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Block copolymers of L-lactide and poly(ethylene glycol) for biomedical applications

P. CERRAI, M. TRICOLI, L. LELLI, G. D. GUERRA
Centro Studi Processi Ionici di Polimerizzazione e Proprietà Fisiche e Tecniche di Sistemi di Macromolecole, CNR, via Diotisalvi 2, I-56126 Pisa, Italy

R. SBARBATI DEL GUERRA
Istituto di Fisiologia Clinica del CNR, via Savi 8, I-56126 Pisa, Italy and Centro Studi Processi Ionici di Polimerizzazione e Proprietà Fisiche e Tecniche di Sistemi di Macromolecole, CNR, via Diotisalvi 2, I-56126 Pisa, Italy

M. G. CASCONE, P. GIUSTI
Dipartimento di Ingegneria Chimica, Chimica Industriale e Scienza dei Materiali, Università di Pisa, I-56126 Pisa, Italy

Poly(L-lactide)–poly(oxyethylene)–poly(L-lactide) block copolymers obtained in bulk, by a ring opening mechanism, from poly(ethylene glycol)s (PEG)s and L-lactide (LA), at 120–140 °C, in the absence of added catalysts are described. By using PEGs with different molecular masses, 3000 and 35 000, respectively, and varying the initial molar ratio LA to PEG, two series of copolymers with different molecular masses, relative length of blocks and hydrophilicity were obtained. Physico-chemical characterization of the copolymers had been previously performed. The morphological characteristics of the copolymers were investigated by means of X-ray diffractometry, optical and scanning electron microscopy. The biological properties of the materials were determined by evaluating their cytotoxicity, cytocompatibility, hemocompatibility and degradability using different standard tests. The results obtained indicate that the block copolymers synthesized may be useful for biomedical applications, in particular as resorbable drug vehicles. The materials are brittle and their mechanical properties are not appropriate for implant devices.

1. Introduction

The synthesis and the properties of poly(ester–ether–ester) block copolymers based on lactides and poly(alkylene glycol)s have been reported in several recent papers [1–5]. Ether blocks poly(ethylene glycol)s (PEG)s or poly(propylene glycol)s (PPG)s of different molecular mass are generally used, while the ester ones are usually formed by ring-opening polymerization of ε-caprolactone, glycolide and L- or D, L-lactide, in the presence of different catalysts. Such copolymers are generally used as biomedical materials, for drug delivery systems, bioresorbable surgical sutures, bone fracture healing devices or selectively biodegradable vascular grafts. For these uses the polymeric materials must be non-toxic, biocompatible and more or less biodegradable. On the contrary, all the block copolymers reported in the literature prepared in the presence of organic catalysts containing heavy metals, like Al, Sb, Sn, Zn etc., can leave residues of doubtful biocompatibility in the final material.

For these reasons, in this paper we report on the biological characterization of triblock poly(L-lactide)-poly(oxyethylene)–poly(L-lactide) copolymers (PLA–POE–PLA) synthesized in bulk without any added catalyst. In particular the paper deals with the biocompatibility of the new copolymers evaluated on the basis of their cytotoxicity and hemocompatibility. The lack of cytotoxic effects, which is a fundamental requisite to assess the biocompatibility of new materials, was checked by means of different standard tests. The hemocompatibility was tested by the contact activation of the coagulation cascade (intrinsic pathway). Finally, the degradability behaviour of some copolymers with different compositions was studied *in vitro* by the hydrolytic method. Similar work based on PEGs and ε-caprolactone is at present in press [6, 7].

2. Materials and methods

2.1. Materials

L-lactide (LA) (Aldrich, > 98%) was recrystallized twice from ethyl acetate washed, with distilled ethyl ether and sublimed in vacuo (m.p. 98 °C).

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Poly(ethylene glycol)s (PEG)s (Merck, zur synthese) with $\bar{M}_n = 3000$ and $\bar{M}_n = 35000$ were used after double recrystallization from anhydrous acetone (m.p. 58 °C and 64 °C, respectively).

