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Synthesis, Material Properties and Biocompatibility of a Novel Self-Crosslinkable Poly(caprolactone fumarate) as an Injectable Tissue Engineering Scaffold

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Abstract

A novel self-crosslinkable and biodegradable macromer poly(caprolactone fumarate) (PCLF) has been developed for guided bone regeneration. This macromer is a copolymer of fumaryl chloride, which contains double bonds for in-situ crosslinking, and poly(ϵ -caprolactone) that has a flexible chain to facilitate self-crosslinkability. PCLF was characterized with Fourier transform infrared (FTIR) spectroscopy, ¹H and ¹³C nuclear magnetic resonance (NMR) spectroscopy, and gel permeation chromatography (GPC). Porous scaffolds were fabricated with sodium chloride particles as the porogen and a chemical initiation system. The PCLF scaffolds were characterized with scanning electron microscopy (SEM) and micro-computed tomography (micro-CT). The cytotoxicity and in vivo biocompatibility of PCLF were also assessed. Our results suggest that this novel copolymer, PCLF, is an injectable, self-crosslinkable, and biocompatible macromer that may be potentially used as a scaffold for tissue engineering applications.

Introduction

The clinical needs for bone regeneration are diverse, and there are roughly 1 million patients who have skeletal defects each year in the United States that require bone graft procedures to achieve union.^{1, 2} These include bony defects resulting from resection of primary and metastatic tumors, bone loss after skeletal trauma, primary and revision total joint arthroplasty with bone deficiency, spinal arthrodesis, and trabecular voids following osteoporotic insufficiency fractures.

Autografts, allografts, and synthetic materials are being used in orthopedic surgery to fill osseous defects. Autografts have a limited supply and morbidity of the harvest site is a concern. The problem with allografts is the risk of disease transfer and immunogenic response from the host tissue. Stress shielding and particulate wear have limited the use of non-degradable polymers such as poly(methyl methacrylate) (PMMA) for filling defects or as a bone cement in orthopedics. Synthetic biodegradable and biocompatible polymers are an ideal replacement material for orthopedic applications because of unlimited supply, minimum risk of disease transfer, and reduced stress shielding and particulate wear due to the coupling of polymer

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degradation with tissue regeneration. Injectable biomaterials coupled with minimally invasive techniques are an attractive alternative for treating irregularly shaped osseous defects with minimum tissue dissection and retraction. Pre-formed scaffolds must be implanted surgically, while injectable scaffolds, which harden in-situ, can be injected, reducing the invasiveness of the implantation procedure.

Injectable bioactive calcium phosphate cements, such as calcium deficient hydroxyapatite, and their blends with synthetic or natural polymers³ that undergo curing and harden in-vivo have been developed⁴. These injectable cements⁵ show no extended inflammatory response, promote bone growth, have compressive mechanical properties higher than that of cancellous bone, remain neutral after setting, and have been tested in preclinical⁶ and clinical trials^{6d, 7}. However, potential drawbacks of calcium phosphate cement are fatigue fracture and their very low strength in shear and tension, despite compressive strength between that of cancellous and cortical bone. This limits the use of bioactive injectable ceramic bone cements to fractures that are subject to relatively uniform compressive loading.^{7b}

Bone graft substitutes that expand in-situ to conform to the geometry of bone defects as a result of the release of carbon dioxide during the polymerization reaction have been developed⁸ based on poly(dioxanone-*co*-glycolide) endcapped with a lysine diisocyanate crosslinker. After injection, the isocyanate groups at the end of each arm of the macromer react with water from the surrounding tissue to form a polyurea/urethane network and carbon dioxide. However, less than optimal interconnectivity of the pore volume by the foaming reaction is a limitation.

A group of photocrosslinkable poly(anhydrides) consisting of polymers of sebacic acid, or its copolymers with either 1,3-bis(*p*-carboxyphenoxy) propane, or 1,6-bis(*p*-carboxyphenoxy) hexane have been developed.⁹ However, the dependence upon light for polymerization creates an extra step during surgery and may limit the use of these materials in deep defects.

Poly(propylene fumarate) (PPF), an unsaturated linear polyester that can be modified or crosslinked through its fumarate double bonds, and chemically crosslinked in-situ via redox initiation has been developed.¹⁰ PPF degrades by simple hydrolysis¹¹ of the ester bonds into the nontoxic products, propylene glycol, poly(acrylic acid-*co*-fumaric acid), and fumaric acid, a substance that occurs naturally in the Krebs's cycle. The in-situ hardening time depends on the crosslinking agent used for producing the PPF network. PPF can be crosslinked with methyl methacrylate (MMA)¹², *N*-vinyl pyrrolidinone (NVP) monomers¹³, or biodegradable macromers such as PPF-diacrylate or poly(ethylene glycol)-diacrylate¹¹ when combined with benzoyl peroxide as a radical initiator. The maximal temperature during setting is 48 °C, much less than that for PMMA bone cement¹⁴. The high concentration (>20 wt% of PPF) of crosslinking agent used for in-situ hardening of PPF, however, may affect the biocompatibility of the injectable system.

