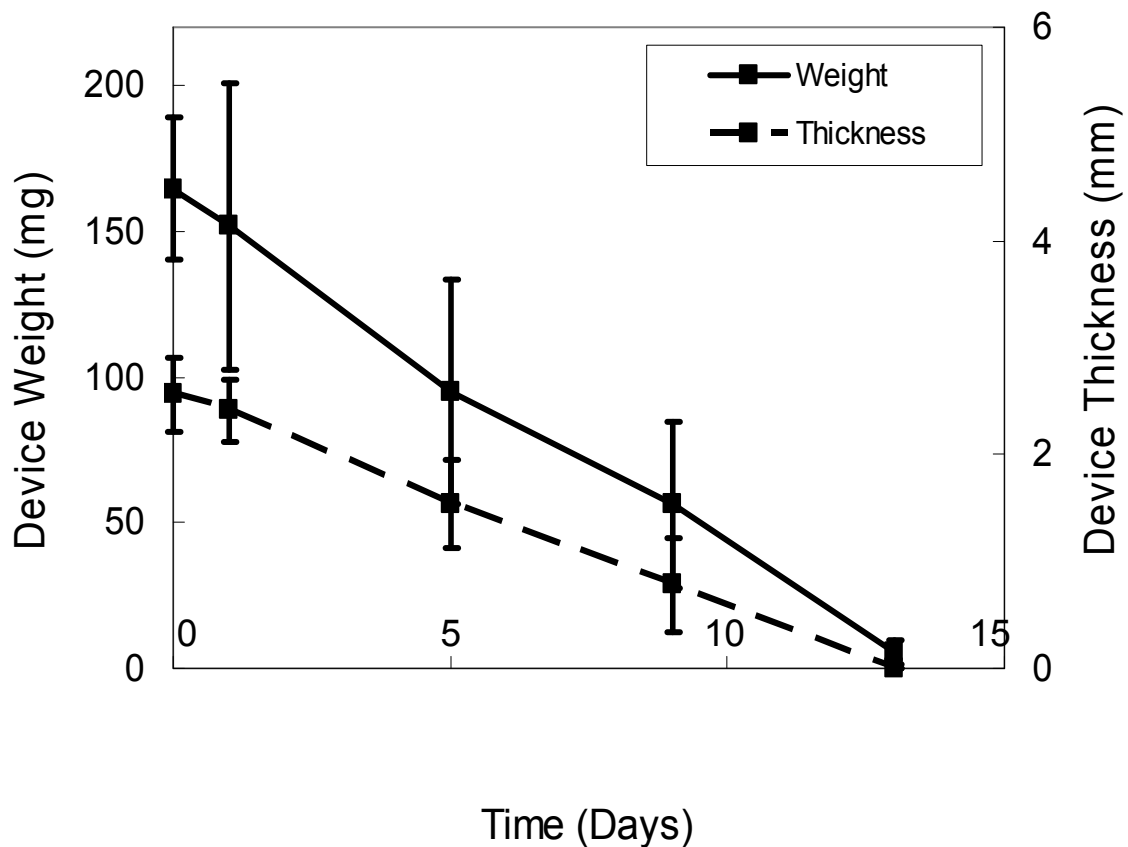
### **3.3.1 Release Profiles and Sample pH**

The CAP/PF-127 polymer degraded by erosion at the one surface

exposed to the aqueous environment. In Figure 3.1, device weight and thickness were decreased linearly at the rate of 11.5 mg/day-15.4 mg/day over the period of the experiments, as expected from zero-order kinetics.

Intermittent release profiles were achieved by alternately layering PTH(1-34)-loaded microspheres and blank (unloaded) microspheres. Figure 3.2 shows both intermittent and sustained release profiles for 10-layer devices. With sustained release devices, the amount of released PTH(1-34) gradually increased over the first 3 days, and afterward the amount of delivered PTH(1-34) remained relatively constant and showed a zero-order release profile. In contrast, intermittent release devices provided five discrete peaks, each separated by 2-3 days. Figure 3.3 shows cumulative release of PTH(1-34) from intermittent and sustained release devices. Sustained release devices were composed of 10 PTH(1-34)-loaded layers, but intermittent release devices were fabricated with 5 PTH(1-34)-loaded and 5 blank layers. Consequently, the total amount of PTH(1-34) released from intermittent release devices was close to half that from sustained release devices ( $p < 0.05$ ).

Figure 3.4 shows the pH in intermittent release samples at each day. All devices in various amounts of PBS were degraded in 13 days, as also shown in the previous figure. When devices were immersed in 5, 10, and 15 ml of PBS, the ranges of pH of samples were 6.64 - 6.88, 6.76 -6.91 and 7.05-7.24, respectively. For samples incubated in 10 and 15 ml PBS, pH was more stable through out the degradation period compared to those incubated in 5 ml, but at day 13, pH was 0.15-0.19 lower than any other period ( $p < 0.05$ ).



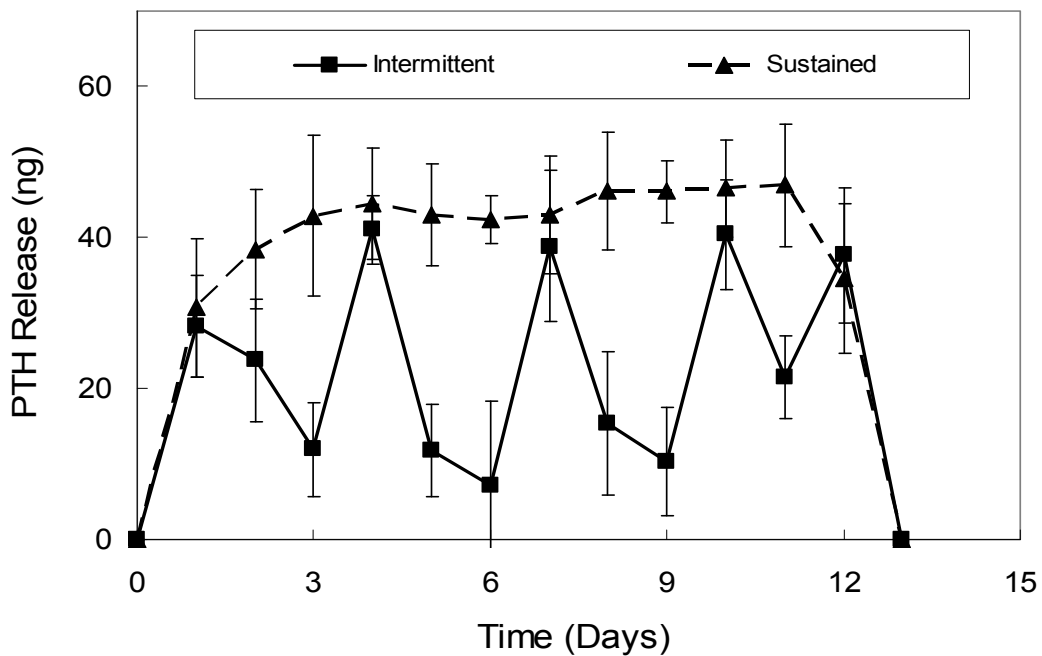
**Figure 3.1. Device thickness and weight changes during degradation of 10-layer release devices.**

### **3.3.2 Effect of Alternating or Constant Concentrations of PTH(1-34) on Osteoblastic Cells**

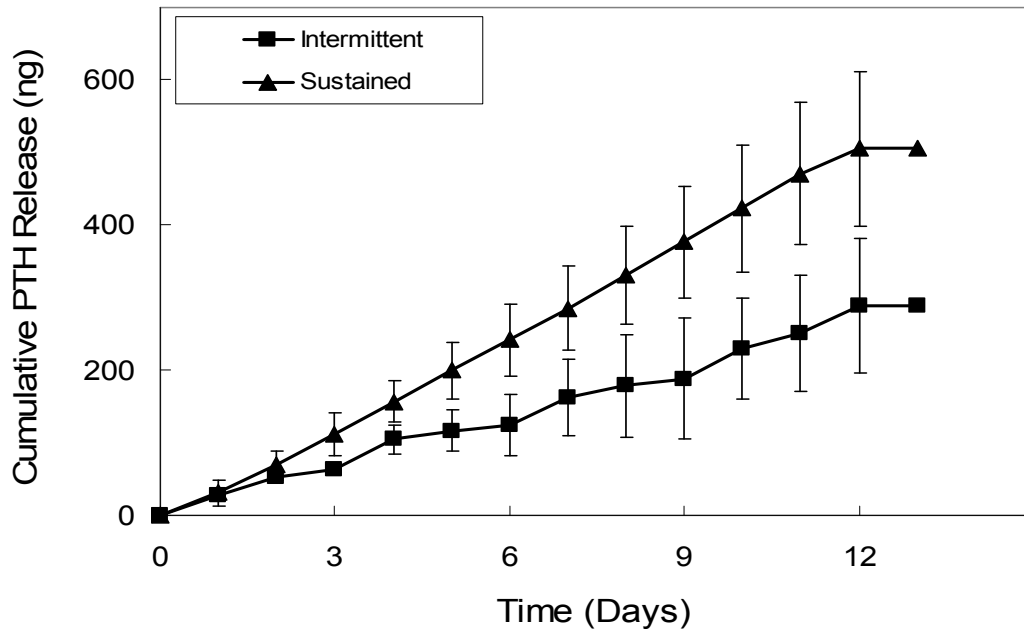
In order to determine proper levels of PTH(1-34) release from the devices and to investigate effects of concentration, and mode of exposure to PTH(1-34), cell responses were investigated. Results for DNA contents in



