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Development of an Injectable, In Situ Crosslinkable, Degradable Polymeric Carrier for Osteogenic Cell Populations

by

Richard Grady Payne

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE DOCTOR OF PHILOSOPHY

APPROVED, THESIS COMMITTEE

Antonios G. Mikos, Ph.D., John W. Cox Professor of Bioengineering and Chemical Engineering, Rice University

Kyriacos Zygourakis, Ph.D., Professor and Chair of Chemical Engineering, Rice University

Michael C. Gustin, Ph.D., Associate Professor of Biochemistry and Cell Biology, Rice University

Alan W. Yasko, M.D., Associate Professor of Surgery, Chief, Section of Orthopaedic Oncology, University of Texas M.D. Anderson Cancer Center

Michael J. Yasgur, M.D., Ph.D., Associate Professor of Orthopaedics and Bioengineering, Mayo Medical School

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Houston, Texas
ABSTRACT

Development of an Injectable, In Situ Crosslinkable, Degradable, Polymeric Carrier for Osteogenic Cell Populations

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Richard G. Payne

An injectable, in situ crosslinkable, degradable polymeric carrier for osteogenic cell populations was developed. Specifically, a system for encapsulating marrow stromal osteoblasts in gelatin microspheres has been implemented with the goal of incorporation into a crosslinking composite based on poly(propylene fumarate) (PPF).

Initially, the microparticle formation procedure was evaluated for effects on the marrow stromal cells. It was determined that the encapsulation procedure had only minor effects on the viability, proliferation, and phenotypic expression through 28 days.

The surfaces of the microparticles were treated to provide mechanical integrity at body temperature. The gelatin microparticles were exposed to two levels of a crosslinker in order to assess the effect of crosslinker concentration on cell viability, proliferation, and phenotypic expression. The results indicated that exposure to a relatively high concentration of the crosslinker (5 mM) for a relatively short amount of time (5 min) produced microparticles which maintained their mechanical integrity in 37 °C media for about one hour before dispersing. It yielded only minor reductions in the measured properties over 28 days. Physical properties of the crosslinked microspheres were measured. Based on these observations, it was concluded that the encapsulation
procedure we had developed was a candidate for use with the crosslinking PPF composite in the next study.

Cells encapsulated in crosslinked microparticles were placed on fully crosslinked PPF composites and on composites in various stages of crosslinking. The results showed that encapsulated cells retained their viability and proliferation to a much greater extent than nonencapsulated cells when placed on crosslinking substrates.

A final study was performed using one of the crosslinking composite addition times, and varying the formulation of the composites by adjusting the polymer to monomer ratio. The results of this 28 day experiment indicated that encapsulation of cells allowed them to remain viable and express the osteoblastic phenotype when placed on crosslinking PPF based composites. Nonencapsulated cells, however, did not retain their viability on those same crosslinking substrates.

The outcome of this work is that the resulting polymeric cell delivery system, which is injectable and *in situ* crosslinkable, holds promise for bone regeneration and orthopaedic tissue engineering.
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CHAPTER 1 INTRODUCTION

In 1994 over 250,000 operations were performed by orthopaedic surgeons which involved bone defects and the need for bone regeneration. (AAOS, 1998) Bone replacement is an important concern due to the large number of patients affected and the monetary cost of treating those patients. An understanding of bone function and physiology, along with bone grafting and replacement options, is essential to the design of bone regeneration strategies.

1.1 Bone Function

Bones have three principal functions. First, they serve as the primary storage depot of calcium ions. These ions are essential to many biological processes, and their concentrations in bodily fluids are tightly controlled. Secondly, the bone marrow serves as the site of production of hematopoietic cells, such as erythrocytes and leukocytes. (Yaszemski et al., 1996a) In addition, bone marrow contains pleuripotent cells called mesenchymal stem cells. These may differentiate into a variety of cell types, including osteoblasts and chondrocytes. (Buckwalter et al., 1995a) While these two functions can be provided by bone in other regions of the skeletal system, it is the third function of bone, that of providing mechanical support for body tissues and sites for muscle attachment, which is the focus of bone transplantation and regeneration efforts. (Yaszemski et al., 1996a) When a bone defect occurs which impairs this third function, only restoration of the mechanical properties at the defect site may resolve the situation.
1.2 Bone Composition

Bone is comprised of two types of substances, extracellular matrix and cells. These groups may be further described and categorized.

1.2.1 Extracellular Matrix

Extracellular matrix (ECM) can comprise as much as 90% of the volume of some bones. The inorganic or mineralized phase of bone is primarily a form of calcium phosphate known as hydroxyapatite, with the chemical formula \( \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 \). The organic portion of the bone ECM is similar to the ECM of fibrous tissues such as tendons and ligaments. The organic phase consists primarily of type I collagen (about 90%), non-collagenous glycoproteins, and bone specific proteoglycans. (Buckwalter et al., 1995a) The ECM bears the mechanical loads, serves to sequester vital mineral ions, and acts as a depot for growth factors.

1.2.2 Bone Cells

There are three types of bone cells. Osteoblasts line the surfaces of bone and secrete new bone matrix. Osteocytes are formed from osteoblasts entrapped in their own matrix. These cells have an extensive network of elongated cell processes, and are thought to have a role in signaling. Osteoclasts are large polynuclear cells which dissolve small volumes of bone, leading to bone resorption. (Buckwalter et al., 1995a) In addition to these cells, bone contains marrow elements, whose function was previously described, and vasculature, which delivers nutrients to and removes wastes from the bone cells.
1.3 Histology

Bone exists in two forms, cortical and cancellous. Cortical bone, also called compact bone, forms the shafts of long bones and the surfaces of flat and short bones. Cortical bone is characterized by low porosity (about 10%) and high density (about 2 g/cm³). Mature cortical bone is anisotropic, with mechanical properties primarily depending on the orientation of parallel cylindrical units called osteons. Cancellous bone is also referred to as trabecular or spongy bone. It is located at the end of long bones and in the interior of flat and short bones. Cancellous bone is less dense (less than 1 g/cm³) than cortical bone, with a much higher porosity (50-90%). This difference is a result of the rod and plate orientation of the inorganic phase of the ECM, which forms an open-celled foam. Mature cancellous bone is also anisotropic. (Buckwalter et al., 1995a; Yaszemski et al., 1996a)

Both cortical and cancellous bones may be present in woven (immature) or lamellar (mature) forms. Woven bone is formed during embryogenesis and fracture callus healing. The ECM of woven bone has a random orientation, and is therefore isotropic. Woven bone is later remodeled into lamellar bone through resorption of mineralized ECM by osteoclasts and deposition of new ECM (osteoid) by osteoblasts, which becomes mineralized. The new lamellar bone possesses orientation dictated by the local mechanical environment, conferring anisotropy upon it. (Buckwalter et al., 1995a; Yaszemski et al., 1996a)

1.4 Bone Formation

Initial bone formation in the embryonic stage occurs through two mechanisms: endochondral and intramembranous ossification. Endochondral ossification of the long
bones starts with undifferentiated cells which produce a cartilage-like matrix and subsequently differentiate into chondrocytes. A periosteal covering surrounds the cylindrical diaphysis and begins to form a thin layer of bone. Some of the cartilaginous regions mineralize, followed by enlargement of the chondrocytes. The cartilage is invaded by vascular buds, which lead to the resorption of the central portion, forming the marrow space. Osteoprogenitor cells migrate into the region with the vascular buds and, after differentiating into osteoblasts, form bone matrix on the calcified cartilage. The new woven bone and mineralized cartilage are later resorbed by osteoclasts. Osteoblasts then deposit mature lamellar bone. (Buckwalter et al., 1995b)

Intramembranous bone formation of flat bones occurs without a cartilaginous precursor. Undifferentiated mesenchymal cells form layers, and produce a tissue which can contain blood vessels, fibroblasts, and preosteoblasts. After differentiating into osteoblasts, these cells deposit spicules of bone matrix. Osteoblasts add more bone woven matrix onto these islands of bone, which is mineralized and eventually remodeled into lamellar bone. (Buckwalter et al., 1995b)

Once bones have formed, they grow to skeletal maturity in two ways. The long bones increase in length via longitudinal growth in a form of endochondral ossification. Cells at the ends of the bones proliferate and secrete cartilaginous matrix, increasing the length of the bone. As newly dividing cells at the ends continue to proliferate, older cells away from the ends become hypertrophic. They secrete vesicles which are the loci of calcium deposition on the cartilage matrix. These regions are then invaded by vasculature bringing cells which further remodel the ECM and deposit bone matrix. This woven bone is later remodeled to form lamellar bone.
The second method of bone enlargement is known as appositional ossification. This occurs when cells from the periostal membrane differentiate into osteoblasts on the surface of the existing bone. They synthesize osteoid in layers, forming lamellar bone, and thus increasing bone girth. This method of bone formation eliminates the use of both cartilaginous intermediates and woven bone. (Buckwalter et al., 1995b)

1.5 Bone Injury

Bone injury can occur in a variety of ways. These include trauma, tumor, disease, and surgery. A common feature of the resulting fractures and osteotomies is that they form an anatomic discontinuity in the affected region. As a result, there is mechanical instability between the fragments, leading to an attempt by the body to repair the injury.

1.6 Bone Repair / Fracture Healing

Repair of fractures and osteotomies can occur through four biological phases of healing. First, inflammation results from factors released by clotting factors at the injury site. Inflammatory cells remove the necrotic tissues, and the second phase of healing – repair – begins. In the reparative stage, a soft callus is formed by cells derived from pluripotent mesenchymal cells. This callus is composed of fibrocartilagenous and organic bone ECM, and provides initial interfragmentary stability. The next stage, modeling, is often grouped with the reparative stage. The modeling phase is characterized by the mineralization of the soft callus to form a hard callus. This hard callus confers mechanical stability to the fracture site. The final stage of indirect fracture repair is remodeling. In this phase, the immature woven bone is resorbed and redeposited as mature lamellar bone. (Brighton et al., 1994; Yaszemski et al., 1996a)
Additionally, it is possible to biomechanically describe the healing of fractures.

Fractures proceed through four stages of biomechanical healing, which can be characterized by the site and stiffness of failures when torsional loads are applied to the fracture site. In stage I, failure occurs with low stiffness at the initial fracture site. The stage II failure also occurs at the fracture site, but with a higher stiffness. Failure in stage III occurs partially through the fracture site and partially through intact bone, with high stiffness. Finally, stage IV failure occurs with high stiffness only through intact bone. (White III et al., 1977)

1.7 Requirements for Repair / Regeneration

As described in the previous section, the completion of the normal healing cascade requires vascularization to bring in inflammatory and preosteoblast cells (Buckwalter et al., 1995a; Kim et al., 1997) and mechanical stability. (Gazdag et al., 1995; Hollinger and Kleinschmidt, 1990) Three other elements necessary for bone regeneration: osteoconduction, osteoinduction, and an osteogenic cell population, (Gazdag et al., 1995) Osteoconduction involves the presence of a substrate or scaffold upon which bone cells may attach, proliferate, and migrate. Osteoinduction involves the presence of signaling molecules which attract osteoblasts or preosteoblasts through recruitment and migration, or leads to the differentiation of uncommitted cells to the osteoblastic lineage. An osteogenic cell population is a population of cells with the potential to make bone, either osteoblasts or osteoblast progenitors.

These five characteristics are required for successful fracture repair. If the body is unable to provide them, they must be exogenously supplied. This is often the case when a bone defect is larger than a certain size. Such a critical size defect will fill with fibrous
tissue, but it will not ossify. (Hollinger and Kleinschmidt, 1990) This condition can also occur after fractures, where it is called a fibrous non-union and can be the result of micromotion or other factors. Additional intervention is then necessary in order for the defect to heal.

1.8 Bone Grafts

Often, the intervention required to repair the bone defects come in the form of bone grafts. These occur in the form of autologous grafts (autografts) or allogologous grafts (allografts).

1.8.1 Autografts

Autografts are bone grafts which are harvested from one site and placed into another site within the same patient. They are considered the gold standard in bone transplantation. (Gazdag et al., 1995; Yaszemski et al., 1996a) The autograft can be cancellous bone, cortical bone, or a combination of the two. It can impart little or no mechanical properties, as in morcellized pieces harvested from the iliac crest, or significant mechanical properties, as in a portion of the fibula. Autografts may possess or induce four of the five of the elements for bone regeneration. An osteoconductive organic and inorganic matrix is coupled with osteoinductive cytokines found in the matrix. Mechanical strength may be present and increases as the graft is incorporated into the host bone and remodeled. Vascularization can be accomplished by revascularization of the graft or by transferring blood vessels with the graft and reanastomosing them at the defect site. The fifth component of regeneration, osteogenic cells, are transplanted with an autograft. However, unless the autograft is reconnected to the blood supply
immediately (Gazdag et al., 1995) it is unlikely that the cells significantly contribute to the regeneration process. (Yaszemski et al., 1996a) The autograft yields superior results because it usually fully integrated and is completely remodeled. (Gazdag et al., 1995) There are drawbacks associated with autografts, however. These include a second surgery site, donor site pain and morbidity, and a limited supply of bone which can be harvested. (Gazdag et al., 1995)

1.8.2 Allografts

Allografts are also commonly used and typically consist of cadaver bone which has been processed to destroy the biologically active portion, leaving only the mineralized phase. This removes the osteoinductive activity, but the osteoconductive scaffold remains, and may supply some mechanical strength, depending on the form of the graft. The allograft is vascularized from the surrounding tissue and repopulated by osteogenic cells. It is subsequently incorporated into the host tissue and remodeling occurs. While there is no limit to the supply, allografts do have drawbacks. There is no osteoinductive effect, it can be rejected and resorbed without remodeling, and, even though the graft is processed, there exists a small possibility of pathogen transfer. (Gazdag et al., 1995)

1.9 Bone Graft Substitutes

In an effort to address the deficiencies of autografts and allografts, several bone graft substitutes have been investigated. These substitutes attempt to add a single element or combination of the elements for necessary bone regeneration. The following sections will describe these substitutes and combination approaches are described under one of the attributes which they are replacing.
1.9.1 Osteoconductive Scaffolds

Natural bone graft substitutes serving as osteoconductive scaffolds include demineralized bone matrix (DBM) and type I collagen. DBM is formed via the acid extraction of bone. The remaining structure is comprised of collagen and noncollagenous proteins in a porous scaffold structure. DBM also contains osteoinductive growth factors which remain after processing. (Bolander and Balian, 1986; Gazdag et al., 1995) Type I collagen, the primary constituent of DMB can be isolated, processed, and formed into scaffolds, which are placed into bone defects. (Pasquier et al., 1996; Yasko et al., 1992) Other natural polymers used in this application include methylhydroxypropylcellulose (Grimandi et al., 1998) and cellulose. (Dupraz et al., 1996) These materials are frequently mixed with a ceramic to form a composite or used as a carrier of osteoinductive growth factors. These natural graft substitutes tend to have poor mechanical properties so graft sites require that additional mechanical stability be supplied.

