Vascularization and tissue infiltration of a biodegradable polyurethane matrix

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Abstract: Urethanes are frequently used in biomedical applications because of their excellent biocompatibility. However, their use has been limited to bioresistant polyurethanes. The aim of this study was to develop a nontoxic biodegradable polyurethane and to test its potential for tissue compatibility. A matrix was synthesized with pentane diisocyanate (PDI) as a hard segment and sucrose as a hydroxyl group donor to obtain a microtextured spongy urethane matrix. The matrix was biodegradable in an aqueous solution at 37°C *in vitro* as well as *in vivo*. The polymer was mechanically stable at body temperatures and exhibited a glass transition temperature (Tg) of 67°C. The porosity of the polymer network was between 10 and 2000 μm, with the majority of pores between 100 and 300 μm in diameter. This porosity was found to be adequate to support the adherence and proliferation of bone-marrow stromal cells (BMSC) and chondrocytes *in vitro*. The degradation products of the polymer were nontoxic to cells *in vitro*. Subdermal implants of the PDI–sucrose matrix did not exhibit toxicity *in vivo* and did not induce an acute inflammatory response in the host. However, some foreign-body giant cells did accumulate around the polymer and in its pores, suggesting its degradation is facilitated by hydrolysis as well as by giant cells. More important, subdermal implants of the polymer allowed marked infiltration of vascular and connective tissue, suggesting the free flow of fluids and nutrients in the implants. Because of the flexibility of the mechanical strength that can be obtained in urethanes and because of the ease with which a porous microtexture can be achieved, this matrix may be useful in many tissue-engineering applications.
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Key words: diisocyanato-2-methylpentane; sucrose; biodegradable polyurethane; biocompatibility; tissue engineering

INTRODUCTION

Polyurethanes by far are the most extensively used polymers in biomedical applications because of their biocompatibility and versatility. However, their application has been limited to bioresistant polymers used in blood-contact devices, such as heart valves, aortic grafts, and dialysis membranes.[1,2] Their feasibility for use in tissue engineering has not been tested. A scaffolding matrix adequate for tissue-engineered grafts requires a vast range of properties, such as biodegradability, biocompatibility, adequate mechanical strength, moldability, and the ability to support adherence and proliferation of cells, to bind proteins, and to allow infiltration of vascular and tissue-specific cells when implanted in a host.[1–3] Polyurethanes possess many of the attributes necessary for tissue-engineering applications, provided these materials can be synthesized into a composition that is biodegradable *in vivo* and that their degradation products are nontoxic.[4–10]

In recent years, biodegradable polyesterurethane foams, synthesized with toluidine diisocyanate (TDI) and poly[(R)-3-hydroxybutyric acid-co-(R)-3-hydroxyvaleric acid]-diol (PHB/HV-diol) or polycaprolactone diol (PCL-diol), have been shown to be compatible substrates for chondrocytes and to support chondrocytic adhesion, cell proliferation, and phenotype, as assessed by collagen type II/I synthesis, *in vitro*.[11] Polyesterurethanes synthesized with lysine diisocyanate (LDI)-based hard segments and polyesters poly(L-lactide) or 50:50 poly(lactide-co-glycolide) are biocompatible *in vitro* and *in vivo*.[7–10,12,13] Simi-

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larly, poly(urethane-urea) matrices with LDI as the hard segment and glucose, glycerol, or polyethylene glycol as soft segments are nontoxic *in vitro* and *in vivo*.[9,10] Biodegradable polyurethanes synthesized by esterification of phenylalanine and 1,4-cyclohexane dimethanol to yield a diester and polymerized with polycaprolactonediol and polyethylene oxide also exhibit biocompatibility *in vitro*.[6] These findings suggest that by changing either hard or soft segments or by changing both, urethanes that exhibit variable degrees of biodegradability and biocompatibility can be synthesized.