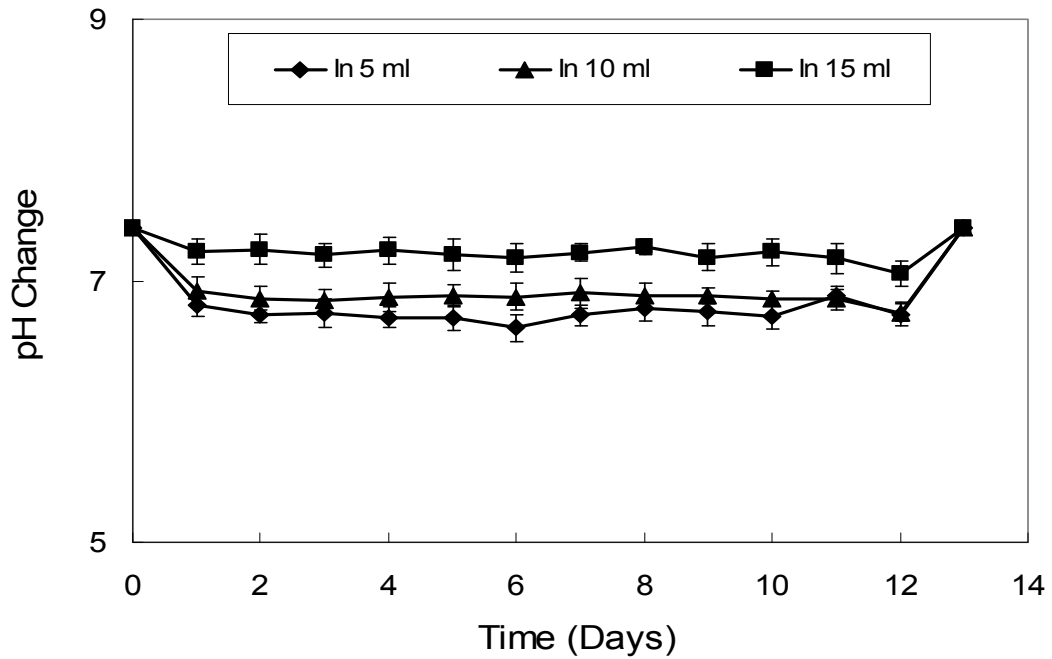
MC3T3-E1 cultures exposed to intermittent or sustained delivery of PTH(1-34) are shown in Figure 3.5. At day 3, DNA contents in osteoblast cultures treated with both sustained and alternating concentrations of PTH(1-34) were 64-86% higher than in controls ( $p < 0.05$ ). At day 7, all groups except the sustained 500 pg/ml showed their highest DNA contents ( $p < 0.05$ ) during culture, however levels in the 5 ng/ml alternating group were slightly higher than the others. At day 10, all groups except the 500 pg/ml sustained showed a slight decrease in DNA content



**Figure 3.2. Instantaneous release of PTH(1-34) from 10-layer intermittent and sustained release devices.**



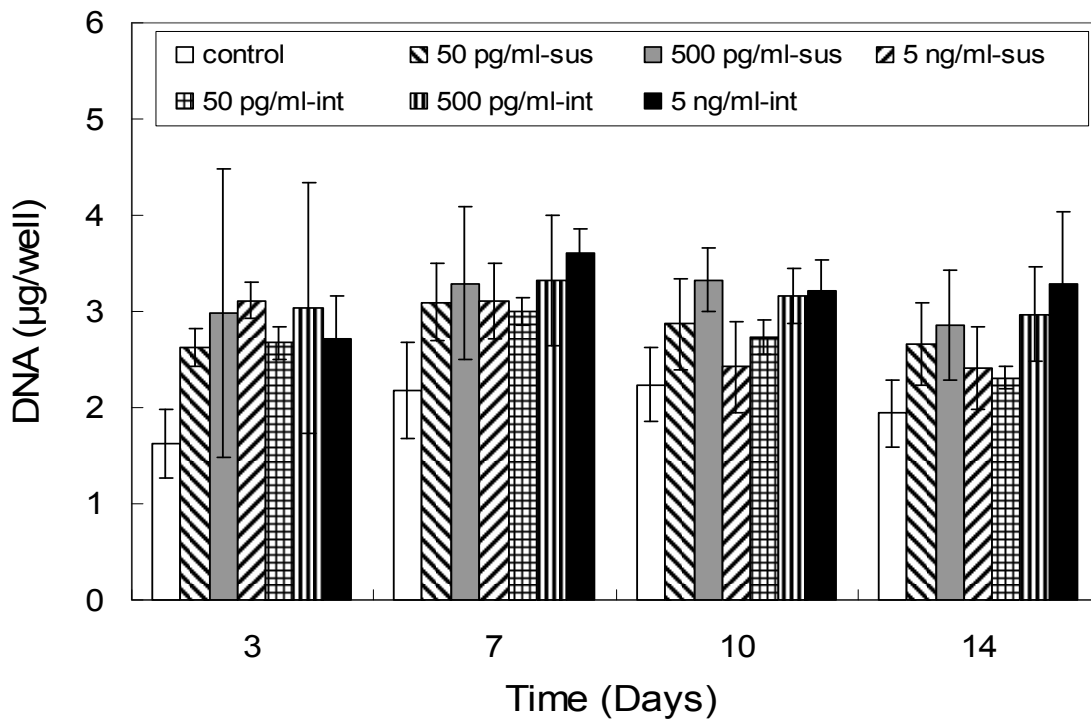
**Figure 3.3. Cumulative release of PTH(1-34) from 10-layer intermittent and sustained release devices.**



**Figure 3.4. Daily pH in sample solution during degradation of 10-layer devices.**

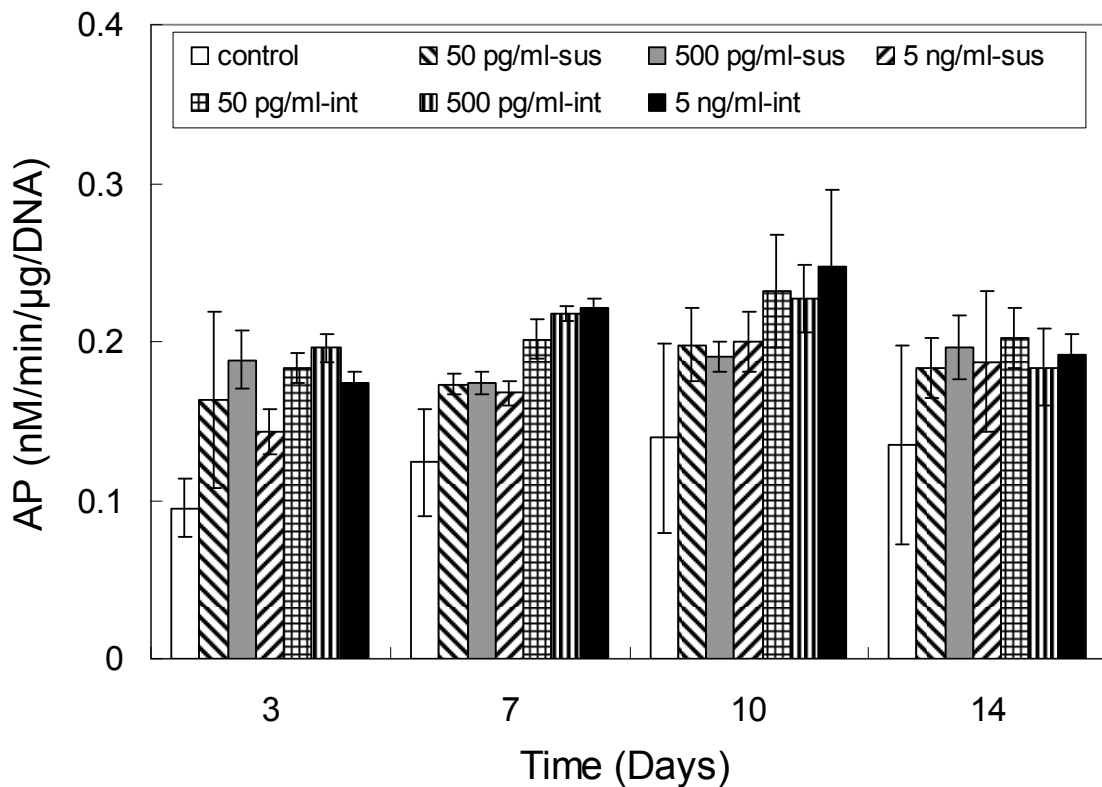
, with a decrease for the 5 ng/ml sustained group. At day 14, both alternating and sustained 5 ng/ml groups had similar DNA contents as 10 days, while other groups had decreased levels.

AP activity in PTH(1-34)-treated cultures is shown in Figure 3.6. At day 3, AP activity was 50-106% higher in PTH(1-34)-treated cultures compared to controls ( $p < 0.05$ ). At day 7, all groups showed increased AP activity except 500 pg/ml sustained. At day 10, all groups showed their highest AP activity, while at day 14, all groups showed decreased AP activity. AP activity in cultures exposed to 5 ng/ml alternating PTH(1-34) had the largest decrease compared to other groups. Cells treated with alternating levels of 5 ng/ml PTH(1-34) showed the highest overall activity at day 10.

