Apatite-Polymer Composites for the Controlled Delivery of Bone Morphogenetic Proteins

by

Tseh-Hwan Yong

S.B. Chemical Engineering
Massachusetts Institute of Technology, 1998

Submitted to the Department of Materials Science and Engineering in Partial Fulfillment of the Requirements for the Degree of

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Abstract

Current treatment of bone defects due to trauma, cancer, or degenerative spine diseases involves the implantation of a bone graft. Autografts, which are harvested from the patient’s own body, are associated with problems of limited availability and surgical morbidity. The use of allografts obtained from donors is also not desirable due to the risks of disease transmission and the costs of maintaining bone banks. The ideal solution would be to regenerate native bone to fill the defects. A group of potent growth factors known as bone morphogenetic proteins (BMPs) have been hailed as alternatives to bone grafts due to their ability to elicit new bone formation. Clinical use of BMPs involves loading the protein solution onto collagen sponges and subsequent implantation. However, these conventional collagen carriers show rapid clearance of BMPs within ~2 weeks, whereas bone healing is a longer process, especially in higher mammals. The poor BMP retention in collagen sponges may explain the greater response variability in higher mammals, ranging from full bone bridging within weeks to no bone union. These sponges are also not capable of tunable or multifactor release that could benefit healing in certain anatomic sites, e.g. avascular sites and prolonged non-unions. Hence, the motivation of this thesis is to develop new carriers that allow more efficacious and flexible delivery of BMPs to achieve bone healing.

The carrier should ideally exhibit (i) sustained release to maintain the response and activity of bone-forming cells, (ii) low initial burst to avoid adverse effects of a bolus administration and to conserve the expensive growth factor, and (iii) tunable release to meter out BMPs at the desired rate. In particular, tunable release and low burst release have long been challenges in controlled delivery systems. A carrier that can offer such temporal control will be highly valuable to the delivery of other therapeutic proteins, drugs and genes as well.

To this end, we have devised a novel composite of two biomaterials with proven track records: poly(lactic-acid-co-glycolic acid) (PLGA) and apatite. The controlled release strategy was based on the use of a biodegradable polymer with acidic degradation products to manipulate the dissolution of the basic apatitic component. Proteins were pre-adsorbed onto the apatitic component such that as the apatite dissolved, proteins were released. Apatite-
PLGA composites were formed into microparticles by a solid-in-oil-in-water emulsion process. In contrast to polymeric microparticles prepared by the conventional water-in-oil-in-water emulsion process, these composite microparticles exhibited zero-order, low burst release. Low burst release was attributed to the affinity of the apatite for the protein; until the apatite was dissolved, the protein was sequestered and prevented from premature release. Accordingly, the use of apatite singly as a carrier would have led to extremely slow release.

A model protein, bovine serum albumin (BSA), and a therapeutic protein, recombinant human BMP-2 (rhBMP-2), were encapsulated in these apatite-PLGA composite particles. The release profile was modified systematically by changing variables that affected polymer degradation and apatite dissolution, such as polymer molecular weight, polymer hydrophobicity, apatite loading, and apatite particle size. An increase in polymer molecular weight, apatite loading or apatite particle size reduced the release rate of both BSA and rhBMP-2. Interestingly, increasing polymer hydrophobicity diminished the release of BSA, but enhanced the release of rhBMP-2. Slower polymer degradation associated with greater polymer hydrophobicity might have decreased the total amount of protein released, but preserved a larger bioactive fraction due to milder pH conditions within the particles. A suitable particle formulation for sustained rhBMP-2 delivery was identified as protein-sCAP-59 kD PLGA.

When rhBMP-2 was encapsulated in these composite microparticles, it was released in a sustained fashion over 100 days. More importantly, the bioactivity of the protein was retained, as evaluated by its ability to induce the differentiation of mesenchymal stem cells toward the osteoblast lineage. Specifically, the levels of osteoblastic phenotype markers such as alkaline phosphatase (ALP) and osteocalcin were found to be significantly elevated compared to the controls. In contrast, rhBMP-2 released from conventional collagen sponges after 2 weeks did not increase the ALP expression over the controls.

Protein-loaded composite microparticles were dispersed in secondary matrices, either gelatin or collagen sponges, for bone tissue engineering. Multifactor release from these scaffolds was possible through the incorporation of different sets of composite microparticles containing different proteins and exhibiting distinct release profiles. Collagen sponges injected with rhBMP-2-loaded composite microparticles were implanted in subcutaneous sites in rats. These composite collagen sponges stimulated a much higher degree of cellularity and vascularity than the controls without BMPs. The increased vascularity might be evidence of the angiogenic activity of rhBMP-2 at low concentrations in vivo.

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Chapter 1 – Background and Motivation

1.1 Bone Regeneration

Bone is a naturally regenerative tissue; it is able to heal from fractures and breaks by recapitulating the embryonic skeletal developmental process. However, an estimated 5–10% of fractures fail to recover properly and proceed to delayed union or nonunion [1]. The repair of bone loss associated with trauma and cancer is also typically not observed. Current treatment involves the implantation of autogenic or allogenic bone grafts, a procedure that an estimated 1.5 million patients undergo in the United States each year [2]. Autografts, long considered the gold standard in bone grafting, are plagued with problems of limited supply, morbidity associated with graft harvest, and variability in fusion success rate. The use of allografts is dampened by the risks of disease transmission and the costs of maintaining bone banks. Synthetic grafts constructed of metals and ceramics are also used, but their mechanical and chemical incompatibility with bone tissue often leads to implant failure [3]. The ideal solution would be to regenerate native bone to fill the defects. A group of potent growth factors known as bone morphogenetic proteins (BMPs) has the ability to elicit new bone formation. These proteins provide a promising alternative to bone grafts, and have garnered much excitement and interest.

1.2 Bone Morphogenetic Proteins as Alternatives to Bone Grafts

BMPs are a class of fourteen cytokines belonging to the transforming growth factor-β (TGF-β) superfamily. They were discovered by Urist in 1965 as the components in demineralized bone matrix responsible for inducing ectopic bone and cartilage formation in muscle and subcutaneous sites of rodents [4, 5]. BMPs induce bone morphogenesis in a multistep cascade reminiscent of embryonic skeletal formation, beginning with the migration of mesenchymal cells, their differentiation into cartilage-forming cells (chondrocytes) or bone-forming cells (osteoblasts), deposition of bone matrix, establishment of functional marrow within the bone, and culminating in remodeling of the bone consistent with the anatomic site and biomechanic environment [1, 4, 6, 7]. In addition to bone induction (osteoinduction), BMPs also play a role in the development of other tissues and organs such as kidney, gut, lung, teeth, heart, limb, and brain [6, 8].
Among the osteoinductive BMPs, BMP-2, BMP-4 and BMP-7 appear to have greater potency [4, 8], and are being produced with high bioactivity and purity via recombinant DNA technology. *In vitro* administration of BMP-2 and BMP-7 to embryonic rat calvarial cells, rat osteosarcoma cells or mouse fibroblasts resulted in enhanced osteogenic activity, as evidenced by differentiation into osteoblasts and elevated expression of bone mineralization proteins. *In vivo* treatment with BMP-2 or BMP-7 augmented the healing of defects in rodents [9, 10], rabbits [11-15], dogs [16-18], sheep [19], and non-human primates [13, 20, 21]. Critical-size defects, which do not heal spontaneously, were bridged within 3 months in primates [20, 21]. These animal studies validate the safety and efficacy of BMP-2 and BMP-7 in promoting orthopedic repair. However, the results from human trials have shown more variation. Geesink *et al.* reported that amongst six patients receiving BMP-7 with a collagen carrier for the treatment of high tibial osteotomy, four showed bridging by 6 weeks, one by 10 weeks, and one showed no bone formation during the course of the study [22]. When similar BMP-7 carriers were implanted in the maxillary sinuses of three patients with maxillary atrophy, only one exhibited satisfactory bone formation, whereas the remaining two showed little bone formation after 6 months [4]. More recently, 450 patients with open tibial fractures were randomly assigned to 3 groups receiving (i) the standard of care (tissue irrigation, débridement, followed by intramedullary nail fixation), (ii) standard of care and 6 mg of rhBMP-2 on a collagen sponge, or (iii) standard of care and 12 mg of rhBMP-2 on a collagen sponge [23]. One year after treatment, 52% of the patients who received the standard of care had healed, compared to 59% and 72% of patients who were further treated with 6 mg and 12 mg of rhBMP-2, respectively. While the value of BMP treatment was undeniable, the results from clinical studies were not as impressive as those observed in animal studies where healing occurred more quickly and more completely. The greater variability and slower response in humans may be attributed to a smaller population of multipotent cells, which are also less responsive than those in smaller animals. It has been proposed that the therapeutic outcomes may be enhanced by carriers capable of (i) delivering BMPs at a rate that matches the responsiveness of the cells or (ii) delivering a suitable cocktail of growth factors to stimulate the cells [24, 25].
1.3 Delivery Systems for Bone Morphogenetic Proteins

In order for BMPs to exert an effect on bone healing, they must be delivered to the defect site. Bolus injections have limited effect because of the rapid clearance of exogenous proteins from the body. To increase retention of BMPs at the defect site, a carrier is needed that has desirable release kinetics. Furthermore, if the volume of bone to be regenerated is large, the carrier has to be combined with a scaffold or serve as a scaffold itself to allow for the cell migration and growth, and the deposition of extracellular matrix [25].

Delivery systems for BMPs have been constructed from a variety of materials, which can be categorized as natural polymers, synthetic polymers, inorganic materials, and composites of the above.

Of the natural polymers, collagen is the most widely used [9, 21, 26, 27] and was the first to be employed commercially for BMP delivery. Demineralized bone matrix (DBM), the material from which Urist originally extracted BMPs, is also commonly used [28, 29] as the residual levels of BMPs, matrix proteins and calcium in DBM could enhance osteoinductivity. Other natural polymers such as gelatin [30-33], hyaluronic acid [34], chitosan [35] and alginate [36, 37] have also been tested. Potential issues with naturally derived materials include batch variations in purity and quality, as well as the risk of disease transmission.

Biodegradable and/or biocompatible synthetic polymers, such as poly(α-hydroxy acids) [38-41], poly(ortho esters) [42], polypropylene fumarate [43] and polyethylene glycol [44], offer benefits of reproducible manufacture and readily tailored functionality. In particular, poly(α-hydroxy acids) comprising lactic acid and glycolic acid are approved by the Federal Drug Administration (FDA), and have been used as suture material since the 1970s. Biodegradable polymers are designed to decompose to non-toxic products in physiological environments. However, some degradation products, such as those from poly(α-hydroxy acids) and polyanhydrides, are acidic and may cause tissue inflammation.

Hydroxyapatite (HAP) [19, 45, 46], calcium phosphate [45, 47, 48], silica [49], bioglass [50, 51] and titanium [52, 53] are some of the inorganic materials that have been used as BMP carriers. The most commonly used is HAP because of its similarity to the mineral component of bone and its osteoconductivity. The osteoconductivity of HAP and calcium phosphate is sometimes attributed to their ability to concentrate growth factors, including BMPs, in the body [54]. The release of proteins from ceramics can be very slow.
and sustained because of the high affinity of certain ceramics for proteins [54-58]. Drawbacks of ceramics include their brittleness and difficulty in processing.

A fruitful combination of the above three classes of materials as composites would allow the harnessing of the benefits of each component. Furthermore, the drawbacks of one material may be countered by another. For example, HAP and calcium phosphate can act as a buffer against the acidity of the degradation products from poly(α-hydroxy acids) [59, 60]. Composites have been produced with enhanced mechanical and handling properties [61-66], improved bioactivity [67-69], optimized biodegradation [59, 60, 70], and microstructures that more closely resemble natural tissues [71, 72]. Some composites that have been explored for BMP delivery include HAP-collagen [17, 26, 73, 74], poly(lactic acid-co-glycolic acid) (PLGA)-calcium phosphate [75], PLGA-cellulose [76, 77], and PLGA-gelatin [14].

![Graph showing release rate of radio-labeled BMP-2 from the rabbit ulna osteotomy site](image)

**Fig. 1.1.** A local pharmacokinetic curve showing release rate of radio-labeled BMP-2 from the rabbit ulna osteotomy site [25]. The various carriers shown were either implanted (♦ hyaluronic acid pad and ▲ collagen sponge) or injected (■ Gelfoam paste and ♣ buffer) into the defect. Reprinted from [25] with permission from Elsevier.

Many of these materials are transformed into BMP carriers by simply mixing in the proteins during processing or by soaking pre-fabricated carriers in the protein solution. Variations in release kinetics amongst the carriers are observed due to inherent differences in the affinity of the materials for BMPs and in the carrier dimensions (Fig. 1.1). However, adjustment of BMP release kinetics with such carriers in order to attain the optimal profile is difficult and has not been accomplished. The optimal BMP release profile may vary with
animal species, age of host, anatomic site, wound history and other factors [25]. For example, slower release rates may be required in more fluid environments where BMP clearance may be faster, and in more compromised sites where the healing response is diminished. Tunable carriers would offer greater potential and flexibility in realizing the desired release profile.

Two products containing BMPs have recently been approved by FDA for spinal fusion: Stryker’s OP-I comprising BMP-7 and Medtronic Sofamor Danek’s INFUSE containing BMP-2. Both products use collagen sponges as the carrier for BMPs; the sponges are loaded by soaking with a BMP solution for 10–20 min. The BMP loading in these sponges is on the order of milligrams per implant, which is several orders of magnitude above the natural occurrence in bone (~ 0.002 mg of BMP-2 can be extracted per kg of powdered bone [78]). The release of BMPs from collagen tends to be rapid: 70–90% of the load is depleted by the first week [29, 34, 79]. However, bone healing is often a much longer process requiring weeks or months, especially in higher mammals with less responsive cells. A possible reason for the use of supra-physiological BMP doses in collagen carriers is the need to overcome the low availability of BMPs at later stages of healing [25] since cellular response to BMPs has been found to increase with dosage as well as time of exposure [77, 80]. Therefore, carriers that are capable of sustained release of lower but still therapeutic levels of BMPs would allow greater efficacy and cost-savings by optimizing the use of these expensive proteins. The presence of BMPs over the entire duration of healing in higher mammals may also reduce the variation in response (see Section 1.2), and enhance the therapeutic outcomes. Furthermore, tunable release or multifactor release at different rates, which current collagen sponge carriers cannot accomplish, may also augment the bone healing response.

1.4 Research Objective

Certain anatomic sites and certain indications, e.g. prolonged non-unions, may require BMP release kinetics that current collagen sponge carriers cannot provide. The objective of our research is to improve the efficacy of BMP delivery for bone healing by developing carriers that can retain and meter out BMPs at the appropriate dose and for a sufficient duration to achieve the desired host response. Ideal release characteristics of such carriers include:
1. Sustained release – to make BMPs available over the entire period of healing, possibly lasting for several weeks to several months.

2. Low burst release – to avoid the adverse effects of an overdose, and to conserve the expensive therapeutic proteins, making the formulation more cost-effective.

3. Tunable release – to allow flexibility in designing release rates for different therapeutic proteins, host species, and anatomic sites.

Other desirable properties of the carrier include biocompatibility, biodegradability, bioactivity, ease of manufacture, and surgical practicability. Such a carrier would also have broader applications in the delivery of other therapeutic proteins and drugs.

To achieve the research objective, the following approach was taken:

- Development of a biocompatible, biodegradable carrier capable of tunable, sustained, and low-burst release (Chapter 2)
- Application of the carrier to the delivery of BMPs (Chapter 3)
- *In vitro* testing to establish bioactivity of released BMPs (Chapter 3)
- Incorporation of BMP carriers into scaffolds capable of supporting *in vivo* cell migration and growth (Chapter 4)
- Ascertaining the safety and efficacy of these scaffolds *in vitro and in vivo* (Chapter 4)

### 1.5 Apatite-Polymer Composites for Delivery of BMPs

Our strategy for a tunable controlled delivery platform is based on the use of a polymer with acidic degradation products to control the dissolution of a basic inorganic substrate on which BMPs have been adsorbed. The release of acid from the hydrolysis of the polymer leads to the dissolution of the inorganic substrate and consequently, the desorption and release of the protein. Hence, protein release is anticipated to be accelerated over passive leaching from the inorganic substrate, yet, more controlled than the release from polymeric microspheres due to the affinity of the substrate for the protein. The release mechanism is depicted in Fig. 1.2.

Systematic modulation of the release profile may be possible by changing variables that affect polymer degradation, subsequent acid generation, and/or inorganic substrate dissolution. These variables include polymer type, polymer molecular weight, polymer composition (including copolymers and blends), inorganic substrate type, inorganic substrate
loading, inorganic substrate particle size, relative proportion of polymer and inorganic substrate, and protein loading on the inorganic substrate. This delivery platform can be applied to any therapeutic agent that can be adsorbed and sequestered on the surface of a basic inorganic material.

![Diagram of composite microparticle](image)

Fig. 1.2. Protein is released from the composite microparticle as a result of polymer hydrolysis that leads to dissolution of the apatite substrate.

Candidates for the polymeric component include polyanhydrides, poly(α-hydroxy acids) and poly(ortho esters), all of which degrade to produce acids. Poly(α-hydroxy acids) comprising lactic acid (LA) and glycolic acid (GA) – PLGA, PLA, and PGA – are attractive candidates because they are FDA-approved, and are commercially available in a wide range of molecular weights. Furthermore, poly(α-hydroxy acids) are bulk eroding polymers, which may be more suitable for this controlled release mechanism as they allow the buildup of acid within composite particles necessary for dissolving the apatitic phase. On the other hand, acidic degradation products from surface eroding polymers, e.g. polyanhydrides, may diffuse away too quickly to encounter the apatitic phase. The pH within degrading PLGA microspheres has been found to be less than 4.7 [81] and as low as 1.5 [82]. Such a pH range should be sufficient to cause the dissolution of a basic material.

A number of basic inorganic materials, such as HAP, carbonated apatite (CAP), calcium phosphate and calcium carbonate, exhibit bioactivity and enhanced integration with host bony tissue [83-88]. These materials are also employed as fillers to enhance the mechanical properties of the softer natural and synthetic polymers [61, 62, 64]. In addition, they have been used to alleviate the acidity created by the degradation of poly(α-hydroxy
acids) [59, 60, 89, 90], which is relevant to our current application. The high affinity of HAP for proteins (see Section 1.3) suggests that HAP should be capable of holding BMPs on its surface without premature release. Nanocrystalline HAP and CAP, the syntheses of which were previously developed in our laboratory [91-93], were selected as the inorganic phase of the composites. CAP differs from HAP (Ca₁₀(PO₄)₆(OH)₂) in that it has carbonate ions substituted in the hydroxyl or phosphate sites in the crystal lattice [94]. CAP is more amorphous, more resorbable, and closer in composition to natural bone mineral.

The dimensions of the carrier would depend on the method of administration, defect site to fill, and impact on release kinetics. The apatite-polymer composite carriers may be prepared as particles, which can be delivered by injection into the bone defect, compressed into pellets for implantation, or dispersed in a secondary matrix that can be formed into tissue engineering scaffolds. In addition, the composites may be fabricated as films or porous, bulk scaffolds.

1.6 References


Chapter 2 – Synthesis, Characterization, and In Vitro Release Profiles of Apatite-Polymer Composite Microparticles for the Controlled Delivery of a Model Protein

2.1 Introduction

Proteins experience short half-lives in vivo on the order of minutes or hours due to enzymatic degradation, evisceration through the reticulo-endothelial system, immunological inactivation, and other pathways [1-3]. The encapsulation of proteins in microparticles protects the proteins from their external environment and slows their release [4]. Methods of preparing microparticles loaded with water-soluble drugs and proteins include water-in-oil-in-water emulsions [5-9], coacervation [10-12], and spray drying [10, 13-15]. The microparticles are typically constructed of polymers; some examples are biodegradable synthetic polymers such as poly(lactic-co-glycolic acid) (PLGA) and polycaprolactone, and natural polymers such as chitosan and gelatin.

Formulating the protein delivery vehicle as microparticles allows direct injection of the particles into the bloodstream or a defect site. Through compression or sintering, microparticles can also be formed into other shapes and sizes [16, 17]. A further application of microparticles is in the release of multiple therapeutic proteins, such as a combination of growth factors to encourage tissue regeneration. Different sets of microparticles, each containing a therapeutic protein and each designed with a distinct release profile, can be dispersed in a bulk matrix or scaffold. For versatile multifactor release, it is important that the release profiles of the microparticles be tunable.

2.1.1 Conventional Double Emulsion Processing

Water-in-oil-in-water (W/O/W) emulsion, also known as double emulsion, is arguably the most frequently cited method of producing polymeric microparticles encapsulating water soluble agents due to its relative processing ease and low equipment demand. This process involves adding an aqueous solution of the therapeutic agent to an organic solution of the polymer. The mixture is agitated by vortexing, homogenization or sonication to form a water-in-oil (W/O) emulsion. This emulsion is then added to a large volume of water supplemented with a surfactant, and agitated again to create a W/O/W emulsion. Solid microspheres are obtained after the organic solvent is removed by extraction or evaporation [8].
While the microparticle yield and protein encapsulation efficiency can be relatively high with this process, it has several limitations. These microparticles typically exhibit high initial burst as a large proportion of the protein is only loosely associated with the microparticles on the surface or in the pores. The release rate after the initial burst is also difficult to predict and control. The effects of a wide range of material and processing parameters on release have been studied, including polymer molecular weight [18], surfactant type [19], ratio of inner and outer water phases [5, 20], addition of hydrophilic or hydrophobic agents [21, 22], and addition of salt to the outer water phase to reduce protein leaching [6]. These studies have highlighted the difficulty in predicting a priori the effect of each parameter on the release profile. In addition, the effect on release is often a change in magnitude, and not a change in rate. A further drawback of the double emulsion process is the exposure of the protein to denaturing organic solvent at the water-oil interface, which may lead to protein unfolding, aggregation and deactivation [23].