In this study, we hypothesized that with simple five carbon chain 2-methyl pentane-diisocyanate (PDI) and sucrose, a biodegradable polyurethane foam can be synthesized for potential application in tissue engineering. Polyurethanes as inherently microporous foams will create a suitable biomimetic environment for cell infiltration and growth *in vitro* and vascular and connective tissue infiltration and growth *in vivo*. Furthermore, we examined their toxicity *in vivo* and *in vitro* to observe their suitability for biomedical applications.

MATERIALS AND METHODS

Materials

1,5-Diisocyanato-2-methylpentane, dimethyl sulfoxide (anhydrous) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Sucrose, P/S solution (10,000 units of penicillin and 10 g of streptomycin/mL of saline), and reagents for histology were purchased form Sigma Chemical Co. (St Louis, MO). The tissue culture medium RPMI 1640 and reagents for molecular biology were obtained from Life Technologies (Grand Island, NY). All reagents were of analytic grade.

Synthesis of PDI–sucrose polymer

Sucrose (5 mmol) dissolved in dimethyl sulfoxide (DMSO) was reacted with PDI (26 mmol) at room temperature for 5 days. The formation of urethane linkages was monitored by FT-IR spectra. Upon disappearence of isocyanate groups, the reaction was terminated by adding water to generate a foam. Typically, 0.5 mL of water were added to 5 g of the prepolymer at room temperature and stirred for 10 min. The polymer then was placed in a vacuum oven at 22°C overnight to enhance foaming and to dry the material.

Analysis of glass transition temperature (Tg) of PDI–sucrose polymer

PDI–Sucrose polymer foam (5 mg) was dried under vacuum at room temperature prior to sealing in aluminum pans. Subsequently, the thermal analysis was performed in a Thermal Analyst 2000 (TA Instruments) with DSC 2910 differential scanning calorimeter. The temperature was increased at a rate of 10°C/min under constant nitrogen purge.

Biodegradability of the PDI–sucrose polymer

Preweighted PDI–Sucrose matrix was incubated in phosphate-buffered saline (PBS, 1 mg of matrix/mL of PBS) at 37°C for 60 days. Every 7 days the polymer was retrieved from the medium, washed briefly, dried, and weighed. The remaining PBS was analyzed for the oligomers and monomers by HPLC size-exclusion column. Simultaneously, in a small aliquot of PBS, the effect of degradation products on the pH was examined. To examine the degradation of polymer *in vivo*, the preweighed implanted polymers (n = 4/mouse) were removed after 3 or 6 weeks. Subsequently, the cells were removed from the polymer by treatment with 1% SDS for 2 days at 37°C. The polymer was washed extensively, dried, and weighed to assess the weight loss.

Cell growth and proliferation *in vitro*

PDI–sucrose foams were sterilized by Sterrad® (low-temperature H_2O_2 gas plasma) for 30 min prior to use. Bone-marrow stromal cells (BMSCs) and chondrocytes were obtained from Balb C mice (Hilltop Inc., Scottdale, PA), isolated, and characterized, as described earlier.[10] The cells were grown in TCM (RPMI 1640 containing 2 m*M* of glutamine, 10% fetal calf serum, and 1% P/S). The cells were harvested by trypsinization, washed twice by centrifugation at 1100X g, and a total of 30 μL of cell suspension of 10^5 cells/10 μL/2 mm^3 was loaded onto polymers prewashed with TCM.

After 7 or 14 days of culture at 37°C, BMSC attachment and proliferation on polymers were analyzed by light and/or scanning electron microscopy. The polymer-containing cells were fixed in 2.5% paraformaldehyde and 2% glutaraldehyde for 30 min, rinsed, and dehydrated in a graded series of ethanol. For light microscopy, the polymer was embedded in JB-4 medium and sectioned at 2 μm of thickness, and stained with hematoxylin and eosin prior to their microscopic analysis. For scanning electron microscopy, the polymer with or without cells was critically point dried and sputter coated with gold/palladium. The polymer was examined under a Joel scanning microscope with an accelerating voltage of 20 kV.

