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Synthesis and characterization of poly(L-lactic acid-co-ethylene oxide-co-aspartic acid) and its interaction with cells

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Abstract Multiblock terpolymer of poly(L-lactic acid)/poly(ethylene oxide)/poly(L-aspartic acid), (PLLA/PEO/PAsp) was synthesized by ring opening polymerization of β-benzyl L-aspartate N-carboxyanhydride, Asp(OBzl)-NCA with α-ω-hydroxy terminated triblock PLLA/PEO/PLLA copolymer.

The resulting multiblock terpolymer was characterized by several techniques including Fourier transform infrared spectroscopy and differential scanning calorimetry. ^{1}H nuclear magnetic resonance spectra indicated the molar ratio of PLLA/PEO/PAsp (OBzl) to be 86/10/4.

Thermal gravimetric analysis and environmental scanning electron microscopy data showed that PLLA/PEO/PAsp had crystalline and brittle structure. In order to improve its mechanical and physical properties, the terpolymer was blended with high molecular weight poly(L-lactic-co-glycolic acid) copolymer, PLGA(85/15) (M_w: 95000 gmol^{-1}) in 25/75 and 50/50 mole ratios.

The hydrolytical degradation properties of these polymers were studied. Degradation experiments were performed during a 48-day period in pH:7.4 phosphate-buffered saline (PBS) at 37°C. The observed molecular weight losses were 91% and 67% for the 25/75 and 50/50 mixtures, respectively. In vitro attachment and growth of L929 mouse fibroblasts on these biopolymers were also investigated. Cell growth experiments indicated that the copolymer blend allowed the attachment and growth of cells.

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Introduction

One of the challenges in the field of tissue engineering is the development of optimal materials for use as scaffolds to support cell growth and tissue regeneration. Poly(L-lactic acid), PLLA and Poly(L-lactic acid-co-glycolic acid), PLGA have been extensively used in clinical applications, such as wound closure, controlled release systems, orthopaedics and tissue reconstruction, as they undergo hydrolytic degradation to non-toxic substances[1–5].However, they lack functional groups which enable further modification of the polymers to improve cell adhesion properties. To overcome this limitation, potentially degradable polyesters that have side chains with functional groups are synthesized and investigated. Veld *et al.* have reported ring opening polymerization of morpholine-2, 5-dione derivatives that provide a method to prepare a wide range of biodegradable polyesteramides[6]. Langer and coworkers have synthesized a copolymer composed of L-Lactic acid and N_ε-carbobenzoxy-L-Lysine units which was further modified to create comb-like graft copolymers[7, 8]. Morita and coworkers reported the synthesis of poly(ε-caprolactone-co-glycolic acid-co-L-serine) copolymer[9] and Ouchi *et al.* reported the preparation of poly(lactic acid-glycolic acid-lysine) terpolymer[10]. Lavik *et al.* have synthesized PLGA-polylysine block copolymer by coupling PLGA to poly(ε-carbobenzoxy-L-lysine)[11]. Mikos and coworkers

reported the preparation of poly(ethylene glycol)-tethered poly(propylene fumarate) and its modification with cell adhesion peptides[12]. Recently, Shinoda and coworkers have synthesized a novel type of amphiphilic biodegradable copolymer from L-aspartic acid and L-lactide[13].

This paper describes the synthesis and characterization of poly(L-lactic acid)/poly(ethylene oxide)/Poly(L-aspartic acid) (PLLA/PEO/PAsp) multiblock terpolymer. We introduced L-aspartate groups as a bioactive moiety to modify PLLA/PEO/PLLA macromer in order to improve biocompatibility and thus to enhance cell affinity of multiblock terpolymer. In addition, the functional groups present in the aspartate blocks could be used to add other polypeptides or proteins into this terpolymer and open a new favorable way for cell seeding purposes. This terpolymer has been blended with high molecular weight poly(L-lactic acid-co-glycolic acid) (PLGA), to improve physical properties. The terpolymer and blends were characterized through FTIR spectroscopy, thermal analysis (DSC and TGA) and environmental scanning electron microscopy (ESEM). Hydrolysis of polymer mixture was performed up to 48 days and hydrolyzed films were studied by using size exclusion chromatography (SEC), and gravimetry. In vitro cell culture experiments were performed using L929 mouse fibroblasts to determine the effects of these polymeric mixtures on cell attachment and growth.

Experimental part

Materials

L-Lactide (LLA) and glycolide were obtained from Polysciences (Warrington, PA). LLA was recrystallized from dry benzene and dried under vacuum at room temperature before use. Stannous octoate, Sn(II)Oct. (Sigma) was used as received. Poly(ethylene oxide) (PEO) (M_n = 300 gmol^{-1}) was purchased from Fluka.

β-benzyl L-aspartate (Sigma) and triphosgene (Aldrich Chemical, Milwaukee,WI) were stored in a drybox in the freezer. HBr (Merck, 33 wt% in acetic acid) was used as received. Triethylamine (Merck, Darmstadt, Germany) was stored over molecular sieves.

THF,hexane and dioxane were purified and dried according to standard procedures. All other solvents were used as received.

Synthesis of N-carboxyanhydride of β-benzyl L-aspartate

The synthesis of β-benzyl L-aspartate N-carboxyanhydride (Asp(OBzl)-NCA) was carried out by using triphosgene[14 – 16]. A suspension of 9.57 g of β-benzyl L-aspartate (Asp(OBzl)) in 100 ml of THF was heated to 40°C in argon atmosphere. A solution of 4.25 g of triphosgene dissolved in 30 ml of THF was added dropwise to the stirred reaction mixture. When the reaction mixture started to become transparent, a stream of dry argon was bubbled through the solution to remove HCl. After the reaction was complete (approximately 2 hours), the mixture was precipitated in anhydrous hexane. Further purification of the obtained Asp(OBzl)-NCA was accomplished by its recrystallization from a mixture of anhydrous THF/hexane immediately followed by drying under vacuum at room temperature . The yield was 92%.

IR (KBr,cm^{-1}): 3312 (amide NH), 3000 (aromatic), 1870–1788 (cyclic anhydride, asym&sym. C = O str.mode), 1728 (ester C = O str.), 1640 (C = O amide I band), 1517 (amide II band) *^{1}H-NMR(DMF-d$_6$, ppm):* δ = 8.95 (CH-$\underline{NH}$-C = O), 7.36–7.37 (Ph), 5.13 ($\underline{CH_2}$-Ph), 4.69 (NH-$\underline{CH}$), 2.8–3.0 (O=C-$\underline{CH_2}$-CH)

Synthesis of PLLA/PEO/PLLA triblock macromer

α-ω-hydroxy terminated poly(L-lactic acid)-polyethylene oxide copolymer (PLLA/PEO) was prepared by a ring opening polymerization method. LLA (7g), and PEO-300 (0.3 g) were reacted at 110°C for 24 h in a vacuum sealed glass tube using Sn(II)Oct. as catalyst (monomer to catalyst, mole ratio, M/C = 1000). After cooling to room temperature, the tube was opened and purification was performed by dissolving the reaction mixture in a small amount of chloroform and dropping into an excess of methanol. The yield was 90%.

(M_w = 7300 gmol^{-1}, M_n = 4600 gmol^{-1}). The structure of the macromer which contains PEO blocks, was characterized by FTIR spectroscopy.

The ^{1}H-NMR measurement in $CDCl_3$ was carried out to determine the composition.

IR(KBr, cm^{-1}) : 3500 (OH), 1745 (C=O), 1454 (CH_3), 1100 (C-O)

^{1}H-NMR (CDCl$_3$, ppm): δ = 1.55 ($\underline{CH_3}$), 3.63 ($\underline{CH_2}$-O), 3.73 ($\underline{CH_2}$-CH$_2$-O-C=O), 4.30 ($\underline{CH_2}$-O-C=O), 5.15 (O=C-$\underline{CH}$-CH$_3$).

Synthesis of Poly(L-lactic acid)/ Poly(ethylene oxide)/ Poly(L-Aspartic acid)

PLLA/ PEO/PAsp Terpolymer.

A total of 1.36 g of PLLA/PEO macromer was dissolved in 15 ml anhydrous dioxane and stirred for 20 min. Then 0.45 g of Asp(OBzl)-NCA, dissolved in 7 ml anhydrous dioxane was added to the macromer solution. The reaction mixture was stirred for 24 h at 40°C under dry nitrogen atmosphere and subsequently precipitated in methanol. The yield was 72%. PLLA/PEO/PAsp(OBzl) was deprotected by stirring

a slurry of the polymer in HBr/HOAc under argon for 2 hours[14 − 16]. The product was then washed with ether and methanol and collected via vacuum filtration. It was then redissolved in $CHCl_3$, neutralized with excess triethylamine, precipitated from methanol, and dried to yield deprotected PLLA/PEO/PAsp. The yield was 70% (M_w=6950 gmol^{-1}, M_n=5200 gmol^{-1}).