2.1.2 Solid-in-Oil-in-Water Processing

To circumvent protein aggregation at the water-oil interface, a semi-aqueous technique has been developed in which the protein is lyophilized and dispersed as a solid powder in the organic polymer solution to create a solid-in-oil (S/O) suspension. This suspension is then emulsified in an aqueous phase to form a solid-in-oil-in-water (S/O/W) suspension [24]. The rationale behind this technique is the observation that dehydrated proteins are less prone to denaturation in anhydrous solvents because conformational changes are kinetically prohibited [25]. Bovine serum albumin (BSA) encapsulated by this technique was found to show little change in secondary conformation from its lyophilized state [24].

2.1.3 Adsorption of Proteins onto Apatite

The S/O/W emulsion method overcomes the problem of protein denaturation at the W/O interface, but does not address issues of high initial burst and poor tunability of release from polymeric microspheres. To reduce initial burst, the protein can be formulated into less soluble forms such as by precipitation with divalent ions [26-28] or adsorption onto a substrate. In the former case, the concentration of the divalent ion, such as zinc, needed to precipitate each type of protein has to be carefully determined. The diffusion or displacement
of the divalent ions leads to solubilization and release of the protein. In the latter case, the substrate used for adsorption should have a high affinity for the protein and a high surface area for adsorption. Protein release is affected by desorption of the protein and/or dissolution of the substrate. A suitable candidate for adsorbing bone morphogenetic proteins (BMPs) is apatite, which has a natural affinity for BMPs. In fact, the osteoconductivity of apatite and calcium phosphate is sometimes attributed to their ability to concentrate growth factors such as BMPs in the body [29]. As a result of this high protein affinity, the release of proteins from apatite and calcium phosphate is typically very slow and sustained [30].

Our strategy for a tunable, controlled delivery platform utilizes a polymer with acidic degradation products, poly(lactic-co-glycolic acid) (PLGA), to affect the dissolution of a basic apatitic substrate on which a protein has been pre-adsorbed. Such a composite can be formulated into microparticles by the S/O/W method. Protein release is anticipated to be accelerated over passive leaching from apatite, yet, more controlled than release from polymeric microspheres due to apatite’s affinity for protein.

2.2 Experimental

2.2.1 Synthesis of Hydroxyapatite and Carbonated Apatite

Nanocrystalline hydroxyapatite (HAP) and carbonated apatite (CAP) were synthesized according to the method developed by Ahn et al. [31-33]. For the synthesis of HAP, 900 ml of 0.167 M Ca(NO₃)₂ (Fluka) and 900 ml of 0.100 M (NH₄)₂HPO₄ (Fluka) were prepared in deionized (DI) water. The pH of the (NH₄)₂HPO₄ solution was raised to 10.4 with ammonium hydroxide. The Ca(NO₃)₂ solution was added to the (NH₄)₂HPO₄ solution at a rate of ~ 3 ml/min to precipitate HAP. The resulting suspension was stirred at room temperature for 72 h. After this aging step, the white precipitate was washed with solutions of decreasing pH, followed by two ethanol washes. The gel was air-dried overnight, then oven-dried at 120°C for 24 h. The dried gel was ground in a heated mortar and calcined at 550°C for 2 h (ramp rate = 4°C/min). After calcination, the HAP powder was sieved through a 45-μm mesh.

CAP was synthesized by the same method but with the following modifications. The carbonate source, (NH₄)HCO₃, was added to the (NH₄)₂HPO₄ solution at a concentration of 0.100 M. After oven drying, the gel was ground and sieved. The powder was not calcined to avoid driving off the carbonate groups at elevated temperatures.
For the synthesis of submicron-sized apatite particles, modifications were made to the above protocol to reduce agglomeration. The concentrations of the Ca(NO$_3$)$_2$ and (NH$_4$)$_2$HPO$_4$/(NH$_4$)HCO$_3$ solutions were reduced 10-fold. Tween 80 (Aldrich) was added as a surfactant to constitute 11 v/v% of the (NH$_4$)$_2$HPO$_4$/(NH$_4$)HCO$_3$ solution. The particles were collected and washed by ultrafiltration instead of centrifugation. Washes with ethanol were not performed due to its incompatibility with the ultrafiltration device. Water was removed by freeze drying to obtain the final product. Calcination, which leads to grain growth, was not performed on these apatite powders. The hydroxyapatite and carbonated apatite thus prepared are referred to as sHAP and sCAP, respectively.

Two types of HAP powder were purchased from Berkeley Advanced Biomaterials, Inc. (BABI) for comparison with our materials. The two products were BABI-HAP-SP and BABI-HAP-N20, with advertised mean particle sizes of 5 μm and 20 nm, respectively.

2.2.2 Characterization of Apatite

Powder X-ray diffraction (XRD) patterns of the various apatite powders were obtained with a Siemens D5000 0-0 diffractometer (45 kV, 40 mA, Cu K$_\alpha$). Grain size analyses were performed on the <002> diffraction peaks using Scherrer’s method. The BET surface area was determined by nitrogen adsorption on a Micromeritics ASAP 2000/2010 Analyzer. Particle size distribution was evaluated using a Horiba CAPA-300 Particle Size Analyzer.

2.2.3 Adsorption of Proteins onto Apatite

Fluorescein isothiocyanate bovine serum albumin (FITC-BSA, Sigma Aldrich) was used as a model protein. The adsorption of FITC-BSA onto apatite was typically conducted as follows. FITC-BSA was dissolved in DI water at a concentration of 0.25 mg/ml, and added to 10 mg of apatite. The suspension was stirred for 16 h at room temperature to allow the adsorption of protein onto the apatite. The resulting BSA-apatite complex was collected by centrifugation, and the supernatant was filtered and saved. Subsequently, the BSA-apatite complex was washed with DI water and lyophilized. The amount of protein adsorbed was determined as the difference between the protein concentration of the initial stock solution and that of the supernatant after adsorption. Protein concentration was analyzed by Coomassie Plus total protein assay (Pierce).
Maximum protein adsorption was determined for HAP, CAP, sHAP, sCAP, BABI-HAP-SP, and BABI-HAP-N20. For each type of apatite, the sample size tested was 3–5. Adsorption isotherms for FITC-BSA onto CAP and HAP at 4°C and room temperature were obtained by varying the concentration of the initial FITC-BSA solution from 0.25 to 6.00 mg/ml. Adsorption times ranging from 0.5 h to 7 d were used to determine the minimum amount of time required for maximum protein adsorption at room temperature.

To explore the possibility of adsorbing other types of proteins, proteins of different isoelectric point (IEP) and size were tested for adsorption onto CAP. These proteins (all from Sigma) included lysozyme (IEP = 11, MW = 14 kD), cytochrome C (IEP = 10, MW = 12.4 kD), and alcohol dehydrogenase (IEP = 5.5, MW = 141 kD). Protein stock solutions of 100–200 μg/ml were prepared with DI water. Each protein solution was added to triplicates of 10 mg of CAP and stirred for 8 h at room temperature. The amount of protein adsorbed was expressed as a percentage of the amount of protein in the original stock solution.

Zeta potential measurements (Brookhaven ZetaPALS Zeta Potential Analyzer) were performed at pH 7 on CAP and HAP before and after the adsorption of FITC-BSA. For each apatite or BSA-apatite complex, the number of specimens tested was 3.

### 2.2.4 Effect of pH on the Dissolution of Apatite and the Release of Adsorbed Proteins

To study the effect of pH on the dissolution of apatite and the release of adsorbed proteins from BSA-apatite complexes, triplicates of 10 mg of either BSA-CAP or BSA-HAP powder was added to microvials containing 1.5 ml of medium. The BSA loading on the apatites was 7.5 w/w% (750 μg of BSA per 10 mg of BSA-apatite complex). The medium was citrate buffer of pH 3, 4 or 5. As a comparison, N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) buffer of physiological pH (pH 7.4) was also used. The vials were incubated in a 37°C water bath for 8 weeks. At pre-determined time intervals of 1, 4, 7, 14, 21, 28, 42 and 56 days, the vials were centrifuged. Half of the supernatant (0.75 ml) was removed and filtered, and replaced with the same volume of fresh buffer. The vials were then returned to the water bath. The supernatant was stored at 4°C until analysis by Coomassie Plus total protein assay for protein concentration. The results were used to construct a cumulative protein release profile for each protein-apatite complex at a specified pH.
2.2.5 Preparation of Composite Microparticles

Composite microparticles were synthesized by a S/O/W emulsion process modified from that described by Castellanos et al. for encapsulating proteins in the solid state [24]. A typical synthesis involved dissolving 250 mg of PLGA (Alkermes) in 2 ml of dichloromethane. To this polymer solution, 10 mg of protein-apatite complex were added and vortexed to create a uniform suspension. The S/O suspension was homogenized in 50 ml of 0.1 w/v% aqueous methyl cellulose solution at 8000 rpm for 2 min at room temperature. The resulting S/O/W suspension was heated at 30°C for 3 h to evaporate dichloromethane and solidify the particles. The particles were collected by centrifugation, washed with DI water, and lyophilized. A schematic of this synthesis is presented in Fig. 2.1. This preparation was varied systemically one parameter at a time to investigate the effect of different material and processing parameters on particle size and morphology, as well as protein release.

![Diagram of the S/O/W process for preparing composite microparticles.](image)

Fig. 2.1. Schematic of the S/O/W process for preparing composite microparticles.
2.2.6 Preparation of PLGA Microspheres by Double Emulsion Processing

As a standard for comparison, the double emulsion technique of Blanco et al. [34] was modified to prepare polymeric microspheres encapsulating proteins. An aqueous solution of FITC-BSA was first created by dissolving 4 mg of the protein in 50 µl of DI water. This aqueous phase was added to an organic phase containing 250 mg of PLGA in 2 ml of dichloromethane. The two phases were homogenized at 8000 rpm for 2 min to form a W/O emulsion. The emulsion was then transferred to 50 ml of aqueous 0.1 w/v% methyl cellulose solution and homogenized at 8000 rpm for another 2 min to create a W/O/W emulsion. This double emulsion was stirred and heated at 30°C for 3 h to drive off dichloromethane. The resulting microparticles were collected by centrifugation, washed 3 times with DI water, and freeze-dried to obtain the final product. A schematic of this synthesis is presented in Fig. 2.2. The key difference between W/O/W and S/O/W processing lies in the protein formulation; the protein is dissolved in an aqueous solution in the former but adsorbed onto solid apatite in the latter.

![Schematic of the W/O/W process for preparing polymeric microparticles.](image)

Fig. 2.2. Schematic of the W/O/W process for preparing polymeric microparticles.
2.2.7 Characterization of Microparticle Morphology and Size

The morphology of the composite microparticles was evaluated by environmental scanning electron microscopy (ESEM, FEI/Philips XL30 FEG) at 6–7 kV. The particle size distribution was determined by measuring the dimensions of a minimum of 100 particles by ESEM.

2.2.8 Evaluation of Encapsulation Efficiency of Composite Microparticles

To evaluate the protein loading in the composite microparticles, the polymeric phase was first dissolved with 0.5 ml of dichloromethane per 10 mg of composite particles. 3 ml of citrate buffer of pH 2 were then added to the organic suspension to dissolve the apatitic phase. Prior testing showed that 3 ml of pH 2 citrate buffer were sufficient to dissolve more than 8 mg of protein-apatite complex. Among all the sets of composite particles that were prepared, the maximum loading of protein-apatite in 10 mg of composite particles was 2.65 mg. The dichloromethane-citrate buffer mixture was vortexed periodically over 2 days to allow protein extraction into the aqueous phase. The aqueous phase was then removed for protein concentration analysis by Coomassie Plus total protein assay. The low pH of the citrate buffer sometimes led to the precipitation of BSA. To dissolve the protein precipitate, 1 ml of 0.1 N NaOH was added. The resulting alkaline protein solution was also assessed for protein concentration. The total protein content in the composite particles was the summation of the quantities of protein measured in the citrate buffer and in the NaOH solution. Hence, the protein loading of the composite particles was calculated as:

\[
\text{Protein Loading} (\%) = \frac{\text{Protein Amt}_{\text{citrate buffer}} + \text{Protein Amt}_{\text{NaOH soin}}}{\text{Mass of Composite Particles}} \times 100
\]

The encapsulation efficiency was calculated as follows:

\[
\text{Encapsulation Efficiency} (\%) = \frac{\text{Measured Protein Loading}}{\text{Theoretical Protein Loading}} \times 100
\]

Each set of composite particles was tested in triplicates.

An alternative method of loading these composite particles with protein was to allow post-synthesis uptake of protein into blank apatite-PLGA composite particles. Both CAP-PLGA and HAP-PLGA composite particles were tested in triplicates. 15 mg of blank apatite-PLGA particles were mixed with 1 mg of FITC-BSA in 1 ml of DI water. The mixtures were
allowed to stir at room temperature for 24 h, and then centrifuged. The protein loading for each sample was derived from the difference between the protein concentration of the original stock solution and that of the supernatant.

2.2.9 Evaluation of In Vitro Protein Release

A known amount of protein-loaded composite microparticles was resuspended in 1.5 ml of BES buffer (pH 7.4) supplemented with 0.02 w/v% sodium azide as a germicide, and incubated at 37°C. Each set of particles was tested in duplicates. At pre-determined time intervals (1, 4, 7, 14, 21, 28, 42 and 56 days, up to 20 weeks), the samples were centrifuged, and 0.75 ml of the supernatant was withdrawn and replaced with 0.75 ml of fresh BES buffer. For samples containing FITC-BSA, the collected supernatant was filtered and assayed for fluorescence, and stored at 4°C until further analysis of protein concentration with the Coomassie Plus total protein assay. The concentration of the protein released at each time point was used to construct cumulative release profiles.

2.2.10 Evaluation of Polymer Degradation

Blank composite microparticles fabricated from PLGA with molecular weight of 6, 13, 24 and 59 kD were tested. The apatite loading was 0.2 mg per mg of PLGA. For each set of particles, 20-mg samples were suspended in 1.5 ml of pH 7.4 BES buffer supplemented with 0.02 w/v% sodium azide. One sample was prepared for each time point at 1, 4, 7, 14, 21 and 28 days. At each time point, the sample was centrifuged and the supernatant was removed. The residue was freeze-dried, dissolovd in tetrahydrofuran, filtered, and analyzed for molecular weight by gel permeation chromatography using Waters 1525 HPLC system and 2414 refractive index detector, equipped with Styrogel® HT3, 4 and 5 columns.

2.2.11 Evaluation of Calcium Release from Composite Microparticles

The calcium concentration of the release media incubated with composite microparticles was analyzed using a fluorescent calcium indicator, Rhod-5N (Molecular Probes). Rhod-5N is a rhodamine-based fluorophore that exhibits enhanced fluorescence (Ex/Em = 551/576) when bound to calcium. It is sensitive in the range of 10 μM to 1 mM calcium.
Calcium release from CAP-PLGA composite microparticles prepared from polymers of different molecular weight was tracked. In addition, bare CAP and one set of BSA-CAP-PLGA (59 kD) composite particles with a BSA loading of 1.67 w/w% were tested. The apatite loading for all sets of composite particles was 0.2 mg per mg of PLGA. For each set of composite particles, duplicates were prepared consisting of 30 mg of particles incubated in 1.5 ml of BES buffer (pH 7.4) at 37°C. 5 mg were used for bare CAP, which were equivalent in amount to the apatite loading in 30 mg of composite particles. The release medium was collected, and renewed at 1, 4, 7, 14, 21, 28 and 42 days. To generate a calcium calibration curve, standards ranging in concentration from 10 μM to 1 mM were prepared using calcium nitrate tetrahydrate (Fluka).

2.3 Results and Discussion

2.3.1 Characterization of Apatite

The preparation of apatite by chemical precipitation resulted in the formation of a pure apatitic phase as shown in Fig. 2.3. The major peaks at 25.88°, 31.77°, 32.20°, 32.90° and 39.82° corresponded to (002), (211), (112), (300) and (310) diffractions of HAP. Calcination of HAP led to higher crystallinity, as indicated by the sharper and narrower peaks. HAP had a much finer grain size than commercial BABI-HAP-SP and BABI-HAP-N20 powders (Table 2.1). CAP, sCAP, and sHAP, which were prepared without calcination, possessed smaller grain sizes than HAP.

HAP and CAP have similar average particle sizes (Table 2.1). The use of surfactant led to sHAP and sCAP with finer particle sizes. The measured particle size of BABI-HAP-SP was close to its advertised particle size of 5 μm, and was in the same range as HAP and CAP. The measured particle size of BABI-HAP-N20 was 30 times larger than its advertised particle size, probably due to agglomeration. Apatite particle size is important as it places a lower limit on the size of the apatite-polymer composite microparticles that can be formed. Smaller apatite particles would facilitate more uniform apatite dispersion in the polymeric matrix, as well as the creation of smaller composite particles without phase separation.

Table 2.2 shows that CAP has a high BET surface area (302 m²/g) and a high pore volume, suggesting that it might be able to sequester a larger amount of protein than HAP. The commercial BABI powders have lower surface areas and pore volumes, and larger pores.
than the apatites we synthesized. The sCAP and sHAP powders gave inconsistent measurements of BET surface areas, likely due to surfactants remaining in their pores. Attempts to drive off the residual surfactants by gentle calcination to 300°C resulted in the discoloration of these powders and reduction in surface areas. Hence, their nitrogen adsorption results were not included in Table 2.2.

Fig. 2.3. XRD patterns of apatites prepared with surfactant (sCAP, sHAP) and without surfactant (CAP, HAP), and commercial BABI-HAP-SP and BABI-HAP-20N powders. The diffraction peak positions of the HAP standard (std) are also included for comparison.

Table 2.1. Grain sizes and particle sizes of apatite powders.

<table>
<thead>
<tr>
<th>Apatite Sample</th>
<th>Grain Size (nm)</th>
<th>Average Particle Size (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAP</td>
<td>27</td>
<td>5.3</td>
</tr>
<tr>
<td>CAP</td>
<td>12</td>
<td>6.8</td>
</tr>
<tr>
<td>sHAP</td>
<td>15</td>
<td>0.7</td>
</tr>
<tr>
<td>sCAP</td>
<td>15</td>
<td>1.0</td>
</tr>
<tr>
<td>BABI-HAP-SP</td>
<td>47</td>
<td>4.5</td>
</tr>
<tr>
<td>BABI-HAP-N20</td>
<td>43</td>
<td>0.6</td>
</tr>
</tbody>
</table>
Table 2.2. BET surface areas, pore volumes and mean pore radii of apatites.

<table>
<thead>
<tr>
<th>Property</th>
<th>CAP</th>
<th>HAP</th>
<th>BABI-HAP-SP</th>
<th>BABI-HAP-N20</th>
</tr>
</thead>
<tbody>
<tr>
<td>BET Surface Area (m²/g)</td>
<td>302</td>
<td>86</td>
<td>48</td>
<td>53</td>
</tr>
<tr>
<td>Pore Volume (cm³/g)</td>
<td>1.24</td>
<td>0.52</td>
<td>0.18</td>
<td>0.27</td>
</tr>
<tr>
<td>Mean Pore Radius (nm)</td>
<td>8.2</td>
<td>12.2</td>
<td>24</td>
<td>36</td>
</tr>
</tbody>
</table>

2.3.2 Protein Adsorption onto Apatite

The maximum FITC-BSA adsorption capacities of various apatites are summarized in Table 2.3. BABI-HAP-SP and BABI-HAP-N20 were found to have lower protein adsorption capacities due to their lower surface areas (Table 2.2). Compared to BABI-HAP-N20, sHAP has approximately the same particle size, but adsorbed more than thrice the amount of protein.

Table 2.3. Protein adsorption capacities of apatites.

<table>
<thead>
<tr>
<th>Apatite Sample</th>
<th>Maximum BSA Adsorption (w/w%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAP</td>
<td>15.9 ± 0.8</td>
</tr>
<tr>
<td>CAP</td>
<td>31.4 ± 1.5</td>
</tr>
<tr>
<td>sHAP</td>
<td>25.3 ± 1.6</td>
</tr>
<tr>
<td>sCAP</td>
<td>21.3 ± 1.2</td>
</tr>
<tr>
<td>BABI-HAP-SP</td>
<td>7.1 ± 0.5</td>
</tr>
<tr>
<td>BABI-HAP-N20</td>
<td>8.0 ± 0.6</td>
</tr>
</tbody>
</table>

FITC-BSA adsorption isotherms of CAP and HAP are shown in Fig. 2.4. Adsorption isotherms at 4°C show saturation at ~ 80% of the BSA levels at room temperature. The value of the plateau was taken as the maximum amount of protein adsorbed for the given mass of apatite. Table 2.3 shows that the maximum amount of FITC-BSA adsorbed onto CAP was 2.4 times higher than that adsorbed onto HAP, although the BET surface area derived from nitrogen adsorption was 3.5 times higher for CAP (Table 2.2). Compared to HAP, CAP has a finer mean pore size of 8.2 nm, which was equivalent to BSA’s hydrodynamic diameter. Thus, the smaller pores in the pore size distribution for CAP might not be accessible for BSA adsorption.
Protein adsorption onto apatite was found to be a rapid process, and maximum adsorption could be achieved within 6 h. A representative adsorption profile is shown in Fig. 2.5. The minimum time required for maximum protein adsorption should be used to reduce processing time and exposure of sensitive proteins to elevated temperatures. However, in the case of FITC-BSA, which is a relatively stable protein, adsorption was typically conducted overnight for 16 h. For the adsorption of BMPs [35], the adsorption time was shortened.

Fig. 2.4. FITC-BSA adsorption isotherms of (a) HAP and (b) CAP at room temperature, and (c) HAP and (d) CAP at 4°C.
Fig. 2.5. Adsorption of FITC-BSA onto HAP with time at room temperature.

The ability of apatite to adsorb other types of proteins of different isoelectric points (IEP) and sizes was examined. The amount of protein adsorbed is expressed as a percentage of the total amount of protein in the initial stock solution. CAP was found to be capable of adsorbing a high percentage of all the proteins tested, with the exception of lysozyme (see Table 2.4). Only 24% of the lysozyme added to the apatite suspension was adsorbed. This lower affinity could be attributed to the rigid structure of lysozyme, which allowed it to only partially unfold during adsorption and make fewer contacts with the substrate [36].