The objective of this research was to develop a new material, poly(caprolactone fumarate) (PCLF), that allows in-situ hardening of the macromer without the use of an additional crosslinking agent, which is potentially toxic. In PCLF, propylene glycol is replaced with poly(ϵ -caprolactone) (PCL), which is biocompatible and FDA-approved for certain clinical uses. Since each caprolactone unit provides four free rotating carbon-carbon bonds, the PCLF chain is much more flexible than the PPF chain above the melting point of the PCL. For example, PCLF with PCL M_n of 760 g.mol⁻¹ and melting point of 48 °C has a viscosity of 360 and 14300 Pa.s at 60 and 37 °C, respectively. This suggests that PCLF can be injected above its melting point but the mixture hardens in-situ first by chemical crosslinking and then by partial crystallization of the PCL units of the copolymer. The increased flexibility of the PCLF above its melting point makes the fumarate double bonds and the ester bonds more accessible for crosslinking and hydrolysis respectively. Since the PCL molecular weight can be easily varied,

PCLF offers another degree of freedom to control the crosslinking, mechanical, and degradation characteristics of the polymer network. The increased mobility of the fumarate double bonds allows PCLF to self-crosslink without the use of an additional crosslinker. The term “self-crosslink” in this report means that the macromer can be crosslinked without the aid of a low molecular weight monofunctional or multifunctional crosslinking agent. The crosslinking reaction still requires initiator and accelerator to initiate and accelerate the polymerization, respectively. The synthesis, material properties, and biocompatibility of PCLF, as a novel injectable system, are described in this report.

Experimental Section

Synthesis

All the chemicals in the present study were purchased from Aldrich (Milwaukee, WI) if there is no additional notation. Three poly(ϵ -caprolactone) (PCL) diols were used for the synthesis of poly(ϵ -caprolactone fumarate) (PCLF) with nominal molecular weight of 530, 1250, and 2000 g.mol⁻¹. The PCLs were dried under vacuum at 60 °C for at least 12 hr before the reaction. Methylene chloride and triethylamine (TEA) were dried and distilled over CaH₂ before the reaction. Fumaryl chloride (FC) was purified by distillation at 161 °C. PCLF was synthesized by polycondensation of PCL with FC in methylene chloride with TEA as a catalyst shown in Scheme 1. Three molar ratios of FC to PCL such as 0.9, 0.95, and 0.995 have been used to investigate its effect on the polycondensation. In a typical reaction, 50 g of PCL was dissolved in 300 ml of methylene chloride under dry nitrogen atmosphere in a three-neck reaction flask. 3.9 ml of FC and 10.0 ml of TEA each dissolved in 25 ml of methylene chloride were added dropwise to the reaction with stirring. The reaction vessel was placed in an ice bath to limit the temperature rise of the exothermic reaction. After the addition of FC and TEA, reaction was continued for 24 hr under ambient conditions.

After completion of the reaction, solvent was removed by rotovaporation. The residue was dissolved in 500 ml of anhydrous ethyl acetate, and the by-product triethylamine hydrochloride salt was removed by filtration. Ethyl acetate was removed by vacuum distillation at 30 °C. The macromer was re-dissolved in methylene chloride and precipitated twice in ethyl ether. It was dried in vacuum (less than 5 mmHg) at ambient temperature for at least 12 hr and stored at – 20 °C until used.

Characterization

Gel permeation chromatography (GPC) was used to determine the molecular weight and polydispersity of the synthesized macromers. The GPC was carried out with a Waters 717 Plus Autosampler GPC system (Waters, Milford, MA) connected to a model 515 HPLC pump and model 2410 refractive index detector. The columns consisted of a styragel HT guard column (7.8×300 mm, Waters) in series with a styragel HR 4E column (7.8×300 mm, Waters). Polymer sample (20 μ l), at a concentration of 20 mg/ml in tetrahydrofuran, was eluted at a flow rate of 1 ml/min. Monodisperse polystyrene standards (Polysciences, Warrington, PA) with M_n of 0.474, 6.69, 18.6, and 38 kg.mol⁻¹, and PI (polydispersity index) of less than 1.1, were used to construct the calibration curve.