Examination of biocompatibility of subdermal PDI–sucrose implants

PDI–sucrose matrices (10 mg each) were implanted subdermally at four sites on the right and left dorsal flanks of 12-week-old female Balb C mice after approval of protocols

from the Institutional Animal Care and Use Committee at the University of Pittsburgh. Aseptic procedures were used for all surgical procedures. The mice were anesthetized with Ketamine (10 μL/10 g of body weight) and the back and flank regions were shaved and cleaned with 70% ethanol. A mid-incision, approximately 1.5 cm in length, was made on the back. Four subcutaneous pockets were created by blunt dissection about 1.5 cm lateral to the midline, and polymers were placed subdermally in the pockets away from the incision line. The skin was closed with surgical staples, which were removed 8 days postsurgery. The mice were sacrificed at 3 and 6 weeks postimplantation and evaluated by gross inspection, by histologic analysis, and by confocal microscopy.

Analysis of vascular tissue infiltration by confocal fluorescence imaging

The mice were anesthetized and 0.05 mL of 0.02 μm-in-diameter microspheres containing red fluorescent dye (FluoSpheres, Molecular Probes, Eugene, OR) were administered intravenously via the tail vein. Fluorescence confocal microscopy was performed using a prototype hand-held confocal imaging probe (Optiscan Pvt. Ltd., Hill, Australia). The imaging probe tip was positioned in contact with the surface of the implant and angles to maintain a flat imaging axis. In this system, Z-axis focusing within the implant involves movement of the internal lens element of the probe relative to the outer coverglass element using a focus wheel on the probe. Laser excitation utilized the combined 488 nm/568 nm spectral lines of a 50 mW air-cooled Krypton-Argon laser. Strong autofluorescence of implants was exhibited following excitation with the above laser lines, and this autofluorescence was visualized as green using a 505-nm longpass filter for detection (Chroma Technology Corp., Brattleboro, VT). Emitted fluorescence from red microspheres contained within the vasculature was detected using a 585-nm longpass filter (Chroma Technology Corp., Brattleboro, VT). The images acquired consisted of single x-y confocal scans at either 512 × 512 or 1024 × 1024 pixel resolution. The image magnification is approximately that of a conventional confocal microscope utilizing a 40X objective lens.

Histologic analysis of implanted PDI–sucrose matrices

The implanted matrices and adjacent tissue were removed *en bloc* and fixed overnight in 2% paraformaldehyde in PBS. The tissues then were rinsed in PBS, dehydrated in a graded series of methanol, and infiltrated with three changes of JB-4 embedding medium (Polysciences, Inc. Warrington, PA) over a 24-h period at 4°C. The tissues were mounted on aluminum stubs, sectioned on a JB-4 microtome with glass knives to 3–5 μ*M* in thickness, and stained with Lee's methylene blue-basic fuchsin. Stained sections were examined for evidence of possible necrosis of tissue, chronic inflammatory reactions, host cell in-growth into the polymer, angiogenesis, and changes in polymer integrity. Microscopic images were captured digitally using an Olympus BX-60 microscope and an Olympus MagniFire camera.

RESULTS

Synthesis and characterization of PDI–sucrose polyurethane

The reaction of sucrose and PDI (1:4 molar ratios) resulted in the formation of PDI–sucrose prepolymer, as demonstrated by the strong absorption band at approximately 1712 cm-1 by FT-IR spectroscopy [Fig. 1(A)]. The absorption peak at 1712 cm-1 is attributed to the formation of $^{-}$NHCOO^{-} groups. Under these experimental conditions, the prepolymer contained sufficient unreacted isocyanate groups ($^{-}$NCO), observed at 2274 cm-1, to avoid the formation of a thermoset-like matrix. The addition of water to the PDI–sucrose prepolymer resulted in the formation of a foamed polymer, with crosslink points forming a network in the matrix [Fig. 1(B)]. These pores were formed due to the liberation of CO_2 during polymerization. The light microscopy of the cross-sectional view of the polymer exhibited sponge-like interconnected pores ranging between 50 and 1000 μm in diameter. However, the majority of pores was between 100 and 300 μm in diameter. These pores provided a large surface area to support cell growth and free fluid flow for circulation of nutrients and other metabolites [Fig. 1(C)]. The glass transition temperature (Tg) of the PDI–sucrose polymer showed that its heat capacity did not change below 67°C [Fig. 1(D)], suggesting its suitability for use in biologic systems.