2.2. Polymerization procedure

In each run the preweighed amounts of PEG and LA were mixed in a pyrex phial connected to a vacuum line. The mixture was heated at 50–60 °C and thoroughly degassed, then the phial sealed off and put in an oven at 120–140 °C. After some days (4 to 8), depending on the initial feed, the phial was opened and the polymeric material recovered as a friable (from PEG 35000) or waxy (from PEG 3000) solid. The polymer was extracted with ethyl ether to eliminate the unreacted LA, dried at 50 °C in vacuo and characterized.

2.3. Characterization tests

The physico-chemical characterization of the copolymers by means of techniques such as viscometry, infrared (IR) and proton magnetic resonance (^{1}H NMR) spectroscopies, differential scanning calorimetry (DSC), dynamic-mechanical thermal analysis (DMTA), and X-ray diffractometry (RX) was carried out as previously reported [8].

Scanning electron microscopy (SEM) of fractured samples was carried out using a Jeol T-300 apparatus.

Optical microscopy for bulk crystallization experiments of thin films was carried out with an Ortholux Pol LEITZ instrument equipped with a FP-52 Mettler hot stage.

2.4. Cytotoxicity and cytocompatibility tests

All the copolymers under study were submitted to both cytotoxicity and cytocompatibility tests.

The cytotoxicity tests were Neutral Red uptake (NR), Kenacid Blue R-binding method (KB) and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (MTT).

The cytocompatibility tests consisted in cell proliferation tests on copolymer films.

A detailed description of both (a) cytotoxicity and (b) cytocompatibility assays has been already published [7]. In brief: (a) Copolymers samples were sterilized by ethylene oxide and extracted in PBS for 5 days at 37 °C. The extracts were sterile filtered and used for 24 h and 72 h NR, KB and MTT assays, using the mouse fibroblast line 3T3.

(b) Films were obtained from copolymers by evaporating 2% (w/w) solutions in $CHCl_3$ on micro-cover glasses (22 × 22 mm) sterilized by ethylene oxide. Human umbilical vein endothelial cells (HUVEC) were seeded on the polymer films at a seeding density of either 1×10^4 cells/cm^2 (proliferation test) or 4×10^4 cells/cm^2 (adhesion test).

HUVEC were trypsinized (0.05% trypsin/0.02% EDTA (v/w)) and counted by means of a haemocytometer either after 6 h (adhesion test) or after 1 week (proliferation test) from seeding.

2.5. Hemocompatibility test

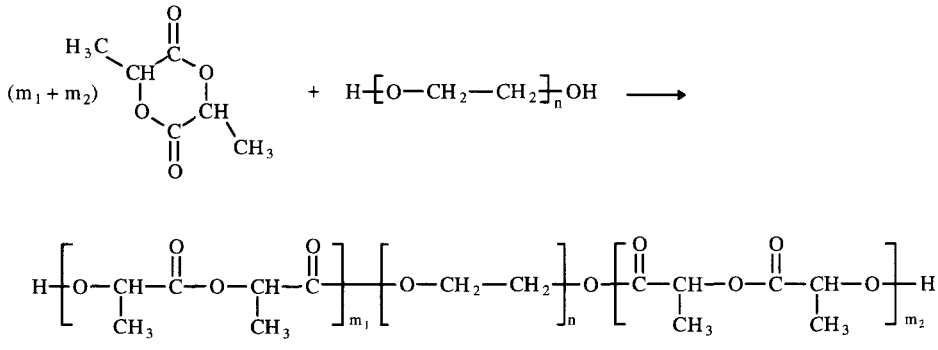
The start of the intrinsic pathway was tested by measuring the activation of plasma prekallikrein (PKK) to kallikrein (KK) [9, 10]. The activation of PKK to KK, caused by the contact of plasma with the copolymers, was determined by the proteolytic reaction between KK and the chromogenic substrate H–D–Pro–Phe–Arg–pNa (S-2302 Kabi Diagnostica), according to a procedure already described in detail [7]. Kallikrein-like activity (KLA) values (units per litre) were obtained from the initial rates by the "initial velocity method" [10, 11].

