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Interaction of fibroblast with poly(*p*-dioxanone) and its degradation products

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In vitro techniques were used to evaluate the interactions between Fibroblastic cells and Poly(*p*-Dioxanone) PPDX and compared its performance with that of other polymeric substrates. *In vitro* biocompatibility was assessed by studying cell adhesion and cell growth of cells on the polymer films themselves as well as in media enriched with the degradation products of PPDX and Poly(glycolic)/Poly(L-lactide 90:10 copolymer (PGLA-910). Our results show that althought all polymers tested were suitable for initial attachment, PPDX proved to be the most favorable surface for cell growth; as cell density after 48 h of culture was similar to that obtained on tissue culture Polystyrene TCPS (control). No signs of cell damage were detected using scanning electron microscopy (SEM) where after 48 h. of cell seeding on PPDX, fibroblast exhibited a confluent cell multilayer similar to TCPS. In addition, the products of the hydrolytic degradation of PPDX had no citotoxic effect on the adherence and proliferation of fibroblastic cell on TCPS. The hydrolytic degradation starts in the amorphous regions, as the tie-chain segments in these regions degrade into fragments causing the pH decrease in buffer solution and weight loss of degradable polymers. The *in vitro* evaluation suggests that PPDX may be candidate biomaterial for the construction a cell-polymer matrix.

1. Introduction

A wide variety of polymeric materials have been evaluated as potential biomaterials due to the great variety of their compositions, structures and properties. In recent years, the development of biodegradable polymers is considered one of the major advances in biomedical materials research, and are currently being employed in a variety of medical and surgical applications, such as the temporary repair and regeneration of tissues [1, 2]. A more recent application has been the use of biodegradable polymers as scaffolds in the development of synthetic graft materials as an alternative to the traditional use of allograft and autograph tissues [3].

This growing interest has led to a new area of research concerned with the development of biomaterials designed to assume the mechanical and other functional roles of the tissues they are replacing, to be biocompatible, a concept based on the interactions between a biological environment and the biomaterial and finally, to degrade *in vivo* in a controlled manner over an implantation period.

The biocompatibility of a test materials has been examined either *in vivo*, via implantation or injection of the test material, or *in vitro*, using appropiate cells that play important role during wound healing and regeneration processes. The latter have the advantage with respect to *in vivo* studies that cell/polymer interactions can be studied without the wound reaction and immune response that normally occurs *in vivo* studies.

In determining the biocompatibility of a given biopolymer there are a number of aspects to be considered. The first is concerned with the topography and composition of the material, surface chemistry has been shown to significantly affect cell adhesion and proliferation [4]. The second is concerned with the type and amount of formed degradation products that can influence the long term properties of the materials and cause cytotoxic effects [5].

In recent years many investigations have reported on the degradation rates and biocompatibility of a variety of bioadsorbable materials [6]. Aliphatic polyesters containing flexible ester bonds such as poly (glycolic acid), poly (L-Lactic Acid), and their copolymers of poly(Lactic and Glycolic acid) appear to be the most promising because of their biocompatibility and variable degradation [7–9]. Another poly-hidroxy-ester with similar characteristics is Polydioxanone (PPDX), this biodegradable material shows in principal adequate molecular structure and properties to be used in tissue regeneration [10, 11]. Moreover, PPDX has been approved by the Food and Drug Administration and is widely used as a suture material in ginecology [1].

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However, no systematic study has been performed to determine the biocompatibility of PPDX. With this objective we have used *in vitro* techniques to evaluate the interactions between fibroblastic cells and PPDX and compared its performance with that of other polymeric substrates. *In vitro* biocompatibility was assessed by studying cell adhesion and cell growth of fibroblastic cells on the polymer film themselves as well in media enriched with the degradation products of PPDX.