Ceramics are inorganic, crystalline, materials that can be formed into a variety of shapes, sizes, and pore morphologies. In orthopaedics, the most commonly used ceramics are hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP). These are osteoconductive scaffolds which possess some mechanical strength. Hydroxyapatite is very similar to the mineral phase of bone and coral, and can be slowly resorbed, during bone remodeling. β-Tricalcium phosphate resorbs somewhat more rapidly, allowing for faster removal of the material. (Jarcho, 1981; Yaszemski et al., 1996a) Ceramic particulates are frequently incorporated into polymeric composites due to their osteoconductive potential. (Dupraz et al., 1996; Grimandi et al., 1998; Maquet and
Jerome, 1997; Pasquier et al., 1996; Peter et al., 1998b; Schmitt et al., 1998; Yaszemski et al., 1996b) A drawback of ceramics include a difficulty shaping the material to exactly fit the defect and the lack of osteoinduction. Other ceramics used in orthopaedic applications include dahlilitie (an injectable calcium phosphate), (Constanz et al., 1995) bioglasses, (Hollinger et al., 1996) and plaster of Paris (calcium sulfate). (Anson, 1996; Pecora et al., 1997; Sottosanti, 1995, 1997)

Synthetic polymeric materials are also used to aid the regeneration of bone. One advantage synthetic polymers have over other materials is that, due to the nature of their repeating-unit structure, many of their properties may be tailored to suit specific applications. Synthetic polymers can be divided into two categories: degradable or nondegradable. The most commonly used polymer in orthopaedic applications is poly(methyl methacrylate) (PMMA), also known as bone cement. It is a synthetic nondegradable polymer which is injectable and polymerizes in situ. It is readily available in a sterile form and possesses mechanical properties between those of cortical and trabecular bone. (Yaszemski et al., 1995) The disadvantages associated with PMMA arise from its nondegradability. As such, it remains in the body and is not resorbed, preventing its replacement with host tissue. It addition, PMMA can be prone to fatigue damage after extended periods of time. (Topoleski et al., 1990) The injectable nature of bone cement is desirable, as it allows the polymer to interdigitate with the interstices of cancellous bone, and to fill irregularly shaped defects.

Degradable synthetic polymers investigated for use in filling bone defects include poly(α-hydroxy esters), poly(orthoesters), polyanhydrides, polyurethanes, (Yaszemski et al., 1996a) poly(dioxanones), (Nichter et al., 1992) poly(caprolactones),
poly(phosphazines), and poly(amine acids). (Hollinger et al., 1996) Most of these materials are formed into porous osteoconductive scaffolds which allow for bone ingrowth and vascularization. Their degradation into biocompatible materials, which are then removed from the body, leads to replacement of the polymer with woven bone. This bone will then be resorbed and redeposited during normal remodeling processes, so no foreign material permanently remains in the body. This is the main advantage that degradable polymers have over non-degradable materials.

Within the degradable polymers group is a subset comprised of injectable materials, which, after injection as a lower viscosity fluid, form hardened scaffolds *in situ*. Often these materials are combined with ceramics to increase their osteoconductive properties. Several investigators have used poly(propylene fumarate) (PPF), an unsaturated linear polyester, in a composite which can be crosslinked at the time of injection by N-vinyl pyrrolidinone. (Domb et al., 1996; Gerhart et al., 1989; Gresser et al., 1995; Peter et al., 1998ab, 1999b; Yaszemski et al., 1996ab) The composites contain benzoyl peroxide and dimethyl toluidine as an initiator and accelerator, sodium chloride as a porogen, and may also contain β-tricalcium phosphate. Schmitt, et al. reported on an injectable bone wax polymer ceramic composite {poly(ε-caprolactone-co-glycolide) and β-TCP} containing a growth factor (TGF-β1) as a material which significantly increased the formation of bone in a defect. (Schmitt et al., 1998) Hollinger et al. also reported on the use of composite which consists of a copolymer of para-dioxanone, glycolate, and crosslinked by a diisocyanate-like compound designated as “lactomer diisocyanate”. (Hollinger et al., 1996) This material is injectable, has a working time of about 10 minutes, and can be mixed with β-TCP or HA. Grimandi et al. describe the in
vitro evaluation of an injectable solution of methylhydroxypropylcellulose mixed with granules of β-TCP and HA. (Grimandi et al., 1998) Pasquier et al. reported an injectable collagen-HA material which, unfortunately, exhibits less bone growth than unfilled controls. (Pasquier et al., 1996) Dupraz, et al. reported the physical microcharacterization of an injectable cellulose-calcium phosphate material and postulate its effectiveness in spine surgery. None of these injectable or moldable composites incorporate cells at the time of implantation.

1.9.2 Osteoinductive Factors

The primary group of osteoinductive factors which have been investigated are the family of bone morphogenetic proteins (BMPs). This group, whose members are all similar to the bone-inducing compound first isolated by Urist and Strates, includes over 40 members. (Hollinger, 1997) Many of them are members of the transforming growth factor-beta (TGF-β) and have the ability to cause bone to form heterotopically. Members of this group may be known by other names, such as osteogenin (BMP-3), and osteogenic proteins-1, -2, and -3 (BMP-7, -8, and -8B). (Hollinger, 1997) In order to be effective in regenerating bone in defect sites, these compounds must be incorporated into a carrier material, such as β-TCP (Urist et al., 1987), inactive bone matrix (Gerhart et al., 1993), poly(lactic acid) (Heckman et al., 1991), type I collagen and saccharide spheres. (Yasko et al., 1992)

1.9.3 Osteogenic cell population

Attempts have been made to deliver osteogenic cells to bone defects. This is frequently accomplished by using a biodegradable scaffold to as a carrier. Ishaug-Riley et al.
implanted porous poly(lactic-co-glycolic acid) (PLGA) scaffolds upon which osteoblasts had been cultured. (Ishaug-Riley et al., 1997a) Upon explantation, a thin layer of bone (300 μm) was formed at the periphery, with no bone formation in the center of the construct. This effect was due to nutrient transport limitations and a lack of early vascularization within the construct. These 95% porous foams had marginal mechanical strength and, while processable into a variety of shapes, were not injectable or moldable in situ. Vacanti and Upton reported a system whereby bone cells were cultured on PGA fibers before implantation. (Vacanti and Upton, 1994) They report some bone formation, but it is not quantitated. This method allows for limited molding and fitting of the fibers, however the initial mechanical properties with this method are inadequate. Caplan and Bruder reported the use of a ceramic cube/osteoblast implant in an attempt to regenerate bone. (Caplan and Bruder, 1997) This method was successful, but the ceramic cube does not allow for moldability. In addition, the dimensions of these cubes are 3 mm on a side. It is unknown if similar results would be generated with a larger construct.

In addition, bone marrow, which contains osteoprogenitor cells and a milieu of proteins, has been delivered (Gazdag et al., 1995), either alone (Connolly et al., 1989) or in combination with inorganic matrix. (Ohgushi et al., 1989)

1.10 Summary

When the mechanical integrity of a bone is compromised, it cannot perform one of the primary functions of skeletal system. This function – to support soft tissues and provide attachment sites for musculature – provides protection for vital organs and permits locomotion. There are five requirements in order for the mechanical integrity to be restored via normal fracture healing mechanisms: mechanical stability, vascularization,
osteocorticotomy, osteoinduction, and an osteogenic cell population. If one of these factors is deficient, intervention is required to heal the defect. This intervention frequently comes in the form of bone grafts, either autografts or allografts. However, the limitations of these techniques have led to the investigation of other bone graft substitutes. There have been a wide range of materials examined for this purpose, including demineralized bone matrix, natural and synthetic polymers, ceramics, osteoinduction factors and cellular components. It would be desirable, however, to develop an injectable, biodegradable, synthetic polymeric composite which will deliver an osteogenic cell population. It is this void which leads to the objectives described in Chapter 3.
CHAPTER 2 POLY(PROPYLENE FUMARATE)

Poly(propylene fumarate) (PPF) is an unsaturated linear polyester. PPF has been suggested for use as a scaffold for guided tissue regeneration, often as part of an injectable bone replacement composite. (Domb et al., 1996; Lewandrowski et al., 1999; Peter et al., 1999a; Yaszemski et al., 1995) PPF has been used for controlled release of antibiotics (Gerhart et al., 1988) and as a substrate for osteoblast culture. (Peter et al., 2000b) In addition, PPF has been modified with poly(ethylene glycol) to form a crosslinkable copolymer. (Suggs et al., 1997, 1998; Suggs and Mikos, 1999a) Degradation of the PPF ester bonds via hydrolysis yields propylene glycol and fumaric acid, (He et al., 2001) which are biocompatible and readily removed from the body. The double bond along the backbone of the polymer permits crosslinking in situ, which causes a moldable composite to harden within 10-15 minutes. (Peter et al., 1999a) Mechanical properties and degradation time of the composite can be controlled by varying the PPF molecular weight. (Peter et al., 1999ab)

Several schemes have been used to synthesize PPF. Gresser et al. reported making PPF via a 2 step process whereby an equimolar reaction mixture of fumaric acid and propylene glycol were combined in an acid catalyzed reaction, first at 250 °C at 760 mmHg and then at 220 °C at 1 mmHg. (Gresser et al., 1995) This reaction can be difficult to control due to stoichiometric imbalance resulting from loss of the volatile propylene glycol.

Domb et al. report synthesis of PPF via direct condensation of complementary trimers. (Domb et al., 1990) Bis(2-hydroxypropyl fumarate) and bis(hydrogen maleate) trimers were formed, then reacted together, producing PPF polymer. Although the
reactants in the condensation reaction are non-volatile, the reaction is still sensitive to unbalanced stoichiometry.

Kharas et al. describe the analysis of several reactions (diethyl fumarate with a variety of diols and catalysts). (Kharas et al., 1997) It was determined that the method with the highest yield was the reaction of diethyl fumarate with 10% molar excess propylene glycol in the presence of an acid catalyst - zinc chloride (ZnCl₂). This reaction required a maximum temperature of 200 °C.

Peter et al. reported synthesis of PPF in a two step reaction (Peter et al., 1999b) using a modification of a method by Yaszemski et al. (Yaszemski et al., 1996b) In the first step fumaryl chloride was reacted with a large excess of propylene glycol in the presence of a proton scavenger to produce the bis(2-hydroxypropyl fumarate) diester intermediate. In the second reaction, this diester intermediate was transesterified at 160 °C, producing PPF with propylene glycol as a byproduct. The advantage of this system is that, since the only reactant in the second step is the diester intermediate, there are no stoichiometric concerns. An intermediate purification step was included to remove the proton scavenger before the transesterification. NMR results have indicated partial loss of the double bonds due to HCl addition. (Peter et al., 1999b) This synthesis method virtually ensures that the resulting polymer is hydroxy-terminated, which presents advantages for performing additional chemistry, such as the covalent linking of peptides to PPF. (Jo et al., 2000)

Our laboratory has developed a synthesis method which is a combination of those described by Kharas et al. (Kharas et al., 1997) and Peter et al. (Peter et al., 1999b). In the presence of ZnCl₂, diethyl fumarate is reacted with a large excess of propylene glycol
to first form the bis(2-hydroxypropyl fumarate) diester. This is then transesterified to form PPF. Using this method, PPF with number average molecular weights of 800-4800 have been synthesized. In a manner similar to that reported by Kharas et al., no intermediate purification step is performed. Lower reaction temperatures also tend to yield fewer undesired side reactions. As a result, NMR analysis indicates that this reaction yields a more pure product than the fumaryl chloride/propylene glycol synthesis method by Peter et al. (Shung et al., 2001)
CHAPTER 3 OBJECTIVES

The goal of this project is to engineer bone through the use of an injectable, biodegradable, polymeric bone substitute containing an osteogenic cell population. Specifically, the focus of the work contained within this thesis was to develop a cell delivery method for use with this composite. It is envisioned that the osteogenic cells will initiate a regeneration cascade and eventually bone will replace the degrading polymer. A highly unsaturated linear polyester, poly(propylene fumarate) (PPF), was developed as a candidate material for this application. As part of a moldable composite, it crosslinks in situ in a physiological environment to restore mechanical stability to a defect region. However, certain changes in the local environment that occur during the crosslinking of the composite appear to be detrimental to cell survival. This necessitates the use of a cell delivery method.

In this application, a successful cell delivery method must meet several design criteria. 1) The cell carrier must preserve the viability and phenotypic expression of the cell population during the composite crosslinking. 2) It must maintain its mechanical integrity during the composite crosslinking. 3) The cell carrier must allow the cells to attach and function in their new environment following the completion of the composite crosslinking. The final criterion requires that the cell delivery system should only contain the cells for a very short time (1 hour or less) before releasing them to interact with their surroundings. We hypothesized that successful cell delivery meeting all the design criteria could be achieved through the temporary encapsulation of cells in gelatin microparticles. We also hypothesized that crosslinking the surfaces of those microparticles will enhance their mechanical stability.
Initially, an encapsulation method was developed and the microparticle size distribution was determined. Then, the effects of gelatin encapsulation on marrow stromal osteoblast viability, proliferation, and phenotypic expression were evaluated. Next, the surfaces of the microparticles were treated with two concentrations of crosslinker to determine the effect of the crosslinker concentration on the viability, proliferation, and phenotypic expression of the encapsulated cells. Characterization of the resulting crosslinked microparticle shells was performed. Specifically, shell thickness and microparticle burst strength were measured for each crosslinker concentration.

Once the encapsulation and surface crosslinking methods were established, our attention turned to the crosslinking PPF composites and their interaction with the microparticle-encapsulated cells. In preparation for the following studies, the onset of gelation and the maximum temperature rise of three PPF composites with varying polymer/monomer ratios were measured. Both the nonencapsulated cells and cells encapsulated in crosslinked microparticles were then placed on thick layers of a PPF composite formulation at various time points during crosslinking, with the aim of determining the effect of this addition time on the cell viability and proliferation. Based on these results, an appropriate addition time point was chosen for the next study, in which encapsulated and nonencapsulated cells were added to thick layers of three composite formulations during crosslinking. We examined the effects of the polymer/monomer ratio on the viability, proliferation, and phenotypic expression of both encapsulated and nonencapsulated cells in an effort to quantify the benefits of temporary gelatin encapsulation as a cell delivery system with an injectable in situ crosslinkable composite formulation.
CHAPTER 4  ENCAPSULATION OF CELLS IN GELATIN MICROPARTICLES

4.1 Background

While cell encapsulation has been well documented in the literature, these efforts have been usually directed toward long term (months or longer) immunoisolation. (Avgoustiniatos and Colton, 1997; Babensee and Sefton, 1996; Lanza et al., 1999; Sefton et al., 1997; Uludag et al., 1994; Zielinski et al., 1997) No information is available from the literature describing approaches for very short term (less than 1 hour) encapsulation. We believe the cells incorporated into a crosslinking polymeric bone substitute only need to be protected from the local environment during polymer crosslinking (approximately 20 min). After that time, the cells will need to interact with their surroundings to attach, proliferate, and proceed to form mineralized tissue. Gelatin, the proposed material for encapsulation, is a biocompatible (Sakai et al., 1998) and biodegradable natural polymer with minimal immunological responses. (Tabata et al., 1998) It possesses thermal gelation characteristics that can be adjusted through concentration and processing.