2.6. Degradation test

The *in vitro* degradability of the copolymers was estimated by the hydrolytic erosion measured by solution viscometry. Different samples (1 × 2 × 0.2 cm) were immersed in deionized water at 37 °C and a part of each sample was used for measuring the intrinsic viscosity [η] at fixed times. [η] was determined with a modified Ubbelohde viscometer in chloroform at 25 °C.

3. Results and discussion

PLA–POE–PLA copolymers were formed by reacting PEG-3000 or PEG-35000 with LA monomer in bulk, under vacuum, at 120–140 °C. The reaction occurs according to a ring-opening mechanism, where the active hydrogen atoms of PEG induce a selective acyl–oxygen cleavage of the lactide ring, so forming two external ester blocks [12, 13]. Using PEGs with different molecular masses (3000 or 35000) and varying the initial LA to PEG molar ratio, copolymers with different molecular weight, composition and average length of blocks were obtained, as shown in the following reaction scheme.



Since the structure of the copolymers involves both hydrophilic (POE) and hydrophobic (PLA) blocks, it was also possible to modulate the degradation characteristics of the final materials by simply varying the molar composition of the feed.

A critical step in the experimental work was the very high purity of PEGs and LA necessary to have relatively high and reproducible yields to PLA–POE–PLA copolymers. Indeed, negative phenomena such as a partial homopolymerization of LA to low molecular weight products or a racemization of LA to give yellowish amorphous materials, may occur. Such phenomena can be reduced only by means of the careful purification already described [8], in a paper concerning in particular the chemical aspects of the synthesis of PLA–POE–PLA copolymers. In spite of good purification, a partial LA racemization was observed in the copolymerization experiments with PEG-3000, which gave amorphous and coloured waxy materials.

The PLA–POE–PLA copolymers investigated in this work are listed in Table I.

All the samples were chemically characterized by an intensive application of different techniques, including viscometry, IR and ^{1}H NMR spectroscopies, DSC and DMTA analyses, RX diffractometry, optical and scanning electron microscopies. The results obtained have been described in detail [8]. Notwithstanding, it may be useful to summarize the main aspects of the physico-chemical investigation.

Copolymer yields between 70% and 90% are obtained within 4 to 8 days, indicating a very slow addition process of LA monomer due to the non-catalysed procedure.

The experiments carried out with a lower initiating PEG concentration (i.e. LA-8, LA-13, LA-9, LA-3) are characterized by LA content and experimental $\bar{M}_n$ of the copolymers markedly lower than the theoretical ones because of unreacted monomer, as indicated by the lower copolymer yield.

The block nature of the copolymers is demonstrated by the results obtained in IR, ^{1}H NMR and DSC measurements. IR and ^{1}H NMR spectra have typical patterns which reflect both qualitatively and quantitatively the molar compositions of the copolymers listed in Table I. DSC analysis shows a continuous melting point depression of POE block with increasing amount of LA units in the samples, so confirming its incorporation into a block copolymer. In copolymers from PEG-35 000 the POE melting endotherm is always present, whereas the PLA endotherm appears only for LA content > 50 mol%; in copolymers from PEG-3000 the POE block seems to crystallize only for OE content > 40 mol%. The different crystallinity of the copolymers depending on their molar composition was confirmed also by the RX analysis of powder samples.

In Fig. 1 typical RX diagrams of samples with different composition are shown, together with those of PLA and PEG homopolymers as reference.

Interesting information concerning the crystallization mechanism of the different copolymers arises from optical and scanning electron microscopy.

Bulk crystallization experiments carried out on thin films of the copolymers permit recognition of different kinds of crystals depending on the copolymer composition. Samples with a high LA content (i.e. LA-3 in Table I) present spherical microcrystals as prevailing, quite similar to those of PLA homopolymer. Conversely, samples with a high OE content (i.e. LA-4) show regular large spherulites like those typical of pure PEG homopolymer. In samples with an intermediate molar composition (i.e. LA-6) large spherulites and microcrystals are both present, as clearly shown in the polarized light micrograph in Fig. 2.

