J Mater Sci: Mater Med (2008) 19:2063–2070
DOI 10.1007/s10856-007-3292-2

In vitro and in vivo degradation of poly(L-lactide-co-glycolide) films and scaffolds

Elzbieta Pamula · Elzbieta Menaszek

Received: 30 June 2007 / Accepted: 25 September 2007 / Published online: 30 October 2007

Abstract Poly(L-lactide-co-glycolide) (PLGA) was synthesized using a biocompatible initiator, zirconium acetylacetonate. In vitro and in vivo degradation properties of PLGA films (produced by solvent casting, 180 μm thick) and PLGA scaffolds (produced by an innovated solvent casting and particulate leaching, 3 mm thick) were evaluated. The samples were either submitted for degradation in phosphate buffered saline (PBS) at 37 °C for 30 weeks, or implanted into rat skeletal muscles for 1, 4, 12, 22 and 30 weeks. The degradation was monitored by scanning electron microscopy, atomic force microscopy, weight loss, and molecular weight changes (in vitro), and by microscopic observations of the materials' morphology after histological staining with May-Grunwald-Giemsa (in vivo). The results show that the films in both conditions degraded much faster than the scaffolds. The scaffolds were dimensionally stable for 23 weeks, while the films lost their integrity after 7 weeks in vitro. The films' degradation was heterogenous—degradation in their central parts was faster than in the surface and subsurface regions due to the increased concentration of the acidic degradation products inside. In the scaffolds, having much thinner pore walls, heterogenous degradation due to the autocatalytic effect was not observed.

E. Pamula (✉)
Department of Biomaterials, Faculty of Materials Science and Ceramics, AGH University of Science and Technology, Al. Mickiewicza 30, Krakow 30-059, Poland
e-mail: epamula@agh.edu.pl

E. Menaszek
Department of Cytobiology and Histochemistry, Faculty of Pharmacy, Jagiellonian University, Collegium Medicum, ul. Medyczna 9, Krakow 30-688, Poland

1 Introduction

Resorbable poly-α-hydroxy acids, namely polyglycolide, polylactides, and their copolymers (PLGA) have been widely studied in various medical, drug delivery, and more recently, tissue engineering applications [1, 2]. The main advantage of such materials is that their degradation proceeds through hydrolysis of their ester bonds into compounds naturally present in the organism (lactic and glycolic acids), which are eventually resorbed, e.g. removed from the body by normal metabolic pathways [3]. Polymer biodegradability is influenced not only by its structure (chemical composition, molecular weight and polydispersity, chain microstructure, crystallinity, etc.), but also environmental conditions (medium, pH, temperature, presence of enzymes, cells and tissues) and implant size and form. PLGA thick samples are thought to be bulk-degrading, where the degradation in the inner parts proceeds faster than at the surface [4–6]. This heterogeneous degradation was attributed to the easier diffusion of soluble oligomers from the surface and their neutralization by the external buffer solution, while in the internal parts of the sample the degradation rate was increased by autocatalysis due to carboxylic endgroups trapped inside [7, 8]. It was already found that PLGA films having a thickness of 100 μm degrade much faster than those with a thickness of 10 μm [9]. Indeed, reducing the thickness of the film minimizes the risk of autocatalytic hydrolysis.

PLGA materials are commercially synthesized with the use of tin compounds (tin octoate, tin ethylhexanoate), because they are very effective initiators of polymerization, yielding high molecular weight polymers in a rather short time [10]. However, it is worth noting that complete elimination of the initiator from the polymer is practically impossible. As a result, during degradation small traces of

the tin compounds are released and may be harmful to the tissues [11]. Therefore, several attempts have been made to use less-toxic compounds—mainly zinc-metal or zinc lactate, calcium and zirconium—to conduct polymerization of the resorbable polymers [10, 12, 13]. To our knowledge, only one medical product synthesized with the use of non-toxic zinc initiator is available in the market so far (Phusiline®) [10].

In our experiments we are studying PLGA materials obtained with a non-toxic initiator, zirconium acetylacetonate. Their chain structure is more segmental, with higher lengths of glycolidyl and lactidyl microblocks, in contrast to the structure of the copolymers synthesized in a conventional way with tin compounds, where those segments are distributed more statistically [13]. As a result, their mechanical properties and resistance to thermal degradation are also enhanced [13]. Such materials were found to be more cytocompatible with osteoblasts and fibroblasts in vitro [14, 15] than those obtained with tin compounds. In vitro studies with osteogenic cells have shown that PLGA porous scaffolds support adhesion and growth of cells [16, 17].

