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The biodegradation of poly(ester-ether-ester) block copolymers in a cellular environment *in vitro*

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In this paper we report about the biodegradation of tri-block poly(ester-ether-ester) copolymers obtained by reaction of preformed poly(ethylene glycol) (PEG) with two different lactone monomers, i.e. ε-caprolactone (CL) and L-lactide (LA). The two series of copolymers will be indicated as PCL-POE-PCL and PLA-POE-PLA. We identified and measured by HPLC analysis the amount of degradation products of the poly(ester-ether-ester) copolymers; three copolymers were tested for each series during 3–8 week experiments. The experiments were carried out both in the presence and absence of fibroblast cell populations. We evaluated at the same time the decrease of copolymer molecular mass after degradation by means of intrinsic viscosity [η] measurements. From the [η] measurements we can conclude that the higher the hydrophilicity of the material, the faster the rate of decrease of its intrinsic viscosity with time. The HPLC results indicate that the amount of the degradation products, i.e., respectively, the monomers 6-hydroxyhexanoic acid and L-lactic acid, is a function of both hydrophilicity of the molecule and the lateral block length. When the fibroblast cell populations were present in the same wells together with the biodegradable copolymers, signs of cellular metabolism of the degraded monomers were detected.

1. Introduction

The development of biodegradable polymers can be considered as one of the major advances in biomedical materials research. Biodegradable materials have numerous applications such as bioresorbable sutures, surgical prostheses and drug delivery systems. As a future perspective the availability of totally or partially biodegradable stable implants would eliminate the need for device removal following use and would allow the regeneration with time of host own tissue. In this paper we report about the biodegradation of tri-block poly(ester-ether-ester) copolymers obtained by reaction of preformed poly(ethylene glycol) (PEG) with two different lactone monomers, i.e. ε-caprolactone (CL) and L-lactide (LA). The two series of copolymers will be indicated as PCL-POE-PCL and PLA-POE-PLA. The synthesis and the physicochemical characterization of the copolymers have been previously reported [1, 2]. Their biocompatibility was also ascertained [2, 3]. Although it is known that hydrolytic breakdown is responsible for poly(ester-ether-ester) block copolymer degradation into metabolizable molecules, very little is known about the degradation kinetics, the amount of released materials, and how the degradation products interact with the cellular metabolism. Furthermore new insight is needed into how the (enzymatic) products of cellular metabolism could interact with the biodegradation process. We identified and measured by HPLC analysis the amount of degradation products of the poly(ester-ether-ester) copolymers; three-four copolymers were tested for each series during 3–8 week experiments. The experiments were carried out both in the presence and absence of fibroblast cell populations. We evaluated at the same time the decrease of copolymer molecular mass after degradation by means of intrinsic viscosity [η] measurements. A test of the cell viability, i.e. lactate dehydrogenase (LDH) release, was carried out.

2. Materials and methods

2.1. Copolymer synthesis

The chemical synthesis of the PCL-POE-PCL and PLA-POE-PLA tri-block copolymers was carried out according to the general scheme shown in Fig. 1. The copolymer formation occurs through a ring-opening mechanism, where the active hydrogen atoms of the preformed PEG determine a selective acyl-oxygen cleavage of the lactone ring. After the initial formation of an intermediate bis-ε-hydroxy-diester, a step-by-step addition of monomeric lactone units occurs with

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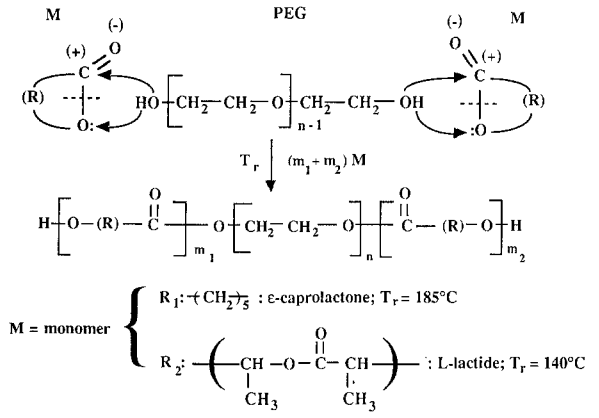

Figure 1 General scheme of reaction for the chemical synthesis of the PCL-POE-PCL and PLA-POE-PLA tri-block copolymers.

formation of two external polyester blocks, the length of which depends on the amount of lactone monomer ($m_1 + m_2$ moles) used in the feed. The syntheses are carried out in bulk, without added catalysts and thermally activating the hydrogen atoms of PEG at suitable reaction temperatures (T_r), different for each monomer. The T_r value for PCL-POE-PCL copolymers was 185 °C, while PLA-POE-PLA copolymers were obtained at $T_r = 140$ °C. Using PEG prepolymers with different molecular mass and varying the initial M to PEG molar ratio, copolymers with different molecular mass, different composition and consequently different average lengths of the ester blocks were obtained.

