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Synthesis of poly(ethylene glycol)-tethered poly(propylene fumarate) and its modification with GRGD peptide

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Abstract

Poly(ethylene glycol) (PEG), a highly biocompatible hydrophilic polyether, was tethered to poly(propylene fumarate) (PPF), a biodegradable polyester. To avoid change in molecular weight distribution of PPF, end hydroxyl groups of PPF were reacted with bis-carboxymethyl PEG after being treated with thionyl chloride. New end carboxyl groups of the PEG-tethered PPF were further reacted with *N*-hydroxysuccinimide (NHS) in the presence of dicyclohexylcarbodiimide (DCC) to couple bioactive molecules. Glutamine and glycine–arginine–glycine–aspartic acid (GRGD) were attached to the PEG-tethered PPF in 50 mM phosphate buffer of pH of 7.4.

Tethering PEG to PPF, activation of the PEG-tethered PPF with NHS, and coupling of peptide and amino acid were characterized by proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy, Fourier transform infrared (FT-IR) spectroscopy and gel permeation chromatography (GPC). The ^{1}H-NMR spectrum of PEG-tethered PPF showed significant PEG proton peaks at 3.5 ppm. The GPC chromatogram of PEG-tethered PPF showed significant increase in molecular weight of the polymer without noticeable unreacted PEG. Characteristic proton peaks of glutamine and GRGD also were observed in proton NMR spectra after the modification of PEG-tethered PPF. The carbonyl stretching bands of amide bonds (amide I bands) were observed in the IR spectra of modified PEG-tethered PPF with glutamine and GRGD. Glutamine and GRGD were coupled to the PEG-tethered PPF with yields of 90 and 99%, respectively, as determined by trinitrobenzene sulfonic acid (TNBS) analysis. The proposed method also will be valuable for the preparation of a triblock copolymer with PEG end blocks and the coupling of biologically active molecules.

Keywords: Poly(ethylene glycol); Poly(propylene fumarate); GRGD peptide

1. Introduction

Poly(propylene fumarate) (PPF), a biodegradable polyester, contains unsaturated fumarate sites which can be crosslinked by monomers such as vinylpyrrolidone (VP) [1–3], methyl methacrylate (MMA) [4] or their mixture [5]. Such in situ crosslinking of PPF was valuable for developing injectable materials that could fill skeletal defects of varying size and shape. Since cured PPF matrices have high mechanical strength, the polymer has been especially promising for orthopedic application [2]. In addition to high mechanical strength of the cured PPF matrices, the possible biodegradation of PPF into non-toxic propylene glycol and fumaric acid is advantageous for designing biomaterials. On the basis of these unique properties, PPF has been formulated as bone cement [1,3–6], an orthopaedic scaffold for bone tissue regeneration [7], and a drug delivery system [8].

To extend its application in biomedical science, PPF has been modified with PEG, a highly flexible hydrophilic polyether, by transesterification [9]. Incorporation of PEG into PPF decreased platelet adhesion on the material for cardiovascular application [10,11]. Mechanical properties of the hydrogel made of poly(propylene fumarate-*co*-ethylene glycol) (P(PF-*co*-EG)) could be controlled by varying the ratio between hydrophilic PEG and hydrophobic PPF [12]. However, the direct transesterification of PEG and PPF in one-pot has a critical disadvantage of the difficulty in controlling the molecular weight distribution of the unsaturated PPF blocks. The prediction of molecular weight change and molecular weight distribution in transesterification requires a complicated mathematical model [13]. Insertion of PEG chains into hydrophobic PPF also can restrict the mobility of the PEG chains, which might result in ineffective prevention of protein adsorption on the basis of steric repulsion [14,15]. Flexible PEG chain is especially

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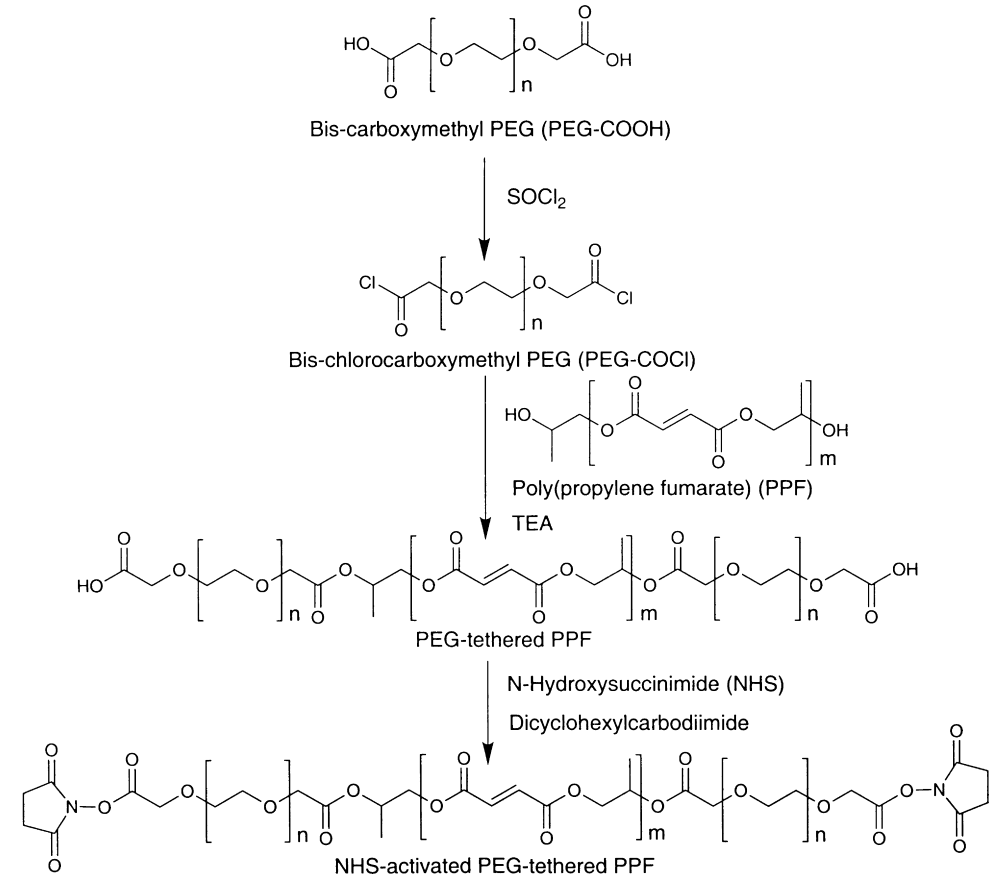