The aim of this study was to determine in vitro (in phosphate-buffered saline, PBS) and in vivo (skeletal muscles of rats) degradation of poly(L-lactide-co-glycolide) synthesized with the use of zirconium initiator. The PLGA was processed into both films and porous scaffolds in order to evaluate the influence of the material's form and microstructure on its degradation kinetics and mechanism. The in vivo study was performed using the muscle tissue model. Skeletal muscles are generally considered to show a more intense inflammatory response than bone and cartilage. Moreover, the bone/cartilage replacing materials are in contact not only with bone or cartilage but also with the surrounding soft tissues (periosteum, connective tissue, muscles) [18]. In addition, it was recently shown that muscles may serve as an in vivo bioreactor, allowing the growth of heterotopic bone and ingrowth of vessels prior to transplantation of the tissue-biomaterial construct into a mandibular defect [19]. Our main attention to in vivo degradation was, however, focused on the changes in properties of the scaffolds and films themselves. Relevant results for inflammatory reactions with histological/immunohistological observations will be given separately in a following paper.

2 Materials and methods

2.1 Materials

Copolymerization of glycolide and L-lactide (Purac, The Netherlands) was performed in bulk with a zirconium acetylacetonate initiator, according to a method described previously [13]. In order to remove non-reacted monomers, the obtained copolymer was dissolved in chloroform, precipitated with cold methanol and finally dried in a vacuum at 50 °C to a constant weight.

The copolymer composition was determined by ^{1}H-NMR measurements and revealed that the molar ratio of L-lactide to glycolide was 85:15. Molecular masses were 50 kDa (M_n) and 105 kDa (M_w), as studied by gel permeation chromatography.

The scaffolds were produced by the modification of a classical solvent casting/particulate leaching technique [20]. Sieved sodium chloride particles (POCh, Gliwice, Poland) of defined grain fraction (400–600 μm), were mixed with 10% (w/v) copolymer solution in methylene chloride (POCh, Gliwice, Poland) in such proportions that a salt volume fraction of 85% was obtained. The mixture was transferred into polypropylene vials (diameter 12 mm, 5 ml volume) and air dried overnight, followed by vacuum treatment at a reduced pressure for 48 h. Next, the vials with the rigid salt/polymer mixture were cut into slices of the thickness of 3 mm and placed into 100 ml of ultra-high-purity water (UHQ-water, produced by Purelab UHQ-PS apparatus, Elga, UK). The water was exchanged several times until the conductivity of the water after washing was about 2 μS/cm. The samples were then dried in a vacuum oven at 35 °C for at least 24 h. Subsequently, 1 ml of methylene chloride was poured into a glass vial (outer diameter 12 mm, 5 ml volume). The vial was covered with a scaffold slice, which was fixed to the vial around its perimeter with a stripe of Parafilm M (Pechiney, Chicago, USA). Afterward, the solvent vapour was forced to flow through the porous scaffold by applying a vacuum of 80 kPa, which took 5–10 min, thereby dissolving some thin pore walls and improving pore interconnectivity. The resulting scaffolds were dried under vacuum for at least 48 h and stored in a desiccator prior to use.

The PLGA films were cast from 10% (w/v) polymer solution in methylene chloride on glass Petri dishes, followed by air drying for 24 h and vacuum drying for the next 72 h. Then, the films were rinsed with UHQ-water for 12 h. Water was exchanged 6 times. Afterwards, the films were air and vacuum dried for 24 and 48 h, respectively, and stored in a desiccator prior to use.

2.2 Degradation conditions in vitro

Degradation of copolymer films and scaffolds was performed at 37 °C in phosphate-buffered saline (PBS) (137 mM NaCl, 24 mM Na_2HPO_4 and 16 mM KH_2PO_4, pH = 7.2). Three scaffolds (12 mm in diameter and 3 mm in thickness) weighing in total 100 mg (±20 mg) were

placed in separate plastic vials containing 30 ml of PBS. Simultaneously, pieces of film (180 μm in thickness) weighing 100 mg (±10 mg) were also placed in separate plastic vials containing 30 ml of PBS. The PBS was changed once a week and the samples were incubated up to 30 weeks. Every week, one vial with scaffolds and one vial with film were taken and the samples were washed 5 times in UHQ-water and dried in air overnight, followed by vacuum drying for at least 24 h.

