Histocompatibility of photocrosslinked polyanhydrides: A novel *in situ* forming orthopaedic biomaterial

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Abstract: Cell–polymer interactions in subcutaneous and bony tissue were examined for a novel class of *in situ* forming and surface eroding polyanhydride networks. Specifically, photopolymerized disks of several polyanhydride compositions were implanted subcutaneously in rats, and the tissue was analyzed for an inflammatory response. The compositions elicited varied histological responses, ranging from highly active cell layers to moderate fibrous capsules, depending on the degrading polymer composition. Furthermore, one composition was photopolymerized in a model orthopaedic defect in the proximal tibia. The feasibility of photopolymerizing the methacrylated monomers *in situ* and the adherence of the photocrosslinked polyanhydride to the medullary canal were examined. © 2002 Wiley Periodicals, Inc. J Biomed Mater Res 64A: 62–69, 2003

Key words: polyanhydrides; orthopaedic biomaterial; photopolymerization; biocompatibility; surface eroding

INTRODUCTION

While metal fixation hardware and poly(methyl methacrylate) (PMMA) cements have established their efficacy among clinicians for numerous orthopaedic procedures, a great deal of interest has emerged in the last two decades toward the development and application of degradable orthopaedic biomaterials. Degradable polymers could provide numerous advantages over currently used nondegrading materials. They can provide initial structural integrity for a weakened bone and subsequently degrade to allow the natural processes of growth and remodeling to occur. Degradable polymers circumvent secondary removal surgeries, and provide flexibility in their chemical and structural design to tailor specific mechanical and degradative properties. For example, polymers can be easily synthesized to match the degradation kinetics of concurrent tissue growth; processed into porous scaffolds with carefully engineered pore morphologies to promote tissue in-growth or for cell seeding; and readily modified to form composite or reinforced implants and fabricated to deliver osteoinductive particles, such as demineralized bone matrix or hydroxyapatite.

To date, the major classes of degradable polymers that have been actively researched as promising orthopaedic biomaterials include poly(α-hydroxy esters),[1–3] polyphosphazenes (PPHOS),[4,5] poly(propylene fumarate) (PPF),[6,7] tyrosine-derived polycarbonates,[8] and polyanhydrides.[9,10] Each of these synthetic polymers is degradable and possesses suitable mechanical properties for certain orthopaedic applications, but has limitations with respect to its degradation mechanism or processing requirements that compromise its ultimate success. To address some of the limitations of the aforementioned degradable materials, we have developed a new class of synthetic monomers that can be reacted *in situ* to form high-strength and surface degrading polymer networks.[11–13] Specifically, we have rationally designed a new class of methacrylate anhydride monomers that can be com-

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bined with a photoinitiator, packed into a defect, and photopolymerized *in situ* to form a densely crosslinked and high-strength polymer scaffold. Subsequently, the anhydride linkages in contact with the aqueous *in vivo* environment (i.e., those at the surface of the implant) are readily hydrolyzed such that the polymer erodes at the surface only. The polymer's high crosslinking density, in combination with its hydrophobic backbone chemistry, prevents diffusion of water into the polymer bulk, further supports the surface erosion mechanism, and allows maintenance of the original mechanical properties with degradation.

Photoinitiated polymerizations overcome many of the limitations of polymerizing *in situ.* Namely, the reactions are rapid and can be controlled to occur over a time frame of seconds to minutes. Under physiological conditions, the photoinitiation rates are fast enough to overcome oxygen inhibition and moisture effects, and do not require elevated temperatures. In addition, the temporal and spatial control of the photoinitiation process allows for precise control of polymer formation by directing and shuttering the light source. This control allows for the possibility of using photopolymerizations to form contourable fixation plates; the light source may be shuttered prematurely, when the system is still flexible, and the polymer could be contoured to a rounded or irregular bone and further irradiated to form a rigid plate.[11] Thus, a range of applications could benefit from an *in situ* forming material, and photopolymerizations provide numerous advantages (e.g., flexibility during implantation) in the orthopaedic arena.

