

# An Investigation of the Cytotoxicity and Histocompatibility of *In Situ* Forming Lactic Acid Based Orthopedic Biomaterials

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**Abstract:** The cytotoxicity and biocompatibility of polymer networks prefabricated from multifunctional lactic acid based oligomers that are being developed for orthopedic applications were assessed through *in vitro* cytotoxicity analysis and subcutaneous implantation. After 7 and 14 days, no significant difference was observed in the relative viability or alkaline phosphatase activity of primary rat calvarial osteoblasts cultured in the presence or absence of degrading polymer networks, indicating that the degradation products had no detrimental effect on the function or activity of the cultured cells. The tissue response to preformed lactic acid networks implanted in rats consisted of a mild inflammatory response with an increase in fibrous capsule thickness and inflammation correlating with faster degrading polymer compositions. This relatively neutral response is indicative of a biocompatible, degradable polymer that has potential medical applications. Finally, porous scaffolds were implanted subcutaneously in rats, and vascularized fibrous tissue infiltration was highly dependent on the scaffold porosity and architecture. This finding indicates that an *in situ* forming porous scaffold of this composition may support the infiltration of surrounding vascularized tissue, and thus be applicable to orthopedic treatments of large bone defects. © 2002 Wiley Periodicals, Inc. *J Biomed Mater Res (Appl Biomater)* 63: 484–491, 2002

**Keywords:** orthopedic biomaterials; biocompatibility; photopolymerization; bone tissue engineering; *in situ* formation

## INTRODUCTION

Limitations of current orthopedic techniques have prompted researchers to investigate alternative strategies for treatment of musculoskeletal impairments. The current decade has been termed the Bone and Joint Decade by the World Health Organization, and the development of treatments for musculoskeletal injuries due to trauma or disease has been outlined as one of the primary research thrusts.<sup>1</sup> With this in mind, tissue engineering is one area that could have a significant impact for future treatment of these musculoskeletal impairments. In general, tissue engineering of bone involves a combination of one or more of the following approaches:<sup>2</sup> (1) the release of osteoinductive factors, such as bone morpho-

genetic proteins (BMPs),<sup>3,4</sup> transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1),<sup>5,6</sup> and, more recently, DNA that delivers the appropriate genes to enhance bone regeneration;<sup>7,8</sup> (2) the study of the development and differentiation of bone-forming cells that could potentially be delivered to the body;<sup>9–11</sup> and (3) the development of osteoconductive scaffolding materials that provide a surface that is conducive to the attachment and proliferation of osteoblasts or osteoblast precursor cells.<sup>12,13</sup>

The focus of many researchers is the development of materials that support the attachment of osteoblasts. Synthetic polymer development has included polymers such as poly( $\alpha$ -hydroxy esters),<sup>14–16</sup> poly(propylene fumarates),<sup>17,18</sup> polycarbonates,<sup>19</sup> and polyanhydrides.<sup>20,21</sup> Although this introduction is not an exhaustive review of current techniques and materials being developed for bone tissue engineering applications, it illustrates the breadth of research in the area.

One beneficial property of these synthetic polymers is the ability to form *in situ* scaffolding materials that could deliver cells or provide a structure for tissue infiltration. Besides eliminating the need for *ex vivo* implant fabrication, there are

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other benefits for *in situ* scaffold formation. For instance, the contact and adhesion between the biomaterial and native bone may be enhanced with a polymer formed directly in the bone defect. This aspect is especially important if the defect is of an unusual shape and size. Ease of implantation during surgery is an additional advantage. With these ideas in mind, multifunctional, degradable oligomers that can be photopolymerized *in vivo* to form degradable polymer networks have recently been developed.<sup>22</sup> The macroscopic properties of the resulting networks, along with the ability to react the oligomers *in situ*, make the materials ideal candidates for degradable scaffolds for musculoskeletal applications.