The relative mass change (M_R) was calculated from the weight of the samples prior and after incubation.

2.3 Materials characterization

The porosity of the scaffolds, P, was calculated from the mass (weighed with a precision of 0.00005 g) and the dimensions of each scaffold (measured with a caliper with a precision of 0.1 mm). Next, the apparent density, ρ_S, of the scaffold was calculated, by dividing the weight by the volume of the scaffold. The porosity was then determined according to the equation:

$$P = 1 - \rho_S/\rho_p \quad (1)$$

where ρ_p is the density of the copolymer (1.3 g/cm^3). The average porosity and confidence interval (at a confidence level $\alpha = 95\%$) were calculated from measurements of 90 individual scaffolds (12 mm in diameter, 3 mm in height).

Molecular masses (M_n, M_w) of the films and scaffolds as a function of degradation were determined by gel permeation chromatography with a Spectra Physics SP 8800 chromatograph (chloroform was used as the eluent; the flow rate was 1 mL/min; Styragel columns and a Shodex SE 61 detector were used).

The microstructure of the films and scaffolds before and after degradation was studied with the use of a scanning electron microscope (JSM 5400, JEOL, Japan; accelerating voltage 15 kV, magnification 50×). Before the analysis, the samples were sputter-coated with a thin carbon layer in order to make them conductive.

Topography measurements of the films were performed with an Explorer atomic force microscope (ThermoMicroscopes, Vecco, USA). Contact mode topographic images were recorded using Si_3N_4 tips with a spring constant of 0.05 N/m and a nominal radius of curvature of 20 nm (Vecco NanoProbe™ Tips, model MLCT-EXMT-A). The images were recorded with a scan area of 100 μm × 100 μm for six randomly chosen places (300 × 300 data points) and with scan rate of 3 lines/s. All images were flattened using a third-order polynominal algorithm provided with the instrument. R_{RMS} roughness was calculated and expressed as means ± standard deviation.

The structure and thermal properties of PLGA after processing into films and scaffolds were analysed by ^{1}H-NMR (Varian Unity Inowa spectrometer) and DSC (DuPont, 1090B), respectively; the experimental details are given elsewhere [21].

2.4 Implantation

The experiment was performed according to the EU ISO 10993-6 guidelines and the study protocol was approved by the local bioethics committee (Krakow, Poland, No 32/OP/2003). A total of 25 adult male rats (inbred Hooded Oxford) divided into 5 groups were used in the experiment. Pieces of the films (4 mm × 2 mm × 0.18 mm) and the scaffolds (4 mm × 2 mm × 3 mm) were implanted into the cut glutei muscles of the anaesthetized rats under sterile conditions. Each animal received two implants: the film was placed into the right muscle, and the scaffold into the left one. The tissues were sutured with PDS*II (6/0, 0.7 metric, Ethicon, UK). After 1, 4, 12, 22 or 30 weeks from the surgery the animals were euthanized by an overdose of Vetbutal. Implants and surrounding muscles were excised, frozen in liquid nitrogen and subsequently cut with a cryostat microtom into sections 6 μm thick. To identify the degradation process of PLGA and infiltrating inflammatory cells, histological reaction by the May-Grünwald Giemsa (MGG) method was carried out. Observations were made using an optical microscope (Olympus BH2, objective 10–40×), and pictures were taken with a digital camera.

Results of morphometric analysis and observations of the inflammation flow, metabolic state of the tissues, processes of tissue development into and around the implant will be presented in our subsequent paper.

3 Results

3.1 Properties of scaffolds and films

Figures 1 and 2a show the morphology and microstructure of polymeric scaffold before degradation registered by a digital camera and by a scanning electron microscope, respectively. The scaffolds had interconnected pores of the size close to the size of porogen particles (400–600 μm). The porosity of the scaffolds was 85 ± 1.1%. The thickness of the scaffolds was 2.9 ± 0.11 mm, while the films had the thickness of 0.18 ± 0.014 mm.