This work describes an investigation to test the hypothesis that rat marrow stromal osteoblasts can be temporarily encapsulated in gelatin microparticles and subsequently retain their viability, proliferation, and phenotypic expression. Studies were also undertaken to characterize the microparticles.

4.2 Materials

4.2.1 Cell Culture

Dulbecco’s Modified Eagle Medium (DMEM) (Gibco, Gaithersburg, MD)
Trypsin/ethylenediaminetetraacetic acid (EDTA) (Gibco, Gaithersburg, MD)
Phosphate buffered saline (PBS) (Gibco, Gaithersburg, MD)
Penicillin/Streptomycin/Fungizone (PSF) (Gibco, Gaithersburg, MD)
Neomycin (Sigma, St. Louis, MO)
Tetracycline (Sigma, St. Louis, MO)
Gentamicin (Sigma, St. Louis, MO)
Dihydroxyvitamin D₃ (Sigma, St. Louis, MO)
Dexamethasone (Sigma, St. Louis, MO)
Fetal bovine serum (FBS) (Gemini, Calabasas, CA)
All remaining chemicals were obtained as cell culture grade (Sigma, St. Louis, MO)

4.2.2 Microparticle Formation

Porcine gelatin [G-1890] (Sigma, St. Louis, MO)
Mineral oil (Acros, Fisher Scientific, Pittsburgh, PA)

4.2.3 Assays

Hoechst 33258 dye for the DNA assay (Polysciences, Warrington, PA)
Tritiated thymidine (Sigma, St. Louis, MO)
Ecolmune scintillation cocktail (ICN, Irvine, CA)
All osteocalcin assay materials (Biomedical Technologies, Stoughton, MA)
Chemiluminescent substrate (0.4 mM disodium 3- (4-methoxyspiro [1,2-dioxetane-3,2'-
(5'-chloro) tricyclo [3.3.1.1³⁷] deca|) -4-yl) phenyl phosphate (CSPD®) with
Sapphire II, CD100RX) (Tropix, Bedford, MA)
Calf intestinal alkaline phosphatase (CIAP) (Promega, Madison, WI)
Tris and Triton X-100 (Fisher Scientific, Pittsburgh, PA)
Additional chemicals needed to make solutions or buffers for the assays were obtained as cell culture grade (Sigma, St. Louis, MO).

4.3 Experimental Design

The cell number was measured via DNA assay and cellular proliferation was measured by $^3$H-thymidine incorporation assay. Early and late differentiation markers of phenotypic expression were measured using alkaline phosphatase, osteocalcin, and mineralization assays. In this study, 0.5 g of microparticles containing encapsulated cells (EC) were dispensed into sample wells on a 12 well tissue culture plate. The seeding density corresponded to $2.03 \times 10^5$ cells/well, or $5.3 \times 10^4$ cells/cm$^2$, approximately enough for a confluent monolayer. (Ishaug et al., 1994) Aliquots containing the same cell number were added directly to wells, without undergoing the encapsulation procedure, as nonencapsulated control cells (NC). Microparticles formed without cells served as negative controls. Samples, positive controls, and negative controls were each seeded into 4 wells for each assay at every time point. The DNA, alkaline phosphatase, and osteocalcin assays were obtained from the same wells, while the $^3$H-thymidine and mineralization assays were obtained from separate wells. Samples were taken on 1, 4, 7, 14, 18, 21, 24, and 28 days for the DNA, and alkaline phosphatase activity assays; on 1, 4, and 7 days for the proliferation assay; on 7, 14, 21, and 28 days for the mineralization assay; and on 7, 14, 18, 21, 24, and 28 days for the osteocalcin level assay.
4.4 Methods

4.4.1 Cell Harvest and Culture

Rat marrow stromal cells were harvested from the hind limbs of 6 week old male Sprague-Dawley rats as previously described. (Ishaug-Riley et al., 1997b) These cells were cultured in T-75 tissue culture flasks in 12 ml primary media (DMEM, 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 0.25 μg/ml amphotericin B (antimycotic), 50 μg/ml gentamicin, 10 μg/ml tetracycline, 100 μg/ml neomycin). The marrow cells were seeded at a density of $5 \times 10^7$ nucleated cells per flask. After 2 days, the media and nonadherent cells were removed and replaced with fresh primary media. Media were changed every other day for 6 additional days, by which time the cells were approximately 80% confluent. After a total of 8 days in culture, the cells were rinsed with PBS, enzymatically lifted with trypsin/EDTA solution (0.5% trypsin, 5.3 mM EDTA•4Na), pelleted via centrifugation, and resuspended in 5 ml of complete media [primary media plus osteogenic supplements (1 x $10^{-8}$ M dexamethasone, 10 mM Na $\beta$-glycerol phosphate, 32 μg/ml L-ascorbic acid 2-phosphate)]. A 50 μl sample of cells was counted with a Coulter Counter measuring sizes between 8 and 32 μm. A volume containing 2.03 $\times 10^7$ cells was pipetted into a 15 ml centrifuge tube. The cells were diluted with complete media to 5 ml to be added to 45 ml gelatin solution.

4.4.2 Gelatin Solution Preparation

A 10% porcine gelatin solution in media was prepared in three stages. First, a 30% w/v aqueous solution was prepared by heating 70 ml ddH$_2$O to 95°C on a hotplate while mixing it with a magnetic stirrer. A total mass of 30 g porcine gelatin was added to the
water in approximately 0.25 g portions. Each portion was allowed to completely dissolve before the addition of the next. Once all of the gelatin was dissolved, the volume of the solution was increased to 100 ml by the addition of ddH₂O. The resulting 30% solution solidified as it cooled to room temperature, and was stored at 4°C. In the second stage, an 11% gelatin solution was prepared from the 30% solution by the following process. DMEM and the 30% gelatin solution were warmed to 50°C. A mass of 33.33 g of the 30% gelatin solution and volume of 56.67 ml DMEM were combined in a beaker. Following thorough mixing, the pH was adjusted to 7.2 by the addition of approximately 10 µl 5M NaOH. The resulting 11% solution was again warmed to 50°C. In a laminar flow hood, a 0.22 µm bottle top filter (Fisher 09-761-50) was affixed to a sterile media bottle. A short length of autoclaved flexible PVC tubing with a sterile inline filter was attached to the vacuum port of the bottle top filter. The entire apparatus was allowed to equilibrate for 20 min in a 37°C warm room. The warm gelatin solution was then sterilized via vacuum filtration in the 37°C room. The apparatus was disassembled in the laminar flow hood. This sterile 11% solution was stored at 4°C until use.

In the final stage of gelatin preparation, 45 ml of the 11% solution at 37°C were diluted with 5 ml of a sterile suspension of cells in DMEM and mixed thoroughly. The resulting cell suspension of 4.06 x 10⁵ cells/ml in a 10% gelatin solution was equilibrated at 37°C.

4.4.3 Microparticle Formation

In a laminar flow hood, the gelatin/cell suspension was placed into two 30 ml syringes, which were fitted with 22G needles. The suspension was allowed to cool to 34°C before being dripped into 400 ml sterile mineral oil at 10°C, which was agitated with a magnetic
stirrer. The hydrophobic interaction with the oil caused the suspension to form droplets, and the temperature change caused the droplets to harden.

Once the microparticles had formed, they were filtered and collected on a cold 80 μm nylon mesh (Spectrum Laboratories, Rancho Dominguez, CA). The microparticles were transferred to a beaker containing 400 ml sterile PBS at 15°C, agitated by a magnetic stirrer. Agitation was stopped; the microparticles were allowed to settle; residual mineral oil floating on the surface was aspirated; and the PBS was decanted. The microparticles were again filtered and collected on another cold 80 μm nylon mesh. The mesh was then placed on sterile absorbent towels on an aluminum tray in an ice bath to help wick away residual PBS while ensuring the microparticles remained cool.

4.4.4 Tissue Culture Plate Preparation and Culture Conditions

The tissue culture wells were preincubated with 0.5 ml 10% FBS for 1 hr in an effort to increase cell attachment. The FBS was removed by aspiration and 1.5 ml complete media were placed in each well prior to cell seeding.

The tissue culture plates were kept in an incubator at 37°C, with 5% CO₂ and 95% relative humidity. Prior to encapsulation, all cells were cultured in primary media; following encapsulation, the samples and controls were cultured in complete media through the end of the study. Each well contained a volume of 2 ml. The media were changed every 2 days, with approximately 0.5 ml media left in the well during the first 2 media changes to allow for complete removal of residual mineral oil. The mineral oil is less dense than media; this method allowed the oil to partition to the surface, reducing its contact with the tissue culture surface. The subsequent media changes involved complete removal and replacement of the entire 2 ml.
4.4.5 Assays

4.4.5.1 Sol-Gel Transition Assay

The sol (flow)-gel (no flow) transition of the 10% gelatin solution was measured using a previously reported procedure. (Jeong et al., 1999) The 10% gelatin was prepared and 1 ml was aliquotted into each of six 5 ml test tubes. The samples were equilibrated in a 4 °C refrigerator. After 24 h, samples were removed and placed into a constant temperature water bath at temperatures ranging from 28 to 32 °C. Following equilibrating for 20 min, the test tubes were inverted. If no fluidity was visually observed in 1 min, the solution was determined to be in a gel state. Temperature was monitored with a mercury thermometer with and accuracy of ± 0.5 °C.

4.4.5.2 Microparticle Size Assay

Freshly formed microparticles were widely spread on petri dishes and imaged using the microscope and camera system described in the mineralization assay (Section 4.4.5.7). The images were then analyzed for microparticle diameter using NIH Image 1.59. Two hundred individual microspheres were imaged and analyzed.

4.4.5.3 DNA Assay

A fluorometric assay was used to determine total DNA content, and thus total cell number. (Peter et al., 1998) At the appropriate time points the wells were rinsed with phosphate buffered saline (PBS) and aspirated 3 times. The samples were then frozen (-40°C) until testing. At that time, all of the plates were thawed and DNA was extracted by subjecting the cultures to 4 freeze-thaw cycles in the presence of 0.5 ml lysis buffer consisting of 25 mM Tris and 0.5% Triton X-100 (pH~8). The cell suspensions were
transferred to 5 ml tubes, and 20 µl were removed for the alkaline phosphatase assay. To
every tube, 1 ml of 10 mM EDTA (pH 12.3) was added, and the samples were sonicated
for 10 minutes in ice water. The tubes were incubated for 20 minutes at 37°C and placed
on ice. The pH was adjusted to approximately 7.0 by adding 0.2 ml KH₂PO₄ (1.0 M) to
each tube. At the time of analysis, 1.5 ml of a 200 ng/ml Hoechst solution was added to
each sample, which was then read on BioRad VersaFlour fluorometer (BioRad, Hercules,
CA) with absorbance filters of 455 nm and excitation filters of 350 nm. Total cell
numbers were determined using cell standard calibration curves, after subtracting
appropriate blanks (values for acellular microspheres were subtracted from the values for
encapsulated cells, values for blank wells were subtracted from nonencapsulated
controls).

4.4.5.4 ³H-Thymidine Assay

Cell proliferation was measured by assaying the incorporation of ³H labeled thymidine.
(Peter et al., 1998) As cells divide and proliferate, the ³H-thymidine is incorporated into
the newly formed DNA. Quiescent cells do not incorporate any thymidine. Thus, the
amount of ³H-thymidine incorporated is proportional to the amount of DNA synthesized,
and if taken over a consistent time period for all cultures, proportional to the rate of DNA
synthesis. Twenty-four hours before the assay timepoint, 7 µl of ³H thymidine solution
were added to the 2 ml of media in each well. At the time of the assay, the media was
aspirated and the wells were rinsed 3 times with PBS. Then 1.0 ml of a trypsin/EDTA
solution was added to each well and the samples were incubated for 30 minutes at 37°C.
The contents of the well were then transferred to a scintillation vial containing 10 ml of
Ecolume scintillation cocktail. Each well was rinsed with 0.5 ml PBS, which was also
added to the scintillation vial. The β emissions were read by a Minaxi β Tricarb 4000 Series Liquid Scintillation Counter (Packard, Laguna Hills, CA) for 2 minutes. Following subtraction of appropriate blanks, the results were normalized with cell number as determined by DNA assay.

4.4.5.5 Alkaline Phosphatase Assay

Alkaline phosphatase (ALPase) activity was measured according to a published method (Blum et al., 2001). The samples used were the same as in the DNA assay. Following the vortexing of the samples in the 5 ml tubes, 20 µl of each sample were removed and added to 100 µl CSPD® substrate in a luminometer tube. The mixture was vortexed and incubated at room temperature for 1 min. Luminescence was read on a luminometer (TD-20/20, Turner Designs, Sunnyvale, CA), which integrated light output for 10 sec after a 2 sec delay. A calibration curve was constructed using calf intestinal alkaline phosphatase in concentrations of from $10^{-3}$ to $10^{-7}$ U/ml. Following subtraction of appropriate blanks, the results were normalized with cell number as determined by DNA assay.

4.4.5.6 Osteocalcin Assay

Osteocalcin secretion by the cells into the media was measured using a radioimmunoassay. Two days prior to the time point, the media in the wells containing the DNA and ALP assay cultures were replaced with serum free osteogenic medium containing dihydroxyvitamin D₃ (1 nM). At the appropriate time points, the medium was removed and frozen at -40°C. A previously published method was followed. (Wake et al., 1998) At the time of the assay, samples were thawed and
standards were prepared in concentrations of 0.03-2.0 ng osteocalcin/tube. Two 100 µl aliquots of each sample and three 100 µl aliquots of each standard were incubated with goat anti-rat osteocalcin antibody (100 µl, 1:50 dilution) and normal goat nonimmune serum (300 µl, 1:40 dilution) on an orbital shaker rotating at 80 rpm at 4°C overnight. On the second day of the assay, 100 µl of 125I-labeled osteocalcin (1:10 dilution) were added to all tubes, which were then incubated overnight under the same conditions. On the third day of the assay, 1 ml of donkey anti-goat IgG (1:50 dilution) was added to each tube. The samples were incubated for 2 hours, then centrifuged at 1500 g for 20 min at 4°C. The supernatant was removed, the pellet was resuspended in ddH2O and centrifuged again. The supernatant was removed, and the radioactivity remaining each tube was measured in a Cobra II gamma counter (Packard, Laguna Hills, CA) for 2 minutes. A calibration curve, from which sample concentrations were read, was generated from the osteocalcin standards. Following subtraction of appropriate blanks, the results were normalized with cell number as determined by DNA assay.