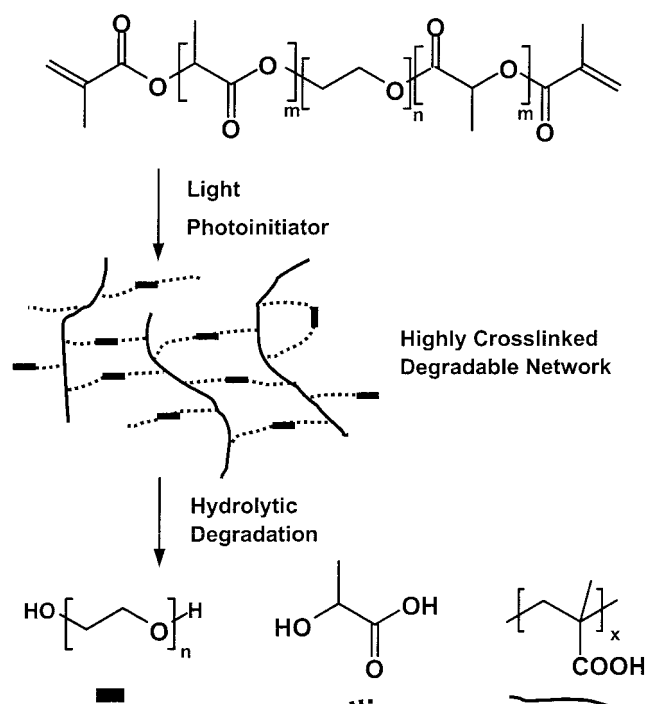
Specifically, *in situ* forming lactic acid based oligomers that react on clinically acceptable time scales to form degradable networks through photoinitiated polymerizations were synthesized and characterized.<sup>22</sup> The ability of osteoblasts to attach and proliferate on these polymers was investigated.<sup>23</sup> Osteoblast growth and viability on these polymers were comparable to that on tissue culture polystyrene (TCPS) and degradable polymer (50:50 PLGA) controls, depending on the oligomer chemistry. Additionally, alkaline phosphatase activity was expressed in osteoblasts attached to films of the synthesized polymers. The results of these studies suggested that the oligomer chemistry plays an important part in polymer osteoconductivity, and thus, one specific oligomer, 2EG10LA, was identified as being the most osteoconductive of the oligomers investigated. This molecule consists of a diethylene glycol core with ~10 lactic acid repeat units on each side, which was functionalized with methacrylate groups to facilitate formation of a highly cross-linked polymer via a photoinitiated polymerization.

In a recent article,<sup>24</sup> Agrawal and Ray outlined many of the desirable properties of a polymeric scaffold for musculoskeletal applications. The first property on the list was that the scaffold must be biocompatible. *In situ* forming lactic acid based polymers possess many desirable properties as a scaffolding material in orthopedics, but the intrinsic biocompatibility of these specific polymers has yet to be investigated. Thus, the first objective of this study was *in vitro* analysis of the toxicity of the degrading polymers in the presence of primary rat calvarial osteoblasts. Next, the *in vivo* cellular response in subcutaneous tissue was examined for both two-dimensional films and three-dimensional porous scaffolds.

## MATERIALS AND METHODS

### Polymer Synthesis and Fabrication

Tetrafunctional lactic acid oligomers were synthesized and purified as described previously,<sup>22</sup> and their general structure is shown in Figure 1. The polymers investigated in this study included poly(2EG10LA) ( $n = 2$ ,  $m = 10$ ) and poly(8EG6LA) ( $n = 8$ ,  $m = 6$ ). These two chemistries were chosen because they resulted in varying network degradation kinetics and mechanical properties<sup>22</sup> and osteoblast attachment.<sup>23</sup> For all studies, a visible light initiating system of 0.5



**Figure 1.** Schematic of multifunctional lactic acid oligomers ( $n$  is the number of ethylene glycol repeat units, and  $m$  is the number of lactic acid repeat units); *in situ* network formation with the addition of light and a photoinitiator; and hydrolytic degradation into the starting oligo(ethylene glycol) core, lactic acid, and poly(methacrylic acid). Compositions studied: 2EG10LA ( $n=2$ ,  $m=10$ ) and 8EG6LA ( $n=8$ ,  $m=6$ ).

wt% camphorquinone and 0.5 wt% ethyl-4-N,N-dimethylaminobenzoate was added directly to the oligomers. Camphorquinone is widely used in the dental field for curing of dimethacrylate composite resins.<sup>25</sup> The oligomer/initiator solution was polymerized between glass slides for 15 min with exposure to  $\sim 10$  mW/cm<sup>2</sup> visible light. Disks with a diameter of 5 mm and a thickness of 1 mm were punched from the polymer films for cytotoxicity studies, and ones with a diameter of 10 mm and a thickness of 1 mm were used for subcutaneous implantation. As a degradable polymer control, 50:50 poly(lactic-co-glycolic acid) (PLGA) was obtained from Birmingham Polymers (Birmingham, AL) and molded into disks of the same dimensions. Porous polymers were prepared with the use of a modification of a previously described<sup>26</sup> poragen leaching technique where salt particles were photoencapsulated in the polymer and leached in deionized water for 24 h. All implanted polymers were sterilized by exposure to ultraviolet light overnight.

### Cell Culture and Cytotoxicity Studies

Primary neonatal rat calvarial osteoblasts were isolated as described in detail previously.<sup>27</sup> Briefly, the calvaria of neonatal rats were dissected, the periosteum was removed, and the calvaria were minced and digested in a collagenase solution. The supernatant from the third 20-min digestion was collected and filtered to obtain the osteoblasts. Osteoblasts

were seeded at a density of approximately  $3 \times 10^4$  cells/cm<sup>2</sup> in 12-well transwell plates (Costar). Cells were cultured in Dulbecco's modified eagle medium (Gibco) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco). After 24 h of culture, the media was changed, and the synthesized polymers were added to the wells. The transwell plates allowed the polymers to degrade while being suspended ~1 mm above the cultured cells, while still submerged in media (2 ml per well). The osteoblasts were cultured for 7 and 14 days in the presence of the degrading polymers. The media were changed every other day throughout the experiment. Cells cultured without a degrading polymer present were used as a control system. The mass loss during polymer degradation in culture was determined by weighing the polymer initially and then drying the polymer in a vacuum oven after the desired amount of degradation.

