

Amido-modified polylactide for potential tissue engineering applications

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Abstract—Poly(ester amide) copolymers based on L-lactide (**2**) and a new depsipeptide (**1**) were prepared by ring opening polymerization in the presence of Sn(Oct)₂ as the catalyst. Variable monomer feed ratios up to 2.3 mol% **1** afforded copolymers containing ester and amido functional groups in the backbone. Lower glass transition temperatures and reduced crystallization kinetics and crystallinity compared to homo-polylactide (PLA) was achieved with low levels of amido incorporation. A reactivity comparison between enchainment of **2** and **1** was determined using *in situ* infrared spectroscopy. An increase in shear viscosity was observed with the increase of **1** content as determined by rheology studies. Cellular compatibility of the co-polymers was investigated by seeding D1 mouse stem cells onto films and characterizing cell morphology by optical microscopy. Preliminary results indicate that these novel materials exhibit reduced cell attachment compared to PLA and, pending further exploration, may have potential use in biomedical applications.

Key words: Polylactide; depsipeptide; poly(ester amide); morpholinedione; tissue engineering.

INTRODUCTION

Over the past few decades, research efforts have focused on the design of thermoplastics using renewable resource agricultural materials rather than traditional petroleum derived plastics. Lactic acid, the precursor for commercial lactide monomer and polylactide (PLA), is obtained from the fermentation of corn or beet sugar. Primarily, PLA is used in food packaging, agricultural films and biomedical appli-

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cations such as sutures [1–9]. Large-scale production of PLA was recently initiated by Cargill Dow Polymers, suggesting potential high market demand in the near future [10–12].

Morpholine-2,5-dione derivatives (depsipeptides) are a class of monomers that have been used to obtain biodegradable copolymers with alternating amido and ester vertebrae. These materials degrade into nontoxic components *in vitro* and *in vivo*; therefore, they have attracted attention for specific biomedical applications. Ring opening polymerization of depsipeptides with lactide, glycolide, or caprolactone have been reported with variable properties compared to the corresponding homopolymers [13, 14].

We have recently prepared a novel depsipeptide monomer, 6,6'-dimethyl-2,5-morpholinedione (**1**) in good overall yield (40%), the synthesis of which has been reported elsewhere (Ref. [15] and data not shown). Copolymerization of the new monomer (**1**) with L-lactide (**2**) was accomplished in the presence of Sn(Oct)₂ catalyst. The thermal characterization of the novel copolymers by Differential Scanning Calorimetry (DSC) revealed that the glass transition temperature and the crystalline melting temperature of the copolymers decreases with the increment of the percentage of **1** in the polymer backbone. Further we have demonstrated that the crystallinity of the copolymers can be decreased by incorporating a small percentage of **1** into the polymer. It has been found that 8% of **1** co-monomer in the main chain resulted in a completely amorphous copolymer (Ref. [15] and data not shown).

Here we report our initial investigations on the relative reactivity of **2** and **1** with a ReactIR™ 1000 reaction analysis system, using attenuated total reflection (ATR) mid-infrared spectroscopy [16–19]. This system has the capability of recording an infrared spectrum of materials that are contacted with the chemically resistant probe, and it can be applied to a range of reaction conditions. Hillmyer and co-workers previously reported the kinetics of the ring opening polymerization of lactides using ReactIR™ spectroscopy for zinc and tin alcoxide catalyst systems [20, 21]. We present detailed FT-IR experimental results for the homo- and co-polymerization of **2** with **1** in this study. In addition, we report on the rheological behavior by measuring the complex viscosity as a function of depsipeptide content. Finally, we assessed the copolymer's potential use as a scaffold for tissue engineering applications by cell adhesion studies.