Cell adherence and colonization

Scanning micrographs of cells cultured on PDI–sucrose polymer for various time intervals showed that following seeding, the cells spread on the polymer surface and gradually adhere to the polymer within 2 to 4 h [Fig. 2(A)]. Continuous culture of BMSCs on polymers for 7 days showed that BMSCs retained their morphology similar in manner to cells grown on tissue culture polystyrene (TCPS). The cells seeded on the surface migrated into the pores of the polymer, suggesting that the porosity of the polymer is adequate for free fluid flow to support cell growth and that pores are interconnected to allow cell migration *in vitro* [Fig. 2(B)]. Furthermore, BMSC proliferation on the PDI–sucrose polymer was approximately 1.6-fold higher than on TCPS [Fig. 2(C)]. BMSCs

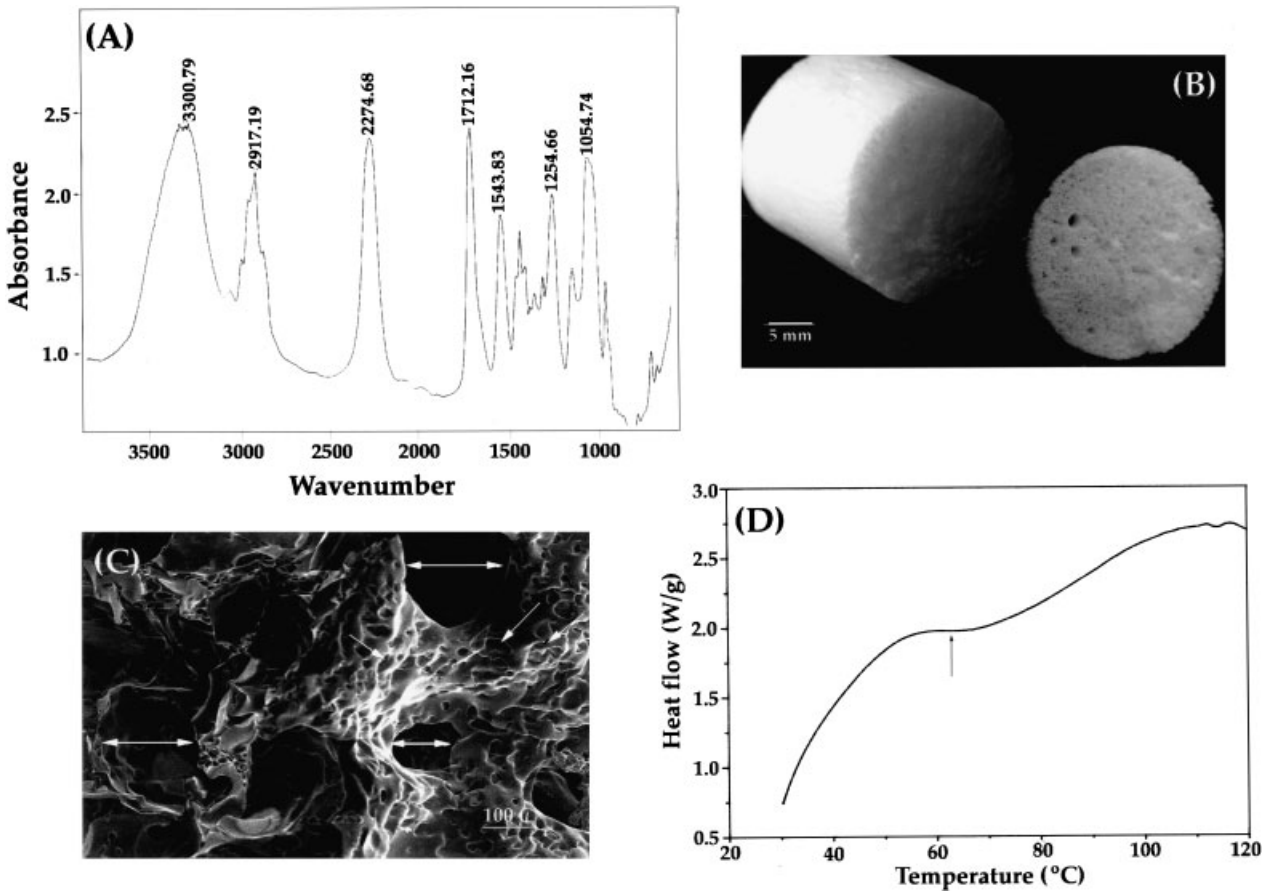

Figure 1. (A) FT-IR spectrum of synthesis of PDI–sucrose polymer exhibiting formation of urethane bonds. (B) Gross morphology of polymer foam and a section of foam showing the porosity. (C) Scanning micrograph of PDI–sucrose exhibiting 100–300-μ*M* pores (double headed arrows) and smaller (single headed arrows) pores (1 to 10 μ*M*) in the matrix dividing the pores. (D) Thermal analysis of PDI–sucrose foam exhibiting the Tg of 65°C.

formed nodules of multiple layers of cells in the pores of the foamed polymer over a period of 14 days, a typical characteristic of BMSC grown on TCPS.

Vascular infiltration in the PDI–sucrose implants

After reflecting the skin, the gross morphologic examination of the subdermal implants showed no signs of tissue necrosis adjacent to the implantation sites at 3 and 6 weeks. Similarly, all sites around the implanted matrix neither were visibly edematous nor did they exhibit signs of inflammation. However, blood vessels were observed around the PDI–sucrose matrix surrounding and penetrating the polymer [Fig. 3(A)].

Determination of the extent of vascularization in pores of the PDI–sucrose matrix by fluorescence confocal microscopy using a hand-held confocal imaging probe showed green autofluorescence of the polymer. Therefore, vascularization within the matrix was followed after intravenous administration of 0.05 mL of Texas-red-conjugated microspheres. These spheres traveled in the circulatory system and enabled visualization of the neovascularization associated with the implanted matrix. After 3 weeks of implantation, blood vessels were visible within the superficial layers of polymer. Additionally, an extensive network of fine capillaries infiltrated within the pores located in the deeper layers of the PDI–sucrose matrix [Fig. 3(B),(C)].

Biocompatibility and tissue infiltration in the PDI–sucrose subdermal implants

Histologic analysis of the subcutaneously implanted matrix confirmed the observations obtained from confocal microscopy. These sections displayed intense vascularization of the polymer with small vessels as well as with capillaries [Fig. 4(A)]. Additionally, extensive ingrowth of connective tissue was apparent within the polymer [Fig. 4(B)]. While the invading connective tissue consisted of fibroblasts and fibrous connective tissue, the presence of foreign-body giant cells also was evident.