SEM microscopy demonstrates further the crystallization characteristics of the copolymers, as shown in Fig. 3, where the typical regular spherulites of POE blocks in LA-4 sample are shown.

TABLE I Experimental data of PLA–POE–PLA copolymers obtained with PEG-3000 and PEG-35 000

Expt	Feed composition		Copolymer composition[a]		Copolymer yield (wt %)	$\bar{M}_n$[a] ($\times 10^{-4}$)	$\bar{M}_n$[b] ($\times 10^{-4}$)	Note
	OE[c] (mol %)	LA[c] (mol %)	OE[c] (mol %)	LA[c] (mol %)				
PEG-3000								
LA-8	20	80	41.0	59.0[d]	70.0	1.010[d]	2.258	waxy
LA-13	30	70	47.5	52.5[d]	78.5	0.838[d]	1.435	waxy
LA-9	40	60	51.2	48.8[d]	79.5	0.770[d]	1.041	waxy
LA-10	50	50	54.1	45.9	83.6	0.720	0.789	friable solid
LA-11	60	40	64.1	35.9	87.5	0.575	0.627	friable solid
LA-12	80	20	81.7	18.3	92.0	0.414	0.423	friable solid
PEG-35 000								
LA-3	20	80	27.3	72.7[d]	72.5	18.930[d]	26.640	solid
LA-5	40	60	42.0	58.0	85.0	10.780	12.080	solid
LA-6	50	50	52.8	47.2	81.4	8.250	9.260	solid
LA-7	60	40	65.3	34.7	86.2	6.830	7.340	solid
LA-4	80	20	81.2	18.8	91.4	4.540	4.940	solid

[a] evaluated from ^{1}H NMR spectra

[b] theoretical values calculated from the feed composition

[c] OE and LA denote the repetitive oxyethylene and lactoyl units, respectively

[d] LA content and experimental $\bar{M}_n$ markedly lower than the theoretical ones for unreacted monomer.