Table 2.4. Adsorption of proteins of different IEP and size onto CAP.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Isoelectric Point</th>
<th>Molecular Weight (kD)</th>
<th>Amt. Adsorbed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC-BSA</td>
<td>4.7</td>
<td>66</td>
<td>99.2</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>11</td>
<td>14</td>
<td>23.7</td>
</tr>
<tr>
<td>Cytochrome C</td>
<td>10</td>
<td>12</td>
<td>96.9</td>
</tr>
<tr>
<td>Alcohol Dehydrogenase</td>
<td>5.5</td>
<td>141</td>
<td>97.5</td>
</tr>
</tbody>
</table>

The zeta potential of CAP at pH 7 was found to be slightly negative (Table 2.5). HAP, which was also negatively charged, had a higher zeta potential. A higher zeta potential would give rise to increased stability of the suspension since the charged particles would repel one another and prevent agglomeration. Hence, a suspension of higher zeta potential could be expected to have finer particle size (see Tables 2.2 and 2.5). Upon the adsorption of BSA
(IEP = 4.7), which was negatively charged at pH 7, the zeta potentials of both CAP and HAP rose in magnitude. The particle sizes of BSA-CAP and BSA-HAP were also found to decrease.

Table 2.5. Zeta potential and particle size of apatites and BSA-apatite complexes.

<table>
<thead>
<tr>
<th>Sample</th>
<th>CAP</th>
<th>HAP</th>
<th>BSA-CAP</th>
<th>BSA-HAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zeta Potential at pH 7 (mV)</td>
<td>-2.82 ± 1.78</td>
<td>-11.19 ± 2.12</td>
<td>-18.59 ± 0.66</td>
<td>-22.16 ± 1.81</td>
</tr>
<tr>
<td>Particle Size (μm)</td>
<td>6.8 ± 1.7</td>
<td>5.3 ± 1.5</td>
<td>5.8 ± 1.8</td>
<td>3.8 ± 2.1</td>
</tr>
</tbody>
</table>

As BSA and apatite were both negatively charged at pH 7, electrostatic interaction was not the driving force for protein adsorption in this case. The surface of bare hydroxyapatite is reported to be hydrophobic, so hydrophobic interactions between BSA and hydroxyapatite could have induced protein adsorption [37, 38]. BSA, a “floppy” protein with high conformational adaptability, could expose its hydrophobic groups for interactions with the hydroxyapatite surface [39]. Other mechanisms of protein adsorption might include ion-exchange and hydrogen bonding. Thus, adsorption onto apatite would not be limited to proteins that were oppositely charged at the pH of interest. Both positively charged proteins (such as cytochrome C and BMPs [35]) and negatively charged proteins (such as BSA and alcohol dehydrogenase) were found to adsorb onto CAP (Table 2.4).

2.3.3 Effect of pH on Protein Release from Protein-Apatite Complexes

The effect of pH on the BSA release from BSA-apatite complexes is shown in Fig. 2.6. It was hypothesized that since apatite was basic, its dissolution in an acidic medium would cause proteins sequestered on its surface or within its pores to be released. In line with our hypothesis, we found that at the physiological pH of 7.4, protein release was very slow and almost negligible over the 8-week test period. One additional data point was obtained at 436 days for pH 7.4, indicating that even after this extended period, only 2.6% and 1.5% of the protein had been released from the BSA-HAP and BSA-CAP complexes, respectively. Hence, under neutral pH conditions, the affinity of apatite for BSA remained high and apatite dissolution was low over more than one year. The stability of ovine albumin in carbonated hydroxyapatite gel over one year has also been reported by Barralet et al. [30].
Lowering of the pH to 5 led to a sharp increase in protein release from both BSA-HAP and BSA-CAP complexes. The BSA-HAP complex released 45% of its protein in the first week, but the release plateaued thereafter. Protein release from BSA-CAP complex was more gradual and constant over the period tested. The difference in release profile could be attributed to the difference in the pore volume of HAP and CAP. HAP, being less porous, held a larger proportion of BSA on its surface, whereas CAP has a more even distribution of BSA on its surface and in its pores. Acid would first erode the surface of the apatite, resulting in an initial burst of protein from BSA-HAP complex. For BSA-CAP complex, the protein would be released more gradually since the acid would have to penetrate through the surface layer of the CAP particle to release those proteins adsorbed deep within the pores.

Further reductions in pH to 4 and 3 were also investigated. At pH 4, two observations were noted: First, the release rate was approximately zero-order after a small initial burst, suggesting that a constant protein release rate was possible in the constant presence or addition of acid. Second, protein release decreased to ~ one-fifth the levels at pH 5 for BSA-CAP complex. A fall in the protein release with decreasing pH was further exemplified by the profile at pH 3. A possible reason for the depressed protein release was a change in electronic interactions between BSA and apatite as their points of zero change were crossed at pH 4.7 and ~ 4 (preliminary experimental data not shown) [40], respectively. At pH 7.4, both BSA and apatite were negatively charged. At pH 3 or 4, BSA was positively charged, whereas apatite was negatively charged or mildly positively charged. Thus, at the lower pHs tested, stronger interactions might have existed between BSA and apatite, leading to a decrease protein desorption and release. Another possibility was that low pH might have induced protein precipitation [41], which became more severe with increasing acidity. Therefore, in the subsequent preparation of polymer-apatite composite microparticles, the pH within the particles should be regulated by the selection of (i) an appropriate proportion of apatite to neutralize the acid, and (ii) a polymer with a suitable degradation profile to avoid overly rapid acid production.
Matsumoto et al. have recently reported similarly enhanced protein release from hydroxyapatite pre-adsorbed with cytochrome C when the pH was reduced from 7 to 4 [42]. However, the protein adsorption capacity of their HAP particles was very low; the maximum cytochrome C adsorbed was only 1.5 w/w%.

2.3.4 Effect of Processing Parameters on Composite Particle Size

ESEM micrographs of four representative sets of composite microparticles are shown in Figs. 2.7 and 2.8. The first pair has an average diameter of ~ 40 μm (Fig. 2.7). Composite particles prepared with sCAP have smoother surfaces, whereas those containing CAP were rougher and more porous.

Fig. 2.8 illustrates the effect of blending in a higher molecular weight polymer. Particles prepared with 13 kD PLGA were smaller (~ 20 μm) and contained a larger proportion of non-spherical debris (Fig. 2.8a). The addition of 24 kD PLGA led to slightly larger (~ 23 μm) and more spherical particles (Fig. 2.8b).
The effects of various processing parameters on the size of the composite particles are summarized in Table 2.6. For each parameter, low and high values around a midpoint were investigated to demonstrate the general trend. Variations that caused higher shear during homogenization led to the creation of smaller particles. For example, increase in homogenization speed and time increased the amount of shear experienced by the suspension. Decrease in polymer solution concentration and polymer molecular weight reduced the viscosity of the organic phase and lowered the particle size concomitantly. Blends of PLGA and poly(lactic acid) (PLA) or polyethylene glycol (PEG) were also examined.
Table 2.6. Effects of processing parameters on composite particle size.

<table>
<thead>
<tr>
<th>Processing/Material Parameter</th>
<th>Particle Size (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard processing conditions as described in Section 2.2.5</td>
<td>41 ± 12</td>
</tr>
<tr>
<td>Increase homogenization speed to 12000 rpm</td>
<td>30 ± 8</td>
</tr>
<tr>
<td>Increase homogenization speed to 16000 rpm</td>
<td>27 ± 8</td>
</tr>
<tr>
<td>Decrease homogenization time to 1 min</td>
<td>55 ± 21</td>
</tr>
<tr>
<td>Increase homogenization time to 4 min</td>
<td>31 ± 10</td>
</tr>
<tr>
<td>Increase CH₂Cl₂ volume to 4 ml</td>
<td>27 ± 8</td>
</tr>
<tr>
<td>Decrease CH₂Cl₂ volume to 1 ml</td>
<td>65 ± 24</td>
</tr>
<tr>
<td>Reduce aqueous surfactant volume to 25 ml</td>
<td>35 ± 12</td>
</tr>
<tr>
<td>Increase aqueous surfactant volume to 100 ml</td>
<td>51 ± 15</td>
</tr>
<tr>
<td>Increase BSA-CAP complex loading to 30 mg</td>
<td>37 ± 13</td>
</tr>
<tr>
<td>Increase BSA-CAP complex loading to 50 mg</td>
<td>37 ± 12</td>
</tr>
<tr>
<td>Decrease apatite particle size to 1 μm (with the use of sCAP)</td>
<td>44 ± 13</td>
</tr>
<tr>
<td>Use of lower molecular weight PLGA (6 kD)</td>
<td>23 ± 7</td>
</tr>
<tr>
<td>Use of higher molecular weight PLGA (59 kD)</td>
<td>45 ± 15</td>
</tr>
<tr>
<td>Blending in 20 w/w% PLA (25 kD)</td>
<td>42 ± 13</td>
</tr>
<tr>
<td>Blending in 60 w/w% PLA (25 kD)</td>
<td>44 ± 12</td>
</tr>
<tr>
<td>Blending in 10 w/w% PEG (3.4 kD)</td>
<td>37 ± 12</td>
</tr>
<tr>
<td>Blending in 30 w/w% PEG (3.4 kD)</td>
<td>23 ± 7</td>
</tr>
</tbody>
</table>

2.3.5 *Encapsulation Efficiency of Protein in Composite Microparticles*

More than 60 formulations of BSA-loaded composite microparticles were prepared, differing in polymer molecular weight, polymer blend, apatite type, apatite particle size, etc. All 60 formulations were tested for encapsulation efficiency, and the average was found to be 69.8% ± 19.9%. The multi-step nature of the extraction process used to determine encapsulation efficiency might have led to under-estimations due to protein loss at each step. However, the average encapsulation efficiency was high, and was comparable to that of particles prepared by the double emulsion technique [18, 21, 43-46]. The encapsulation efficiency of selected formulations is shown in Table 2.7.
Table 2.7. Encapsulation efficiency of selected BSA-loaded composite microparticles.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Theoretical BSA Loading</th>
<th>Encapsulation Efficiency§</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA 6 kD BSA-CAP (15 w/w%) 1.5 w/w%</td>
<td>77.1%</td>
<td></td>
</tr>
<tr>
<td>PLGA 24 kD BSA-CAP (15 w/w%) 1.5 w/w%</td>
<td>52.1%</td>
<td></td>
</tr>
<tr>
<td>PLGA 24 kD BSA-CAP (8 w/w%) 2.2 w/w%</td>
<td>68.7%</td>
<td></td>
</tr>
<tr>
<td>PLGA 24 kD BSA-HAP (20 w/w%) 2.0 w/w%</td>
<td>73.8%</td>
<td></td>
</tr>
<tr>
<td>PLGA 59 kD BSA-CAP (15 w/w%) 1.5 w/w%</td>
<td>50.9%</td>
<td></td>
</tr>
<tr>
<td>PLA 10-15 kD BSA-CAP (15 w/w%) 1.5 w/w%</td>
<td>57.9%</td>
<td></td>
</tr>
<tr>
<td>PLGA + PEG (7:3) 24 kD, 70 kD BSA-CAP (9 w/w%) 0.9 w/w%</td>
<td>94.0%</td>
<td></td>
</tr>
</tbody>
</table>

* Values in parentheses referred to the weight percent of the BSA-apatite complex in the composite microparticles.
† Weight percent of BSA in the composite microparticles.
§ Mean values for n = 3.

The maximum amount of protein that could be loaded into the CAP-PLGA and HAP-PLGA composite particles post-synthesis was 0.26 and 0.28 w/w%, respectively. If the apatite powders had been pre-adsorbed with protein, the maximum theoretical protein loading would have been 5.3 and 2.7 w/w% for CAP-PLGA and HAP-PLGA particles, respectively. PLGA is not known to have a particularly high affinity for proteins. Thus, the less effective protein uptake into the composite particles post-synthesis was expected, since the apatite particles would then be embedded in the PLGA matrix and be less accessible for protein adsorption. Hence, the pre-adsorption of protein onto apatite was a crucial step in ensuring adequate and appropriate protein loading for the composite particles to be used in controlled delivery applications.

2.3.6 Effect of Material and Processing Parameters on the In Vitro Release from Composite Microparticles

2.3.6.1 Polymer Molecular Weight

The molecular weight of a biodegradable polymer is expected to influence its degradation rate and lifetime. Apatite-polymer composite particles were prepared from PLGA of different molecular weights ranging from 6 kD to 59 kD. The onset of rapid protein
release was observed to occur at different times depending on the molecular weight of the polymer used (Fig. 2.9a). For particles prepared with short-chained, 6 kD and 13 kD PLGA, the rapid release phase began immediately. An upturn in the release profile was observed after ~3 weeks for particles synthesized with 24 kD PLGA, whereas protein release remained gradual up to 8 weeks for particles prepared with 59 kD PLGA. Such control of release rate by polymer molecular weight might be useful in sequential release, such as to direct the debut of a protein in a wound healing process.

![Graph a](image1.png)

**Fig. 2.9.** Protein release from composite particles loaded with 15 μg of FITC-BSA per mg of carrier. Particles were prepared using CAP and (a) PLGA of different molecular weights and (b) blends of PLGA of different molecular weights. Data shown are mean values for n = 2.

Polymers of different molecular weights could also be blended together to modulate the acid production over time. For example, rapid protein release from PLGA of low molecular weights (6 kD and 13 kD) was slowed by blending an equi-proportion of 24 kD PLGA (Fig. 2.9b).

2.3.6.2 *Polymer Hydrophobicity*

2.3.6.2.1 Blends of PLGA and PLA

Composite particles were synthesized from blends of PLGA and PLA. As the proportion of PLA in the composite particles was increased, the release rate decreased (Fig. 2.10). Lactic acid contains a methyl group that augments its hydrophobicity relative to
glycolic acid, hence, PLA is more hydrophobic than PLGA. Since it is a homopolymer, PLA is also more crystalline than PLGA, which is a random 50:50 copolymer of lactic acid and glycolic acid. An increase in polymer hydrophobicity would delay water penetration, polymer degradation and acid production, and consequently, reduce protein release. This parameter could be varied by using different types, blends, or compositions of polymers. For example, the lactic acid to glycolic acid ratio in PLGA could be varied to obtain a range of hydrophobicities and degradation rates.

![Graph](image)

Fig. 2.10. Effect of PLA incorporation on protein release. The CAP-polymer composite particles contained 15 μg of FITC-BSA per mg of carrier. The molecular weights of PLGA and PLA were 24 kD and 25 kD, respectively. Data shown are mean values for n = 2.

2.3.6.2.2 Blends of PLGA and PEG

PEG, a hydrophilic, biocompatible and non-degradable polymer, was blended with PLGA in the synthesis of composite particles. Increasing the PEG content led to an increase in the protein release (Fig. 2.11). All release profiles showed an upturn at ~ 21 days, which coincided with the degradation of 24 kD PLGA.

The hydrophilicity of PEG was expected to draw water into the particles where the PEG chains resided. In addition, PEG might have leached out of the polymer matrix, leaving behind pores [47]. The aqueous channels thus formed would facilitate water penetration and PLGA hydrolysis, as well as protein diffusion out of the particles. Therefore, the incorporation of a hydrophilic polymer increased the protein release.
Fig. 2.11. Effect of PEG incorporation on protein release. The CAP-polymer composite particles contained 9 µg of FITC-BSA per mg of carrier. Molecular weights of PLGA and PEG were 24 kD and 3.4 kD, respectively. Data shown are mean values for n = 2.

2.3.6.3 Apatite Particle Size

BSA-apatite complexes prepared with sCAP (1 µm) and CAP (7 µm) were incorporated into the composite particles. Reducing the apatite particle size resulted in a protein release profile that more closely approximated zero-order for a longer period of time (Fig. 2.12). In contrast, the release plateaued after the first week for composite particles containing the larger, 7-µm CAP particles.

For a given amount of acid produced by polymer degradation, a certain amount of apatite would dissolve. Consequently, the protein adsorbed on that amount of apatite would be released. Compared to CAP (which has a porosity of 1.24 cm³/g and a mean pore size of 8.2 nm), sCAP has a higher surface/volume ratio, and a lower porosity (0.27 cm³/g) and mean pore size (4.2 nm). Thus, most of the protein molecules would be expected to be adsorbed on the surface of sCAP particles, whereas protein molecules would be adsorbed both on the surface as well as within the pores of the CAP particles. To free the protein adsorbed within the pores, a larger volume of apatite would have to be dissolved; this might explain the slower rate of protein release from CAP after its initial protein release phase. By controlling the apatite particle size and the available adsorption sites, the protein release profile could be modulated to achieve extended zero-order release.
Fig. 2.12. Effect of carbonated apatite particle size on protein release. The composite particles were prepared with 59 kD PLGA and contained 18 μg of FITC-BSA per mg of carrier. Data shown are mean values for n = 2.

2.3.6.4 Addition of Buffering Apatite

In addition to protein-apatite complexes, varying amounts of bare apatite with no adsorbed protein could be loaded into the composite particles. As the proportion of this “buffering apatite” was increased, the protein release rate decreased (Fig. 2.13). Protein release was dependent on apatite dissolution by acidic polymer degradation products. By incorporating buffering apatite into the composite particles, a portion of the acidity was neutralized by these bare apatite particles rather than by the protein-adsorbed apatite particles. As a result, less protein was released for a given amount of acid produced.
Fig. 2.13. Effect of buffering apatite on protein release, expressed as (a) cumulative mass of BSA released per mg of carrier and (b) cumulative percentage of BSA released. The composite particles were fabricated from 24 kD PLGA (250 mg) and contained (♦) 9, (■) 8.4 and (▲) 7.6 µg of FITC-BSA per mg of carrier (theoretical loading). Results in (b) are based on BSA content determined by extraction. Data shown are mean values for n = 2.

2.3.6.5  Protein Loading on Apatite

BSA-apatite complexes were prepared by adsorbing 9 and 20 w/w% of FITC-BSA onto CAP. They were incorporated into composite particles (labeled “9 w/w% BSA in BSA-CAP” and “20 w/w% BSA in BSA-CAP” in Fig. 2.14) such that each set of particles contained the same mass of BSA-CAP and essentially the same mass of apatite, but the BSA content in the “20 w/w%” particles was twice that of the “9 w/w%” particles. Fig. 2.14 shows that as the protein loading on apatite was increased, the amount released was increased. However, the release profile of the “20 w/w%” particles was sharper initially and then leveled off, which deviated from the typical release profile of composite particles prepared with 24 kD PLGA, where the upturn occurred after ~ 21 days. The higher initial burst might be indicative of multilayer instead of monolayer adsorption of proteins on CAP. By applying the Langmuir adsorption isotherm to the protein adsorption data (Section 2.3.2), it was estimated that a protein loading of ~ 28 w/w% would constitute a monolayer coverage on CAP if all the BET surface area were available for protein adsorption. However, a portion of the CAP’s BET surface area would be occluded due to pores that were smaller than the size of BSA molecules (Section 2.3.1). Hence, we postulated that a protein loading of 20 w/w% could
have exceeded monolayer coverage. Proteins that were adsorbed in multilayers would be released more quickly and in larger quantity upon dissolution of the underlying apatite.

Fig. 2.14. Effect of protein loading in BSA-CAP complex on protein release. The composite particles labeled “9 w/w% BSA in BSA-CAP” and “20 w/w% BSA in BSA-CAP” contained 9 and 18 μg of FITC-BSA per mg of carrier, respectively (theoretical loading). Results in (b) are based on BSA content determined by extraction. PLGA molecular weight was 24 kD. Data shown are mean values for n = 2.

2.3.6.6 BSA-Apatite Complex Loading in Particles

Different amounts of BSA-CAP with the same BSA loading (9 w/w%) were introduced to 24 kD PLGA to form composite particles. Increasing the BSA-CAP loading diminished the amount of BSA released (Fig. 2.15a). The polymer-apatite composite particles behaved very differently from the polymeric particles prepared by the W/O/W emulsification method; the latter demonstrated faster and increased protein release in the absence of apatite particles when the protein loading was increased [45, 48]. This was because for a given BSA loading on apatite, increasing the BSA-CAP content in the composite particles would give rise to increasing apatite/polymer content, so that less acid would be produced from polymer degradation per unit mass of carrier to dissolve a larger amount of apatite. In addition, the alleviation of acidity by larger amounts of apatite per unit mass of carrier would diminish the autocatalytic effect of acid on polymer hydrolysis. Hence, the overall effect of increasing BSA-CAP content was a reduction in protein release from the composite particles.
If the BSA-CAP loading was decreased to a very low level, the complex would be quickly depleted by acid evolution from PLGA such that at later time points, even with the continual degradation of PLGA, there would be no CAP to dissolve and no further protein release. On the other hand, if the proportion of BSA-CAP complex was increased, some of the complex might remain after complete PLGA degradation. Plotting the percentage cumulative release from such composite particles would lead to seemingly low release (Fig. 2.15b). For our experiments, we have chosen to overload rather than underload protein-apatite complexes so that protein was continually released and tracked over the course of the study. In actual applications, the complex loading can be adjusted so that a high percentage of the precious therapeutic protein gets released before complete polymer degradation.

2.3.7 Comparison of Protein Release from Polymeric Microspheres Prepared by Double Emulsion Method

The release profiles of polymeric microspheres prepared by the double emulsion method (Fig. 2.16a) show similarities to those obtained by other researchers [47] (Fig. 2.16b). For the polymeric microspheres, protein release typically began with an initial burst,
sometimes as high as 55% [19, 47], due to the solubilization of protein that was loosely adsorbed on the surface or in the pores of the particles. Protein release would then reach a plateau, as the physically entrapped proteins slowly diffused through tortuous channels in the particles for release. In the later stages, an upturn in the release profile might occur due to polymer degradation, releasing proteins that were polymer-bound. Hence, the early stages of protein release were diffusion-controlled, and the later stages were degradation-controlled. Our 4-week release profiles did not reach the degradation-controlled phase. They showed that the addition of a more hydrophobic polymer, PLA, reduced the initial burst. However, the release rates for PLGA and PLGA-PLA particles were remarkably similar despite their difference in composition. In addition, the similarity between our profiles and those of Cleek et al., who used PEG to modify the profiles, further illustrated the limitation in using material or processing parameters to alter the release rates of polymeric microparticles.