Before incubation in PBS, the films were transparent and smooth at macroscopic level (Fig. 1). However, at nanometric scale, as can be seen in the AFM topographical image (Fig. 3a), wave-like features less than 25 nm in height were observed. Such topographical features have

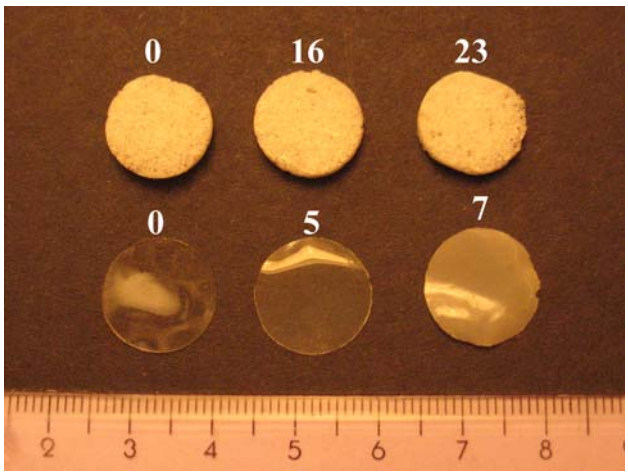

Fig. 1 Gross morphology of PLGA scaffolds and films before and after incubation in PBS: scaffolds in upper row, degradation period 0, 16, 23 weeks; films in lower row, degradation period 0, 5, 7 weeks

been often observed on the surface of amorphous polymeric films made by slip casting, and they are created during solvent evaporation. The R_{RMS} roughness of the PLGA surface estimated for 100 μm × 100 μm scan areas was 3.8 ± 0.7 nm.

Processing into scaffolds or films did not have an impact on copolymer chain microstructure nor the presence of residual monomers, as determined by ^{1}H-NMR (data not presented). Both PLGA scaffolds and films were amorphous polymers with glass transition temperature of 57 °C, similar to that reported previously by Dobrzynski et al. [13] and Pamula et al. [21].

3.2 In vitro degradation

Figure 1 presents morphology of the scaffolds and the films as a function of incubation time in PBS. The scaffolds were dimensionally stable for 23 weeks of incubation in PBS before starting to lose integrity. The films were transparent and dimensionally stable for 4 weeks; after week 5 they started to become opaque, and at week 7 the films changed colour to white and started to crumble.

As can be seen in the SEM picture after 24 weeks of incubation (Fig. 2b) morphological changes in the scaffolds were visible: the pore size changed, some pore walls disappeared, some fibre-like structures appeared among the pores, and the scaffolds collapsed. Cross-sections of the films after 5 weeks of incubation in PBS revealed that the outer regions of the films were less degraded than those of inner parts (Fig. 2c, d). It is worth noting that inside the films, pores tens of micrometers in diameter were created. On the PLGA films protuberances of up to 300 nm in height were observed on the surface after incubation in PBS (Fig. 3b). The R_{RMS} roughness of the PLGA surface on week 5 of in vitro degradation, estimated for 100 μm × 100 μm scan areas, was 16.4 ± 3.7 nm. Note that wave-like structures typical of the films before degradation were still visible and the roughness of the

