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Segmented Polyurethanes for Medical Applications: Synthesis, Characterization and *in vitro* Enzymatic Degradation Studies

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Summary: Degradable segmented poly(ether-ester-urethanes) of variable segment chemistry and content were synthesized and characterized. Polycaprolactone diol, a series of poly(ether-ester) block copolymers, and a diisocyanate giving non toxic degradation products were used to form the prepolymer. Cyclohexane dimethanol and a L-phenylalanine–based diester (Phe diester) were used as chain extenders. The influence of α-chimotrypsin on the degradation was investigated by exposing the polymers to buffer and enzyme solutions for 12 days. The SEM, SEC, and gravimetric results showed that a significant erosion of the Phe diester containing polymer compared with the control polyurethane occurred in the presence of the enzyme but not in a normal buffer solution.

Keywords: block copolymer; degradation; enzyme; film casting; polyurethanes

Introduction

There is a growing interest in the design of biodegradable polymers for specialized applications such as tissue engineering or controlled-release formulations of drugs. New versatile polymers are needed to fulfil the different requirements within these fields of applications.

A vast majority of biodegradable polymers developed for uses in clinical applications belongs to the polyester family [1-3]. Polyesters have been largely used in controlled drug delivery [4-6] and also have been considered for tissue engineering applications [7,8].

Segmented polyurethanes have received great attention as they possess a broad range of chemical and physical properties, due to the possibility of easily varying their chemical composition; moreover it is known that the blood coagulation is less severe for polyurethanes than for other materials. One of the most important problems in their use as biodegradable materials is the release

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of toxic diamines from conventional isocyanates. A L-lysine derived isocyanate [9,10] was applied in this work to overcome this problem because its perspective hydrolysis product, L-lysine, is not harmful to the living system.

Recently, Skarja and Woodhouse [11] described the synthesis of amino-phenyl-acetic acid 4-(2-amino-2-phenyl-acetoxymethyl)-cyclohexylmethyl ester (Phe diester) which permits to introduce easily hydrolyzable ester bonds in the polymer backbone. Moreover, Phe diester containing polyurethane-ureas undergo fast enzymatic erosion releasing degradation products presumably non toxic and readily metabolized *in vivo*. As reported by the authors, when polyethylene glycol (PEG) was used as soft segment the obtained polyurethane-ureas were completely amorphous materials, deformed easily, and very difficult to handle.

In this work, the synthesis of segmented polyurethanes and polyurethane-ureas containing the above mentioned building blocks and PEG containing soft segments (low-molecular-weight tri-block polycaprolactone-polyethylenglycol-polycaprolactone copolymers, PCL-PEG-PCL) is described. High–molecular-weight biocompatible tri-block copolymers synthesis through a non-catalyzed route was reported by Cerrai *et al.* [12] and their biocompatibility demonstrated [13, 14]. The introduction of soft segments of variable chemistry and composition leads to generate polymers that exhibit a diverse range of physical and chemical properties. Degradation studies in buffer or chymotrypsin solution were carried out to evaluate the specificity of enzyme-mediated polymer degradation and erosion.

Materials and methods

Materials

Where not specifically indicated, all reagents and solvents were purchased from usual commercial sources in the best quality available. The tri-block PCL-PEG-PCL copolymers, used as soft segment, were obtained from PEG (Merck, $\overline{M}_n$=600) and ε-caprolactone monomer (Fluka, distilled from CaH_2 prior to use) as described by Cerrai *et al.* [12]. The method reported by Storey *et al.* [10] was used to synthesize the ethyl-2,6-diisocyanatohexanoate (LDI). Phe diester was prepared according to Skarja and Woodhouse [11]. The control polyurethanes were synthesized using 1,4-cyclohexane dimethanol (CDM) as chain extender (Aldrich, mixture *cis* and *trans*). Dibutyltin dilaurate was obtained from Fluka.