2. Materials and methods

2.1. Materials

The films tested in were: tissue culture Polystyrene (TCPS) as a positive control, Poly (2-oxy-ethoxy-acetyl) or Poly(*p*-dioxanone) (PPDX), Poly(glycolic)/Poly(L-lactide) 90:10 copolymer (PGLA-910), Poly (ehyleneterephthalate) (PET) Kodapack 7352, semi-rigid Poly(vinylidene chloride) (PVC) and Ultra High Molecular Weight Poly(ethylene) (UHMWPE). Polymer films were melt-compressed and total surface area of the disc samples was in the order of 10 mm in diameter and 0.15 mm thin. The materials were degreased, cleaned and sterilized in ethanol for 5 min at 25°C.

2.2. Cell culture

The cells used for assessment of the material were the Monkey derived Fibroblast cell line (VERO) and MDCK. Both cells lines were grown in Dulbeccos Modified Eagles Medium DMEM (GIBCO Laboratories) supplemented with 10% fetal bovine serum (GIBCO Laboratories). The cells were maintained at 37°C in a fully humidified atmosphere at 5% CO_2 in air and sub-cultured every four days by dissociating with 0.05% trypsin (GIBCO Laboratories) in phosphate buffered saline (PBS).

2.3. Attachment Assay

The different test substrates were positioned on the bottom of sterile 24-well culture plates (Corning Science Products). Fibroblastic cells were collected after trypsinization by centrifugation at 750 rpm for 3 min, resuspended in cell culture medium, to a plating concentration of 1×10^4 cells per well in a total volume of 2 ml. After seeding the cultures were incubated for 6 h. at 37°C in 5% CO_2 air. At the end of this period the wells were washed twice with PBS to remove nonattached cells. The number of viable cells attached on the different substrates was evaluated by 3-(4,5 dimethylthiazol 2-yl)-2-5 diphenyl tetrazolium bromide (MTT) assays (PROMEGA). In this test MTT-formazan production was used to evaluate succinic dehydrogenase enzyme activity in the mitochondria of the cells, which also indicates the cell number. The amount of formazan was quantified by measuring its optical density at 560 nm. A calibration curve was obtained measuring absorbance vs. cell number and allowed the calculation of cell number. Two runs of experiments were carried out. In each run all materials were present in triplicate. As a reference material cells seeded on TCPS were included in each run.

2.4. Proliferation Assay

Fibroblastic cell suspensions, containing 5×10^3 cells/well were seeded on the experimental substrates as described previously for the cell attachment assay and incubated at 37°C in 5% CO_2/air atmosphere for 6, 24 and 48 h. At the end of the incubation period, the non-attached cells were removed by washing with PBS. The number of cells attached to the different materials at the different periods of incubation was evaluated by MTT assays as previously described.

2.5. Scanning electron microscope (SEM)

Fibroblastic cell suspensions 2.5×10^3 cells were added to each sample as previously described and incubated for 4 and 48 h. The attached cells were fixed *in situ* with a 2.5% solution of Gluteraldehyde in 0.1% Sodium Cacodylate buffer for 30 min at 4°C and rinsed twice with PBS for 15 min at 4°C. Post fixation was made in 1% OsO_4 in Sabatini buffer at pH 7.4 for 60 min at 4°C. After this period the samples were washed twice with the same buffer for 5 min followed by dehydration in a graded series of methyl ketone (30, 50, 70, 90, 95 and 100%) for 10 min in each solution at 4°C. Following the fixation and dehydration process the samples were introduced in a critical point drying (Hitachi HCP-2) for 30 min. Finally, the specimens were mounted and coated with gold-palladium (Balzers metallizer) and examined on an Electron Microscope (Phillips 505-SEM).

2.6. Degradation assays

In order to determine the hydrolytic degradation of PPDX and PGLA-910, the suture-films samples were introduced in a test tube in a sterile environment containing a buffer phosphate solution (0.2 M) at pH 7.44 and 37°C as previously described [10]. Samples were previously dry and weighed on a Mettler analytical balance (AE 200). The hydrolysis was followed by weight lost in a week periods; and pH was monitored with an Orion pH-meter (model 420A).

2.7. Assays of cell viability in the presence of degradation products

Fibroblastic cells were sub cultured into 24 flat-bottomed culture plates at a density of 5×10^3 cell/ml of media. Following a period of 4 h., the culture media was exchanged with the same media containing the degradation products of PPDX and PGLA-910 films obtained as previously described. Citotoxicity was measured at 6, 24 and 48 h. with the MTT assay.