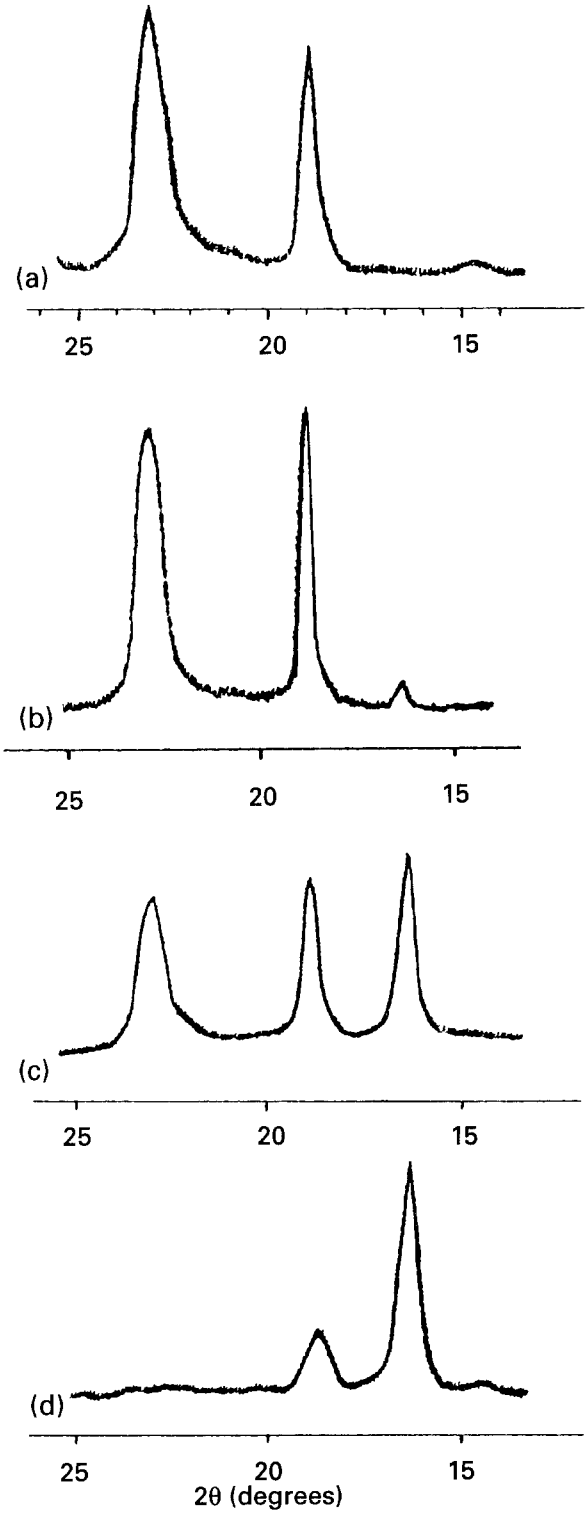


Figure 1 Powder X-ray diagrams of PEG and PLA homopolymers and two PLA–POE–PLA copolymers with different molar composition ((a) PEG; (b) LA-4; (c) LA-5; (d) PLA).

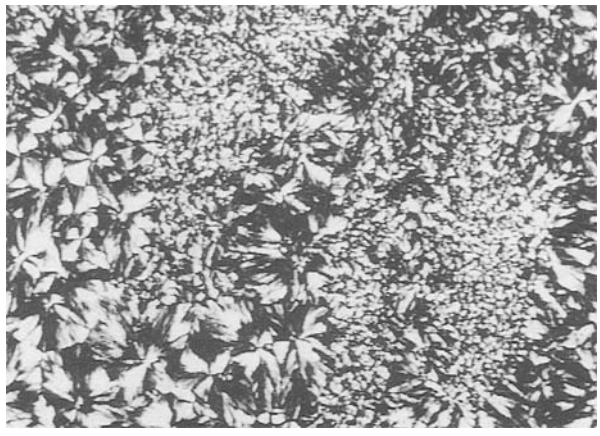


Figure 2 Polarized light micrograph of isothermal bulk crystallization at 30 °C of the PLA–POE–PLA copolymer LA-6 in Table I (1.33 cm corresponds to 100 μm).

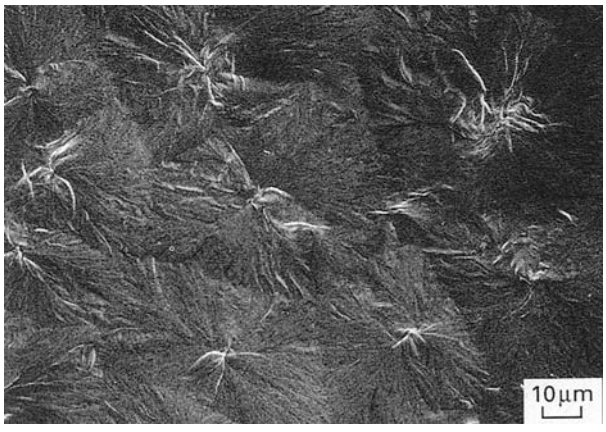


Figure 3 Scanning electron micrograph (SEM) of the PLA–POE –PLA copolymer LA-4 in Table I.

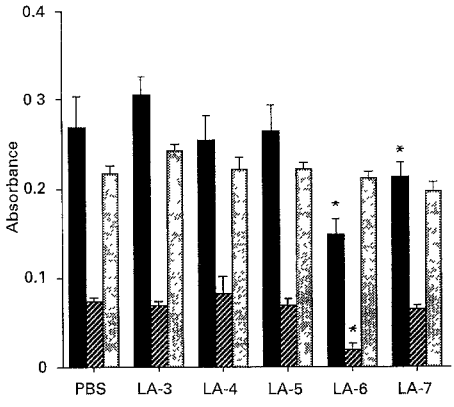


Figure 4 Cytotoxicity tests of the PLA–POE–PLA copolymers obtained with PEG-35 000. Comparison with the negative control (PBS) is shown (* $p < 0.05$, Fisher test). ■ NR; ▨ MTT; ▩ KB.