The relative toxicity of the polymer degradation products on the cells was assessed with an MTT viability assay. A solution of 1% MTT in serum-free media was added to the wells and incubated for 4 h. An equal amount of 0.04 N HCl in spectrograde isopropanol was added to the wells and incubated for 30 min on an orbital shaker. The absorbance of this solution was measured at 560 nm on a Perkin Elmer Lambda 40 spectrophotometer. The cell viability was normalized to the viability of osteoblasts cultured without a degrading polymer present.

Alkaline phosphatase (ALP) activity was determined using a commercially available kit (Sigma, Kit 245). Briefly, 20  $\mu$ l of lysate solution was incubated with p-nitrophenyl phosphate at room temperature for 5 min. The absorbance was measured at 405 nm in 1-min increments, and the slope of the absorbance versus time plot was used to calculate the ALP activity. The total protein was determined with the use of a commercially available Bio-rad Protein Assay, where 250  $\mu$ l of the lysate solution was incubated with 750  $\mu$ l of the Bio-rad solution (diluted one part dye reagent concentrate with four parts deionized water). The ALP activity is reported as a value normalized to the total protein. All experiments were performed in triplicate and reported as the mean and the standard deviation of the mean.

### Subcutaneous Implantation

To assess the inflammatory response, polymers were implanted subcutaneously, according to approved IACUC protocols, in Sprague-Dawley rats (Harlan) weighing 375–400 g. NIH guidelines for the care and use of laboratory animals (NIH Publication No. 85-23 Rev. 1985) were observed. Rats were anesthetized by intramuscular injection of a mixture of ketamine HCl (87 mg/kg body wt) and xylazine (13 mg/kg body wt). Two midline incisions were made in the dorsum of the rats. Subcutaneous pockets were made with blunt dissection, polymer disks [poly(2EG10LA), poly(8EG6LA), and PLGA] were implanted (four disks per animal), and the incisions were closed with sterile wound clips. After 14, 28, and 56 days, rats were sacrificed and the polymers ( $n=3$  per composition per time point) and surrounding tissue were

harvested for histological analysis. Specimens were fixed in formalin for 24 h, dehydrated, and embedded in paraffin. Samples were sectioned (5  $\mu$ m), and stained with hematoxylin and eosin according to standard protocols.

## RESULTS AND DISCUSSION

The biocompatibility of networks synthesized from multifunctional lactic acid based oligomers that can be formed *in situ* via a photoinitiated polymerization was assessed in this study through cytotoxicity studies and subcutaneous implantation of prefabricated networks of selected polymer compositions. These polymer networks possess many of the desired properties for scaffolding materials for musculoskeletal applications, and thus, a study of the polymer biocompatibility, especially as a function of degradation, is an essential step in the development of these networks for orthopedic applications.

The general structure of the multifunctional lactic acid oligomer is shown in Figure 1. The oligo(ethylene glycol) core is of low molecular weight and serves only to present terminal hydroxy end groups for subsequent modification with two methacrylate groups. These divinyl macromers can then be reacted via a photoinitiated chain polymerization to produce relatively highly cross-linked networks. Upon hydrolysis, these networks degrade into the starting oligo(ethylene glycol) core molecule, lactic acid, and poly(methacrylic acid). The bulk of the network is composed of lactic acid. The poly(methacrylic acid) chains are of relatively low molecular weight, which is controlled by the initiation rate.<sup>28</sup> The biocompatibility of the primary degradation product, lactic acid, is one reason why poly(lactic acid) has been so widely used in the biomedical industry with applications ranging from degradable sutures, implant hardware, and scaffolding materials for tissue engineering.

### Cytotoxicity Studies

The viability of primary rat calvarial osteoblasts in the presence of degrading 50:50 PLGA, poly(2EG10LA), and poly(8EG6LA) is shown in Figure 2(a). The viability, as determined with an MTT assay, was normalized to the viability of cells cultured on TCPS, and thus, values close to 1 are indicative of nontoxic cell culture conditions. As observed in Figure 2(a), the viability is nearly 1 for all the degradable polymer compositions investigated over the time course of the experiment (2 weeks) with no significant differences between the degrading polymer compositions. The corresponding polymer mass loss (i.e., mass of released degradation products) is outlined in Table I for each of the polymer compositions after 7 and 14 days. Poly(2EG10LA) lost 4.3 and 9.5 % mass after 7 and 14 days degradation, respectively, whereas poly(8EG6LA) lost 13.0 and 16.4% mass after 7 and 14 days, respectively. These differences in degradation kinetics and mass erosion were attributed to differences in the starting oligomer chemistry and, conse-

quently, differences in polymer hydrophobicity and diffusivity of water and degradation products through the network. As expected the more hydrophilic poly(8EG6LA) networks degraded at the greatest rate; however, the faster release of degradation products did not negatively impact osteoblast viability.