Fig. 1. Synthetic reactions for tethering PEG to PPF and preparing the succinimidyl ester of PEG-tethered PPF for peptide attachment.

valuable when the PEG chain is employed as spacer to attach bioactive molecules such as peptides and proteins. For effective coupling of those molecules into polymeric substrates, hydrophilic PEG chain should be tethered as end blocks. In this system, tethered PEG chains may facilitate the specific interaction between ligand and ligate.

The particular peptide sequence plays a ubiquitous role on cell adhesion process. The peptide sequence arginine–glycine–aspartic acid (RGD) has been established as a minimal peptide sequence responsible for integrin/ligand interaction on many adhesive proteins such as fibronectin, vitronectin, collagen, and laminin [16]. Therefore, synthetic RGD peptides have been immobilized into polymeric materials to improve specific cell attachment [17–19]. Cook et al. modified poly(lactic acid-*co*-lysine) with RGD peptide for possible application as a temporary scaffold for cell transplantation [20]. Hern and Hubbell incorporated RGD-peptide sequences into PEG diacrylate networks by photopolymerization and reported that RGD peptide required a certain hydrophilic spacer for specific cell spreading into nonadhesive PEG diacrylate matrices [21].

According to previous studies, the coupling of a cell adhesion peptide into PPF matrices for use as scaffold for tissue regeneration requires hydrophilic spacers. Here we report a novel method to tether PEG to PPF ends for peptide coupling without changing the molecular weight distribution of the PPF block. This method utilizes bis-carboxymethyl PEG (PEG-COOH) since the carboxyl group can be easily transformed to a highly reactive acid chloride. PEG-carbonyl chloride (PEG-COCl) reacts readily with PPF and forms PPF copolymer with PEG end blocks. The remaining end carboxyl groups of PEG-tethered PPF can be used for further modification with cell adhesion peptides. This novel method will be valuable for the preparation of PPF copolymer with PEG end groups and its modification for biological purposes.

2. Experimental

2.1. Materials

Fumaryl chloride, propylene glycol, and calcium hydride were purchased from Acros (Pittsburgh, PA) and used after distillation. Thionyl chloride (2 M in methylene chloride), bis-carboxymethyl PEG ($M_n = 600$), triethylamine, *N*-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), anhydrous benzene and anhydrous dimethyl formamide were obtained from Aldrich (Milwaukee, WI). Glutamine and trinitrobenzene sulfonic acid (TNBS) were from