2.2. Biodegradation assay

3T3 cells of a fibroblast cell line were seeded on to six-well culture plates (seeding density = 5×10^3 cells per cm^2) and cultured in complete cell culture medium for 4 days. Pre-weighed copolymer specimens (CL16, CL24, CL27, CL28 [2] and LA3, LA4, LA5 [3]), sterilized by ethylene oxide, were laid on to polycarbonate microporous (pore size 3 μm) inserts (Transwell, Costar, I) hanging from the top of the culture wells, allowing study of the cell–material interactions. In different experiments the copolymers were either in the form of powder or bars made by powder compression. Cell culture medium (3 ml) was added to cover the cells and the material specimens completely. Twice a week at regular time intervals, cell culture medium was completely withdrawn from the wells at fixed times, replaced with fresh medium, stored at − 20 °C, and used for HPLC analysis.

2.3. [η] measurements

Portions of the copolymer specimens were taken from *ad hoc* wells at fixed times and [η] determined by means of an Ubbelhode viscometer.

2.4. HPLC analysis

The amounts (g/l) of either 6-hydroxyhexanoic acid or L-lactic acid, the degradation products of, respectively, PCL-POE-PCL and PLA-POE-PLA copolymers, were quantified by HPLC analysis. A Perkin-Elmer 410 LC pump was used with a 25 cm C18-Beckman Ultrasphere IP column. Measures were performed at 210 nm.

2.5. LDH assay

LDH activity (U/l) in the culture medium was measured photometrically as the rate of decline of the concentration of NADH reacting with sodium pyruvate; this rate is directly proportional to the LDH activity in the sample material (Merckotest®, Merck, D).

2.6. Cell-free experiments

A similar protocol was used to perform cell-free experiments in which no cells were seeded on to the lower wells and the materials specimens were kept under sterile distilled water.

3. Results

3.1. [η] measurements

The decrease of copolymer molecular mass was estimated by [η] measurements at different times. The CL bar specimens in water did not show significant intrinsic viscosity changes for CL16, while the same parameter decreased in both CL24 and CL28 but with a different time course (Fig. 2a), which seems to indicate faster degradation of CL24. In the same CL series, but with powder specimens, the faster degradation of CL24 compared to CL28 (Fig. 2b) was confirmed. In Fig. 2b copolymer C27, which has the same molecular mass as CL16 but different copolymer composition [2], is shown for comparison. In the LA bar specimen series in distilled water, LA4 showed the fastest rate of intrinsic viscosity decrease followed by LA5, while LA3 was characterized by a slower and constant decrease with time (Fig. 3a). The presence of viable fibroblast populations in the same wells with the copolymers seemed to induce a slowing down of the rate of [η] decrease for both the CL (data not shown) and the LA series (Fig. 3a, b). The rate of [η] decrease of LA4, the most hydrophilic of the LA series, was very rapid, falling to very small figures both in water and with the cells (Fig. 3a, b).

3.2. HPLC analysis

The chemical products released by degradation depend on the polyester blocks present in the copolymer under study. The chemical nature of the products obtained after degradation was tested by a HPLC technique. For the PCL-POE-PCL copolymers the expected degradation compound was the 6-hydroxyhexanoic acid, $HO-(CH_2)_5-COOH$, while from PLA-POE-PLA copolymers lactic acid, $CH_3-CHOH-COOH$, is released. HPLC analysis revealed that 6-hydroxyhexanoic acid was detected after 20 min, and L-lactic acid after 4.6 min. Since the peak areas are proportional to the compound concentration in the sample, we could identify and quantify the

