

ANGIOGENIC COMPETENCY OF BIODEGRADABLE HYDROGELS FABRICATED FROM POLYETHYLENE GLYCOL-CROSSLINKED TYROSINE-DERIVED POLYCARBONATES

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Abstract

Synthetic biomaterials can be used as instructive biological milieus to guide cellular behaviour and function. To further realize this application, we synthesized a series of structurally similar hydrogels and tested their ability to modulate angiogenesis. Hydrogels were synthesized from poly(DTE-co- $x\%$ DT carbonate) crosslinked by $y\%$ poly(ethylene glycol) (PEG). Hydrogel desaminotyrosyl tyrosine (DT) contents ($x\%$) ranged from 10-100%, and crosslink densities ($y\%$ PEG-crosslinker) ranged from 5-80%. The hydrogels were fashioned into porous scaffolds with highly interconnected macro- and micro-pore (>100 and <10 μm in diameter, respectively) architecture using poly(DTE-co-10%DT carbonate) crosslinked with 8% PEG. Under physiological conditions (*in vitro*), the hydrogels degraded into three major products: desaminotyrosyl-tyrosine ethyl ester (DTE), desaminotyrosyl tyrosine (DT), and poly(ethylene glycol)-di-DT-hydrazide (PEG-di-DT hydrazide). Increasing either DT content or crosslink density brought quickened degradation. Because DT and DTE, two of the three major degradation products, have not demonstrated any noticeable cytotoxicity or angiogenic effect in previous studies, we measured the cytotoxicity of PEG-di-DT hydrazide, the third major degradation product. We found that PEG-di-DT hydrazide only displayed significant cytotoxicity at the high concentration of 100 mg/mL. Interestingly, PEG-di-DT hydrazide and its further degradation product PEG-dihydrazide stimulated *in vitro* endothelial cell migration and tubulogenesis, which is comparable to results found with FGF- β treatment. Subcutaneous implantation of the PEG-crosslinked poly(DTE-co-10%DT carbonate) scaffolds into the backs of rats elicited greater tissue growth over time and superior vascularization than poly(DTE carbonate) implantation. These results show that this new class of biomaterials has a strong potential to modulate angiogenesis.

Keywords: Tyrosine-derived polycarbonates, PEG-crosslink, hydrogel, degradation, angiogenesis.

Introduction

Tyrosine-derived polycarbonates are a group of homologous carbonate-amide copolymers individually differing only in the length of their respective alkyl ester pendant chains (Fig. 1) (Ertel and Kohn, 1994). Various polymers of desaminotyrosyl-tyrosine alkyl esters (DTR) have been extensively studied and characterized as tissue compatible, strong, hydrophobic, and slow degrading materials (Ertel and Kohn, 1994; James *et al.*, 1999; Yu and Kohn, 1999). Out of the DTR polymers studied, Poly desaminotyrosyl-tyrosine ethyl ester (DTE) carbonate has shown the most promise for biocompatibility. Recently, the incorporation of free carboxylate pendent chains, desaminotyrosyl tyrosine (DT), into poly(DTR carbonate)s has been shown to increase the degradation rate (Abramson, 2002).

A series of copolymers with the general formula poly(DTE-co- $x\%$ DT carbonate) were created with DT contents ranging 5-100 mole%. This polymer series possesses higher degradation rates than the poly(DTR carbonate)s, while still maintaining the mechanical properties and processability of poly(DTR carbonate)s. Through semicarbazide linkage, poly(DTE-co- $x\%$ DT carbonate)s can be chemically crosslinked with poly(ethylene glycol)-dihydrazide (PEG-dihydrazide) (Fig. 1b) at the free acid groups of the DT monomer. The incorporation of PEG increases the hydrolytically degradable property (Zhao and Harris, 1998) of the polymer by increasing the hydrophilicity. Altering the level of PEG crosslinking significantly changes the material properties. By increasing the amount of crosslinking, the polymer scaffolds can change from a stiff, hydrophobic material into a soft, hydrophilic hydrogel.

Angiogenesis is the growth of new blood vessels from the existing vascular system (Bishop *et al.*, 1999; Folkman and Shing, 1992; Lutolf and Hubbell, 2005). This process occurs naturally during reproduction, wound repair, placental development, and the foreign body response. New methods of modulating and directing angiogenesis, including the utilization of endothelial growth factors or the use of polymeric biomaterials, provide new treatment options for the fields of regenerative medicine and wound healing (Black *et al.*, 1999; Cassell *et al.*, 2002; Tello-Montoliu *et al.*, 2006; Yla-Herttuala *et al.*, 2007). For biomaterial tissue scaffolds, it is crucial that the scaffolds permit proper vascularization so as to provide functional tissue with adequate nutrients and oxygen (Jain *et al.*, 1997; Sieminski and Gooch, 2000). Endothelial cells (ECs) grown on a biopolymeric matrix curl, associate,

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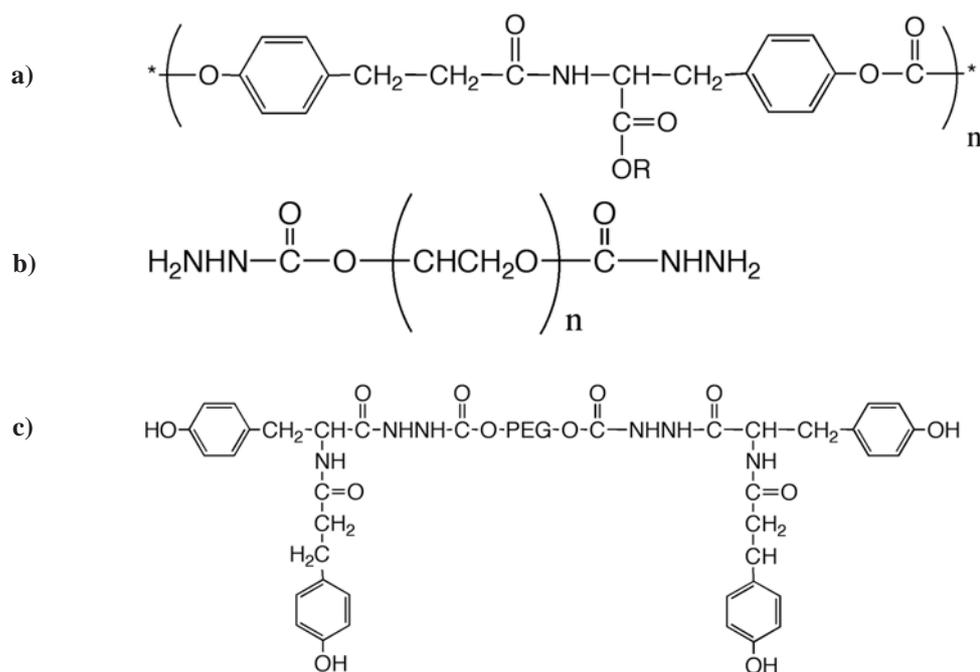


Figure 1. Polymer structure. **a)** Tyrosine-derived polycarbonates: the monomer is desaminotyrosyl-tyrosine alkyl ester (DTR). Alkyl ester (R) pendent chain can be designed to ethyl (DTE), butyl (DTB), hexyl (DTH) and octyl (DTO). **b)** PEG-dihydrazide. **c)** Model compound, PEG-di-DT hydrazide.

and elongate into tubule structures that resemble immature budding capillaries during angiogenesis (Bachetti and Morbidelli, 2000). EC proliferation and migration are crucial events in angiogenesis (Bernardini *et al.*, 2005).