¹H and ¹³C NMR (Nuclear Magnetic Resonance) spectra were recorded with a Bruker Avance 500 MHz system (Bruker Analytik GmbH, Rheinstetten, Germany) at ambient temperature to confirm the presence of the fumarate group in the macromer. Polymer solutions for NMR were prepared with deuterated chloroform at a concentration of 50 mg/ml containing trimethyl silane (TMS) as the internal standard.

An FTS-40 Fourier transform infrared spectrophotometer (FTIR) (Bio-Rad, Hercules, CA) was used to measure the absorption of PCLF in the region from 1000 to 4000 cm⁻¹. A thin film of

the polymer was cast on a CaF₂ disk (Wilmad Glass, Buena, NJ) with dimensions of 3 mm×32 mm and the spectrum was collected under a dry nitrogen atmosphere with 16 scans and a resolution of 4 cm⁻¹. A drop of PCLF in acetone solution (50 mg of PCLF in 1 ml of acetone) was placed on the CaF₂ disk and dried at ambient conditions for 30 min. It was then dried in vacuum at ambient temperature for 30 min, and finally heated to 60 °C to remove any residual solvent in the film.

The melting point (T_m) of PCLF samples was measured by Differential Scanning Calorimetry (DSC; model 2910, TA Instruments, DE). The sample was first melted by heating to 100 °C and then cooling to ambient temperature at a rate of 5 °C/min. T_m was determined as the exothermal peak in the cooling cycle.

X-ray diffraction was used to measure the crystallinity of PCL and PCLF. Data was collected with a Bruker-AXS microdiffractometer (Bruker Elektronik GmbH, Rheinstetten, Germany) with copper radiation, a graphite incident-beam monochromator, 0.8 mm point collimation system, and a two-dimensional multiwire area detector. For data collection, angles ranging from 5 to 35° were covered and data collection time was 100 s. Sample area detector frames were integrated with a step-size of 0.04° and plotted versus intensity. Percent crystallinity was determined using a JADE profile fitting software (Materials Data Inc., Livermore, CA) with a linear background and a pseudo-Voigt peak fitting parameters. Percent crystallinity was determined as the ratio of the area under the crystalline peaks to the area of the entire pattern.

The temperature and time dependence of the viscosity and shear modulus of PCLF samples were measured by rheometry to evaluate injectability, hardening, and crosslinking characteristics. The measurements were obtained with an AR2000 Rheometer (TA Instruments, DE) using a parallel plate geometry. The upper portion was a 40 mm diameter stainless steel plate. The PCLF, upon mixing the reaction components, has a consistency and level of pressure required for injection (thumb pressure on a syringe) similar to those of the PMMA bone cement used clinically. The PCLF reaction components (2 g) were injected through a syringe onto the center of the peltier plate, which was the lower portion of the geometry. The plate was then heated to 60 °C to melt the sample. The upper plate was lowered until a gap of 0.75 mm was formed between the upper and lower plates. A sinusoidal shear strain profile was exerted on the sample via the upper cylinder and the force required to strain the sample along with the complex viscosity and complex modulus was monitored. The experiments were conducted with a peak sinusoidal shear strain of 0.1%. The completion of the crosslinking reaction was evaluated by rheometry as shown in Figure 1. Figure 1 shows the shear modulus of the PCLF with M_n of 3680 g.mol⁻¹ as a function of crosslinking time at 40 °C. The shear modulus of the sample increased by three orders of magnitude from 10 Pa to 10 kPa as the sample was crosslinked. According to Figure 1, the sample had reached more than 90% of the final shear modulus after 1 hr of crosslinking at 40 °C. Therefore, PCLF samples were crosslinked for 1 hr at 40 °C in a convention oven.

PCLF Crosslinking

PCLF was crosslinked by free radical polymerization with benzoyl peroxide (BPO) and dimethyl toluidine (DMT) as the radical initiator and accelerator, respectively. The initiator solution was prepared by dissolving 50 mg of BPO in 0.25 ml of 1-vinyl-2-pyrrolidinone (NVP). The accelerator solution was prepared by mixing 40 µl of DMT in 0.96 ml of methylene chloride. 50 µl and 40 µl of initiator and accelerator solutions per gram of PCLF were used for crosslinking. The amounts of the initiator and accelerator solutions were chosen such that the minimum working time and the maximum crosslinking time would be 5 min and 1 hr, respectively. Working time is the minimum time required to prepare and inject the polymerizing mixture. The crosslinking time is defined as the time for greater than 90% completion of the crosslinking reaction, as evaluated by rheometry. The fraction of NVP as a