In addition to developing an *in situ* polymerizable orthopaedic biomaterial, we have designed this novel class of polymers to degrade by a surface erosion mechanism. We hypothesize that a surface degradation mechanism may be ideal for many orthopaedic applications, where maintenance of strength with degradation and a gradual transfer of load from the degrading implant to the healing bone are essential. Specifically, surface erosion allows only the surface of the polymer to degrade and recede; the remainder of the implant retains its original structure. Surface erosion may also improve the overall material histocompatibility, since the degradation products eroded from the polymer surface can diffuse from the implant in a controlled manner with time. In contrast, polymers that degrade homogeneously (e.g., polyesters), demonstrate a lag-stage effect, generating high concentrations of acidic degradation products with complete polymer dissolution, which has been shown to elicit an inflammatory response.[14,15]

In this contribution, we present the first evaluation of the *in vivo* biocompatibility of this new class of photopolymerized polyanhydrides. Specifically, the general biocompatibility of several polyanhydride compositions was assessed from subcutaneous implant studies. In addition, the feasibility of photopolymerizing the methacrylated monomers in a tibia defect and the adherence of the photocrosslinked polyanhydrides to the medullary canal were examined.

MATERIALS AND METHODS

Materials

The photoreactive anhydride monomers and linear polyanhydrides were derived from materials that have shown their safe application in drug delivery formulations. Linear polyanhydrides of sebacic acid and 1,3-bis(*p*-carboxyphenoxy) propane (CPP) were approved by the Food and Drug Administration in 1996 as controlled release devices for local delivery to brain tumors.[16]

Methacrylated anhydride monomers were synthesized as described elsewhere.[12]Linear polyanhydrides were synthesized from CPP and 1,6-bis(*p*-carboxyphenoxy) hexane (CPH), and purified as presented elsewhere.[17] Two linear polyanhydride compositions were used in this study: poly-(CPH), a homopolymer of CPH, and poly(CPP:CPH), a 50:50 molar copolymer of CPP and CPH. A photopolymerizable, monovinyl monomer based on cholesterol was synthesized[18] to enhance the biocompatibility and overall hydrophobicity of the polyanhydride networks.

Subcutaneous implants

General polymer biocompatibility was assessed by subcutaneous implantation of polymer disks (d = ~12 mm, t = ~1.4 mm) in rats. Sample compositions were evaluated in triplicate for two formulations, described in Table I. The semi-interpenetrating network (semi-IPN) compositions, Formulation 1 and Formulation 2, were chosen because they exhibit ideal handling properties (i.e., moldable putties at room temperatures) and varied rates of degradation that are suitable for orthopaedic applications.[11] The polyanhydride disks were photocrosslinked under optimum polymerization conditions to ensure complete reaction of functional groups.[13] Specifically, 0.1 wt % 2,2-dimethoxy-2-

TABLE I
Polymer Formulations and Respective Degradation Rates

Formulation	Polymer Composition	Surface Degradation Rate (mm/h)
Control 1	Poly(lactic acid) (MW ~15,000)	NA
Formulation 1	75/25 (w/w) poly(MSA)/poly(CPH)	1.9×10^{-3}
Formulation 2	50/50 (w/w) poly(MSA)/poly(CPP:CPH)	5.4×10^{-4}
Formulation 3	22/78 (w/w) poly(MC/MSA)	3.0×10^{-4}

phenylacetophenone (DMPA) was dissolved in each monomer solution, and the composition photopolymerized with UV light (25–100 mW/cm^2) for ~30 min per side.

Sprague-Dawley rats averaging 450 g were used and fed laboratory rodent diet and tap water *ad libitum*. The animals were kept in a facility accredited for animal care, and the office of protection of research sources guidelines for the care and handling of laboratory animals was followed. The rats were weighed and anesthetized by intramuscular injection of ketamine HCl (87 mg/kg body wt) and xylazine (13 mg/kg body wt).[19] After the anesthesia had taken effect, the entire back of the rat was shaved and sterilely prepared. Two dorsal midline incisions were made through the skin layer ~2 cm in length and ~2–3 cm apart. A subcutaneous pocket was created laterally from each incision using blunt dissection techniques. A sterile polymer disk was placed in each pocket (4 pockets per animal), and the incisions were closed using sterile wound staples. At least 3 polymer disks were used per time point and composition, and compression molded poly(lactic acid) (PLA; MW ~ 15,000, Polysciences) disks served as controls (Control 1, Table I).