3. Results

3.1. Cell attachment

The number of attached cells was measured after incubation (6 h.) with the MTT test as described in Materials and Methods. As shown in Fig. 1, no significant differences was observed in the number of cell retained to PPDX, PGLA-910, PET and UHMWPE after incubation period. The number of cells attached to the different materials was similar to the adhesion of cell on the reference tissue culture plastic (TCPS). Contrary

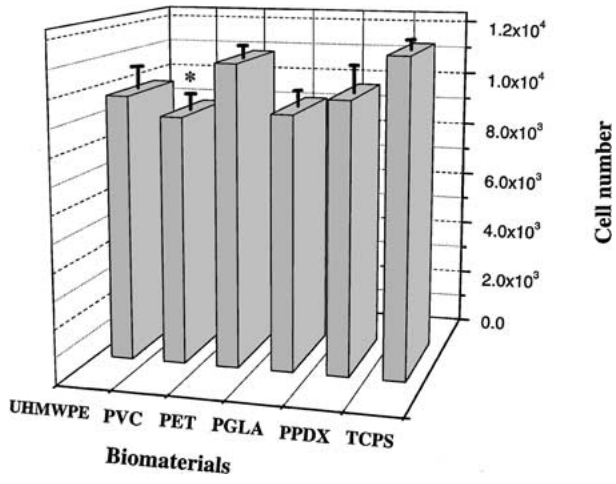

Figure 1 Attachment of Fibroblast on different polymers or on standard plastic culture after 6 h. The date are the mean ± sem of 3 cultures; *indicates a significant difference with plastic culture ($p < 0.05$).

to these results, significant differences were observed with PVC, where a 22% less attachment as compare to TCPS is found.

3.2. Cell proliferation

The growth kinetics of fibroblasts (VERO) in cells culture on the various substrate materials are shown in Fig. 2. As shown after 6 h. of platting the cells the density is similar among the different materials. However at 24 h., cell growth on all surfaces were comparable to cells cultured on TCPS, except for PVC where a significant difference in cell growth (about 80% of TCPS) is observed. After 48 h., the differences in cell growth on the materials is apparent; the highest growth is observed for PPDX (about 91% of TCPS) and the lowest one is observed for PVC (52% of TCPS).

3.3. Cell morphology (SEM)

Fig. 3, shows electron micrographs of fibroblasts cultured for 48 h. on the different polymers surfaces. On PPDX and PET (Fig. 3b and c), fibroblastic cells exhibited a flattened spindle-like appearance developed into a monolayer similar to cells cultured on TCPS (Fig. 3a). By the other hand, cells grown on PGLA-910, although exhibiting a flattened appearance had signs of cell damage (Fig. 3d). In agreement with the previous results [11], cells cultured in PVC exhibited minimal spreading forming cell aggregates (Fig. 3e).

3.4. Hydrolytic degradation of PPDX and PGLA-910

Biopolymers are degraded by a hydrolytic process that generally lead to the liberation of low molecular weight molecules [5]. One of the evaluations performed to study the behavior of PPDX and PGLA-910 during a hydrolytic process was to determine weight loss and pH changes over a period of 12 weeks. Therefore, both polymers were introduced in test tubes containing buffer solution and weight loss and pH changes were monitored every week as described in Materials and Methods. Fig. 4 shows the percentage of the retained weight obtained during the hydrolytic process for PPDX and PGLA-910. The difference in hydrolysis rate between PPDX and PGLA-910 is apparent. For instance after 12 weeks PPDX retained a 58% of weight and PGLA-910 only 38%.

To verify the occurrence of a hydrolytic process, the pH of the buffer solutions which containing theses polymers was measured and the result obtained over a 12 week period are shown in Fig. 5. As observe, the pH change paralelled the retained weight vs. Time plot. As expected from the relative hydrolysis rate, the pH decreased was more for PGLA-910 than PPDX: The action of the media increases due to the formation of acids of low molecular weight (degradation products).