MATERIALS AND METHODS

Materials

L-Lactide was generously provided by Poly-Med (Pendleton, SC, USA) and Ortec (Easley, SC, USA), and was recrystallized from ethyl acetate and vacuum dried prior to use. New monomer 6,6'-dimethyl 2,5-morpholinedione (**1**) was synthesized as described elsewhere (Ref. [15] and data not shown). Stannous 2-ethylhexanoate (Aldrich) was stored under nitrogen prior to use. House nitrogen filtered through

a Drierite filter/dryer was used for all synthetic operations. Other chemicals and reagents were purchased from Aldrich or Fisher Scientific and used as received unless otherwise stated.

Co-polymerization

A general procedure for copolymerization of **1** and **2** is described below. Monomer **1** (0.2 g, 1.4 mmol), **2** (0.8 g, 5.6 mmol) and Sn(Oct)₂ 70 μ l of 0.01 mol l⁻¹ solution in anhydrous toluene was transferred to an ampoule. After evaporation of the solvents, the tube was sealed in vacuum and kept at 130°C in an oil bath. After 24 h, the crude product was dissolved in chloroform and precipitated into methanol. The resulting polymer was dried *in vacuo*. Yield: 85%. ¹H-NMR (500 MHz, CDCl₃) δ (ppm): 6.60 (s, 1H), 5.1 (m, 2H), 4.6 (s, 2H), 1.6 (d, 12H). ¹³C-NMR (500 MHz, CDCl₃) δ (ppm): 172.0, 168.0, 71.9, 69.0, 57.1, 24.8, 16.0. FT-IR (film) (cm⁻¹): 1174, 1181, 1208, 1522, 1685, 1760, 1852, 1900, 3400.

Gel-permeation chromatography (GPC)

GPC samples were prepared in HPLC grade CHCl₃ (6 mg in 1 ml of CHCl₃). GPC data were collected using a Waters 2690 Alliance System equipped with two consecutive Polymer Labs PLGel 5 mm Mixed-D and Mixed-E columns with refractive index detection at 35°C.

In situ ReactIR spectroscopy (ReactIR™)

In situ mid-FT-IR spectra for the polymerizations were collected using an ASI Applied Systems ReactIR™ 1000 reaction analysis system equipped with a light conduit and DiComp (diamond composite) insertion probe. The probe was inserted into the reaction flask containing the predetermined amount of the monomers and the initiator (monomer/initiator = 100:1), and it was sealed under nitrogen. The flask was heated to the desired temperature using heating tape and spectra were collected every 2 min (8 scans with 4 cm⁻¹ resolution) for 1 h and every 5 min (128 scans with 4 cm⁻¹ resolution) for the next 3 h. After collecting the spectra, the data was further analyzed with ReactIR™ (version 2.2) software. Due to overlapping of peaks of interest, integrating the area was difficult. Therefore, the peak height with a specified baseline was used for the calculations.

Rheology

The rheological properties of poly(**1-co-2**) were investigated using a Rheometric Scientific ARES Rotational Rheometer equipped with 8-mm parallel plates and a 0.3-mm gap between the plates. A nitrogen atmosphere was employed and a dynamic frequency sweep was used to measure the complex viscosity as a function of frequency. Dynamic strain sweeps were performed to establish the linear viscoelastic regime. All tests were repeated a minimum of three times to insure reproducibility.

Table 1.

Composition and molecular weight data for the co-polymers.

Entry	Poly(1-co-2) ^a (% monomer 1)	M_n^b ($\times 10^{-3}$)	M_w/M_n^b
PLA-a	0	52.6	1.2
Poly(1-co-2)-b	0.1	33.0	1.6
Poly(1-co-2)-c	0.9	40.6	1.5
Poly(1-co-2)-d	1.3	37.0	1.6
Poly(1-co-2)-e	2.3	40.0	1.7

^a Determined by ¹H-NMR.^b GPC in chloroform vs. polystyrene.

Cell seeding experiments

Three films of each material type were sized to fit a 12-well tissue culture polystyrene plate, using an arch punch. All films were cleaned with ethanol and rinsed with sterile medium prior to use. The materials included poly(**1-co-2**)-c, poly(**1-co-2**)-e and PLA-a (Table 1). The six surfaces were seeded to 20% confluence using D1 mouse stem cells. Teflon rings were used to immobilize each film, resulting in a cell seeding density of approx. 32 000 cells per well. Dulbecco's Modified Eagle's Medium, supplemented with 10% fetal bovine serum, 1% fungizone and 1% antibiotic-antimycotic, was used for the incubation period. The cultures on all polymer surfaces were maintained for 10 days. Representative photographs of all cellular surfaces were acquired using an inverted microscope.