IR(KBr, cm^{-1}) : 3343 (NH str.), 3015 (aromatic CH str.), 1740 (C=O ester str.), 1680 (C = O amide I band), 1580(amide II band).

^{1}*H-NMR (CDCl*$_3$*, ppm):* δ = 1.55 ($\underline{CH_3}$), 3.63 ($\underline{CH_2}$-O), 5.15 (O = C-$\underline{CH}$-CH_3), 5.02–5.06 and 5.27–5.31(O-$\underline{CH_2}$-Ph), 7.21 (Ph).

Synthesis of poly(L-lactic-co-glycolic acid) copolymer

Poly(L-lactic-co-glycolic acid) (PLGA) copolymer was prepared by the ring opening polymerization of L-lactide and glycolide in the presence of Sn(II)Oct. as the catalyst[17]. A solution of Sn(II)Oct. in dry chloroform and 10 g of (LA+GA) monomer mixture were added to a reaction tube (monomer/catalyst, mole ratio, M/C = 1000). The feed molar ratio of the L-lactide/glycolide was 85/15. The solvent was removed in vacuum, and the tube was sealed and immersed in a silicone oil bath at 115°C. The polymerization was allowed to proceed for 24 hours. The product was dissolved in a small amount of chloroform and precipitated in excess methanol. The conversion to copolymer was 75% (M_n = 45500 gmol^{-1}, M_w = 95000 gmol^{-1}). This copolymer was characterized by using ^{1}H-NMR and DSC techniques.

^{1}*H-NMR (CDCl*$_3$*, ppm):* δ = 5.17 (O = C-$\underline{CH}$-CH_3), 4.77($\underline{CH_2}$-C=O), 1.6($\underline{CH_3}$) DSC:T_g= 60°C, T_m = 155°C

Characterization

The molecular weights of polymers were determined with size exclusion chromatography (SEC) using a Waters styragel column HT6F and Waters 410 differential refractometer detector. THF was used as the eluting solvent at a flow rate of 1 ml / min and polystyrene standards were used to generate calibration curves and calculate the molecular weights.

^{1}H-NMR spectra of the N-carboxyanhydride of β-benzyl L-aspartate were obtained on a Bruker AC 200L spectrometer at 200 MHz. ^{1}H-NMR spectra of the triblock macromer and terpolymer were obtained on a Mercury-VX 400 BB model spectrometer at 400 MHz. All spectra were taken in deuterated chloroform at 20°C.

The infrared spectra were recorded on Perkin Elmer Spectrum One Model FTIR spectrometer.

Differential scanning calorimetry (DSC) analyses of the polymers were obtained using a Perkin Elmer DSC Pyris 1 Model device. Samples were run under a nitrogen atmosphere from 30°C to 250°C with a heating rate of 10°C/min. Transition temperature measurements were taken from the second heating run. Thermogravimetric analyses (TGA) of the polymers were performed using Perkin Elmer Thermogravimetric Analyzer Pyris 1 TGA Model. Samples were run from 30°C to 650°C with a heating rate of 10°C/min.

Porous film fabrication and characterization

Porous polymeric films were prepared by mixing PLGA(85/15) and PLA/PEO/PAsp terpolymers in two different compositions (75/25; 50/50) using a solvent casting particulate leaching technique[5]. The two polymers were dissolved in chloroform to yield a 10%(w/v) solution. The polymer/salt ratio was 0.18 for this study. 1 ml of polymer solution was placed into Teflon molds (diameter = 22 mm, height = 10 mm), packed with 0.567 g sodium chloride particles sieved to a size between 200 and 400 μm. The solvent was subsequently allowed to evaporate and the entrapped salt particles were removed by immersing the films in distilled water for 48 h. Porous polymeric films (1mm in thickness) were lyophilized to remove the residual water.

Degradation studies

The *in vitro* degradation studies of (PLLA/PEO/PAsp)/PLGA blends were performed at 37 °C in phosphate buffered saline (PBS, pH = 7.4). Dry porous films were prepared as described above and were placed in vials containing 15 ml PBS. The vials were incubated at 37 °C up to 50 days and films were removed at certain time intervals. The PBS solution was replaced every 60 hours. At each time interval, samples were removed, washed extensively with water and dried at 30 °C in vacuo until a constant weight was reached. The degree of degradation was estimated from the mass loss and molecular weight loss by SEC.

Cell culture

L929 mouse fibroblasts were grown in DMEM/F12 medium containing 10% fetal calf serum, 100 IU/ml penicillin and 0.1 mg/ml streptomycin (Culture medium, Biological Industries, Israel) at 37°C in a 5% CO_2 incubator. Cells were subcultured at a 1:6 ratio upon reaching confluence. Experiments were performed with cells from passages 14–19.

Cell growth experiments

These were performed in 6-well tissue culture polystyrene plates (Techno Plastic Products, Switzerland). The biopolymers that were moulded into 22-mm diameter, 1-mm thick porous films as described above, were placed into 100 %

ethanol for 1 hr for sterilisation prior to the experiments. The ethanol was subsequently removed from the films by rinsing in sterile PBS for 30 min at room temperature, this procedure was repeated 3 times. In parallel experiments, 22-mm diameter round glass coverslips (sterilised with a flame) were coated with sterile fibronectin (40 μg/ml) or gelatin (1% in H_2O) solutions. Immediately before cell seeding, the polymers and coated glass coverslips were incubated with 0.5 ml of culture medium described above for 45 min in the incubator. For cell seeding, the cells growing on cell culture plates were suspended using trypsin-EDTA solution (Biological Industries) and counted using a haemocytometer. 100 μL of cell suspension containing 150,000 cells was placed onto the biopolymers or glass coverslips prepared as described above. These were placed into 6 well plates and allowed to attach to the materials for one hour. The growth of cells on tissue culture polystyrene was observed by placing the 100 μL cell suspension directly into the wells. At the end of this incubation period, 2 ml of culture medium was added to each well and cells were grown in a CO_2 incubator for another 72 hours.

The amount of cells present on the surfaces of the materials was quantified using a neutral red uptake assay[18]. Cells grown on the above materials were washed once with sterile PBS and incubated in sterile PBS containing 2% FCS and 0.001% neutral red (Sigma, St.Louis, MO) for 2 hours at 37°C in the CO_2 incubator, during which the dye is internalized into viable cells by an active process.This was followed by a brief wash with 4 % formaldehyde in PBS, which enabled the cells to be fixed. The internalized dye was solubilized in 1 ml of 50% EtOH, 1% acetic acid in H_2O. The amount of entrapped dye was measured spectrophotometrically at 540 nm, the absorbance is proportional to the number of viable cells in each well.

ESEM experiments

The sample surface micro-characteristics and morphological topology of a set of images were taken on the Philips XL30 ESEM FEG environmental scanning electron microscope which makes it possible to examine materials in their natural, uncoated state at low vacuum around 0.6–0.8 Torr and at 10 kV. During in-situ testing of uncoated samples, the absence of charge formation in this system, provides an obvious advantage. The sample morphologies were investigated at room temperature, revealing their natural surface characteristics by using a special technique (gaseous low vacuum mode) that allows charge-free, gaseous secondary electrons.

In addition, certain routines of the ESEM fractograph analysis of chemically pre-fixed (with Butadiene-based emulsion) fractured sections, enabled the characterization of the fracture morphology. This was used to discriminate the surface details. Microstructural information was presented in the form of three-dimensional images (‘volumes’) of biopolymer sections.

To investigate cell attachment onto the biopolymers, cells were grown on these materials for 72 hours as described above and subsequently fixed in 2.5% glutaraldehyde in PBS for 2 hours and stored in sterile PBS until being used for ESEM experiments.

The different fractured sections were investigated for the presence of cells. To this aim, ESEM optimized for low voltage operation and fitted with a computerized image analysis system was used. The ESEM–digital images were utilized to determine the average pore size and to examine the differences in surface details in the polymers.

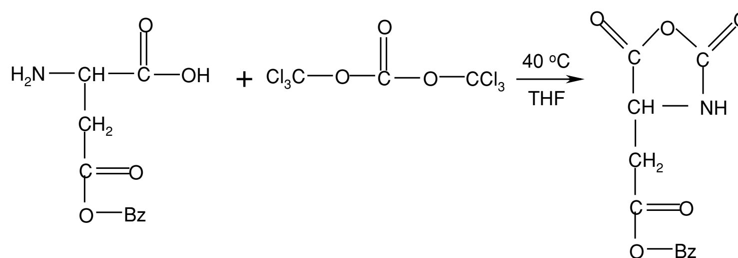
Results and discussion

Characterization of monomers and macromers

The synthesis of β-benzyl L-aspartate N-carboxyanhydride (Asp(OBzl)-NCA) was carried out by reacting the amino acid with triphosgene[14, 19] (Scheme 1). A 92% yield was observed following purification. The FTIR and ^{1}H-NMR spectra confirmed the expected structure.