Polymer synthesis and film casting

Segmented polyurethanes were obtained as follows: the soft segment was dissolved in 1,2-dichloroethane (DCE) and the solution dried azeotropically by refluxing under nitrogen over activated molecular sieves. LDI was reacted with the polycaprolactone diol (PCL diol) or the tri-block copolymers at 85°C, at a 2:1 molar ratio, in anhydrous DCE, to form the prepolymer. The prepolymer reaction proceeded for 150 min in the presence of dibutyltin dilaurate. The reaction temperature was subsequently reduced to 25°C and the chain extender, previously dissolved in DCE and dried azeotropically, was added at a 1:1 molar ratio with respect to the prepolymer. Chain extension reaction was carried out for approximately 18h and then stopped by the addition of methanol. The polymer was obtained by precipitation in petroleum ether, then collected by filtration, and dried at 35°C under reduced pressure. Low-molecular weight impurities were removed by rinsing with methanol. Polyurethane films were prepared by casting a 4% w/v polymer solution in chloroform ($CHCl_3$, 20 ml) into level 5.5 cm diameter poly(tetrafluoroethylene) dishes at room temperature. The cast films were removed from the dishes after 48h, dried at 35°C for 48h to eliminate any residual solvent, and stored in a desiccator.

Polymer characterization

Molecular weight and molecular weight distribution of the polymers were measured by size exclusion chromatography (SEC) using a PL Gel Mixed-C column, polystyrene standards, and chloroform as eluting agent.

Differential Scanning Calorimetry (melting points, T_m, glass transitions, T_g, and melting enthalpies, ΔH) was performed with a Perkin Elmer DSC-7 instrument for tri-block copolymer analysis (scan rates of 10°C min^{-1}, temperature range from -100 to +100°C, aluminium pans) and a Mettler Toledo Star System for polyurethane and PCL diol analysis (scan rates of 5°C min^{-1}, temperature range from -100 to +100°C, aluminium pans).

Film samples were examined with a Leitz Ortholux II POL-BK polarizing microscope.

Film samples were analyzed by a JEOL JSM 300 scanning electron microscope at 12 kV on samples sputter coated with gold.

Wide angle X-ray diffraction measurements (WAXS) were carried out with a Siemens 810 Cristallo Flex diffractometer, using Cu Kα radiation.

Water contact angles were measured with an optical contact angle meter (CAM 200, KSV instruments LTD). Samples were spin coated on glass slides, using a polymer solution of 4% w/v in $CHCl_3$. The angles were determined on 15 measurements for each polymer substrate and the average values were calculated.

Bulk hydrophilicity of the polyurethanes was quantified by measuring the amount of water that each polymer absorbed at 37°C. Polymer film samples with a surface area of 1x1 cm were weighed and placed in 20 ml deionized water containing glass vials and the vials placed in a shaking water bath at 37 (±0.1)°C. The film samples were removed after 48h, the surfaces dried and the samples weighed. To evaluate the degree of water absorption, the samples were weighed, dried under vacuum to constant weight, and then weighed again. The mass loss during drying was assumed to be equal to the water absorption.

Degradation experiments

The degradation experiments took place in 15 ml plastic vials in a mechanically shaken water bath at 37(±0.1)°C. Cast film samples were cut into square samples (1.5x1.5 cm), weighed, and placed in plastic vials containing 10 ml of chymotrypsin solution (500 U ml^{-1} in 0.036 M Tris with 0.045 M $CaCl_2$ and 0.02% $NaNH_3$, pH 8.0) or buffer solution (0.036 M Tris with 0.045 M $CaCl_2$ and 0.02% $NaNH_3$, pH 8.0). The samples were incubated from 2 to 12 days and the enzyme solution changed every 48h. Samples were withdrawn after 2, 4, 6, 8, 10, and 12 days and placed in 15 ml of 1% (v/v) solution of Triton X-100 to remove reversibly bound enzyme. The retrieved samples were then rinsed with deionized water, gently dried with soft absorbent paper, weighed, placed in a desiccator to thoroughly dry, and reweighed to determine mass loss due to the erosion.

Results and discussion

Tri-block PCL-PEG-PCL copolymer characterization

The experimental data concerning the molar composition, the molecular weights, and calorimetric analysis of the obtained copolymers are collected in Table 1. All the PCL-PEG-PCL tri-block copolymers are semi-crystalline, even if they show a quite different behaviour depending on molar composition. The crystallization pattern of different copolymers has been investigated by RX and DSC measurements and the ether-ester nature was proved by IR and ^1H-NMR spectroscopies.