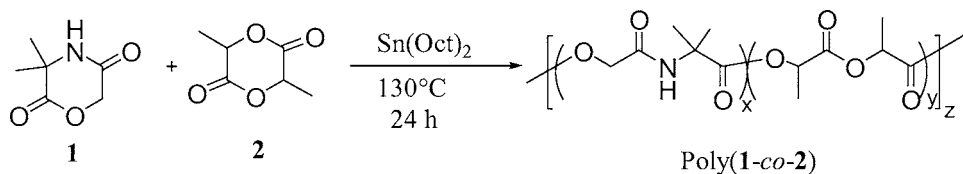
RESULTS AND DISCUSSION

Synthesis

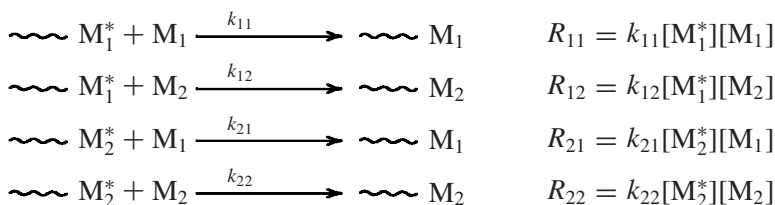
We have reported the synthesis of the novel depsipeptide monomer **1** and the structural and thermal characterization of its copolymer with **2** [15, 16]. The general synthetic route to copolymerize **1** and **2** to obtain poly(**1-co-2**) is shown in Scheme 1.

The ring-opening co-polymerization of **1** and **2** was performed in glass ampoules under high vacuum catalyzed by Sn(Oct)₂. A series of copolymers containing **1** and **2** was prepared at 1–10 g scale in 85–90% yield. The molecular weights of the polymers were measured using GPC relative to polystyrene standards. The molecular weight data collected for poly(**1-co-2**) with different percentages of **1** in the polymer backbone are summarized in Table 1.

The most commonly used catalyst for the ring-opening polymerization of lactide, Sn(Oct)₂, exhibited excellent activity in the co-polymerization. It is reported to be an efficient catalyst, which allows complete conversion of monomers even at a very high monomer/catalyst ratio (typically 10 000:1) with minimum racemiza-



Scheme 1. Random co-polymerization to poly(1-co-2).



$[M_1^*]$, $[M_2^*]$ = concentration of the active propagating polymer having a **1** or **2** at the end. $[M_1]$, $[M_2]$ = **1** or **2** monomer concentration.

Scheme 2. Kinetic expressions and assumptions for the homopolymerization and copolymerization of **2** with **1**.

tion [16–18]. The number average molecular weights of the copolymers ranged from 33 000–52 000 with relatively narrow polydispersity.

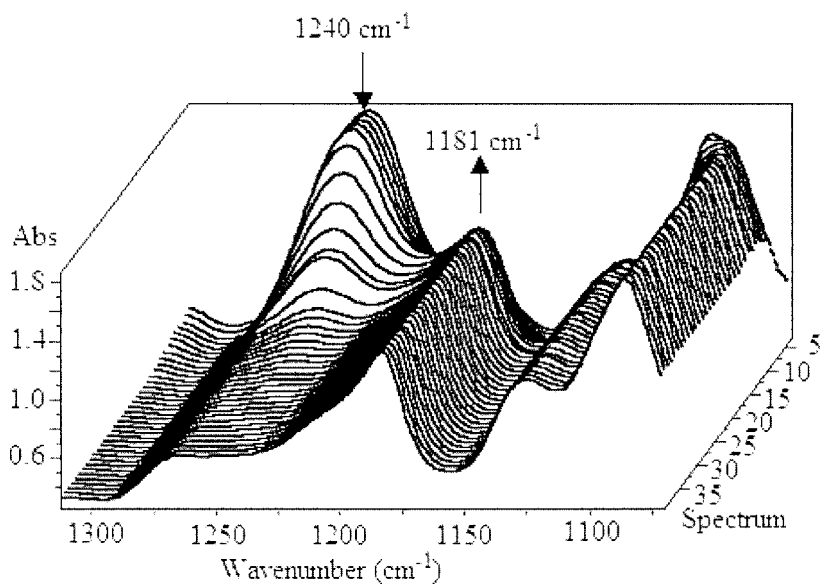
In situ FT-IR spectroscopy (ReactIR™)