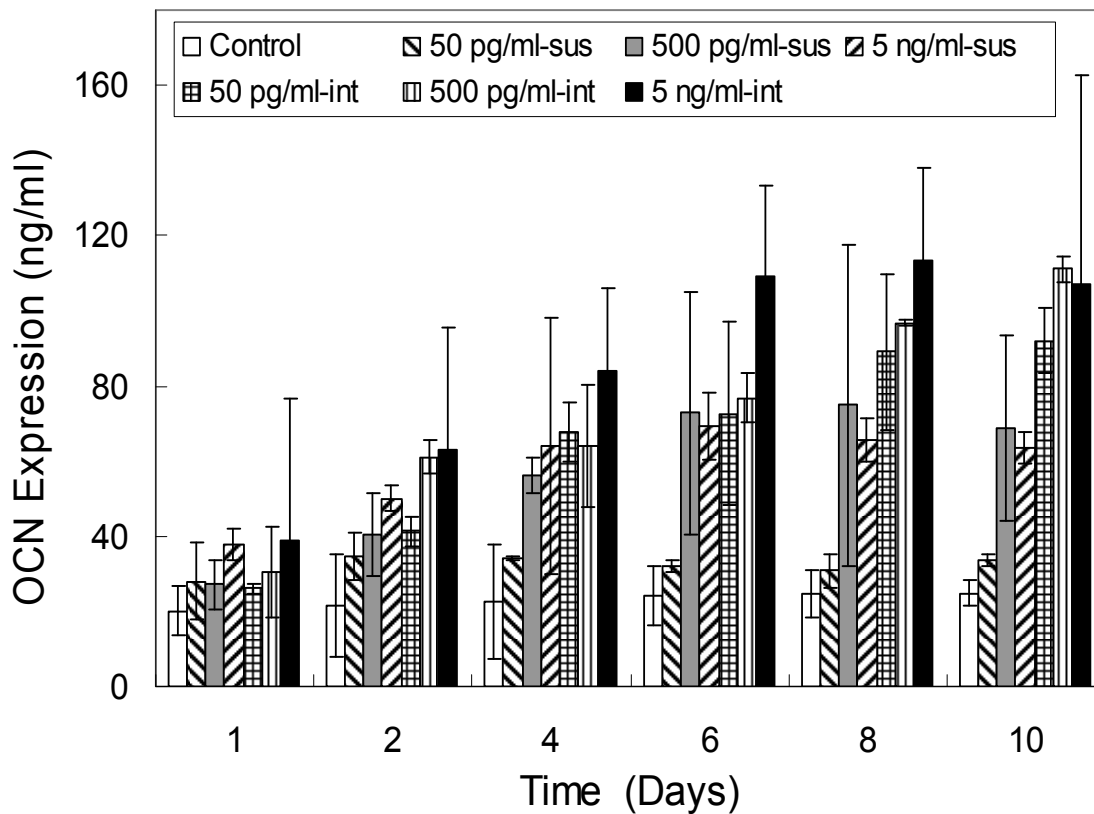


**Figure 3.5. DNA content in cultures exposed to sustained and intermittent PTH(1-34).**

In Figure 3.7, all experimental groups showed increased OCN contents compared to control groups ( $p < 0.05$ ). At day 1, there was no significant difference in OCN secretion between sustained and alternating groups in the same concentration, but from days 2 to 10, cultures treated with alternating levels of PTH(1-34) stimulated significantly greater (14-267%) osteocalcin secretion compared to cells constantly treated with the same concentration ( $p < 0.05$ ). Cultures exposed to alternating PTH(1-34) showed increasing OCN secretion through the period of culture (10 days), but OCN secretion in cultures treated with constant concentrations of PTH(1-34) plateaued earlier (by 6 days).



**Figure 3.6. AP activity in cultures exposed to sustained and intermittent PTH(1-34).**



**Figure 3.7. Osteocalcin secretion for osteoblasts exposed to sustained and intermittent PTH(1-34). ??did you check statistics for sus groups at 4 and 6 days??**

### 3.4 DISCUSSION

Blends of CAP and PF-127 form an association polymer that undergoes surface erosion and shows zero-order release [16,46]. A number of ether sites in PF-127 and carboxylic acid groups in CAP form hydrogen bonds. When these hydrogen bonds are exposed to aqueous solution, carboxyl groups start to deprotonate, and the material degrades. The deprotonation makes the surrounding solution acidic. Acidic conditions can have adverse effects on bone

and bone cells. Decreasing pH reduces expression of ALP and OCN in bone marrow stromal cells [126]. In particular, pH below 6.6 caused two- to three-fold decreased ALP expression. Another group showed that acidic conditions up-regulates PTH/PTHrP receptors in osteoblast-like cells [127]. In order to prevent local acidosis, and reduced ALP and OCN expression, pH at the site of a degrading CAP/PF-127 implant should be kept above 6.6 [128]. Our samples had slightly acidic pH, down to 6.64 when incubated in the smallest volume of PBS. In larger volumes, release supernatants were nearly neutral. Continuous clearance of degradation products *in vivo* would be expected to minimize pH changes. Preliminary studies in a rat model did not show adverse effects.

By modifying surface area, layer thickness, and alternately loading layers in the devices, PTH(1-34) release can be controlled. Unlike the cumulative release profile of sustained release devices, alternating devices showed relatively flatter regions, which reflect erosion of unloaded layers. Directional control of delivery was provided by coating with a more hydrophobic biodegradable polymer, in this case poly(lactide-co-glycolide). Thus, only one surface was exposed to PBS. Theoretically, the lowest points in the intermittent release profiles should have reached zero PTH(1-34) because the blank layers did not contain peptide. However, low but measurable concentrations were detected. It is believed that when fabricating the multilayered devices, some microspheres adjacent to loaded and blank layers became intermingled under pressure and sampling interval also affect to the result. Our devices were even 10 layer device with 13 day of degradation time. Ideally, sampling interval should

have been 1.3 day or more frequent sampling such as 3 times or 4 times a day. By 1 day sampling interval, we can closely calculate the trend of release profile of the devices but somewhat difficult to figure out exact release profile.

The plasma half life of intact PTH(1–34) is less than 11 min [36]. The fast metabolic degradation of PTH requires multiple administrations of PTH necessary to keep the effective concentration in the plasma [78]. Normally, the physiological PTH level in plasma is 500 +/- 30 pg/ml, and there is no significant difference according to age or sex [129]. Our work provided a maximum 20 times higher PTH(1-34) concentration. The release devices were not intended for systemic delivery but for local delivery. Encapsulated PTH(1-34) can be protected from degradation factors, such as oxidization, water, and enzymes, until released from devices and then it is available to travel a short distance to the target area. Consequently, local delivery of PTH(1-34) enables enhancement of osteoblastic activity and bone formation with smaller amounts of PTH(1-34) compared to systemic delivery of PTH(1-34).

Many approaches to intermittent PTH delivery have been investigated, such as injections, oral dosing, pulmonary inhalation, and transdermal delivery [35-41]. Most of these studies are focusing on systemic delivery for treatment of osteoporosis, whereas relatively few studies have looked at local delivery of PTH(1-34) for repair of focal bone defects. Pulmonary inhalation of 18-20 µg PTH(1-34) gave a plasma concentration of approximately 45 ng/ml, but the duration was less than 120 min [41]. Consequently, continuous inhalations were required for achieving and maintaining effective concentrations. PLGA

microspheres incorporating PTH(1-34) were developed for local delivery [78]. Although the microspheres sustained delivery of the peptide for more than 11 weeks, it is difficult to achieve pulsatile release with this system. Recently, Liu et al. described a system for pulsatile delivery of PTH(1-34) [35]. PTH(1-34)/alginate films were alternated with polyanhydride layers inside a PLLA container. Release profiles showed 4 sharp peaks, with PTH(1-34) concentrations of 40-65 ng/mL, over a period of 4 days. Our devices provided 5 discrete peaks with PTH(1-34) concentrations of 21-41 ng/ml for 12 days.

Comparing alternating and sustained exposure to the same PTH(1-34) concentration, intermittent exposure stimulated higher osteoblastic activity. This could be expected, because continuous exposure down-regulates expression of its receptor in target cells [130,131]. PTH at physiological concentration is a potent suppressor of osteoblast differentiation; it is thought that PTH prevents differentiation of preosteoblasts into osteoblasts [132]. The results in these studies correspond to our bioactivity results.

### **3.5 CONCLUSION**

This work describes devices for local intermittent, delivery of PTH(1-34) without injection. By alternately loading microspheres with and without PTH(1-34), intermittent release of PTH(1-34) was achieved using the association polymer system of CAP and PF-127. A slightly acidic condition that comes from erosion of the polymer may up-regulate expression of PTH receptors in cells. *In vitro* bioactivity experiments showed that either intermittently or sustained



exposing osteoblastic cells to PTH(1-34) enhanced their activity, but intermittent exposure resulted in enhanced cellular activity. These devices warrant further development for enhancing localized formation of bone.