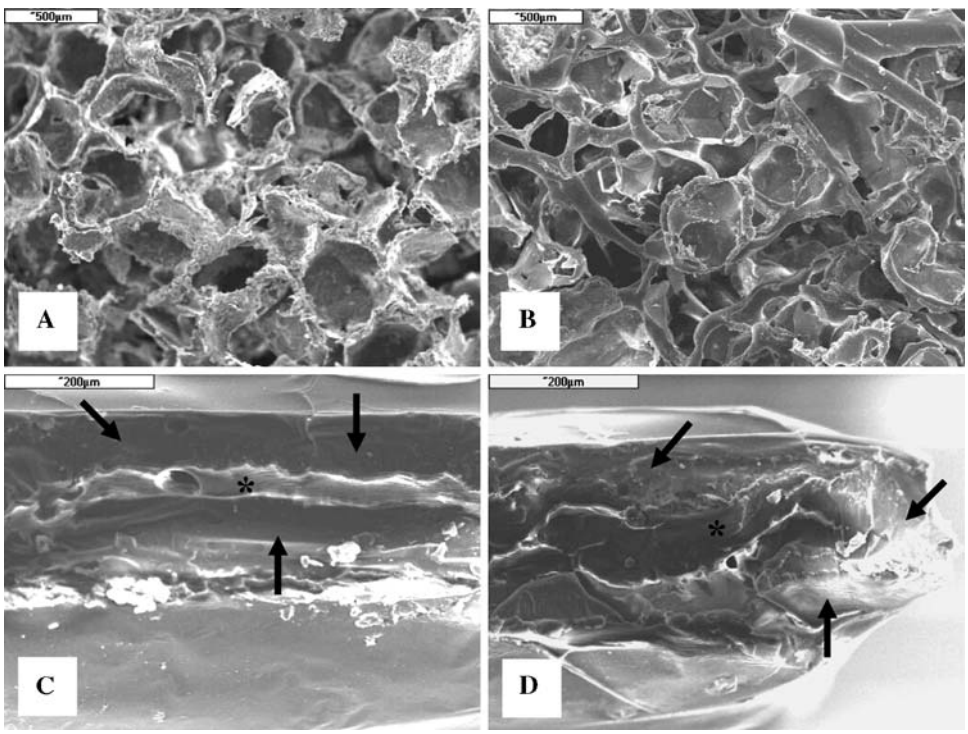

Fig. 2 SEM microphotographs of PLGA scaffolds and films: (**a**) initial scaffold, (**b**) scaffold after degradation in PBS for 24 weeks, (**c**, **d**) cross-sections of the films after 5 weeks in PBS. Arrows indicate less-degraded regions, while asterisks indicate more-degraded central parts of the films. Original magnification 50× (scaffolds) and 200× (films)

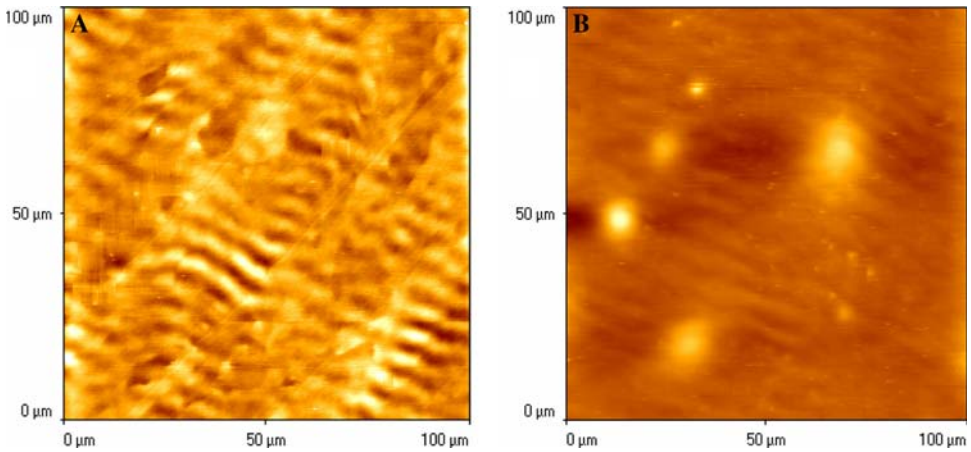

Fig. 3 AFM topographical images (scan area 100 µm × 100 µm) of PLGA films: (**a**) before degradation (z = 25 nm), and (**b**) after 5 weeks in PBS (z = 300 nm)

regions between protuberances was similar to that before degradation.

Figures 4a and 5 present variations of the number-average molecular weight (M_n) and mass change (M_R) of the PLGA films and scaffolds as a function of incubation time in PBS, respectively. The molecular weight of the films decreased linearly with degradation time up to week 7, and it remained constant until the end of the experiment. On the other hand, the molecular weight of the scaffolds was constant for the first 6 weeks, followed by a linear decrease up to week 26. A slight increase in polydispersity indices (from 1.9 to 3.1) as a function of degradation was detected; however, the molecular weight distribution, in both films and scaffolds, remained monomodal (Fig. 4b).