![Graph](image)

Fig. 2.16. Release of (a) BSA from PLGA (24 kD) and 2:3 PLGA (24 kD)-PLA (25 kD) microparticles prepared in-house by the double emulsion method, and (b) FITC-IgG from PLGA microparticles blended with 0, 1, 2 and 5 w/w% of PEG [47]. Data shown in (a) are mean values for n = 2. Reprinted from [47] with permission from Elsevier.

### 2.3.8 Degradation of the Polymeric Phase of Composite Microparticles

Acid release from blank CAP-PLGA composite particles was estimated by tracking the molecular weight of the remaining polymer by GPC, and converting the mass lost into the amount of acid released (Fig. 2.17). The conversion was performed using the approximation
that a two-fold reduction in molecular weight (which would suggest the breakage of a polymer chain into two) corresponded to the release of one acid molecule.

![Graph of acid release](image)

**Fig. 2.17.** Release of acid from the degradation of PLGA of different molecular weights in blank CAP-PLGA composite particles, n = 1.

6 kD and 13 kD PLGA degraded rapidly, as indicated by the steepness of the slope in Fig. 2.17. Polymers of higher molecular weights degraded more gradually, giving rise to a more constant rate of acid production. The increase in slope at later time points for PLGA of different molecular weights might be due to an autocatalytic effect. Ester hydrolysis can be catalyzed by acid or base, and hence, the accumulation of acidic degradation products in the particles would accelerate the rate of hydrolysis [49].

These profiles bear relation to the profiles in Fig. 2.9 illustrating the effect of polymer molecular weight on protein release. Lower molecular weight polymers that degraded more quickly, as indicated by the steeper gradient of the profiles in Fig. 2.17, also showed faster protein release. For particles fabricated from 24 kD and 59 kD PLGA, the change in the gradient of the acid evolution profile appears to coincide with the upturn in the protein release profile.

### 2.3.9 Calcium Release from Composite Microparticles

Subsequent to polymer degradation and acid release, apatite particles that were embedded in the polymer matrix would dissolve to produce calcium and phosphate ions. The
release of calcium ions was detected with a calcium-sensitive fluorophore (Rhod-5N). A caveat to the use of this fluorophore was its sensitivity to the pH and salt concentration of the medium. Hence, even with a calibration curve, only estimations of the calcium concentrations could be obtained. Nevertheless, the fluorophore was useful in confirming the presence of calcium and the relative change in its concentration with time. The results for blank CAP-PLGA microparticles synthesized with 13 kD and 59 kD PLGA are shown in Fig. 2.18. Comparisons were made with 59 kD BSA-CAP-PLGA microparticles and with bare CAP.

![Graph showing calcium release from microparticles](image)

**Fig. 2.18.** Calcium release from microparticles prepared with (*) CAP-13 kD PLGA, (×) CAP-59 kD PLGA, (▲) BSA-CAP-59 kD PLGA, and (■) bare CAP. The CAP loading in the composite particles was 16.67 w/w%; an equivalent amount of bare CAP was employed in ■. Data shown are mean values for n = 2.

For the 59 kD CAP-PLGA microparticles, the calcium release was gradual and bore similarity to the protein release profile (Fig. 2.9a). The presence of BSA on CAP did not appear to affect the calcium dissolution rate. However, it was unclear if the increased magnitude of calcium release was due to a real enhancement in the presence of BSA or a change in Rhod-5N fluorescence in the presence of BSA.

The 13 kD CAP-PLGA microparticles showed a sharp rise in calcium dissolution in the first week, followed by a plateau. This resembled its protein release profile (Fig. 2.9a). Although acid continued to be released past the first week (see Fig. 2.17), there appeared to be
little calcium or protein release. An unusual observation was the fall in the cumulative
calcium release profile after 7 days, which suggested that calcium was re-precipitated out of
the release medium, possibly due to interactions with the polymer’s anionic groups or due to
pH shifts. The calcium indicator was able to confirm enhanced release of calcium from the
composite microparticles over the passive leaching of calcium ions from bare CAP.

Comparisons amongst the acid, calcium and protein release profiles of the composite
microparticles confirmed that as the apatite particles were dissolved by the acid from polymer
degradation, proteins were released into the medium.

2.4 Summary

Composite microparticles of a biodegradable polymer (PLGA) and a basic inorganic
substrate (apatite) were developed as protein delivery vehicles. Controlled release was
accomplished through the interplay of polymer degradation to form acidic products and
dissolution of the basic apatitic phase. Consequently, protein that was pre-adsorbed onto
apatite was released.

The uniqueness of the controlled release strategy lies not only in the material
composition, but also in the way the individual components were combined. A key step was
the adsorption of protein onto apatite, thereby preventing its premature release until the
underlying apatitic substrate was dissolved or the protein-apatite interactions were weakened.
As discussed in Section 2.3.3, BSA-apatite interactions were sufficiently strong to frustrate
protein release for more than a year. Without such interactions to retain the proteins,
diffusion out of the carrier particles could be rapid and result in an initial burst, as was the
case for polymeric microparticles prepared by the W/O/W technique (Fig. 2.16). Therefore,
an essential criterion for the use of this release strategy was the adsorption of the therapeutic
agent onto apatite. The high affinity of apatite for proteins of different sizes and IEPs was
demonstrated in Section 2.3.2. An exception, however, was lysozyme. Hence, it is important
to verify the affinity of apatite for the therapeutic protein to be delivered.

The premise that acids from polymer degradation would dissolve the apatitic phase
and promote protein release was supported by the study of the effect of pH on BSA-apatite
complexes (Section 2.3.3). A reduction in pH to 5 caused a substantial increase in BSA
release. However, further pH reductions had the opposite effect, which could be attributed to
the stronger electronic interactions between BSA and apatite as BSA became progressively more positively charged below its IEP (4.7), or to the precipitation of BSA. The augmentation in Ca release when CAP or BSA-CAP was encapsulated in PLGA was also evidence that acid evolution from PLGA degradation accelerated apatite dissolution (Section 2.3.9).

Protein release from these apatite-polymer composite microparticles could be modified by parameters that influence polymer degradation and/or apatite dissolution. Amongst the factors studied, those that affect polymer degradation include polymer molecular weight and polymer hydrophobicity, and those that affect apatite dissolution include apatite loading and apatite particle size. Polymer molecular weight was found to determine the onset of the accelerated phase of release; the use of a low molecular weight polymer led to an immediate upturn in the release profile (burst release). An increase in polymer hydrophobicity through the addition of PLA was responsible for slowing aggregate polymer degradation and consequently, reducing the protein release rate. When a non-degradable, hydrophilic polymer, PEG, was blended into the particles, the magnitude of protein release increased whereas the release rate remained approximately the same. PEG encouraged water penetration throughout the composite particles, enabling degradation of a larger proportion of PLGA without changing the polymer degradation rate significantly.

Apatite loading in the composite particles was altered by adding bare apatite in addition to the protein-apatite complex or by adding different proportions of the protein-apatite complex. In both cases, increasing the apatitic content of the composite particles meant that more acid had to be produced for apatite dissolution to provide for protein release. The alleviation of acidity by apatite would also lessen the autocatalytic effect of acid on polymer hydrolysis, further reducing the amount of acidic degradation products formed. The result was a decrease in protein release rate.

Apatite particle size was observed to have a significant effect on protein release. The use of sub-micron-sized apatite particles enhanced low-burst protein release that was zero-order for the first 4 weeks. The enhancement of protein release could be attributed to a surface effect: smaller apatite particles would sequester a larger proportion of proteins on their surface than within the pores of the apatite; proteins on the surface would be more readily released when apatite was eroded from the surface. Zero-order release would be encouraged
by a constant rate of acid production and apatite dissolution, as well as homogeneity of protein distribution on apatite. In the experiment, the same polymer (59 kD PLGA) was used to encapsulate protein-apatite complexes formed from either sCAP (1 μm) or CAP (7 μm). The degradation rate of 59 kD PLGA was found to be relatively constant over 4 weeks (Fig. 2.17). The dissolution rates of sCAP and CAP might differ, but it would be reasonable to assume that each apatite would dissolve by a fixed amount in response to a given amount of acid (preliminary experimental data not shown). Thus, protein distribution on apatite seemed to be the factor driving zero-order release in this case. Protein concentration likely decreased sharply from the surface to the interior of the particle for the protein-apatite complex prepared from CAP. Uniform protein distribution might be promoted by using very small (nanometer-sized) and non-porous apatite particles, or large, highly porous apatite particles with large pores.

With an understanding of the modulation of protein release by the above factors, we can attempt to design protein release profiles by selecting specific parameters. For example, to attain low burst, zero-order and sustained release of reasonable magnitude that might be suitable for BMP delivery, the following combination could be used: high molecular weight polymer (e.g. 59 kD PLGA) of intermediate hydrophobicity (little or no PLA added) encapsulating a protein-sCAP complex with no added bare apatite. An intermediate level of apatite loading could be used, e.g. 0.08 mg of apatite per mg of polymer. To increase the magnitude of release, PEG could be blended in, whereas to alter the rate of release, PLA or bare apatite could be introduced in the appropriate amounts. The next chapter describes the application of composite particles of selected formulations to the delivery of recombinant human BMP-2.

### 2.5 References


[35] Yong TH, Ying JY. Apatite-polymer composite microparticles for the controlled delivery of bone morphogenetic proteins. To be submitted to Biomaterials.


Chapter 3 – Application of Apatite-Polymer Composite Microparticles to the Controlled Delivery of BMPs

3.1 Introduction

In the previous chapter, the potential of apatite-PLGA composite microparticles for protein delivery was established. The goal of this chapter is to explore the application of these composite microparticles to the delivery of bone morphogenetic proteins (BMPs), specifically, BMP-2. Primary criteria in the delivery of therapeutic proteins include (1) safety and biocompatibility of the carriers, and (2) preservation of biological activity (bioactivity) of the therapeutic protein. When these criteria are met, tunability of release from the carriers would provide added versatility and efficacy over conventional carriers, such as collagen sponges.

3.1.1 Osteoinductive Effect of BMPs

As their name implies, BMPs promote bone formation by inducing other cell types to differentiate into bone cells (osteoblasts) that deposit the matrix material constituting bony tissue [1-7]. BMPs begin their signaling cascade by binding to type I and type II transmembrane serine/threonine kinase receptors. The resulting ligand/receptor complex phosphorylates intracellular signaling proteins, Smad 1, 5 and 8. The activated Smad proteins heterodimerize with Smad 4, and translocate into the nucleus where they regulate gene expression associated with osteogenesis [1, 7-11]. Genes that are increased in expression include those that code for osteocalcin, type I collagen, bone sialoprotein and alkaline phosphatase (ALP). These proteins are typically expressed by osteoblasts during matrix deposition and mineralization, and are considered markers of the osteoblastic phenotype. In particular, ALP and osteocalcin were chosen as markers in our studies. ALP is a glycosylated enzyme attached on the surfaces of osteoblasts. It is able to cleave phosphates off proteins, and is involved in the regulation of extracellular phosphate concentration. An increase in ALP activity is associated with the onset of mineralization [11, 12]. Osteocalcin is a small gamma-carboxylated protein that binds to apatite with high affinity. It is produced by osteoblasts during mineralization, and hence, appears at a later stage than ALP. Osteocalcin is believed to be a regulator of bone formation and mineral maturation; its absence in knockout mice has been linked to excessive calcification [12].
BMP-induced osteoblastic differentiation of uncommitted mesenchymal stem cells and osteoprogenitor cells has been widely demonstrated [6, 13-21]. To assess the bioactivity of BMPs, we selected a pluripotent murine embryonic fibroblast cell line, C3H10T1/2, which can be induced to differentiate along osteoblast, chondrocyte, adipocyte or myoblast lineages, depending on the signals provided [16, 18, 20]. BMPs promote the development of the osteoblastic phenotype in these cells, which can be assessed by an increased expression of ALP and osteocalcin.

3.2 Experimental

3.2.1 Adsorption of BMPs onto Apatite

Recombinant human bone morphogenetic proteins (rhBMPs) were purchased from R&D Systems. The capacity of apatite for adsorbing BMPs was tested using three members of the BMP family, rhBMP-2, rhBMP-4 and rhBMP-6. Each rhBMP was added to 5 mg of carbonated apatite (CAP) in the amounts of 0, 2.5, 5.0 or 10.0 μg in 1 ml of deionized (DI) water. Duplicates of each combination were tested. The suspensions were stirred at room temperature for 8 h, and then centrifuged. The supernatant was collected and filtered. The amount of rhBMP adsorbed was determined by measuring the difference in concentration between the original stock solution and the supernatant. RhBMP concentration was evaluated with a sandwich enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) specific to each rhBMP. In general, the assay consisted of adding 50 μl of standards and test samples in duplicates to a 96-well plate coated with anti-BMP monoclonal antibody. The plate was incubated at room temperature for 2 h and washed. Biotinylated anti-BMP antibody was then added, and the plate was further incubated for 2 h. Unbound antibody was removed by washing, following which streptavidin-horse radish peroxidase was introduced. After 30 min, color was developed by the addition of hydrogen peroxide and tetramethylbenzidine. Absorbance was read at 450 nm using a UV-Vis microplate reader (VersaMax, Molecular Devices) with wavelength correction at 570 nm.

Only small quantities of rhBMPs were tested for adsorption onto apatite as their potency suggested that only tiny amounts were needed to achieve efficacy in subsequent experiments. For example, the ED_{50} of rhBMP-2 for the induction of ALP activity in ATDC5 chondrogenic cells is 100-300 ng/ml according to the manufacturer, R&D Systems. (ED_{50} or
effective dose 50 is the amount required to achieve a specific effect in 50% of the cell or animal population.) The high cost of these cytokines (~ US$40/μg) was also taken into consideration in the planning of the experiments so as to maximize utility and conservation.

For the preparation of rhBMP-apatite complexes for encapsulation in composite microparticles, a protein mixture of 1.4 w/w% rhBMP and 98.6 w/w% BSA was used. As the amount of rhBMPs required was small, the intention was for BSA to cap some of the adsorption sites, especially in the pores of the apatite, so that rhBMPs, which are less than one-third the size of BSA, would not tunnel too deep into the apatite particle and result in delayed release. Adsorption was conducted under aseptic conditions in a biosafety cabinet. Sterile, cell culture grade water (Sigma) was used to prepare the protein solutions, which were subsequently sterile-filtered. The apatite was either sterilized with 70% ethanol and lyophilized, or sterilized by UV irradiation. A typical adsorption consisted of adding 40 μg of rhBMP-2 and 2800 μg of BSA to 20 mg of CAP in 10 ml of water. After adsorption for 8 h, the suspension was lyophilized. Washing of the rhBMP-apatite complex to remove any free protein was not performed in order to minimize loss of these expensive proteins. Although the unadsorbed proteins may lead to an initial burst during release, some studies have shown that an initial burst of BMP may be desirable in jump-starting osteoinduction by chemotactically attracting cells to the wound site. A sustained release of BMPs is then required to differentiate the cells into osteoblasts, and to maintain their phenotype for bone deposition [22].

3.2.2 Aseptic Preparation of rhBMP-2-Loaded Composite Microparticles

Of the rhBMPs tested, rhBMP-2 has been more extensively studied in the literature, and has been reported to have higher potency [1, 6, 8]. Hence, it was used in the majority of the formulations. Composite microparticles encapsulating rhBMP-2 were synthesized under aseptic conditions by a solid-in-oil-in-water (S/O/W) emulsion process similar to that described in Section 2.2.5 [23]. A typical synthesis involved dissolving 250 mg of PLGA (Alkermes) in 2 ml of dichloromethane. The polymer solution was sterile-filtered through a 0.45-μm Teflon membrane into a vial containing the rhBMP-2-apatite complex. The mixture was vortexed to create a solid-in-oil suspension, which was then transferred to 50 ml of 0.1 w/v% methyl cellulose solution that had been autoclaved. A S/O/W suspension was formed.
by homogenizing at 8000 rpm for 2 min at room temperature. To solidify the composite particles, dichloromethane was evaporated by heating the suspension with stirring at 30°C for 3 h. The particles were collected by centrifugation, washed with sterile water, and freeze dried.

3.2.3 Estimation of the Magnitude of Initial Burst from Composite Microparticles

BMPs that were not adsorbed onto apatite were expected to contribute to burst release when the composite microparticles were introduced to an aqueous medium. To estimate the amount of free rhBMP-2 in the microparticles, we used a similar procedure as that described in Section 2.2.8 [23] for determining the protein encapsulation efficiency of composite microparticles. The polymeric phase of the composite particles was dissolved with 0.5 ml of dichloromethane per 10 mg of particles. 2 ml of DI water were added to the organic suspension to extract free protein. The mixture was vortexed periodically over 6 h to facilitate extraction, and then centrifuged. Protein associated with apatite settled as a residue, whereas free protein was dissolved in the supernatant. The rhBMP-2 concentration of the filtered supernatant was evaluated by ELISA, and was used to calculate the amount of free rhBMP-2 per mg of composite particles. Each set of particles was tested in duplicates.

RhBMP-2 encapsulation efficiency of the composite particles was not determined as preliminary experiments suggested that the low pH (pH 2) of the citrate buffer used to dissolve the apatitic phase led to denaturation/deactivation of the rhBMP-2 molecules and very poor detection by ELISA.

3.2.4 Evaluation of In Vitro Release

Release studies were conducted in Eagle’s basal medium (BME; Sigma) supplemented with 10 v/v% heat-inactivated fetal bovine serum (Invitrogen) and 1 v/v% antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin; ATCC). This medium will be referred to as ‘complete BME’. Each set of rhBMP-2-loaded composite microparticles was suspended at a concentration of 20 mg/ml in complete BME in 2 sample vials, and incubated at 37°C. At pre-determined time intervals (1, 4, 7, 10, 14, 18, 22, 26, 30 days, up to 100 days), the vials were centrifuged, and the supernatant was withdrawn completely and replaced with the same volume of fresh medium. The collected supernatant was filtered and stored at -20°C until
evaluation by an rhBMP-2 ELISA kit. The concentration of the protein released at each time point was used to construct cumulative release profiles.

In addition to complete BME, serum-free BME and BES buffer, both of pH 7.4, were tested as release media.

3.2.5 Cellular Response

A murine pluripotent embryonic fibroblast cell line, C3H10T1/2, was obtained from ATCC. The cell line was maintained in complete BME. Cells were expanded in T-75 flasks at a density of 2000 cells/cm² with two medium renewals per week. Subculturing was performed before cells reached confluence due to the sensitivity of these cells to contact inhibition of cell division. 0.25% trypsin/0.53 mM EDTA (ATCC) was used to dissociate cells, which were then plated onto 24-well plates for in vitro experiments. Cells from the 11th to 15th passages were used.

3.2.5.1 Effect of Release Medium Collected at Each Time Point

Different sets of rhBMP-2-loaded composite microparticles were incubated at 37°C in complete BME at a concentration of 20 mg/ml. Blank composite particles prepared under the same processing and material parameters but without rhBMP-2 served as the controls. The medium was renewed every 3-4 days as described in Section 3.2.4. The release medium collected at each time point was stored at -20°C until incubation with cells. As a positive reference, Helistat® collagen sponges (Integra Life Sciences) were soaked with rhBMP-2 solution for 10–15 min based on protocols in clinical use [24, 25]. Sponges weighing ~ 7 mg apiece were cut and were each loaded with 2.0 μg of rhBMP-2. Loaded and blank (control) sponges were then transferred into 2 ml of complete BME per sponge for release studies.

ALP activity was determined by a modification of the protocol of Lowry et al. [26]. C3H10T1/2 cells were seeded on 24-well plates at a density of 6000 cells/cm² (~ 12,000 cells/well). The cells were allowed to adhere overnight for 16 h. The medium was aspirated and replaced with 0.5 ml per well of release medium from a specific time point. For each time point, 5 wells were prepared. In addition, complete BME enriched with 0–2 μg/ml of rhBMP-2 was incubated with cells. For each rhBMP-2 concentration, 5 wells of cells were
tested, each containing 0.5 ml of enriched BME. This experiment was intended to establish the bioactivity of rhBMP-2, as well as to evaluate dose-dependent cellular response.

After 4 days of culture, the cells were rinsed twice with phosphate buffered saline (Gibco), and the plates were blotted dry. The cells were lysed according to the protocol provided by R&D Systems. 100 µl of 0.1% Triton X-100 solution (Sigma) containing 150 mM of NaCl and 3 mM of NaHCO₃ (pH 9.3) were added to each well. The plates were incubated at 37°C for 30 min. 25 µl of the cell lysate were assayed in duplicate for ALP activity using 100 µl of p-nitrophenyl phosphate substrate solution (pNPP; Sigma) in 96-well plates. The absorbance at 405 nm was read every 5 min for 30 min at 37°C. ALP cleaves the phosphate off pNPP to form p-nitrophenol. Using Beer's Law and an extinction coefficient of 18.45 cm·L/mmol for p-nitrophenol, the amount of p-nitrophenol (in nmol) formed per minute was determined. ALP activity was normalized by the protein concentration of the lysate as determined by total protein assay (Pierce) and expressed as nmol/min·mg protein. ALP activity induced by the medium collected at each time point was plotted as a function of time.

3.2.5.2 Effect of Prolonged Exposure to Release Medium

Based on the results from in vitro release and induced ALP activity at each time point, a set of composite microparticles with a sustained release profile of bioactive rhBMP-2 was chosen. This set of composite particles was constructed of 59 kD PLGA and 0.08 mg of sCAP per mg of PLGA. RhBMP-2 loading in the particles was 145 ng per mg carrier. The effect of prolonged exposure of C3H10T1/2 cells to release medium from this set of particles was studied. As before, the composite microparticles were incubated at 37°C in complete BME at a concentration of 20 mg/ml. C3H10T1/2 cells were seeded in 24-well plates at a density of 6000 cells/cm². Twice per week, the medium in the wells was aspirated and replaced with 0.5 ml of filtered release medium collected from the particles. Release medium from blank composite particles served as the control. For comparison, complete BME systems enriched with fixed concentrations of rhBMP-2 (10 and 100 ng/ml; 0.5 ml per well) were used. Cell culture was conducted for 8 weeks in all groups.