Several strategies have been employed to induce vascularization with biomaterial implants, including the incorporation of biological moieties like growth factors, the functionalization of materials to absorb angiogenic factors to the implant site, and the use of cell lines that secrete angiogenic substances (Eckardt *et al.*, 2003; Kidd *et al.*, 2002; Tabata *et al.*, 1998). However, there are some problems with current strategies. For instance, undesired tissue growth and increased vessel permeability are potential side effects; there is also a general lack of information regarding other biological activities relating to these strategies. Therefore, there is great need for a new synthetic polymer with controlled and predictable angiogenic activity based on the material properties.

One reason why clinical researchers go to such lengths to increase new blood vessel growth around an implant is that capillaries act as highways to transport cell metabolites to and from the site and aid in inflammatory cell action. The disruptive action of surgically implanting a material into the body creates trauma. If an implant site is better suited for dealing with inflammatory cells and the body's natural healing response because of increased blood flow due to angiogenesis, there is a better chance that implant patency will remain preserved. In other words, a tissue engineered implant should be able to perform its function effectively (i.e. vascular grafts remain open, bone grafts support mechanical loading, and native tissue infiltration should occur on a timescale to match the degradation of degradable polymer scaffolds) for the lifetime of the implant. For tissue engineered scaffolds used as dermal

equivalents, some researchers realized that seeding endothelial cells directly into the scaffold with keratinocytes led to better vascularization of the natural polymer scaffolds and resulted in greater cell growth.

To further expand the properties and the utility of the tyrosine-derived polycarbonates, we fabricated a series of biodegradable hydrogels from PEG-crosslinked poly(DTE-co-DT carbonate)s. The degradation profile of the resulting materials exemplified unique, controlled, predictable kinetics. The hydrogels and their degradation products exhibited suitable biocompatibility and great potential to modulate angiogenesis.

Methods

Polymer synthesis, scaffold fabrication, and characterization

Materials

N, N-dimethylformamide (DMF), poly(ethylene glycol) (PEG), Amberlyst 15, Trizma buffer solution, and Diff Quik staining were obtained from Sigma-Aldrich (St. Louis, MO, USA). N-hydroxysuccinimide (NHS), triethylamine (TEA), hydrazine, and hydroxybenzotriazole (HOBt) were obtained from Acros Organics (Morris Plains, NJ, USA). N-ethyl-N'-dimethylaminopropyl carbodiimide.HCl (EDCI) was obtained from Tanabe Research Laboratories U.S.A., Inc. (San Diego, CA). HPLC grade solvents were used for HPLC analysis and other work unless otherwise stated. DMF and 1-methylpyrrolidinone (Omnisolv, EM Science, Gibbstown, NJ, USA) were purified by treatment with Amberlyst 15 to remove basic impurities.

Table 1. Stoichiometric amounts of PEG-dihydrazide and EDCI used to fabricate non-porous and porous scaffolds. (Unit: mg)

Hydrogels	Non-porous scaffolds		Porous scaffolds	
	PEG-diH	EDCI	PEG-diH	EDCI
10D/8P	446	80	67	12
15D/12P	670	120	101	18
20D/16P	900	162	135	24
25D/5P	282	51	84	15
25D/20P	1130	203	168	30
50D/20P	1140	207	172	31
100D/20P	1200	215	179	32
100D/80P	4770	862	716	129

Preparation of the crosslinker, PEG-dihydrazide

One hundred grams of PEG ($M_w=2000$ g/mol, 0.05 mol) was dissolved in 1L of toluene and then dried azeotropically; 150 mL of 20% phosgene in toluene was then added. The mixture was then stirred overnight to produce PEG-dichloroformate (excess phosgene and toluene were distilled off). One hundred mL of methylene chloride and 300 mL of dry toluene were then added to the residue. Seventeen grams of N-hydroxysuccinimide (NHS) (0.15 mol) and 15 grams of triethylamine (TEA) (0.15 mol) were subsequently added to the residue; stirring resumed for 2 hours at 0°C. Next, 15.7 mL of Hydrazine (0.50 mol) was added to the reaction mixture, which was stirred for an additional 12 hours. The precipitate of TEA×HCl was removed by filtration; the filtrate was concentrated to a volume of 200 mL; and PEG-dihydrazide was precipitated with 600 mL of diethyl ether. Then, the desired product, PEG-dihydrazide, was isolated by filtration, recrystallized using 800 mL of isopropanol (IPA), and finally dried under vacuum at room temperature. ¹H NMR was used to determine the structure and purity of the product.

Crosslinking and scaffold fabrication

Poly(DTE-co-X%DT carbonate)s were synthesized as previously described (Abramson, 2002). For the scaffold, 0.3 g of the polymer was dissolved in 3 mL of N-Methylpyrrolidone (NMP), and the corresponding amounts of PEG-dihydrazide and EDCI/methylene chloride were added (Table 1). After two minutes of mixing in a vortex mixer, the solution was poured into a Teflon dish 5 cm in diameter. To prepare porous scaffolds, 10 g of sieved NaCl crystals (212-425 μm) (Sung *et al.*, 2004) were placed into the Teflon dish; then, the polymer solution was carefully poured over the salt bed. To produce micropores less than 10 μm in diameter, the sample dish was immersed in liquid nitrogen for 30 minutes to induce phase separation (Levene, 1999). The scaffolds were repeatedly washed with deionized water to remove NMP, NaCl, and other by-products. By varying polymer DT content and PEG-

dihydrazide stoichiometry, a series of hydrogels with differing degrees of crosslinking (from 5-80%) and DT percents (from 10-100%) were prepared. The hydrogel composition is presented as xD/yP where x represents the %DT and y represents the degree of crosslinking (%PEG-dihydrazide). For example, 10D/8P represents poly(DTE-co-10% DT carbonate) with 8% crosslinking. This nomenclature will be used throughout the manuscript.