good solvent for the initiator in the polymerizing mixture was less than 4% of the weight of PCLF. 1 g of PCLF was mixed with 5.0 g of porogen in a scintillation vial, corresponding to 75% by volume porosity. The polymerizing scaffold was transferred into a 5mm x 18mm Teflon mold and pressed manually to maximize packing. The mold was placed in a convection oven at 40°C for 1 hr to facilitate crosslinking. An oven was used to crosslink the samples at a temperature at or near the physiological temperature. After crosslinking, the mold was cooled to ambient temperature, scaffolds were removed from the mold, and cylindrical specimens with diameter and length of 5 mm by 8 mm were cut with an Isomet low speed saw (Buehler, Lake Bluff, IL). The salt was leached out by placing the scaffolds in distilled water for 3 days, during which time water changes occurred every 12 hr. The scaffolds were dried in vacuum of less than 5 mmHg for at least 12 hr.

Scaffold Pore Morphology

A cold field-emission scanning electron microscopy (S-4700, Hitachi Instruments Inc., Tokyo, Japan) was used to examine the pore morphology of the scaffolds. Each scaffold was fractured at liquid nitrogen temperature to expose a flat internal section of the scaffold. The sample was mounted on an SEM stub (Ted Pella, Redding, CA, USA) using Hanid-Tak putty (Super Glue Corp., Rancho Cucamonga, CA) and it was painted with colloidal silver to electrically ground the assembly. It was dried overnight in a vacuum desiccator. The assembly was sputter coated with gold-palladium using a Bio-Rad/Polaron E5400 High Resolution sputter coater (Bio-Rad/Polaron, Cambridge, MA) for 30 s at 90 mA. The samples were viewed with SEM at 5 kV accelerating voltage.

A micro-computed tomography (micro-CT) scanner was custom-built in the Physiological Imaging Research laboratory at the Mayo Clinic (Rochester, MN) from commercially available components. The scanner generates 3-D images consisting of up to a billion cubic voxels. The specimen was positioned close to the crystal and rotated in several hundred equiangular steps around 360° between each x-ray exposure and its accompanying charge coupled device recording. A comprehensive 3-D image display and analysis program, (Analyze®, Mayo Foundation, Mayo Clinic, Rochester, MN) was employed to quantitatively assess the polymer scaffold microstructure. An operator-selected threshold of intensities segmented images in order to separate voxels representing regions of differing density. Images were then analyzed to obtain volume fractions of each material, to measure interconnectivity, and to obtain size distributions of isolated pores.

Cytocompatibility

PCLF films were prepared, as described in the scaffold fabrication section, by self-crosslinking PCLF without the salt as the porogen. To prepare films, the mixture was pressed at 40 °C with 275 lb.in⁻². Disks with diameter of 5 mm were cut from the films using a cork-borer. Disks were sterilized by transferring to excess 70% ethanol overnight with shaking and washed with PBS at least three times before adding to 12-well plates. ASTM F813-01 was used for direct contact cell evaluation of the PCLF scaffolds. Immortalized hFOB (human fetal osteoblasts) cells were used. The cryopreserved cells were thawed and plated on polystyrene flasks in media containing DMEM/F12 and sodium bicarbonate, 10% v/v fetal bovine serum (FBS), and 150 mg of Geneticin. After plating, the suspension was incubated for 12 hr in a 5% CO₂, 95% relative humidity incubator at 34 °C. The cytotoxicity evaluation proceeded by seeding the cells in a 12-well plate at a density of 2×10⁴ cells.cm⁻² in 300 µl.cm⁻² of primary media in the presence of a sterile 5 mm×0.5 mm PCLF disk. The PCLF disk, floating in the media, was exposed to the hFOB cells for 48 h. Next, the disks were removed, cells were washed with PBS, fresh media was added to each well, and incubated for an additional 24 hr. hFOB cells plated with the same density without exposure to PCLF was used as the positive control. The

cells were viewed with an Axiovert 25 Zeiss light microscope (Carl Zeiss, Germany). The cells were counted with a haemocytometer using a Trypan Blue stain.

In Vivo Implantation

Compatibility of crosslinked PCLF in vivo was evaluated by subcutaneous implantation in 12 female Sprague Dawley rats (250–300 g average weight; Harlan, Indianapolis, IN). Disks 8 mm×0.8 mm were cut from a crosslinked PCLF film using a cork-borer, sterilized in excess 70% ethanol, washed with PBS, and implanted subcutaneously in the left rear haunch of the rats. Groups included three crosslinked PCLF samples prepared from three different PCLs and an expanded poly(tetrafluoroethylene) (Gore artificial dura; W. L. Gore & Associates, Newark, DE) as the negative control. The animals were anesthetized with 80 and 5 mg/kg ketamine and xylazine, respectively. One month after implantation, the animals were sacrificed and samples with the surrounding tissue were removed. Samples were dehydrated in sequential ethanol solution and Hemo-De, embedded in paraffin, and sectioned. Sections were stained with H&E for histological examination. Samples were treated with the monoclonal antibody Mouse Anti-Human CD68 (macrophage clone KP-1, Code No. M 0814, Dako Corporation, Carpinteria, CA) to observe the presence of macrophages.