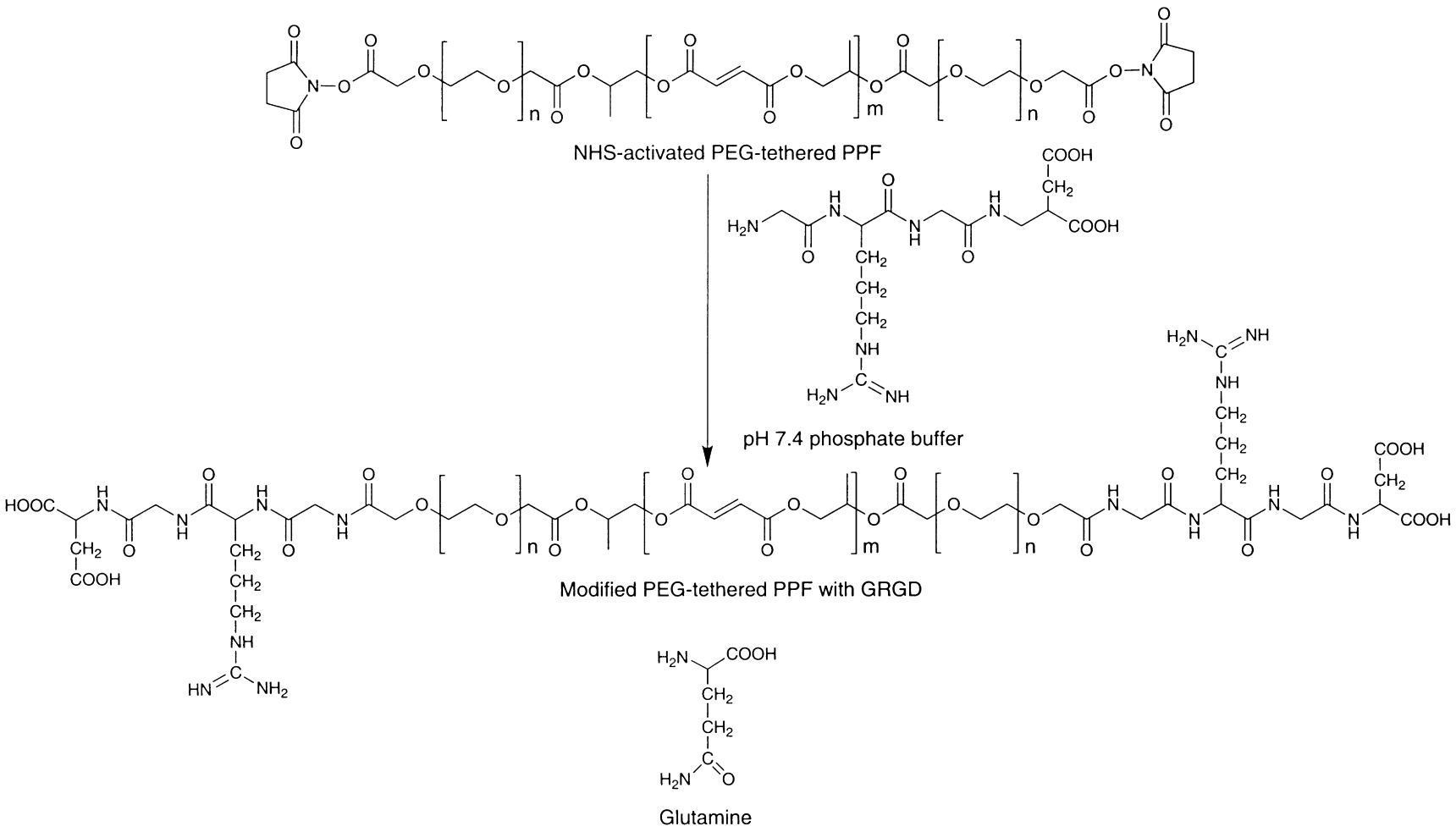

Fig. 2. Reaction scheme to modify PEG-tethered PPF with GRGD and the chemical structure of glutamine.

Sigma (St. Louis, MO). Glycine–arginine–glycine–aspartic acid (GRGD) was purchased from Bachem California Inc. (Torrance, CA). Methylene chloride for organic reactions was purified by distillation after refluxing 4 h over calcium hydride. The other solvents were purchased from Aldrich as reagent grade and used as received.

2.2. Methods

2.2.1. Preparation of PPF

PPF was prepared by the method described by Peter et al. [22] with minor modification. Briefly, oligo(propylene fumarate) was obtained by dropwise addition of fumaryl chloride into a three-neck round bottomed flask charged with propylene glycol, potassium carbonate, and methylene chloride. The molar ratio of fumaryl chloride to propylene glycol and potassium carbonate was 1:3:2.5. Fumaryl chloride was added to propylene glycol solution in an ice bath while agitating with a mechanical stirrer. After the reaction, the mixture was added to water to remove the remaining propylene glycol and potassium carbonate. The organic phase was separated and was dried with anhydrous sodium sulfate. After solvent removal by rotovaporation, the molecular weight of the oligomer was increased by transesterification at 160°C at 0.25 torr. Hydroquinone was added to prevent thermal crosslinking. Transesterification was performed for 12 h and the molecular weight change was monitored by gel permeation chromatography using polystyrene standards for a relative calibration curve. The obtained PPF was purified by precipitation from methylene chloride with ether.

2.2.2. Tethering PPF with PEG

Purified PPF ($M_n = 2900$) was tethered to PEG as described in Fig. 1. Thirty grams of PEG-COOH of molecular weight 600 was dried by azeotropic distillation of 100 ml out of 150 ml of anhydrous benzene. After adding 0.1 mol of thionyl chloride to the residual PEG-COOH, the solution was stirred overnight at 70°C to form PEG-COCl. The remaining thionyl chloride was removed by distillation under reduced pressure and further removed by distilling after adding another 60 ml of anhydrous benzene. Conversion of COOH to COCl was about 89% by NMR spectroscopy. Twenty grams of previously dried PPF and 16 ml of triethylamine in 100 ml of anhydrous methylene chloride were added dropwise to 20% PEG-COCl solution in anhydrous methylene chloride in an ice bath. After stirring 6 h at room temperature, the solvent was removed by rotovaporation and the residue was dissolved into 200 ml of deionized distilled water (DDW). After adjusting the pH of the solution to 2.0 with 1 N HCl, the tethered PPF with PEG was extracted three 200 ml portions of methylene chloride. The extract was neutralized with triethylamine, dried with 20 g of anhydrous sodium sulfate, and concentrated by rotovaporation. Free PEG-COOH was removed by washing several times with isopropanol. Finally 16.18 g of PEG-tethered PPF were obtained.

2.2.3. Activation of PEG-tethered PPF with N-hydroxysuccinimide

Sixteen grams of PEG-tethered PPF were dried by azeotropic distillation with 100 ml of anhydrous benzene. After dissolving PEG-tethered PPF and 3.0 g of NHS (0.026 mol) in 150 ml of anhydrous methylene chloride, 7.0 g of DCC (0.034 mol) were added. After reacting overnight at room temperature, precipitated dicyclohexyl urea was filtered out and the solution including product was concentrated by rotovaporation. NHS-activated PEG-tethered PPF was purified further by precipitation with anhydrous ether after dissolving in 100 ml of anhydrous ethyl acetate. Activated polymer was dried under vacuum and kept in a refrigerator.

2.2.4. Attachment of glutamine and GRGD into PEG-tethered PPF

Glutamine and GRGD were coupled to PEG-tethered PPF as outlined in Fig. 2. Five milligrams of glutamine or GRGD were dissolved in 3.5 ml of 50 mM pH 7.4 phosphate buffer. After dissolving 270 or 55 mg of NHS-activated PEG-tethered PPF in 1.5 ml of anhydrous dimethylformamide for glutamine and GRGD, respectively, the polymer solution was added dropwise to the amino acid or peptide solution in an ice bath and stirred for another 2 h. The solution was transferred into a regenerated cellulose ester dialysis membrane (MWCO = 2000, Spectrum) and dialyzed by using deionized distilled water for 2 days with periodic medium changes. After dialysis, the solution of modified PPF with GRGD was immediately frozen with liquid nitrogen and lyophilized overnight. GRGD coupling to PPF was characterized by ^{1}H-NMR (Bruker AC 250 NMR spectrometer) after dissolving in D_2O–CD_3OD (1:2) solution. Coupling of glutamine and GRGD was analyzed by using the TNBS method established by Snyder and Sobocinsky [23].