Homopolymerization of **2** as well as the copolymerization of **1** and **2** were monitored utilizing *in situ* FT-IR analysis. Kinetic assumptions utilized for these reactions are summarized in Scheme 2. The carbonyl absorption for pure **2** generally appears at 1759 cm^{-1} as a narrow strong peak [19]. The peaks at 2995 and 2945 cm^{-1} correspond to $-\text{CH}_3$ and $-\text{CH}$ -groups of the lactide moieties. During homopolymerization of **2**, the peak at 1240 cm^{-1} from lactide ring stretching disappeared, and a new peak at 1181 cm^{-1} appeared due to the forming PLA. It was used for kinetic analysis using *in situ* FT-IR. The waterfall plot for this region is shown in Fig. 1a. The preliminary analysis for the homopolymerization of **2** exhibits a linear first-order kinetic plot ($k_{\text{obs}} = 0.08 \text{ min}^{-1}$) (Fig. 2a).

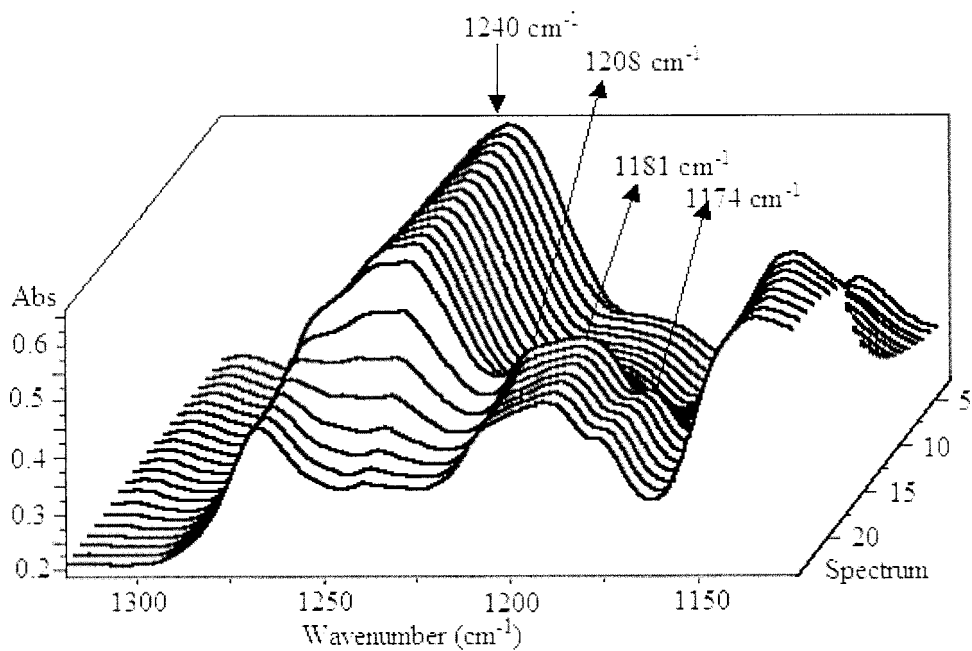
For homopolymerization of **2**, the rate of polymerization is:

$$\begin{aligned}
 R_{22} &= -\frac{d[\mathbf{2}]}{dt} = k_{22}[M_2^*][M_2], \\
 R_{22} &= \frac{d[-M_2]}{dt} = k_{22}[-M_2] = \frac{d[\text{PLA}]}{dt} = k_{22}[\text{PLA}],
 \end{aligned}$$

where k_{22} = absolute propagation rate constant and $[-M_2] = [\text{PLA}] = \text{PLA}$ concentration.