The alkaline phosphatase (ALP) activity was also measured for cells under the same culture conditions as the viability experiments, and the results are shown in Figure 2(b). Alkaline phosphatase is an important marker of osteoblast phenotype and differentiation, and thus is essential in determining the effects of the degradation products on the cultured osteoblasts. There was no significant difference in the ALP expression between the three polymer compositions. Additionally, an increase in ALP was observed between 7 and 14 days for osteoblasts cultured in the presence of the degrading polymers. Therefore, *in vitro* results showed no significant toxic effects on cultured cells by the synthesized polymers over a 2-week period when up to 16.4% of the polymer degradation products have been released. There was no investigation of markers for late osteoblastic differentiation (e.g., calcium deposition or osteocalcin secretion) in this study, but it was previously shown<sup>23</sup> that osteoblasts seeded onto films of poly(2EG10LA) deposited minerals similarly to

**TABLE I. Percent Polymer Mass Loss from Polymer Disks During Cytotoxicity Study**

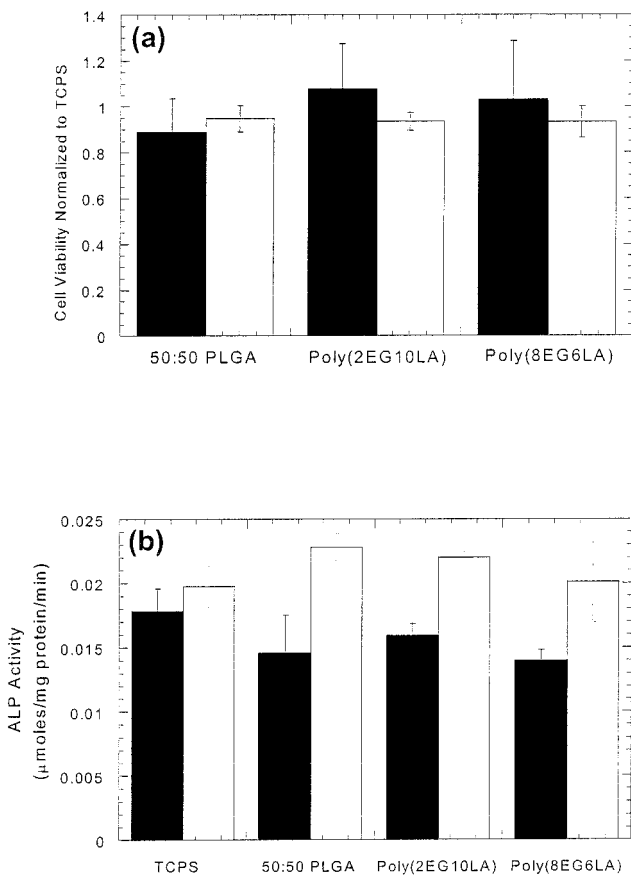
Polymer Composition	Mass Loss (%)	
	7 days	14 days
50:50 PLGA	7.2 ± 4.3	15.3 ± 5.3
Poly(2EG10LA)	4.3 ± 2.3	9.5 ± 5.3
Poly(8EG6LA)	13.0 ± 0.9	16.4 ± 1.8

osteoblasts seeded on control surfaces of 50:50 PLGA and TCPS.

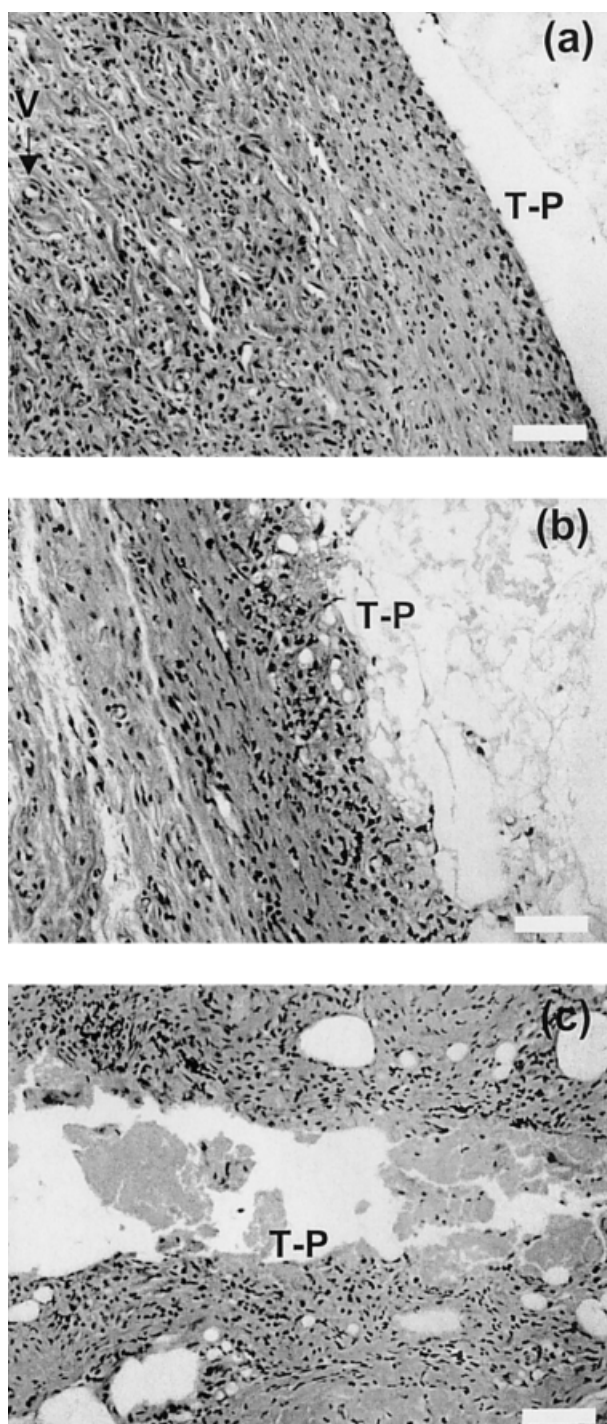
### Subcutaneous Implantation: Polymer Films