2.2.5. Characterization of polymer and modified polymer

FT-IR spectra were obtained on a Nicolet 500 spectrometer (Madison, WI). Samples were dissolved in $CDCl_3$ and placed on a calcium fluoride window (Aldrich, Milwaukee, WI). After forming a thin film by evaporating solvent with nitrogen gas, sixteen scans were collected at a 4 cm^{-1} resolution by using the calcium fluoride window as a reference.

NMR spectra were obtained on Bruker AC 250 using $CDCl_3$, D_2O, CD_3OD, and pyridine-d_5 as solvents. Proton NMR spectra of GRGD and glutamine were recorded in D_2O while those of modified PEG-tethered PPF with GRGD and glutamine were in D_2O–pyridine-d_5 (8:2) and D_2O–CD_3OD (1:2) solutions respectively since the solubility in a single solvent was apparently low.

Gel permeation chromatography was used to characterize PPF and PEG-tethered PPF. A Phenogel guard column (50 × 7.8 mm, 5 μm, mixed bed, Phenomenex, Torrance,

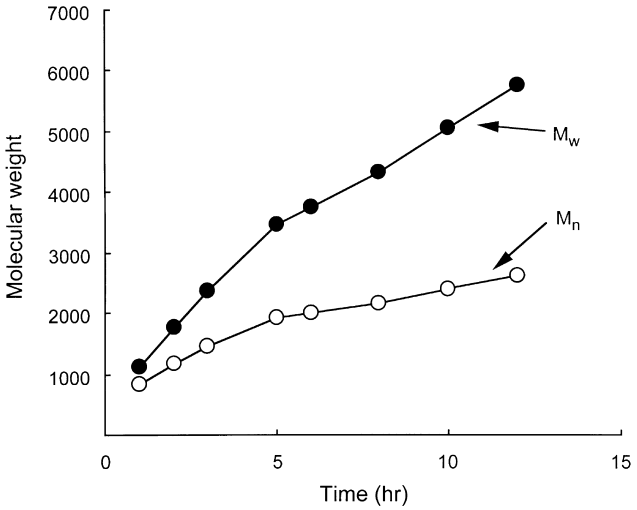

Fig. 3. Representative plots of temporal increase of number-average molecular weight (M_n) and weight-average molecular weight (M_w) during a transesterification.

CA) and a Phenogel column (50 × 7.8 mm, 5 μm, mixed bed, Phenomenex) were used to elute the samples at 1 ml/min chloroform flow rate. Polystyrene standards were used to obtain a calibration curve for calculating the polymer molecular weight.

3. Results and discussion

3.1. Preparation of PPF and PEG tethering

For tethering PEG, PPF was prepared by the transesterification of oligo(propylene fumarate). The number-average molecular weight (M_n) of PPF increased up to about 2900 while the weight-average molecular weight (M_w) went up to 5300 (Fig. 3). The M_n of the PPF was also determined by end-group analysis with NMR spectroscopy after reacting end hydroxyl groups with trifluroacetic anhydride as 2700. (The value determined by GPC was used in the molar ratio calculation.) During the transesterification, a main variable determining molecular weight was time. The temperature was kept below 200°C to prevent side reactions such as spontaneous crosslinking and addition to fumarate double bonds. By proton NMR spectroscopy, methyl, methylene, and methine proton peaks from propylene glycol appeared at 1.3, 4.2, and 5.3 ppm, respectively (data not shown but available from the NMR spectrum of PEG-tethered PPF in Fig. 4C). The NMR spectrum of prepared PPF was consistent with those reported previously [1,22].

As the first step to tether PEG to PPF, PEG-COOH was transformed into reactive PEG-COCl as shown in Fig. 1. The carboxyl groups of PEG-COOH were easily converted to the highly reactive acid chloride (–COCl) by reaction with thionyl chloride. Fig. 4B shows the change in the NMR spectrum of PEG-COOH upon reaction with thionyl chloride. As Fig. 4A and B shows, a methylene (–**CH$_2$**–COOH) proton peak shifted from 4.1 to 4.5 ppm upon replacement of OH by the electron-withdrawing chloride. The peak areas showed that the conversion was 89%. PEG-COOH of molecular weight 600 and 250 are commercially available. PEG of other molecular weights also can be tethered to PPF after converting PEG to PEG-COOH by the method previously reported by Royer and Anatharmaiah [24]. Instead of modifying the functionality of PEG, the functionality of PPF can be changed to an acid chloride by first carboxylating PPF with succinic anhydride. However, this alternative has a major problem that the ester bonds of PPF are labile to thionyl chloride. Prepared PEG-COCl of molecular weight 600 was reacted with PPF in the presence of triethylamine. As shown in Fig. 4C, significant proton peaks from PEG appeared at 3.6 ppm in addition to proton peaks from PPF as a result of PEG tethering. According to integration of methyl (–**CH$_3$**) peaks of PPF and methylene (–**CH$_2$CH$_2$**–) proton peaks from PEG, more than two PEG blocks were tethered to PPF. On the basis of the molecular weight of PPF (M_n = 2900), molecular weight of PPF repeating unit (M_r = 155) and PEG-COOH (M_n = 600), ratios of PEG methylene proton to PPF methyl protons of PEG–PPF–PEG triblock copolymer and PEG–PPF diblock copolymer should be about 2:1 and 1:1, respectively. From the NMR spectrum of PEG-tethered PPF, the proton ratio was 2.6:1. The main reason for the difference in proton ratios between an ideal triblock copolymer and the obtained copolymer might be the molecular weight distribution of PEG-COOH and possible overestimation of molecular weight of PPF. As seen in Fig. 5, the molecular weight distribution of commercially available PEG-COOH of molecular weight 600 was not normal. However, relative molecular weights from Table 1 strongly supported the formation of PEG–PPF–PEG triblock copolymer. By GPC analysis using polystyrene standards, relative molecular weight (number-average molecular weight, M_n) of PEG-COOH and PEG-tethered PPF were 1300 and 5900, respectively. Considering the molecular weight of PPF as 2900, molecular weights of PEG–PPF and PEG–PPF–PEG would be 4200 and 5500. In comparison with calculated molecular weights of di- or tri-block copolymers, the obtained molecular weight of copolymer, 5900, was very close to that of triblock copolymer, 5500. In the chromatogram, unreacted PEG was not observed. The molecular weight of PEG-COOH was overestimated by using polystyrene standards, which might be due to different hydrodynamic behavior of PEG from polystyrene because of conformational differences.