Results and Discussion

The molecular weights of PCLs used in the present study were 340, 760, and 1200 g.mol⁻¹ with PI of 1.7, 1.8, and 1.8, respectively, as shown in Table 1. The influence of FC/PCL ratio on molecular weight and polydispersity of PCLF has been studied. For PCL with M_n of 340 g.mol⁻¹ M_n of PCLF remained around 3150 g.mol⁻¹ with PI of 2.9 as the FC/PCL ratio increased from 0.9 to 0.95 and 0.995. For PCL with M_n of 760, as the FC/PCL ratio increased from 0.9 to 0.95 and 0.995, the PCLF molecular weight changed from 3680 to 5540 and 6100 g.mol⁻¹ with PI of 2.3, 3.1, and 3.9, respectively. For PCL with M_n of 1200, as the FC/PCL ratio increased from 0.9 to 0.95 and 0.995, the PCLF molecular weight changed from 3870 to 7020 and 7140 g.mol⁻¹ with PI of 2.6, 4.1, and 4.0, respectively. The FC/PCL ratio did not affect the PCLF molecular weight for PCL of 40 g.mol⁻¹. On the other hand, PCLF molecular weight increased for PCL of 760 and 1200 g.mol⁻¹ as the FC/PCL ratio approached unity.

Three PCLF samples studied in this paper were prepared using a constant FC/PCL ratio of 0.9. As shown in Table 1, PCLs with M_n of 340, 760, and 1200 g.mol⁻¹ produced PCLF copolymers with molecular weights of 3150, 3680, and 3870 g.mol⁻¹ with PI of 2.9, 2.3, and 2.6, respectively, indicating that PCLF is more polydisperse after copolymerization. When PCL molecular weight was increased from 340 to 1200 g.mol⁻¹, a significant increase in PCLF molecular weight was not observed.

The FTIR spectra of PCL with M_n of 340 g.mol⁻¹ before and after the reaction with fumaryl chloride are shown in Figure 2. The absorption bands with peak positions at 2944 and 2860 cm⁻¹, common to the spectrum of PCL and PCLF, are due to the asymmetrical stretching (ν_{as} CH₂) and symmetrical stretching (ν_s CH₂) of the methylene groups of PCL. The absorption band at 1730 cm⁻¹, common to the spectrum of PCL and PCLF, is due to the carbonyl (C=O) vibration of PCL and fumarate. This band is stronger in the PCLF spectrum because the carbonyl groups of PCL as well as those of fumaryl chloride contribute to the absorption band. The weak absorption bands with peak positions at 1260 and 1300 cm⁻¹ are due to the C-H rocking vibration of the disubstituted fumarate group and at 1645 cm⁻¹ due to C=C stretching, which are absent in the spectrum of PCL. A relatively strong absorption band with peak position at 1160 cm⁻¹, present in PCL and PCLF spectra, is due to asymmetric coupled vibrations of C-C(=O)-O and O-C-C groups of PCL.

The ^1H NMR spectrum of PCLF with PCL M_n of 340 g.mol^{-1} is shown in Figure 3. The corresponding PCL spectrum is also shown as an inset. The chemical shifts with peak positions at 1.38 and 1.65 ppm are due to methylene protons of PCL attached to two methylene ($-\text{CH}_2$) groups or one methylene group and one ($-\text{CH}_2\text{O}$) group, respectively. The chemical shift with peak position at 2.3 ppm is due to the methylene protons of PCL attached to one ($-\text{CH}_2$) and one carboxyl ($-\text{COOR}$) group. The chemical shift centered at 3.6 ppm is due to terminal methylene hydrogens of PCL attached to a $-\text{CH}_2$ and a hydroxyl group ($-\text{OH}$). The chemical shift centered at 4.1 ppm is due to non-terminal methylene protons attached to a $-\text{CH}_2$ and a $-\text{OCOR}$. The relatively small peaks centered at 3.1 and 4.35 ppm are due to the methylene protons of the residual ethoxy groups in PCL. The chemical shift with peak position at 6.8 ppm is due to protons of fumarate group of PCLF, which is absent in the spectrum of PCL. Since the chemical shift of the fumarate protons is below 7.0 ppm, the fumarate group in the copolymer is in the *cis*-configuration. Two additional chemical shifts at 134 and 165 ppm in ^{13}C NMR spectrum (not shown here) of PCLF compared to that of PCL are due to carbons on double bonds and carbonyl groups in fumarate segments, respectively.