At specified time points (14, 28, and 56 days) after surgery, the rats were sacrificed by CO_2 asphyxiation, and the implants and surrounding tissue harvested. The explants were fixed with 10% buffered formalin solution, dehydrated to wax, and sectioned to 5–10 μm with a microtome (Leica RM2125). The sections were stained with hematoxylin (Electron Microscopy Sciences) and eosin (Electron Microscopy Sciences) for histological analysis.

Model bone defect

Defects were created in rat tibias to determine the feasibility of *in situ* forming the crosslinked polyanhydrides in bone and to examine the influence of the *in vivo* polymerization reaction on local bone tissue. Sprague-Dawley rats averaging 450 g in weight were used for the bone model and maintained as described previously. Using aseptic techniques and general anesthesia (i.e., ketamine HCl and xylazine with the aforementioned doses), the anteromedial tibial metaphasis was exposed via a ~2 cm longitudinal incision. A 2.3 mm diameter hole was drilled through the near cortex and underlying trabecular bone using a hand-press drill.

A reactive monomer solution, Formulation 3, consisting of 22/78 (w/w) methacrylated cholesterol (MC)/ methacrylated sebacic acid (MSA) was chosen for the bone defect studies based on the rapid reaction of this composition to form a highly crosslinked and slowly degrading network. Furthermore, this putty was easily packed into the defects and photocured *in situ* using a visible light initiating system. Specifically, the monomer composition was polymerized with 0.25 wt % camphorquinone (CQ; Aldrich), 0.25 wt % triethanolamine (TEA; Aldrich) and 25–50 mW/cm^2 blue light (DenMat, Marathon Two, Model 3940). Unfilled defects were used as controls. At specified time points (3, 5, and 7 days) postsurgery, the animals were sacrificed by CO_2 asphyxiation, the tibias were harvested, and the defect sites examined grossly. The bone was fixed in 10% formalin buffer solution, decalcified in 10% formic acid, and cut down to include the defect with minimal bone tissue on each side. Finally, the bones were dehydrated through graded ethanol to wax and sectioned to 5–10 μm sections. Bone sections were stained with hematoxylin and eosin and Masson's trichrome.[20]

RESULTS AND DISCUSSION

General histocompatibility

Prefabricated polyanhydride implants were placed subcutaneously in rats and the surrounding tissue analyzed for an inflammatory response. Abbreviated names for cells and tissue landmarks are listed in Table II, and used for labels in the following micrographs of subcutaneous and bone tissue. For the tissue sections presented, the polymer–tissue interface is carefully labeled, as well as cell types, for reference.

It has been thought that a typical, "acceptable" response to inert biomaterials includes the formation of a fibrous tissue with many fibroblasts and few phagocytic leukocytes (i.e., lymphocytes, neutrophils, macrophages). However, degradable materials typically elicit a greater inflammatory response due to the continued stimulus of the degradation products. Thus, PLA disks were implanted as a degradable control material. In contrast to the surface eroding polyanhydrides, PLA degrades homogeneously by hydrolysis of ester linkages. The degradation products (i.e., lactic acid) can be metabolized and excreted normally, and as a result, PLA is widely used in many medical applications. PLA controls were evaluated at 14 and 28 days, and Figure 1 shows the cellular response at the polymer–tissue interface (P-T) at 14 [Fig. 1(A)] and 28 [Fig. 1(B)] days. In general, the PLA samples exhibited mild to moderate inflammatory responses representative of a degrading material.