Synthesis of the model degradation compound PEG-di-DT Hydrazide

Over an ice-water bath, 4.5 mmol of DT, 2.36 mmol of PEG-dihydrazide, 0.45 mmol of HOBt, and 10 mL of NMP in methylene chloride were mixed and reacted for 1 hour. Next, 4.72 mmol of EDCI was reacted to the mixture by stirring for 24 hours. The reaction mixture was then washed with a solution of 5% sodium bicarbonate, 0.2 M hydrochloric acid, and 20% sodium chloride in water. The organic phase was dried over magnesium sulphate, filtered, and then evaporated. We dissolved the resulting oil in IPA (50 mL) under heat and then crystallized the product at -20°C. The PEG-di-DT hydrazide yellow solid was washed with IPA and dried under vacuum at 40°C.

Scaffold imaging

Hydrogels were prepared for Scanning Electron Microscope (SEM) analysis by immersion in liquid nitrogen until frozen and were then freeze-fractured. Prior to SEM analysis, samples were dried under vacuum for 24 hours, mounted on aluminium studs with adhesive paper, and sputter-coated with gold/palladium under vacuum for 120 seconds. Samples were visualized on an AMRAY 1830-I Scanning Electron Microscope at 20 kV. For optical coherence tomography (OCT), samples were placed in a test tube and evacuated for 10 min to remove air. Distilled water was introduced while the samples were still under vacuum. The scaffolds were placed on a Petri dish and immersed in distilled water for imaging. Samples were imaged on an OCT using the 26X objective having a resolution of 21 μm in the x direction.

Degradation

Water uptake

Dried, non-porous hydrogels 8 mm in diameter and 2 mm thick were weighed ($weight_{dry}$), incubated in water at room temperature for 48 hours, and re-weighed to obtain the wet weight ($weight_{wet}$). The percent water uptake ($n=5$) was calculated as:

$$\%wateruptake = \frac{weight_{wet} - weight_{dry}}{weight_{dry}} \times 100 \quad (1)$$

Mass loss

Non-porous hydrogel scaffolds were weighed ($weight_{init}$) and then incubated at 37°C with a Trizma buffer solution (pH 7.4, Sigma) that was modified by the addition of HCl, NaCl, KCl, NaHCO₃, MgCl₂·6H₂O, MgSO₄, K₂HPO₄, and CaCl₂ to simulate the composition of body fluid (Labazzo, 2004). Hydrogels were vacuum-dried for two weeks and re-weighed ($weight_{tp}$) at nine different time-points: 3 days, 1, 2, 3, 4, 6, 12, 18, and 24 weeks. Percent mass loss ($n=5$) was calculated as:

$$\%massloss = \frac{weight_{init} - weight_{tp}}{weight_{init}} \times 100 \quad (2)$$

Analysis of degradation product

By using HPLC (Perkin-Elmer, Norwalk, CA, USA), buffers collected from the mass loss measurements at like time points were analyzed, and the cumulative release (mg) of DT from non-porous scaffolds was measured. Further degradation of PEG-di-DT hydrazide was analyzed after incubation at 37°C with the modified Trizma buffer solution as described above. Desaminotyrosine (DAT) was used as an internal standard.

In vitro and *in vivo* biological evaluation

For biological evaluation, all materials were UV-sterilized for 30 minutes before use; solutions, which could not be sterilized, were filtered through a 0.22 μm bacterial filter (Becton Dickinson Labware Europe, Le Pont de Cles, France). The hydrogels were prepared under aseptic conditions and UV-sterilized for 30 minutes before implantation.

In vitro cytotoxicity, migration, and tubulogenesis

Rat lung fibroblasts (RLF-6) were treated for 24 hours with varying concentrations of PEG-di-DT hydrazide, PEG, or lactic acid. Cytotoxicity was determined by using an MTS assay (Promega, Madison, WI) and following the instructions provided by the supplier (Malich *et al.*, 1997). Human aortic endothelial cells (HAEC) purchased from Cambrex (Walkersville, MD, USA) were cultured in EGM-2 medium according to the supplier's instructions. HAEC were derived from 3 individual donors. Cell cultures (P4-P6) were maintained at 37°C, 5% CO₂ and 100% humidity. Migration assays were performed using a Costar microchemotaxis transwell chamber. The wells were coated with 10 μg/mL fibronectin (Calbiochem, San Diego, CA, USA). HAEC were seeded onto the transwells with one of the target molecules and then incubated for 6 hours. The migrated cells were stained with Diff Quik nucleus staining (Sigma) and counted under a light microscope.

Basic fibroblast growth factor β (FGF-β, 0.1 μg/ml) was treated as a positive control (Bachetti and Morbidelli, 2000; Black *et al.*, 1999; Cassell *et al.*, 2002; Tabata *et al.*, 1998).

We performed an *in vitro* matrigel tubulogenesis assay. We first incubated matrigel for complete gelation in a 96-well plate at 37°C for 30 minutes. HAECs (2 × 10⁴/well) were then seeded to the matrigel and treated with a target molecule for 18 hours (Montanez *et al.*, 2002). The cells were stained with Diff Quick staining (Sigma) and imaged under a light microscope with a 4X objective lens. All images were processed semi-automatically using Microsuite software (ANALYSIS, Olympus, Münster, Germany). The ANALYSIS software enabled us to draw lines following the tubulogenic feature on the image and automatically calculated the interactive length of tubules. The quantitative data were then exported as an Excel format. FGF-β (1 μg/ml) was used as a positive control.

All the *in vitro* assays were performed using 4 replicates of each substrate type ($n=4$), the minimum sample number suggested by Power Analysis with which statistical significance was valid. We statistically analyzed samples by comparison to the control condition treated by only culture media using one-way ANOVA testing with Dunnett *post hoc* analysis (confidence interval 95% or $p \leq 0.05$).

In vivo histological analysis

Porous 10D/8P and poly(DTE carbonate) scaffolds were implanted subcutaneously into the backs of rats ($n=4$) and harvested 1, 3, 8, and 15 weeks post implantation. We stained the samples using haematoxylin and eosin (H&E) for histological analysis.

Results

Synthesis, fabrication, and characterization

We synthesized PEG-dihydrazide, the chemical crosslinker, and obtained a yield of 81%. We verified the chemical structure of the crosslinker using ¹H NMR. The values in ¹H NMR (CDCl₃) δ ppm relative to TMS were 6.71 (broad multiplet, 2H, NHNH₂), 4.27 (m, 4H, CH₂-O-C=O), and 3.64 (m, 176H, internal CH₂s of PEG and NHNH₂).