The α-ω-hydroxy terminated triblock PLLA/PEO/PLLA macromer was synthesized by reacting excess amounts of LLA in bulk with PEO-300, and Sn(II)Oct as a catalyst. The yield of copolymer was 90% and M_n of 4600 g/mol. The structure of copolymer may be ideally represented by

Scheme 1 Synthesis of β-benzyl L-aspartate of N-carboxyanhydride (Asp(OBzl)-NCA).



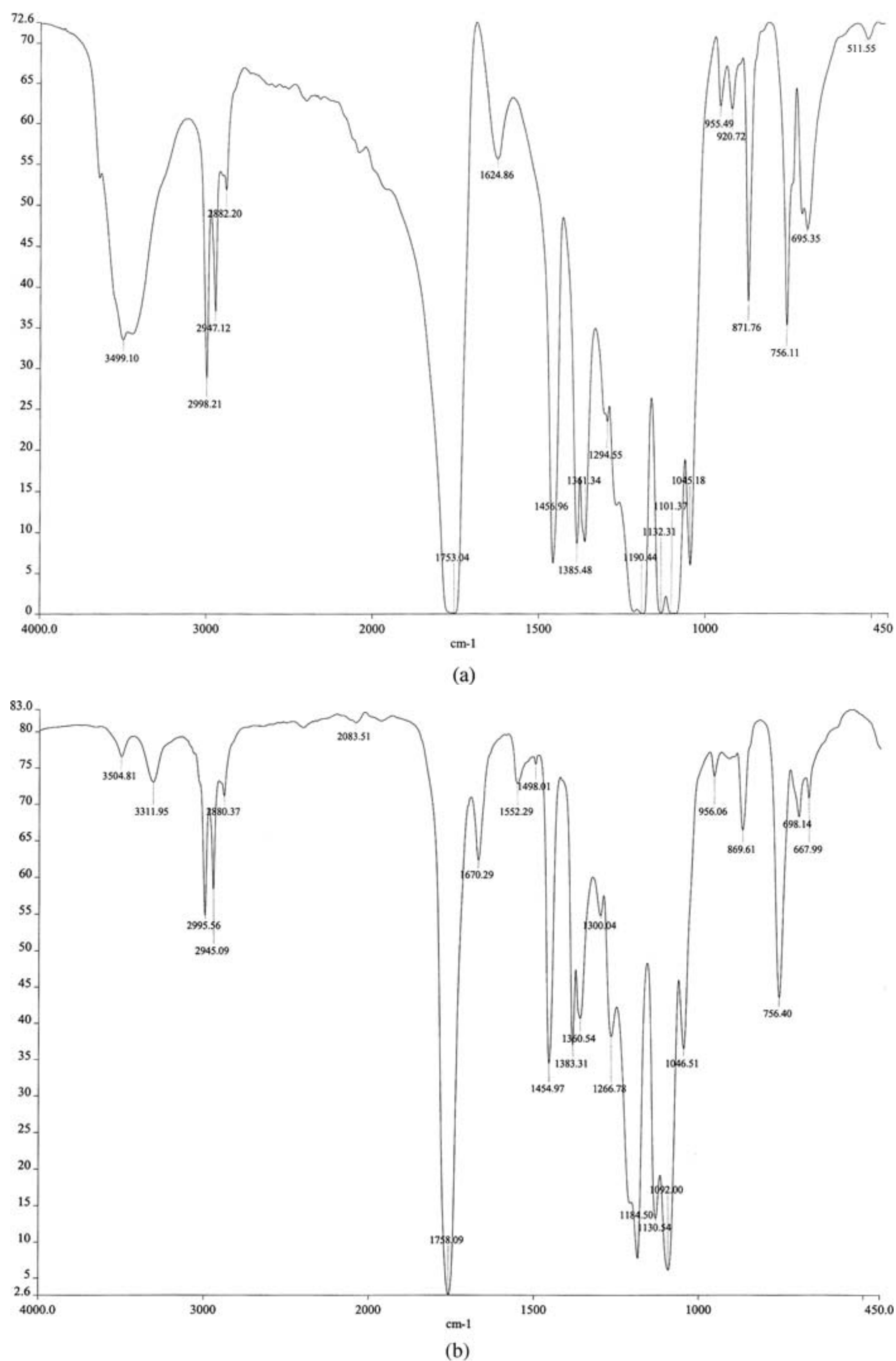


Fig. 1 FTIR spectra of the PLLA/PEO/PLLA triblock macromer (a) and PLLA/PEO/PAsp(OBzl) terpolymer (b).

a central PEO block ($M_n = 300$) having two PLLA arms ($M_n = 2150\ gmol^{-1}$). Fig. 1a shows the FTIR spectrum of PLLA/PEO/PLLA triblock macromer. All characteristic bands of this compound exist in the spectrum. Fig. 2a shows the NMR spectrum of the macromer.

The copolymer composition was calculated from the ratios of integral intensities of methyne protons of the lactic acid repeat units at 5.15 ppm to the methylene protons of polyethylene oxide (PEO) repeat units at 3.63 ppm in the ^{1}H-NMR spectra (Fig. 2a). ^{1}H-NMR spectra indicated a molar ratio of lactyl/oxy ethylene = 78/22.

Characterization of PLLA/PEO/PAsp terpolymers

Terpolymer of β-benzyl L-aspartate N-carboxyanhydride (Asp(OBzl)-NCA) with α-ω-hydroxy terminated PLLA/PEO/PLLA macromer was synthesized as described in Scheme 2. The stoichiometric molar ratio, macromer/Asp(OBzl)-NCA = 15/85 was used in this

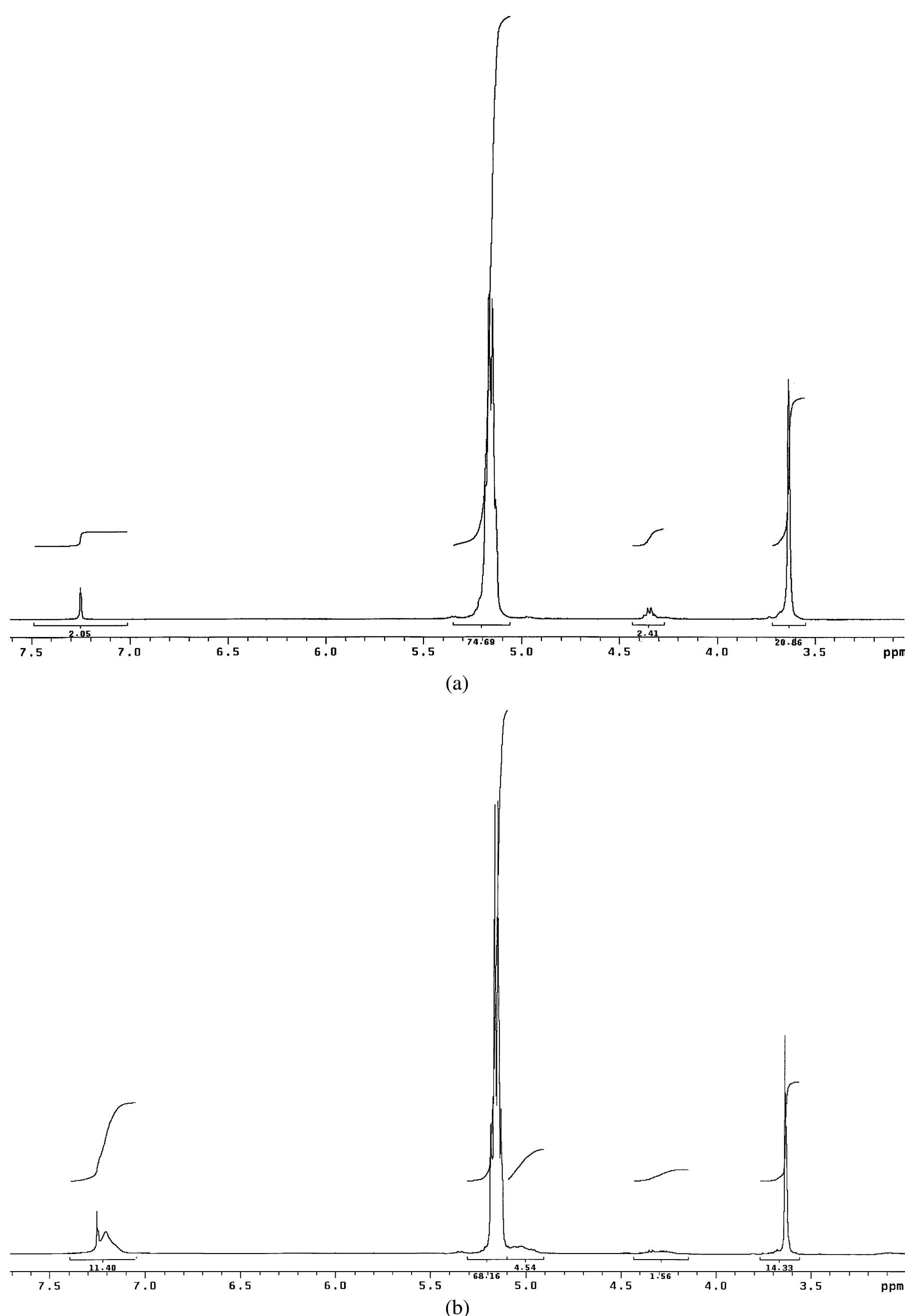
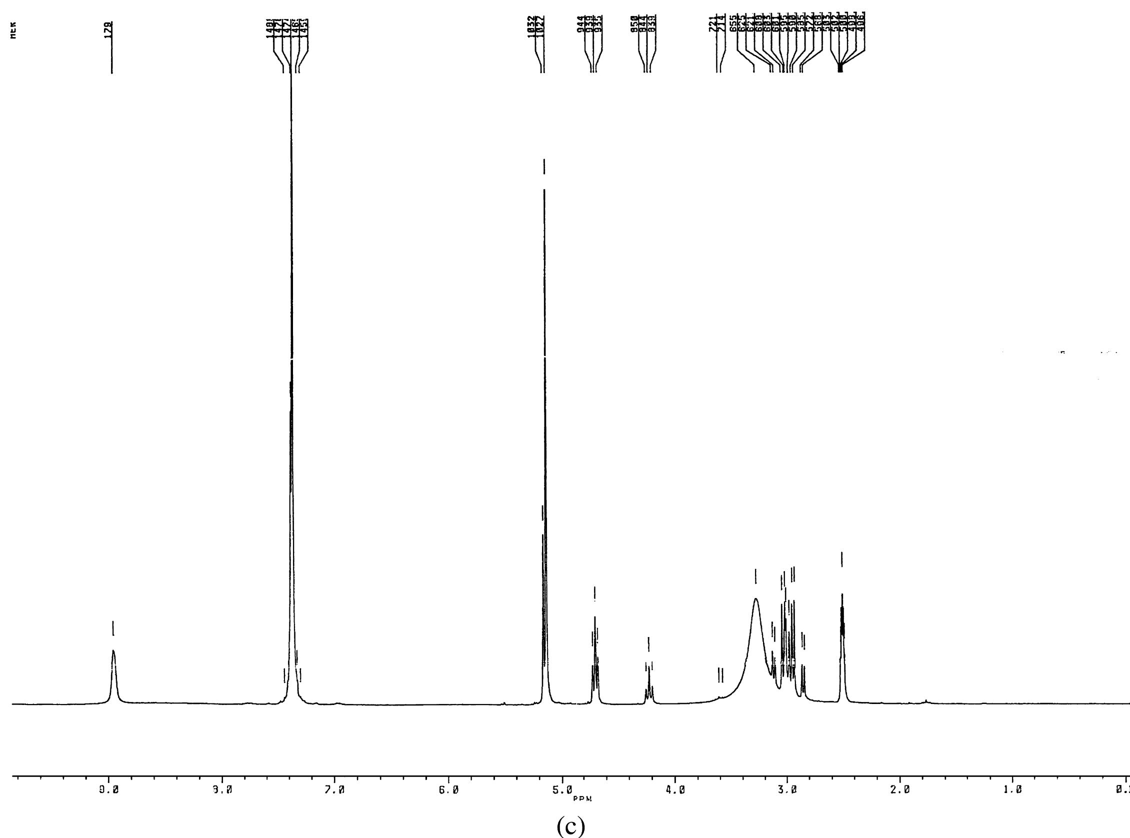