## Chapter Four

### ALTERNATING RELEASE OF DIFFERENT BIOACTIVE MOLECULES

#### 4.1 INTRODUCTION

Controlled release technology enables delivery of a variety of biomolecules at the proper time, in the appropriate amount, and to the desired site. Most commonly, investigators seek to release one drug at constant concentrations for extended periods. However, such zero-order kinetics can be undesirable. For example, parathyroid hormone (1-34) (PTH(1-34)) can have either anabolic or catabolic effects on bone, depending on the mode of delivery. Daily administration of low doses of PTH stimulates bone formation and increases bone mass in humans and animals *in vivo* [7,12,14], and it enhances proliferation of primary osteoblastic cells [61,62] and diverse effects on osteoblast differentiation depending on differentiation stage [14,63]. In contrast, continuous administration of PTH has a catabolic effect [8,9,15,64].

Simvastatin (Sim), a member of the statin family of drugs known for lowering serum cholesterol, also has been reported to stimulate bone formation [1]. According to Mundy's group, treatment of cell cultures with Sim induced expression of bone morphogenetic protein 2 (BMP-2), and daily subcutaneous injections of Sim stimulated bone formation on the calvaria of mice. Other studies showed that oral dosing with simvastatin increased cancellous bone volume in

rats [51], and it also increased transverse area of fracture callus as well as mechanical properties compared to controls [2].

Regeneration of bony defects can be complex and involves sequential cascades of events [80]. Furthermore, these sites may face the threat of microbial infection, especially compound fractures and periodontal lesions. Infections in bony sites can be difficult to treat. Treatment may require multiple surgeries, prolonged antibiotic treatment, and long-term complications. Therefore, every effort is required to prevent this problem with early treatment [81]. Cecropin B (CB) is a naturally occurring cationic, amphipathic peptide derived from moths that has broad spectrum activity against not only certain gram-positive bacteria but also gram-negative bacteria, fungi, and parasites [84-86]. CB has an EC<sub>50</sub> (half maximal effective concentration) from 4 to 80 µg/ml, depending on the microorganism [86,90,91].

Currently, therapies for tissue regeneration in the body mainly rely on the delivery of single biomolecules. Microspheres, nanospheres, and porous scaffolds comprised of numerous polymers, such as polylactides, polyglycolides, poly(lactide-co-glycolides), polyanhydrides, and polyorthoesters, have been developed for delivery of one biomolecule at a time [21-24,41,78,133,134], but few studies have been consulted with multiple biomolecules [92,93,135,136]. However, multiple signals are required to drive the regeneration process to completion in a timely and efficient manner, and consequently the success of current efforts releasing a single biomolecule to the defect site may be limited [92]. An approach to overcome limitations of single molecule delivery is release

of multiple molecules with distinct kinetics to trigger the desired tissue responses.

In previous studies, we developed a controlled release system based on blends of cellulose acetate phthalate (CAP) and Pluronic F-127 (PF-127) for intermittent release of small and large biomolecules [16,46]. The CAP/PF-127 polymer system can be used for programmed drug delivery, with the rate, duration, and amount of released drug controlled by blending ratio, number of layers, and thickness [16,46,47]. The objective of this research was to further develop the CAP/PF-127 system for alternating delivery of multiple biomolecules and to investigate effects on osteoblastic cells.

## **4.2 MATERIALS AND METHODS**

### **4.2.1 CAP/PF-127 Microspheres**

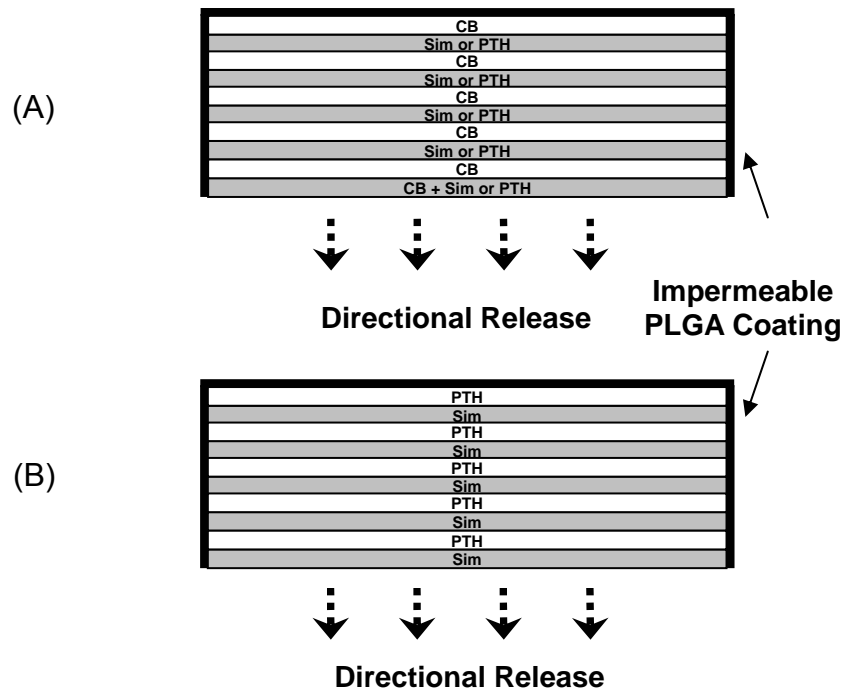
Cecropin-B (Anaspec, San Jose, CA) and PTH(1-34) (Bachem Torrance, CA) were labeled with Alexa Fluor 350 Carboxylic acid, succinimidyl ester (Invitrogen, Eugene, OR) and Alexa Fluor 488 Carboxylic acid, succinimidyl ester (Invitrogen), respectively, to enable measurement of the released amounts. CAP (Fluka, Buchs, Switzerland) and PF-127 (Sigma, St Louis, MO) microspheres were made by a water-acetone-oil-water (W/A/O/W) triple emulsion process. CAP and PF-127 polymer blend (7:3 weight fraction of CAP to PF-127) was dissolved in acetone. One ml of phosphate-buffered saline (PBS), pH 7.4, containing CB (250 or 500 µg/ml), PTH(1-34) (50 or 100 µg/ml), or Sim (420 or 840 µg/ml; Aldrich, Milwaukee, WI) was added to the polymer solution, homogenized into corn oil, and then sonicated for 20 seconds. The CAP/PF-127

suspension and 5% Triton X-100 solution were added to deionized water and then stirred for 5 minutes to harden the microspheres. Following collection, microspheres were washed, filtered, and lyophilized overnight.

#### **4.2.2 Release Devices**

Release devices were fabricated using a pressure-sintering process and consolidated in acetone vapor. The first layer of CB/Sim and CB/PTH devices was made with 20 mg of mixed UV-sterilized microspheres (10 mg of each type). They were then placed in the wells of a Delrin mold (diameter, 6.2 mm; depth, 20 mm) and consolidated by applying 20 MPa pressure for 5 sec. Next, microspheres containing only CB were added on top of the first layer, and pressure was reapplied. The third layer consisted of only Sim-containing microspheres for CB/Sim release devices or PTH-loaded microspheres for CB/PTH devices. Ten-layer devices were made by repeatedly alternating CB and Sim or PTH(1-34). Sim/PTH release devices were made by alternating layers of PTH- and Sim-containing microspheres.

Figure 4.1 depicts the design of the three devices. To provide directional control of drug release, the bottom and sides of the devices were coated three times with 10% poly(lactic-co-glycolic acid) (75:25,  $M_w \sim 75$  kDa; Alkermes, Cincinnati, OH) solution in methylene chloride.



**Figure 4.1. Schematic representation of (A) CB/Sim and CB/PTH devices and (B) Sim/PTH devices.**

#### **4.2.3 Characterization of Microspheres**

Drug content was determined by dissolving 100 mg of microspheres in 5 ml of PBS followed by precipitation of CAP in the solution with 50  $\mu$ l of 0.1 M hydrochloric acid. After centrifugation at 190 G-force (g) for 5 minutes, samples were placed into 96-well assay plates, along with solutions of Sim, fluorophore-labeled CB, and fluorophore-labeled PTH. Each standard was made by serial diluting from a known stock solution. The concentrations of released Sim, CB,

and PTH(1-34) were determined fluorometrically using a SpectraMAX Gemini XS (PTH(1-34):  $\lambda_{\text{ex}}=346$  nm,  $\lambda_{\text{em}}=442$  nm; CB:  $\lambda_{\text{ex}}=495$  nm,  $\lambda_{\text{em}}=519$  nm; and Sim:  $\lambda_{\text{ex}}=390$  nm,  $\lambda_{\text{em}}=413$  nm). Mean diameter of microspheres was measured using a Partica LA-950 Laser Diffraction Particle Size Analyzer.

#### **4.2.4 *In Vitro* Release**

Samples were immersed in 5 ml of PBS, pH 7.4, and incubated at 37°C. Supernatant was collected and replaced daily to maintain a constant volume. To allow quantification of released biomolecules, CAP was precipitated from the supernatant by treatment with 50  $\mu$ l of 0.1 M hydrochloric acid, and fluorescence was measured as described in the previous section. In order to investigate pH changes, devices were immersed in 15 ml PBS at 37°C, and the pH was measured each day.

#### **4.2.5 Cell Culture**

MC3T3-E1 preosteoblastic cells (CRL-2593; ATCC, Manassas, VA) were seeded at a density of 15,000/cm<sup>2</sup> into 24-well tissue culture plates in  $\alpha$ -Minimum Essential Medium (MEM) containing 10% fetal bovine serum (GIBCO/Invitrogen, Carlsbad, CA), 50  $\mu$ g/ml ascorbic acid (Sigma), 5 mM  $\beta$ -glycerophosphate (Sigma), 1  $\mu$ g/ml CB, 100 pM-1  $\mu$ M Sim, and 500 pg/ml-50 ng/ml PTH(1-34). In CB/Sim, CB/PTH, and Sim/PTH release cultures, one set of cells was alternately exposed to CB and Sim, CB and PTH, and Sim and PTH(1-34), respectively. In control cultures, medium was changed on the same schedule, but it did not contain biomolecules.