3.5. Effect of degradation products on cell viability of cultured fibroblasts

To study the effect of degradation products on cell viability, two different fibroblastic cell lines were tested,

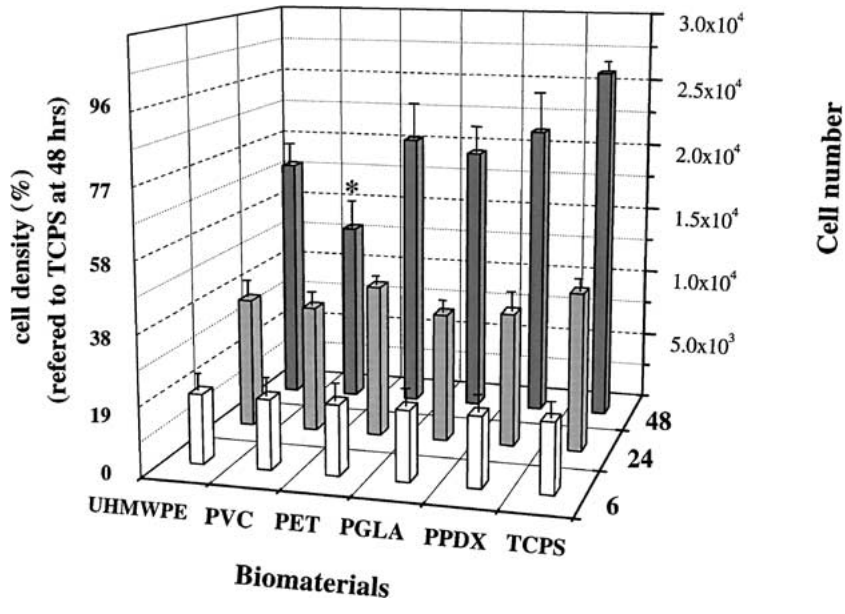

Figure 2 Proliferation of Fibroblast cells on different polymers or on standard plastic culture after 6, 24 and 48 h. of culture. The date are the mean ± sem of 3 cultures; * indicates a significant difference with plastic culture at the corresponding time point ($p < 0.05$).