TABLE II
Abbreviated Names for Cell Types and Tissue Landmarks in Histology

Cell Type	Abbreviation
Adipocyte (fat cell)	A
Fibroblast	FB
Foreign body giant cell	FBG
Macrophage	MA
Marker	**Abbreviation**
Active layer	AL
Fibrous layer	FL
Muscle	MU
Polymer–tissue interface	P-T
Vascular structure	V
Sectioning artifact	AR
Cortical bone	CB
Fibrous callus	FC

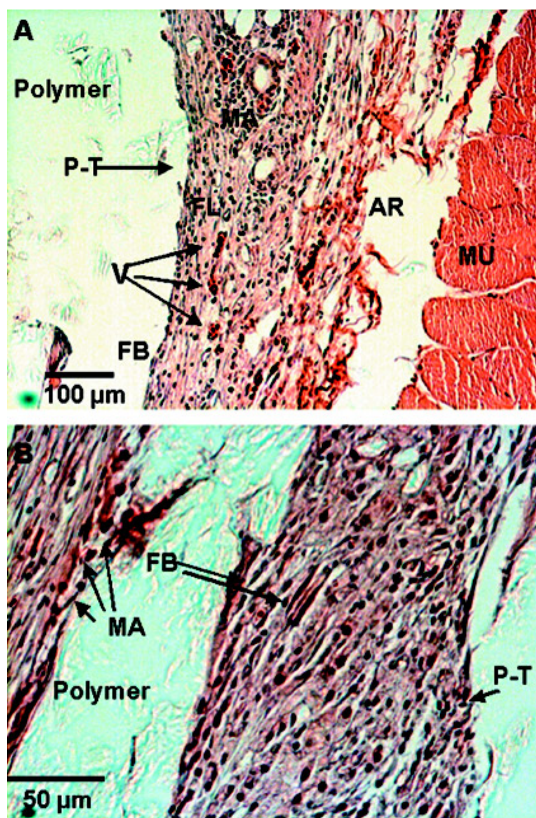

Figure 1. Micrographs illustrating the hematoxylin and eosin stained tissue adjacent to poly(lactic acid) controls at 14 (A) and 28 (B) days. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 1(A) shows a mildly organized and oriented fibroblastic layer (FL) immediately adjacent to the PLA implant. The cells in this layer are primarily fibroblasts (FB) with a few wandering macrophages (MA). Slightly further from the polymer–tissue interface (i.e., 50–75 μm from the PLA surface), the cell density increases and the number of free macrophages also increases. In this region, there are numerous small capillaries (V) present, filtering the inflammatory cells into the local tissue. Even further from the tissue–polymer interface (i.e., ~150 μm), the cells become more fibroblastic and are loosely oriented in parallel to the polymer surface.

At later time points, as represented in Figure 1(B), the active cellular layer becomes more aggressive, particularly because the cells have begun to penetrate into the degrading polymer. In Figure 1(B), the bulk of the PLA implant is located on the right side of the micrograph; however, there is a PLA particle that has been engulfed by tissue on the left of the micrograph. Furthermore, the tissue adjacent to the polymer surfaces has roughly the same number of macrophages and fibroblasts. The fibroblasts are only slightly oriented, and the macrophages present are actively engulfing PLA as indicated by the presence of several foreign body giant cells (FBG). At this time point, the number of blood vessels has diminished. Overall, the *in vivo* response to the PLA controls was considered mild to moderate.

The polyanhydride compositions chosen for subcutaneous implantation and compared to the PLA controls were based on their desirable degradation kinetics and preliminary *in vitro* studies, suggesting their cytocompatibility with osteoblasts. Two polyanhydride (semi-IPN) compositions comprised of crosslinked MSA and hydrophobic, linear polyanhydrides were examined. These compositions showed promise as orthopaedic biomaterials, in terms of repeatable material synthesis, ease in handling and processing, and acceptable degradation kinetics. The hydrophobic linear polyanhydrides [e.g., poly(CPH) and poly(CPP:CPH)] in Formulations 1 and 2 significantly reduce the rate of degradation compared to a homopolymer network of poly(MSA). For example, an ~1 mm thick disk of MSA polymerized with 50 wt % poly(CPP:CPH) degrades in ~2 months, compared to ~2 days for a similar disk of poly(MSA) alone. In addition, the linear polymers provide advantages with respect to controlling the initial viscosity and reducing the polymerization exotherm. Disks of the semi-IPN compositions were implanted subcutaneously and evaluated in triplicate at 14, 28, and 56 days.