#### **4.2.6 DNA and AP Assays**

After 3, 7, 10, and 14 days of culture, cells were harvested and then lysed by sonication in high salt solution (0.05M NaH<sub>2</sub>PO<sub>4</sub>, 2M NaCl, and 2mM EDTA). DNA standards were prepared by serial diluting calf thymus DNA in the high salt solution. Hoechst 33258 (final concentration, 0.5 µg/ml; Sigma) was added to DNA standards and samples and allowed to react in the dark for 10 minutes [98]. DNA content of the lysates was quantified by measuring fluorescence ( $\lambda_{ex}$ =356 nm,  $\lambda_{em}$ =458 nm).

Alkaline phosphatase (AP) activity was determined by a colorimetric assay. Lysates were incubated with substrate solution prepared by dissolving 10 mM of Sigma 104 phosphatase substrate in Sigma 221 alkaline buffer solution. After 30 minutes, 0.25 N NaOH was added to each well to immediately stop enzyme activity. Absorbance at 410 nm was measured with an MR5000 microplate reader (Dynatech Laboratories, Chantilly, VA), and the amount of substrate cleaved was determined using  $\epsilon=1.7 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ . Activity was expressed as nM of substrate cleaved per minute and then normalized by DNA content.

#### **4.2.7 Statistical Analysis**

All data were analyzed by analysis of variance (ANOVA) using the computer application InStat (Graphpad Software, San Diego, CA). Post-hoc comparisons were made using the Tukey-Kramer Comparison Test when the p-value was significant ( $p < 0.05$ ).



## 4.3 RESULTS

### 4.3.1 Characterization of Microspheres

Drug content and encapsulation efficiency of three different biomolecule-containing microspheres are shown in Table 1. Drug content per g polymer and encapsulation efficiencies of CB, Sim, and PTH(1-34) microspheres were 11.2, 85.1, and 111  $\mu\text{g}$  and 53.1( $p<0.05$ ), 47.2( $p<0.05$ ), and 39%( $p<0.05$ ), respectively. As shown in Table 2, the mean size of blank, CB-, Sim-, and PTH(1-34)-loaded microspheres was 104, 161, 141, and 159  $\mu\text{m}$ , respectively. Compared to blank microspheres, CB, Sim, and PTH(1-34) microspheres were 56.3% ( $p<0.05$ ), 37.3% ( $p<0.05$ ), and 54.4%( $p<0.05$ ) respectively, larger in mean size.

**Table 4.1. Drug content and encapsulation efficiency for the three types of microspheres.**

	Initial CAP/PF-127 (g)	Drug Added ( $\mu\text{g}$ )	M.S. Harvested (g) / Yield (%)	Total Drug Content ( $\mu\text{g}$ ) / E.E.* (%)	Drug Content per g polymer ( $\mu\text{g}$ )
PTH	1.4/0.6 = 2	50	1.654 $\pm$ 0.124 / 82.7 $\pm$ 7.5	24.6 $\pm$ 9.1 / 39 $\pm$ 36.9	11.2
Cecropin B	1.4/0.6 = 2	250	1.563 $\pm$ 0.215 / 78.2 $\pm$ 13.7	132.8 $\pm$ 17.5 / 53.1 $\pm$ 13.1	85.12
Simvastatin	1.4/0.6 = 2	420	1.788 $\pm$ 0.298 / 89.4 $\pm$ 16.7	198.6 $\pm$ 18.7 / 47.2 $\pm$ 9.4	111.1

\* encapsulation efficiency. Data are the mean of four replicates

**Table 4.2. Sizes of the three types of microspheres.**

	Blank	Cecropin B	PTH	Simvastatin
Mean Size ( $\mu\text{m}$ )	103.92	161.16	159.08	141.18
Median Size ( $\mu\text{m}$ )	117.72	168.87	184.34	123.245
Std. Dev ( $\mu\text{m}$ )	67.894	113.31	90.161	89.848

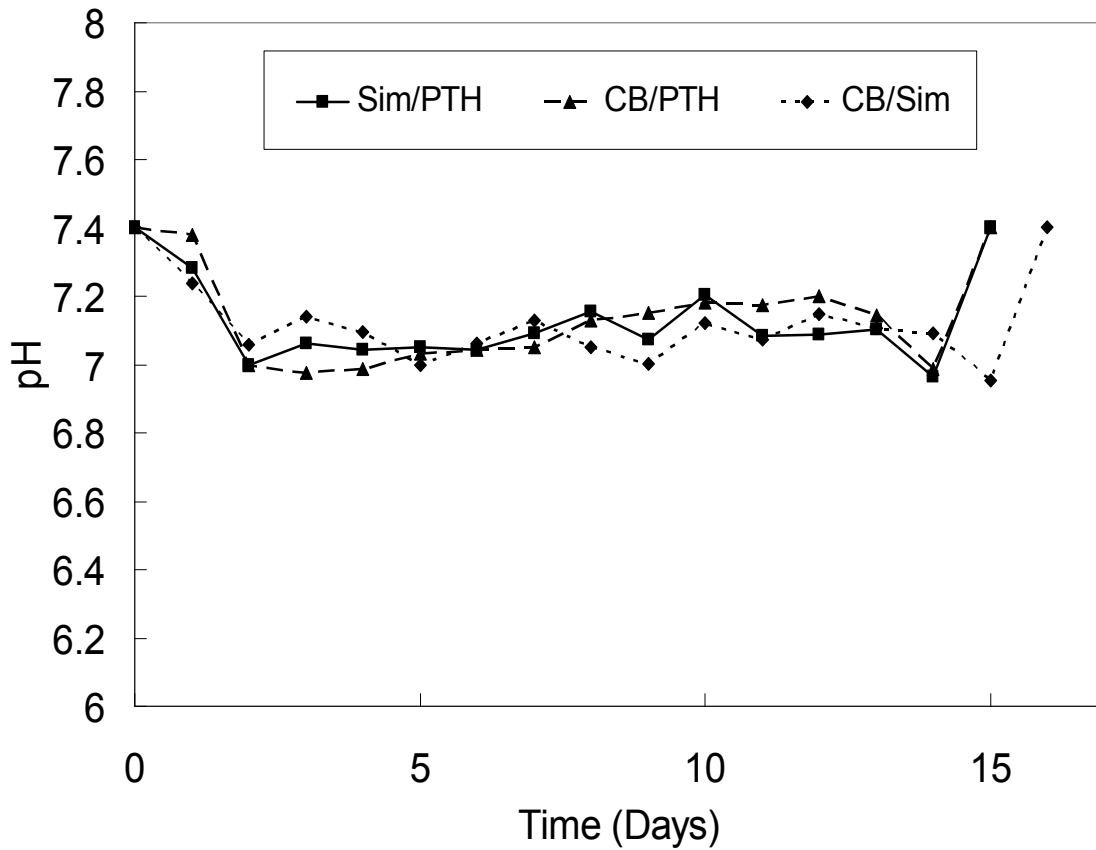
Refractive Indices : D.I. water, 1.333; CAP, 1.475. Data are the mean of four replicates

### 4.3.2 Sample pH and Release Profiles

Figure 4.2 shows the pH at each day for the three different devices developed. CB/PTH devices were degraded in 15 days, and CB/Sim and Sim/PTH devices degraded in 16 days. Solution pH decreased from 7.4 to 6.96-7.04 over the first 1-2 days and then was stable until the last time points, when the devices fully eroded. The ranges of pH for CB/Sim, CB/PTH, and Sim/PTH devices were 6.95-7.23, 6.97-7.37 and 6.96-7.28, respectively. Alternating release profiles were achieved by sequentially layering microspheres loaded with different biomolecules.