Fig. 2 ^{1}H-NMR spectra of the PLLA/PEO/PLLA triblock macromer (a); PLLA/PEO/PAsp(OBzl) terpolymer (b) and N-carboxyanhydride of β-benzyl L-aspartate (c) in $CDCl_3$.

procedure. The initiation mechanism of the ring opening polymerization of the protected NCA may occur via nucleophilic attack of the hydroxyl group of macromer on the carbonyl of the anhydride group in the ring. The terpolymer's architecture is of the multiblock type with well defined length of the ester-ether-aminoacid segments. The multiblock terpolymer was obtained in high yield, 70% as a white powder which was readily soluble in chloroform and THF. The M_n value of PLLA/PEO/PAsp(OBzl) terpolymer ($M_n = 6500$ gmol^{-1}) was obtained by SEC. The molecular weight distribution curve was rather narrow as indicated by the polydispersity index value of 1.35. As reported by Hrkach et al. [16] the primary amine initiated ring opening polymerization of NCA allows good control over the degree of polymerization when compared with alcohol and water based nucleophilic initiators. In our case, the low molecular weight of the polymer formed by the ring opening polymerization of the Asp(OBzl)-NCA is probably due to the lower initiation efficiency of hydroxyl terminated macromer. Moreover, the protected COOBzl side groups may also sterically affect the polymerization. The lower polymerizability and the slower kinetics may be the other reasons for the low molecular weight of the multiblock terpolymer. Due to the lower molecular weight of the terpolymer, films prepared



(c)

Fig. 2 *Cont.*

by solution casting from chloroform were observed to be brittle.

The molecular structure of the terpolymer was confirmed by FTIR and ^{1}H-NMR spectroscopy measurements. The FTIR spectra obtained on polymer films (Fig. 1b) show characteristic amide bands at 1670 cm^{-1} and 1550 cm^{-1}, aromatic C-H stretching band at 3000 cm^{-1}, ester bands at 1760 cm^{-1} and absorption of the C-O-C ether group at 1100 cm^{-1}. Comparison of FTIR spectra in Fig. 1a and Fig. 1b indicate that the hydroxy groups at 3500 cm^{-1}(Fig. 1a) from the PLLA/PEO/PLLA macromer have mostly disappeared and a characteristic N-H stretching band appeared at 3312 cm^{-1} (Fig. 1b).

^{1}H-NMR spectra of the PLLA/PEO macromer and PLLA/PEO/PAsp(OBzl) terpolymer are shown in Figures 2a and 2b respectively. In Fig. 2a, one can observe that the sharp peak at 3.63 ppm represents methylene groups adjacent to the oxygen atom in the polyoxyethylene block. The band at 4.35 ppm represents the resonance of the methylene group bonded to the ester oxygen. The resonance of methine groups in the lactic acid units are at 5.15 ppm. In this spectrum, due to the high molecular weight of polymeric material, the resonance of terminal hydroxy protons which are present at such a small concentration is not observed. In Figure 2b, the formation of a terpolymer structure was confirmed by the presence of phenyl protons at 7.21 ppm and methylene protons of the OBzl group at 5.02–5.06 and 5.27–5.31 ppm (Fig. 2b). The composition was determined by comparing the integral intensities of methyne hydrogens of PLLA blocks at 5.12–5.22 ppm and –CH_2-O- hydrogens of oxyethylene sequences at 3.63 ppm with those of methylene hydrogens of the OBzl group at 5.02–5.06 and 5.27–5.31 ppm. Molar ratio of PLLA/PEO/PAsp(OBzl) = 86/10/4 was obtained by using integral intensity values of ^{1}H-NMR spectrum. The calculated values of y, x and n from the stoichiometry and the molecular weight of the terpolymer (see scheme 2) were 15, 7 and 4 respectively. This discrepancy between the measured and calculated molar ratios of components results from overlapping peaks of CH_2 and CH groups in the NMR spectra of the macromer and terpolymer (see Fig. 2b and Fig. 2c).

Subsequently, the deprotection of the OBzl group was carried out with 25% hydrogen bromide/acetic acid treatment[15]. The yield of completely deprotected terpolymer was about 70%. ^{1}H-NMR analysis of the resulting terpolymers demonstrated complete removal of the

Scheme 2 Synthesis of PLLA/PEO/PAsp(OBzl) Terpolymer.