At 1, 2, 3, 4, 6 and 8 weeks, cells in 4 wells for each group were lysed, and the ALP activity of the cell lysate was measured. Each week, the osteocalcin level of the conditioned
medium of 4 wells in each group was assayed by sandwich ELISA (Biomedical Technologies, Inc.). The procedure for this ELISA was similar to that of the rhBMP-2 assay.

Cell proliferation was evaluated for C3H10T1/2 cells exposed to fixed concentrations of rhBMP-2 (10 or 100 ng/ml), or to release medium collected from blank or rhBMP-2-loaded composite microparticles. Cell counting was performed using the CyQUANT® assay (Molecular Probes). At one week intervals and for each group, 4 wells of cells were detached with 0.25% trypsin and centrifuged. The cell pellets were lysed by freeze-thaw cycles in a lysis buffer, and digested with RNase (1.35 Kunitz units/ml; Sigma) for 1 h at room temperature to reduce interference from RNA. Green fluorescent CyQUANT® DNA binding dye was then added, and the fluorescence was read at 485/538 nm (ex/em) using a microplate reader (fMax, Molecular Devices). DNA and cell number standard curves were generated to quantify the amount of DNA (and associated fluorescence) per cell. This value (~ 6.5 pg of DNA per cell) subsequently allowed for the determination of the number of cells in each test sample.

3.2.5.3 Statistical Analysis

Statistical analysis was conducted using analysis of variance (ANOVA) with respect to treatment or time, followed by Tukey-Kramer HSD post hoc test for multiple comparisons (JMP v.5.0.1). Data were plotted as mean ± standard deviation. A significance level of p < 0.05 was used.

3.3 Results and Discussion

3.3.1 Adsorption of rhBMPs onto Apatite

The affinity of CAP for rhBMP-2, rhBMP-4 and rhBMP-6 was found to be high. For all BMP loadings tested (2.5, 5.0 and 10.0 µg of BMP per 5 mg of CAP), the amount of BMP remaining in the supernatant after adsorption was negligible (Table 3.1). The co-adsorption of 10 µg of BMP and 700 µg of FITC-BSA onto 5 mg of CAP, which constituted a total protein loading of 12.4 w/w% on CAP, also led to the complete adsorption of both BMP and FITC-BSA.
Table 3.1. Adsorption of rhBMPs onto 5 mg of CAP.

<table>
<thead>
<tr>
<th>BMP Member</th>
<th>Amount of Protein Added (µg)</th>
<th>Amount of BMP in Supernatant (ng)</th>
<th>Amount of BMP Adsorbed (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rhBMP-2</td>
<td>2.5</td>
<td>0.0</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>0.0</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>0.0</td>
<td>10.0</td>
</tr>
<tr>
<td>rhBMP-6</td>
<td>2.5</td>
<td>0.0</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>0.0</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>0.9</td>
<td>10.0</td>
</tr>
<tr>
<td>rhBMP-6 (+ BSA)</td>
<td>10.0 (+700.0)</td>
<td>25.4</td>
<td>9.97</td>
</tr>
</tbody>
</table>

3.3.2 Estimation of Proportion of Unbound rhBMPs in Composite Microparticles

The percentage of rhBMP-2 that was not bound to apatite, and hence, extractable by water, was found to be 0.06–0.58 w/w% of the theoretical rhBMP-2 loading in the composite microparticles. (The actual rhBMP-2 loading was not determined because the low pH required for dissolving the apatitic phase led to denaturation of rhBMP-2, as discussed in Section 3.2.3.) We surmised that the unbound rhBMP-2 contributed to burst release. Although seemingly small, this proportion of rhBMP-2 was significant when compared to the actual amount of growth factor released. Depending on the particle formulation, composite microparticles released between 2.5 w/w% and 15 w/w% of the theoretical BMP loading over 4 weeks (Section 3.3.3). Therefore, an initial burst was expected to be discernible in the release profiles of rhBMP-2 from the composite microparticles. In contrast, BSA-loaded composite particles described in Chapter 2 did not exhibit significant burst release because the BSA-apatite complexes were washed repeatedly to remove unbound BSA before polymer encapsulation.

3.3.3 Effect of Material and Processing Parameters on the In Vitro Release of rhBMP-2 from Composite Microparticles

Based on the in vitro release results reported in Chapter 2, a composite particle formulation of protein-sCAP-59 kD PLGA was hypothesized to be suitable for BMP delivery. However, since BMP-2 is a smaller molecule (26 kD) and has a very different IEP (~9) from BSA, it is reasonable to expect differences in the release profiles of the two proteins. Hence, we verified the effect on rhBMP-2 release of some of the parameters that were described in Section 2.3.6.
3.3.3.1 Polymer Molecular Weight

Polymers of lower molecular weight degraded more quickly, and hence, enhanced the release of proteins from composite microparticles. This observation, which was discussed in Section 2.3.6.1 for composite particles encapsulating BSA [23], was further verified with particles loaded with rhBMP-2 (see Fig. 3.1). By using an equi-portion blend of PLGA of different molecular weights, the production of acidic degradation products was equalized over time, leading to a more gradual and sustained release of rhBMP-2.

![Graph showing effect of PLGA molecular weight on rhBMP-2 release from composite microparticles. CAP loading = 0.08 mg/mg of polymer; rhBMP-2 loading = 65 ng/mg of carrier. Data shown are mean values for n = 2.](image)

Fig. 3.1. Effect of PLGA molecular weight on rhBMP-2 release from composite microparticles. CAP loading = 0.08 mg/mg of polymer; rhBMP-2 loading = 65 ng/mg of carrier. Data shown are mean values for n = 2.

3.3.3.2 Apatite Particle Size

RhbMP-2 was adsorbed onto sCAP (1 μm) and CAP (7 μm). The rhBMP-2-apatite complexes formed were subsequently incorporated into separate sets of composite microparticles. Apatite particle size was found to have a pronounced effect on rhBMP-2 release, similar to that on the release of BSA (Section 2.3.6.3) [23]. The smaller apatite particles presented a larger proportion of rhBMP-2 on their surface than within their pores due to their higher surface/volume ratio. As the apatitic surface was eroded by acidic degradation products, sCAP released larger amounts of rhBMP-2 than CAP, accounting for the difference in release profiles shown in Fig. 3.2.
Fig. 3.2. Release of rhBMP-2 from composite particles fabricated from 59 kD PLGA, and (●) 1-μm sCAP and (■) 7-μm CAP. Apatite loading = 0.08 mg/mg of polymer; rhBMP-2 loading = 65 ng/mg of carrier. Data shown are mean values for n = 2.

3.3.3.3 Apatite Loading

When the amount of rhBMP-2 was held constant while the loading of sCAP in the composite microparticles was increased from 18 to 45 mg per 250 mg of polymer, rhBMP-2 release was found to decrease (Fig. 3.3). This result was in agreement with the observations reported in Section 2.3.6.4 for BSA release [23]. For the release of a fixed amount of protein from apatite, a greater extent of polymer degradation would be needed to dissolve a higher apatite loading. In addition, the apatite served as a buffer, mitigating the acidity within the composite particles, and diminishing the autocatalytic effect of pH on polymer hydrolysis. Hence, the apatite served towards dampening the protein release.
Fig 3.3. Effect of sCAP loading (per 250 mg of polymer) on rhBMP-2 release. Composite particles were fabricated from 59 kD PLGA, and contained 65 ng of rhBMP-2/mg of carrier. Data shown are mean values for n = 2.

**3.3.3.4 Polymer Hydrophobicity**

Increasing polymer hydrophobicity by the addition of PLA led to a surprising observation: rhBMP-2 release was enhanced with increasing polymer hydrophobicity (Fig. 3.4), contrary to the results for BSA release discussed in Section 2.3.6.2.1 [23]. Polymer hydrophobicity was expected to reduce water penetration and polymer degradation, and hence, decrease protein release. The unexpected result could be explained by considering the release of structurally intact, biologically active protein versus the total release of both intact and degraded protein. The determination of rhBMP-2 concentration by ELISA was highly specific; rhBMP-2 molecules had to be of the appropriate conformation for binding to antibodies. Denatured rhBMP-2 molecules were not detected, as confirmed by our previous experience with evaluating the encapsulation efficiency of composite particles whereby rhBMP-2 was extracted in citrate buffers of low pH (Section 3.2.3) – the low pH led to rhBMP-2 denaturation and poor detection by ELISA. Therefore, higher polymer hydrophobicity could have led to a less aggressive pH environment within the composite microparticles, contributing to less protein denaturation and a greater release of bioactive rhBMP-2.
3.3.3.5 Release Medium

The medium in which composite particles were incubated had a significant impact on rhBMP-2 release (Fig. 3.5). BES buffer, which was low in ionic strength and contained no proteins or amino acids, showed the highest release. Release was considerably diminished in complete BME, which contained a host of amino acids, serum proteins, salts, antibiotics and other supplements. The variation in rhBMP-2 release might arise from differences in polymer degradation, apatite dissolution and/or protein deactivation in the different media. To test the first of these possibilities, degradation of the polymeric component of composite microparticles in BES buffer and complete BME was tracked by GPC, and found to be similar (Fig. 3.6). Differences in apatite dissolution were not examined due to the difficulty in monitoring calcium release in Ca-rich cell culture medium. The possibility of enzymatic degradation of rhBMP-2 by serum proteases contained in complete BME was checked by comparing rhBMP-2 release in serum-free BME. In the latter, rhBMP-2 release was further suppressed (see Figure 3.5), suggesting that other factors, such as protein precipitation due to high ionic strength, were at work. This result highlighted the difficulty in extrapolating in vitro data for in vivo applications since the in vivo milieu would be even more complex. All
Release studies discussed in this chapter were performed in complete BME unless otherwise specified.

**Fig. 3.5.** RhBMP-2 release in (■) BES buffer, (*) complete BME, and (●) serum-free BME, all at pH 7.4. Composite microparticles were fabricated from 59 kD PLGA. sCAP loading = 0.08 mg/mg of polymer; rhBMP-2 loading = 65 ng/mg of carrier. Data shown are mean values for n = 2.

**Fig. 3.6.** Change in PLGA molecular weight with time in (●) BES buffer and (■) complete BME, both at pH 7.4. Composite microparticles were fabricated from 59 kD PLGA. sCAP loading = 0.08 mg/mg of polymer.
3.3.4 Effect of Conditioned Medium Collected at Each Time Point

The induction of ALP activity in C3H10T1/2 cells by different concentrations of rhBMP-2 over 4 days was examined. Increasing the rhBMP-2 concentration from 0 ng/well to 200 ng/well (400 ng/ml) was found to elevate ALP activity (Fig. 3.7). The effect reached saturation at a rhBMP-2 concentration of 200 ng/well. Thus, there appeared to be an optimum dosage of rhBMP-2 for evoking ALP expression, which was related to the commitment of undifferentiated cells to the osteoblast lineage.

![Graph of Fig. 3.7](image)

Fig. 3.7. Effect of rhBMP-2 concentration on induced ALP activity in C3H10T1/2 cells. Each well contained 0.5 ml of medium; 50 ng/well is equivalent to 100 ng/ml of rhBMP-2. Asterisks denote statistically significant differences of \( p < 0.05 \) compared to the control (0 ng of rhBMP-2/well). Data shown are mean ± standard deviation (SD) for \( n = 5 \).

Levels of ALP activity induced by release media collected from rhBMP-2-loaded composite microparticles are illustrated in Figs. 3.8–3.10. Cumulative release is given in units of ‘ng/well’ to reflect the actual amounts to which the cells were exposed, and to facilitate comparison with Fig. 3.7.

Fig. 3.8 shows that the release media collected over a period of 100 days were able to induce elevated ALP expression in C3H10T1/2 cells. These encouraging results suggested that the released rhBMP-2 retained at least part of its biological activity, and that the release of bioactive rhBMP-2 over extended periods of time was possible. The experiment was halted at 100 days, but this set of composite microparticles could conceivably release
bioactive rhBMP-2 for even longer periods of time since the cumulative release and ALP inductive capacity of rhBMP-2 appeared to be experiencing an upturn at 100 days (Fig. 3.8).

![Graph showing ALP activity and release levels over time](image)

Fig. 3.8. ALP activity induced by release medium collected at each time point from rhBMP-2-loaded composite microparticles fabricated from 59 kD PLGA. sCAP loading = 0.08 mg/mg of PLGA; rhBMP-2 loading = 145 ng/mg of carrier. Asterisks denote statistically significant differences of p < 0.05 compared to control (blank composite particles) at each time point. Data shown are mean ± SD for n = 5.

When a higher apatite loading was used in the composite microparticles, rhBMP-2 release was dampened, and the capacity for ALP induction declined within the first week (Fig. 3.9). In contrast, when rhBMP-2 release was augmented and prolonged by using a 3:2 blend of PLGA and PLA in the composite microparticles, ALP expression was significantly elevated throughout the course of the study (Fig. 3.10).
Fig. 3.9. ALP activity induced by release medium collected at each time point from rhBMP-2-loaded composite microparticles fabricated from 59 kD PLGA. sCAP loading = 0.18 mg/mg PLGA; rhBMP-2 loading = 145 ng/mg of carrier. Asterisks denote statistically significant differences of p < 0.05 compared to control (blank composite particles) at each time point. Data shown are mean ± SD for n = 5.

Fig. 3.10. ALP activity induced by release medium collected at each time point from rhBMP-2-loaded composite microparticles fabricated from a 3:2 blend of 59 kD PLGA and 25 kD PLA. sCAP loading = 0.08 mg/mg of polymer; rhBMP-2 loading = 145 ng/mg of carrier. Asterisks denote statistically significant differences of p < 0.05 compared to control (blank composite particles) at each time point. Data shown are mean ± SD for n = 5.
Fig. 3.11. ALP activity induced by release medium collected at each time point from rhBMP-2-loaded Helistat sponges. rhBMP-2 loading = 286 ng/mg of carrier. Asterisks denote statistically significant differences of p < 0.05 compared to control (empty sponge) at each time point. Data shown are mean ± SD for n = 5.

However, the induction of ALP activity was not a simple function of the amount of rhBMP-2 released. This was made apparent by our experiments with Helistat® collagen sponges, which were the conventional carriers of BMPs used in spinal fusions. RhBMP-2 release from Helistat® sponges (Fig. 3.11) was similar to that from the composite microparticles in Fig. 3.10. Nevertheless, the ALP expression level was lower in the former, and tapered off after 2 weeks despite the continued release of rhBMP-2. The cause of the lower bioactivity of rhBMP-2 released from these sponges was unclear. The experiment was repeated to validate these results; the same drop-off in ALP activity was observed in the second trial as well. In comparison, the set of composite microparticles formulated from a 3:2 blend of PLGA and PLA (Fig. 3.10) appeared to have remarkable bioactivity, and should hold promise for future in vitro and in vivo investigations.

The higher bioactivity of rhBMP-2 released from composite microparticles was possibly due to synergy between rhBMP-2 and the calcium and phosphate ions released from apatite dissolution. These ions alone were not sufficient to increase ALP activity, as inferred
from the levels induced by release media from blank composite particles. However, calcium and phosphate might have boosted the activity of rhBMP-2 by priming the cells for osteoblastic differentiation. High extracellular calcium levels have been reported to stimulate osteoblastic proliferation and differentiation through mediation by calcium-sensing receptors (CaRs) [27, 28].

3.3.5 Effect of Prolonged Exposure to Conditioned Medium

3.3.5.1 Measurement of Induced Alkaline Phosphatase Activity

Compared against controls with no exposure to rhBMP-2, prolonged exposure of C3H10T1/2 cells to the growth factor resulted in elevated levels of ALP expression (Fig. 3.12). However, the increase was not monotonic; ALP activity reached a maximum at ~ 2 weeks, and then dipped to levels that were maintained over the remainder of the experiment. Similar profiles were reported by Shea et al., who studied the effect of BMP-7 on C3H10T1/2 cells, and observed a peak in ALP activity at 8 days [18]. In general, ALP expression would rise with increasing osteoblast differentiation from stem cells to osteoprogenitors to preosteoblasts and finally to osteoblasts. However, ALP activity would then decrease with the progression of mineralization [29]. The culture of C3H10T1/2 cells with release media collected from rhBMP-2-loaded composite microparticles produced a similar effect. ALP activity was considerably raised in the first 2 weeks, and then slowly declined to levels comparable to the control at the end of 8 weeks (Fig. 3.13). Fig. 3.12 also shows a dose-dependent response to rhBMP-2; 50 ng of rhBMP-2/well induced higher levels of ALP activity than 5 ng/well.
Fig. 3.12. Effect of length of exposure to rhBMP-2 on ALP activity. RhBMP-2 concentrations of 0 (control), 5 and 50 ng/well were used. Asterisks denote statistically significant differences of p < 0.05 compared to control at each time point. Data shown are mean ± SD for n = 4.

Fig. 3.13. Effect on ALP activity of prolonged exposure to release media collected from rhBMP-2-loaded composite microparticles fabricated from 59 kD PLGA. sCAP loading = 0.08 mg/mg of PLGA; rhBMP-2 loading = 145 ng/mg of carrier. Asterisks denote statistically significant differences of p < 0.05 compared to control (blank particles) at each time point. Data shown are mean ± SD for n = 4.
3.3.5.2 Measurement of Osteocalcin Expression

Osteocalcin is a later stage marker of osteoblast differentiation associated with matrix maturation and mineralization [12]. RhBMP-2 was found to upregulate osteocalcin expression in C3H10T1/2 cells in a time- and dose-dependent manner (Fig. 3.14). Control cells with no exposure to rhBMP-2 showed low osteocalcin levels, particularly at the start of the experiment. 5 ng of rhBMP-2/well produced a weak response, which was statistically insignificant (p > 0.05) over the 8-week study. A more robust response was obtained with 50 ng of rhBMP-2/well; osteocalcin levels were doubled in the first 3 weeks, held steady for the next 4 weeks, and then peaked again at week 8.

Release media collected from rhBMP-2-loaded composite microparticles had a remarkable effect on osteocalcin upregulation. Osteocalcin levels increased steadily with time over 4 weeks, then showed an exponential rise from weeks 4 to 7 (Fig. 3.15). The level reached at week 7 was 10-fold that of the response to rhBMP-2-enriched BME (50 ng/well) shown in Fig. 3.14. These results suggested that the cells were experiencing a significant amount of mineralization activity, indicative of an osteoblastic phenotype.

![Fig. 3.14. Effect of length of exposure to rhBMP-2 on osteocalcin levels. RhBMP-2 concentrations of 0 (control), 5 and 50 ng/well were used. Asterisks denote statistically significant differences of p < 0.05 compared to control at each time point. Data shown are mean ± SD for n = 4.](image-url)
Fig. 3.15. Effect on osteocalcin levels of prolonged exposure to release media collected from rhBMP-2-loaded composite microparticles fabricated from 59 kD PLGA. sCAP loading = 0.08 mg/mg of PLGA; rhBMP-2 loading = 145 ng/mg of carrier. Asterisks denote statistically significant differences of p < 0.05 compared to control (blank particles) at each time point. Data shown are mean ± SD for n = 4.

3.3.5.3 Evaluation of Cell Proliferation

RhBMP-2 appeared to have a proliferative effect on C3H10T1/2 cells. In the first 4 weeks, treatment with 50 ng of rhBMP-2 per well led to cell numbers that surpassed those of treatment with 0 ng (control) and 5 ng of rhBMP-2 per well (Fig. 3.16). By week 5, however, cell numbers for all 3 groups reached similar levels and began to decline, suggesting that diffusion and spatial limitations of static cell culture were being manifested.

Release media collected from rhBMP-2-loaded composite microparticles also induced significant increases in cell number (Fig. 3.17), demonstrating the proliferative capacity of the released rhBMP-2. Puleo proposed that rhBMP-2 overrides the contact inhibition of C3H10T1/2 cells, allowing them to grow in multilayers necessary for in vitro mineralization [17]. He found that the withdrawal of rhBMP-2 led to a decrease in cell number, purportedly due to the loss of the ability to maintain cell multilayers.
Fig. 3.16. Effect of length of exposure to rhBMP-2 on cell number. RhBMP-2 concentrations of 0 (control), 5 and 50 ng/well were used. Asterisks denote statistically significant differences of $p < 0.05$ compared to control at each time point. Data shown are mean ± SD for $n = 4$.

Fig. 3.17. Effect on cell number of prolonged exposure to release media collected from rhBMP-2-loaded composite microparticles fabricated from 59 kD PLGA. sCAP loading = 0.08 mg/mg of PLGA; rhBMP-2 loading = 145 ng/mg of carrier. Asterisks denote statistically significant differences of $p < 0.05$ compared to blank particles at each time point. Data shown are mean ± SD for $n = 4$. 

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Fig. 3.17 further shows that between weeks 2 and 6, cell population was smaller for cultures using release media collected from blank composite particles than for cultures with no exposure to composite particles or rhBMP-2 (control). This difference in cell proliferation might be caused by composite particles promoting the adsorption or precipitation of proteins in the cell culture medium, rendering these proteins unavailable for utilization by cells. Another possibility was the lower pH caused by acidic PLGA degradation products being less conducive to cell proliferation. However, the fact that cell number did increase albeit slowly over 8 weeks suggested that the composite microparticles did not compromise cell viability. More importantly, the delivery of rhBMP-2 with these composite microparticles led to significant cell proliferation, overwhelming any negative impact the composite particles might have had alone. Hence, these particles, especially in combination with growth factors, were biocompatible, i.e. they were non-toxic and generated an appropriate cell response.