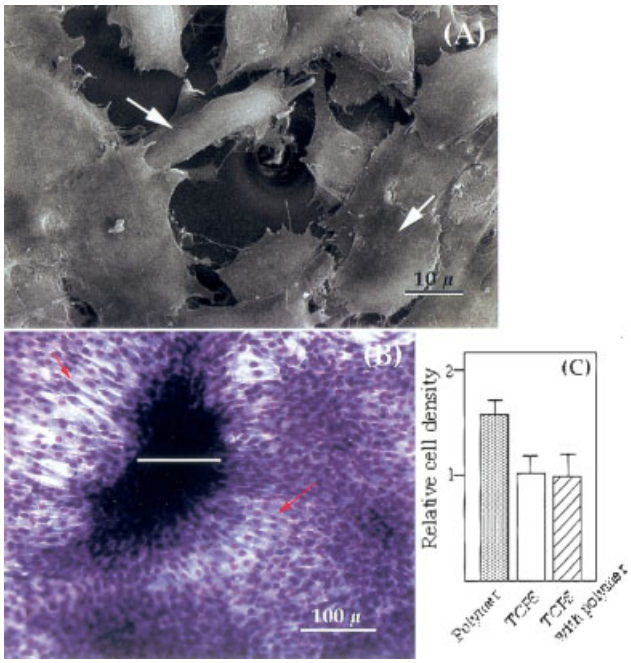

Figure 2. (A) Scanning micrograph of PDI–sucrose polymer exhibiting the attachment and spreading of BMSCs *in vitro* after 10 days of culture. (B) Light microscopy of a PDI–sucrose foam surface exhibiting proliferation of BMSCs (red arrows) over a period of 14 days. The white bar indicates a large pore in which cells are infiltrating. (C) Relative density of cell growth on PDI–sucrose polymer, TCPS alone, and on TCPS that contained polymer. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Biodegradability of PDI–sucrose polymer *in vitro* and *in vivo*

Degradation analysis of PDI–sucrose polymer *in vitro* exhibited approximately a 19 ± 4% mass loss in aqueous solution over a period of 60 days. This degradation was rapid during the initial 20 days and slowed down thereafter [Fig. 4(C)]. The degradation products of PDI–sucrose did not change the pH of the PBS over a period of 60 days at 37°C (data not shown). The analysis of *in vivo* degradation revealed that 20 ± 4% of the PDI–sucrose matrix was degraded in the first 3 weeks and an additional 38 ± 6% in the following 3 weeks [Fig. 4(D)].

DISCUSSION AND CONCLUSIONS

The current trend in biomaterial development is to develop biodegradable matrices that encourage angiogenesis as well as support the cells of the tissue it is intended to replace. With this in mind, we have synthesized a biodegradable biomaterial by reacting PDI and sucrose into a polymeric form. During the process of its polymerization, the water molecules react with