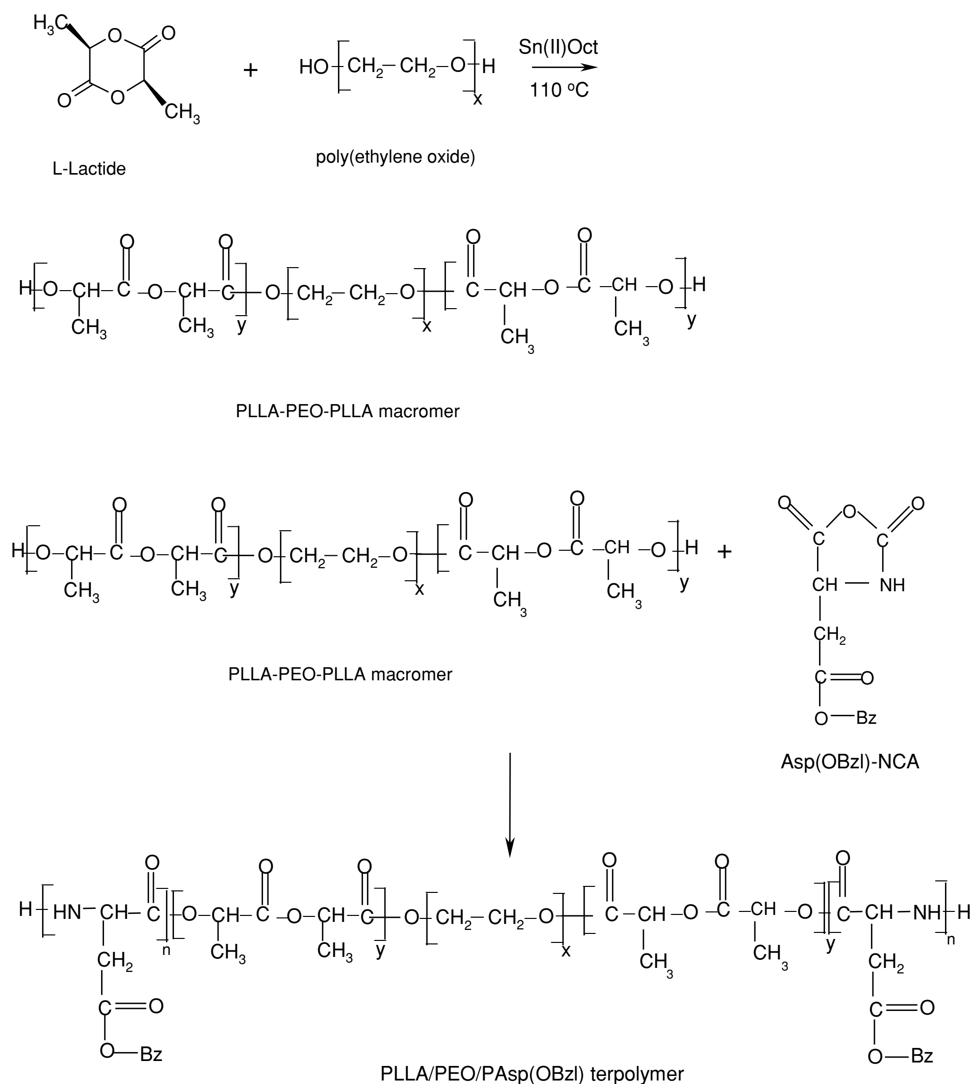


Table 1 SEC results for the polymers

Polymer	M_n(g/mol)	M_w(g/mol)	P.D
PLLA/PEO/PLLA	4600	7300	1.58
PLLA/PEO/PAsp(OBzl)	6500	8750	1.35
PLLA/PEO/PAsp	5200	6950	1.34

protecting groups by the absence of proton signals of the benzyl group at 5.02–5.06, 5.27–5.31 and 7.21 ppm.

Table 1 presents the number average and weight average molecular weights for PLLA/PEO/PLLA macromer, the protected PLLA/PEO/PAsp(OBzl) and deprotected PLLA/PEO/PAsp terpolymer. Each polymer exhibited a single peak in the SEC. The SEC analysis of the deprotected copolymer suggested that main chain cleavage did not occur under our deprotection reaction conditions.

Thermal behavior of the terpolymers were examined by DSC and TGA. The DSC thermograms of PLLA/PEO/PLLA macromer and PLLA/PEO/PAsp(OBzl) terpolymer are presented in Fig. 3. Both polymers are semicrystalline, as shown by the presence of melting endotherms in their DSC traces. For the PLLA/PEO/PLLA macromer, the second heating run showed (Fig. 3a) the T_m values at 145 °C that corresponds to the L-lactyl blocks. The second heating run also reveals a cold crystallization process at 80 °C. This peak is attributed to the crystallization of PLLA block. We could not observe the low T_m value of the PEO block in this DSC study. The thermal behavior of PLLA/PEO/PAsp(OBzl) is very similar to that of PLLA-PEO-PLLA triblock macromer. In the second heating run, the sample exhibits a broad crystallization peak at a lower temperature ($T_c = 67$°C) compared with the parent triblock macromer. However, the T_m values (140 °C and 146 °C) are higher than that of triblock macromer and this shift is probably due to

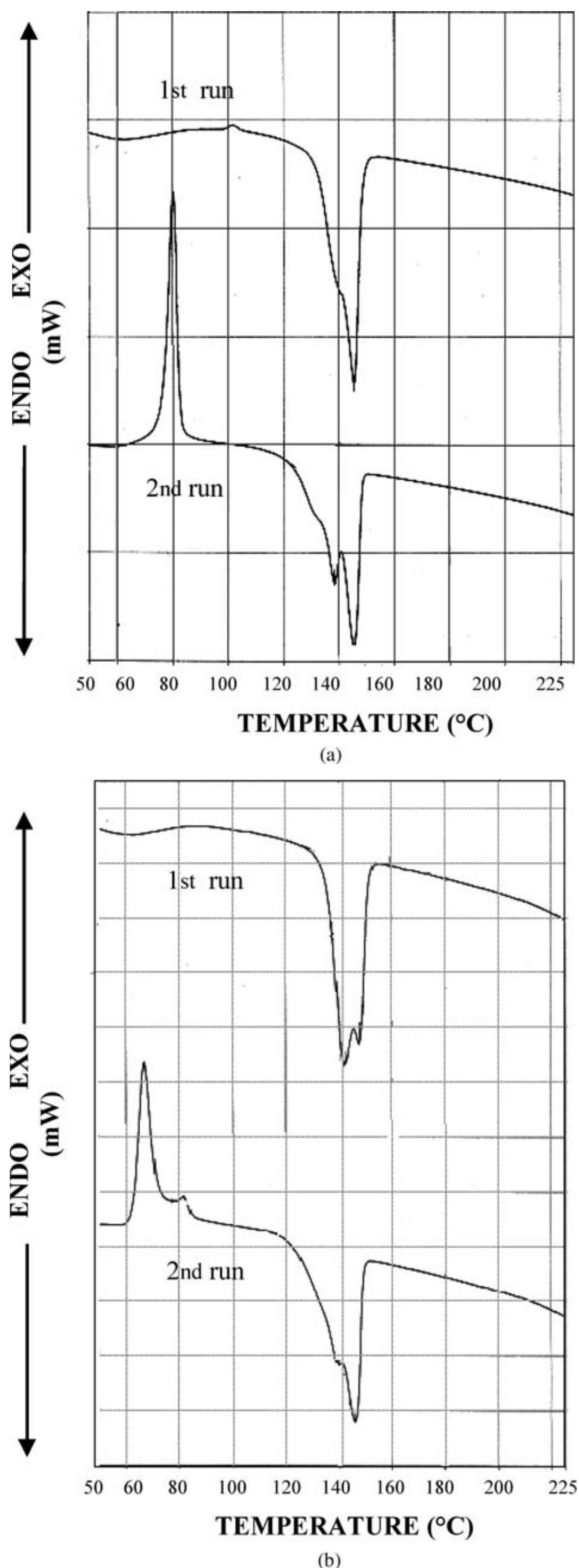


Fig. 3 DSC thermograms of the PLLA/PEO/PLLA triblock macromer (a) and PLLA/PEO/PAsp(OBzl) terpolymer (b).

Table 2 TGA data of the polymers.

Sample	T_p^a(ΓC)	Weight Loss (wt.-%)
PLLA/PEO/PLLA	329	95
	411	4
	520 (ash)	1
PLLA/PEO/PAsp(OBzl)	323	91
	411	6
	552 (ash)	3
PLLA/PEO/PAsp	120	5
	360	94
	650 (ash)	1

incorporation of aspartic acid units into the polymer backbone. The deprotected PLLA/PEO/PAsp triblock copolymer shows similar thermal behaviour compared with protected PLLA/PEO/PAsp(OBzl).

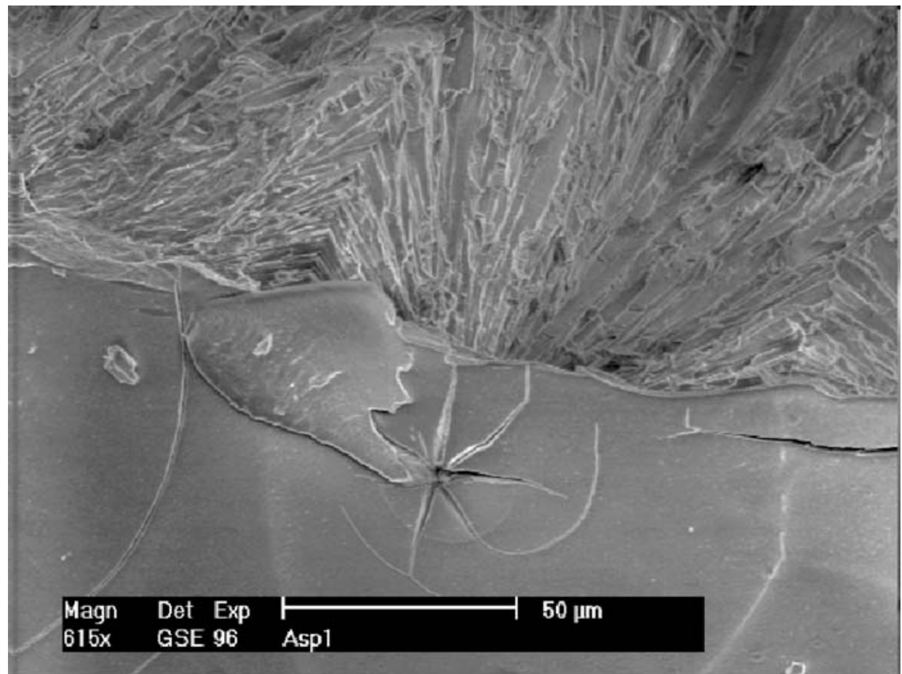
Thermal stability was evaluated by performing the thermogravimetric analysis, in the range of 30 °C to 650 °C and at a scanning rate of 10 °C/min under nitrogen atmosphere. In Table II, the temperature at each inflection point (T_p) and total weight loss are reported. Both PLLA/PEO/PLLA triblock macromer and PLLA/PEO/PAsp(OBzl) show similar behavior. It is clear that samples have a significant weight loss at around 325 °C (91–95%). However, the deprotected sample showed a small weight loss at 120 °C implying the loss of moisture (5%), and a significant weight loss at 360 °C (94%).