The DSC curves in Figure 4 show the melting points (T_m s) of the PCLF samples with different PCL molecular weights. The PCLFs with PCL molecular weights of 340, 760, and 1200 g.mol^{-1} had T_m s of 36, 49, and 56°C , respectively. The PCLF sample with PCL M_n of 340 g.mol^{-1} is a wax but the samples with PCL M_n of 760 and 1200 g.mol^{-1} are solid at ambient to physiological temperature. The PCLFs with PCL molecular weights of 760 and 1200 g.mol^{-1} can potentially be injected at temperatures above 49 and 56°C , respectively, and harden in-situ as the samples return to the physiological temperature of 37°C . The change in percent crystallinity of PCL, PCLF, and crosslinked PCLF (PCLF-X) with molecular weight is shown in Figure 5. The crystallinity of PCL increased from 13 to 46 and 57% as the molecular weight increased from 340 to 760 and 1200 g.mol^{-1} , respectively; for PCLF it increased from 8 to 43 and 52%, respectively; and for PCLF-X it increased from 6 to 35 and 47%, respectively. For all the molecular weights, the crystallinity of PCL decreased slightly by 2–5% after copolymerization with fumaryl chloride and that of PCLF decreased by 2–8% after crosslinking. For all molecular weights, copolymerization with fumaryl chloride and crosslinking resulted in small but statistically significant reductions in percent crystallinity. The PCLs with M_n of 340 and 1200 g.mol^{-1} showed the highest (52% reduction) and the lowest (16% reduction) percent crystallinity after crosslinking. The molecular weight of PCL had a strong influence on crystallinity. For example, for PCLF, percent crystallinity increased from 8 ± 1 to 43 ± 2 and $52 \pm 2\%$ as the PCL M_n increased from 340 to 760 and 1200 g.mol^{-1} , respectively. These results clearly show that PCL retains significant fraction of its crystallinity after copolymerization and even after crosslinking.

Preliminary degradation studies of the PCLF macromer in phosphate buffer saline (PBS) at 37°C have been done for PCLF with PCL molecular weight of 340, 760, and 1200 g.mol^{-1} , respectively. For example, for PCLF with PCL M_n of 340 g.mol^{-1} , the weight-average molecular weight (M_w) decreased gradually to 92%, 69%, 57%, and 40% of the initial M_w as the incubation time was increased from 0 to 1, 2, 3, and 6 months, respectively. The detailed investigation on degradation mechanism is being performed.

The changes in storage modulus G' as the PCLF samples were cooled from above their melting points to physiological temperature of 37°C in the absence of crosslinking is shown in Figure 6. According to this figure, for PCLF with PCL M_n of 340 g.mol^{-1} , there was a relatively small increase in shear modulus from 3 to 4 Pa as the sample was cooled. For PCLFs with PCL M_n of 760 and 1200 g.mol^{-1} , the shear modulus showed a very large increase from 0.5 kPa to 1.5 kPa and from 1 kPa to 1 MPa, respectively, upon cooling from 57°C to 37°C , respectively. The PCLF sample with M_n of 1200 g.mol^{-1} showed the highest increase (1000 fold) in G' upon cooling.

Figure 7 shows the storage shear modulus of the PCLF with PCL molecular weight of 760 g.mol^{-1} when the temperature is lowered from 60°C to 37°C while crosslinking in the presence of initiator and accelerator with concentrations of 1% and 0.15% by weight of PCLF, respectively. The PCLF mixture before injection has viscosity and consistency similar to the PMMA bone cement used clinically and similar level of pressure is required for injection. After injection, the shear modulus increases quickly by an order of magnitude and continues to increase with time as the PCLF crosslinks chemically and crystallization occurs. After 1 hr of crosslinking at 37°C , G' increased from 4 Pa to 10 kPa, 1.5 kPa to 40 kPa, and 1.0 MPa to 1.2 MPa for PCLFs with PCL M_n of 340, 760, and 1200 g.mol^{-1} , corresponding to fold increase of 2500, 27, and 1.2, respectively (data not shown). As the molecular weight of PCLF was increased, crosslinking was less effective in increasing G' . This can be explained by the fact that with increasing PCLF molecular weight, the sample becomes more rigid at 37°C , the diffusivity of reactive molecules in the sample decreases, and the crosslinking reaction becomes diffusion-limited.