(a)



(b)

Figure 1. Ester region of the waterfall plot for homopolymerization of 2 (a) and copolymerization of 1 and 2 (b) by *in situ* FT-IR spectroscopy.

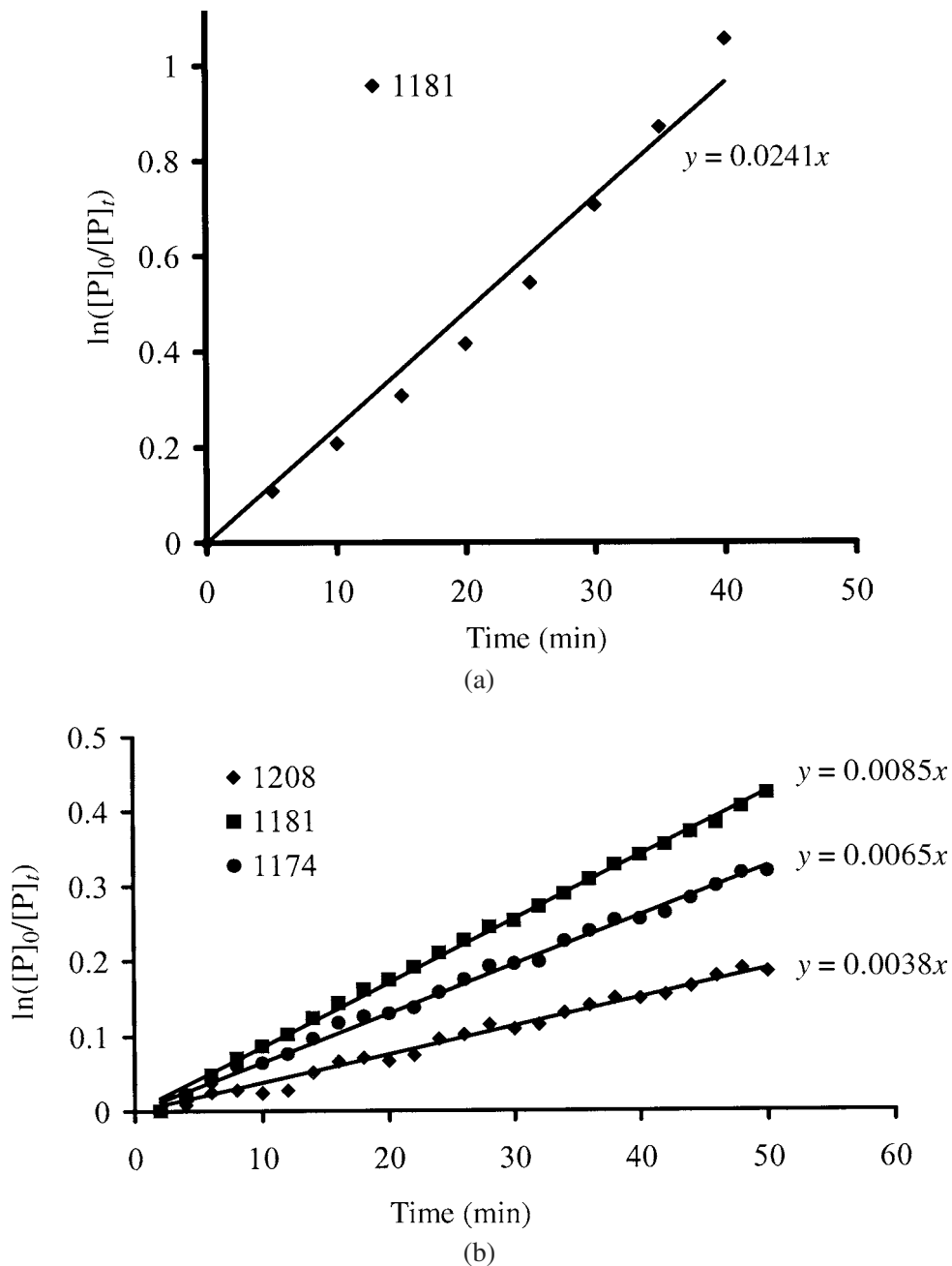


Figure 2. First-order kinetic plots for homopolymerization of **2** (a) and copolymerization of **1** and **2** (b) determined by FT-IR peak height at 1174, 1181 and 1208 cm^{-1} .

The first-order rate constant is expressed as:

$$k_{\text{obs}(2)} = k_{22},$$

$$k_{\text{obs}(2)} = (\ln[[\text{PLA}]_0/[\text{PLA}]_t])/t = \ln([\text{P}]_0/[\text{P}]_t)/t,$$

where $[\text{P}]$ = peak height at 1881 cm^{-1} for PLA determined by ReactIR. For the copolymerization of **1** and **2**, kinetic plots of $\ln([\text{P}]_0/[\text{P}]_t)$ vs time for the peaks at 1174 , 1181 and 1208 cm^{-1} would correlate to the relative rates for the reactions between **1,2**; **2,2** and **1,1** and provide k_{obs} for $k_{21} + k_{12}$ (assuming $k_{21} = k_{12}$), k_{22} and k_{11} , respectively.

Homopolymerization and co-polymerization is performed using the same experimental conditions and monomer: initiator ratio (100:1) in melt at 130°C . In addition to the peak at 1181 cm^{-1} , two additional peaks were observed at 1174 and 1208 cm^{-1} that could be assigned to **1-2** and **1-1** ester linkages in the copolymer. The FT-IR waterfall plot for this region is shown in Fig. 1b and a first-order dependence for each peak was observed as shown in Fig. 2b. The k_{obs} values obtained using these respective plots were 0.0065 , 0.0085 and 0.0035 min^{-1} . This observation illustrates that a growing chain terminated by **2** prefers to add another **2** unit and a chain terminated by **1** prefers to add another **2** rather than adding a **1** unit. These values are in good agreement with the polymer structure given in Scheme 1, which strongly suggests a copolymer with a random distribution of monomer **1** amido linkages.