Formulation 1 invoked a severe inflammatory response both at early and late time points. One explanation for the heightened inflammatory reaction compared to the PLA controls is that this polymer composition degrades rapidly; an ~1 mm thick disk of this composition is completely degraded *in vitro* in ~20 days (i.e., the degradation front velocity is 1.9×10^{-3} mm/h). Figure 2 shows the cellular response at the polymer interface at 14 [Fig. 2(A)] and 56 [Fig. 2(B)] days. At 14 days postimplantation, the tissue adhering to the polymer implant was infiltrated with many inflammatory cells: lymphocytes (L), macrophages, and even foreign body giant cells. The few collagen fibrils in the encasing tissue were disoriented and loosely arranged between large quantities of small and large blood vessels.

At 56 days, the tissue surrounding the implant had infiltrated the polymer and begun to engulf large polymer particles. In addition, the heightened number of vascular structures present at 14 days is not apparent at 56 days. Figure 2(B) illustrates the aggressive macrophage response to the polymer particulates. Here, macrophages have fused together to form multinucleated foreign body giant cells, and these cells are phagocytosing the polymer particles. Even at this late time point (8 weeks), the majority of the cells in the tissue surrounding the polymer implant are highly active, inflammatory cells.

One possible explanation for the severe inflammatory reaction seen in the tissue encasing this implant

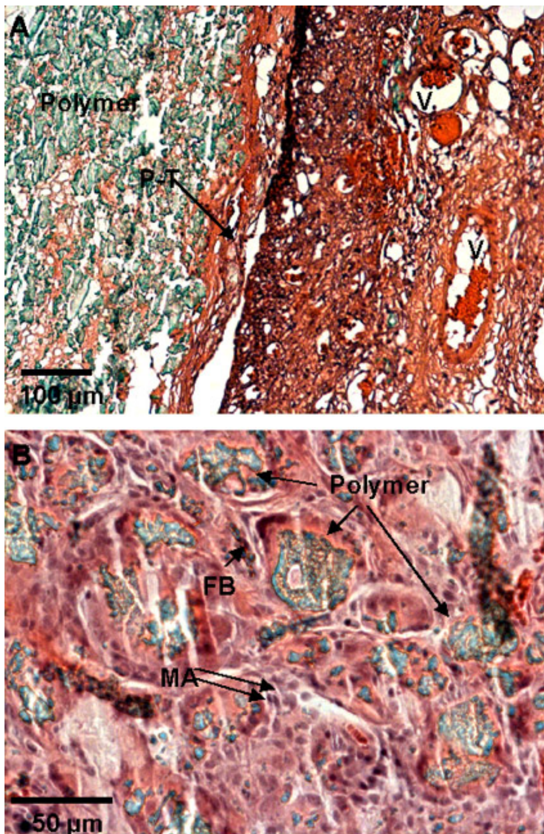

Figure 2. Hematoxylin and eosin stained micrographs of the tissue reaction to Formulation 1 implanted for 14 (A) and 56 (B) days in subcutaneous tissue. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