Figure 4 presents both the surface and fractured section views of PLLA/PEO/PAsp terpolymer. The terpolymer has a smooth surface texture, as shown by ESEM micrographs (Fig. 4a). A star-shaped fracture zone on the polymer microsection is seen, adjacent to the locus of failure. A typical fracture morphology is exhibited as the crack propagates and accelarates away from the failure locus. More detailed fracture morphology can be seen in Fig 4b. Fractured surface has an oriented texture. This is probably due to the semicrystalline nature of the polymer sample. Briefly, the ESEM study indicated that PLLA/PEO/PAsp terpolymer with semicrystalline nature and poor elongation properties exhibited brittle fracture morphology.

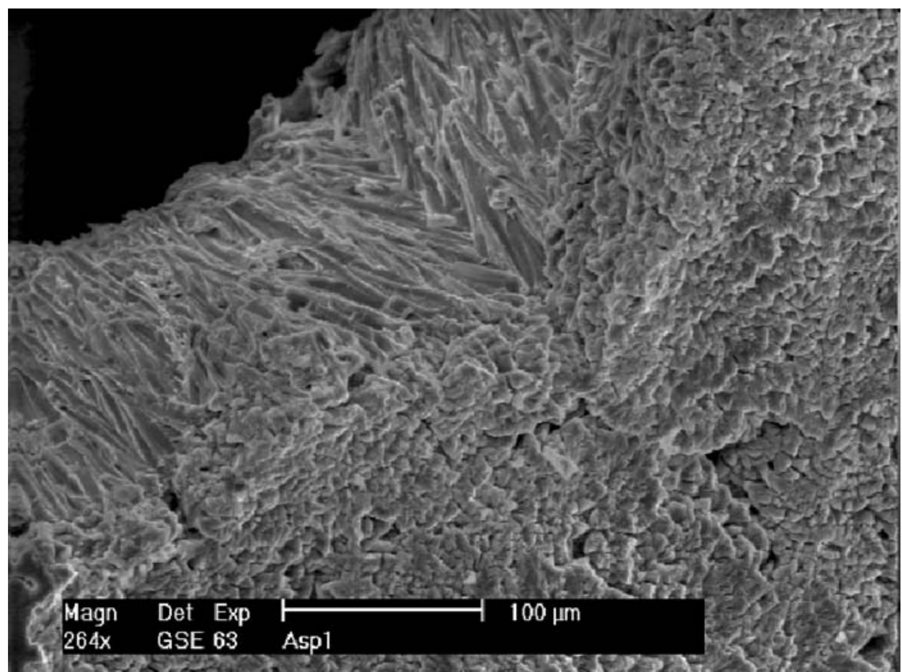
Degradation

Degradation properties of a scaffold material are of crucial importance in the long term success of a tissue-engineered cell/polymer construct.

As mentioned previously, the main goal of this work is to prepare a new polymer that will support the cell growth better compared with PLGA's performance. Due to the low molecular weight of the PLLA/PEO/PAsp terpolymer, it formed mechanically poor and brittle films. For this reason it was impossible to use this polymer alone for cell seeding. To



(a)



(b)

Fig. 4 ESEM micrographs of PLLA/PEO/PAsp terpolymer (a) cross-section and side surface details, (b) fractured surface details.

overcome this difficulty, blends of PLLA/PEO/PAsp (86/10/4) and high molecular weight PLGA(85/15) in two different compositions (25/75 and 50/50) were prepared.

Blending techniques are an extremely promising approach which can improve the properties of the polymer. Another objective of this work was to study the effect of the second component on the properties and hydrolytic degradation. A particulate leaching-solvent evaporation technique[5] was utilized for this purpose.

The hydrolytic degradation of these porous polymeric mixtures were investigated in PBS at pH = 7.4 and 37 °C. Porous scaffolds with an open pore structure are often desirable in many tissue engineering applications in order to maximize cell seeding, attachment, growth, extracellular matrix production and tissue ingrowth.

The degradation of porous PLGA polymers have been studied. The *in vitro* degradation kinetics of PLGA 85:15 polymers were reported to be independent of pore

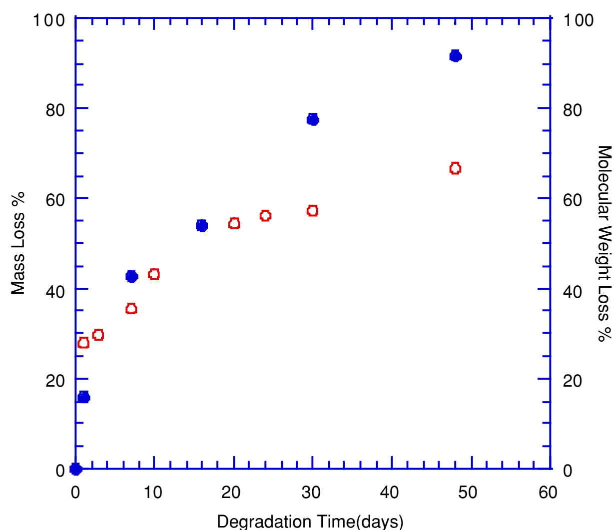


Fig. 5 Mass and Molecular weight loss for the 25/75 (PLLA/PEO/PAsp)/PLGA mixture: (O) mass loss; (•) M_w loss.

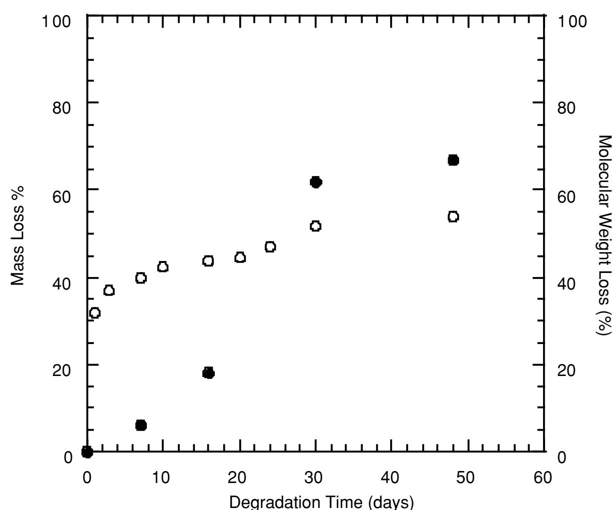


Fig. 6 Mass and Molecular weight loss for the 50/50 (PLLA/PEO/PAsp)/PLGA mixture: (O) mass loss ; (•) M_w loss.

morphology with no significant variation in polymer weight, thickness, pore distribution and morphology during degradation[20].

The molecular weight and mass losses (%) for the mixtures (PLLA/PEO/PAsp)/PLGA 25/75 and 50/50 are shown in Figs. 5 and 6, respectively. These figures show that about 30% mass loss were observed during the first few days before there is any significant molecular weight loss. This loss is due to a leachable component of the sample.

These results indicate that the degradation occurs in bulk by chain scission of PLGA component in the mixture. For (PLLA/PEO/PAsp)/PLGA 25/75 sample, about 67% of the material weight was lost in 48 days, while the weight loss of (PLLA/PEG/PAsp)/PLGA 50/50 sample was only 54% in the same time interval. This difference in mass loss is due to the content of the PLGA in the mixture. The molecular weights steadily decreased with the degradation and the molecular weight loss was 91% and 67% after 48 days for 25/75 and 50/50 mixtures, respectively. The appearance of the polymeric mixtures changed from a slightly translucent material to a white brittle material over the course of 48 days.