For all of the polymer compositions investigated in this study, no grossly apparent tissue necrosis or inflammation was observed from implantation up to 8 weeks postimplantation. As a baseline reference, hematoxylin and eosin stained histological sections for the 50:50 PLGA degradable polymer control are shown in Figure 3 for implants removed 14, 28, and 56 days postimplantation. After 14 days of implantation [Figure 3(a)], a moderate acute and chronic inflammatory response was observed near the degrading 50:50 PLGA implant. The tissue surrounding the implant consisted mainly of unorganized connective tissue with macrophages and foreign body giant (FBG) cells. Mature fibrous tissue was not yet seen at this time point. After 28 days [Figure 3(b)], a maturing fibrous capsule was formed around the implant, with mild chronic inflammation. An increase in FBG cells was seen, especially around dislodged fragments of the degrading polymer. Fibrous tissue had begun to infiltrate the bulk of the polymer. After 56 days [Figure 3(c)], the polymer was almost completely degraded, with very little polymer remaining in the implantation site. A high density of chronic inflammatory cells was still observed near the remaining polymer. The expedited polymer degradation and, consequently, the enhanced rate of mass loss (due to the high glycolic acid concentration in the polymer) provide one reasonable explanation for the inflammatory response observed in this system.

Photomicrographs of the material-tissue interface for poly(2EG10LA) are shown in Figure 4. After 14 days [Figure 4(a)], a thin loosely organized fibrous capsule was formed around the material with minimal acute or chronic inflammation. Granulation tissue was present around the implant consisting of fibroblasts and newly formed blood vessels. After 28 days [Figure 4(b)], the fibrous capsule was thickened, with minimal chronic inflammation near the implant. Few FBG cells were observed, only around rare foci of detached polymer. A well-developed, dense fibrous capsule with minimal chronic inflammation was seen after 56 days [Figure 4(c)]. Very little infiltration of the degrading polymer by fibrous tissue was seen, with a distinct border between the polymer and surrounding subcutaneous tissue. The inflammatory response to this polymer was relatively mild and consistent with the typical response to implantation of a biocompatible material.



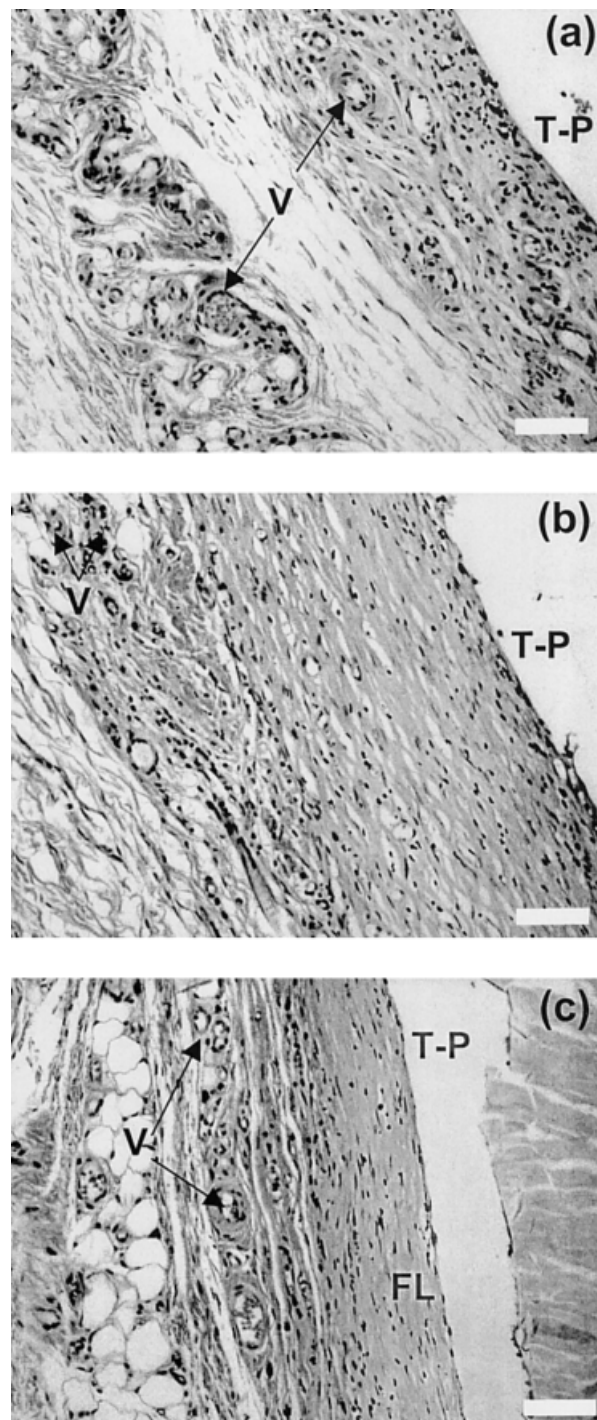
**Figure 2.** (a) Relative cell viability and (b) alkaline phosphatase activity of rat calvarial osteoblasts in the presence of degrading 50:50 PLGA, poly(2EG10LA), and poly(8EG6LA) after 7 (black) and 14 (white) days.