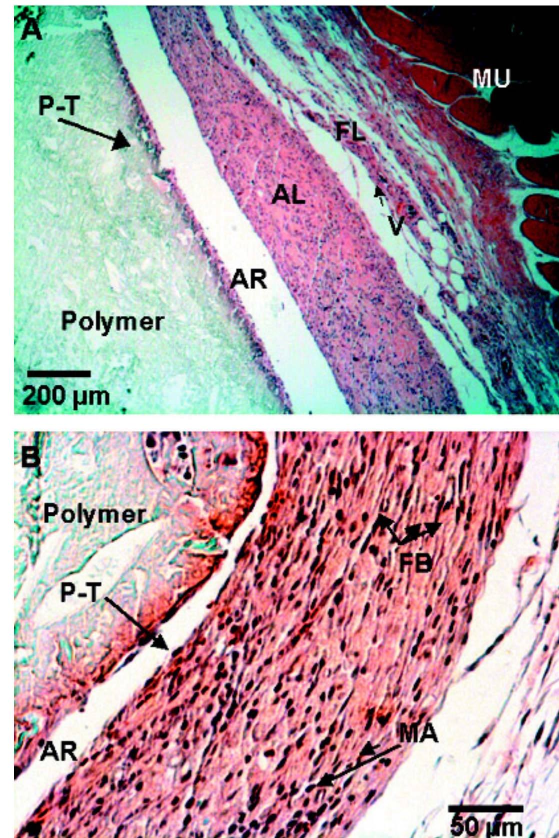

Figure 3. Hematoxylin and eosin stained micrographs at low (A) and high (B) magnification of the tissue–polymer interface for a disk of Formulation 2 implanted subcutaneously for 28 days. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

composition is that the rate of degradation of the polymer was exceeding the ability of the tissue to process and remove the degradation products. Acidic degradation products are released as the polyanhydride implants are broken down, which may result in local pH changes and promote inflammatory cell infiltration. To test this hypothesis, a second polyanhydride composition, Formulation 2, which was similar in chemistry from the aforementioned formulation, yet degrades much more slowly (by a factor of 3.5), was examined histologically at identical time points.

After 4 and 8 weeks, the tissue response to Formulation 2 was examined, and representative histological sections are shown in Figures 3 and 4. Using hematoxylin and eosin stains, the polyanhydride network images slightly green and is clearly evident in the 5 μm sections. In general, the overall response to the polymer implant can be classified as a moderate inflammatory response, with portions of highly cellular, active regions and mild fibrotic areas at 4 weeks.

The polymer–tissue interfaces for two samples of Formulation 2 evaluated at 28 days are shown in Figure 3. The micrographs represent the varied cellular capsule surrounding this semi-IPN material. The top micrograph indicates a highly cellular "active" layer (AL) surrounded by a loose connective tissue capsule. The active layer consists mainly of active macrophages and fibroblasts. Some necrotic tissue or wound exudate is also present in the active layer. In the loose fibrous capsule adjacent to the highly cellular layer, there are numerous healthy fibroblasts dispersed in a collagen network oriented parallel to the surface of the polymer. The bottom micrograph [Fig. 3(B)] illustrates the response to Formulation 2 at higher magnification, comprised of healthy fibroblasts with a few, dispersed macrophages.

The tissue response to Formulation 2 was also examined at 8 weeks, and micrographs of two regions of the cellular capsule at the implant interface are shown in Figure 4. The top micrograph [Fig. 4(A)] shows a low magnification image of the dense cellular active layer, surrounded by a highly fibrous connective tissue capsule. After examining the time sequence of tissue encapsulation for this implant composition, it was evident that the active layer grows to a thickness of ~250 μm at 28 days, and continues to thicken as the polymer disk degrades and decreases in thickness. In some areas of the polymer–tissue interface, there was a significantly thinner active layer (i.e., 50 μm); how-