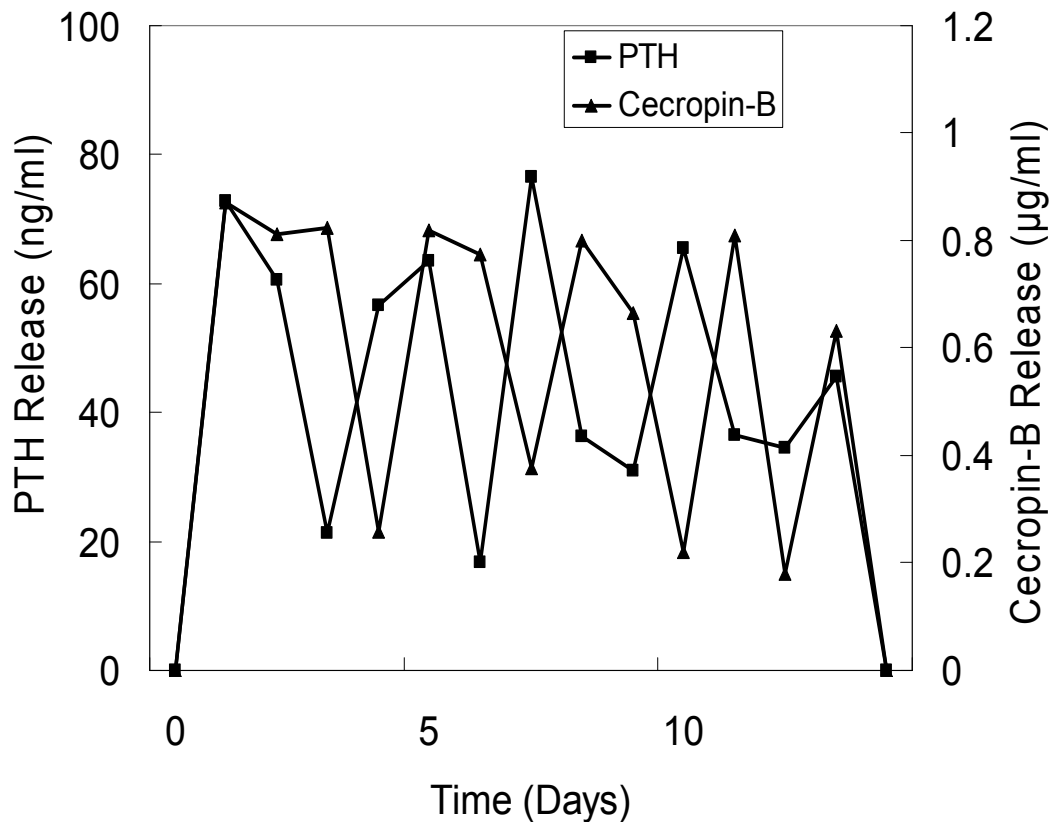
Figure 4.3 shows release profiles for devices designed to alternately release CB and PTH(1-34). Overall, five discrete release peaks were observed each for CB and PTH(1-34). To prevent early stage infection, CB release was maintained for three days at the beginning. During the release period, CB showed uniform peak concentrations (0.68-0.94  $\mu\text{g/ml}$ ), and PTH(1-34) also had relatively even maximum release (59-78  $\text{ng/ml}$ ), except for the last peak when integrity of the devices was lost. Figure 4.4 shows release characteristics of

devices alternately fabricated with CB and Sim. Again, prolonged CB release was observed over the first three days followed by five discrete release peaks for each component over a 15 day period. The range of concentration of CB and Sim are 0.76-0.95  $\mu\text{g/ml}$  and 1.26 -1.56  $\mu\text{M}$ , respectively.



**Figure 4.2.** pH in sample solutions during degradation of 10-layer devices. Data are the mean of six replicates. Error bars, which ranged from 11-28% of the mean, are not shown to prevent obscuring the curves.

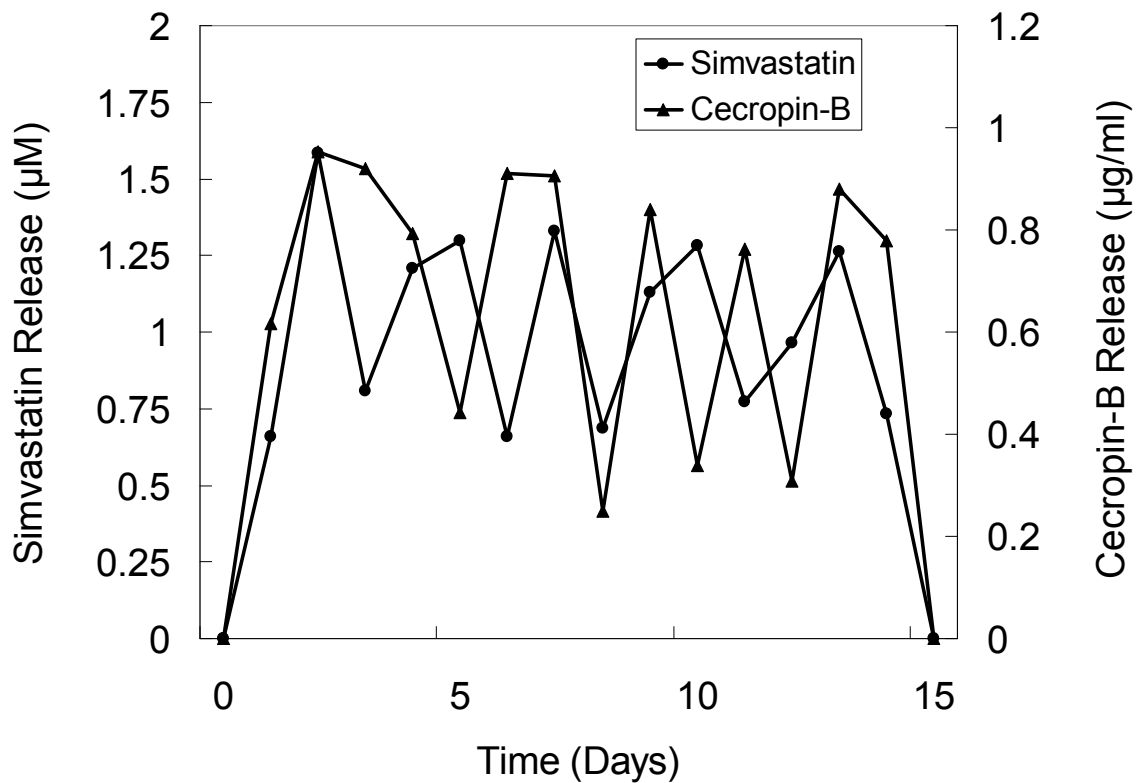
Figure 4.5 shows results for devices alternately loaded with Sim and PTH(1-34). Each release profile had 5 distinguishable peaks. In the Sim release profile, the first two peaks were sharp and high (1.58-1.65  $\mu\text{M}$ ), but the next two were relatively broad and gave lower concentrations (0.97-1.12  $\mu\text{M}$ ) than the first two. PTH(1-34) peaks showed relatively even release for the first four peaks (77.2-83.16 ng/ml), but a lower peak was observed for the late one (54.1 ng/ml).



**Figure 4.3. Release profile from 10-layered CB/PTH devices. Data are the mean of six replicates. Error bars, which ranged from 11-31% of the mean, are not shown to prevent obscuring the curves.**

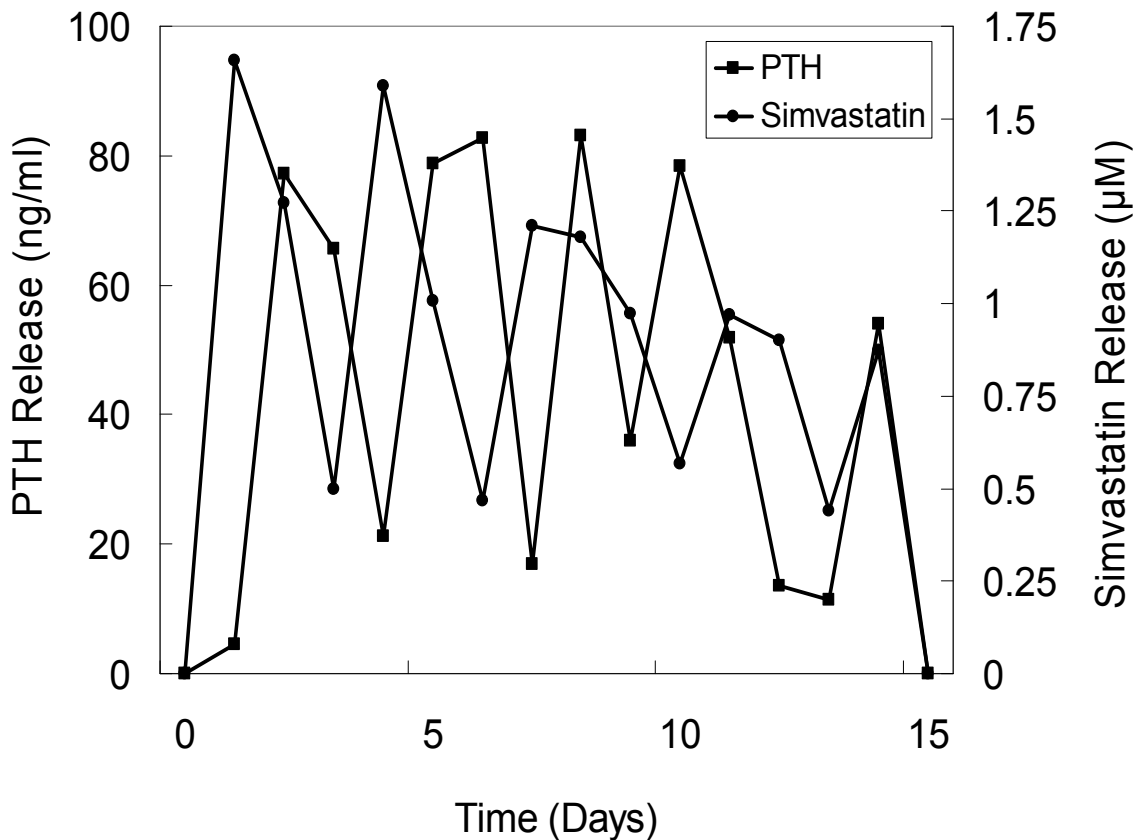
### 4.3.3 Effect of Combined Delivery of Biomolecules on Osteoblastic Cells

In Figure 4.6 a, DNA contents in all MC3T3-E1 cultures increased until the seventh day and then showed relatively unchanging levels. Cells exposed to 1  $\mu\text{g/ml}$  CB with 10 nM Sim showed higher DNA content (5.64  $\mu\text{g/ml}$ ) than any other group ( $p < 0.05$ ). AP activity in all groups increased through the tenth day and then decreased, except for the 1  $\mu\text{M}$  Sim group (Figure 4.6 b). As was observed for DNA content, the 10 nM Sim group had higher AP activity than any other group ( $p < 0.05$ ). Unlike DNA content, however, AP activity was higher for the 1  $\mu\text{M}$  Sim group compared to controls ( $p < 0.05$ ).