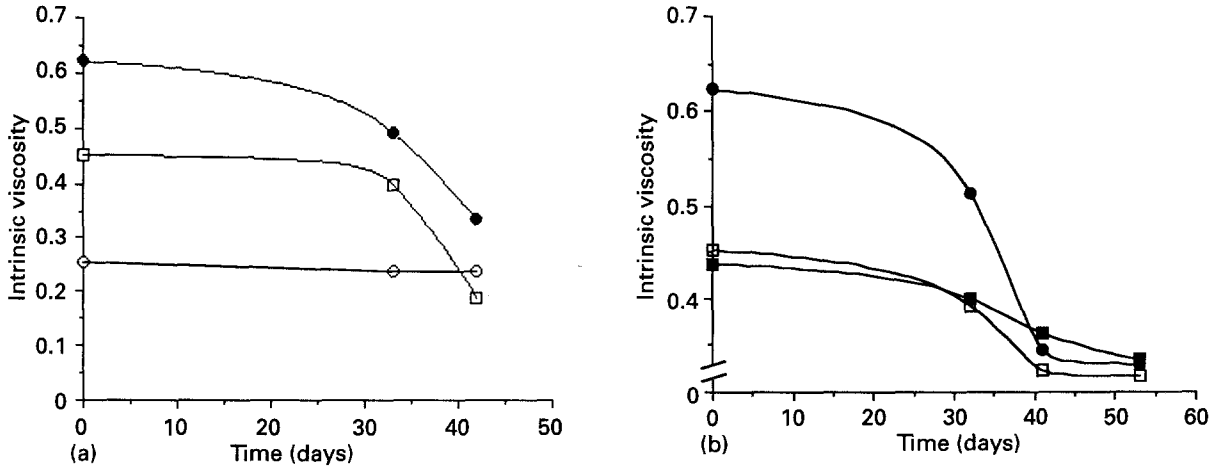

Figure 2 Viscometric analysis of block copolymers PCL-POE-PCL during degradation in water. (a) Compressed copolymer bars (● CL24; □ CL 28; ○ CL16) and (b) powder specimens (● CL24; □ CL28; ■ CL27) were kept in wells in contact with water, for, respectively, 42 and 54 days. At given time intervals the copolymers were tested for intrinsic viscosity [η]. Copolymers CL 16 (a) and CL27 (b) were available for analysis only in the form of bars and powder, respectively.

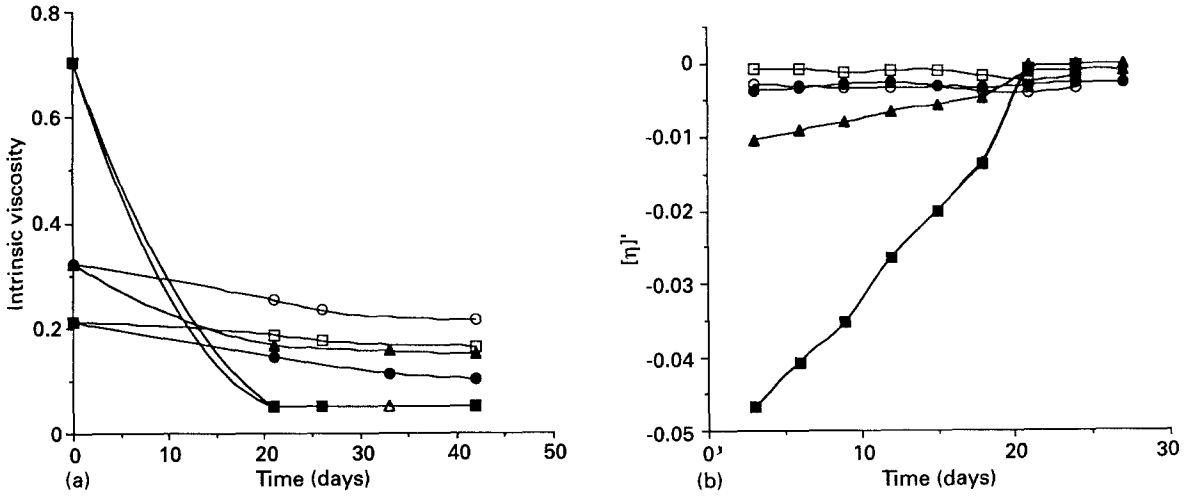

Figure 3 Viscometric analysis of block copolymers PLA-POE-PLA during degradation both in water (c) and in the presence of fibroblast cell populations (T). In (a) the viscometric curves both in the presence (T) and absence (c) of cells are compared. In (b) the first derivatives of the interpolated curves as shown in (a) are given for all the copolymers of the series. □ LA3 T or T'; ■ LA4 T or T'; ○ LA 5 T or T'; ● LA 3 c or c'; △ LA 4 c or c'; ▲ LA 5 c or c'.