The clinical scenario for the use of PCLF will be similar to that used for the injection and polymerization of PMMA bone cement. The PMMA bone cement can reach temperatures of greater than 100°C during polymerization.¹⁴ It has been in use for over 40 years, and although the Orthopaedic and Neurosurgical literature does not contain reports of tissue necrosis associated with PMMA use, its polymerization temperature is well above the temperature considered dangerous for tissues (about 50°C).¹⁴ Surgeons are cautious with PMMA application near vital structures, such as the spinal cord in cases where PMMA is used for vertebral body reconstruction. The PCLF crosslinks at temperatures well below those for PMMA polymerization (we accomplished crosslinking at 60°C in this study). Appropriate surgical technique and care will need to be exercised in the clinical application of the PCLF crosslinking reaction, as it is in the use of more highly exothermic PMMA polymerization reaction.

Porous scaffolds were fabricated from the PCLF samples with sodium chloride salt crystals of $400\mu\text{m}$ average size as the porogen. The concentration of BPO and DMT was 1% and 0.15% by weight of PCLF, respectively. Figure 8a shows the SEM micrograph of a freeze-fractured cross-section of a porous PCLF scaffold with 75% pore volume. The PCLF used in the fabrication of this scaffold had M_n and PI of 3680 g.mol^{-1} and 2.3, respectively. The SEM picture shows a highly porous and interconnected solid scaffold. A longitudinal section of the same scaffold, obtained by micro-CT, shown in Figure 8b, confirms the formation of a three-dimensional solid, porous and interconnected PCLF scaffold. In Figure 8b, the dark and gray areas are the pore and crosslinked PCLF spaces, respectively. The bright white dots in the picture are the salt particles remained in the scaffold after soaking in water. Before soaking in water, all of the pore volume was occupied by salt crystals. The area of bright dots is a very small fraction of the total pore area. Therefore, more than 99% of the salt was removed by soaking in water. It should be noted that swelling of the scaffold is not required for dissolving and leaching the salt crystals because the pore volume was interconnected, as shown in Figure 8b.

Less than 4% by weight NVP crosslinker was used to dissolve the BPO initiator to prepare the scaffolds. The reactivity of NVP is orders of magnitude faster than the PCLF macromer because of the smaller molecular size. The crosslinked PCLF was swollen in a good solvent such as methylene chloride to extract the sol fraction. The sol fraction in all cases was not significant (less than 1%) of the initial dry weight of the sample. NVP oligomers are formed very early in the reaction (because of higher reactivity) which reacted with fumarate groups to become part of the crosslinked network.

Figure 9 shows the cell viability of linear and crosslinked PCLF after 48 hr exposure to hFOB cells. The fraction of viable cells normalized to the positive control for linear PCLF with M_n of 760 and 1200 g.mol⁻¹ was 1.01 ± 0.04 and 0.89 ± 0.03 , respectively, and for crosslinked PCLF with M_n of 340, 760 and 1200 g.mol⁻¹ was 0.99 ± 0.07 , 0.99 ± 0.06 , and 1.02 ± 0.03 , respectively. All linear and crosslinked PCLF samples showed excellent compatibility to human osteoblasts with no statistically significant difference among the five groups.

No sign of chronic infection in the blood, abnormalities in the renal capsules or kidney glomerulus, or interstitial thickening around the blood vessels were observed one month after implantation of PCLF disks (data not shown). Figure 10 shows a typical section of the implanted PCLF sample with M_n of 760 g.mol⁻¹ in (a) and the surrounding tissue in (b). No activated macrophages were observed in the surrounding tissue. The thickness of the fibrous capsule around the sample was less than 100 μ m, which was comparable to Gore artificial dura as the negative control. These results suggest good biocompatibility of the implanted PCLF disks.

The cytocompatibility studies and in-vivo implantation studies do not exactly represent the in-situ crosslinking situation but these tests provide very useful information about possible leaching of unreacted compounds from the disk after crosslinking of the matrix. It should also be noted that most of the components of the crosslinking reaction such as methylene chloride, BP, DMT, and PCLF are insoluble in aqueous physiological media. As a result, their concentration in the surrounding tissue after injection and before completion of crosslinking is relatively small. If the unreacted BPO, DMT, and NVP diffuse out, their release rate depends on the degradation characteristics of the PCLF matrix. The fraction of BPO, DMT, and NVP in the PCLF matrix were 1%, 0.16%, and 4%, respectively. Most of these compounds react and become part of the crosslinked network. The unreacted fraction (assuming maximum 10% from the rheometry experiments) will be released over at least a six-month period (degradation time of the PCLF). The release rate for BPO, DMT, and NVP will be relatively minute at 64, 256, and 10 pg/sec. Further studies are needed to assess the affect of these concentrations on cell viability and tissue biocompatibility.