4.4.5.7 Mineralization Assay

Mineralization was measured by staining wells with a Von Kossa silver nitrate solution. (Ishaug et al., 1994) Samples were rinsed with PBS 3 times, fixed with 10% neutral-buffered formalin for 12 hours at 37°C, rinsed with ddH2O, and frozen at -20°C. At the time of the assay, the plates were thawed, stained with a 5% silver nitrate solution and exposed to UV light (λ = 365 nm, 4 mW/cm²) for 5 min. The wells were then rinsed with ddH2O, treated with 5% aqueous sodium thiosulfate for 1 min to remove unreacted silver nitrate, rinsed again with ddH2O, and air dried. This assay stains calcium phosphate deposits black. Images of wells were viewed with a black and white CCD video camera
(Sony, Model XC-77) through a Jenna 250-CF stereo light microscope (Micro-Tech Instruments, Dallas, TX). Output of the video camera was routed to a Power Macintosh 8500/T20 with a Scion Averaging Frame Grabber (Scion Corporation, Frederick MD, Model AG5). A mosaic of 9 by 12 images was collected using a custom built motorized stage controlled by a LabView 2.0.6 computer program. The mosaic had dimensions of 9.4 x 9.4 mm, corresponding to 5880 x 5880 pixels. The image was analyzed with a Scion 1.59 image analysis program (a modified version of NIH Image 1.59). The image was compressed to 60% of its original size for storage and processing. A threshold was determined which removed all background noise from 8 stained wells, which had been seeded with blank microparticles. This threshold was imposed on all images, which were converted into binary images and analyzed for number of black pixels. The results are represented as % surface mineralization.

4.5 Statistics

All assay results are reported as mean ± standard deviation, n=4. A one-factor analysis of variance (ANOVA) with blocking was utilized to assess the difference between treatments. The blocking variable was time of assay, thus isolating only the differences between treatments (EC vs NC) over the course of the assay. The assumption of independence across time was evaluated using the Durbin-Watson test on ordered mean residuals for each time point. The response variables are log-transformed values of DNA, ALP, OC, and mineralization. The log transformation was used to stabilize the variance of the responses. A separate ANOVA was run on each of the four responses. Each ANOVA was followed by a linear contrast post-hoc test on comparison between treatments. Linear contrasts estimate and test linear functions of the parameters by
multiplying a vector $L$ by the parameter estimate vector $b$ resulting in $Lb$. The adjusted mean difference was estimated along with standard errors and two-tailed $p$-values for significance of difference between means. The null hypothesis is $H_0 : Lb = 0$ (vs $H_1 : Lb < 0$) is equivalent to $H_0 : \mu_1 - \mu_2 = 0$. We reject the null hypothesis for $p > 0.05$. (Johnson and Wichern, 1988)

Since the results of the $^3$H-thymidine assay are normalized by cell numbers derived from a different set of samples, a randomization test approach was taken. All possible ratios of a particular time and treatment were calculated. Then four samples were chosen with replacement from the inner quartile range of those ratios. These samples were used in an analysis of variance from which a $p$ value was calculated. This sample - analysis procedure was repeated 100 times, returning a result of "significant difference" if the average $p$ value was less than 0.05.

4.6 Results

4.6.1 Sol-Gel Transition Results

The results of the sol-gel transition assay are presented in Table 4.1. The 10% gelatin solution in DMEM exhibited a sol-gel transition between 30 and 31 °C.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Flow / No Flow</th>
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<tbody>
<tr>
<td>28</td>
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<td>30</td>
<td>No Flow</td>
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<tr>
<td>31</td>
<td>No Flow</td>
</tr>
<tr>
<td>32</td>
<td>No Flow</td>
</tr>
</tbody>
</table>
4.6.2 Microparticle Size Results

The average diameter of the microparticles was 630 ± 394 µm, as determined by image analysis. At the seeding density of $4.06 \times 10^5$ cells/ml, a microparticle with the average diameter of 630 µm would contain 53 cells.

4.6.3 DNA Results

Cells were successfully encapsulated in gelatin microparticles. This is supported by the results of the DNA assay shown in Figure 4.1. Cells were present throughout the study for both the encapsulated cells (EC) and non-encapsulated control cells (NC). The cell number for both groups followed similar trends, increasing with time from 32,700 ± 5200 cells/cm² (NC) and 17,700 ± 1800 cells/cm² on day 1 to 122,000 ± 16300 cells/cm² (NC) and 88900 ± 16600 cells/cm² (EC) on day 14, at which time they appeared to plateau.
Figure 4.1 Cell number of encapsulated cells (EC) and nonencapsulated control cells (NC). Error bars represent means ± SD for n=4.
The results of the DNA assay show that the cell numbers on Day 1 for the EC and NC were lower than the seeded 53,000 cells/cm², with the EC values lower than those for the NC. However, a large portion of the cells remained viable during the process. Across all time points, the EC cell numbers were statistically lower than the NC (α=0.05).

4.6.4 ³H-Thymidine Results

Cellular proliferation was monitored by measuring the amount of incorporated ³H-thymidine. This assay measures the β-emissions of ³H labeled thymidine incorporated into newly synthesized DNA, thus it is correlated to the rate of proliferation. Results were normalized by the cell number results from the DNA assay, and are reported as emission counts per minute per cell (cpm/cell). As shown in Figure 4.2, both treatments demonstrated the highest incorporation on Day 1 (NC = 0.63 ± 0.11 cpm/cell, EC = 0.78 ± 0.36 cpm/cell), followed by decreasing values for days 4, 7, and 14.
Figure 4.2 Cellular proliferation of encapsulated cells (EC) and nonencapsulated control cells (NC). Error bars represent means ± SD for n=4.
The results of this assay confirm that the cells retain their proliferative potential and indicate that the EC proliferated in a manner similar to that of the NC. There is no statistical difference between the values for EC and NC ($\alpha = 0.05$).

4.6.5 Alkaline Phosphatase Results

Alkaline phosphatase (ALP) activity, an early marker of osteoblastic differentiation, was measured via luminescent assay. The ALP activity results were normalized with the cell number results from the DNA assay, yielding ALP activity/cell. As shown in Figure 4.3, both NC and EC demonstrated peak values \(2.77 \pm 1.22 \times 10^{-6} \, \mu \text{mol/min/cell (NC)}, 2.33 \pm 0.85 \times 10^{-6} \, \mu \text{mol/min/cell (EC)}\) on day 14.
Figure 4.3  Alkaline phosphatase activity of encapsulated cells (EC) and nonencapsulated control cells (NC). Error bars represent means ± SD for n=4.
The results indicate that the values for the EC and NC were similar throughout the study. Both NC and EC displayed increasing values that peaked on day 14 before decreasing. Such temporal profiles of ALP activity are typical for differentiating osteoblasts, (Lian and Stein, 1993) and indicate that the marrow stromal osteoblasts retained their ability to express ALP activity following encapsulation in gelatin microparticles. Across all time points, the EC ALP activity was statistically lower than the NC (α=0.05).

4.6.6 Osteocalcin Results

Osteocalcin (OC) is a protein which can be measured in the culture media of differentiating osteoblasts, and serves as a late marker of osteoblast phenotype. OC values from the assay are normalized by cell number results from the DNA assay. OC levels were similar for EC and NC through 28 days. As seen in Figure 4.4, the OC levels for both treatments peaked on day 18 with values of $1.91 \pm 0.31 \times 10^{-5}$ ng / cell (EC) and $2.35 \pm 0.75 \times 10^{-5}$ ng / cell (NC), decreasing through the remainder of the study.
Figure 4.4 Osteocalcin levels of encapsulated cells (EC) and nonencapsulated control cells (NC). Error bars represent means ± SD for n=4.
This trend is typical of differentiating osteoblasts. (Lian and Stein, 1993) Across all time points, the EC osteocalcin levels were statistically lower than the NC ($\alpha=0.05$).

4.6.7 Mineralization Results

The mineralization assay provides a means to quantitatively measure the deposition of mineralized matrix. Mineralization was measured via imaging following treatment with Von Kossa stain. The resulting fraction of imaged pixels registering "black" is interpreted and reported as % surface mineralization. The results of this assay show increasing mineral deposition throughout the course of the study by both EC and NC, with substantial deposition by day 28. As shown in Figure 4.5, values were similar for both treatments throughout the 28 day study, yielding final values of $38.9 \pm 14.7$ % surface mineralization (EC) and $53.8 \pm 17.9$ % surface mineralization (NC).
Figure 4.5 Mineralization of encapsulated cells (EC) and nonencapsulated control cells (NC). Error bars represent means ± SD for n=4.
There is no statistical difference between the values for EC and NC ($\alpha=0.05$). It should be realized that this assay only measures the two-dimensional surface area covered by such mineralized deposits. It does not gauge the three-dimensional nature or quality of the deposits. However, for the purpose of identifying the presence of mineralization and demonstrating increased deposition with time, this assay was satisfactory.

4.7 Discussion

To succeed as a method to insulate cells from environmental effects during processing, an encapsulation procedure must maintain the viability of the cell population and not impact its phenotypic expression. The experiments reported here were designed to determine if rat marrow stromal osteoblasts can be temporarily encapsulated in gelatin microparticles while retaining their viability, proliferation, and phenotype. The data suggest that viability and phenotypic expression of encapsulated cells are preserved, though in slightly lower quantities than nonencapsulated controls. This work is the first to demonstrate the ability to temporarily encapsulate cells.

Microparticles were formed with an average diameter of 630 ± 394 $\mu$m. Future studies beyond the scope of this thesis will address how varying the size of the microparticles will affect the viability of the encapsulated cells.

The values for the EC were generally lower than for the NC (11% lower across all assays). There may be several reasons that contribute to this result. If any residual mineral oil were to come in contact with the tissue culture surface, it would adsorb, preventing cell attachment in the affected area. This would reduce the available area for cell growth and therefore the total cell number. Steps were taken to minimize the amount
of residual oil, and to promote the partitioning of the oil to the surface of the media in the culture well, where it was eventually removed during media changes. However, the possibility of the oil contacting the surface remained. A second factor which could contribute to the reduction in cell number and phenotypic markers is the temperature shock resulting from the addition of warm cell/gelatin suspension (34 °C) to the cool mineral oil (10 °C). This step was necessary for gelation to occur, and the temperature drop was reduced as much as possible while ensuring adequate microparticle formation. In addition, an increase in resistance to gas diffusion to the cells may occur while the microparticles are suspended in the agitated mineral oil. This would result in a depletion of oxygen and a build up of carbon dioxide. Not only are there fewer cells in the EC wells, but even when the phenotypic markers are normalized on a per cell basis, the values are lower than the NC, indicating decreased osteoblastic activity of the cell population.

Small decreases in cell number and phenotypic expression were seen following encapsulation. These reductions are acceptable because these studies demonstrate that the significant quantity of cells that do remain viable, continue to proliferate, and express markers typically seen during osteoblastic differentiation. This novel technique has the potential to allow for delivery of cells in a wider variety of methods than currently available, such as an injectable carrier, as proposed in this thesis.

4.8 Conclusions

These results demonstrate that marrow stromal osteoblasts do survive encapsulation in gelatin microparticles. The cells retain their proliferative potential and phenotypic
expression, although at a slightly lower level than nonencapsulated control cells. This study indicates that this method of temporary encapsulation of cells warrants further investigation as a way to protect cells from short term environmental effects, such as those which might be present during polymer crosslinking. This may allow such cells to be implanted as part of an injectable polymer/cell composite.
CHAPTER 5 CROSSLINKED SURFACES OF GELATIN MICROPARTICLES

5.1 Background

Based on the study presented in the previous chapter, encapsulation of cells in gelatin microparticles appears to be a promising method to deliver cells while protecting them from environmental factors during polymer crosslinking. However, untreated gelatin microspheres formed via the methods described in Chapter 4, when raised to body temperature, would undergo reverse thermal gelation and become fluid. We hypothesize that by crosslinking a thin layer on the surface of the microparticles, they will be able to maintain their mechanical integrity at 37 °C. Furthermore, we believe that this can be executed in such a way that the microparticles will only remain intact for about one hour. Since the duration of the crosslinking reaction of the proposed injectable bone substitute is about 20 min, the microparticles will remain whole to protect the cells during the entire time that harmful environmental conditions persist. We hypothesize that after one hour, the crosslinked surfaces will degrade sufficiently via hydrolytic and enzymatic degradation to release the interior gelatin solution. This would allow the cells contained within to then interact with the crosslinked composite and the surrounding physiological fluid.

Since gelatin is a denatured form of collagen, it will participate in protein chemistry reactions. One such reaction commonly used is amide formation resulting from an N-hydroxysuccinimide ester (NHS-ester) reacting with primary amines. Dithiobis(succinimidylpropionate) (DSP) is a water-insoluble, homobifunctional NHS-ester which is used to crosslink proteins via the ε-amines of lysine residues and the
terminal α-amines. DSP is frequently used for intracellular and intramembrane conjugation. Since this compound is water-insoluble, it should not penetrate much into the interior of the microparticle. Thus treatment of a gelatin microparticle with DSP should result in a crosslinked layer on the surface of the microparticle.

This study attempted to determine the effects of exposure of the gelatin microparticle surfaces to two concentrations of a crosslinking agent on the viability, proliferation, and phenotypic expression of the encapsulated cells. Additionally, studies were undertaken to determine physical characteristics of the crosslinked microparticles.

5.2 Materials

Materials described in section 4.2 on page 20.

5.3 Experimental Design

The experimental design was the same as the previous experiment (Chapter 4), with the exception that instead of encapsulated cells, nonencapsulated control cells, and blank microparticles, the treatment groups consisted of encapsulated cells in microparticles which were crosslinked via exposure to 1mM DSP solution for 15 min and 5mM DSP solution for 5 min. These combinations of reaction times and crosslinker concentrations resulted in both groups of microparticles dispersing approximately 60 minutes after placement into 37 °C media, as observed by release of a tracer dye. Uncrosslinked controls - encapsulated cells in microspheres which were not exposed to a crosslinking solution - were also used. These uncrosslinked controls experienced the same treatment as the encapsulated cells in the previous study. The same assays were performed at the same time points as in the first experiment.
5.4 Methods

Cell harvest and culture and gelatin solution preparation were described in sections 4.4.1 and 4.4.2 on page 23. Tissue culture preparation and culture conditions were the same as described in 4.4.4 on page 25.

5.4.1 Microparticle Formation / Gelatin Crosslinking

The uncrosslinked microparticles in this study were formed the same way as the microparticles in section 4.2.2. The crosslinked microparticles were formed similarly, with the exception that the appropriate amount of dithiobis(succinimidyldithio) (DSP) (Pierce, Rockford, IL) was added to the mineral oil prior to the addition of the gelatin. Then the microparticles were agitated in the mineral oil for the assigned amount of time (5 or 15 min) before they were removed by filtration and collected. The remainder of the microparticle preparation was the same as in Chapter 4.

5.4.2 Microparticle Burst Strength

The burst strengths of the crosslinked microparticles were measured using an AR1000 Rheometer (TA Instruments). A 0.8 cm cylindrical mandrel was immersed in corn syrup to a gap length of 300 µm. The corn syrup was used because it possesses sufficient viscosity to transfer appropriate shear stresses to the microparticles. It was contained by a 1 cm high, 5 cm inner diameter Teflon ring sitting on the rheometer base. The entire system was maintained at 40°C, well above the sol-gel transition of the gelatin. The microparticles, which had been made with ~1% FITC-dextran as a fluorescent marker, were placed into the syrup and allowed to thermally equilibrate for 2 min. Upon rotation of the mandrel, a shear field developed in the corn syrup. The shear stress was gradually
increased by 200 Pa/min in 50 Pa increments until the microparticle burst, as evidenced by release of the inner fluid containing the fluorescent marker illuminated by UV light. Three runs were completed for each DSP concentration.