We prepared the model degradation product, PEG-di-DT hydrazide (Fig. 1c), with a yield of 42%; HPLC analysis identified the purity of this compound to be 100%. The values in ¹H NMR (DMSO) δ ppm relative to TMS were 9.86 and 9.16 (singlet, 2H, OHs), 9.13–9.09 (s, 4H, -NHNH-), 8.03 (doublet, 2H, NH-CH), 7.02–6.59 (m, 16H, aromatic Hs), 4.46 (m, 2H, CH), 4.09 (m, 4H, terminal CH₂ of PEG), 3.3 (m, 176, internal CH₂s of PEG), 2.75 (m, 4H, CH-CH₂), and 2.99–2.25 (m, 8H, CH₂-CH₂-C=O).

The pore architecture of the 10D/8P hydrogel scaffold was observed with SEM and OCT (Fig. 2). The SEM images show the macro-pores, formed by NaCl porogens, to be greater than 100 μm in diameter (Fig. 2a). The micro-pores less than 10 μm in diameter were produced by phase separation (Fig. 2b). OCT imaging shows a highly overlapped porous area (red colour) within a primarily non-porous area (green colour) (Fig. 2c), indicating an interconnected pore structure.

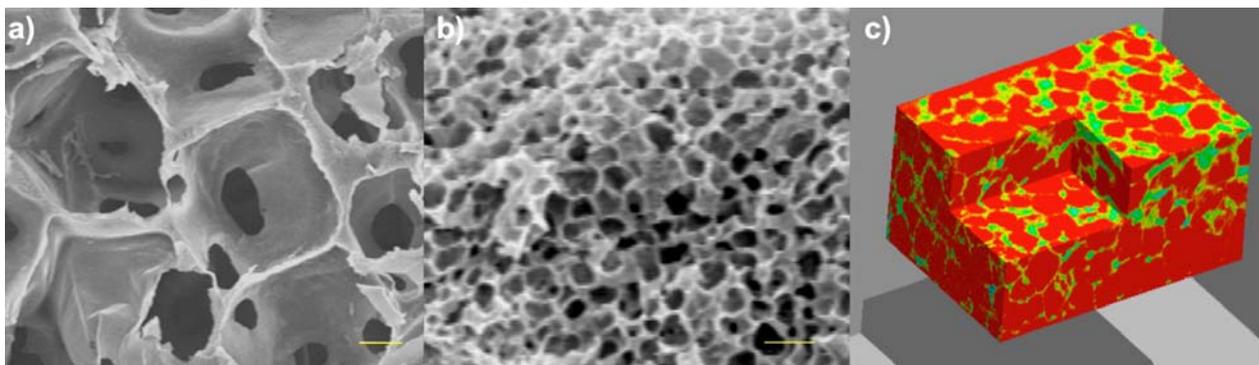


Figure 2. Pore architecture of the 10D/8P hydrogel scaffold. SEM images demonstrate **a)** macropores (Bar = 100 μm) and **b)** micro-pores (Bar=10 μm). **c)** OCT image shows the interconnected pores. Red and green colour represents the porous and the non-porous area of the scaffold, respectively.

The design of these hydrogels provides for four distinct parameters that can be widely modified. The first parameter is the amount of free carboxylate pendent groups designated as DT. The DT content can vary from 0-100 mole%; this indicates that none or all of the polymer repeat units carry a carboxylic acid group. The second parameter is the theoretical crosslink density (TCD). The TCD is determined by the amount of PEG-dihydrazide added to the reaction mixture during the crosslinking reaction. For example, when poly(DTE-co-50%DT carbonate) was selected as the base polymer, 50 mole% of all the polymer repeat units were DT, while 50 mole% were unreactive DTE. For the crosslinking reaction, we used an amount of PEG-dihydrazide equivalent to 40 mole% of the repeat units of poly(DTE-co-50%DT carbonate) present in the reaction mixture. The resulting hydrogel had, theoretically, a PEG-dihydrazide crosslink at 40 mole% of all repeat units. A residual amount, 10 mole%, of the repeat units still carried a reactive carboxylate group (DT). The third design parameter is the molecular weight of the PEG chain used in the crosslinking reaction. The last parameter is the structure of the alkyl ester pendent chain used to block potentially reactive carboxylate groups. For this study, the DT content in the polymers ranged from 10-100%, the TCD values ranged from 5-80%, the PEG molecular weight was kept constant at 2000 g/mol, and the ethyl ester was used for all hydrogels studied.

Degradation

In order to exclude the influence of pores on degradation and focus solely on chemical composition, only non-porous films were tested in the degradation study. The degree of swelling ranged from 27-407%. Out of the 8 hydrogels tested, the greatest amount of water uptake was observed with the 100D/80P and 100D/20P hydrogels (Fig. 3a). We used a 2-way ANOVA to determine the cause of the variation among the paired samples; DT content was found to be a significant contributor to the disparities in water uptake ($p < 0.0001$). In contrast, the crosslink density alone was not shown to significantly impact water uptake ($p > 0.1$). In fact, crosslinked polymers were only shown to deviate from the control's water uptake percentage when they contained DT, indicating that the primary determinant of water uptake was the DT content. This is especially

obvious in the hydrogels that possessed equal degrees of crosslinking but had different DT contents (i.e. 100D/20P, 50D/20P, 25D/20P) (Fig 3a). Because DT has free acid groups that contribute to water uptake (Kim *et al.*, 1999), decreasing the DT content is suspected to decrease polymer water uptake.

We measured the scaffold's percent mass loss over time in order to determine the degree of degradation into water-soluble fragments (Fig. 3b). The deviations and the large errors in Figure 3b could be attributed to the rapid erosion of the degrading hydrogels, which makes accurate weighing of samples at each point in time difficult. The samples with high DT content (50% and 100% DT) lost most of their mass within 28 days. We analyzed the incubation solution by means of HPLC to determine the amount of DT released from the non-porous scaffolds. Figure 3c shows the cumulative DT release in mg calculated over time. Similar in nature to the mass loss results, the samples with high DT content (50% or 100% DT) were found to release DTs rapidly for the first 21 days before exhibiting a deceleration in DT release. All of the other samples released DTs rapidly in a log phase manner within the first 40 days before entering a lag phase in DT release; a small amount of DT remained constantly present on the HPLC for the entire duration of the study. The 15D/12P hydrogel released fewer DTs than the other samples with higher DT contents, confirming DT's effect on degradation.