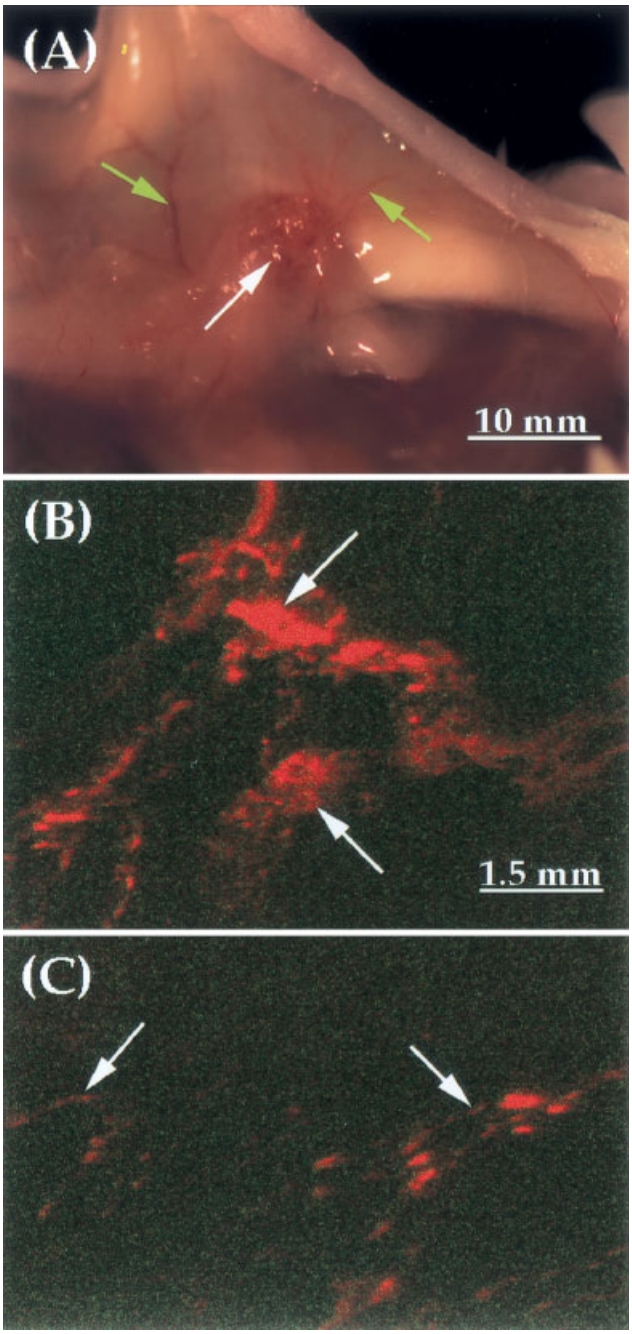

Figure 3. (A) Gross examination of subdermally implanted PDI–sucrose (white arrow) exhibiting absence of tissue necrosis, redness, and edema around the polymer 6 weeks postimplantation. Green arrows indicate capillaries growing towards the polymers. Confocal microscopic examination of vascularization (white arrows) in the (B) superficial layers and (C) deeper layers of subdermally implanted polymer after 3 weeks. The polymer is seen as green because of their autofluorescence, and capillaries are stained red because of the intravenous injection of 0.2 μm of Fluorospheres containing the fluorescent red dye Rhodamine isothiocyanate. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