Infrared (IR) spectra were especially valuable for the characterization of PEG-tethered PPF. The IR spectrum of PPF showed the following characteristics bands in Fig. 6A: –OH stretch at 3400 cm^{-1}, C–H stretch of –CH=CH– at 3080 cm^{-1}, ester carbonyl at 1720 cm^{-1}, and –C=C stretch at 1646 cm^{-1}. After tethering PEG to PPF, spectral changes were observed as seen in Fig. 6B: disappearing –OH band at

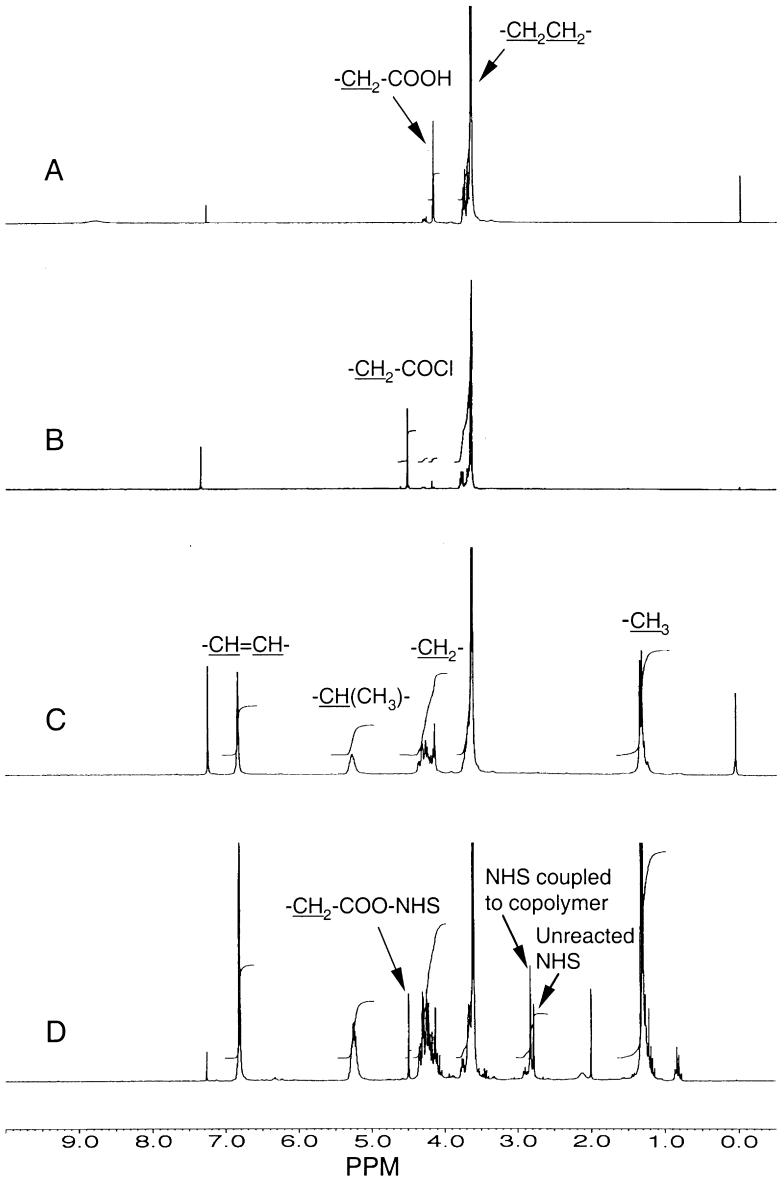

Fig. 4. Proton NMR spectra of: (A) bis-carboxymethyl PEG (PEG-COOH); (B) PEG-COCl; (C) PEG-tethered-PPF; and (D) succinimidyl ester of PEG-tethered PPF in $CDCl_3$.

3400 cm^{-1}, building up more C–H stretching band at 2870 cm^{-1}, and C–O–C stretching at 1120 cm^{-1}. It was apparent from GPC and IR data that PEG was successfully tethered to PPF.

In the reaction to prepare PEG-tethered PPF, the molar ratio of PEG-COCl to PPF was 7.2:1. Taking into consideration the conversion of PEG-COOH into PEG-COCl, the ratio of reactive functional groups between –COCl and –OH was 6.4:1. An excess amount of PEG-COCl was employed to prevent the formation of copolymer with more than two PPF blocks. To prepare PPF copolymer with PEG end blocks, PPF should be added into PEG-COCl solution since the opposite order of addition is more likely form copolymer of more than two PPF blocks. One important advantage for this method on the preparation of poly(propylene fumarate-*co*-ethylene glycol) (P(PF-*co*-EG)) is that reaction occurs only at the end hydroxyl groups of PPF. Therefore, this method does not alter the molecular weight distribution of the PPF block as transesterification does. Using the reaction scheme presented in Fig. 1, PEG–PPF–PEG triblock copolymer also can be prepared by using monomethoxy-PEG (m-PEG). m-PEG can be converted into m-PEG-COOG by the same method for the preparation of PEG-COOH. The method employed here is versatile enough to modify other polymers with hydroxyl groups such as poly(hydroxyethyl methacrylate), poly(vinyl alcohol), poly caprolactone diol, polybutadiene diol, and even cellulose derivatives.