3.4 Summary

Apatite-PLGA composite microparticles were applied to the delivery of rhBMP-2. A comparison with the results in Section 2.3.6 [23] shows that despite the size and charge differences between rhBMP-2 and BSA, polymer molecular weight, apatite loading and apatite particle size had similar effects on the release of both proteins. However, an unexpected observation was the increase in rhBMP-2 release with increasing polymer hydrophobicity created by the addition of PLA (Fig. 3.4). The slower degradation of PLA would have contributed to less acid generation and less severe pH conditions within composite microparticles, which might have assuaged the denaturation of rhBMP-2 molecules. Therefore, while the total amount of rhBMP-2 released might be lower with the introduction of PLA, the amount of structurally intact rhBMP-2 released was higher. Herein lies the difference between rhBMP-2 detection by ELISA, which is sensitive to the conformation of the protein, and BSA detection by Coomassie total protein assay, which is sensitive to certain amino acid residues (primarily arginine) of proteins and does not discriminate readily between denatured and intact proteins. Hence, the Coomassie assay measured the total amount of protein (BSA) released, whereas ELISA measured the fraction of rhBMP-2 with sufficiently preserved conformations. Coomassie was not used to measure the total concentration of
rhBMP-2 released since the concentrations, on the order of ~ ng/ml, were below its detection limit (~ 0.5 μg/ml).

The bioactivity of rhBMP-2 released from composites microparticles was assessed by measuring the expression of osteoblastic markers, ALP and osteocalcin, in C3H10T1/2 murine pluripotent fibroblasts after a certain period of exposure to release medium collected from the composite particles. In the first set of experiments, cells were exposed for 4 days to release medium from a particular time point. This experimental design facilitated screening of different sets of particles and minimized the amount of composite particles needed. In the second set of experiments, cells were cultured for 8 weeks during which the culture medium was continually replenished with release medium collected from a selected set of particles with the formulation, rhBMP-2-sCAP-59 kD PLGA. In the first set of experiments, the elevation of ALP activity in response to release media implied that the released rhBMP-2 had retained at least part of its bioactivity. Furthermore, rhBMP-2 released after 70 days, and even at 100 days appeared to be active, demonstrating the potential of apatite-PLGA composite particles for releasing protein in the bioactive form over extended periods of time. The expression profiles of ALP and osteocalcin, which were tracked in the second experiment, were in agreement with those reported in the literature. ALP, an early stage marker, was found to peak at 2 weeks and then decline (Fig. 3.13), whereas osteocalcin, a later stage marker, was upregulated significantly after 4 weeks (Fig. 3.15).

A notable observation was the remarkable bioactivity of composite particles prepared with rhBMP-2-sCAP and a 3:2 blend of 59 kD PLGA and 25 kD PLA. Even slight levels of rhBMP-2 released from these particles were able to markedly increase ALP activity in C3H10T1/2 cells (Fig. 3.10). Hence, this particle formulation might be superior to the rhBMP-2-sCAP-59 kD PLGA formulation that was proposed before the unusual effect of PLA was discovered.

Helista® collagen sponges, which were used as a positive reference, were found to release rhBMP-2 with a higher initial burst than composite particles (16.7% vs. < 2.7%). Release media collected from these sponges after 2 weeks were not able to raise ALP activity above the control levels (Fig. 3.11). However, the same amount of rhBMP-2 contained in release media from composite particles had an inductive effect (Figs. 3.8 and 3.11). The higher bioactivity of composite particles might be ascribed to possible synergy between
rhBMP-2 and Ca^{2+}/PO_{4}^{3-} ions released from apatite dissolution. In the absence of rhBMP-2, these ions, as released from blank composite particles, did not exhibit an inductive effect. A synergistic effect of Ca^{2+}/PO_{4}^{3-} seemed reasonable since elevated calcium concentrations are present *in vivo* during the resorptive phase of bone remodeling and might act on calcium-sensing receptors to initiate steps for depositing bone. The Ca^{2+}/PO_{4}^{3-} ions might also have contributed to raising the levels of osteocalcin expressed by cells continually exposed to release media from composite particles (Fig. 3.14). The strikingly high levels, indicative of significant mineralization, were 6–12 times higher than the levels expressed by cells continually exposed to 50 ng of rhBMP-2 per well (100 ng/ml). Over 8 weeks, 50 ng of rhBMP-2 replenished twice a week added up to a cumulative exposure to 750 ng of rhBMP-2 per well of cells. In contrast, the total amount of rhBMP-2 released by the composite particles, and hence, the total amount that the cells were exposed to over 8 weeks was less than 40 ng per well (see Fig. 3.8). This served to highlight the remarkable bioactivity of the rhBMP-2-loaded composite particles.

### 3.5 References


Chapter 4 – Incorporation of Apatite-Polymer Composite Microparticles into Bulk Matrices

4.1 Introduction

Chapter 3 [1] demonstrated that apatite-polymer composite microparticles [2] are capable of extended release of bioactive rhBMP-2. Being on the order of tens of microns, these microparticles are small enough to be injected into bone fractures and cracks. To fill large bone defects, the particles have to be formed into bulk implants. One possibility is to compress the particles into the desired shape and size. However, the compaction would disrupt the microparticles, create low porosity (less than 20%), and alter the release profile of the therapeutic agent [3-5]. Laurencin et al. have sintered microparticles of 100–300 μm to form highly interconnected scaffolds with 30–40% porosity [6, 7]. These scaffolds are capable of supporting osteoblast attachment and proliferation throughout the pore system. One concern, however, is that the heat treatment may cause protein denaturation; their system, which did not contain proteins, was sintered at ~150°C.

An attractive option is to disperse protein-loaded composite microparticles in a secondary matrix that constitutes the tissue engineering scaffold. The composite microparticles would serve as carriers of growth factors, while the scaffold would provide the desired mechanical and chemical properties for cell adhesion, migration, and proliferation. Elisseeff et al. have applied such a concept to cartilage engineering by co-encapsulating chondrocytes and PLGA microspheres containing insulin growth factor-1 and transforming growth factor-β in PLA-PEG hydrogels formed by photopolymerization [8, 9]. The release profiles of the growth factors were not specifically controlled, but their presence in the hydrogel led to increased cell proliferation.

Similarly, Woo et al. dispersed rhBMP-2-loaded PLGA microspheres in carboxymethylcellulose [10]. The release profile of the system was adjusted by varying the proportion of “immediate release” and “sustained release” microspheres in the implant.

The greater tunability of our composite microparticles should allow increased flexibility in the design of growth factor release from tissue engineering scaffolds. Multifactor release at distinct rates can be achieved by dispersing different combinations of composite microparticles containing different growth factors in a single scaffold.
4.1.1 Tissue Engineering Scaffolds

Desirable characteristics of scaffolds for bone tissue engineering include (i) biocompatibility – for favorable interactions with cells, (ii) biodegradability – to allow ultimate replacement by natural bone, (iii) high porosity and pore interconnectivity – for cell migration and vascularization, and (iv) mechanical integrity – to maintain a stable 3-dimensional scaffold to support cell growth at the defect site and to prevent encroachment of soft tissue [11-14].

The dispersion of BMP-loaded apatite-polymer composite microparticles in the scaffold should preferably be conducted under aqueous conditions since the polymeric constituent of the microparticles, PLGA, is soluble in many organic solvents. Hence, biocompatible and biodegradable hydrogels were selected as the scaffold matrix. The particles could be dispersed in an aqueous solution of the polymer, which would be subsequently crosslinked to entrap the particles in the hydrogel network. Although these matrices have limited mechanical strength, they would be able to maintain their shape if the network density were high enough. Particle loading in the hydrogels might also enhance their mechanical integrity. Hydrogels of interest included naturally derived materials such as gelatin [15], chitosan [16], cellulose [17], dextran [18] and alginate [19], and synthetic gels such as PLGA-PEG [20] and PLGA-polyvinyl alcohol (PLGA-PVA) [21]. Protein delivery from hydrogels is often rapid due to facile diffusion through the aqueous channels, and due to the low affinity of some gels (e.g. PEG) for proteins [22]. This rapid diffusion could be countered by encapsulating the proteins in composite microparticles before dispersion in the gel.

Gelatin and collagen were selected for their biosafety [15] and their similarity to the extracellular matrix of bone. Composite gelatin scaffolds were prepared by crosslinking a suspension of BMP-loaded microparticles in gelatin. The commercially available and clinically used Helistat® collagen sponge (Integra Life Sciences) was also used as the scaffold. BMP-loaded microparticles were introduced into the collagen sponges by injection. The use of collagen sponges would facilitate comparison with the current clinical method. In addition, the low pH required for collagen dissolution in water, which might accelerate PLGA hydrolysis, could be avoided with the prefabricated collagen sponges.
4.1.1.1 Gelatin Scaffolds

Gelatin is derived from collagen. Type A and type B gelatins refer to gelatins derived by acidic and basic denaturation of collagen, respectively. Type A gelatin has an isoelectric point (IEP) of \(~ 7–9\) as opposed to \(~ 3–5\) for type B gelatin. BMPs typically have IEPs of \(~ 9\). Hence, at the physiological pH of 7.4, type A gelatin and BMPs are positively charged, whereas type B gelatin is negatively charged. Through electrostatic interactions, type B gelatin can retain BMPs, and indeed, such interactions are utilized for delayed protein release from gelatin [15, 23]. However, our intention was for the scaffold to sequester the composite particles and interfere minimally with protein release. Therefore, a scaffold material with low interactions with BMPs was desired, and type A gelatin was selected for our experiments.

Gelatin could be chemically crosslinked by aldehydes or carbodiimides. More highly crosslinked gels have lower water content and would undergo slower enzymatic degradation in the body [15]. To extend the life span \(in vivo\), a gel with higher crosslinking and higher molecular weight should be chosen [24]. Porous gels with large pore sizes could be created by controlling the freeze drying process [25]. A slower freezing rate would promote growth rather than nucleation of ice crystals, leading to larger pores after sublimation of the ice.

4.1.1.2 Collagen Sponges

Helistat\textsuperscript{®} absorbable collagen sponges, originally designed as hemostatic agents, are prepared from collagen obtained from the bovine Achilles tendon. These porous sponges are weakly crosslinked and are resorbed within \(~ 8\) weeks after subcutaneous implantation in rats, according to the product insert.

4.2 Experimental

4.2.1 Preparation of Composite Gelatin Scaffolds

Apatite-PLGA composite microparticles loaded with either BSA (model protein) or rhBMP-2 (therapeutic protein) were prepared under aseptic conditions according to the procedure in Section 3.2.2 [1]. Cell culture grade gelatin powder (Type A, 300 Bloom; Sigma) was added to sterile water to form a 10 w/v% solution. The gelatin solution was sterilized by autoclaving. A typical synthesis involved adding 250 mg of protein-loaded apatite-PLGA composite particles to 5 ml of gelatin solution, giving a particle loading of 33.3 w/w%. The
mixture was vortexed briefly, and 0.5 ml was transferred to a chamber on a chamber slide (Lab-Tek™ II, Nunc; 8 chambers total, ~ 0.7 cm²/chamber).

The gelatin mixture was crosslinked with either glutaraldehyde or N-ethyl-N’-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC; Sigma) and N-hydroxysuccinimide (NHS; Sigma). Glutaraldehyde bridges the amino groups on gelatin to form an imine linkage whereas EDAC/NHS catalyzes the reaction between a carboxyl group and an amino group to form an amide linkage. In the case of crosslinking by glutaraldehyde, 20 µl of a 25 w/v% aqueous glutaraldehyde solution was added to the gelatin solution, resulting in a concentration of 10 w/w% glutaraldehyde in gelatin. The gel was left to set at room temperature for 3 h. It was then removed from the chamber slide and washed 3 times with 5 w/v% glycine in water. Glycine was used to cap any unreacted glutaraldehyde, which is highly cytotoxic. The glycine washes were followed by 3 aqueous washes. All washes took at least 1 h at 37°C. Crosslinking by EDAC/NHS was conducted by adding 50 µl of 0.70 mM NHS and 50 µl of 0.98 mM EDAC to 0.5 ml of gelatin suspension in the chamber. Crosslinking was allowed to proceed at room temperature for 2 h, after which the composite gels were rinsed 3 times with water to remove the non-toxic side-product, urea, and unreacted EDAC/NHS. The composite gels were frozen at −20°C overnight and then freeze dried for 2 days.

For dual release of proteins from these composite gels, co-encapsulation of two sets of BSA-loaded composite particles was performed. Fast-releasing FITC-BSA-loaded composite particles were constructed from 250 mg of 6 kD PLGA and 50 mg of sCAP. Tetramethyl rhodamine isothiocyanate (TRITC)-BSA-loaded composite particles were fabricated from 250 mg of 59 kD PLGA and 50 mg of sCAP, and had a slower release profile. Protein loading in both sets of particles was 20 µg/mg of particle. The two sets of particles were dispersed in equal proportion in gelatin at a particle loading of 28.5 w/w% prior to crosslinking.

RhBMP-2-loaded composite particles constructed from 250 mg of 59 kD PLGA and 40 mg of sCAP were also loaded into the composite gels at a particle loading of 28.5 w/w%. RhBMP-2 loading was 145 ng per mg of particle. The details of each system are included with the results in Section 4.3.
4.2.2 Preparation of Composite Collagen Sponges

Helisat® collagen sponges were cut into the desired size. For characterization, larger pieces of ~13 mm x 13 mm x 6 mm and ~14 mg were used. For in vitro studies, sponges of ~6 mm x 13 mm x 4 mm and ~2.3 mg were used. Protein-loaded composite microparticles were suspended in sterile water at a concentration of 0.03–0.1 g/ml. Using a 21-gauge needle, the suspension was carefully injected along the midline of each collagen sponge. Only one set of composite particles was introduced into the sponges for the release of a single protein; whereas for dual release of proteins, two sets of protein-loaded composite particles were mixed in a single suspension before injection. The resulting composite collagen sponges were frozen at −20°C overnight and freeze dried for 2 days.

Particle loading in the sponges was varied from 0 to 78.5 w/w%. A particle loading above 50 w/w% implies a larger proportion of particles than matrix; for example, the loading of 78.5 w/w% was derived from 8.4 mg of particles in 2.3 mg of sponge. These low-density sponges were able to contain more particles than their own mass. Blank composite particles as well as composite particles encapsulating FITC-BSA, rhBMP-2 or rhBMP-6 were injected into the sponges. The details of each system are noted with the results in Section 4.3.

It was apparent that this method of loading collagen sponges with composite particles suffered from inefficiencies due to loss of particles in the dead volume of the syringe and needle, and possible leakage of particles from the sponge during injection. To obtain an average particle loading efficiency, 12 pre-weighed sponges were injected with a fixed mass of blank composite particles. After freeze drying, the composite sponges were weighed to determine the mass of particles loaded and the particle loading efficiency.

4.2.3 Characterization of Scaffolds and Sponges

4.2.3.1 Mechanical Testing

Composite gelatin scaffolds and composite collagen sponges were swollen in water. The wet gels and sponges were trimmed to rectangular blocks and their dimensions were measured with a pair of vernier calipers. For each type of gel or sponge, a minimum of 3 specimen blocks were tested with a Zwick/Roell series Z010 machine equipped with a 10-kN load cell. A pre-load stress of 5 N and a crosshead speed of 1 mm/min were used. The machine recorded loading and displacement data, which were converted into stress-strain
curves for estimating the compressive modulus of the specimens.

4.2.3.2 Porosity Measurements

The pore size distribution and porosity of composite gelatin scaffolds and composite collagen sponges were assessed by mercury porosimetry (Quantachrome PoreMaster 33). Pore sizes below 10 μm and greater than 200 μm were not detected with this equipment due to the experimental settings used. For each type of gel or sponge, a minimum of 2 specimens was tested.

4.2.4 Evaluation of Release Kinetics

4.2.4.1 Release of BSA

For each type of composite gelatin scaffold or composite collagen sponge tested, two pieces of the gel or sponge were placed in separate 1.5-ml centrifuge tubes (i.e. n = 2). Composite gelatin scaffolds were pre-swollen with 10 μl of sterile water per mg of scaffold. 1 ml of BES buffer at pH 7.4, which was sufficient to keep the gels and sponges submerged, was added to each tube. The tubes were incubated at 37°C. At 1, 4, 7, 14, 18, 22, 26 days, up to 42 days, 0.75 ml of release medium was withdrawn from the tubes and replaced with 0.75 ml of fresh buffer. The fluorescence of the release medium was measured at 485/538 nm (ex/em) for FITC-BSA and 544/590 (ex/em) for TRITC-BSA. Using standard calibration curves, fluorescence was converted to protein concentration, which was used to construct cumulative release profiles.

4.2.4.2 Release of rhBMP-2

For the release of rhBMP-2 from the gels and sponges, the same procedure as above was used except for the following modifications. The release medium was 1 ml of complete BME instead of BES buffer. At each time point, the release medium was withdrawn entirely, and replaced with 1 ml of fresh complete BME. RhBMP-2 concentration in the release medium was determined with an ELISA kit.

4.2.5 Cellular Response

Following the evaluation of release kinetics, it was decided that in vitro testing with
pluripotent murine embryonic fibroblasts (C3H10T1/2 line; ATCC) would be conducted on composite collagen sponges only.

4.2.5.1 Evaluation of Cell Viability by Microscopy

To evaluate cytotoxicity, collagen sponges injected with BSA-loaded composite particles were used. It was postulated that any cytotoxicity would stem from the materials involved (collagen, PLGA, apatite, BSA, remnant solvent and surfactant), and that BMPs were unlikely to have a negative influence on cell viability. Therefore, in the interest of conserving the expensive cytokine, BSA was used in place of rhBMP-2.

Each piece of composite sponge was placed in a well on a 24-well plate. C3H10T1/2 cells were seeded directly onto the sponges by slowly dripping ~ 50 μl of complete BME containing 50,000 cells onto the dry composite sponges. This small volume was chosen to ensure that the sponge soaked up the entire cell solution with minimal spillage onto the plate. The sponges were incubated at 37°C for 30 min to allow cell attachment before 1 ml of complete BME was added to the well in which the sponge was contained. Cells seeded in 24-well plates with no exposure to the composite sponges served as controls. The medium in all wells was renewed twice a week.

Cell viability was evaluated using a Live/Dead cytotoxicity kit (Molecular Probes). The basis of this assay was the use of two dye agents, calcein AM and ethidium homodimer, to distinguish between live and dead cells. Cell-permeant calcein AM was converted to bright green fluorescent calcein (495/515 ex/em) by enzymes in live cells, whereas ethidium homodimer entered dead cells with damaged membranes, bound to nucleic acids, and experienced a significant increase in red fluorescence (495/635 ex/em). Thus, live and dead cells could be detected simultaneously by their different colors with a fluorescence microscope.

At day 4 and day 7 of culture, 3 wells of each group were aspirated and washed 2 times with Dulbecco’s phosphate buffered saline (DPBS; Sigma). 100 μl of a 0.5-μM calcein AM/ethidium homodimer dye solution were added to each well, and the plate was incubated at 37°C for 40 min. The wells were then visualized using fluorescence microscopy (Olympus FV 300 laser scanning confocal microscope with IX-71 base unit).
4.2.5.2 *Evaluation of Cell Proliferation*

Collagen sponges injected with BSA-loaded composite particles were evaluated for cell proliferation. This study was complementary to the visualization of cell viability described in the previous section. Cells were seeded by two methods. The first method was described in Section 4.2.5.1, whereby cells were seeded onto the sponges directly. The second method involved seeding cells at a density of 6000 cells/cm² in each well of a 24-well plate, and allowing cells to attach overnight before the composite collagen sponge was added to each well. In this case, the cells were attached to the bottom of the plate, and not to the sponge, which floated above the cell layer. Cells with no exposure to composite sponges served as controls.

Cell culture was conducted for 3 weeks for all groups. Each week, cells in 3 wells of each group (except controls, where n = 4) were lysed and analyzed for cell number by the CyQUANT assay according to the procedure described in Section 3.2.5.2 [1]. For samples whereby cells were seeded onto the sponge, the sponge was transferred to lysis buffer and subjected to freeze-thaw cycles to release cellular contents. Hence, cells that had grown onto the bottom of the plate were not included in the cell count.

4.2.5.3 *Assessment of Osteoblastic Markers*

Four groups of composite collagen sponges were tested: (i) sponges injected with rhBMP-2-loaded composite particles, (ii) sponges injected with rhBMP-6-loaded composite particles, (iii) sponges injected with an equi-mixture of rhBMP-2-loaded and rhBMP-6-loaded composite particles, and (iv) sponges injected with blank composite particles. Each sponge contained the same mass of composite particles; the particle loading was ~ 66 w/w% (4.4 mg of particles per 2.3 mg of sponge). For sponges injected with rhBMP-containing composite particles, the total amount of rhBMP was 638 ng per sponge. All sets of composite particles were prepared from 250 mg of 59 kD PLGA and 20 mg of sCAP, and contained 145 ng of BMP per mg of particle.

C3H10T1/2 cells were seeded in 24-well plates at a density of 6000 cells/cm² with 1 ml of complete BME per well. After 1 day of culture, a composite sponge was introduced into each well. The plates were incubated at 37°C. The medium was renewed twice per week. Every week for 6 weeks, 4 wells of each group were analyzed for ALP activity and
osteocalcin level according to the procedures described in Sections 3.2.5.1 and 3.2.5.2, respectively [1]. In addition, to compare the in vitro potency of rhBMP-6 versus rhBMP-2, C3H10T1/2 cells were cultured in complete BME enriched with different concentrations of rhBMP-6 ranging from 0 to 1000 ng/well for 4 days. For each concentration, 5 wells of cells were tested. ALP activity was compared to that induced with rhBMP-2 at the same concentration (using results from Section 3.3.4 [1]).

4.2.5.4 Statistical Analysis

Statistical analysis was conducted using analysis of variance (ANOVA) with respect to treatment or time, followed by Tukey-Kramer HSD post hoc test for multiple comparisons (JMP v.5.0.1). Data were plotted as mean ± standard deviation. A significance level of p < 0.05 was used.

4.2.6 Preliminary In Vivo Testing in a Rat Ectopic Model

Two groups of BMP-loaded collagen scaffolds were tested. The dimensions of the Helistat® collagen sponges used were ~ 6 mm × 4 mm × 13 mm, weighing ~ 2.3 mg. The first group comprised of sponges injected with ~ 2.4 mg of rhBMP-2-loaded composite microparticles (with ~ 1.4 μg of rhBMP-2 per mg of particle). Thus, the total amount of rhBMP-2 in the scaffold was ~ 3 μg. The second group was a positive reference, prepared by soaking the collagen sponges with 3 ptg of rhBMP-2 for 15–20 min. Controls were either blank Helistat® sponges (without composite particles) or sponges containing ~ 2.4 mg of blank composite microparticles.