ESEM Studies

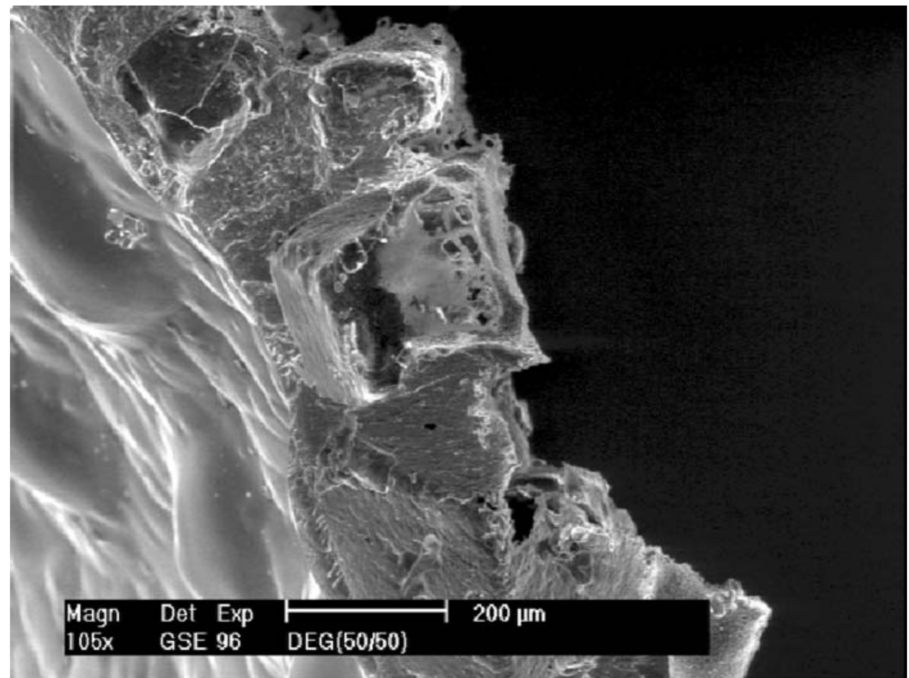
ESEM examination of (PLLA/PEO/PAsp)/PLGA blends is shown in Fig. 7a–d. Highly porous structures can be seen in the cross section of (PLLA/PEO/PAsp)/PLGA 50/50 (Fig. 7a) and 25/75 (Fig. 7b) blends. The porous sponges were prepared to perform cell seeding experiments and to follow cell growth. Detailed surface morphology at higher magnification can be seen in Figure 7c. The sizes of the pores depend on the size range of the salt particles that are used in the particulate leaching technique. Figure 7d depicts the appearance of polymeric films following 10 days of degradation, where the films can be observed as being broken into small pieces. ESEM images reveal micro-voids approximately 10 μm in diameter on the surface of each degraded specimen.

In Fig. 8, ESEM examination of (PLLA/PEO/PAsp)/PLGA 25/75 blends without (a) and with (b) cells 72 h after seeding is shown. A group of L929 cells attached to a surface inside a pore in the blend structure, with a significant number of cells residing on the polymer surface.

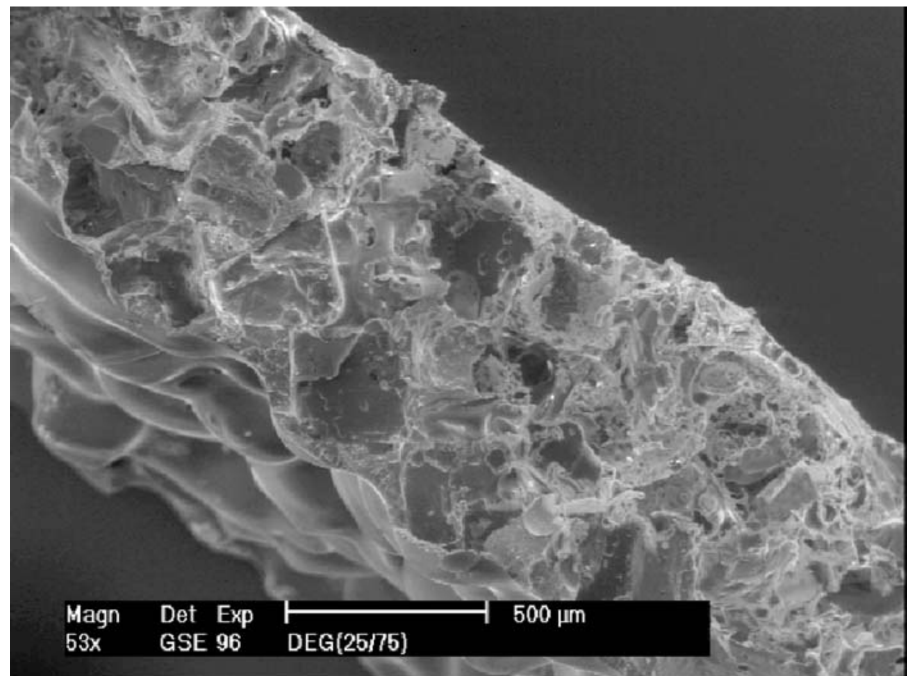
Viability of cells

Polyesters of α-hydroxy acids, poly(L-lactic acid) (PLA), poly(glycolic acid) (PGA), and copolymers of poly(lactic-co-glycolic acid) (PLGA) are widely used in tissue enginnering. One of the challanges in the field of tissue engineering is the development of biomaterial/cell interactions. In order to promote cellular adhesion, polymers are chemically modified. Poly(amino acid) also is an attractive candidate for the fabrication of tissue-engineering matrices [21].

An experiment in which cell viability was measured and compared on different materials is shown in Fig. 9. L929 cells placed on TC Plastic, gelatin or fibronectin rapidly proliferate, giving high absorbance values after 72 hours. The films obtained by using the 50/50 blend were not strong enough to be used for cell culture experiments as they disintegrated in cell culture medium. This can be seen in Fig. 7d. Therefore, cell culture experiments were done utilizing the (PLLA/PEO/PAsp)/PLGA 25/75 blend. PLGA (85/15) copolymer or 25/75 blend allowed the attachment and proliferation of a significant amount of cells, albeit much less than the above substrates, which are regarded as best materials for cell attachment and culture. The number of L929 cells



(a)



(b)

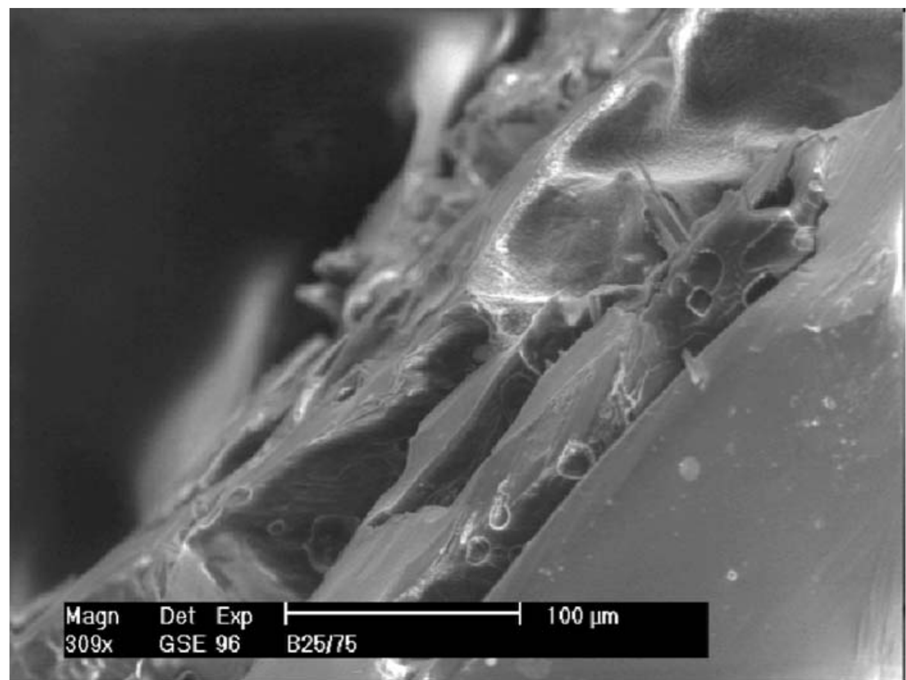
Fig. 7 ESEM micrographs of (PLLA/PEO/PAsp)/PLGA blends.(a) 50/50 blend before degradation, (b) 25/75 blend before degradation.

on the 25/75 blend was slightly higher than the 85/15 PLGA copolymer.