5.4.3 Microparticle Shell Thickness

The thicknesses of the crosslinked shells of the microparticles treated with DSP were measured. Each microparticle was punctured with a 22G needle and was placed into a vial containing 37 °C water. The vials were placed on a shaker table rotating at 60 RPM in a warm room (37 °C). After 1 hour, all of the uncrosslinked gelatin in the center of the microparticle had dissolved and leached away through the hole in the surface of the microparticle. The remaining crosslinked gelatin shell was serially transferred to solutions of 33%, 66%, and 100% Freezing Medium (Tissue-Tek O.C.T. Compound Embedding Medium, 4583). The shell in 100% freezing medium was placed in a 1cm x 1cm x 3 cm cuvette and frozen at -20 °C. The frozen sample was then transferred to a mounting stage and sectioned using a Microm HM 500 M microtome cryostat. The microparticle section with the largest diameter was placed on a glass slide.

Images of the sections, which at this point resembled rings, were captured with a Nikon eclipse E600 Microscope and ImgVGA-Plus v1.40 software. Using Scion Image 1.62, the width of the shell was measured at 8 locations around the circumference, every 45 degrees. The measurements were calibrated using a stage micrometer. Twenty microparticles were analyzed for each DSP concentration.
5.4.4 Assays

The DNA, $^3$H-thymidine, alkaline phosphatase activity, osteocalcin, and mineralization assays were performed as described in section 4.4.5.3 on pages 26 through 29.

5.5 Statistics

As in Chapter 4 all assay results are reported as mean ± standard deviation, n=4. The statistical analysis of the assays was performed as described in section 4.5. The microparticle burst strengths were compared using a two-tailed t-test at $\alpha=0.10$. Using stratified sampling to account for intershell and intrashell variations, means and standard deviations for the microparticle shell thicknesses were calculated. (Cochran, 1977)

5.6 Results

5.6.1 Microparticle Burst Strength

As depicted in Figure 5.1, the burst strength of the 5 mM microparticles was 2880 ± 1060 Pa and that of the 1 mM microparticles was 1380 ± 680 Pa. The 5 mM microparticles were statistically higher than the 1 mM microparticles ($\alpha=0.10$).
Figure 5.1 Burst strength of microparticles exposed to two levels of crosslinker. Error bars represent means ± SD for n=3.
5.6.2 Microparticle Shell Thickness

The thickness of the shells of microparticles crosslinked with 5 mM DSP was 77 $\pm$ 44 $\mu$m, while the thickness of those crosslinked with 1 mM DSP was 74 $\pm$ 28, as shown in Figure 5.2. The irregular nature of the thickness of the microparticle shell can be seen in Figure 5.3, a typical 5 mM shell image.
Figure 5.2 Thickness of microparticle shells treated with two levels of crosslinker. Error bars represent means ± SD for n=20.
Figure 5.3 A typical image of a cryosection of a microparticle shell indicates the irregular thickness of the shell.
5.6.3 DNA Results

The results indicate that the cells encapsulated in microparticles crosslinked with 5 mM DSP for 5 min (referred to as 5 mM cells) survived the encapsulation process well, whereas those crosslinked with 1 mM DSP for 15 min (referred to as 1 mM cells) did not. The DNA assay results, shown in Figure 5.4, indicate that the encapsulated controls (EC) cell number was 27,400 ± 11,700 cells/cm² on Day 1, rising to 122,700 ± 9100 cells/cm² on day 18, where it remained relatively constant. The 5 mM cells had cell numbers of 13,000 ± 7300 cells/cm² on day 1, increasing to 74,300 ± 15,100 cells/cm² on day 18. The 1 mM cells had day 1 cell numbers of 6700 ± 3200 cells/cm², with the maximum cell number on day 28 of 23,100 ± 2900 cells/cm².
Figure 5.4  Cell number of cells encapsulated in microparticles and exposed to three levels of crosslinker: No Crosslinker (Control), 1 mM Crosslinker, or 5 mM Crosslinker. Error bars represent means ± SD for n=4.
The DNA assay indicates that the cells encapsulated in all of the microparticles survived. As in 0, all of the cell numbers on day 1 were lower than the seeded 53,000 cells/cm². The control cells had the highest number of cells, followed by the 5 mM and 1 mM cells. Both the control cells and the 5 mM cells increased with time, plateauing around day 18. However, the number of 1 mM cells remained low and relatively unchanged from the Day 1 values. Over the course of the study, the number of control cells was statistically higher than the number of 5 mM cells, which was statistically higher than the number of 1 mM cells (α=0.05).

5.6.4 3H-Thymidine Results

The cellular proliferation assay, when normalized by results of the DNA assay, indicates that the 5mM and control microparticle cells had similar values for cellular proliferation through the study, decreasing from Day 1 through Day 14. The EC cells had 3H-thymidine incorporation values of 0.74 ± 0.47 cpm/cell on day 1, while the 5 mM cells had 3H-thymidine incorporation of 0.67 ± 0.53 cpm/cell on day 1. The 1 mM cells, as shown in Figure 5.5, had proliferation rates of 0.13 ± 0.08 cpm/cell on Day 1. Proliferation rates for all treatments decreased through the remainder of the study. There was no statistical difference between EC and 5 mM cells (α=0.05). The proliferation rate of 1 mM cells was low throughout the study, significantly lower than the 5 mM and control cells.
Figure 5.5 Proliferation of cells encapsulated in microparticles and exposed to three levels of crosslinker: No Crosslinker (Control), 1 mM Crosslinker, or 5 mM Crosslinker. Error bars represent means ± SD for n=4.
5.6.5 Alkaline Phosphatase Results

Figure 5.6 shows the results of the ALP assay as normalized by the DNA assay results. The EC and 5 mM cells had peak ALP activities on day 18, with values of $2.70 \pm 0.29 \times 10^{-6}$ µmol/min/cell (EC) and $1.60 \pm 0.30 \times 10^{-6}$ µmol/min/cell (5 mM), after which they decreased. The 1 mM cells had peak values of $0.72 \pm 0.71 \times 10^{-6}$ µmol/min/cell. The levels of the control cells were statistically higher ($\alpha=0.05$) than the 5 mM cells. The ALP activity of the 1 mM cells was low but variable throughout the study.
Figure 5.6 Alkaline phosphatase activity of cells encapsulated in microparticles and exposed to three levels of crosslinker: No Crosslinker (Control), 1 mM Crosslinker, or 5 mM Crosslinker. Error bars represent means ± SD for n=4.
5.6.6 Osteocalcin Results

In a manner similar to the ALP assay, the normalized OC levels of both the 5 mM and control cells increased to a peak on day 21, after which they decreased. The cells exhibited values of $3.80 \pm 1.70 \times 10^{-5}$ ng / cell (EC) and $1.48 \pm 0.54 \times 10^{-5}$ ng / cell (5 mM), as shown in Figure 5.7. The maximum values for the 1 mM cells occurred on day 7 ($1.53 \pm 0.78 \times 10^{-5}$ ng / cell). The values for the control cells were statistically higher ($\alpha=0.05$) than the 5 mM cells. The OC levels of the 1 mM cells were erratic and relatively low throughout the study.
Figure 5.7 Osteocalcin levels of cells encapsulated in microparticles and exposed to three levels of crosslinker: No Crosslinker (Control), 1 mM Crosslinker, or 5 mM Crosslinker. Error bars represent means ± SD for n=4.
5.6.7 Mineralization Results

Mineralization, as measured by von Kossa assay increased with time to 44.5 ± 11.7 % surface coverage for the EC cells and 30.1 ± 12.7 % surface coverage for the 5 mM cells on day 28. As seen in Figure 5.8, the 1 mM cells show virtually no mineralization (1.2 ± 0.9 % coverage) on day 28. Mineralization deposition in the control wells was statistically higher (α=0.05) than the 5 mM cells. There was essentially no mineralization in the wells containing the 1 mM cells.
Figure 5.8 Mineralization of cells encapsulated in microparticles and exposed to three levels of crosslinker: No Crosslinker (Control), 1 mM Crosslinker, or 5 mM Crosslinker. Error bars represent means ± SD for n=4.
5.7 Discussion

In order for microparticles containing cells to be useful in a clinical setting, they must be mechanically stable at body temperature during the 20 minutes of composite crosslinking. To this end, the surfaces of the gelatin microparticles were treated with two concentrations of a crosslinker - 5 mM and 1 mM DSP in an attempt to determine the effects of exposure of the microparticle surfaces to those concentrations of a crosslinking agent on the viability, proliferation, and phenotypic expression of the encapsulated cells. Additionally, studies were undertaken to determine physical characteristics of the crosslinked microparticles.

The exposure time of the microparticles to these concentrations was varied so that, in both treatments, the resulting microparticle shell dispersed in media after approximately 60 minutes. The 5 mM DSP reacted with the gelatin microparticles for 5 minutes and the 1 mM DSP reacted with the microparticles for 15 minutes. These microparticles were compared with encapsulated cells not exposed to DSP, the same treatment received by the EC in Chapter 4. We investigated the affect of two crosslinker concentrations on the viability, proliferation, and phenotypic expression of the cells encapsulated through 28 days of culture. The data suggest that exposure of the microparticles to 5 mM DSP for 5 min allows the encapsulated cells to maintain most of their viability, proliferation and phenotypic expression.

This work is the first to demonstrate the ability to temporarily encapsulate marrow stromal cells in a gelatin carrier with a crosslinked surface. As in the previous study, Chapter 4, the assay values reported for control and 5 mM cells were similar to previously reported values. (Peter et al., 1998) In addition, the values for the
encapsulated cells in this study were very similar to the encapsulated cell controls in the previous study. One difference seen between the studies is that the differentiation of the marrow stromal cells to osteoblasts seems to be delayed by about 3 days in this study, as compared to the previous one. In Chapter 4, the ALP activity of the cells peaked on day 14, and the OC levels peaked on day 18. In this study, the ALP activities peaked on day 18 and the OC levels on day 21. The cells used in these studies were primary rat marrow stromal cells, and thus heterogeneous populations. The cells used in the previous study were harvested and encapsulated separately from those used in the current study. The use of control cells in both studies affords internal consistency and allows for the conclusion that this apparent delay in differentiation was due to factors other than those being investigated.

In this study, the cells encapsulated in gelatin crosslinked with 5 mM DSP had lower values for cell number and phenotypic markers than the control cells which were encapsulated in gelatin but not crosslinked. Since both groups of cells underwent the encapsulation process, other explanations for this difference must be explored. The DSP crosslinked thicknesses of approximately 75 μm of the outer surfaces of the microspheres. It is likely that any cell within this region would have had its surface proteins crosslinked, rendering it unviable. In addition, even though DSP is nonwater soluble, it must diffuse into the microparticle, if only in very small amounts to cause crosslinking to that depth. This small concentration may have toxicity sufficient to lead to some cell death. Also, the crosslinked microparticles stayed in the mineral oil longer than the uncrosslinked controls. The effects of the mechanisms mentioned in Chapter 4, gas diffusion limitations and thermal shock, would be enhanced by this increased
exposure. These mechanisms appear to be more likely when the results of the 1 mM crosslinked microparticles are considered. Few of cells therein encapsulated survived. Visual inspection revealed low numbers of cells present, which either remained rounded or spread in an uncharacteristic manner. These cells were exposed to a lower concentration of DSP, but for a longer time. The failure of the 1 mM cells to increase in number or express the osteoblastic phenotype was an unexpected and surprising result. A reasonable deduction to make would be that it was not the increase in DSP which had a beneficial effect, but rather the longer exposure time required by the lower concentration which had a detrimental effect on the cell viability and differentiation. We recognize the limitations of this study in determining the mechanisms which led to these unanticipated results. Future studies, outside the scope of this thesis, will address the effects of temperature, chemical environment, and microparticle size on the viability and proliferation of encapsulated marrow stromal cells.

We believe that there was little osteoblastic phenotypic expression by the 1 mM cells. This is evidenced by the almost total lack of mineralization. The nonzero, erratic values seen in the results of the ALP and OC assays result from those low values being normalized by low cell numbers. Both the numerators and denominators were small and near the low end of detectability. Thus, slight deviations could have resulted in the artifacts seen in these results.

Physical measurements of the crosslinked microparticle shells were made. The thicknesses of the microparticle shells were measured for both crosslinking treatments. The average thicknesses were about the same, and the standard deviations of the thicknesses were rather large. The uneven nature of the shell can be observed in the
micrograph. Visual inspection of the microparticles prior to processing indicated a smooth surface, while the images of the cross sections of the crosslinked microparticles show an uneven, rough surface. This discrepancy is most likely an artifact of the sample freezing and processing. It appears as though the surface folded slightly to accommodate a change in the circumference. However, the comparisons between the 1 mM and the 5 mM should remain valid because any changes should have occurred similarly in all samples.

Another possible explanation for the condition of the microparticle shell is that when the interior, uncrosslinked gelatin was leached for 1 hr in PBS at 37 °C, some degradation of the shell may have occurred. When placed in media containing 10% FBS at 37 °C, the microparticles lost their mechanical integrity after 1 hr, much more rapidly than they did in PBS. While some of the loss of integrity is probably due to hydrolytic degradation, it is likely that there are additional factors contained in the media and FBS which contribute to the accelerated degradation of the microparticle shells.

The burst strength of the microparticles was evaluated using a rheometer. The microparticles crosslinked with 5 mM DSP for 5 min withstood significantly more shear than those crosslinked with the 1 mM DSP for 15 min. These two experiments suggest that while the thickness of the crosslinked region was independent of crosslinker concentration, the strength of the shell was higher when formed by the higher concentration of crosslinker.

Slight decreases in cell number and phenotypic expression were seen in the 5 mM as compared to noncrosslinked encapsulated controls. These reductions are acceptable because this study demonstrates that the significant quantity of cells that do remain viable
continue to proliferate and express markers typically seen during osteoblastic differentiation.

This study suggests that encapsulation of cells in microparticles crosslinked via exposure to 5 mM DSP for 5 min holds promise for temporarily protecting cells from toxic local environments of crosslinking polymeric composites.

5.8 Conclusions

These results demonstrate that marrow stromal osteoblasts do survive encapsulation in crosslinked gelatin microparticles (exposure to 5 mM DSP for 5 min). These cells retain their proliferative potential and phenotypic expression. Exposure to 1mM DSP for 15 min led to severely limited viability and expression. This unexpected outcome is likely the result of the increased crosslinking time, not the decreased crosslinker concentration. This study indicates that this method (5 mM DSP for 5 min) of temporary encapsulation of cells warrants further investigation as a way to protect cells from short term environmental effects, such as those which might be present during polymer crosslinking. This would allow such cells to be implanted as part of an injectable polymer/cell composite.
CHAPTER 6  ENCAPSULATED CELLS ON CROSSLINKING PPF: VARYING THE CROSSLINKING TIME

6.1 Background

A review of the literature indicates that cells have never been successfully incorporated into an injectable, *in situ* crosslinkable polymeric bone substitute. Previous experience in our laboratory suggests that marrow stromal osteoblasts incorporated directly into crosslinking PPF composites do not survive (unpublished data), while fully crosslinked PPF composites make excellent substrates for cell attachment and growth. (Peter *et al.*, 2000b)

A central hypothesis of the work presented in this thesis is that a cell delivery system is necessary to protect the cells during composite crosslinking. Since the previous study indicates that cells have successfully been encapsulated in crosslinked microparticles, we may test this hypothesis. This short, one week study also provides a starting point for a longer, four week study described in Chapter 7.