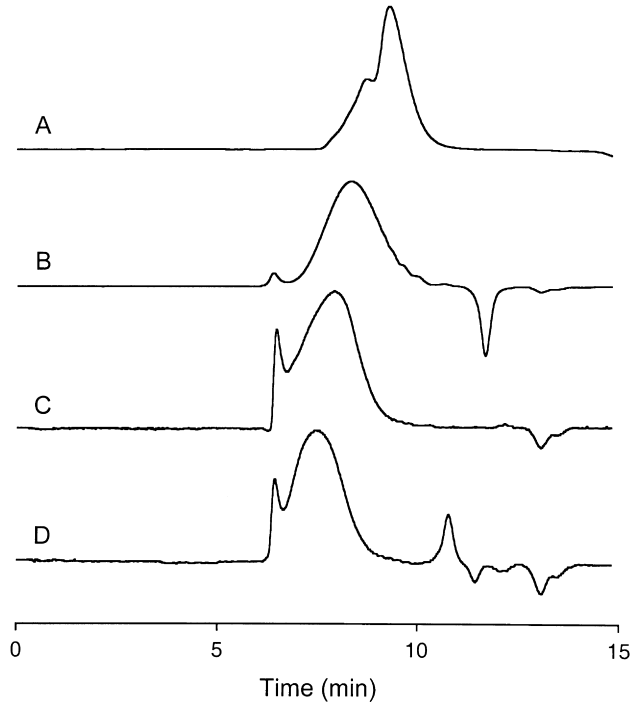

Fig. 5. GPC chromatograms of: (A) PEG-COOH; (B) PPF; (C) PEG-tethered PPF; and (D) succinimidyl ester of PEG-tethered PPF.

The type of copolymers prepared will be valuable for biological applications since tethered PEG can improve the solubility and biocompatibility of PPF. Moreover, end-tethered PEG blocks of a copolymer retain high flexibility that helps to prevent protein adsorption on the basis of steric repulsion [14,15]. PEG end blocks are especially valuable as flexible spacers for immobilization of biological molecules such as peptides and proteins. For the coupling of such molecules, certain functional groups are necessary. In the case of the copolymer obtained from PEG-COOH, the remaining end carboxyl groups can be converted directly to the necessary functional groups.

3.2. Activation of PEG-tethered PPF with N-hydroxysuccinimide and coupling of glutamine and GRGD

The PEG-tethered PPF was activated with NHS in the presence of DCC at room temperature. In Fig. 4D, the

Table 1
Molecular weights of PPF, bis-carboxymethyl PEG, PEG-tethered PPF, and the succinimidyl ester of PEG-tethered PPF obtained by GPC. Number-average molecular weight (M_n) and weight-average molecular weight (M_w) were based on polystyrene standards in chloroform

	M_n	M_w
Bis-carboxymethyl, PEG 600	1300	2000
PPF	2900	5300
PEG-tethered PPF	2900	9900
Succinimidyl ester of PEG tethered PPF	7600	11 600

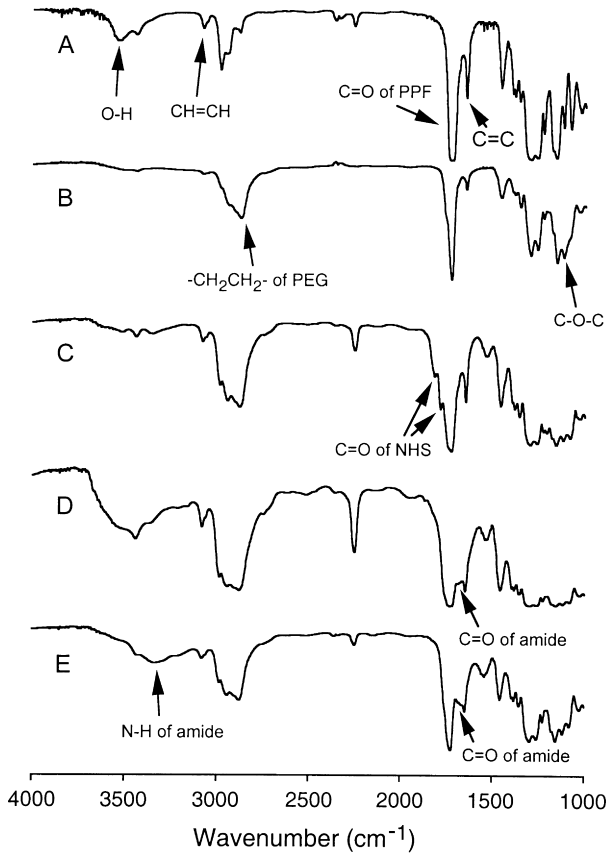

Fig. 6. FT-IR spectra of: (A) PPF; (B) PEG-tethered PPF; (C) succinimidyl ester of PEG-tethered PFF; (D) modified PEG-tethered PPF with glutamine; and (E) modified PEG-tethered PPF with GRGD.

NMR spectrum of the *N*-hydroxysuccinimidyl ester of PEG-tethered PPF showed a proton peak of succinimide at 2.83 ppm in addition to proton peaks from PPF and PEG. Another noticeable change in the NMR spectrum was the shift of the methylenecarboxy protons from 4.1 to 4.5 ppm because of the succinimidyl group. A singlet at 2.78 ppm represents the presence of unreacted *N*-hydroxysuccinimide, which cannot be completely removed even by precipitation from dry ethyl acetate by ethyl ether. Fig. 5C shows the GPC chromatogram of the succinimidyl ester of PEG-tethered PPF. Comparing with PEG-tethered PPF, the molecular weight distribution of the activated copolymer shifted toward higher values, possibly because NHS attached to both ends and the purification process removed low molecular weight copolymer. The obtained molecular weight of succinimidyl ester of PEG-tethered PPF by GPC was 7600, which is significantly higher than that of PEG-tethered PPF of 5900. However, the shape of the molecular weight distribution of the copolymer was close to that of PPF even after purification (Fig. 5C).