Relative mass changes of the scaffolds and the films followed the same tendency up to the fourth week of incubation. Afterwards, the mass of the films decreased more sharply (up to 12%) on week 7, and the films started to lose their integrity. Scaffold mass remained relatively constant up to week 16. Thereafter, it started to decrease, losing as much as 25% by week 23; the scaffolds became very fragile and started to crumble, which made it more and more difficult to continue making mass measurements.

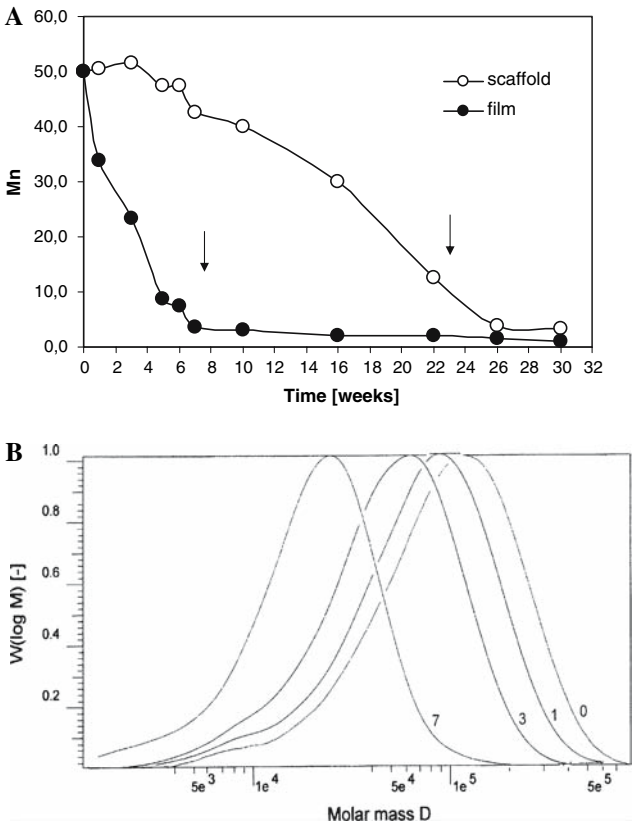

Fig. 4 Variations of the number-average molecular weight (M_n) of PLGA scaffolds and films as a function of incubation time in PBS. Arrows indicate loss of integrity (**a**). Gel permeation chromatograms of PLGA films before (0) and after degradation in PBS for 1, 3 and 7 weeks (**b**)

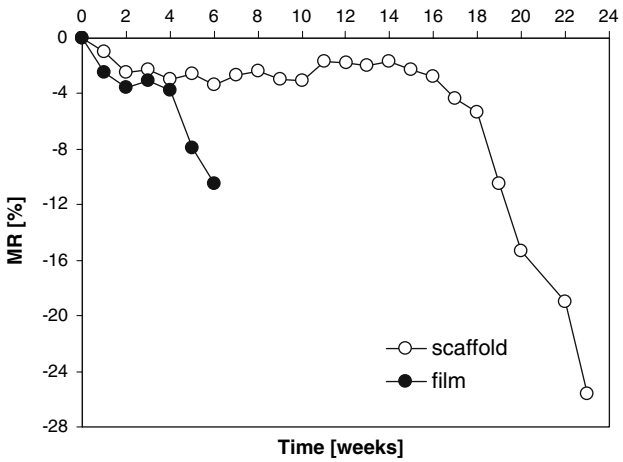

Fig. 5 Relative mass changes of PLGA scaffolds and films as a function of degradation in PBS