Table 1: Experimental results concerning PCL-PEG-PCL three-block copolymers (C1 and C2) and PCL diol. OE: molar percentage of repetitive oxyethylene units; CL: molar percentage of repetitive oxycaproyl units.

Polymer	OE	CL	$\overline{M}_n$ [a]	$\overline{M}_n$ [b]	T_m /°C	ΔH [c] /Jg^{-1}	ΔH [d] /Jg^{-1}
C1	50	50	2200	2200	4; 37; 46	110	107
C2	35	65	3500	3300	7; 43; 52	23	98
PCL diol	/	100	/	1250	51	/	67

[a] Number-average molecular weight, theoretical values calculated from feed composition.
[b] From ^{1}H-NMR spectra.
[c] ΔH related to the melting of PEG block normalized with respect to PEG content.
[d] ΔH related to the melting of PCL blocks normalized with respect to PCL content.

Polyurethane characterization

Polyurethane molecular weights as measured by SEC are shown in Table 2. Molecular weight polydispersity presents low values for all polyurethanes. The PCL containing polymers exhibit higher molecular weights than the tri-block copolymers based polyurethanes. This finding can reflect the presence of contaminating water during the synthesis due to the highly hydrophilic nature of PEG. Water will competitively react with the diisocyanate limiting ultimate polymer molecular weight.

Table 2: Experimental results concerning polyurethanes.

Polymer	$\overline{M}_n$	$\overline{M}_w/\overline{M}_n$	T_g/°C	T_m/°C	ΔH [a]/Jg^{-1}
PCL diol/CDM	42300	2.07	-26,4	42.1	41.6
PCL diol/Phe	33200	2.43	-25.8	45.7	37.7
C1/CDM	16700	1.84	-46.4	47.4	41.4
C1/Phe	23900	2.01	-40.7	49.0	46.8
C2/CDM	19400	1.79	-46.6	52.4	70.3
C2/Phe	30200	2.02	-46.3	50.5	66.3

[a] ΔH normalized with respect to wt % soft segment in polyurethanes.

The major DSC events observed for all materials are summarized in Tab 2. All the samples show endotherms corresponding to PCL crystalline melting phenomena, appearing at 42-52°C, while the crystallization of the PEG segment is absent. No evidence of hard segment crystallinity is detected for any of the polymers. LDI is an asymmetric compound and also contains an ethyl ester side chain, both factors may inhibit chain packing in the hard segment.

DSC data of PCL diol, PCL diol/CDM, and PCL diol/Phe suggest that the crystallizability of PCL segment is significantly affected by the links with the hard segment. Moreover, Tab 2 shows that polyurethane crystallinity diminished with soft segment molecular weight. Reducing soft segment length was hypothesized to reduce polyurethane crystallinity due to an increasing frequency of disrupting hard segments along the polymer chain and reducing crystallizable PCL block concentration [15].

Fig 1-2 presents and compares the wide angle X-ray diffractograms of selected polyurethanes with their soft segment. The position of the crystal reflection in the diffractograms (2θ = 21.2, 21.8, and 23.5) agrees with those of PCL diol. This result confirms that the crystalline phase which develops in the polyurethanes is associated with crystallization of the block PCL in the soft segment. The diffractograms also indicate that the connection of the soft segment with the hard segment only decreases the crystallizability of the PCL block but has no effect on the crystal structure, as observed by Li *et al.* [16].

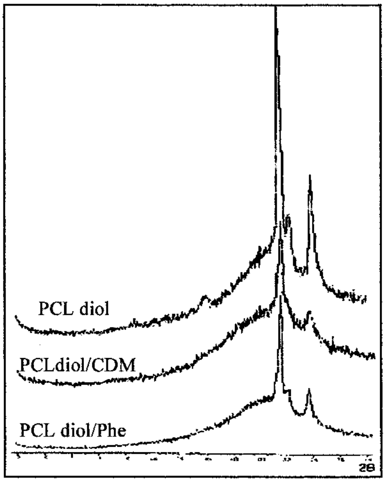

Figure 1: WAXS patterns of PCL diol and the corresponding polyurethanes.