By using the HPLC to analyze the incubation solution, which housed the porous hydrogel scaffolds, we determined the retention times of the three major degradation products – DT, DTE, and PEG-di-DT hydrazide – from the different porous hydrogels. The HPLC retention time for a component in a sample is determined by measuring the time it takes for the peak representing the component to appear after the sample is injected. The retention time values, which are automatically calculated by the HPLC, will generally vary slightly. We found the DT retention times to range from 6.4 to 6.9 minutes for the different samples. The next product to be released, DTE held retention times of 8.7 and 8.9 minutes from the 50D/40P and 25D/20P hydrogels, respectively. No DTE was detected in the 100% DT hydrogel. PEG-di-DT hydrazide, the final product to be released, held

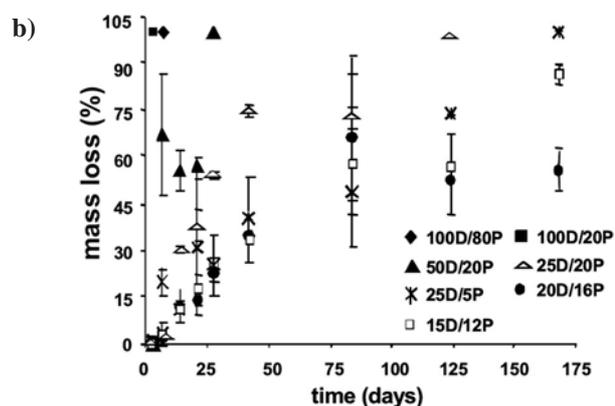
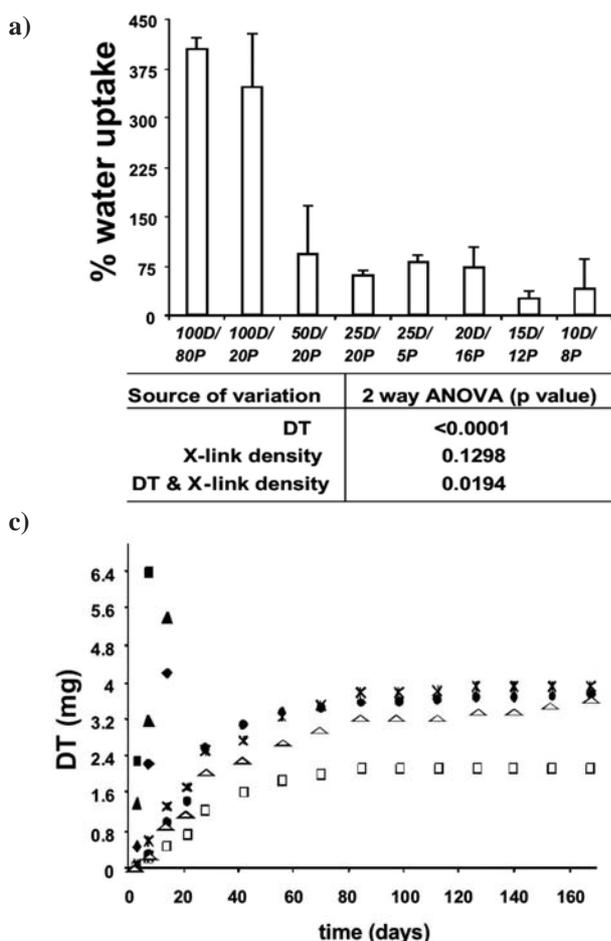


Figure 3. Degradation properties of non-porous hydrogel scaffolds. **a)** Percent water uptake of scaffolds for 48 hours. The source of the variation for percent water uptake was analyzed using two-way ANOVA. **b)** Mass loss and **c)** cumulative release of degraded DT from non-porous scaffolds measured over 168 days.

retention times ranging from 13.0 to 13.7 minutes. The further degradation of PEG-di-DT hydrazide was analyzed using the HPLC chromatogram, which showed no sign of any degradation products for approximately two weeks before it indicated degradation by a peak. Our analysis using the standard compounds determined this peak to be DT-NHNH₂. The extended incubation of DT-NHNH₂ at 37°C eventually revealed a DT peak on the HPLC. After about six and a half weeks, the DT-NHNH₂ was nearly undetectable, and the DT peak continued to rise over the remainder of the study.

***In vitro* and *in vivo* biological evaluation**

Because DT and DTE demonstrated no noticeable cytotoxicity or angiogenic effect in our previous studies (Ertel and Kohn, 1994; James *et al.*, 1999; Yu and Kohn, 1999), the present study focused on the cytotoxic and angiogenic effect of PEG-di-DT hydrazide and its further degradation product, PEG-dihydrazide. Figure 4a illustrates the results of our measure of cytotoxicity with an MTS assay. PEG-di-DT hydrazide and PEG were observed to significantly reduce RLF-6 viability when compared to the media ($p < 0.05$) but only when present at the highest concentration (100 mg/mL). However, at concentrations above 6.25 mg/mL, lactic acid lowered the MTS value to the baseline to indicate significantly greater cytotoxicity than that of the media ($p < 0.05$).

We studied the effect of the degradation products on HAEC migration and compared their effect to that of the medium control (Figs. 4b and 4c). We began by testing

the dose response of HAEC migration to PEG-di-DT hydrazide treatment (Fig. 4b). FGF- β was used as a positive control to induce migration. HAEC migration increased in response to the increase in PEG-di-DT hydrazide concentration from 0 to 50,000 $\mu\text{g/mL}$. At concentrations of 1,000 $\mu\text{g/mL}$ and above, ECs migrated significantly more than when they were incubated in the media free of PEG-di-DT hydrazide ($p < 0.05$). EC migration was comparable to the FGF- β treatment at 0.1 $\mu\text{g/mL}$; there was no statistical difference in EC migration between the PEG-di-DT hydrazide treated media and the media with 0.1 $\mu\text{g/mL}$ FGF- β . To determine which, if any, of the PEG-di-DT hydrazide degradation products was responsible for the chemotaxis of EC migration, we individually tested the EC migratory influence of PEG, PEG-dihydrazide, and DT (Fig. 4c). Neither DT (5 mg/mL) nor PEG (1 mg/mL) treatment was found to significantly induce cell migration, but when cells were treated with PEG-dihydrazide at 1 mg/mL, migration was induced to roughly 2.4 times that of the medium control ($p < 0.05$).

We analyzed the effects of FGF- β , PEG-dihydrazide, and PEG on tubulogenesis by measuring tube length (Fig. 4d). Figure 4d demonstrates the length and structure of the tubules formed on the matrigel. We found that ECs treated with PEG-dihydrazide (1 mg/ml) organized into tubular-like structures; these results were compatible to those with FGF- β treatment (1 $\mu\text{g/mL}$). In contrast, unorganized tubular structure was observed in ECs treated with PEG as well as with culture media. We used the interactive length-measuring feature of the microscope to