Dynamic-mechanical thermal analysis (DMTA) revealed that each copolymer has only one T_g (glass transition temperature) whose value is a function of the molar composition and is intermediate between those of the PEG and PLA homopolymers (− 32 °C and 55 °C, respectively). T_g values are relatively high and around or above room temperature (ranging from − 4 °C to 38 °C), so the materials are waxy or brittle and their mechanical properties not good for implant devices. This is valid for the copolymers obtained from PEG 35 000 and, to a greater extent, for those prepared from PEG 3000 also, which have low molecular weights (4000 to 23 000 Dalton) and are waxy with low melting temperatures (40–50 °C).

In order to establish the biomedical field where the PLA–POE–PLA copolymers synthesized might be used, their biocompatibility was investigated.

Concerning their cytotoxicity, the copolymers obtained from PEG-35 000 showed good behaviour when their extracts were brought in contact with 3T3 cells for 24 (data not shown) or 72 h (Fig. 4).

A one-factor ANOVA was performed, for the different tests, to assay whether the independent variable factor type of polymer significantly influenced the results, expressed as absorbance. The result of this analysis was significant for the NR assay ($p = 0.0001$). In particular, if each copolymer was compared with the negative control (NC), we found that both LA-6 and LA-7 were more toxic than NC at lysosomal level (Fisher test, $p < 0.05$). The mitochondrial activity was also influenced by the copolymer extract (one-factor ANOVA, $p < 0.001$). A negative behaviour was observed for the LA-6 copolymer ($p < 0.05$).

No difference was found between the total protein content of the cells in treated wells compared with the controls (KB test).

The copolymers LA-10, LA-11 and LA-12, obtained from PEG-3000 were more cytotoxic than those of the first series at both lysosomal (NR) and mitochondrial (MTT) levels ($p < 0.05$), when compared with the negative controls.

The cytocompatibility test gave for both series negative results. The copolymer films of the first series swelled when the cell culture medium was added, while those of the second series either broke (LA-8, LA-13) or were soluble to various degrees (LA-9, LA-10, LA-11, LA-12) in the acqueous culture medium solution. No adhesion or proliferation test was therefore possible.

The general poor qualities of the copolymers obtained from PEG-3000 may be tentatively ascribed to the marked racemization of L-lactide observed during synthesis, which gave non-stereoregular amorphous PLA sequences. The high solubility of their films in acqueous medium was to be expected for polymers which have a relatively high hydrophilicity and low molecular weights.

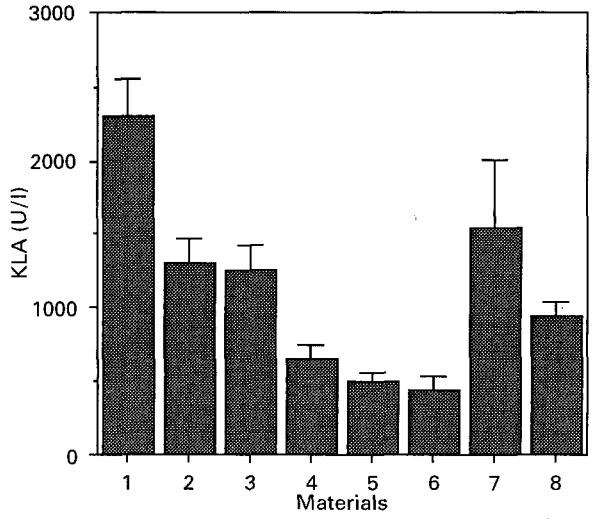


Figure 5 Kallicrein-like activity (KLA) induced from PLA–POE–PLA copolymers with different composition, compared with KLA values of glass and silicone; 1. glass; 2. PLA homopolymer; 3. LA-3 sample in Table I; 4. LA-5; 5. LA-6; 6. LA-7; 7. LA-4; 8. silicone.

With regard to the hemocompatibility of the materials, Fig. 5 shows the KLA induced in the plasma by the copolymers obtained from PEG-35 000, compared with that induced by PLA homopolymer (100% mole LA), borosilicate glass (as a high-activation reference) and silicone (as a low-activation reference). Each point is the mean value of five experiments.