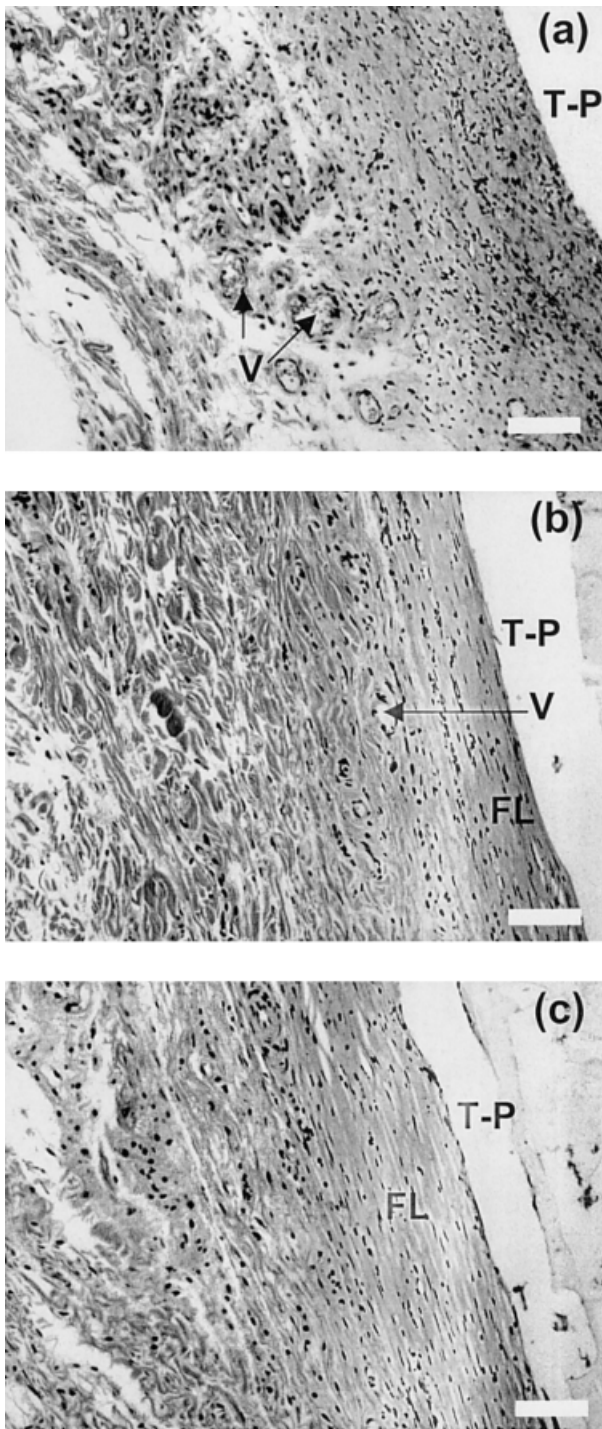
**Figure 3.** Hematoxylin and eosin stained sections ( $5\ \mu\text{m}$ ) of 50:50 PLGA and surrounding subcutaneous tissue: (a) 14 days, (b) 28 days, and (c) 56 days postimplantation (bar =  $100\ \mu\text{m}$ ). T-P = tissue/polymer interface and V = vascular structures.