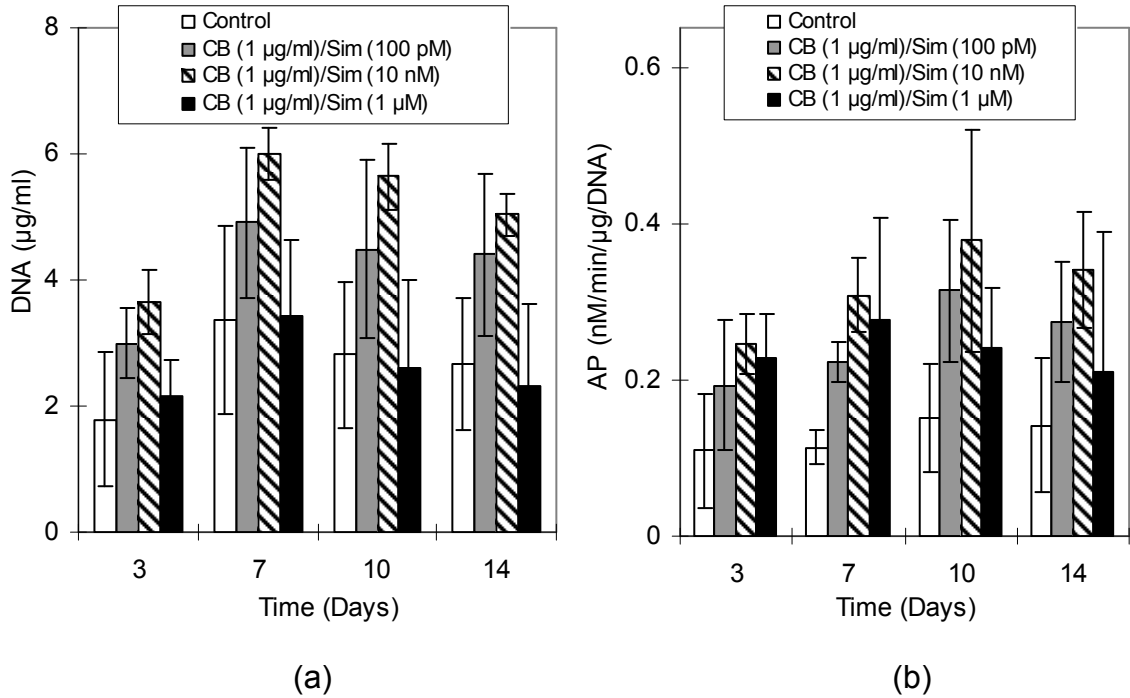


**Figure 4.4.** Release profile from 10-layered CB/Sim devices. Data are the mean of six replicates. Error bars, which ranged from 23-34% of the mean, are not shown to prevent obscuring the curves.

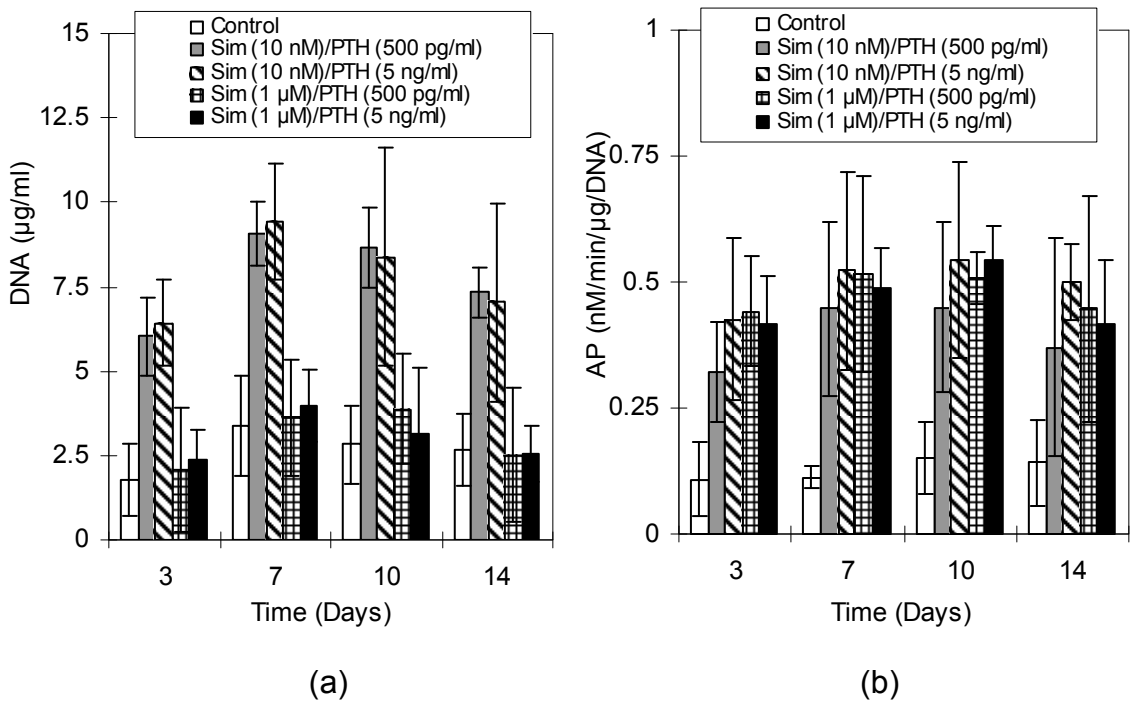
As shown in Figure 4.7 a, all CB and PTH(1-34) treatment groups had higher amounts of DNA (2.99-7.06  $\mu\text{g}/\text{ml}$ ) than did the control group (1.78- 3.37  $\mu\text{g}/\text{ml}$ ) ( $p < 0.05$ ). Although not significantly different, cultures treated with 1  $\mu\text{g}/\text{ml}$  CB and 5 ng/ml PTH(1-34) showed slightly higher DNA content until day 10. As seen for DNA contents, all CB and PTH(1-34) treatment groups had higher AP activity (Figure 4.7 b) (0.26-0.4 nM/min/ $\mu\text{g}/\text{DNA}$ ) than did the control group (0.11-0.15 nM/min/ $\mu\text{g}/\text{DNA}$ ) ( $p < 0.05$ ). All groups increased through the tenth day and then decreased slightly.



**Figure 4.5.** Release profile from 10-layered Sim/PTH devices. Data are the mean of six replicates. Error bars, which ranged from 7-22% of the mean, are not shown to prevent obscuring the curves.

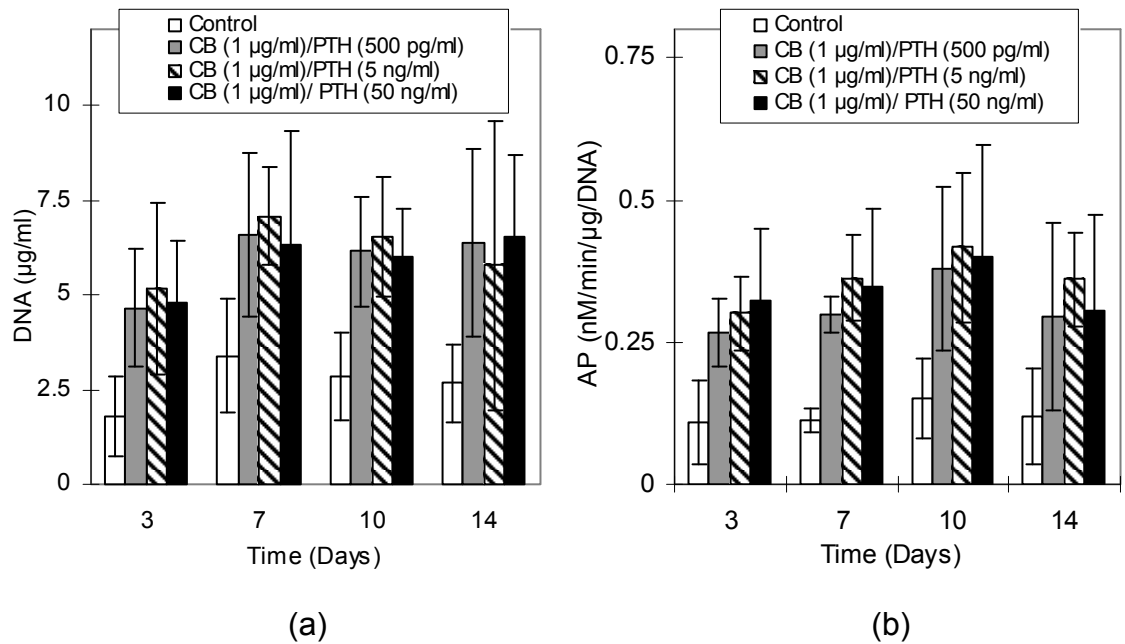


**Figure 4.6 (a) DNA content and (b) AP activity in cultures exposed to alternating CB and Sim.**



**Figure 4.7. (a) DNA content and (b) AP activity in cultures exposed to alternating CB and PTH.**

In cultures exposed to alternating Sim and PTH(1-34), DNA contents mainly depended on Sim concentration (Figure 4.8 a). DNA contents for cells treated with 1  $\mu\text{M}$  Sim were slightly higher than controls, whereas treatment with 10 nM Sim resulted in 163-259% higher amounts of DNA than the controls ( $p < 0.05$ ). Furthermore, these two groups (10 nM Sim plus either 500 pg/ml or 5 ng/ml PTH(1-34)) showed 39.8-101% and 14.6-40.3% higher DNA content than CB/Sim and CB/PTH groups, respectively ( $p < 0.05$ ). Alternating Sim and PTH cultures showed higher AP activity than did the control groups (Figure 4.8 b) ( $p < 0.05$ ). Furthermore, even 1  $\mu\text{M}$  Sim treatment stimulated higher AP activity than in control groups throughout the two week experiments.