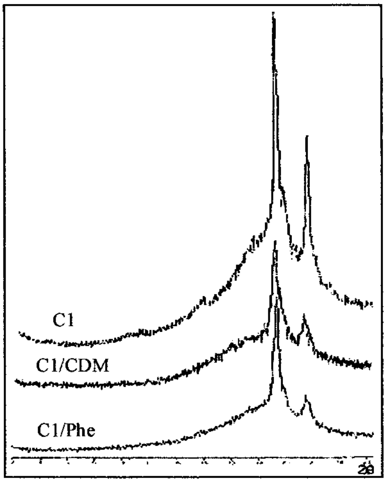

Figure 2: WAXS patterns of C1 macrodiol and the corresponding polyurethanes.

Fig 3 shows optical micrographs of film samples taken at room temperature The figures display the presence of spherulitic structures with different shapes and sizes, due to the crystallization resulting from the PCL component in the soft segment. Polyurethane series exhibits a crystalline phase more homogeneous than the respective polyurethane-urea; moreover, the comparison between the C1/CDM and C2/CDM samples shows that the spherulites become larger as the soft segment length increases.

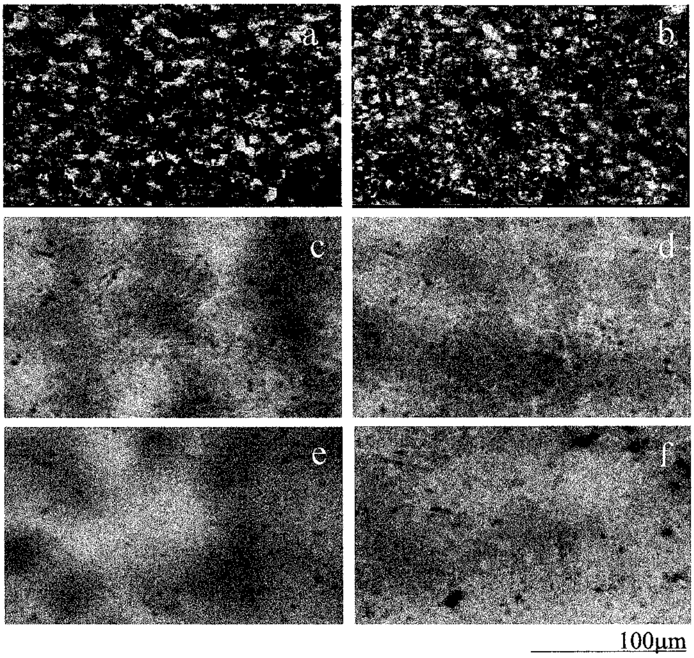

Figure 3: Optical micrographs of solution-cast samples: PCL diol/CDM (a); PCL diol/Phe (b); C1/CDM (c); C1/Phe (d); C2/CDM (e); C2/Phe (f).

To identify the bulk hydrophilicity of the polymers, water absorption measurements were carried out by placing the film samples in deionized water at 37°C for 48h. As expected, Tab 3 shows that water absorption is higher for PCL-PEG-PCL based polyurethanes than the PCL based ones. Moreover, water absorption increases with the PEG content, due to the high hydrophilic behaviour of PEG. Comparison of the CDM and Phe diester containing polymers indicates that the greater hydrophobicity of the diester chain extender imparts a lower water uptake.

Concerning water contact angle data (Tab 3), it is well known that the values obtained may be influenced by the fabrication procedure of samples and the kind of substrate; in this paper measurements were performed on samples prepared by spin coating to gain preliminary indications of their behaviour in influencing the process of cell attachment. The results were in fair agreement with the chemical composition of the polymers. The high surface hydrophilicity hindered a reliable determination of the water contact angle for the C1/CDM sample. Most of the results listed in Table 3 seems to be favourable for cell adhesion [17].

Table 3: Water Contact Angle and water absorption data of polyurethane series.