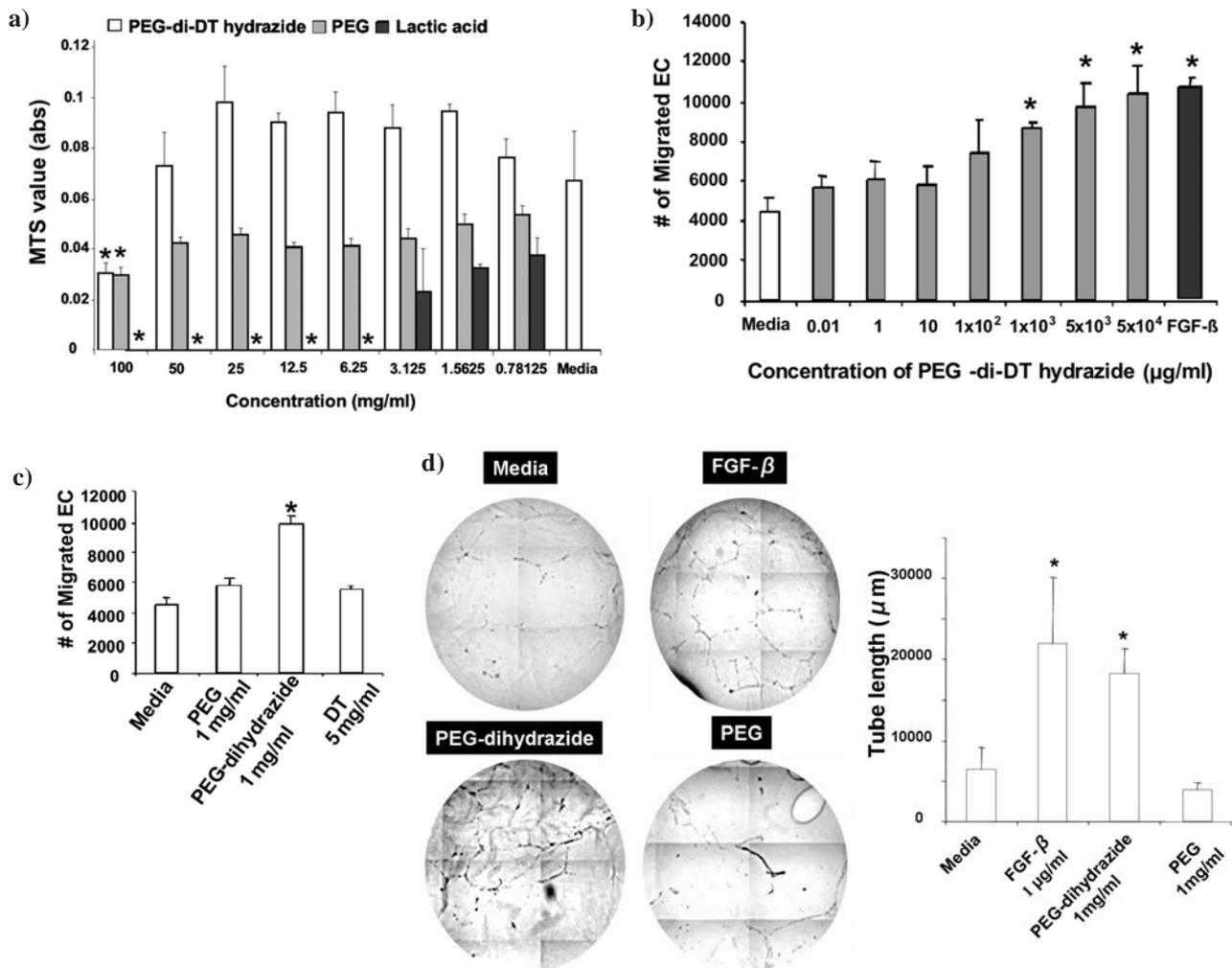


Figure 4. Effect of degradation products on cytotoxicity, EC migration, and tubulogenesis. **a)** Cytotoxicity of the degradation products analyzed with an MTS assay with RLF-6 cell line. Higher MTS values indicate greater cell viability. The effect of **b)** PEG-di-DT in a series of dilution and **c)** its further degradation components, PEG, PEG-dihydrazide, and DT on HAEC migration analyzed based on trans-well migration assay. **d)** Tubular structure and tube length measurements formatted by HAEC in Matrigel. FGF- β and the media (0 mg/ml) were used as controls. * $P < 0.05$ vs. the media.

determine the length of the formed tubules. Cells treated with FGF- β (1 $\mu\text{g/ml}$) or PEG-dihydrazide (1 mg/ml) had significantly longer tubes than those treated with the media ($p < 0.05$), indicating greater angiogenic activity. PEG (1 mg/ml) did not induce tubulogenesis.

Our H&E histological observation of the subcutaneously implanted porous 10D/8P hydrogel revealed a gradual increase in tissue infiltration over the 15-week period (Fig. 5a). One-week post implantation, we observed the presence of fibrous capsules. The empty white space indicated areas of non-degraded hydrogel that were most likely present because the polymers were dissolved during histological processing. Eight weeks after implantation, the scaffold was almost completely infiltrated. Our most interesting high-magnification observation was the presence of more blood vessels in the 10D/8P scaffolds than in the poly (DTE carbonate) scaffolds (Fig. 5b). The vessels noted here were observed within the infiltrated tissue over the 15 weeks. “Fingers” of blood vessels appeared (indicated by the yellow arrows), which seemed to lead to tissue ingrowths towards the centre of the scaffold.

Discussion

In the present study, we fabricated a series of biodegradable hydrogels from poly(DTE-co- $x\%$ DT carbonate) crosslinked by $y\%$ poly(ethylene glycol) (PEG). The degradation profile of the resulting materials exemplified unique, controlled, predictable kinetics. The hydrogels and their degradation products exhibited suitable biocompatibility. Interestingly, PEG-di-DT hydrazide and its further degradation product PEG-dihydrazide stimulated *in vitro* angiogenic activities of endothelial cells. PEG-crosslinked poly(DTE-co-10%DT carbonate) exhibited a strong potential to modulate angiogenesis.

The samples with high PEG content (at least 20% PEG) exhibited accelerated mass loss, even with 25% DT (Fig. 3b). In previous studies, it has been shown that the incorporation of the hydrophilic PEG segments facilitates the solubilization of larger fragments and increases the rate of mass loss (Bezemer *et al.*, 2000). The presence of PEG also increases the polymer’s accessibility to water and, consequently, ensures degradation in the bulk of the matrices.