**Figure 4.8. (a) DNA content and (b) AP activity in cultures exposed to alternating Sim and PTH.**



#### 4.4 DISCUSSION

The present study demonstrates alternating delivery of multiple biomolecules and shows that such alternating concentrations increase proliferation and early osteoblastic activity, which is critical to enhancing bone formation. The devices were based on reports of intermittent delivery of Sim and PTH(1-34) both promoting osteoblastic responses and bone formation [1,3,15,37,51,66,137]. The erodible polymeric devices, developed to allow intermittent release of a single molecule, alternating release of different molecules, or sequential release of multiple molecules, may offer a powerful approach in bone regeneration as well as in other medical therapies that involve multiple biological cascades.

Drug loading and encapsulation efficiency are affected by volume of organic solvent used in dissolving the polymer [138,139] and the initial drug concentration. The smaller volume of organic solvent in the system promotes precipitation of polymer. Drug loading increases with decreasing organic solvent volume and with increasing drug concentration. The size of CAP/PF-127 microspheres used for the release devices can be controlled by modifying the emulsification process, such as by altering agitation velocity, time, temperature, and the parameters that influence the kinematic viscosity type, including concentration of polymer and emulsifier [140]. In our experiments, microsphere sizes ranged from 104 to 161  $\mu\text{m}$ . It is thought that the differences of homogenization speed, water phase temperature, and types of drug are main reasons.

Blends of CAP, which is a well known enteric coating material in the pharmaceutical industry, and a nonionic surfactant, PF-127, which is used for stabilizing proteins and peptides, form an association polymer that undergoes surface erosion and shows zero-order release [16,46]. A number of ether sites in PF-127 and carboxylic acid groups in CAP form hydrogen bonds. When exposed to PBS, carboxyl groups on CAP become deprotonated, and hydrogen bonds with ether groups in PF-127 are consequently lost, resulting in surface erosion of the material.

When CAP is dissolved in aqueous solution, deprotonation results in locally decreased pH. Measurement of pH showed only slight acidification below PBS's initial pH of 7.4. Supernatants remained close to neutral, with the lowest pH of just 6.95 occurring at the last time point sampled when the material was finally completely eroded. Disthabanchong et al. reported that a slightly acidic condition (pH 7.1) up-regulated PTH/PTHrP receptors and increased PTH binding to osteoblast-like cells compared to pH 7.4 [141]. In our experiments, the slight local acidification would not be expected to adversely affect differentiation of mesenchymal cells, which is reported to occur at pH [141] and may even up-regulate expression of PTH receptors by cells. However, according to Brandao-Burch' group, at pH 6,9, acidosis reduced mineralization of bone nodules, osteoblast alkaline phosphatase activity was reduced, and downregulated messenger ribonucleic acid (mRNA) for alkaline phosphatase [142].

Two of the biomolecules used were incorporated because of their osteotropic effects. Many *in vitro* and *in vivo* studies have been conducted to

explain the anabolic effect of intermittent infusion of PTH on bone formation. It is thought that PTH enhances proliferation of primary osteoblastic cells from humans and animals [10,65]. In addition, PTH exerts diverse effects on osteoblast differentiation depending on differentiation stage [13,67,68], and it stimulates alkaline phosphatase (AP) activity in the mouse osteoblastic cell line MC3T3-E1 [68]. Simvastatin promotes bone formation both *in vitro* and *in vivo* in animal models associated with increasing BMP-2 transcription [48,51,110].

Devices used in this study were fabricated to fluctuate around daily between maximum and minimum release of drug and it was expected to be changed in a regular way. Ideally, the minimum value of each profile would decrease to zero, because the adjacent layers do not contain the same biomolecules. However, low but measurable concentrations of drug were detected. It is thought that during fabrication of the multilayered devices, some microspheres in adjacent loaded and blank layers became intermingled under pressure, resulting in “contamination” of the drug-free layer. If absolutely zero concentration between peaks was needed, thicker blank layers could be made, although at the expense of increased overall thickness of the device. In some release profiles, the last one or two peaks were lower than the first peaks because the drug could be diffused out before the device degraded through the PLGA coating. To maintain proper range of concentrations, drug loading of microspheres used for the last layers could be increased, if necessary, and more frequent sample collection-not once a day-would give more precise information about release profiles.

All cultures treated with intermittent PTH showed increasing DNA content and AP activity. Unlike intermittent PTH delivery, exposure to 1  $\mu$ M of simvastatin had an adverse effect on DNA content compared to other concentrations. This result corresponds to our previous findings [46]. Sim/PTH delivery devices showed higher DNA content and AP activity effect compared to CB/Sim, CB/PTH devices. This phenomenon results in an additive effect of Sim and PTH. The mechanism of Sim on bone formation is different from that of PTH. PTH stimulates multiple intracellular signal pathways, mediated by cAMP, and activates both protein kinase A (PKA) and C (PKC). However, the roles of each signal transduction system in osteoblast proliferation and differentiation are unknown [10]. Sim induces BMP-2 transcription through the inhibition of protein prenylation in osteoblasts and inhibits HMG-CoA reductase activity, resulting in the depletion of mevalonate in osteoblasts [51]

One of the polymeric multiple biomolecules delivery systems used a scaffolds and drug loading methods into scaffolds is that one molecule is mixed in scaffold and another drug was encapsulated into microspheres. In this method Mooney's group investigated dual growth factors delivery using PLGA scaffold for 40 days. Another approach was made by Mikos' group; they encapsulated dual growth factors and crosslinked them each other[94]. They seek for the target of traditional controlled release system-simultaneous sustained release system, whereas our devices were aimed at short term intermittent delivery.

## 4.5 CONCLUSION

By alternatingly CAP and PF-127 microspheres loaded with CB and Sim, CB and PTH(1-34), or Sim and PTH(1-34), intermittent release with five distinguishable peaks for each of the two molecules were achieved. *In vitro* experiments showed that alternating delivery enhanced bioactivity of osteoblastic cells. Furthermore, alternating treatment with Sim and PTH(1-34) had an additive effect on cell responses. Overall, CAP/PF-127 polymer is a useful system for alternating, intermittent, or sequential delivery of multiple molecules, such as could be used for treatment of local bone defects.

## **CONCLUSION**

Using the association polymer system of CAP and PF-127, intermittent release profiles of Sim, PTH (1-34), Sim/CB, PTH/CB, and Sim/PTH were achieved. Release profiles can be controlled by altering CAP/PF-127 blending ratio, number of layers, and erosion conditions. From the results of these studies we find many evidences to support the hypothesis that cell responses to Sim and PTH containing devices depended on the mode of drug release. Sim treatments promoted osteoblastic activities and bone formation and PTH treatments enhanced osteoblastic activities compared to control cultures. Intermittently delivered Sim showed a greater anabolic effect *in vitro* and *in vivo* than sustained delivery. Combined devices with CB/Sim, CB/PTH and Sim/PTH showed five distinct peaks for each drug. All cultures treated with multiple molecules showed enhanced osteoblastic activity, and cultures with alternating Sim and PTH showed higher osteoblastic activities than Sim or PTH alone. It was clear that there was additive effect in Sim and PTH combined treatments. Consequently, these devices may lead to better treatments for local bone regeneration.

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**Book Review**

Yasuhiko Iwasaki, Kazuhiko Ishihara, Nobuo Nakabayashi, Gilson Khang, Ju Hyeong Jeon, Jin Whan Lee, and Hai Bang Lee, "Preparation of gradient surfaces grafted with phospholipid polymer and evaluation of their blood compatibility," In Advances in Biomaterials Science I, T. Akaike, T. Okano, M. Akashi, M. Terano and N. Yui Eds., CMC Co., LTD., Tokyo, 1997, pp 91-100

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  8. Ju Hyeong Jeon, Ward T. Piepgrass, Yi-Ling Lin, Mark V. Thomas, David A. Puleo. "Localize Intermittent Delivery of Simvastatin Acid Stimulates Bone Formation *in vivo*". Submitted (2007)
  9. Ju Hyeong Jeon and David A. Puleo "Intermittent Release of Parathyroid Hormone (1-34) Biodegradable Device Enhances Osteoblastic Activities". Submitted(2007)
  10. Ju Hyeong Jeon and David A. Puleo "Alternating Release of Different Bioactive Molecules." Submitted (2007)

### **Presentations**

1. Ju Hyeong Jeon, Sun Hang Cho, and Hai Bang Lee, "Improvement of cell attachment and proliferation onto plasma-treated poly(lactide-co-glycolide) Surfaces," '96 Fall Meeting of The Korean Society for Biomaterials,, October 11-12, 1996, Taejon, Korea.
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