Polymer	Contact Angle/ deg	Mass increase/ %
PCL diol/CDM	80 ± 3	2.5
PCL diol/Phe	86 ± 2	1.9
C1/CDM	n.d.	11.0
C1/Phe	67 ± 5	8.4
C2/CDM	62 ± 3	6.6
C2/Phe	70 ± 2	4.3

Degradation and erosion experiments

C1/Phe and the control polyurethane (C1/CDM) were exposed to buffer and chymotrypsin solutions for 12 days. Degradation (defined as the polymer chain scission process) and erosion (defined as the loss of material owing to monomers and oligomers leaving the polymer) were analyzed by measuring mass loss and change in molecular weight. The mass loss profiles obtained are shown in Fig 4.

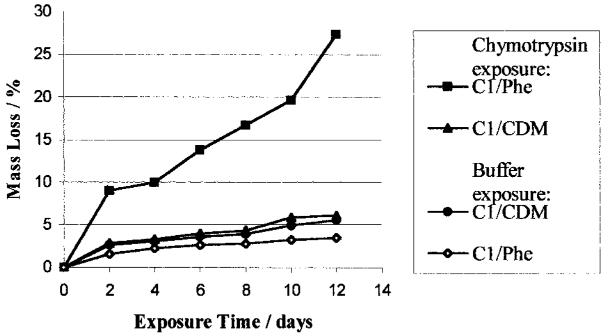

Figure 4: Mass loss profiles for C1/CDM and C1/Phe after chymotrypsin and buffer exposure.

In both cases, the highest tendency to erosion is observed when the polyurethanes were exposed to the enzymatic solution, in agreement with data previously reported by Skarja [18]. The higher erosion noted for C1/Phe, when incubated in chymotripsin solution has been attributed primarily to the inclusion of phenylalanine residues in the polymer backbone adjacent to hydrolyzable ester linkages, probably promoting a specific interaction with enzyme's active site. As indicated by Skarja, bulk hydrophilicity is the parameter primarily determining polymer erosion in buffer solution, moreover C1/CDM samples exhibit a slightly higher mass loss than C1/Phe ones.

Changes in the surface and cross-sectional morphology were investigated by SEM in polyurethane samples after 12 days of exposure to chymotrypsin and buffer solutions. The micrographs (Fig 5a and 5c) showed an obvious C1/Phe surface alteration after enzyme exposure. In Fig 6a and 6c, low morphological alterations confined to the surface can be noted for C1/CDM samples after 12 days of enzyme or buffer exposure, according to mass loss measurements. It could be expected that introduction of hydrophilic PEG chain enhances the water diffusion inside the polymer, accelerating the cleavage of the hydrolizable bonds in the macromolecular chain via a bulk mechanism. In contrast, in any case no relevant differences to the initially bulk texture were observed. This can be reflective of the low exposure time. In enzymatic solution, passive bulk degradation was hypothesized to proceed competitively with enzymatic surface degradation. The comparison of Fig 5b and 5d furnishes a clear indication that the enzyme catalyzed degradation is more efficient since it leads to an evident modification of the sample morphology, starting from the surface to the bulk, which is absent for control films.

Polymer degradation was determined by performing SEC analysis on samples exposed for 4, 8, and 12 days. No significant changes with time in relative number-average molecular weight can be detected for C1/CDM and C1/Phe, after buffer treatment, and for C1/CDM, after enzyme exposure. In these cases, the alteration was surface-limited as noted by SEM analysis and the degradation of the surface region was not measurable by SEC, which analyses bulk properties. In contrast, C1/Phe samples demonstrated (Fig 7) a decreasing weight-average molecular weight with increasing chymotrypsin exposure. Enzymatic degradation occours via a surface erosion [19], then the bulk degradation probably is resulted from the marqued surface alteration that gave rise to deep cracks spreading in the section of film samples, as noted by cross-sectional micrographs.