Histological results for the final polymer composition with increased hydrophilicity and degradation rate, poly(8EG6LA), are shown in Figure 5. The overall response to this material more closely resembled the 50:50 PLGA implant than poly(2EG10LA) and is likely linked to the faster degradation rate and mass loss compared to the poly(2EG10LA). At the

earliest time point of 14 days [Figure 5(a)], mild acute and chronic inflammation with edema was observed near the polymer. Neovascularization and a high density of chronic inflammatory cells with occasional neutrophils were observed near the polymer. After 28 days [Figure 5(b)], the inflammation had decreased, and characteristics of a maturing fibrous



**Figure 4.** Hematoxylin and eosin stained sections ( $5\ \mu\text{m}$ ) of poly(2EG10LA) and surrounding subcutaneous tissue: (a) 14 days, (b) 28 days, and (c) 56 days postimplantation (bar =  $100\ \mu\text{m}$ ). T-P = tissue/polymer interface, V = vascular structures, and FL = fibrous layer.



**Figure 5.** Hematoxylin and eosin stained sections ( $5\ \mu\text{m}$ ) of poly(8EG6LA) and surrounding subcutaneous tissue: (a) 14 days, (b) 28 days, and (c) 56 days postimplantation (bar =  $100\ \mu\text{m}$ ). T-P = tissue/polymer interface, V = vascular structures, and FL = fibrous layer.

capsule including alignment of fibroblasts and collagen near the polymer surface were seen. Infiltration of cells into the polymer was observed with some particles broken off from the implant. After 56 days [Figure 5(c)], a thicker fibrous capsule surrounded the implant. Additionally, polymer particles had broken off and were being engulfed by FBG cells.

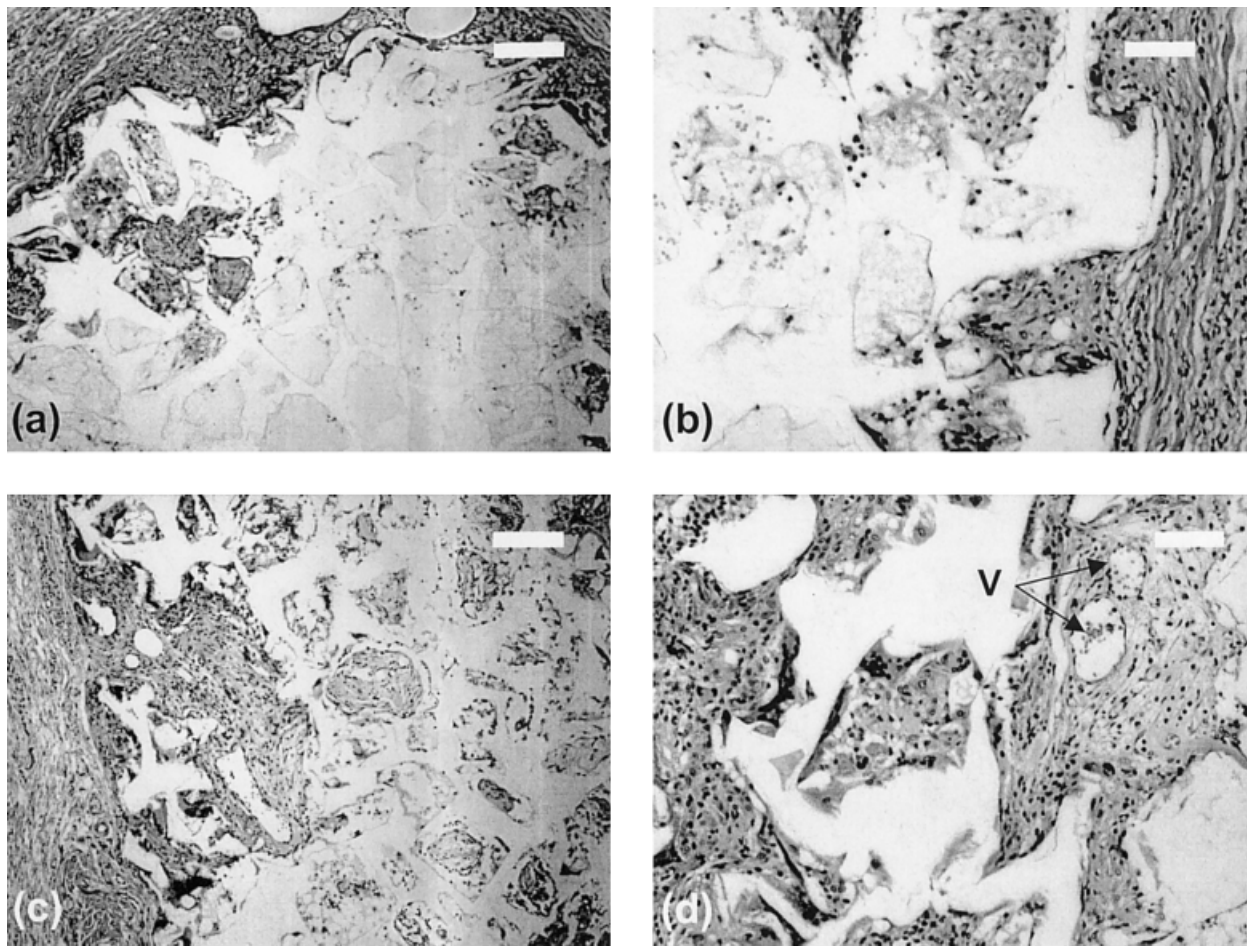
The overall response of the implanted polymers ranged from a very mild response to a moderate inflammatory response with  $50:50\ \text{PLGA} > \text{poly}(8\text{EG}6\text{LA}) > \text{poly}(2\text{EG}10\text{LA})$  in terms of the magnitude of inflammation (i.e., undesirable material response) observed in this study. The response is directly related to the degradation behavior and release of degradation products from the polymer. Although the response to  $50:50\ \text{PLGA}$  was the least desirable of the materials investigated, changing to a more slowly degrading  $75:25\ \text{PLGA}$  or  $\text{PLA}$  polymer could minimize this response.  $50:50\ \text{PLGA}$  was chosen as a control because the degradation rate was more similar to  $\text{poly}(2\text{EG}10\text{LA})$  and  $\text{poly}(8\text{EG}6\text{LA})$  than the more slowly degrading  $75:25\ \text{PLGA}$  and  $\text{PLA}$ . Previously published *in vitro* degradation experiments<sup>22</sup> showed that  $\text{poly}(2\text{EG}10\text{LA})$  loses  $\sim 28\%$  mass after 8 weeks of degradation in phosphate buffered saline, whereas  $\text{poly}(8\text{EG}6\text{LA})$  loses  $\sim 56\%$  of the initial polymer mass. This two-fold increase in degradation and release of degradation products could explain the differences in the biocompatibility response between these polymers.