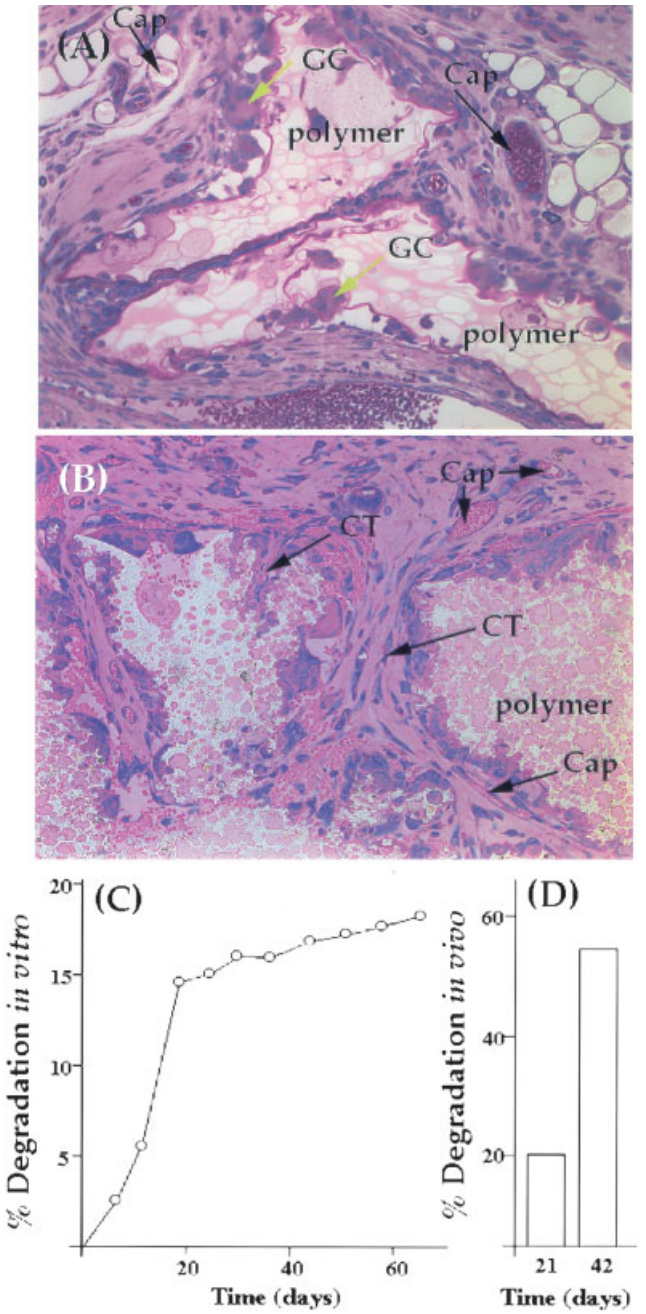

Figure 4. Histologic analysis of PDI–sucrose implants after 3 weeks showing (A) vascular tissue and (B) connective tissue infiltrating the pores of the implants. Cap: capillaries; CT: connective tissue; GC: foreign-body giant cells. Degradation rates of PDI–sucrose at 37°C in phosphate-buffered saline *in vitro* (C) and as subdermal implants *in vivo* (D). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the free isocyanates to generate foam while liberating CO_2, which, when trapped in the polymer, gives it its porosity. This porosity under controlled mechanical stirring results in the formation of a foam, with the majority of pore sizes between 100 and 300 μm. This porosity is shown to be near optimal for tissue growth.[14,15] Furthermore, a biologically suitable polymer also must possess a high enough glass transition temperature to provide stable structural rigidity during its use.[1,2] The Tg of 67°C indicates that this biomaterial will not be physically altered by the body temperature of 37°C.

The PDI–sucrose polymer dissociated in oligomers of PDI–sucrose and in monomers of 1,5-diamino 2-methylpentane and sucrose *in vitro*. The toxicity of 1,5-diamino 2-methylpentane *in vivo* is as yet unknown. However, due to the presence of enzymes such as deaminases in the tissues, the possibility of further degradation of 1,5-diamino 2-methylpentane into pentane hydrocarbons cannot be ruled out. *In vivo*, pentane itself has been shown to be a nontoxic and a nonimmunogenic molecule.[16,17] On the other hand, sucrose is native to the body environment and is utilized by the cells as fructose and glucose found naturally in the tissues.

Additionally, the dissociation of PDI–sucrose does not alter the pH of the macroenviroment of the polymer during its degradation. Whether these products alter the pH of the microenvironment surrounding the polymer is not clear. However, the facts that BMSCs and chondrocytes adhere and proliferate on the polymer better than on TCPS *in vitro*, and that polymer implants do not show any visible signs of inflammation *in vivo*, imply that the possibility of vast pH changes due to polymer degradation is unlikely. These results are further strengthened by the fact that the cells (approximately 40%) that migrate form the polymer to the polystyrene surface in the plate exhibit cell growth similar to the cells grown in TCPS, implying that the milieu containing degradation products of the polymer is nontoxic and does not affect cell survival or proliferation *in vitro*.