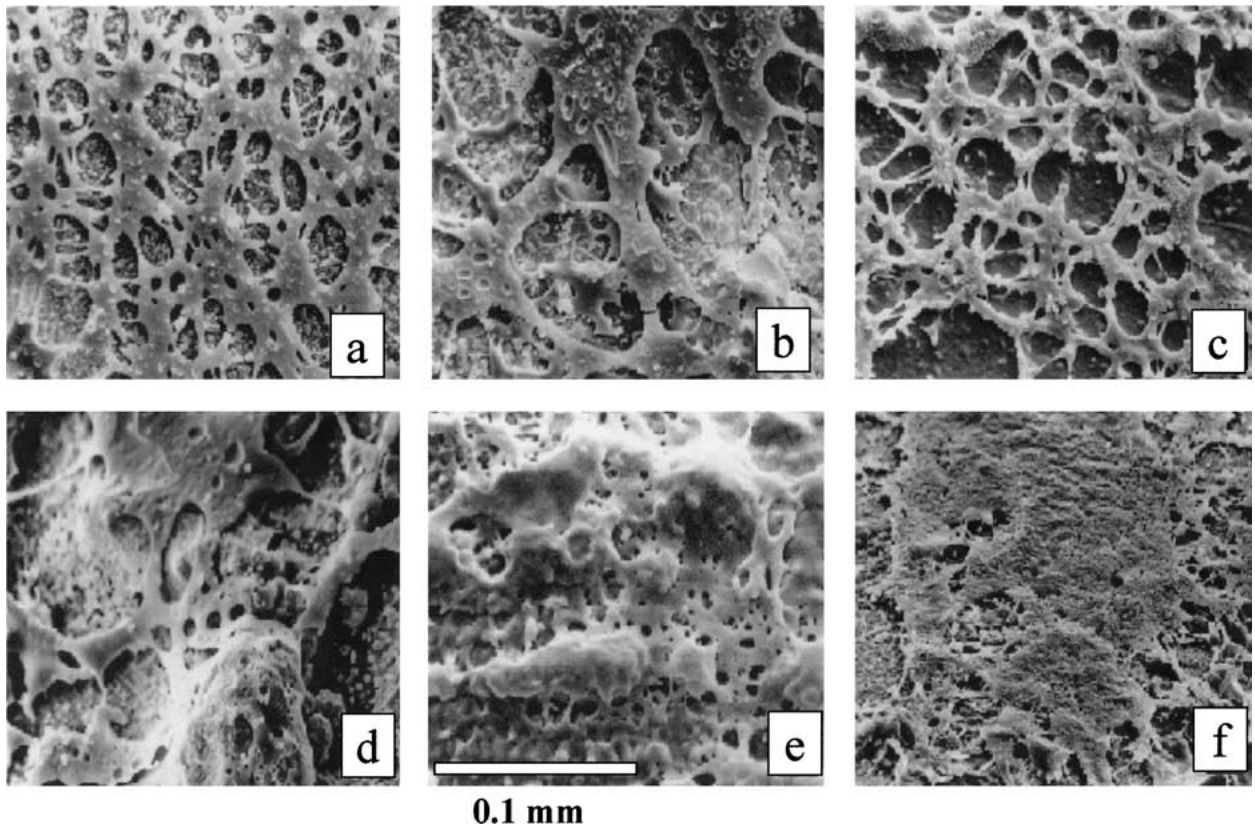

Figure 3 SEM Micrographs of Fibroblast cultured on the different polymers: (b) PPDX; (c) PGLA-910; (d) PET; (e) UHMWPE; (f) PVC and on standard plastic culture (a) after 48 h of culture.

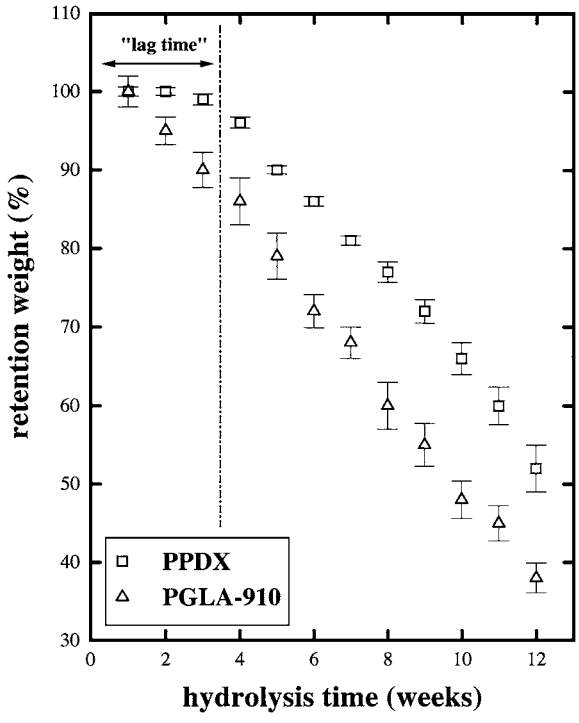

Figure 4 PPDX and PGLA-910 retention weight in a hydrolytic degradation time.