degradation products per week during the 3–8 week experiments. The amounts of both 6-hydroxyhexanoic and L-lactic acid present in the assayed wells varied in quantity and in time between the different copolymers. The following results are related to the cell-free experiments. Signs of lateral block erosion in CL16, which was not suggested by [η] analysis (Fig. 2a), were present mainly during the first week, with a gradual slowing down. The amounts of CL24 and CL28 degradation products were high at the beginning of the analysis, then there was a decrease with time followed, only for CL24, by a new wave of increase during weeks 5 and 6 (Fig. 4a).

The amount of L-lactic acid detected and measured in our samples after the first week was the highest for LA5 followed by LA4 and LA3. During the following weeks a decrease of the relative amounts of released L-lactic acid was observed in all the specimens (Fig. 5a).

The degradation of the copolymers in the presence of fibroblast populations is shown in Fig. 4b and 5b. In the PCL-POE-PCL series the CL16 time course did not seem to be altered even if the amounts of 6-hydroxyhexanoic acid detected are slightly lower than in the controls (Fig. 4a,b). CL24 presented during the first weeks lower degradation than in the control wells (Fig. 4b). CL28, in contrast, showed a higher degradation during the first week, followed later by a decrease (Fig. 4b). The PLA-POE-PLA series degradation in the presence of cells is shown in Fig. 5b. For LA5 the amount of L-lactic acid is lower than in the controls, mainly after the first week. This holds particularly for LA4 where at the third and fourth weeks the L-acid amounts were lower than those released by the fibroblasts in the absence of the copolymers (Fig. 5b).

3.3. LDH measurements

The LDH measurements (data not shown) did not exhibit signs of cytotoxicity due to the presence of the copolymers, thus confirming their excellent biocom-

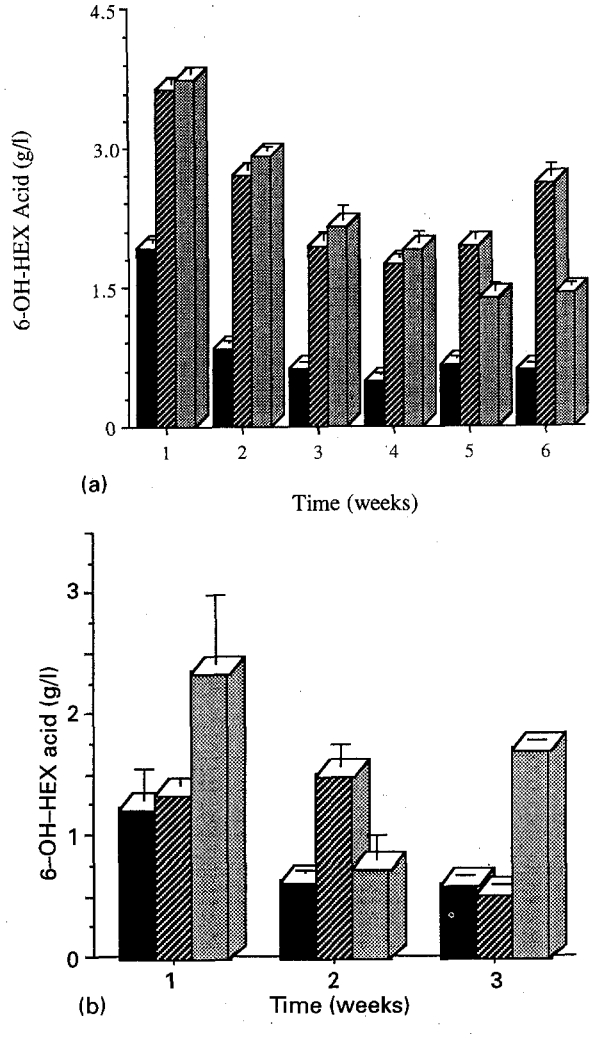

Figure 4 HPLC analysis of PCL-POE-PCL degradation. In (a) the release of 6-hydroxyhexanoic acid in time is given for the copolymers in water (controls), while in (b) the degradation in culture medium and in the presence of fibroblast cell populations is shown. The data are the mean (± SE) of two determinations. ■ CL16; ▨ CL 24; ▩ CL28.