This study seeks to answer the following questions. 1) When placed on a crosslinked PPF composite, does the encapsulation of marrow stromal osteoblasts influence cell viability and proliferation, as compared to nonencapsulated controls? 2) What effect does time of addition of marrow stromal osteoblasts to a crosslinking PPF composite have on cell viability and proliferation? 3) What effect does the presence of aqueous microparticles have on the ability of PPF composites to crosslink?

6.2 Materials

Poly(Propylene Fumarate) Synthesis
Propylene Glycol (PG) (Acros, Pittsburgh, PA)

Diethyl Fumarate (DEF) (Acros, Pittsburgh, PA)

Zinc Chloride (ZnCl₂) (Fisher, Pittsburgh, PA)

Hydroquinone (Hq) (Fisher, Pittsburgh, PA)

Methylene Chloride (Fisher, Pittsburgh, PA)

Sodium Sulfate (Fisher, Pittsburgh, PA)

Sodium Chloride (Fisher, Pittsburgh, PA)

Cell culture, microparticle formation, assays as described in section 4.2.

6.3 Experimental Design

In this experiment, cells were seeded into crosslinking PPF composites. The four treatment groups included: nonencapsulated control cells, cells encapsulated in crosslinked microparticles, acellular crosslinked microparticles (negative control) and, acellular wells (negative control).

PPF was synthesized and formulated into a composite that possessed favorable handling properties while maintaining a fairly low polymer:monomer ratio (PPF:N-VP) of 1:0.1. The initiator and accelerator levels were adjusted to produce a composite which could be crosslinked approximately ten minutes after initiation.

As in previous experimental designs, (Sec. 4.3, 5.3), rat marrow stromal cells were harvested and cultured for 8 days in primary media. They were then lifted and encapsulated in gelatin microparticles. The surfaces of the microparticles were crosslinked via exposure to 5 mM DSP for 5 min. This concentration - time combination yielded microparticles with cells that had the highest viability and phenotypic expression in the previous study (Chapter 5).
The cell density in the microparticles corresponded to $2.03 \times 10^6$ cells per 0.5 g gelatin (equivalent to a confluent monolayer). The nonencapsulated control cell density corresponded to the same number of cells ($2.03 \times 10^6$) in 0.05 ml media. This ten fold difference in cell density allowed the same number of nonencapsulated control cells and encapsulated cells to be added to the wells such that the volume of the solution around the encapsulated cells would be ten times higher. This was done because the gelatin microspheres, in addition to delivering cells, can act as a porogen in the final composite, a condition not afforded to the nonencapsulated cells. The absorption of heat generated during the crosslinking reaction by the gelatin solution may be one of the major benefits of this cell delivery system.

Crosslinking of the PPF composite was initiated and 1 g per well was dispensed into 12 well plates. Cells were added to the crosslinking composites in separate wells. The cells were added at 2, 4, 6, 8, and 10 min following the initiation of crosslinking. In addition, cells were placed on fully crosslinked (for 30 min) composite and tissue culture polystyrene (TCPS). Blank crosslinked composites and acellular microparticles added to the composites 5 minutes after initiation served as negative controls. Enough samples were seeded to allow evaluation of 4 wells for cell number and 4 wells for proliferation at 1, 4, and 7 days for all treatments and addition times. One set of treatments was seeded at 5 minutes after crosslinking and on fully crosslinked composites for an MTT assay on Day 1.
6.4 Methods

6.4.1 PPF Synthesis

6.4.1.1 First Reaction

This synthesis of poly(propylene fumarate) (PPF) has been described. (Shung et al., 2001) As mentioned in Chapter 2, this method is a combination of previously reported methods. (Kharas et al., 1997; Peter et al.) In order to obtain the quantity of PPF necessary for these studies, four runs of the first reaction were performed on consecutive days. For each run, diethyl fumarate (DEF) and propylene glycol (PG) were combined in a 1:3 molar ratio in a 1L round bottom 3-neck reaction vessel. Zinc chloride (ZnCl₂), a catalyst, was added at a molar ratio of 0.01:1 ZnCl₂:DEF, and hydroquinone (Hq) was added in a molar ratio of 0.002:1 Hq:DEF. Hydroquinone was used to inhibit undesired reactions such as thermal crosslinking. A total of 1091 g of DEF were used during the four reactions.

The reactants were agitated by an overhead mechanical stirrer. A cold water condenser with thermometer was attached to one of the reaction vessel necks; at its outlet, a round bottom flask was placed on ice to collect the condensate (ethyl alcohol). The system was slowly purged with purified nitrogen through the third neck of the reaction vessel. A bubbler at the outlet of the system monitored nitrogen flow. At the start of the reaction, the reaction vessel was submerged in an oil bath, whose temperature was raised to 100 °C. The temperature was slowly raised over the course of 5 hours to 150 °C. Care was taken to maintain the vapor temperature below 100 °C. When condensate ceased to form, the first step of the reaction was complete. The diester
intermediate, bis(2-hydroxypropyl fumarate) (BHPF) had been formed. The reaction mixture was allowed to cool to room temperature for storage at 4 °C.

6.4.1.2 Second Reaction

The products of the first reaction (BHPF, excess PG) were combined in a 3 L, 3-neck reaction flask with overhead mechanical stirrer, cold water condenser, and nitrogen inlet. This was a larger version of the configuration used in the first reaction. The system was purged with nitrogen gas. In the course of 45 minutes the temperature was raised to 100 °C and the pressure was reduced to approximately 0.1 torr. At this point, the unreacted PG was distilled and condensed. Once full vacuum was achieved, the temperature was raised to 140 °C over 2 hours. Propylene glycol produced during this transesterification was removed and condensed, resulting in the formation of PPF. The temperature was maintained at 140 °C for 2 hours, then raised to 150 °C for 1 hour, after which the system was purged with nitrogen and allowed to cool to room temperature.

6.4.1.3 Purification

The PPF was dissolved in an equal volume of methylene chloride. The solution was shaken with, and the organic phase collected from, equal volumes of the following: 5% HCl solution in ddH₂O ddH₂O (2X) Brine (ddH₂O saturated with sodium chloride) (2X) Ethyl Ether (Fisher, Pittsburgh, PA)
Sodium sulfate was added to the organic phase, agitated, and filtered. At this stage of the purification, the PPF/methylene chloride solution may be added dropwise to cold ethyl ether. This removes the hydroquinone as the PPF precipitates, but also results in a significant loss of product. This step was ignored and the hydroquinone was left in the product to inhibit side reactions and increase shelf life. The remaining solvent was removed via rotavapor followed by vacuum drying. The product was stored at -20 °C until use.

6.4.2 PPF Characterization - Gel Permeation Chromatography (GPC)

The molecular weight distribution of the PPF polymer was assessed using GPC with a differential refractometer (Waters, Model 410, Milford MA). Samples were dissolved in HPLC grade chloroform and eluted through a Phenogel guard column (50 x 7.8 mm, 5 μm, mixed bed, Phenomenex, Torrance, CA) and a Phenogel column (300 x 7.8 mm 5 μm, mixed bed, Phenomenex) with a separation range up to 40,000. The elution rate was 1 ml/min in chloroform. Polystyrene standards (Polysciences, Warrington, PA) were used to generate a calibration curve for calculating the PPF molecular weight distribution.

6.4.3 Cell Harvest and Culture, Gelatin Preparation, and Cell Encapsulation

The harvest and culture of rat marrow stromal cells were previously described in section 4.4.1. Preparation of the gelatin solution was described in section 4.4.2, and the cell encapsulation procedure was described in section 5.4.1.

6.4.4 PPF Crosslinking / Microparticle Seeding

The base formulation of this PPF composite is found in Table 6.1.
<table>
<thead>
<tr>
<th>Table 6.1 Formulation of PPF Composite</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Poly(propylene fumarate) (PPF)</strong></td>
</tr>
<tr>
<td><strong>N-vinyl pyrrolidinone (N-VP)</strong></td>
</tr>
<tr>
<td><strong>Benzoyl Peroxide (BP)</strong></td>
</tr>
<tr>
<td><strong>Dimethyl p-toluidine (DMT)</strong></td>
</tr>
</tbody>
</table>

The PPF, N-VP, and BP were sterilized by placing them individually in transparent glass vials and exposing them to UV light in a laminar flow hood. The N-VP and BP were exposed for 2 hours, the PPF overnight. Based on toxicity data, (Sax and Lewis, 1989) it was assumed that the DMT was sterile as received. The components were prepared by combining the PPF, the DMT, and half of the N-VP into one vial, and the BP and the other half of the N-VP in another vial. A total of 8 g PPF were mixed at one time. At the time of use, the crosslinking reaction was initiated in a laminar flow hood by pipetting the BP/N-VP solution into the PPF/N-VP/DMT mixture. The decomposition of the BP into peroxide radicals, accelerated by the DMT, initiated the radical polymerization. After brief mixing, the composite was loaded into a sterile 10 cc syringe. Into each of 8 wells of a 12 well plate, 1 g composite was dispensed. The composite spread to cover the bottom of the well, forming a thick layer. At the designated times, 0.5 g microparticles were placed onto the layer of composite in 4 of the wells. The microparticles were spread to achieve a single layer on the composite. A volume of 50 µl of cell suspension was added to the other 4 wells at the same times. At the early addition times, the microparticles partially embedded in the layer of composite, but were never covered by the composite. At later times, the microparticles remained primarily on top of the
composite. Media was added to the wells 30 minutes after the cells were placed on the composites. This was done to more closely simulate the environment the cells and microparticles may see when placed into a three dimensional construct in vivo. In a three dimensional construct, the microparticles would be surrounded by polymer, without access to bodily fluid. By waiting for 30 min before adding media, the microparticles were allowed to experience the full effect of the crosslinking composites.

6.4.5 Cell Culture

Media changes were performed in a manner described previously (Section 4.2.3).

6.4.6 Composite Temperature Profile

The crosslinking composite temperature profile was obtained by a method previously described. (Peter et al., 1999a) Briefly, the composite components described in Table 6.1 were vigorously mixed and placed into a cylindrical Teflon mold held in a 37° water bath. The outer dimensions of the mold were 20 mm in height, 20 mm in diameter. Inner dimensions measured 15 mm in height and 10 mm in diameter. The temperature was recorded throughout the crosslinking reaction via thermocouple inserted 8 mm into the mold. Readings were obtained every 5 seconds until 2 min after the peak temperature. Five samples were measured.

6.4.7 Composite Gel Point

The gel point of the PPF based composites were measured using a method similar to that previously reported (Peter et al., 1999a) using a rheometer (Model AR1000, TA Instruments). The gel point was defined as the time corresponding to the formation of an infinite polymer network in which all of the chains are bound together at a minimum of
one site. (Odian, 1991) At the gel point, the polymer viscosity change with time asymptotically approaches infinity. For these experiments, the gel point represents the onset of the sudden increase of the viscosity of the curing composite paste. Practically, this point represents the point at which the material ceases to possess properties suitable for manipulation. Five samples of the PPF composite were measured. Following initiation of the crosslinking reaction via vigorous mixing, the composite was placed in a Teflon ring (10 mm diameter, 15 mm high) on the temperature controlled rheometer stage (37 °C). A cylindrical stainless steel mandrel (8 mm diameter) with face parallel to the stage was lowered 5 mm into the composite. An oscillatory program consisting of a time sweep at 1 Hz at an applied stress of 10 Pa was used to assess viscosity during composite crosslinking.

6.4.8 Assays

The DNA and $^3$H-thymidine assays were performed as described in section 4.4.5.3

6.4.8.1 MTT Assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed in a manner similar to that described by previously. (Suggs et al., 1999b) This technique stains active mitochondria blue, and was used in this study as a qualitative measure of cellular viability on the PPF substrates. Cells on these substrates can be difficult to visualize via light microscope.

Briefly, MTT was dissolved in PBS to form a stock solution of 5 mg/ml. Twelve hours after seeding onto the substrates, the media were removed from each well and replaced with 200 μl sterile stock solution and 2 ml indicator free media. The cultures
were incubated in darkness for 4 hrs, a which time the media were aspirated and the culture rinsed with PBS. The cultures were allowed to air dry overnight and were imaged with a digital camera.

6.5 Statistics

As in Chapters 4 and 5, all assay results are reported as mean ± standard deviation, n=4. The statistical analysis of the assays was performed as described in section 4.5.

6.6 Results

6.6.1 Gel Permeation Chromatography (GPC) Results

The molecular weights of the poly(propylene fumarate) (PPF) were measured via GPC. The PPF had an $M_n$ of 2180 and $M_w$ of 3450, with resulting PI of 1.58, as measured against polystyrene standards.

6.6.2 Composite Temperature Profile Results

The average maximum temperature recorded for the crosslinking PPF composite was $47.5 \pm 2.9 \, ^{\circ}C$. A typical temperature profile is shown in Figure 6.1.
Max Temp = 45.7 °C at 562 s

Figure 6.1 Typical temperature profile of crosslinking PPF composite
6.6.3 Composite Gel Point Results

The average onset of gelation for the crosslinking PPF composite was 619 ±15 s. A typical plot of the viscous portion of complex viscosity (\(\eta'\)) and the elastic modulus (\(G'\)) is shown in Figure 6.2.
Figure 6.2 Typical plot of increasing viscosity of crosslinking PPF composite. The viscous portion of complex viscosity ($\eta'$) and elastic modulus ($G'$) are shown as a function of time (s).
6.6.4 DNA Results

The results of the DNA assay of the nonencapsulated cells (NC) are presented in Figure 6.3. The data indicate that the cells seeded at 2, 4, and 6 min after composite crosslinking initiation had virtually no cells present throughout the 7 day study. The cells added at 8 min had values of $3100 \pm 1400$ cells/cm$^2$ on day 1, increasing to $24900 \pm 11400$ cells/cm$^2$ in day 7. These values were significantly lower ($\alpha=0.05$) than those for the 10 min addition time ($13300 \pm 3300$ cells/cm$^2$ on day 1, and $53400 \pm 14100$ cells/cm$^2$ on day 7) and the fully crosslinked composite ($1880 \pm 4900$ cells/cm$^2$ on day 1, and $65700 \pm 8100$ cells/cm$^2$ on day 7). The cell number of unencapsulated cells on TCPS were significantly higher ($\alpha=0.05$) than the 10 min and fully crosslinked composites with values of $31500 \pm 6200$ cells/cm$^2$ on day 1 and $79400 \pm 15900$ cells/cm$^2$ on day 7.
Figure 6.3 Cell numbers of nonencapsulated cells (NC) on crosslinking PPF.
Figure 6.4 describes the behavior of the encapsulated cells (EC) on crosslinking PPF. While very few cells were present on day 1 (1300 ± 600 cells/cm²) and day 4 (900 ± 400 cells/cm²) for the cells placed on crosslinking composites 2 minutes after initiation, by day 7 more cells were present (8000 ± 3200 cells/cm²). These values are still rather low. Significantly larger (α=0.05) were the cell numbers on the 4 min composite (3100 ± 700 cells/cm² on day 1, 22200 ± 6100 cells/cm² on day 7). These values were significantly lower than those for the 6, 8, 10 min crosslinking time and the fully crosslinked substrates. These values were all approximately 9000 cells/cm² on day 1 and 40000 on day 7. Significantly higher (α=0.05) were the encapsulated cell numbers on TCPS (15100 ± 3700 cells/cm² on day 1, 62200 ± 7200 cells/cm² on day 7).
Figure 6.4 Cell numbers of encapsulated cells (EC) on crosslinking PPF.
These results indicate that the EC survived on the 4 and 6 min, and to a smaller extent 2 min, crosslinking composite, whereas the NC did not. There is also a trend of increasing cell number with addition time for both EC and NC.