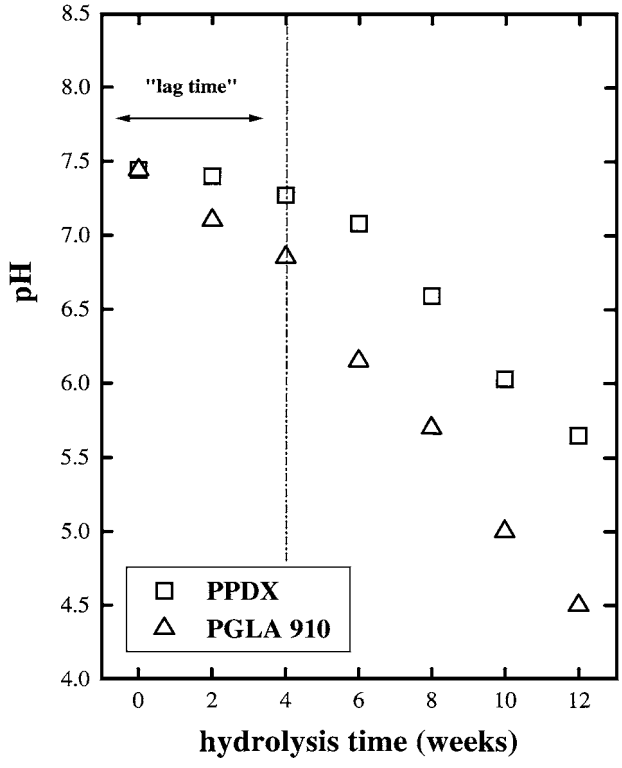

Figure 5 pH changes measured in a buffer solution by hydrolysis of PPDX and PGLA-910.

VERO and MDCK. Cells were seeded into 24 flat-bottomed culture plates and after a period of 4 h., the culture media (MEM) was exchanged with media containing the 12 week degradation products of PPDX or PGLA-910 obtained as previously described in Section 3.4. Citotoxicity of the degradation products was measured by MTT assay at 6, 24 and 48 h. after the exchange. Fig. 6 shows no significant decrease in the number of cells when both cell lines were cultivated in the absence (control) or presence of the degradation products of PPDX or PGLA-910.

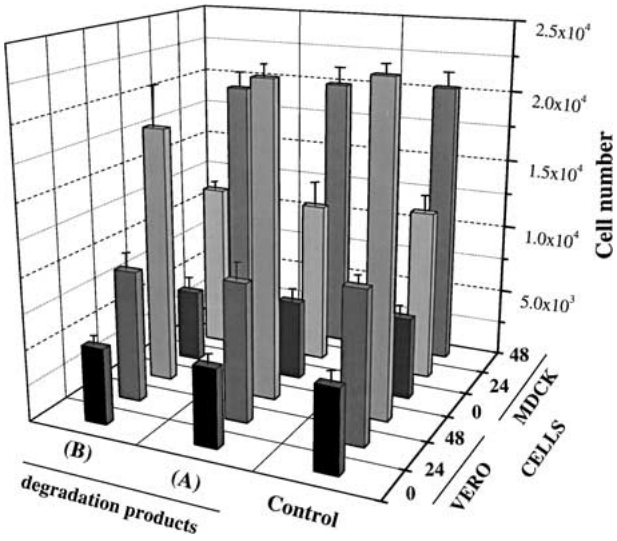

Figure 6 Growth of VERO and MDCK cells on standard plastic cultured in the absence or presence on the degradation products of (A) PPDX or (B) PGLA-910.

4. Discussion

The cellular interactions and the citotoxicity of the degradation products are the most important factors determining the biocompatibility of biomaterials and therefore these factors must be evaluated in the development of degradable polymers [4, 5, 8]. *In vitro* investigations have previously been shown to be suitable for elucidating several aspects of tissue response to implant materials without the interferences and complications encountered in animal models where results at the cellular level are difficult to interpret [6, 11].

In the present study, *in vitro* methods were successfully used to determine the biocompatibility of PPDX in comparison with a variety of different polymers. Our results show that although all polymers tested were suitable for initial cell attachment, there were significant differences when studying the kinetics of cell growth over a period of 48 h. For instance, PPDX proved to be the most favorable surface for cell growth, as revealed by cell density after 48 h. of culture. Under these conditions, scanning electron micrographs showed the presence of flat cells indicative of cell adhesion as well as substrate coverage. In the case of the PGLA-910 matrix, although the number of cells attached to the matrix at 6 h. post-seeding were not significantly different to the values obtained with PPDX, cell number decreased at 48 h. when compared to the values obtained with PPDX. In agreement with these results, electron micrographs taken at 48 h. showed certain damage of the cell seeded on this polymer. A possible explanation of these results could be that cells may initially adhere to PGLA-910 but as the surface undergoes modifications as a consequence of hydrolytic degradation, cells eventually separate from the substrate. Moreover, it was observed in SEM analysis that few adherent cells on the polymer surface did not exhibit cytoplasmic spreading or other evidence of activation. A more severe response is observed in the PVC case, in which it has been proposed [11] that its cytotoxicity destroyed the proper functions of the fibroblast, resulting in premature death cells before they could adhere to the foreing body surface.