Rheology

The complex viscosity, η^* , as a function of frequency for PLA-a, poly(**1-co-2**)-b and poly(**1-co-2**)-e is shown in Fig. 3. The dynamic frequency sweep was performed at 180°C over a frequency range from 1 rad/s to 500 rad/s . The samples exhibit a typical Newtonian plateau at low and intermediate shear rates. In addition, all of the samples exhibit shear thinning behavior at frequencies exceeding 200 rad/s . The high frequencies necessary for the onset of shear thinning are typical for the relatively low molecular weight polymers employed in this study.

By synthesizing and studying materials with similar molecular weights (M_n approx. $35\,000$), it is possible to isolate the effects of copolymer composition on the viscosity. We observe that with increasing depsipeptide concentration, the complex viscosity increases, probably due to the effect of the additional $-\text{CH}_3$ groups in the depsipeptide relative to the lactide and the increased hydrogen bonding from the amido ($-\text{NH-CO-}$) linkage. This increase in viscosity occurs although there is no significant variation in the melting temperature for these copolymers.

To investigate polymer degradation in an air atmosphere, a dynamic time sweep was performed on poly(**1-co-2**)-b (Fig. 4). The test was performed at a temperature of 180°C and a frequency of 10 rad/s for 2 h. The complex viscosity decreases continually with time, indicating degradation of the polymer sample.