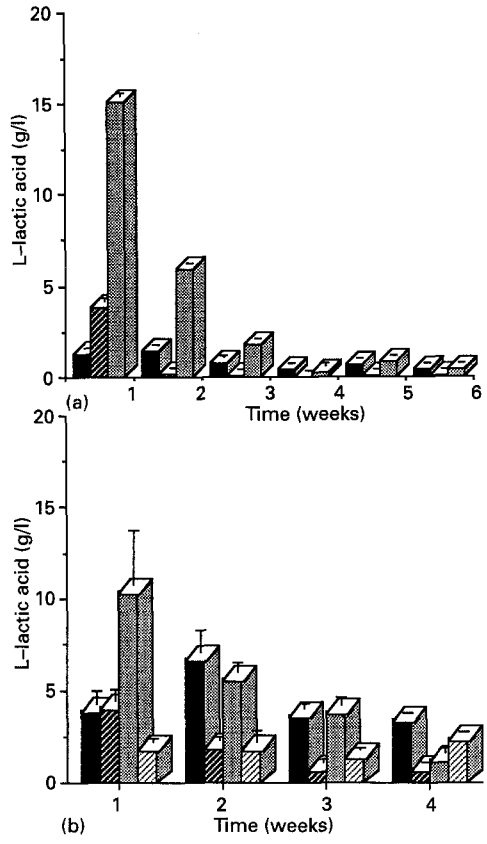

Figure 5 HPLC analysis of PLA-POE-PLA degradation. In (a) the release of L-lactic acid in time is given for the copolymers in water (controls), while in (b) the degradation in culture medium and in the presence of fibroblast cell populations is shown. The production of L-lactic acid by the control fibroblast populations not kept in contact with the copolymers is indicated as cells. The data are the mean (± SE) of two determinations. ■ LA 3; ▨ LA 4; ▩ LA 5; ▨ cells.

patibility [2, 3]. On the contrary, there seems to be a decrease in LDH activity in the wells where the copolymers were present.

4. Discussion

Depending on their chemical structure, the blocks in the copolymers may have different degrees of hydrophilicity, so that at different molar compositions of the copolymer a higher or lower hydrophilicity may occur. The degree of hydrophilicity of the blocks involved may be assumed as:

$$-(CH_2-CH_2-O)- \;>\; -(CO-CHCH_3-O-CO-CHCH_3-O)- \;>\; -(CO-(CH_2)_5-O)-$$

POE PLA PCL

In the copolymers having a higher degree of hydrophilicity, the diffusion of water is facilitated and the hydrolytic erosion of copolymer will be enhanced, when the same is maintained in contact for prolonged time with either water or cell culture medium. If we were to score for hydrophilicity the copolymers under study we would find: CL24 > CL28 > CL27 > CL16 and LA4 > LA5 > LA3.

In order to investigate the degradation behaviour of the different copolymers, [η] measurements have been carried out with time in $CHCl_3$ solutions. From the [η] measurements we can conclude that the higher the hydrophilicity of the material, the faster the rate of decrease of its intrinsic viscosity with time. Even if the viscometry method is generally not very suitable to evaluate the molecular mass of copolymers having blocks with different chemical nature and different polydispersity [4], relative viscometric changes with time for each sample, as shown in Figs. 2 and 3, can reasonably be assumed as indicative of the degradation. As to the correlation between the [η] measured at time $t = 0$ (Fig. 3) and the molecular weights $\bar{M}_n$ derived from the NMR spectra [2, 3], we have found substantial disagreement. The $\bar{M}_n$ values increase as expected from 4.54×10^4 to 18.93×10^4 from LA4 to LA3 copolymers, by increasing the LA lactide content

and consequently the ester blocks length. Conversely, the [η] values seem independent of the LA content and increase by increasing the oxyethylenic (OE) central block. [η] is 0.3 for the LA3 and 0.7 for the LA4 sample (Fig. 3). These experimental results can tentatively be explained by admitting that the solute–solvent interaction in the copolymer solutions is "effective" only for the oxyethylenic blocks, but "very low" for the lactidic ones. The scarce non-specific interaction between the lactidic sequences and chloroform seems to be confirmed by the low increase of [η] in lactide homopolymers with molecular mass 2000 and 100 000, respectively. The [η] values do not increase linearly with molecular mass, being 0.15 for the first one and 0.60 for the second one (unpublished results). A similar behaviour can be postulated for the PCL-POE-PCL copolymers.