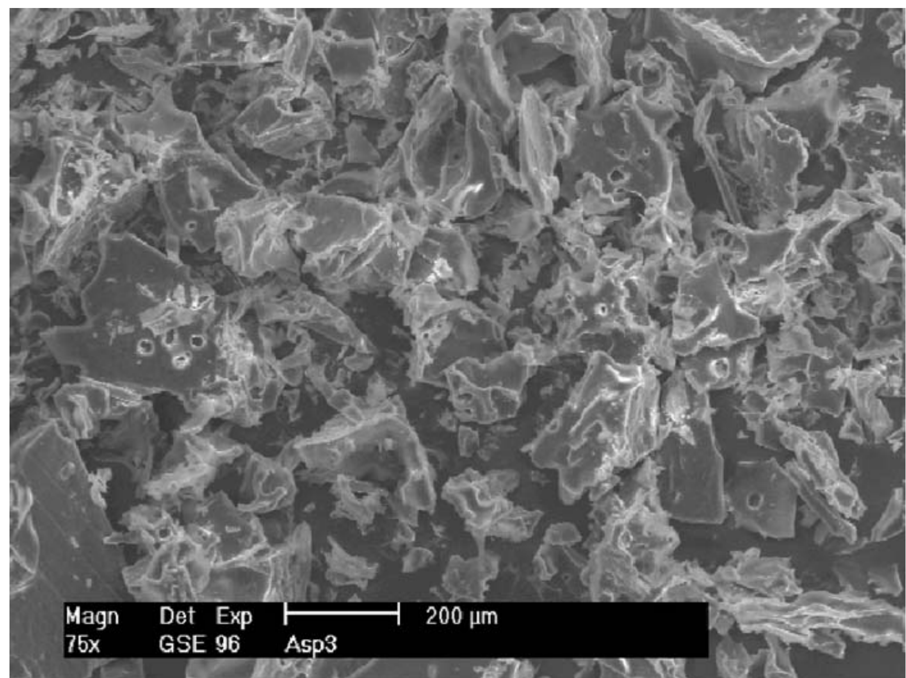
Conclusions

We have synthesized a new multiblock terpolymer (PLLA/PEO/PAsp) with well defined length of the ester-ether-amino acid segments. The structure, morphology and thermal behaviours of this terpolymer have been investigated. The results of DSC and ESEM showed that (PLLA/PEO/PAsp) is crystalline and its surface structure is brittle. Due to its inherent brittle behaviour, we have blended it with a high molecular weight copolymer (PLGA) in two different compositions (25/75 and 50/50). The hydrolytical degradation of polymeric blends was performed up to 48

Fig. 7 *Cont*. (c) A closer view of 25/75 blend, (d) 50/50 blend after degradation of 10 days.



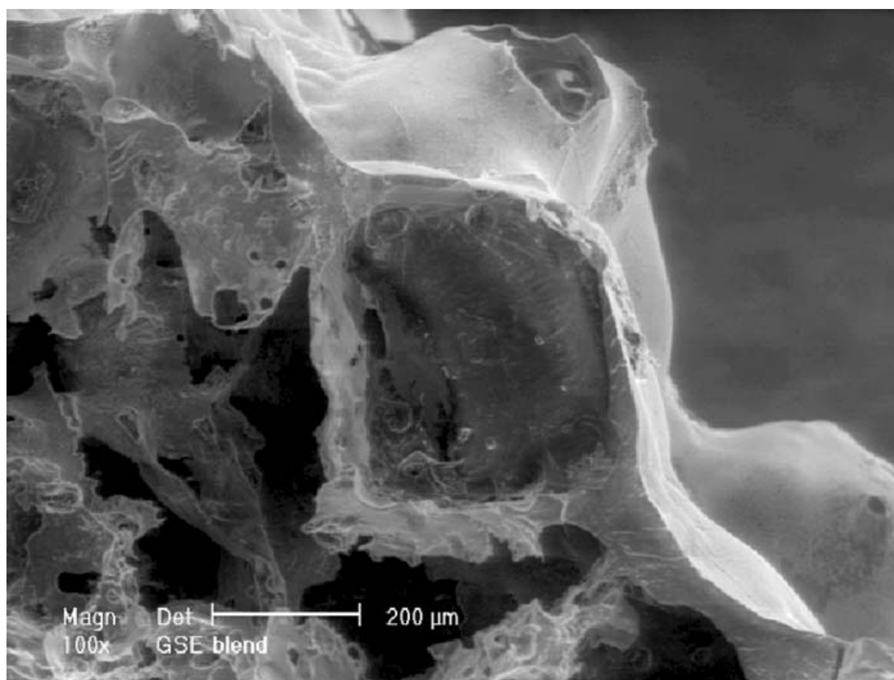
(c)



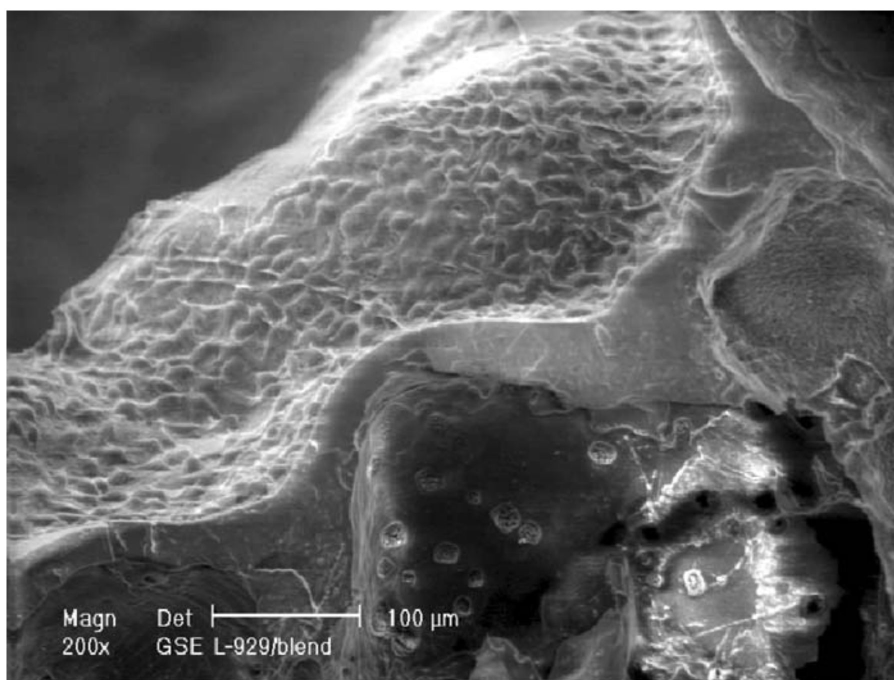
(d)

days and the results indicated that the degradation occurs by chain scission. For the 25/75 (PLLA/PEO/PAsp)/(PLGA) sample, the mass loss and molecular weight loss is faster than the 50/50 (PLLA/PEO/PAsp)/(PLGA) sample because of the high (PLGA) content in the mixture. ESEM studies showed that, after 48 days of degradation, the polymeric porous films were broken into small pieces. The cell seeding experiments showed that cell growth supporting characteristics of the 25/75 (PLLA/PEO/PAsp)/(PLGA) blend was slightly higher than the 85/15 PLGA copolymer which was used in producing the blend. The introduction of an L-aspartate group into terpolymer was successful. However, the molecular weight of resulting terpolymer was rather short to use this material for scaffold purposes in cell growth experiments. The preparation of terpolymer samples having higher molecular weight is under progress. The functional group present in the

Fig. 8 ESEM micrograph showing the cross section of the (a) 25/75 blend without cells, (b) blend 72 hours following seeding with L929 cells, showing a group of L929 cells attached to a surface inside a pore in the blend structure as well as the outer surface of the blend.



(a)



(b)

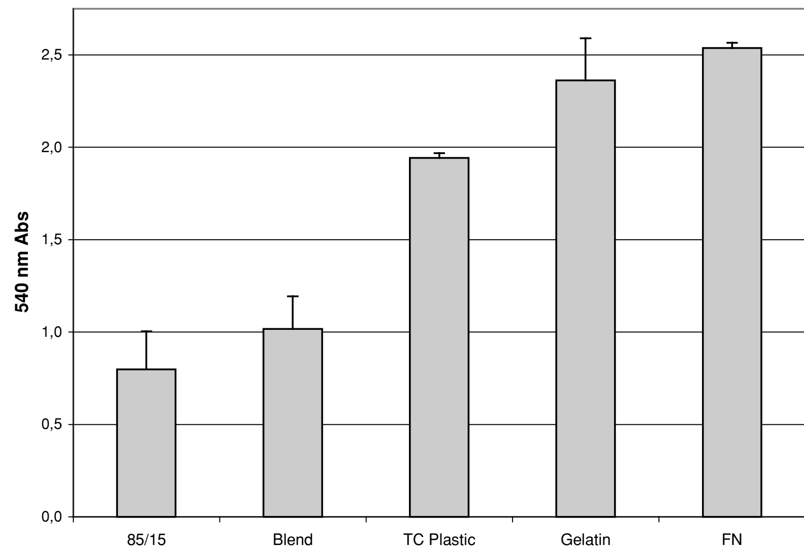


Fig. 9 Cell viability assay by neutral red uptake. 150,000 L929 cells were seeded per 22 mm diameter polymer surface or glass coverslip and grown for 72 hours in a CO_2 incubator as described in Materials and Methods. TC Plastic; tissue culture plastic, FN; fibronectin, 85/15; poly(l-lactic-co-glycolic acid) copolymer, Blend; (PLLA/PEG/PAsp)/PLGA 25/75 mixture.

aspartate blocks will be used to add other polypeptides or proteins into this terpolymer.

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