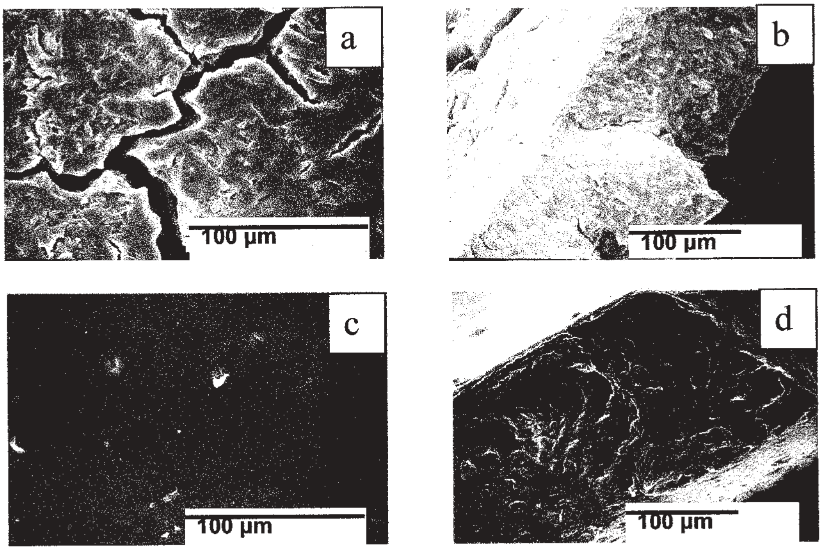

Figure 5: SEM of C1/Phe sample after 12 days of enzyme (a, b) and buffer exposure (c, d).

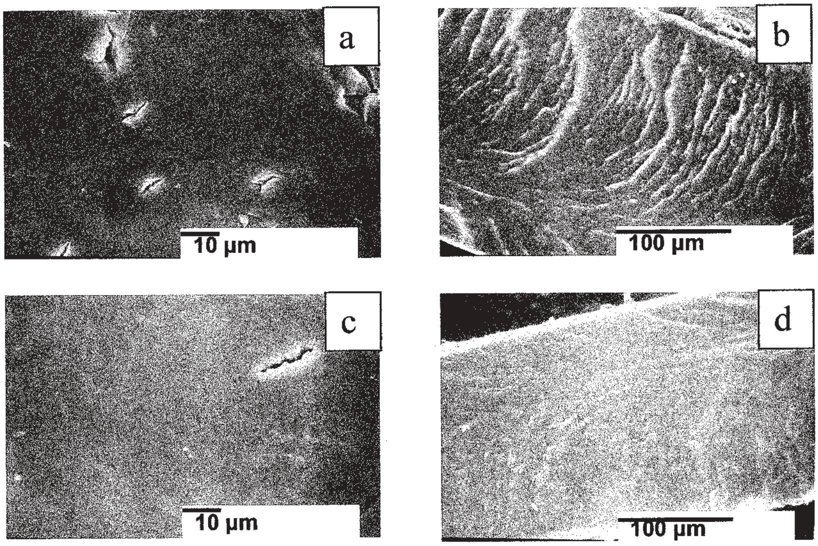

Figure 6: Surface and cross-sectional scanning electron micrographs of C1/CDM sample after 12 days of chymotrypsin (a, b) and buffer exposure (c, d).

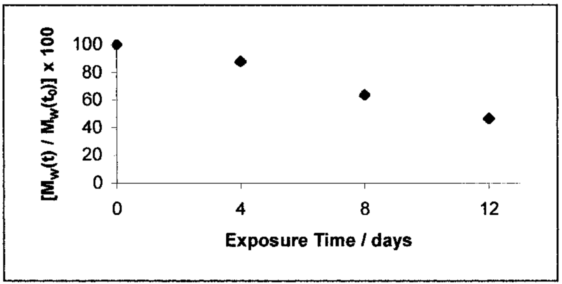

Figure 7: Change with time in relative $\overline{M}_w$ of C1/Phe film for chymotrypsin exposure.

Conclusion

A series of polyurethanes and polyurethane-ureas for medical applications was prepared by using biocompatible constituent blocks and was characterized. Introduction of tri-block copolymers in segmented chain leads to new polymers with tunable thermal properties, crystallinity and crystallites phase distribution, bulk hydrophilicity, and degradation characteristics. Preliminary tests need to be further confirmed in order to propose these materials in biomedical applications.

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