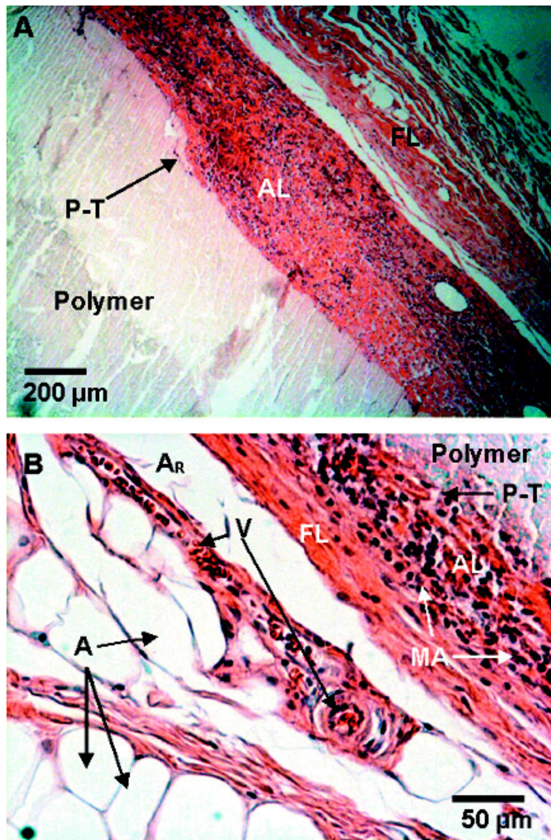

Figure 4. Hematoxylin and eosin stained micrographs at low (A) and high (B) magnification of the tissue capsule surrounding Formulation 2 implanted subcutaneously for 8 weeks. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ever, in every active layer, there was a loose network of fibroblasts with macrophages present. Furthermore, there was a moderate amount of vascularization surrounding the nearby tissue, as typified by the vessel running parallel to the polymer shown in Figure 4(B).

After 8 weeks, the response to Formulation 2 was moderately inflammatory and a significant improvement over Formulation 1, the previously described semi-IPN composition. The improved tissue response is thought to coincide with the slower release of acidic degradation products. In addition, the response seen with this implant composition was comparable to the PLA controls, indicating that the response to this new degradable biomaterial is similar to that observed for other FDA-approved degrading implants.

Tissue response to the *in vivo* photopolymerization

Numerous orthopaedic applications currently use thermally initiated polymerizations to react methyl methacrylate-based bone cements *in vivo*. While this method of reaction has been well characterized and utilized clinically, photopolymerizations may overcome many of the disadvantages of thermal polymerizations.[11] However, the ability to *in situ* form a photopolymerized material in a bone defect has not been demonstrated, nor has the effect of a photopolymerization reaction on bone tissue been characterized. Therefore, a model bone defect was created in the proximal rat tibia, and a suitable monomer-initiator formulation (Formulation 3, Table I) was packed into the defect and photopolymerized *in vivo*.

Formulation 3 was selected based on its handling properties, the degradation rate of the resulting polymer, and the time scale and rate of the polymerization reaction. The system was a comonomer composition of 22/78 MC/MSA photoinitiated with 0.25 wt % camphorquinone and 0.25 wt % triethanolamine. In air and at 25°C, this formulation polymerizes in ~300 s to greater than 95% conversion by exposure to ~30 mW/cm^2 of 400–500 nm visible light. In addition, this system degrades relatively slowly; the degradation front moves at ~3×10^{-4} mm/h. Finally, the viscosity of this system is similar to a putty, which makes handling simple and packing into a defect convenient.

Note that this defect would heal if left untreated, but the objective of this study was to determine whether the polymer can be fabricated *in vivo,* and that its presence and the polymerization do not adversely affect the healing process. Thus, following short time points (i.e., 3, 5, and 7 days), the tibias were harvested, and the bone was examined histologically to determine the effects of the photopolymerization reaction on local bony tissue. In addition, the ability of the implant to adhere to the bone and maintain its shape was grossly examined.

Figure 5 compares cross-sections of tibias that received the defect and polymer treatment evaluated after 3, 5, and 7 days, and compared to a control tibia at 7 days, which received only a defect with no treatment. The micrographs show that the polymer filled the medullary canal completely in all three polymer-treated defects. In the control defect, the medullary canal contains pieces of bone that remained after drilling out the defect. Histological analysis of the bone fragments in the control defects reveals that they are being resorbed by osteoclasts. In addition, fibrous calluses are forming across the defects for all cases. After 3 days postimplantation, there is a small fibrous callous, consisting of few collagen fibrils; however, the callus laid down by local fibroblasts becomes increasingly thicker with time. At 7 days, a thick capsule has developed around the drilled defect, and there are numerous regions of new bone growth. The presence of pink staining spicules of bone demonstrates that neither the photopolymerization reaction nor the polymer implant has hindered the bone's normal remodeling and healing. Similarly, there are areas of new