Conclusions

A novel semi-crystalline poly(caprolactone fumarate) (PCLF) macromer that self-crosslinks in the absence of a crosslinking agent has been synthesized. The presence of chemical shifts at 6.8 ppm in the ¹H NMR spectrum, two additional chemical shifts at 134 and 165 ppm in ¹³C NMR spectrum, and the absorption bands with peak positions at 1260, 1300, and 1645 cm⁻¹ in the FTIR spectrum clearly indicate that fumarate groups are incorporated in PCL. This semi-crystalline copolymer has a softening point between 45–55 °C depending on PCL molecular weight and degree of copolymerization. Above its softening point, the copolymer is a liquid and it can be used as an injectable matrix to fill irregularly shaped defects. As the matrix cools to physiological temperature of 37 °C, the copolymer self-crosslinks and hardens in-situ physically by crystallization and chemically by free radical polymerization of unsaturated bonds of the fumarate groups. The macromer was characterized with respect to chain length distribution, crystallinity, rate of crosslinking, and tissue compatibility. Molecular weight of PCLF depended on that of poly(ϵ -caprolactone) (PCL) used in the synthesis. The M_n of PCLF increased from 3150, 3680, and 3870 g.mol⁻¹ as that of PCL increased from 340 to 760 and 1200 g.mol⁻¹. The polydispersity index of PCLF (2.6) was significantly higher than that of PCL (1.7) for all molecular weights. The degree of crystallinity of PCLF depended on PCL molecular weight. For example, as the PCL molecular weight increased from 340 to 760 and 1200 g.mol⁻¹, percent crystallinity of PCLF increased from 8 ± 1 to 43 ± 2 and $52 \pm 2\%$, respectively. The melting point of PCLF, measured by DSC, was 36, 49, and 56 °C, for PCL molecular weights of 340, 760, and 1200 g.mol⁻¹, respectively. Tissue compatibility was

evaluated by subcutaneous implantation in Sprague Dawley rats for 4 weeks. The PCLF disk and surrounding tissue were removed and examined histologically by H&E staining. Monoclonal antibody specific to allograft inflammatory factor (AIF-1) was used for immunohistochemistry. No inflammatory response was observed in the surrounding tissue. The thickness of the fibrous capsule around the sample was less than 100 μm , which was comparable to Gore artificial dura as the negative control. The biocompatible PCLF macromer is potentially useful as a self-crosslinkable scaffold for tissue regeneration.

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Figure 1. Shear modulus of the PCLF with M_n of 3680 g.mol^{-1} as a function of crosslinking time at 40°C .

Figure 2.
FTIR spectra of PCLF and its parent PCL with M_n of 760 g.mol⁻¹.

Figure 3. ^1H NMR spectrum (in CDCl_3) of PCLF with PCL M_n of 760 g.mol^{-1} . Inset: ^1H NMR spectrum (in CDCl_3) of PCL of 760 g.mol^{-1} .

Figure 4. DSC curves of the PCLFs with PCL molecular weights of 340, 760, and 1200 g.mol⁻¹.

Figure 5. Percent crystallinity of PCL, PCLF, and crosslinked PCLF (PCLF-X) for different PCL molecular weights.

Figure 6.

The changes in storage modulus G' (solid lines) as three PCLF samples with PCL M_n of 340, 760, and 1200 g.mol^{-1} were cooled from above their melting points to physiological temperature of 37 °C in the absence of crosslinking. The temperature change is depicted as a dashed line.

Figure 7.

The storage shear modulus of the PCLF with PCL molecular weight of 760 g.mol^{-1} when the temperature is lowered from 60°C to 37°C while crosslinking in the presence of initiator and accelerator with concentrations of 1% and 0.15% by weight of PCLF, respectively.

(a)

Figure 8.

(a) Scanning electron micrograph of the cross-section and (b) micro-computed tomography of a longitudinal section of a PCLF scaffold with PCLF M_n of 3680 g.mol^{-1} and 75% by volume salt content.

Figure 9. Cell viability of linear (L) and crosslinked (X) PCLF samples with PCL M_n of 340, 760, and 1200 $\text{g}\cdot\text{mol}^{-1}$ after 48 hr exposure to hFOB cells.

Figure 10. A typical section of the implanted PCLF sample with M_n of 760 g.mol^{-1} (a) and the surrounding tissue (b).

Scheme 1.

Table 1

Number average (M_n) and polydispersity index (PI) of the synthesized PCLF polymers and their parent PCLs with FC to PCL molar ratio of 0.9.

Sample	FC/PCL ratio	PCL M_n (g.mol ⁻¹)	PI	PCLF M_n (g.mol ⁻¹)	PI
1	0.9	340	1.7	3150	2.9
2	0.9	760	1.8	3680	2.3
3	0.9	1200	1.8	3870	2.6