The biocompatibility of the polymer was analyzed following its subdermal implantations in mice. The gross microscopic appearance of the subdermally implanted polymer suggests that the polymer and its degradation products are nontoxic. This is evidenced by the marked absence of tissue necrosis in the area adjacent to the polymer. Furthermore, lack of redness or edema around the implanted polymer also indicate that the polymer may not induce acute inflammatory responses. Histologic analysis also revealed a lack of accumulation of neutrophils or other types of granulocytic cells, suggesting that the polymer does not induce an acute inflammatory or allergic reaction. Nevertheless, some foreign-body giant cells were apparent around the polymer and in the pores of the polymer, suggesting that the degradation of PDI–sucrose involves hydrolysis as well as elimination by foreign-body giant cells. This also may explain the faster degradation rates of the polymer *in vivo* as compared to *in vitro* conditions.

The ability to allow vascularization is one of the prerequisites for scaffolding matrices for their use in

tissue-engineering applications.[18,19] Synthetic polymers of solid consistency allow only limited vascularization within the polymer and thus require layering or foaming of the polymer to permit angiogenesis. PDI–sucrose polymer as a urethane is inherently a foam, giving interconnected pores, which allows markedly extensive vascularization. These findings suggest that PDI–sucrose polymer readily permits free fluid flow and availability of nutrients to the tissue located in the deeper layers of the polymer. Additionally, PDI–sucrose allows ingrowth of the connective tissue surrounding the polymer parallel to the vascularization.

In summary, data presented in this report strengthen our hypothesis that by altering the hard and soft segment chemistry, poly(urethane-urea) matrices provide major attributes essential in a scaffolding matrix used for tissue-engineering applications. This matrix allows vascularization and tissue infiltration, and it is nontoxic, biocompatible, biodegradable, and moldable. The potential usefulness of these polymers lies in the versatility of their synthesis into a variety of mechanical strengths and forms. These attributes merit further investigations of application of this matrix in experimental tissue grafts.

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