The *N*-hydroxysuccinimidyl ester of the copolymer was used for the coupling of glutamine and GRGD without further purification since the small amount of remaining NHS does not affect the coupling reaction in a buffered

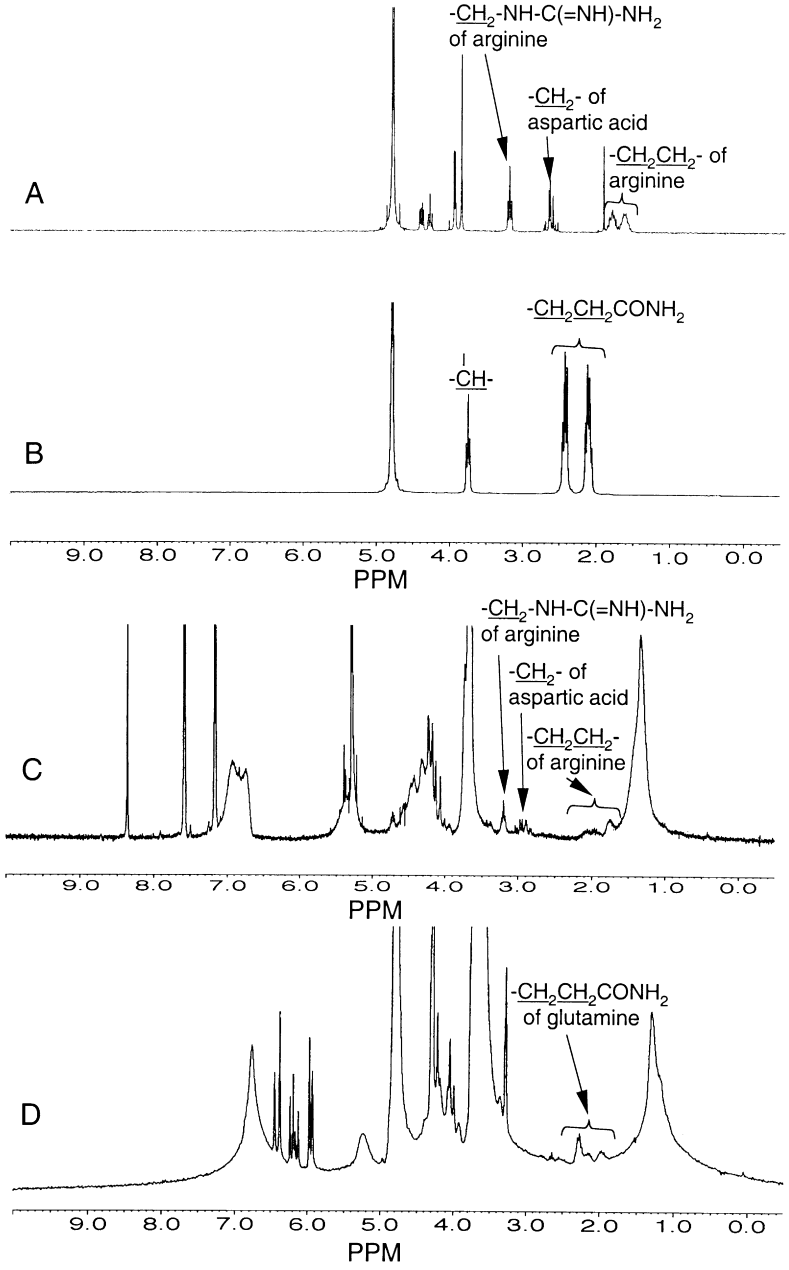

Fig. 7. Proton NMR spectra of: (A) GRGD; and (B) glutamine were recorded in D_2O while those of modified PEG-tethered PPF with (C) GRGD; and (D) glutamine were in D_2O–pyridine-d_5 (8:2) and D_2O–CD_3OD (1:2) solutions, respectively.

solution. Because PPF with carboxymethyl-NHS is highly reactive, glutamine and GRGD were attached to this copolymer at pH 7.4. Normally, the pH for aminolysis using *N*-hydroxysuccinimidyl ester of PEG succinate ranges from 8.0 to 9.0. The higher reactivity toward amines of carboxymethyl-NHS than succinyl-NHS to amines required lowering the pH of the reaction medium. After coupling glutamine and GRGD to PEG-tethered PPF, characteristic proton peaks from glutamine and GRGD were observed in the NMR spectra. Mixtures of D_2O and CD_3OD (1:2) and D_2O–pyridine-d_5 (4:1) were used as solvent for NMR spectroscopy because the modified copolymers were not readily soluble in a single solvent. As seen in Fig. 7D, the NMR spectrum of PEG-tethered PPF modified with glutamine showed broad multiples at glutamine (–$\mathbf{CH_2}$–$\mathbf{CH_2}$–C(=O)NH_2) ranging from 1.8 to 2.5 ppm. After coupling GRGD onto the polymer, three distinct proton peaks from the peptide were identified in NMR spectrum. These peaks were broad multiplets due to the –$\mathbf{CH_2}$–$\mathbf{CH_2}$–CH_2–NH–C(=NH)–NH_2 group of arginine ranging from 1.5 to 2.0 ppm, a multiplet of –CH_2–COOH of aspartic acid at 2.8 ppm, and a triplet of –CH_2–$\mathbf{CH_2}$–CH_2–NH–C(=NH)–NH_2 of arginine at 3.1 ppm (Fig. 7C). The proton peaks were assigned on the basis of the NMR spectra of