Animal studies were conducted in collaboration with the Institute of Bioengineering and Nanotechnology in Singapore in accordance with the Institute’s guidelines for the care and use of animals. Male Wistar rats of 7–8 weeks old were anesthetized with an intraperitoneal injection of sodium pentobarbital (~ 50 mg/kg) before each procedure. The back of each rat was wiped with iodine and 70% ethanol solution. Four incisions, each ~ 1 cm long, were made on either side of the rat lateral to the spine along the dorsum. Four sponges of the same group were inserted into the subcutaneous pouches on one side of the rat, while the other side received the appropriate control (either blank sponge or sponge with blank particles). Each experimental group consisted of 6 rats. After surgery, food was given
ad libitum. No adverse clinical reactions were observed, and all surgical incisions healed without infection.

At time points of 1, 2 and 4 weeks, 2 rats from each group were anesthetized and subjected to radiography. The rats were sacrificed, and the collagen scaffolds were removed and fixed in 10% formaldehyde solution. The specimens were dehydrated, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Histological sections were examined under a light microscope (Leica).

Based on results from radiography and histology, a second experiment was conducted to determine the minimum dosage of rhBMP-2 and rhBMP-6 required to induce ectopic bone formation in vivo. To increase the persistence time of the collagen sponges in vivo, the extent of crosslinking of the sponges was increased by soaking them in 10 ml of a solution of 0.35 mM EDAC and 0.49 mM NHS for 1 h. The sponges were then washed with sterile water and freeze-dried. RhBMP-2 and rhBMP-6 were tested independently at dosages of 2, 5, 10, 20 and 50 μg per sponge. Intramuscular sites, which were more vascularized and might be more responsive than subcutaneous sites, were tested. Each BMP-containing sponge was implanted in one quadriceps of a rat, while the other quadriceps was implanted with a control. For each dosage, two rats were tested. At 2, 4, 6 and 8 weeks, the rats were anesthetized and X-rayed to evaluate the development, if any, of a radiopaque area indicative of bone formation.

4.3 Results and Discussion

4.3.1 Mechanical Properties of Scaffolds

A typical stress-strain curve for composite gelatin scaffolds is shown in Fig. 4.1. The slope of the initial linear segment of the curve was taken as the compressive modulus of the sample.
Fig. 4.1. Typical stress-strain curve for composite gelatin scaffolds. The composite particle loading of this sample was 33 w/w%.

The compressive moduli of gelatin scaffolds containing different amounts of composite microparticles are presented in Fig. 4.2. The results indicated that the introduction of composite particles at low weight percentages led to a reduction in compressive modulus. As the composite particle loading was raised to 33 w/w% and 43 w/w%, the modulus increased two-fold compared to that of blank gelatin scaffolds. The composite particles were believed to act as non-adherent fillers because of the poor bonding between hydrophilic gelatin and hydrophobic PLGA. Fillers that were capable of strong interactions with the matrix would be more effective enhancers of mechanical properties [26]. However, even without such interfacial interactions, an increase in the volume fraction of the composite particles was expected to increase the modulus of the composite scaffold because the particles were stiffer than gelatin [27-29]. The higher modulus also stemmed from the lower porosity of the scaffold with increasing particle loading (see Section 4.3.2.). The apparent anomaly at low particle loading was possibly due to the differential swelling of gelatin and composite particles [30], resulting in the disruption of the gelatin network and the possible introduction of cracks. However, beyond a critical particle loading, the composite particles were able to stiffen the matrix. Other factors that might influence mechanical properties but were not investigated included composite particle size and particle morphology. In this study, the composite particles used were spherical and polydisperse with an average size of 45 ± 15 μm.
Fig. 4.2. Compressive moduli of composite gelatin scaffolds with different composite particle loadings. ‘Composite particle loading’ referred to the weight percent of composite particles with respect to the total weight of the composite gelatin. For example, a scaffold weighing 150 mg with a composite particle loading of 33 w/w% was comprised of 100 mg of gelatin and 50 mg of composite particles. Data shown are mean ± standard deviation (SD) for n = 3.

Helistat® collagen sponges were found to exhibit very poor mechanical properties. When wet, the sponges showed a compressive modulus of only 11.5 ± 8.3 kPa. A composite particle loading of 33 w/w% did not significantly alter the modulus of the sponge (7.4 ± 4.8 kPa).

4.3.2 Porosity and Pore Size Distribution of Scaffolds

To allow for cell migration and proliferation, tissue scaffolds must be porous [12, 25, 31]. Higher porosity would accommodate more cells in the scaffold and enhance cell distribution, but would impact mechanical properties adversely and reduce surface area for cell attachment [6, 31]. The porosities of composite gelatin scaffolds and composite collagen sponges were measured by mercury porosimetry. As the composite particle loading in the gelatin scaffold was increased from 0 to 33 w/w%, porosity was reduced from 72.0% to 35.5% (Table 4.1). The porosity of Helistat® collagen sponges, however, appeared to be unaffected by the presence of composite particles. The particles occluded only a small fraction of the sponge, leaving the porosity and structure of the rest of the sponge unchanged.
Table 4.1. Porosity of composite gelatin scaffolds and composite collagen sponges.

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>Composite Particle Loading (w/w%)</th>
<th>Porosity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelatin</td>
<td>0</td>
<td>72.0</td>
</tr>
<tr>
<td>Gelatin</td>
<td>10</td>
<td>63.3</td>
</tr>
<tr>
<td>Gelatin</td>
<td>33</td>
<td>35.5</td>
</tr>
<tr>
<td>Helistat® Collagen</td>
<td>0</td>
<td>58.9</td>
</tr>
<tr>
<td>Helistat® Collagen</td>
<td>10</td>
<td>57.3</td>
</tr>
<tr>
<td>Helistat® Collagen</td>
<td>33</td>
<td>57.6</td>
</tr>
</tbody>
</table>

Fig. 4.3. Pore size distributions of composite gelatin scaffolds loaded with (a) 0 w/w%, (b) 10 w/w%, (c) 33 w/w% and (d) 43 w/w% of composite microparticles.
For the ingrowth of bone cells, the optimal pore size has been suggested to be in the range of 200–400 \( \mu \text{m} \) [12, 31]. Unfortunately, the Quantachrome PoreMaster could only detect pores up to a maximum of 200 \( \mu \text{m} \) at the settings that were used. However, the presence of pores larger than 200 \( \mu \text{m} \) in composite gelatin scaffolds could be deduced from examining the graphs in Fig. 4.3. With increasing composite particle loading, the fraction of pores larger than 100 \( \mu \text{m} \) seemed to increase. This could be due to occlusion of some of the larger pores by the composite particles, bringing their pore size down to the range measurable by the instrument. The presence of pores greater than 200 \( \mu \text{m} \) was confirmed by environmental scanning electron microscopy (ESEM) (Fig. 4.4). Thus, the gelatin scaffolds possessed pores that would be suitable for the migration of bone cells. Fig. 4.4 also shows composite microparticles dispersed in the gelatin scaffold. These particles were detected throughout the scaffold by electron microscopy.

![Fig. 4.4. ESEM micrographs of a composite gelatin scaffold (10 w/w% particles) showing (a) a pore greater than 200 \( \mu \text{m} \), and (b) composite microparticles dispersed in the scaffold.](image)

The pore size distribution of Helistat® sponges remained essentially the same despite the introduction of increasing amounts of composite microparticles (Fig. 4.5). It appeared that neither porosity (Table 4.1) nor pore size distribution (Fig. 4.5) were much affected by the presence of the particles, which were confined to a small region of the sponge (particles were typically injected along the midline of the sponge). A further observation was that Helistat® collagen sponges had a wide range of pore sizes (Fig. 4.5), and contained large pores (> 100 \( \mu \text{m} \)) that would allow the migration of bone cells. Such pores were also detected by ESEM (Fig. 4.6a). The micrograph in Fig. 4.6b shows the composite microparticles that
had been injected into the sponge along its midline. Regions away from the midline were devoid of particles. These particles were weakly held in the collagen matrix, and left behind spherical impressions when they were dislodged by the sectioning of the sponge.

![Graphs showing pore size distributions](image)

Fig. 4.5. Pore size distributions of composite Helistat® sponges loaded with (a) 0 w/w%, (b) 10 w/w% and (c) 33 w/w% of composite microparticles.
4.3.3 In Vitro Release of Proteins from Scaffolds

4.3.3.1 Composite Gelatin Scaffolds

Two sets of composite microparticles were prepared, each with different release kinetics. One set encapsulating FITC-BSA was fabricated from 6 kD PLGA and sCAP, and had a fast release profile [2]. The other set encapsulating TRITC-BSA was prepared from 59 kD PLGA and sCAP, and exhibited a slower release [2]. The two sets of composite particles were dispersed in separate gelatin scaffolds ('solo gels'), as well as combined in equi-proportions in a single gelatin scaffold ('mixed gel'). The release profiles of BSA from the composite particles, 'solo gels', and 'mixed gels' are shown in Fig. 4.7.

Entrapment of the composite particles in a gelatin scaffold led to protein release of lower magnitude. The gel posed an additional diffusion barrier to the proteins, and served to smoothen out the release profile, particularly for the set of composite particles encapsulating FITC-BSA with a significant initial burst. The co-entrapment of two sets of composite particles in a single gelatin scaffold demonstrated the feasibility of dual protein release. FITC-BSA and TRITC-BSA were released from the 'mixed gel' with distinct release profiles. Whether the particles were combined in a 'mixed gel' or kept separate in 'solo gels' made little difference to their release profiles. Each set of composite particles functioned essentially on its own in gelatin. Hence, the aggregate protein release profile of a scaffold is the sum of its parts, which facilitates the design and testing of multifactor release.
Fig. 4.7. BSA release from composite particles and composite gelatin scaffolds. Protein release was normalized by the mass of the corresponding set of composite particles. Solid curves indicate release from the composite particles alone. Composite particles encapsulating FITC-BSA were fabricated from 250 mg of 6 kD PLGA and 50 mg of sCAP. Composite particles encapsulating TRITC-BSA were fabricated from 250 mg of 59 kD PLGA and 50 mg of sCAP. ‘Solo gel’ refers to release from composite gelatin scaffolds containing one set of composite particles, whereas ‘mixed gel’ refers to release from composite gelatin scaffolds containing an equi-mixture of the two sets of composite particles. The particle loading (28.5 w/w%), and the protein content (20 µg per mg of particle, 200 µg per gel) of ‘solo gels’ and ‘mixed gels’ were the same. Data shown are mean values for n = 2.

Composite particles encapsulating rhBMP-2 were also dispersed in these gelatin scaffolds. However, BMP release was found to be nearly undetectable by ELISA. It is suspected that the proteins were denatured by the crosslinking reaction during gel formation. Crosslinking involved the reaction of amino (and carboxyl) groups on peptides; hence, gelatin and BMPs were similarly susceptible. It was hoped that the BMPs might be shielded from the crosslinkers by their encapsulation in composite particles, but it appeared that the crosslinkers, which were small molecules, were able to permeate into the composite particles. Using glutaraldehyde and EDAC/NHS as crosslinkers led to non-release of rhBMP-2. As further confirmation, release medium from these rhBMP-2-loaded composite gelatin scaffolds were used to culture C3H10T1/2 cells. No inductive effect was found, indicating the absence of bioactive rhBMP-2 in the release medium. This finding highlighted a potential pitfall of using proteins with no biological activity, such as BSA, as surrogates in release studies. The
detection of proteins in release media would not constitute a positive event unless their structure and biological activity were successfully preserved.

Since the gelatin scaffolds were deemed to be unsuitable for BMP delivery, no further experiments were conducted on these scaffolds. However, if a milder crosslinking reaction could be devised, these scaffolds would have potential in multifactor release. Alternatively, protein-loaded composite particles could be dispersed in hydrogels of other chemical compositions formed under more benign conditions. For example, Anseth et al. have dispersed polymeric microparticles containing insulin growth factor-1 and transforming growth factor-β in PEG hydrogels formed by photopolymerization for cartilage tissue engineering [8].

4.3.3.2 Composite Collagen Sponges

Composite microparticles encapsulating either FITC-BSA or rhBMP-2 were injected into porous Helistat® collagen sponges. Since the sponges were prefabricated, loading of these sponges with composite particles did not involve a potentially denaturing crosslinking reaction. The efficiency of loading these sponges with composite particles was estimated to be 49 ± 26% based on the weighing of 12 sponges before and after particle injection and freeze drying.

Releases of FITC-BSA and rhBMP-2 from composite collagen sponges are shown in Figs. 4.8 and 4.9, respectively. As the composite particles were not embedded in the collagen matrix but were held in the voids of the sponge (see Fig. 4.6b), proteins released from the composite particles could diffuse rapidly out of the porous sponge. Consequently, the releases of FITC-BSA and rhBMP-2 from the composite collagen sponges closely matched their respective releases from the composite particles alone.
Fig. 4.8. BSA release from (♦) composite particles and (▲) composite collagen sponge. Protein release was normalized by the mass of the composite particles. Composite particles encapsulating FITC-BSA were fabricated from 250 mg of 6 kD PLGA and 50 mg of sCAP. The particle loading in the sponge was 78.5 w/w% (8.4 mg of particles in 2.3 mg of sponge), and BSA content was 25 μg/mg particle. Data shown are mean values for n = 2.

Fig. 4.9. RhBMP-2 release from (♦) composite particles and (▲) composite collagen sponge. Protein release was normalized by the mass of the composite particles. Composite particles encapsulating rhBMP-2 were fabricated from 150 mg of 59 kD PLGA, 100 mg of 20 kD PLA, and 20 mg of sCAP. The particle loading in the sponge was 68.5 w/w% (5 mg of particles in 2.3 mg of sponge), and rhBMP-2 content was 145 ng/mg particle. Data shown are mean values for n = 2.
4.3.4 Cellular Response

4.3.4.1 Evaluation of Cell Viability by Microscopy

Viable cells were detected on the outer perimeters of the composite collagen sponges at days 4 and 7 (Fig. 4.10). The interior of the sponges was essentially devoid of cells despite the seeding of 50,000 cells per sponge. One explanation for the sparse cell population might be the sieving effect of the sponge. As the pores were not large enough, cells were prevented from percolating into the center of the sponge and were mostly left on the surface. Cells that did not adhere well to the sponge before the medium addition were eventually washed onto the bottom of the well. An examination of the bottom of the wells did reveal a confluent layer of cells at day 7. Even if the interior of the sponge had been seeded with cells initially, diffusion limitations of such a static cell culture might have reduced cell viability. Beyond 3.5 mm from the nutrient supply, cell survival has been reported to be poor [32, 33]. For this reason, dynamic cell culture conditions such as in perfusion bioreactors, which enhance transport of nutrients and removal of metabolic wastes, are used to create tissue constructs ex vivo [34-36].

Fig. 4.10. Fluorescent cell viability staining of cell-seeded composite collagen sponges at (a) 4 days (20× magnification) and (b) 7 days (10× magnification). Live C3H10T1/2 cells appeared green, whereas dead cells and composite particles were stained red. In each figure, the top left quadrant displays green fluorescence, the top right quadrant displays red fluorescence, the bottom left quadrant shows the light micrograph of the scaffold, and the bottom right quadrant shows the superposition of red and green fluorescent images.
Cells that did attach to the scaffold appeared viable, and very few dead cells were detected (Fig. 4.10). In addition, the composite particles were stained red by the ethidium homodimer dye, allowing their visualization in the collagen sponge.

4.3.4.2 Evaluation of Cell Proliferation

For cells seeded onto the bottom of wells, the presence of composite collagen sponges in the medium did not affect cell proliferation. Over 3 weeks of culture, cell number exceeded or was comparable to that of wells with no exposure to collagen sponges (Fig. 4.11). No cytotoxic substances appeared to have leached from the sponges.

![Graph showing cell proliferation](image)

Fig. 4.11. Number of C3H10T1/2 cells growing (■) on composite collagen sponges or (○) on the bottom of wells containing composite collagen sponges. ( □) Controls were cells with no exposure to composite collagen sponges. Asterisks denote statistically significant differences of p < 0.05 compared to control. Data shown are mean ± SD for n = 3 (samples) or n = 4 (controls).

In the case of sponges directly seeded with cells, the cell population on the sponges was one-fifth of that on the bottom of wells in the other 2 groups, despite the much higher seeding density of the former (50,000 cells/sponge vs. 12,000 cells/well). The lower cell proliferation on the sponges might be due to inefficient seeding and poor cell survival associated with diffusion limitations, as discussed in the previous section. The number of cells growing on the composite collagen sponge 7 days after seeding was ~ 13,200, which
translated into a cell seeding efficiency of 26%. This number was probably an over-estimation of the actual seeding efficiency since it encompassed 7 days of cell proliferation on the sponge.

### 4.3.4.3 Measurement of Induced Alkaline Phosphatase Activity

To determine the effect of the composite collagen sponges on osteoblastic differentiation, C3H10T1/2 cells were plated in 24-well plates, and sponges were subsequently introduced to the wells. The sponges were not in direct contact with the cells, but released BMPs into the milieu, simulating what cells a distance away from the implant might experience in vivo. Cells were not seeded directly onto the sponges due to the low cell proliferation (see Section 4.3.4.2).

Four types of composite collagen sponges were investigated: (i) sponges containing rhBMP-2-loaded composite particles, (ii) sponges containing rhBMP-6-loaded composite particles, (iii) sponges containing an equi-mixture of rhBMP-2-loaded and rhBMP-6-loaded composite particles, and (iv) sponges containing blank composite particles. The particle loading was the same in all sponges. The composite particle formulation was also the same in terms of the amount and type of polymer and apatite used. Fig. 4.12 shows the effect on ALP activity of prolonged cell culture in the presence of the composite collagen sponges. Within each group, the significant variation in particle loading in the sponges (~ 49 ± 26%) led to large experimental variance in cellular response. For example, ALP activity levels for the sponge containing rhBMP-2 and rhBMP-6 at day 7 and for the sponge containing rhBMP-2 at day 14 seemed to be considerably higher than that of the control, and yet were not statistically significant by ANOVA and Tukey-Kramer post hoc test because of the small sample size (n = 3) and large experimental variance. In general, ALP activity of BMP-loaded sponges was elevated over controls, particularly for sponges containing rhBMP-2 in the first 2 weeks. Since ALP is an early osteoblastic marker, its levels are expected to diminish with time (Section 3.3.5.1 [1]).
Fig. 4.12. ALP activity induced by prolonged exposure to composite collagen sponges containing composite particles loaded with (■) rhBMP-2, (△) rhBMP-6, (●) rhBMP-2 and rhBMP-6, and (○) no BMP (control). Particles were fabricated from 250 mg of 59 kD PLGA and 20 mg of sCAP. BMP content was 145 ng/mg particle. Particle loading in the sponges was 66 w/w%. Asterisks denote statistically significant differences of p < 0.05 compared to control. Data shown are mean ± SD for n = 4.

Fig. 4.12 suggests that rhBMP-2 was more potent than rhBMP-6 in the stimulation of ALP activity over the first 2 weeks of the experiment. However, Cheng et al. reported that in their experiments, ALP activity induced by BMP-6 was almost 5 times that induced by BMP-2 [37]. Instead of adding growth factors exogenously to the culture medium, Cheng et al. used recombinant adenoviruses expressing various BMPs to transfect C3H10T1/2 cells, leading to the endogenous production of the growth factors. The expression level of the growth factors was unclear and unavailable for our comparison. In addition, the bioactivity of growth factors produced endogenously was likely to be different from that added exogenously, since the commercially obtained proteins were subjected to multiple processing steps.

To compare the bioactivities of rhBMP-2 and rhBMP-6 as received from R&D Systems, the induction of ALP activity in C3H10T1/2 cells by different concentrations of the two growth factors was examined. Both growth factors showed a dose-dependent effect; the optimum dosage was ~ 400 ng/well at which rhBMP-6 had a markedly stronger effect than rhBMP-2 (Fig. 4.13). However, at almost all other concentrations, rhBMP-2 stimulated a
slightly higher effect, though the difference was not statistically significant. Based on these results, a conclusion could not be reached on the relative bioactivity of rhBMP-2 versus rhBMP-6, but it likely depended on their concentrations in the environment.

![Graph showing ALP activity](image)

**Fig. 4.13.** Effects of (■) rhBMP-2 and (□) rhBMP-6 concentrations on induced ALP activity in C3H10T1/2 cells. Each well was seeded with 12,000 cells and contained 0.5 ml of medium. Asterisks denote statistically significant differences of $p < 0.05$ compared to control (0 ng/well of rhBMP-2 or rhBMP-6). Hashes denote statistically significant differences of $p < 0.05$ between rhBMP-2 and rhBMP-6 treatment of the same concentration. Data shown are mean ± SD for $n = 5$.

### 4.3.4.4 Measurement of Osteocalcin Expression

Exposure to composite collagen sponges releasing rhBMPs led to elevated levels of osteocalcin after 7 days (Fig. 4.14). In contrast, control cells cultured in the presence of blank composite collagen sponges expressed osteocalcin weakly over the course of the experiment. The osteoinductive effect of BMPs was more discernible by the evaluation of osteocalcin as the osteoblastic marker than by ALP activity.

In agreement with the ALP activity assessment (Fig. 4.12), rhBMP-6 showed lower inductivity than rhBMP-2. Osteocalcin levels were consistently lower for cells treated with rhBMP-6. There also appeared to be little or no synergy between BMP-2 and BMP-6 in the induction of the osteoblastic phenotype at the dosages used. The selection of this combination of growth factors was arbitrary and was based on availability in our laboratory. A better
choice might have been rhBMP-4 and vascular endothelial growth factor (VEGF) [38]. In Peng et al.’s study, VEGF alone did not induce bone formation, but by promoting vascularization, and through its supposed anti-apoptotic effect, VEGF aided in enhancing nutrient circulation, cell survival, cell recruitment, and ultimately, bone healing [38]. Another possible combination would be transforming growth factor-β3 and rhBMP-2, which was reported to evoke endochondral ossification in a synergistic fashion [39]. Currently, to determine an optimal combination of growth factors to use in bone tissue engineering, multiple hit-or-miss experiments have to be performed. Further elucidation of the bone healing process, especially the signaling pathways involved, might facilitate a more informed development of the appropriate cocktail to use.