6.6.5 $^3$H-Thymidine Results

Cellular proliferation was measured by $^3$H-thymidine incorporation, normalized by results of the DNA assay. As seen in Figure 6.5, the NC placed on composite 2, 4, and 6 min after crosslinking initiation exhibited no proliferation. The assay values for the 8 and 10 min cultures, along with the fully crosslinked and TCPS cultures, were approximately the same, decreasing with time from about 0.65 cpm/cell on day 1 to about 0.05 cpm/cell on day 7.
Figure 6.5 Proliferation of nonencapsulated cells (NC) on crosslinking PPF.
Figure 6.6 describes the proliferation of EC on crosslinking PPF composites. The values for the EC placed on the composite 2 min after crosslinking initiation has values of $0.13 \pm 0.08 \text{ cpm/cell (day 1)}$ decreasing to $0.05 \pm 0.04 \text{ cpm/cell (day 7)}$. These values are significantly lower ($\alpha=0.05$) than those for the remaining data points. The day 1 $^3\text{H}$-thymidine uptake values for the remaining conditions range from $0.44 \pm 0.29 \text{ cpm/cell (4 min)}$ to $0.75 \pm 0.51 \text{ cpm/cell (8 min)}$. The values decrease through the course of the assay to between $0.05 \pm 0.04 \text{ cpm/cell (4 min)}$ to $0.18 \pm 0.07 \text{ (8 min)}$ on day 7.

These data suggest that the EC proliferated on the 2, 4, and 6 min crosslinking composites, while the NC did not. They also indicate that there was no difference in the EC values for the 4, 6, 8, and 10 min crosslinking time, the fully crosslinked composite and TCPS samples. The 8 and 10 min crosslinking time, the fully crosslinked composites and TCPS samples for the NC were also the same.
Figure 6.6 Proliferation of encapsulated cells (EC) on crosslinking PPF.
6.6.6 MTT Results

As shown in Figure 6.7, the EC on the right stained much more intensely than the NC on the left. This 12 hr time point reinforces the DNA results indicating that many more EC survive the exposure to crosslinking composite than NC do.
Figure 6.7 MTT stained cells seeded on PPF composites crosslinking for 5 min. Nonencapsulated cells (A) are shown on the left, encapsulated cells (B) are shown on the right.
6.7 Discussion

This study was designed to address three questions. 1) When placed on a crosslinked PPF composite, does the encapsulation of marrow stromal osteoblasts influence cell viability and proliferation, as compared to nonencapsulated controls? 2) What effect does time of addition of marrow stromal osteoblasts to a crosslinking PPF composite have on cell viability and proliferation? 3) What effect does the presence of aqueous microparticles have on the ability of PPF composites to crosslink? In response to the first question, the data indicate that the encapsulation of cells increases their viability and proliferation, compared to nonencapsulated cells, at early time points. Secondly, the time of addition to the crosslinking composite has a great effect on the viability and proliferation of both the encapsulated and nonencapsulated cells. Cells added to composites soon after initiation of crosslinking had lower cell numbers and thymidine incorporation when compared to cells added at later times. Finally, no evidence was observed to indicate that the presence of the microparticles had an effect on the crosslinking of the composites.

This study is the first to demonstrate the effectiveness of a cell carrier in increasing cell viability and proliferation of cells placed on actively crosslinking, injectable, biodegradable bone substitute. It also elucidates the optimal times for adding the encapsulated cells to this crosslinking composite while maintaining suitable material handling properties.

The volume of crosslinking composite that could be mixed at a given time was constrained by handling and dispensing limitations. Therefore, the number of cells that
could be dispensed at a time was also limited. This led to a variation in the time that the cells (both EC and NC) remained out of the incubator. This issue was addressed in two ways. First, two runs of cell encapsulations were carried out, the first of which was seeded for the DNA assay and the second for the proliferation assay. Secondly, the EC and NC on any single plate were all seeded at the same time. For example, all of the cells seeded for the Day 4 \(^{3}H\)-thymidine assay on composites crosslinking for 6 minutes were seeded on the same plate at the same time. The order of seeding was randomized with respect to addition time point and day of assay.

Several points suggest discussion. The most apparent distinction is in the cell number results between the EC and NC where the EC survived when added to crosslinking composite at 4 and 6 min, whereas the NC did not. Possible factors leading to cell death of the NC include exposure to toxic species combined with temperature rise. The gelatin microparticles may provide resistance to diffusion of both the toxic species and heat.

Initially, the crosslinking PPF composite consists of uncrosslinked PPF, unreacted NVP, BP and DMT. Although the toxic NVP is miscible with aqueous solutions, the extra volume of the gelatin microparticle, which surrounds the EC but not the NC, may provide enough diffusion resistance to the NVP to maintain the viability of the EC. The NVP is consumed during the reaction, either incorporated into the polymeric network or formed into nontoxic poly(vinyl pyrrolidinone). Therefore it would be present in low concentrations at the later time points, which may have a minimal effect on the cells placed on those crosslinking composites. The same reasoning may be applied to the radicals generated by the decomposing BP and the DMT. The gelatin may act as a
diffusional barrier to both species. The peroxide radicals are consumed during the course of the reaction and the toxic DMT is present in very small quantities. Based on the temperature profile of the crosslinking composite, it is likely that the gelatin microparticle would act as a heat sink, absorbing some of the heat generated during the reaction. Since the aqueous microparticle is approximately 90% water, it would have a heat capacity approaching that of pure water. The volume of the microparticles surrounding the cells is ten times larger than the media surrounding the NC. The microparticles would insulate and protect the EC from the heat released to a much greater extent than the media around the NC.

Another difference that is apparent from the results generated in this study is the lower values of encapsulated cell numbers compared to the nonencapsulated cell numbers on TCPS and fully crosslinked composites. These results are consistent with the results of studies described in Chapter 4, which indicate that the encapsulation of cells yields lower cell numbers than nonencapsulated control cells when placed on TCPS. It follows that the same mechanisms would lead to similar cell behavior on the fully crosslinked composite.

In addition, the cell numbers of both the EC and NC on fully crosslinked composites are lower than their counterparts cultured on TCPS. Initially, this would seem to contradict findings by Peter et. al, who describe no difference in cell number of NC cultured on TCPS and PPF/β-TCP composites. (Peter et al., 2000ab) However, the composites used in those studies were prepared differently than composites used here. In the studies by Peter et. al, the composites were polymerized in a mold and allowed to cure for 24 hr. Then the composites were sectioned into 1.5 mm thick disks and leached
in ddH₂O for 4 days to remove any soluble factors. The disks were then sterilized before being seeded with cells. This contrasts to the system described here, where the sterile PPF composite was crosslinked directly into the tissue culture wells. No processing or leaching took place prior to the cells being placed on the substrate. In this study any soluble cytotoxic components remaining after crosslinking, which subsequently leached into the cell culture media, would have deleterious effects on the cells. These components would have been removed by the leaching step in the studies by Peter, et. al. Since the encapsulation was designed to protect the cells on a temporary basis, about 1 hour, it would not protect the cells from exposure to materials leached into the media over the course of several days. Currently, studies are underway in our laboratory to assess the leachable products of crosslinked PPF-based composites. Even so, the high cell number on the fully crosslinked composite substrates indicate that, while not as optimal as TCPS, PPF composites are acceptable cell culture surfaces.

A final set of issues which bear discussion include the logistics of the proposed end use of this method. The overall goal of this field of research is not to grow cells in culture plates in an incubator, but to place them into a human patient. It is envisioned that, in the manner used here, the uncrosslinked composite (presterilized via UV exposure or, more likely, through sterile processing), and sterile microspheres containing cells (prepared in a sterile laminar flow hood, as described above) should be brought together in the sterile field of an operating table. There they can be mixed at the appropriate times and placed into the patient. One advantageous aspect of using a synthetic polymeric composite system is that properties such as crosslinking time may be adjusted by varying the composition of the system. The effective result of this is that if it is determined that a
longer working time in the sterile field would be preferred, the formulation could be adjusted to achieve the desired crosslinking time. The same trends described by these results should apply to systems formulated to have different crosslinking times.

It is envisioned that this system could be used in a variety of ways, depending on the application and situation. For example, there are cases where it will become apparent that a surgery will need to be performed in the future and a bone defect will result. In this case, a bone marrow aspirate could be taken well before the surgery. The cell samples may be sent to a central processing facility to be expanded \textit{in vitro}. The cells may be encapsulated and preserved at this facility, possibly through cryopreservation. (Note: the effect of freezing on encapsulated cells has not been determined.) The encapsulated cells could then be shipped back to the hospital to be available for implantation with the composite. In another hypothetical case, that of a trauma victim with a bone defect requiring surgery, a marrow aspirate could be taken early during the surgery, sealed in the sterile field, and handed off to a nearby lab containing a centrifuge and sterile laminar flow hood. The marrow stromal cells could be isolated, encapsulated, and sealed for return to the sterile field. The microparticles containing the cells could then be incorporated with the crosslinking composite, filling the bone defect. These are two examples of how this technology could eventually be used in "real world" situations.

Based on the data generated in this experiment, in future studies addition of cells and microparticles to crosslinking PPF composites will occur at 6 min after crosslinking initiation. Addition of cells at this time point resulted in the largest difference between EC and NC.
6.8 Conclusions

These results are the first to demonstrate that temporary cell encapsulation is effective in increasing viability and proliferation of cells placed on an actively crosslinking, injectable, biodegradable bone substitute. The addition time of cells (both encapsulated and nonencapsulated) to a crosslinking PPF composite has a large effect on cell viability and proliferation. Cells which were added to crosslinking composites later had higher viability, and more encapsulated cells survived at earlier time points than nonencapsulated cells. The addition time of 6 minutes post-initiation yielded the largest differences between encapsulated and nonencapsulated cells, and is used in the studies presented in Chapter 7. There were no indications that the presence of the microspheres affected the crosslinking of the composite.
CHAPTER 7  ENCAPSULATED CELLS ON CROSSLINKING PPF: VARYING PPF:NVP RATIO

7.1 Background

This study was designed to answer two questions. First, does encapsulation into crosslinked gelatin microspheres affect phenotypic expression and long term viability of marrow stromal osteoblasts placed on a crosslinking PPF based composite, as compared to nonencapsulated cells? Second, does the ratio of PPF to NVP in the composite affect the viability, proliferation, and phenotypic expression of encapsulated marrow stromal osteoblasts, as compared to nonencapsulated controls?

7.2 Materials

Materials used were described in section 6.2. Additional materials for the calcium assay included:

- Calcium Kit 587-A  (Sigma, St. Louis, MO)
- Calcium Standard  (Sigma, St. Louis, MO)
- Acetic Acid  (Sigma, St. Louis, MO)

7.3 Experimental Design

The experimental design is a combination of those described in Chapters 5 and 6. This study uses the DNA, $^3$H-thymidine, alkaline phosphatase (ALP), osteocalcin (OC), and calcium assays to investigate the viability, proliferation, and phenotypic expression through 28 days of both cells encapsulated in crosslinked microparticles and nonencapsulated cells.
The cells were placed on three types of substrates - tissue culture polystyrene, fully crosslinked PPF composites, and PPF composites which have been crosslinking for 6 min. These substrates were chosen based on the results in Chapter 6, which indicate that the 6 min crosslinking time results in a significant increase in the viability and proliferation of the encapsulated cells (EC) as compared to nonencapsulated cells (NC).

An additional factor tested in this study was the PPF to NVP ratio in the composites. Three levels were investigated, with PPF:NVP ratios of 1:0.5 (high), 1:0.1 (medium), and 1:0.05 (low). The medium ratio is the same composition that was used in the previous study (Chapter 6). The initiator and accelerator concentrations were adjusted so that each composite crosslinked in about 10 min, as confirmed via rheometry. Temperature profiles of each crosslinking composite, along with maximum crosslinking temperatures, were measured.

As in Chapter 6, 2.03 x 10^6 cells were added to each well, either in 0.5 ml microparticles (EC) or 0.05 ml media (NC), yielding approximately a confluent monolayer in the 12-well plates. In a manner similar to Chapters 4 and 5, four wells per time point per substrate type per composite ratio were seeded for each assay. The DNA, ALP, and OC samples were taken from the same set of wells. The \(^3\)H-thymidine and calcium assays both used separate sets of wells. DNA and AP were measured on days 1, 4, 7, 14, 21, and 28. The \(^3\)H-thymidine was assayed on days 1, 4, and 7. Calcium and OC were analyzed on days 7, 14, 21, and 28.

7.4 Methods

Poly(propylene fumarate) was synthesized in the manner described in 6.4.1. Cells were harvested and cultured as described in section 4.4.1. Preparation of the gelatin solution
was described in section 4.4.2. The cell encapsulation procedure was described in section 5.4.1.

7.4.1 PPF Composite Crosslinking / Microparticle Seeding

The PPF composites were prepared as in section 6.4.4. Composites with three ratios of PPF to the crosslinker NVP were formed. The formulations are shown in Table 7.1. The medium ratio composite is the one used in Chapter 6. After the ratios were set, the amount of BP and DMT in each formulation was adjusted to achieve crosslinking times of about 10 minutes. This was confirmed by rheology.

<table>
<thead>
<tr>
<th>Component</th>
<th>High Ratio</th>
<th>Medium Ratio</th>
<th>Low Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(propylene fumarate) (PPF)</td>
<td>1 g</td>
<td>1 g</td>
<td>1 g</td>
</tr>
<tr>
<td>N-vinyl pyrrolidinone (N-VP)</td>
<td>0.5 g</td>
<td>0.1 g</td>
<td>0.05 g</td>
</tr>
<tr>
<td>Benzoyl Peroxide (BP)</td>
<td>0.0009 g</td>
<td>0.0015 g</td>
<td>0.0035 g</td>
</tr>
<tr>
<td>Dimethyl p-toluidine (DMT)</td>
<td>2.0 μl</td>
<td>2.5 μl</td>
<td>4.5 μl</td>
</tr>
</tbody>
</table>

Table 7.1 Three PPF composite formulations with different PPF:NVP ratios

Microparticles were seeded onto the 6 minute crosslinking composites and the fully crosslinked composites as described in section 6.4.4.