Although the intended applications of these polymers are for orthopedics, a subcutaneous tissue biocompatibility model was chosen to assess the inflammatory response to the polymer chemistries since the highly cellular subcutaneous tissue is a widely used model and should more clearly represent the inflammatory reaction compared to mineralized bone. Although these studies focused on prefabricated implants, in order to investigate specifically the material chemistry and not the *in vivo* polymerization reaction, previous studies indicate no adverse reaction in a bone defect when photopolymerization was used to form the polymer networks *in vivo*.<sup>20</sup>

### Subcutaneous Implantation: 3D Porous Scaffolds

As a final assessment of the compatibility of these polymers for tissue-engineering applications, prefabricated porous scaffolds were implanted subcutaneously to examine tissue and cellular infiltration. Representative histological sections 14 days postimplantation are shown in Figure 6 for scaffolds fabricated with  $\sim 70$  and  $80\%$  porosity in the size range of  $150\text{--}300\ \mu\text{m}$ . Fibrous tissue infiltration was more prominent in the higher-porosity scaffold, indicating that the  $70\%$  porous scaffold has a less interconnected structure. Sections were taken at the center of the implant to ensure that radial infiltration was similar in all histological sections. Additionally, as the pore size of the scaffold was increased, the general thickness of the pore walls was increased and thus, the scaffold becomes less interconnected. Tissue infiltration decreased as the pore diameter was increased from the range of  $150\text{--}300\ \mu\text{m}$  to  $300\text{--}600\ \mu\text{m}$  (histology not shown).

Vascular structures [magnified in Figure 6(d)] are prominent within the pores of the material. One of the major benefits of *in situ* forming scaffolds as a treatment option for large bone defects is the potential for infiltration of native vascular structures into the depths of the scaffold. Lack of vascularization is a problem that has led to failure of treat-



**Figure 6.** Hematoxylin and eosin stained sections (5  $\mu\text{m}$ ) of subcutaneous tissue infiltration in three-dimensional porous scaffolds fabricated from 2EG10LA oligomer and 70 wt% 150–300- $\mu\text{m}$  salt particles [(a), bar = 200  $\mu\text{m}$  and (b), bar = 100  $\mu\text{m}$ ] or 80 wt% 150–300- $\mu\text{m}$  salt particles [(c), bar = 200  $\mu\text{m}$  and (d), bar = 100  $\mu\text{m}$ .] V = vascular structures.

ments such as autografts and allografts. Thus, synthetic polymeric scaffolds that support or encourage vascularization may provide additional benefits over current treatment options.

## CONCLUSIONS

The cytotoxicity and biocompatibility of networks fabricated from multifunctional lactic acid based oligomers were assessed. *In vitro* cytotoxicity experiments showed no difference in osteoblast viability when cultured with or without degrading networks present over a 2-week culture period. For polymer disks implanted subcutaneously, the inflammatory response ranged from mild to moderate, depending on the degradation rate of the polymer. One specific composition, poly(2EG10LA), had the most favorable response. Additionally, porous scaffolds that allow for infiltration of vascularized tissue were fabricated. Overall, these results indicate that polymers fabricated from multifunctional lactic acid oligomers can be designed to elicit a biocompatible response, a

trait that is very important in the development of a material for biological applications.

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