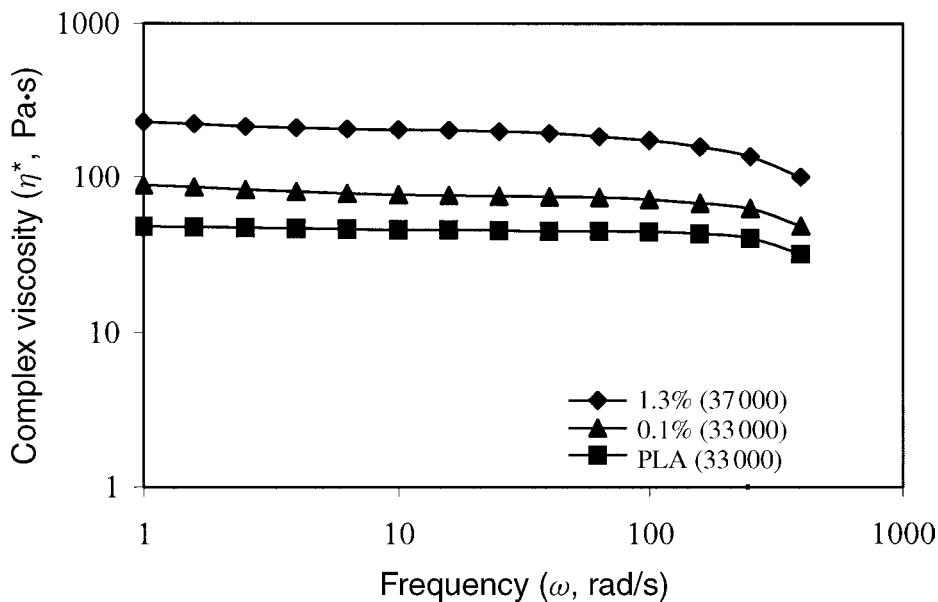


Figure 3. Complex viscosity as a function of copolymer composition.

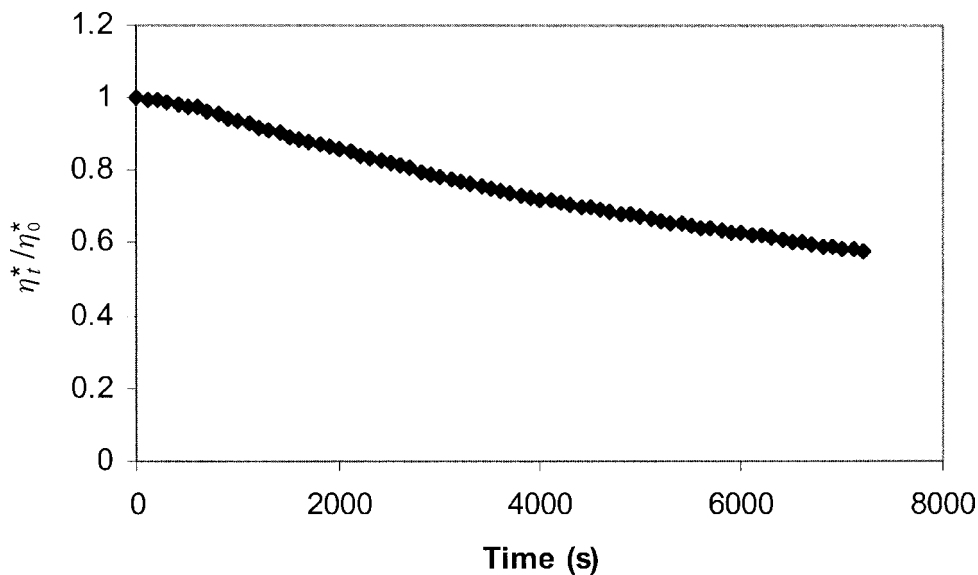
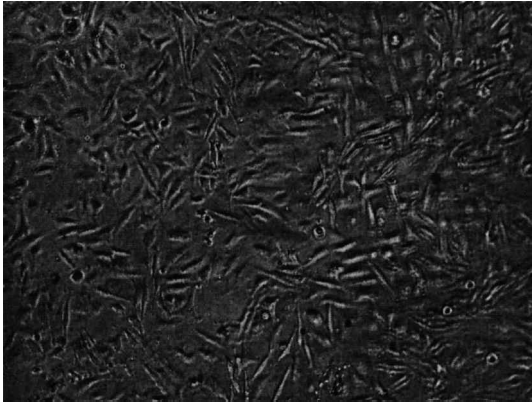