In considering the performance of a synthetic matrix, the initial attachment of cells to an implanted surface is of considerable significance since many cellular functions including cell migration, division and differentiation are known to depend in part on cell adhesion and therefore will influence the subsequent tissue response to the material [11–13]. In recent years there has been an extensive effort to understand the role played by surface topography [14, 15] and surface chemistry in determining the ability of different material to bind cells. Regarding the later aspect, although substrate contact angle has been found to be an important factor in initial cell adhesion to a substrate [16, 17] recent studies find no correlation between the contact angle with water and adhesion suggesting that possibly cells attached to the polymers are not in direct contact with the surface but rather are in indirect contact through adhesive plasma proteins such as fibronectin and vitronectin adsorbed to the surface of the material and later through adherence proteins produced by the cells themselves [18, 19]. Therefore differences in the growth rate among materials may result from the difference in the nature and amount of the protein film that adsorbs onto the surface of the material or from the difference in the amount of proteins synthesized by the cells on the substrate during proliferation. This itself will be a consequence of the physico-chemical properties of the underlying surface.

In addition to cellular interactions between cells and the polymer, the interaction of low molecular weight compounds as degradation products is one of the most important factors determining the biocompatibility of biomaterials and, therefore, must be considered in the development of degradable biocompatible polymers. The weight loss and pH changes observed when measuring the degradation of PPDX and PGLA-910 clearly show the formation of acid low molecular weight degradation products that can diffuse through the crystalline polymer blocks and dissolve in the hydrolysis medium. The results obtained in this study showed that PPDX was more resistant to hydrolytic attack as compared to values reported for other biodegradable polymers such as PGA and PGLA-910 [20, 21]. In the present work similar differences were observed with PGLA-910. Under the same conditions PGLA-910, showed higher weight loss and pH changes when compared to PPDX. In both polymers a "lag time" is observed where no effect on pH and weight loss occurs. This might be related with the time it takes to remove the amorphous regions and in PGLA-910 case is very short.

The hydrolytic degradation starts in the amorphous regions, as the tie-chain segments in these regions degrade into fragments. This chain scission results in a lesser degree of entanglement of long-chain molecules located in the amorphous regions; therefore, the remaining undegraded chain segments in the amorphous region obtain better chain mobility; they can move and reorganize themselves from a disordered to an ordered state [1, 10]. For this reason during the lag time neither weight loss nor changes in pH measured were observed, specially in PPDX (Figs 4 and 5). When all the

amorphous regions have been removed by hydrolysis, the second stage of degradations starts, the size of the fragments reaches the stage that can be dissolved into the buffer medium [1, 10]. This dissolutions removes the fragments from the amorphous regions and a weight loss results and pH decreases indicating that in both cases the hydrolytic reaction produces acid low molecular weight degradation products. The differences between PPDX and PGLA-910 is probably due to differences in their chemical structure, where the ester bond in PPDX in relation to PGLA-910 has been replaced by a stable bond like the ether moiety [10]. This would lead to a longer retention of properties with respect to time. To ascertain the effect of the degradation products of PPDX and PGLA-910 on cell growth, fibroblastic cells were seeded on TCPS in the presence of media containing the degradation products of either both polymers. After 48 h. the pH of the modified media was 7.40 for PPDX and 6.97 in the case of PGLA-910. This slight difference in pH of the media may affect cell growth as previously demonstrated by other authors were slight changes in pH can drastically effect cellular behavior [22, 23].

5. Conclusions

In summary, based on cell culture investigations we conclude that PPDX fulfills an important criteria of biocompatibility, namely good levels of cell adhesion, cell growth. Furthermore, our results show that PPDX not only provides cell support but at the same time slowly degrades observing no citoxicity of the degradation products, a crucial point in deciding the applicability of degradable polymers.

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