The activation induced by all the copolymers is, as expected, much lower than that induced by the glass. The copolymer LA-3 (80% LA, 20% OE units) shows a KLA higher than that of silicone, but near to that of PLA homopolymer (100% LA units). A better behaviour is presented by LA-5, LA-6 and LA-7 copolymers with an intermediate LA content (60% to 40%), which have a KLA much lower than that induced by silicone. Moreover, a statistical treatment of the data based on the Student t-test shows a highly significant difference ($p < 0.01$) between LA-6 and LA-7 copolymers and silicone. Surprisingly, the LA-4 sample (20% LA, 80% OE units) has a KLA value much higher than that of silicone. This can reasonably be considered due to the high hydrophilicity of the sample (80% OE), which results in significant diffusion of water through the copolymer film, which breaks up, giving an unreliable KLA determination.

Concerning the copolymers obtained from PEG-3000, they have a PKK activation independent of their composition and even higher than that of pure poly(L-lactide) homopolymer. As previously said, this anomalous behaviour may be tentatively attributed to the non-controlled structure of the copolymers because of the intense racemization process observed.

In Fig. 6 the degradability *in vitro* of the LA-3 and LA-4 copolymers of Table I is compared with that of PLA homopolymer. The degradation rate of both copolymers is higher than that of PLA and increases with the OE content (LA-4 > LA-3). Increasing the relative content of the hydrophilic POE block facilitates the diffusion of water and the hydrolytic erosion of the copolymer is enhanced.

This result is in agreement with the results of other authors [3] and confirms the possibility of modulating the degradation rate of the materials by varying their composition.

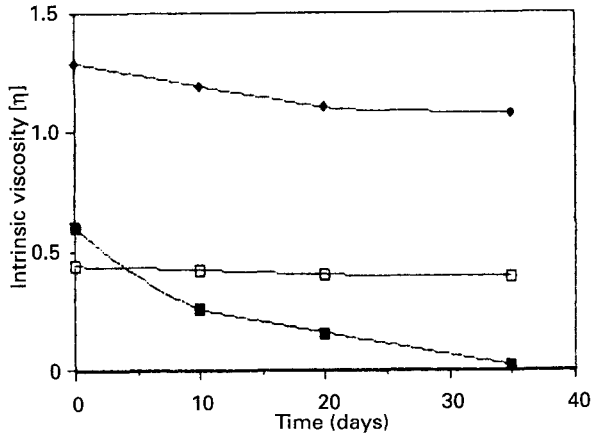


Figure 6 Degradation test of PLA–POE–PLA copolymers *in vitro* at 37 °C in water. Dependence of intrinsic viscosity [η] (dL/g) on time (◆ LA-3; □ PLA; ■ LA-4).

4. Conclusions

Poly(L-lactide)–poly(oxyethylene)–poly(L-lactide) triblock copolymers with different molecular mass and hydrophilicity were prepared by a direct synthetic procedure in the absence of potentially toxic organometallic catalysts.

The physico-chemical characterization of the PLA–POE–PLA copolymers obtained from PEG-35 000 was previously performed by different instrumental techniques [8].

The results obtained by means of X-ray diffractometry, optical and scanning electron microscopies, permitted us to obtain information about the crystallinity of the copolymers and to correlate their morphology with molar composition.

Concerning the biological properties, the results obtained indicate that the copolymers from PEG-35 000 can be considered biocompatible, since they are hemocompatible and non-cytotoxic.

The properties of the copolymers from PEG-3000 are quite different, since they are amorphous, waxy and rather cytotoxic. This behaviour must be ascribed to, besides the low molecular weight of the copolymers, the undesired racemization process of L-lactide monomer during synthesis.

Concerning the physico-mechanical characteristics of the materials, all the copolymers have one T_g (glass transition temperature), which is relatively high and around or above room temperature. Consequently, the materials are brittle or waxy and unsuitable for implant devices.

Conversely, the properties of both series of copolymers, which can be modulated by varying the feed composition, seem to suggest a potential application as a polymer matrix in drug delivery systems. That is supported by the results obtained by different authors [2, 3] with similar block copolymers. Experimental work on this aspect is in progress.

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