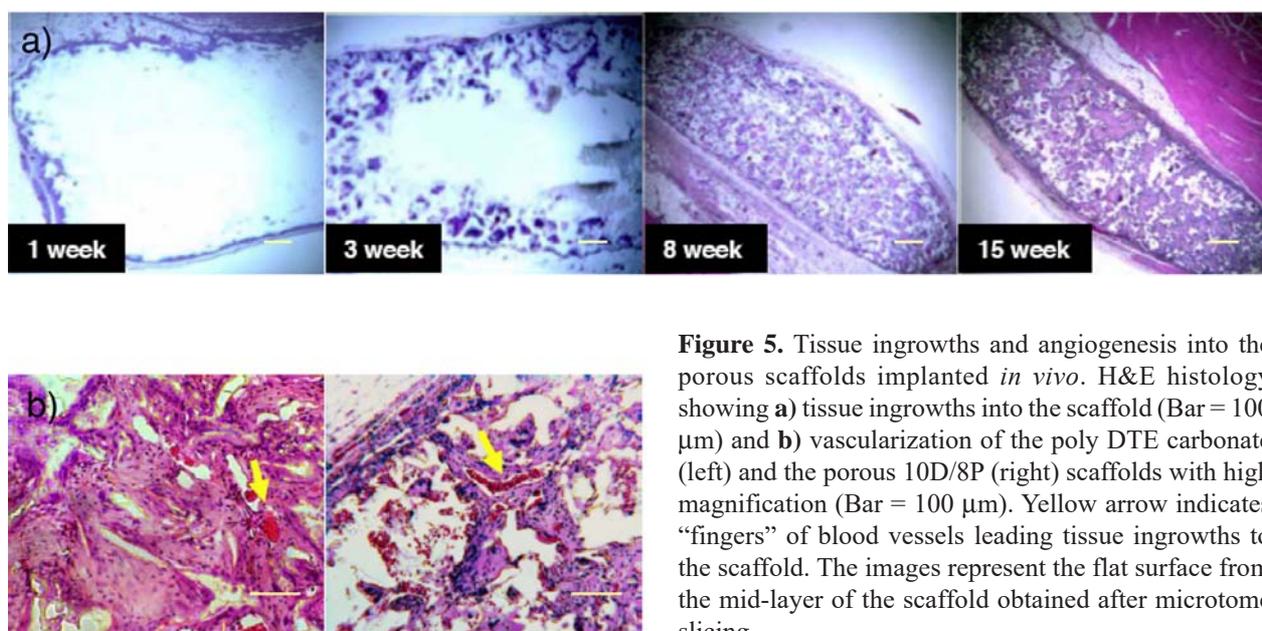


Figure 5. Tissue ingrowths and angiogenesis into the porous scaffolds implanted *in vivo*. H&E histology showing **a)** tissue ingrowths into the scaffold (Bar = 100 μ m) and **b)** vascularization of the poly DTE carbonate (left) and the porous 10D/8P (right) scaffolds with high magnification (Bar = 100 μ m). Yellow arrow indicates “fingers” of blood vessels leading tissue ingrowths to the scaffold. The images represent the flat surface from the mid-layer of the scaffold obtained after microtome slicing.

In contradiction to the finding of Zhao and Harris (Zhao and Harris, 1998), the PEG-crosslink density alone was not shown to significantly impact water uptake ($p > 0.1$) (Fig. 3a). We believe that this is due to the fact that increasing crosslinking is tied to increasing PEG content in the scaffolds, two factors that have opposite effects on water uptake (increased crosslinking, generally, decreases water uptake, whereas increasing PEG content increases water uptake).

The results from HPLC analysis of the incubation solution (Fig. 3c) indicate that the main mechanism of the initial degradation does not involve cleavage of the crosslinking unit but cleavage of the carbonate bonds in the polymer backbone, which are susceptible to water attack (Abramson, 2002). Since not all of the carboxylic acid groups in the polymer backbone are crosslinked, there are two sources for the DT release: the uncrosslinked carboxylic acids and PEG-di-DT hydrazide, which itself continues to degrade after being released as a product of degradation. The presence of the DT-NHNH₂ peak signified that the crosslinking unit eventually degraded by cleavage of the amide bond between DT and PEG-dihydrazide.

Only at the highest concentration (100 mg/mL) did PEG-di-DT hydrazide and PEG reduce RFL-6 viability when compared to the media ($p < 0.05$) (Fig. 4a). In their cytotoxicity study of the CHITOXAN™ hydrogel, Chellat *et al.* noted that the inhibition of cell growth started at 5 mg/mL when they treated its degradation product (Chellat *et al.*, 2000). In other studies, PGA and PLA demonstrated cytotoxicity at concentrations of 87 mg/mL and 72 mg/mL, respectively (Taylor *et al.*, 1994). We calculated that the theoretical maximum amount of PEG-di-DT hydrazide that could be released from the most quickly degrading hydrogel, 100D/80P, is roughly 36 mg. The minimum release from the slowest degrading hydrogel, 10D/8P, was calculated to be about 4–5 mg. Both of these calculations assume hydrogels are 8 mm in diameter and 2 mm thick. Within the range of all the above-mentioned

concentrations, PEG-di-DT hydrazide did not demonstrate any cytotoxicity in comparison to the culture medium control.

It seems that the angiogenic effect of degradation products on *in vitro* EC migration and tube formation resides in the PEG-dihydrazide component. Previous studies on heparin affinity regulatory peptide (HARP) demonstrated that the COOH and NH₂ terminals modulated angiogenesis in a dose-dependent and statistically significant manner (Papadimitriou *et al.*, 2000; Papadimitriou *et al.*, 2001). Researchers hypothesized that the two charged termini interact with cell surface molecules to facilitate HARP binding to its receptor and bring about a change in angiogenesis. Therefore, it is possible that the R-NHNH₂ domains of the degradation products PEG-di-DT hydrazide and PEG-dihydrazide are responsible for modulating EC migration and tubulogenesis. Further studies are necessary to confirm this hypothesis.

The *in vitro* cytotoxicity was assayed to investigate the biocompatibility of the degradation products. This *in vitro* assay provided a rapid and easy screening of polymer/cell interactions without the interference from wound response normally seen *in vivo* (Attawia *et al.*, 1995). The MTS assay measured cell viability to indicate not only the potency of the compound, but also the minimum dose responsible for inducing cell death. However, *in vitro* assays cannot confirm the compound's complete biocompatibility because they lack many of the complex physiological parameters provided by an *in vivo* environment. Nevertheless, our *in vivo* histological evaluation also indicated no noticeable cytotoxicity for the hydrogels or their degradation products.

Angiogenesis in matrixes of biomaterial scaffolds is a complex multi-step process that includes the initial steps of matrix degradation around endothelial cells, migration of endothelial cells into newly formed interstices, and proliferation of migrating endothelial cells (mainly at growth sprouts). The final step in angiogenesis includes the interaction between adjacent endothelial cells to form

a new lumen (i.e. tubulogenesis) and supporting basement membrane (extracellular matrix) and the simultaneous reinforcement of endothelial cells provided by muscle cell pericytes (Gupta and Qin, 2003). It has been established that controlling the degree of angiogenesis and the amount of biomaterial implant vascularization leads to better implant integration from autologous tissue infiltration of the graft. If a polymer scaffold has angiogenic properties, then this increases the likelihood that the implant will be successful since vessel arborization is integral to wound healing and graft survival. Researchers have been engineering new ways to increase angiogenesis by using a combination of biomaterials with controlled drug release systems, co-cultures with ECM and growth factor producing cells, and growth factor injections. The new class of biomaterials examined in this study, however, demonstrates a strong potential to modulate angiogenesis without being combined with the aforementioned additional systems.