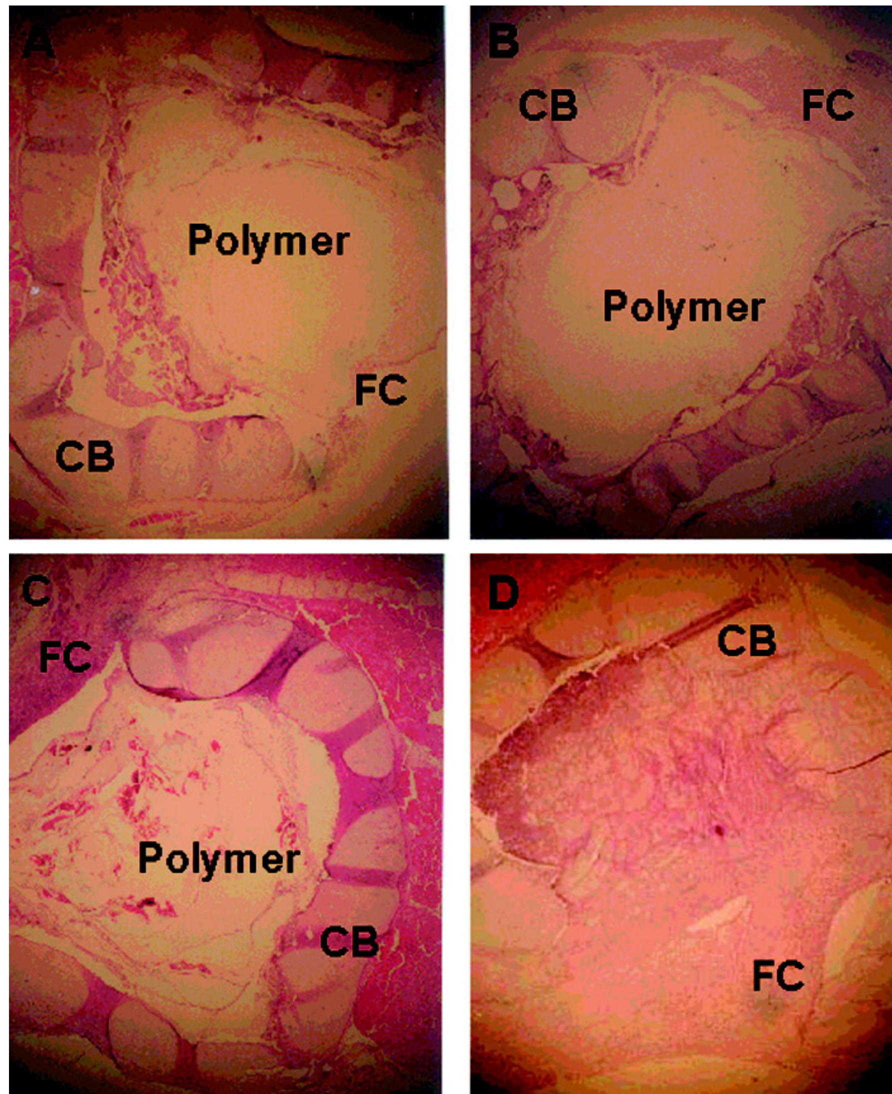

Figure 5. Hematoxylin and eosin stained cross-sections of tibias treated with MC/MSA for 3 (A), 5 (B), and 7 (C) days compared to the sham control at 7 days (D). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

bone growth in the callus forming around the control defect also at 7 days.

CONCLUSIONS

Histological evaluation of subcutaneously implanted disks of polyanhydride semi-interpenetrating networks revealed an active cellular response to the degrading polymer matrices. Formulation 2, 50/50 poly(MSA)/poly(CPP:CPH), encouraged a cellular response that was similar to poly(lactic acid) controls. The decrease in inflammation correlated with the decrease in degradation rate, and consequently, the decrease in release of acid degradation products. Furthermore, a model defect created in the proximal tibia was used to assess the effects of the photopolymerization reaction on local bony tissue and determine the feasibility of forming crosslinked polyanhydride networks *in situ*. The presence of new bone spicules in the fibrous callous indicates healing of the polymer-treated defect at 7 days with no adverse effects from the photopolymerization reaction. Overall, the inflammatory response and the reaction of *in vivo* formation in a bone defect of these polyanhydride networks indicate that these materials have a biocompatibility response that is acceptable for an *in situ* forming orthopaedic biomaterial.

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