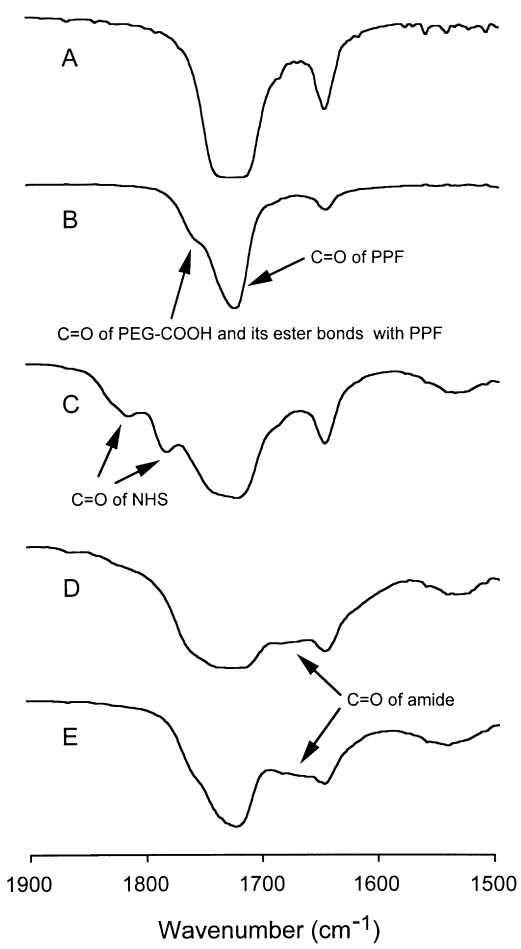

Fig. 8. The carbonyl stretching region in FT-IR spectra was expanded for identifying amide I band. (A)–(E) represents PPF, PEG-tethered PPF, succinimidyl ester of PEG-tethered PPF, modified PEG-tethered PPF with glutamine, and modified PEG-tethered PPF with GRGD, respectively.

GRGD and glutamine in D_2O that are presented in Fig. 7A and B. Glutamine was chosen as a model amino acid for the modification of PEG-tethered PPF because it has proton peaks from the side group that do not overlap with those of the PPF copolymer.

Modified PEG-tethered PPF was characterized by IR spectroscopy in addition to that by a NMR spectroscopy. In comparison with the IR spectrum of PEG-tethered PPF (Fig. 6B), that of copolymer modified with glutamine and GRGD showed differences in the N–H stretching region (from 3100 to 3450 cm^{-1}) and carbonyl stretching region (from 1600 to 1800 cm^{-1}) (Fig. 6D and E). Copolymers modified with glutamine and GRGD showed characteristic N–H stretching bands ranging from 3100 to 3450 cm^{-1} attributed to the amide bonds formed between end carboxylic acid groups and the amine group of glutamine or GRGD. The N–H stretching band of the copolymer modified with GRGD was broad but more readily identifiable than that of the copolymer modified with glutamine because the modification with the peptide resulted in four amide bonds. Fig. 8 shows expanded carbonyl stretching bands of copolymer with or without modification by glutamine and GRGD. The change in the carbonyl stretching region was more obvious in Fig. 8 than in Fig. 6. IR spectra of modified copolymers with glutamine and GRGD showed carbonyl stretching bands, amide I bands, that ranged from 1660 to 1720 cm^{-1}. The amide I bands partially overlapped with the carbonyl stretching (1720 cm^{-1}) and –C=C stretching band 1646 cm^{-1}) of PPF. IR spectral changes in both regions also indicated a successful coupling of glutamine and GRGD to PEG-tethered PPF.

However, NMR spectra presented in Fig. 7 were only qualitative since the polymeric proton peaks were broadened. For the quantitative analysis of the coupling, unreacted amino groups were analyzed by using trinitrobenzene sulfonic acid (TNBS). Calculated yields of coupling according to TNBS analyses were 90 and 99% for glutamine and GRGD, respectively. Based on the work by Hern and Hubbell [21], these results are reasonably acceptable. They reported that the succinimidyl ester of acryloyl PEG of molecular weight 3400 could couple tyrosine–arginine–aspartic acid–glycine–serine (YRDGS) with 85% yields according to a primary amine analysis with fluoroaldehyde. Higher yields with GRGD and glutamine than YRDGS can be explained by less steric hindrance for the amino groups of glutamine and glycine than tyrosine. The analysis with TNBS could lead a higher estimation on account of interference by coexisting substances. However, the control reactions with DMF and *N*-hydroxysuccinimide did not cause any apparent change in UV absorbance at the wavelength for quantification, 420 nm. PPF did not show any significant UV absorbance at 420 nm at the concentration of copolymer for quantification. Thus both spectroscopic characterization and quantitative analysis of unreacted amino groups with TNBS indicated an effective coupling of glutamine and GRGD. The presence of trace amount of free peptide or amino acid even after purification by dialysis is only a minor concern.

The succinimidyl ester of PEG-tethered PPF can be modified by other bioactive molecules including proteins. Modified PEG-tethered PPF with a cell adhesion peptide can be useful for the preparation of polymeric scaffolds for tissue regeneration. The peptides that remain incorporated after in situ polymerization of the fumarate double bonds will be effective for the specific cell attachment on the scaffold. The hydrophilic flexible PEG spacer will facilitate the interaction of cells with peptides. On the basis of high mechanical strength of PPF matrices, copolymer modified with peptides that are specific to bone cells will be valuable for bone and dental tissue engineering.

4. Conclusions

PEG, a highly biocompatible polyether, was tethered to PPF, a biodegradable polyester, by using bis-carboxymethyl

PEG. Tethered PPF was activated with *N*-hydroxysuccinimide to couple a cell adhesion peptide. GRGD, a model peptide, was successfully attached to PEG-tethered PPF. The peptide coupled to the end of PEG-tethered PPF can be incorporated into polymeric scaffolds by in situ polymerization since the PPF block has polymerizable fumarate double bonds.

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