The HPLC results for both series of copolymers, as shown in Figs 4 and 5, indicate that the PLA-POE-PLA series, which is the most hydrophilic, degrades faster than the PCL-POE-PCL series. Copolymer LA4 (copolymer composition: 81.2 mol % OE and 18.8 mol % LA) and copolymer CL24 (copolymer composition: 68.3 mol % OE and 31.7 mol % CL) (Figs. 4 and 5) [2, 3], are similar in that both are with short lateral blocks in percentage. If we compare them we notice that, while in LA4 the release of lactic acid monomer is the highest during the first week and goes rapidly to small figures, CL24 release continues at higher and almost constant levels during the whole experiment.

In the PCL-POE-PCL series in water (Fig. 4a) the degradation trend in time is quite uniform during the first four weeks for all the samples. CL16 which has long lateral blocks (79 mol % CL) [2] releases 6-hydroxyhexanoic acid in small quantities compared, for example, to CL24 (14 mol % CL) and CL28 (48 mol % CL) [2]. In the same series, but in the presence of cells, CL16 does not change its degradation time course, which correlates well with the viscometric analysis (Fig. 2) and the mechanical properties of the copolymer [2].

In the PLA-POE-PLA series in water we notice that LA3, which is the most hydrophobic, releases during the first week the least quantity of lactic acid monomer, compared with the others. LA5 possesses intermediate characteristics of hydrophilicity and lateral block length compared with LA4 and LA3. LA5 releases during the first weeks the highest lactic acid monomer quantity. This might be explained by the combined action of the relative hydrophilicity and of the lateral block length which facilitates the hydrolytic attack.

Our data are in accordance with recent published results [5]. The hydrolytic degradation of our copolymers seems to be characterized by a first phase due to the diffusion of water into the molecules and by a second phase due to the hydrolytic scission of the lateral blocks.

The fibroblast populations release lactic acid, as shown by the controls without the presence of the copolymers (Fig. 5b). Therefore in the samples where the cells were put in contact with the copolymers, the amount of lactic acid measured by HPLC should be considered as the resultant of both cellular production and copolymer degradation and cellular metabolic activity.

The results shown in Fig. 5b indicate the presence of an active L-lactic acid metabolism. For example the amounts of lactic acid detected in the LA4-containing wells at weeks 3 and 4 (Fig. 5b) are lower than would be expected in the presence of cells only.

It has to be carefully evaluated whether the LDH data might be interpreted in the sense of a metabolic recycling of the degradation products. Lactate is a dead end in cellular metabolism. It must go back to pyruvate before it can be metabolized and this reaction is catalysed by LDH. The metabolic pathway of 6-hydroxyhexanoic acid, as far as we know, has not yet been investigated and deserves further study. Further work is needed to give a more complete insight into the cellular metabolic pattern of both L-lactic and 6-hydroxyhexanoic acids.

Acknowledgements

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References

1. P. CERRAI and M. TRICOLI, *Makromol. Chem. Rapid Commun.* **14** (1993) 529.
2. P. CERRAI, G. D. GUERRA, L. LELLI, M. TRICOLI, R. SBARBATI DEL GUERRA, M. G. CASCONE and P. GIUSTI, *J. Mater. Sci. Mater. Med.* **5** (1994) 33.
3. P. CERRAI, M. TRICOLI, L. LELLI, G. D. GUERRA, R. SBARBATI DEL GUERRA, M. G. CASCONE and P. GIUSTI, MIM 20 (7/5/94).
4. G. RIESS, T. HURTREZ and P. BAHADUZ, "Encylopaedia of Polymer Science Engineering" (Wiley, New York, 1985) p. 324.
5. S. S. SHAH, K. J. ZHU and C. G. PITT, *J. Biomater. Sci. Polymer Edn* **5** (1994) 421.