7.4.2 Cell Culture

Media was added to the wells 30 minutes after the cells were placed on the composites. Media changes were performed in a manner described previously (Section 4.2.3).
7.4.3 Composite Temperature Profile

The method of measuring the composite temperature profiles was described in section 6.4.6 on page 76. Five samples of each of the high and low ratio PPF composite formulations described in Table 7.1 were tested. The data for the medium ratio composite were taken from Chapter 6.

7.4.4 Composite Gel Point

The method of measuring the composite gel points was described in 6.4.7 on page 76. Five samples of each of the high and low ratio PPF composite formulations described in Table 7.1 were tested. The data for the medium ratio composite were taken from Chapter 6.

7.4.5 Assays

The DNA, $^3$H-thymidine, alkaline phosphatase activity, and osteocalcin assays were performed as described in section 4.4.5.3

7.4.5.1 Calcium Assay

The calcium assay was performed in a manner similar to one previously reported. (Jaiswal et al., 1997) Briefly, media was aspirated from the cell culture wells which were rinsed two times with PBS. One ml of 0.5 M acetic acid was placed in each well and incubated overnight on a shaker table (60 RPM). Calcium was determined from the supernatant according to the manufacturer's instructions contained in Sigma kit #587. Absorbances were read at 576 nm 10 minutes after the addition of the reagents. Total
calcium was calculated from prepared standards and normalized by the results of the DNA assay.

7.5 Statistics

As in Chapter 4 all assay results are reported as mean ± standard deviation, n=4. The statistical analysis of the assays was performed as described in section 4.5.

The maximum crosslinking temperatures for the three composite ratios were compared using a two-tailed t-test.

7.6 Results

7.6.1 Composite Temperature Profile Results

The temperature profiles and maximum temperatures reached were measured for the three crosslinking PPF composites. The maximum temperature results, displayed in Figure 7.1, are 47.8 ± 2.7 °C for the low ratio, 47.5 ± 2.9 °C for the medium ratio (as reported in Chapter 6), and 50.7 ± 3.8 °C for the high ratio. The results indicate no difference between the PPF:NVP ratios.
Figure 7.1 Maximum temperatures of crosslinking PPF composites. Error bars represent means ± SD.
7.6.2 Composite Gel Point Results

The crosslinking composite gelation profiles were measured for the three crosslinking PPF composites. The onsets of gelation, as displayed in Figure 7.2, are 608 ± 24 s for the low ratio, 619 ± 15 s for the medium ratio (as reported in Chapter 6), and 598 ± 19 °C for the high ratio. The results indicate no difference between the PPF:NVP ratios.
Figure 7.2 Onsets of gelation of crosslinking PPF composites. Error bars represent means ± SD.
7.6.3 DNA Results

The DNA results depicted in Figure 7.5 through Figure 7.3 indicate that encapsulated cells (EC) survive and increase in number when placed on all crosslinking composites, while the nonencapsulated cells (NC) do not. Aside from the NC on crosslinking composites, the cells in all treatments increased with time, leveling off around Days 14 to 21. The NC cells on crosslinking composites showed minimal cell number throughout the assay on all PPF:NVP ratios. In addition, the cells on fully crosslinked PPF composites were statistically lower than those on TCPS ($\alpha=0.5$) and EC on TCPS and fully crosslinked composites were statistically lower than NC on the same substrates. The encapsulated cell number on crosslinked composites of high PPF:NVP ratio were significantly lower ($\alpha=0.05$) than those on medium and low ratios. These differences were only apparent at the early time points of days 1 and 4.
Figure 7.3 DNA assay of encapsulated cells (A) and nonencapsulated cells (B) on low PPF:NVP ratio composites.
Figure 7.4 DNA assay of encapsulated cells (A) and nonencapsulated cells (B) on medium PPF:NVP ratio composites
Figure 7.5 DNA assay of encapsulated cells (A) and nonencapsulated cells (B) on high PPF:NVP ratio composites
7.6.4 $^3$H-Thymidine Results

Figures 7.6 through Figure 7.8 describe the $^3$H-thymidine incorporation of the seeded cells, as normalized by the results of the DNA assay. The NC on all crosslinking PPF composites displayed virtually no proliferation. Cellular proliferation of all of the other treatments decreased with time through the 14 days which were investigated. There were no statistical differences between any of the remaining treatments.
Figure 7.6 Proliferation assay of encapsulated cells (A) and nonencapsulated cells (B) on low PPF:NVP ratio composites.
Figure 7.7 Proliferation assay of encapsulated cells (A) and nonencapsulated cells (B) on medium PPF:NVP ratio composites
Figure 7.8 Proliferation assay of encapsulated cells (A) and nonencapsulated cells (B) on low PPF:NVP ratio composites
7.6.5 Alkaline Phosphatase Results

The alkaline phosphatase (ALP) activity, normalized by cell numbers from DNA assay, are represented in Figure 7.9 through Figure 7.11. Because there were essentially no nonencapsulated cells remaining on the crosslinking composites, there was virtually no ALP activity in those wells. All of the remaining treatments peaked on day 14 before decreasing through day 28. The ALP activity values for EC were statistically lower than NC on TCPS and all ratios of fully crosslinked composites. There was no statistical difference between cells on the fully crosslinked composites and those on TCPS, nor was there a difference in the values of EC on crosslinking composites and EC on fully crosslinked composite or TCPS for any ratio.
Figure 7.9 Alkaline phosphatase assay of encapsulated cells (A) and nonencapsulated cells (B) on high PPF:NVP ratio composites
Figure 7.10 Alkaline phosphatase assay of encapsulated cells (A) and nonencapsulated cells (B) on medium PPF:NVP ratio composites
Figure 7.11 Alkaline phosphatase assay of encapsulated cells (A) and nonencapsulated cells (B) on low PPF:NVP ratio composites
7.6.6 Osteocalcin Results

The results of the osteocalcin (OC) assay were normalized by the cell number results from the DNA assay. The results show that for the NC on crosslinking PPF, as in the other assays, the minimal number of cells yield minimal OC levels (as seen in Figure 7.12 through Figure 7.14). All other treatments increase to day 2. For some treatments, the values peak on day 21 (i.e. all cells on TCPS, EC on crosslinking composite - medium ratio), while others stay relatively constant (NC of fully crosslinked, high ratio composite) or increase (EC on fully crosslinked and crosslinking low ratio composites) on Day 28. Overall, the OC levels of the cells on TCPS were higher than on fully crosslinked or crosslinking PPF composites ($\alpha=0.05$). There was no significant difference between EC on fully crosslinked and crosslinking composites across all ratios.
Figure 7.12 Osteocalcin assay of encapsulated cells (A) and nonencapsulated cells (B) on low PPF:NVP ratio composites
Figure 7.13 Osteocalcin assay of encapsulated cells (A) and nonencapsulated cells (B) on medium PPF:NVP ratio composites
Figure 7.14 Osteocalcin assay of encapsulated cells (A) and nonencapsulated cells (B) on low PPF:NVP ratio composites
7.6.7 Calcium Results

The results from the calcium assay were normalized by cell number as determined from the DNA assay. As expected, no calcium deposition was detected in NC on crosslinked PPF composites (as seen in Figure 7.15 through Figure 7.17). Calcium levels for all other treatments increased through the duration of the assay. Excluding the NC on crosslinking composites, there were no differences between cells on different PPF:NVP ratios or between cells on the different substrates. The calcium levels deposited by EC were statistically lower than those deposited by NC ($\alpha=0.05$).
Figure 7.15 Calcium assay of encapsulated cells (A) and nonencapsulated cells (B) on low PPF:NVP ratio composites
Figure 7.16 Calcium assay of encapsulated cells (A) and nonencapsulated cells (B) on medium PPF:NVP ratio composites
Figure 7.17 Calcium assay of encapsulated cells (A) and nonencapsulated cells (B) on high PPF:NVP ratio composites
7.7 Discussion

This study attempted to address two questions. First, does encapsulation into crosslinked gelatin microspheres affect phenotypic expression and long term viability of marrow stromal osteoblasts placed on a crosslinking PPF based composite, as compared to nonencapsulated cells? Based on the results of the DNA assay, encapsulation allows for a great increase in the long term viability of marrow stromal osteoblasts as compared to nonencapsulated controls. Similarly, alkaline phosphatase, osteocalcin, and calcium assays indicate that the phenotypic expression is maintained through 28 days to a much higher extent for encapsulated cells than nonencapsulated cells on crosslinking composites. The second goal of this paper was to determine if the ratio of PPF to NVP in the composite affects the viability, proliferation, and phenotypic expression of encapsulated marrow stromal osteoblasts cultured upon it, as compared to nonencapsulated controls. The results indicate that only the cell number of cells that had been encapsulated and placed on crosslinking composite were affected by the PPF to NVP ratio, and only at early time points.

This study is the first to demonstrate the effectiveness of a cell carrier in preserving the viability, proliferation, and phenotypic expression through 28 days of marrow stromal cells placed on a crosslinking degradable polymeric bone substitute. This study also demonstrates that fully crosslinked, unleached PPF composite is an excellent substrate for cell growth and osteoblastic expression.

As in the study described in Chapter 6, the varying amount of time which the cells were out of the incubator was addressed by performing three encapsulations: one each for the DNA/ALP/OC assays, the $^3$H-thymidine assays, and one for the calcium assays. In
addition, all of the cells for a particular assay, for a particular time point, on a particular composite formulation were seeded at the same time. For example, four wells each of the encapsulated cells (EC) and nonencapsulated cells (NC) were seeded at the same time on crosslinking composite with a high ratio for day 21 calcium assay. The order of plates seeded was randomized with respect to day of assay and substrate.

Many of the same phenomena seen in this study were apparent in the previous study described in Chapter 6. Encapsulation may provide a barrier to diffusion of toxic species and heat which are present early in the crosslinking reactions but which dissipate or are consumed by the end of the reactions. In addition, EC on TCPS were lower than NC on TCPS. In such cases where no protection from environmental elements is necessary, the encapsulation procedure yields slightly lower cell numbers and phenotypic expression. This was described in Chapters 4 and 5. Also, cell number for both EC and NC on fully crosslinked PPF was lower then cell number on TCPS. As mentioned in Chapter 6, soluble material possessing some toxicity may leach from the composite into the culture media, affecting their viability. All of these observations made in Chapter 6 in relation to the 7 day study apply this longer 28 day study and can be extended from viability and proliferation to phenotypic expression (ALP, OC, and calcium assays).

An interesting comparison to examine is cell number as a function of PPF to NVP ratio on the crosslinking composite. The day 1 EC values increase as the ratio decreases. This effect is only seen at early time points. Later time points show no difference in cell number. This suggests two things. First, the higher concentration of the NVP in the crosslinking composite has a harmful effect on cells added to it. Secondly, after the composite is crosslinked, the composition of the substrate, with respect to PPF to NVP
ratio, has little effect on the viability. This is supported by the observation that the ratio has no detectable effect on the phenotypic expression (ALP, OC, calcium assays) which are assessed at later time points.

7.8 Conclusions

These results demonstrate that temporary encapsulated of marrow stromal cells have much higher long term viability and phenotypic expression when placed on actively crosslinking PPF composites, as compared to nonencapsulated cells. In addition, within the parameters investigated, there was virtually no difference in long term viability and phenotypic expression of encapsulated cells placed on composites with varying PPF/N-VP ratios. This study indicates that encapsulating marrow stromal cells in gelatin microparticles with crosslinked surfaces is a method which has potential for use as a cell delivery method in bone tissue engineering.
CHAPTER 8 CONCLUSIONS

An injectable, *in situ* crosslinkable, degradable polymeric carrier for osteogenic cell populations was developed. Specifically, a system for encapsulating marrow stromal osteoblasts in gelatin microspheres has been implemented with the goal of incorporation into a crosslinking composite based on poly(propylene fumarate) (PPF).

PPF has been investigated as an injectable degradable biomaterial for use as a bone substitute. It can also act as an *in situ* polymerizable polymeric scaffold for guided bone regeneration. The goal of the work described in this thesis was to allow for delivery of viable osteogenic cells with this novel material. Direct incorporation of marrow stromal cells into crosslinking PPF composites has resulted in cell death. We designed this encapsulation method to protect the embedded cells from local environmental effects during the short (20 min) composite crosslinking and subsequently release the cells to interact with the fully crosslinked composite and surrounding environment.

Initially, the microparticle formation procedure was evaluated for effects on the viability, proliferation, and phenotypic expression of marrow stromal cells. The encapsulated cells were assayed for cell number, $^3$H-thymidine incorporation, and three osteoblastic markers - alkaline phosphatase activity, osteocalcin, and mineralization. The cells were cultured for 28 days and compared to nonencapsulated control cells.

Once it was determined that the encapsulation procedure had only minor effects on the measured properties, a system to treat the surfaces of the microparticles in order to provide mechanical integrity at body temperature was investigated. The gelatin microparticles were exposed to two levels of a crosslinker in order to assess the effect of crosslinker concentration on cell viability, proliferation, and phenotypic expression. The
results indicated that exposure to a relatively high concentration of the crosslinker (5 mM) for a relatively short amount of time (5 min) yielded only minor reductions in the measured properties over 28 days. This crosslinker exposure time - concentration combination yielded microparticles which maintained their mechanical integrity in 37 °C media for about one hour before dispersing. Physical properties of the crosslinked microspheres were measured.

Based on these observations, it was concluded that the encapsulation procedure we had developed was a candidate for use with the crosslinking PPF composite in the next study. We placed the cells encapsulated in crosslinked microparticles on PPF composites. Microparticles were placed on fully crosslinked composites and on crosslinking composites at various times after the crosslinking reactions were initiated. The viability and proliferation of these cells was measured through 7 days and compared to nonencapsulated cells placed on the same substrates. The results of the study showed that the encapsulated cells retained their viability and proliferation to a much greater extent than the nonencapsulated cells when placed on crosslinking substrates soon after initiation.

These results led to a longer study that was performed using one of the crosslinking composite addition times, and varying the formulation of the composites by adjusting the polymer to monomer ratio. This 28 day study explored the viability, proliferation, and osteoblastic phenotype of encapsulated cells compared to nonencapsulated cells. The results of this experiment indicated that encapsulation of cells allowed them to remain viable and express the osteoblastic phenotype when placed
on crosslinking PPF based composites. Nonencapsulated cells, however, did not retain their viability on those same crosslinking substrates.

This work does suggest a direction for further investigation in this field. This includes a systematic examination of the effects of temperature and chemical environment on the viability, proliferation, and phenotypic expression of encapsulated marrow stromal cells. These efforts, such as dose-response studies, may elucidate the mechanisms of reduced viability observed both in unencapsulated cells placed on crosslinking composites and in the exposure of the microparticles to certain crosslinking agent – crosslinking time combinations, i.e. the 1 mM DSP for 15 min. Additional studies could investigate the effects of microparticle size and cell concentration on viability and proliferation as well as bone formation in in vivo experiments.

The outcome of this work is that the resulting polymeric cell delivery system, which is injectable and in situ crosslinkable, holds promise for bone regeneration and orthopaedic tissue engineering.
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