One area in which this angiogenic hydrogel scaffold is applicable is in the musculoskeletal system, where vascularization is a key factor for the development of normal bone and cartilage (Ballara *et al.*, 1999). The formation of new blood vessels is crucial during bone development in the embryo, rapid bone growth, and fracture healing, all of which involve vascularization of cartilage (Gerber and Ferrara, 2000). During endochondral bone ossification (the formation of bone from cartilage), calcified cartilage allows for vascular invasion, which initiates the replacement of cartilage by bone (Harper and Klagsbrun, 1999). The cartilage, usually an avascular tissue, hypertrophies and becomes infiltrated with blood vessels which erode the tissue, producing a scaffold on which osteoblasts settle and produce bone (Ballara *et al.*, 1999). During fracture repair, angiogenesis is essential for proper healing (Hausman *et al.*, 2001) and occurs in response to bone injury or pathological conditions (Gerber and Ferrara, 2000). In particular, the angiogenic property of these hydrogels would be useful to regenerate bone tissue for defects larger than 2mm.

The effect of the DT content and PEG-crosslink density on *in vivo* angiogenic activities is a consideration for future examination. Therefore, we plan to expand the hydrogel types by varying the DT content and/or PEG-crosslink density for the next set of *in vivo* studies. Additional *in vivo* models, including the rabbit cornea assay and the chick chorioallantoic membrane assay, will be used for these future studies.

Conclusion

A family of PEG-crosslinked, tyrosine-derived hydrogels was synthesized based on poly(DTE-co-x% DT carbonate), which provided sites for chemical crosslinking with PEG-dihydrazide. We generated a series of hydrogels with varying DT contents and crosslinking levels. The porous 10D/8P hydrogel scaffolds possessed fairly interconnected macro- and micro-pores. DT and PEG contents were the major chemistry factors contributing to water uptake and degradation. The hydrogels degraded into the three major

release products: DT, DTE, and PEG-di-DT hydrazide. PEG-di-DT hydrazide was found to further degrade and release DT, which logically leads to the possibility that hydrazine may also be released. PEG-di-DT hydrazide was shown to induce *in vitro* EC migration and tube formation while exhibiting no noticeable cytotoxicity except at the highest concentration (100mg/ml). Interestingly, PEG-dihydrazide was equally as active as PEG-di-DT hydrazide at the same concentrations. Therefore, it is likely that the biological activity of PEG-di-DT hydrazide resides in the PEG-dihydrazide component. In our *in vivo* H&E histological evaluation, the 10D/8P hydrogel demonstrated a gradual increase in the tissue infiltration over the 15-week time period and seemed to effectively promote blood vessel growth. These results indicate that PEG-crosslinked poly(DTE-co-10%DT carbonate) shows great promise as a biomaterial that could ensure proper vascularization around the implantation site and aid in preserving implant patency by improving angiogenesis.

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Discussion with Reviewers

S. Fuchs: Individual cell types react differently to cytotoxic compounds. The authors tested the cytotoxic reaction of fibroblasts towards one of the degradation products PEG-di-DT hydrazide. How do endothelial cells react towards the tested compound or in extraction test according to the ISO guideline to the mixture of the single degradation products of the proposed hydrogels?

Authors: We agree with the reviewer's comment that ISO-based cytotoxic values of individual cell types in response to PEG-di-DT might be different. Rat lung fibroblasts (RLF-6) were used for our cytotoxicity test because this fibroblast type has been used extensively in previous studies investigating the cytotoxic effects of biomaterial compounds. As we described in the Results section, since

DT and DTE demonstrated no noticeable cytotoxicity or angiogenic effect in our previous studies (Ertel and Kohn, 1994; James *et al.*, 1999; Yu and Kohn, 1999), the present study focused on the cytotoxic and angiogenic effect of PEG-di-DT hydrazide and its further degradation product, PEG-dihydrazide. The results from the assays of EC migration under a series of conditions varying concentration and type of model degradation compounds, including PEG-di-DT hydrazide, demonstrated indirectly that all the tested compounds had no cytotoxic effect upon human aortic endothelial cells within the tested range of concentration. The range of concentration of an individual compound was determined following the guidelines established in ISO 10993 and the US FDA blue book memorandum (#G95-1). Multiple lines of evidence from the tests of *in vitro* tubulogenesis and *in vivo* tissue ingrowth/angiogenesis point out the fact that all of the single and mixture compounds are not cytotoxic.

S. Fuchs: *In vivo* evaluation: What was the reason to choose specifically 10D/8P hydrogels for *in vivo* evaluation? Do the authors have any additional information about the influence of the desaminotyrosyl tyrosine content or the cross-link density on the outcome of the vascularization process *in vivo* or *in vitro*? This information would be essential to understand how PEG-cross-linked tyrosine-derived polycarbonates hydrogels biomaterial *per se* could modulate angiogenesis in further applications.

Authors: In this study, we used only 10D/8P and poly(DTE carbonate) scaffolds for implantation. However, we plan to perform further *in vivo* studies on the implantation of other types of hydrogels with varying

desaminotyrosyl tyrosine contents and/or cross link densities, as the reviewer suggested, to better understand how they influence angiogenic processes. We agree with the reviewer's comment that this information would be valuable in improving our understanding of how PEG-cross-linked tyrosine-derived polycarbonate hydrogel biomaterials could modulate angiogenesis in further applications.

D. Eglin: What is the application that the authors foreseen for such polymeric scaffold? Would it be in bone tissue engineering?

Authors: One area in which this angiogenic hydrogel scaffold is applicable is in the musculoskeletal system, where vascularization is a key factor for the development of normal bone and cartilage (Ballara *et al.*, 1999). The formation of new blood vessels is crucial during bone development in the embryo, rapid bone growth, and fracture healing, all of which involve vascularization of cartilage (Gerber and Ferrara, 2000). During endochondral bone ossification (formation of bone from cartilage), calcified cartilage allows for vascular invasion, which initiates the replacement of cartilage by bone (Harper and Klagsbrun, 1999). The cartilage, usually an avascular tissue, hypertrophies and becomes infiltrated with blood vessels which erode the tissue, producing a scaffold on which osteoblasts settle and produce bone (Ballara *et al.*, 1999). During fracture repair, angiogenesis is essential for proper healing (Hausman *et al.*, 2001) and occurs in response to bone injury or pathological conditions (Gerber and Ferrara, 2000). In particular, the angiogenic property of these hydrogels would be useful to regenerate bone tissue for defects larger than 2mm.