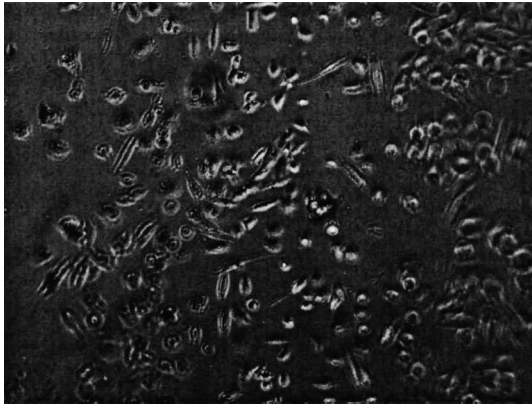
Figure 4. Isothermal complex viscosity as a function of time in air at 180°C.

Cell seeding experiments

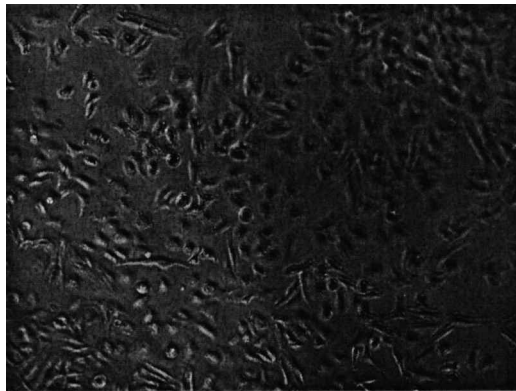
Figure 5 illustrates our preliminary cell seeding results for the various films. The spindle-shaped cells on the PLA-a films were dense near the center, but only grew sporadically outward. Although the cells are healthy in appearance, they did not



(a)



(b)



(c)

Figure 5. Optical micrographs ($\times 10$ magnification) of cells on polymer films for PLA-a (a), poly(1-co-2)-c (b) and poly(1-co-2)-e (c).

appear to proliferate or migrate on the surface during the 10-day study. The cells seeded on the poly(**1-co-2**)-e films were dense in a few widespread areas. They were spindle-shaped as well, but many rounded cells were also observed, indicating a lower state of viability with increased amounts of **1**. The cellular organization on these films appears to be random and little proliferation or migration occurred over the duration of the study.

The poly(**1-co-2**)-c films presented cells that formed dense bands in two of the three wells. Few cells were located outside of these bands. The heterogeneity in adhesion implies that there are slight variations in the consistency of the copolymers, either with respect to the bulk or the surface. All three replicates of the poly(**1-co-2**)-c films had more rounded cells than the other two surfaces. Polylactide is a hydrophobic material and thus does not attract high cell adhesion without surface modification or treatment, therefore, the low level of proliferation is not surprising [22–24]. The lower state of viability on the copolymers may be due in part to the increased polydispersity index associated with these systems, causing a more dynamic surface with which the cells must interact. It may be possible to increase the cell viability by extracting the monomeric and oligomeric species following synthesis and/or film processing [22–24].

CONCLUSIONS

Novel depsipeptide monomer dimethyl-2,5-morpholinedione was copolymerized with L-lactide to obtain amido functionalized copolymers by standard polymerization techniques. The relative reactivity of monomers **1** and **2** was determined by *in situ* ReactIR[™] spectroscopy to be **2-2** \ggg **1-2** > **1-1**. Shear viscosity was altered by varying the depsipeptide content in the copolymer. Cell adhesion studies showed that adhesion of D1 mouse stem cells on copolymer films of monomers **2** and **1** was attenuated compared to pure PLA films. Materials of this type, that appear to be nontoxic but do not appear to induce high cell proliferation, may be extremely useful in regenerating tissues that mandate high mechanical integrity and low cell volume (e.g. cartilage or spinal disc).

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