![Graph](image)

Fig. 4.14. Osteocalcin levels induced by prolonged exposure to composite collagen sponges containing composite particles loaded with (■) rhBMP-2, (○) rhBMP-6, ( □) rhBMP-2 and rhBMP-6, and (□) no BMP (control). Particles were fabricated from 250 mg of 59 kD PLGA and 20 mg of sCAP. BMP content was 145 ng/mg particle. Particle loading in the sponges was 66 w/w%. Data shown are mean ± SD for n = 4.

### 4.3.5 Preliminary In Vivo Testing

After 1 and 2 weeks, collagen scaffolds were excised from the rats. Scaffolds containing rhBMP-2, either encapsulated within composite microparticles or directly loaded onto the sponge, were fully resorbed at 4 weeks. Only remnants of the control – blank composite collagen sponges and blank collagen sponges – were found at 4 weeks. The
explants from week 1 and 2 were stained with hematoxylin and cosin (H&E) (Figs. 4.15 and 4.16). In the histological sections, nuclei appeared blue/blue-black, cytoplasm appeared pink, red blood cells were red, and the collagen scaffold was stained brown (Mayer’s hematoxylin) or blue (Harris’ hematoxylin).

Fig. 4.15 shows histological sections of excised composite collagen sponges, which had been injected with composite particles. Fig. 4.16 shows histological sections of excised collagen sponges without composite particles. The presence of rhBMP-2 in the sponges in both groups led to greater cellularity and vascularity, which were evident 1 week post implantation. The higher density of nuclei and lower proportion of scaffold remnants indicated greater tissue ingrowth. The presence of red blood cells and the distinctive outline of blood vessels particularly in the 2-week samples suggested that angiogenesis had occurred in the rhBMP-2-loaded sponges. The controls without rhBMP-2 showed tissue lining the perimeter of the scaffolds, but the centers remained largely undegraded and unpopulated.

Examination of the histology slides under higher magnification revealed the presence of inflammatory cells, such as macrophages and lymphocytes, in areas adjacent to the scaffold material for both BMP-loaded and control sponges. An inflammatory response to the scaffold material was expected, as it was xenographic (bovine origin). Most of the cells in the infiltrated tissue were fibroblasts, and the inflammatory response was relatively mild. In sponges containing composite particles, inflammatory cells were also found in the perimeter of some of the voids where the composite particles had resided (the particles were dissolved during the preparation of the histology slides). Regions resembling granulation tissue, which would typically be present in the early stages of a tissue healing response, were also observed in BMP-loaded sponges.

No significant difference could be discerned between collagen sponges with rhBMP-2-loaded composite particles and collagen sponges directly loaded with rhBMP-2. Both groups showed approximately the same amount of tissue ingrowth and vascularization, suggesting that rhBMP-2 delivered from the composite microparticles had comparable bioactivity to the protein released from the sponges loaded by conventional method. Staining by von Kossa for calcium deposits was negative for both groups, implying that no bone formation had occurred. A possible explanation was that the rhBMP-2 dosage (3 μg) was too low for osteoinduction. Osteoinduction has been shown to be dose-dependent, and to occur at
dosages as low as 1 μg of rhBMP-2 [40-43]. It was postulated that a lower but still efficacious dosage of rhBMP-2 would lead to more perceptible differences in the osteoinductivity of composite collagen sponges and conventionally loaded collagen sponges. Hence, a dosage of 3 μg was chosen. However, the bioactivity of the protein we employed might have been lower than those used by other groups, necessitating a higher dosage.

In addition, the rapid resorption of the collagen sponges excluded observations at later time points when the effect of more sustained BMP delivery might be apparent. Without the scaffold material, it was extremely difficult to locate in the rats the few milligrams of composite particles, which were likely undegraded at 4 weeks. Thus, scaffolds with longer persistence times are needed to contain the particles.

The vascularity observed in the explants might be an indication of the angiogenic effect of rhBMP-2. BMPs have been reported to promote angiogenesis both in vitro and in vivo [44-47]. This step, which precedes bone formation, is crucial to bone healing as the vasculature supplies stem cells, nutrients, mineral elements, and cytokines needed for osteogenesis. Of particular interest is the study by Deckers et al. showing that BMPs stimulate angiogenesis through enhancing the production of vascular endothelial growth factor A (VEGF-A) by osteoblastic cells [45]. VEGF-A subsequently acts on endothelial cells that form blood vessels. They found that treatment of 25 ng/ml and 100 ng/ml of rhBMP-2, rhBMP-4 or rhBMP-6, but not 10 ng/ml, led to calcium deposition by preosteoblasts. However, all 3 doses stimulated the production of VEGF-A after 72 h of treatment. Within 14 days, VEGF-A levels of cells treated with 10 ng/ml and 25 ng/ml of BMP-4 became indistinguishable [45]. These results suggest that low doses of BMPs may be sufficient for initiating angiogenesis but not osteogenesis, and may explain the vascularity of the BMP-loaded sponges that we observed. Although the 3 μg dose we had used was too low to induce ectopic bone formation, the angiogenic activity of rhBMP-2, which might occur at lower doses, led to increased vascularity that was evident by histology.
Fig. 4.15. H&E staining of excised composite collagen sponges at 1 week (a-d) and 2 weeks (e, f). Micrographs for rhBMP-2-loaded composite collagen sponges are in the left column (a, c, and e); micrographs for blank composite collagen sponges are in the right column (b, d, f). Magnification: $10 \times$ (a, b, e, and f) and $20 \times$ (c and d). Mayer’s hematoxylin was used for all sections except (e), which was stained with Harris’ hematoxylin.
Fig. 4.16. H&E staining of excised collagen sponges at 1 week (a-d) and 2 weeks (e, f). Micrographs for rhBMP-2-loaded collagen sponges are in the left column (a, c, and e); micrographs for blank collagen sponges are in the right column (b, d, f). Magnification: 10× (a, b, e, and f) and 20× (c and d). Mayer’s hematoxylin was used for all sections except (f), which was stained with Harris’ hematoxylin.
4.3.5.1 Evaluation of Dosage Required for Ectopic Bone Formation

To find the minimum dosage required for osteoinduction in an ectopic site, rhBMP-2 and rhBMP-6 were separately loaded into collagen sponges (no composite particles) at different concentrations and implanted in the quadriceps of rats. Intramuscular sites were more vascularized, and might promote faster cellular response. Radiographs of the rats at 2 and 4 weeks are shown in Figs. 4.17 and 4.18 for rhBMP-2 treatment and rhBMP-6 treatment, respectively. Radiopaque areas were deemed to signify mineralization, although bone formation has to be confirmed by histological examination. The results indicated that the minimum dosage associated with radiopacity at 2 weeks was 10 μg of rhBMP-2 or 50 μg of rhBMP-6. At 4 weeks, 5 μg of rhBMP-2 or 10 μg of rhBMP-6 was able to produce radiopacity. These values exceeded the dosage of 3 μg that was used in our initial study (Section 4.3.5), and should be used for future studies. The lower effective dosage of rhBMP-2 than rhBMP-6 confirmed the in vitro results (Figs. 4.12 and 4.14) that rhBMP-2 was the more potent of the two growth factors.
Fig. 4.17. Radiographs at 2 weeks (left column) and 4 weeks (right column) of rats implanted with collagen sponges loaded with rhBMP-2 of (a, b) 2 μg, (c, d) 5 μg, (e, f) 10 μg, (g, h) 20 μg, and (i) 50 μg. Black rings highlight radiopaque areas.
Fig. 4.18. Radiographs at 2 weeks (left column) and 4 weeks (right column) of rats implanted with collagen sponges loaded with rhBMP-6 of (a, b) 2 μg, (c, d) 5 μg, (e, f) 10 μg, (g, h) 20 μg, and (i) 50 μg. Black rings highlight radiopaque areas.
4.4 Summary

Protein-loaded apatite-PLGA composite microparticles that we have prepared are sufficiently small for injection into fractures and small voids in bone. However, for larger bone defects, the particles need to be formulated into a bulk material that can fill the defect space. We have chosen to disperse the composite particles in secondary matrices of either gelatin or collagen. The intention was for the protein-loaded composite particles to deliver molecular signals to guide bone formation, while the matrices would provide suitable porosity, mechanical integrity, and attachment sites for cells. If so desired, multifactor release could be realized by incorporating different sets of composite particles with distinct profiles and growth factors in a single scaffold.

Composite gelatin scaffolds were prepared by crosslinking a suspension of protein-loaded composite particles in a gelatin solution. The encapsulation of a model protein, BSA, led to promising results: dual delivery of FITC-BSA and TRITC-BSA at different rates with a single scaffold. However, when rhBMP-2-loaded composite particles were dispersed in the scaffolds, no protein release was detected by ELISA and no osteoinductive effect was seen when the release medium was used to culture C3H10T1/2 cells. It appeared that the crosslinking reaction used to form the gelatin network was deleterious to rhBMP-2. Glutaraldehyde or EDAC/NHS was used for crosslinking the amino (and carboxyl) groups on gelatin chains. Those functional groups exist on almost all proteins, hence, rhBMP-2 was also susceptible to crosslinking and denaturation. Encapsulation of rhBMP-2 in composite particles could not shield the proteins from the crosslinkers, possibly because the composite particles were porous (see Fig. 2.7). These results also point to a caveat in using model proteins with no measurable bioactivity, such as BSA, as surrogates for therapeutic proteins. A commonly used model protein with bioactivity is lysozyme, whose ability to hydrolyze β-1,4 glycosidic linkages in the cell walls of *m. lysodeikticus* can be assessed. However, the adsorption of lysozyme onto apatite was previously found to be low (see Table 2.4, [2]), which might limit its encapsulation efficiency in composite particles and might not provide for the release of a sufficient amount for testing.

Protein-loaded composite particles were also incorporated into pre-fabricated Helistat® collagen sponges by injection along the midline of the sponges. Due to losses in the dead volume of the syringe and leakage during injection, only ~50% of the particles drawn into the
syringe were actually deposited in the sponge. Particle distribution was non-uniform and was concentrated in the midline of the sponge, as was expected for such a method of loading. These sponges are commercially used as carriers of rhBMP-2 in spinal fusion, and have been proven to be suitable substrates for cell adhesion, migration and proliferation. However, they were found to be mechanically very weak (compressive modulus ~ 10 kPa), and hence, might not hold up well in load-bearing defects. In spinal fusion, mechanical strength is provided by metal cages into which BMP-loaded Helistol® sponges are inserted before implantation. Nevertheless, the use of these pre-fabricated sponges in containing composite particles did not involve a potentially denaturing crosslinking reaction, and allowed the rapid diffusion of the proteins out of the sponges once they were released from the composite particles.

The safety and efficacy of collagen sponges injected with BMP-loaded composite particles were verified in vitro. ALP and osteocalcin levels were elevated by sponges containing rhBMP-2, rhBMP-6, or an equi-mixture of the two growth factors loaded in composite particles. Osteoinductivity was more readily distinguishable by the assessment of osteocalcin as the osteoblastic marker. RhBMP-2 appeared to possess higher activity than rhBMP-6, contrary to a recent study by Cheng et al. [37]. However, others have cited BMP-2 and BMP-7 as the most potent of the fourteen BMPs [48, 49]. These two proteins are also the only two members of the BMP family currently approved by FDA for use in orthopedic applications.

Preliminary in vivo studies were conducted with rats involving subcutaneous implantation of collagen sponges injected with BMP-loaded composite particles (composite sponges) and conventionally loaded collagen sponges. An ectopic model was used to disaggregate osteoinduction from osteoconduction. Bone formation in an orthotopic model might be due to existing osteoblasts migrating into the scaffold and laying down new matrix (osteocoduction), or due to stem cells and pre-osteoblasts differentiating into osteoblasts that then deposit new bone (osteoiduction). Since the activity of BMPs is osteoinductive, an ectopic model would serve to evaluate the bioactivity of BMPs delivered by the sponges in vivo.

Both groups of BMP-loaded sponges (either conventionally loaded or composite sponges) showed significantly enhanced cellularity and vascularity over blank controls at 1 and 2 weeks post implantation. Although no mineralization was observed in the BMP-loaded
sponges by von Kossa staining and radiography, the marked difference in tissue infiltration and vascularization between samples and controls suggested that the BMPs were bioactive in vivo. The rhBMP-2 dosage used, 3 μg, might have been too low to induce ectopic bone formation. Studies have shown that the angiogenic effect of BMPs might be manifested at lower doses than required for osteogenic activity [45]. Therefore, the increased vascularization that was observed might be an indication of rhBMP-2 activity at low concentrations.

Our suspicion that the 3 μg dosage was insufficient for evoking osteogenesis was confirmed by a subsequent investigation showing that 10 μg of rhBMP-2 or 50 μg of rhBMP-6 were needed to induce radiopacity of the implants at 2 weeks. At 4 weeks, lower dosages of 5 μg of rhBMP-2 or 10 μg of rhBMP-6 were able to elicit a similar response. This study also affirmed the higher potency of rhBMP-2 over rhBMP-6.

In this preliminary in vivo study, comparable results were obtained with conventionally loaded sponges and composite sponges. With the composite sponges, we had hoped to demonstrate that a more sustained release of rhBMP-2 might produce a different pattern or amount of bone formation. Unfortunately, the complete resorption of the collagen sponges by 4 weeks excluded observations at later time points. Moreover, as aforementioned, the BMP dosage used was probably inadequate for ectopic bone formation even after an extended period of time. For future in vivo studies, scaffolds with longer persistence times and higher BMP dosages would aid in the evaluation of the effect of different BMP release kinetics on osteogenesis.

4.5 References


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Chapter 5 – Recommendations for Future Work

5.1 Further Investigation of Release Mechanism

Protein release from apatite-PLGA composites is dependent on the interplay of polymer degradation and apatite dissolution. If left to passive leaching from apatite-protein complexes, the release of protein as well as calcium ions would be very slow and small in magnitude (see Sections 2.3.3 and 2.3.9 [1]). To facilitate the design of release profiles, the causal relationships between polymer degradation, the local pH environment, apatite dissolution, and protein bioactivity need to be better understood. Estimations of the pH within the degrading composite microparticles could be accomplished by co-encapsulating pH-sensitive fluorophores in the particles, and visualizing the fluorescence intensity profiles across the particles with a confocal microscope [2]. Our measurements of the pH of the incubation medium (BES buffer or complete BME) suggested that the external environment was sufficiently buffered and maintained at pH levels of ~ 6–7. Fu et al. noted a distinct difference between the interior and exterior pH environment of PLGA microspheres [2]. Hence, it is the pH within degrading composite microparticles that determines the extent of apatite dissolution, and this pH should be tracked. The results should then be correlated with data on polymer degradation as evaluated by GPC, release of calcium ions as measured by calcium-sensitive dyes (e.g. Arsenazo III) or inductively coupled plasma mass spectrometry (ICP-MS), and protein release as determined by ELISA. In addition, the structure of proteins released from the composite microparticles could be assessed by gel electrophoresis, and their bioactivity evaluated by binding assays.

The assembling of these various pieces of information could help determine, for example, the extent of polymer degradation required to generate a particular pH, and the effect of the pH environment within degrading composite microparticles on the degree of apatite dissolution and the bioactivity of proteins. In particular, assessment of protein structure and bioactivity might shed some light on the difference in potency of rhBMP-2 delivered from composite microparticles and Helistat® collagen sponges (see Section 3.3.4 [3]).
5.2 Exploration of Additional Material Parameters

A number of parameters were used to adjust protein release through their effect on polymer degradation and/or apatite dissolution. It would be fruitful to explore additional material parameters, such as new types of polymer and basic inorganic phase. Only poly(α-hydroxy acids) were studied in this thesis; other acid-producing biodegradable polymers, such as poly(ortho esters), poly(anhydrides) and even newly designed synthetic polymers, would possess different degradation rates and could alter protein release rates. Since a constant rate of acid production would facilitate zero-order release, a polymer that undergoes steady degradation with time would be an attractive candidate.

Apatite was used as the basic inorganic phase because its dissolution results in the release of calcium and phosphate, which could be beneficial to bone formation. Other non-toxic basic inorganic materials that would release ions that are natural substituents in bone apatite include magnesium oxide and zinc oxide [4, 5]. These materials could have different solubilities and affinities for proteins, and could provide another parameter for tailoring the protein release rate.

5.3 Incorporation of Composite Microparticles into Tissue Engineering Scaffolds

In the preparation of composite gelatin scaffolds, rhBMPs were denatured by the crosslinking reaction (see Section 4.3.3.1 [6]). However, the potential of such scaffolds in multifactor release is attractive. Scaffolds that can be created without deleterious chemical reactions should be used to encapsulate protein-loaded composite microparticles. Candidate materials include PEG-based hydrogels synthesized by photopolymerization [7], and alginate hydrogels crosslinked by divalent ions [8, 9]. Tissue engineering scaffolds with greater mechanical strength are also highly desirable. Examples include collagen-apatite [10, 11] and chitin-apatite scaffolds [12].

The optimal combination of growth factors to be delivered from these scaffolds is not yet clear. Current studies suggest that synergies exist between BMPs and VEGF or TGF-β3 in bone healing [13, 14]. These combinations could be tested with our composite scaffolds while awaiting the elucidation of the signaling pathways in bone healing and the growth factors involved.
5.4 **In Vivo Studies**

Preliminary *in vivo* studies in this thesis were performed with BMP concentrations that were too low to induce ectopic bone formation in rats (see Section 4.3.5 [6]). These studies should be repeated with rhBMP-2 dosages of at least 10 μg (see Section 4.3.5.1 [5]). The effect of the rhBMP-2 release profile on bone formation could also be examined by incorporating different sets of composite microparticles exhibiting fast, intermediate and slow release, with accompanying evaluation of rhBMP-2 pharmacokinetics. Pharmacokinetic data would facilitate comparison between our composites and other carriers, and elucidate the effect of pharmacokinetics on osteoinduction.

In addition to radiographic and histological examination, biochemical assessment of the explants for markers such as alkaline phosphatase and osteocalcin would provide further evidence for osteoblastic differentiation.

A particular composite microparticle formulation was found to be exceptional in inducing elevated levels of alkaline phosphatase *in vitro*, which warrants further investigation *in vitro* and *in vivo*. This formulation was a 3:2 blend of 59 kD PLGA and 25 kD PLA with a sCAP loading of 0.08 mg per mg of polymer (see Section 3.3.4 [3]).

5.5 **Use of Composites in Other Shapes and Sizes**

Apatite-PLGA composites have been formed into bulk scaffolds and thin films (data not shown). The dimension of the composite, through its impact on diffusional length, is another variable in protein release that should be examined. Furthermore, bulk scaffolds have varying porosity and pore sizes, which influence water penetration and protein release. For the application of composite thin films as coatings, their homogeneity and their ability to adhere to different substrates (e.g. plastics, ceramics and metals) should be further studied.

5.6 **Application to Other Therapeutic Agents**

Any therapeutic agent that can be adsorbed onto apatite and withheld from premature release is a candidate for tunable release from these apatite-polymer composites. We foresee the gainful application of our composite microparticles to the delivery of other growth factors, such as VEGF, IGF, and TGF-β. Gene delivery might be possible with the encapsulation of
DNA-calcium phosphate complexes [15] in these composites. Small organic molecules, such as ascorbic acid and chlorhexidine have been found to adsorb onto hydroxyapatite [16]; hence, the delivery of small drug molecules should also be explored.

5.7 References


Chapter 6 – Conclusions

Apatite-PLGA composite microparticles were developed as a new controlled delivery platform for proteins. The release strategy was based on the hydrolysis of the biodegradable polymer (PLGA) in an aqueous medium in vitro or in vivo to form acidic degradation products, which would dissolve the basic inorganic phase (apatite), leading to the desorption and release of the proteins. Apatite-PLGA composite microparticles encapsulating BSA as a model protein or rhBMP-2 as the therapeutic protein were fabricated by a solid-in-oil-in-water emulsion process. The protein release profiles of these particles were varied by adjusting parameters that affected the polymer degradation and/or apatite dissolution, such as polymer molecular weight, polymer hydrophobicity, apatite particle size, apatite loading in the particles, and protein loading on the apatite. Increases in polymer molecular weight, apatite particle size and apatite loading were found to reduce the rate of protein release. A decrease in polymer hydrophobicity through the incorporation of a more hydrophilic, non-degradable polymer (PEG) was observed to increase the magnitude, but not the rate of protein release. In contrast, supplementation with a more hydrophobic, biodegradable polymer (PLA) led to diminished BSA release, but enhanced rhBMP-2 release. The effect was likely a decrease in the total amount of protein released, but an increase in the bioactive fraction preserved due to milder pH conditions fostered by the slower degrading hydrophobic PLA. A composite particle formulation of protein-sCAP-59 kD PLGA was identified as suitable for sustained rhBMP-2 delivery.

In vitro testing indicated that rhBMP-2 released from these composite microparticles was able to induce elevated expression of alkaline phosphatase and osteocalcin, markers of osteoblastic differentiation, in a mesenchymal stem cell line. Bioactive rhBMP-2 was detected at late stages of the release studies – upward of 70 days – and was found to be more potent when released from the composite microparticles than from conventional collagen sponges. In addition, the composite carriers were determined to be non-toxic and to support cell proliferation.

Protein-loaded composite microparticles were dispersed in secondary matrices – gelatin and collagen sponge – for bone tissue engineering. These matrices could be loaded with multiple sets of composite microparticles, each possessing a different release profile and
encapsulating a different growth factor. However, the crosslinking reaction used to form the gelatin scaffolds led to denaturation of rhBMP-2. Prefabricated collagen sponges injected with rhBMP-2-loaded and/or rhBMP-6-loaded composite particles were found to induce elevated levels of alkaline phosphatase and osteocalcin in vitro. Preliminary in vivo studies indicated enhanced cellularity and vascularity when composite collagen sponges containing rhBMPs were implanted in subcutaneous sites in rats. In contrast, blank composite collagen sponges displayed little tissue ingrowth and scaffold degradation.
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