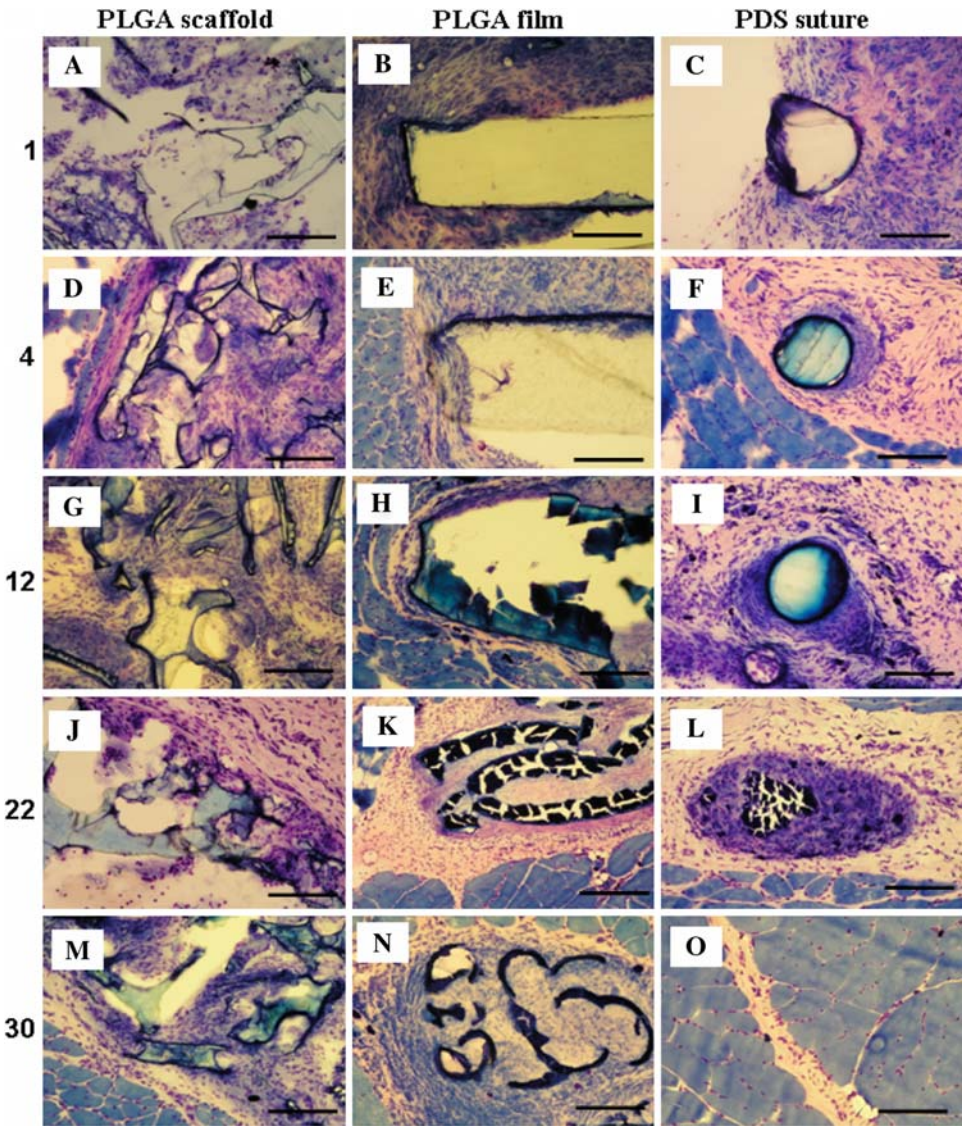

Fig. 6 PLGA scaffolds (**a**, **d**, **g**, **j**, **m**), PLGA films (**b**, **e**, **h**, **k**, **n**), and PDS sutures (**c**, **f**, **i**, **l**, **o**) after implantation in the glutei muscles of rats for 1 week (**a**, **b**, **c**), 4 weeks (**d**, **e**, **f**), 12 weeks (**g**, **h**, **i**), 22 weeks (**j**, **k**, **l**) and 30 weeks (**m**, **n**, **o**); MGG staining, optical microscope Olympus BH2, objective 20×, bar = 100 μm

3.3 In vivo degradation

Figure 6 presents the cross-sections of the scaffolds, films, and PDS sutures implanted into the glutei skeletal muscle of rats for 1, 4, 12, 22 and 30 weeks after histological staining with May-Grunwald-Giemsa (MGG). This staining is acid sensitive, and was thus especially effective in visualising the acidic degradation by-products within the resorbable polymers, as previously reported by Schwach and Vert [10].

One *week* after implantation, the structure of all materials seemed not to be changed, and an influx of cells, mainly neutrophils and monocytes/macrophages, indicated that early inflammation processes had started.

Four *weeks* after implantation, the sutures changed colour to blue, due to stain absorption, while the scaffolds and the films looked unaltered. All the implants were surrounded by granulation tissue.

Twelve *weeks* after implantation, some parts of the scaffolds showed slightly higher absorption of the stain. Fragmentation of the films was observed. In contrast to the scaffolds, much higher stain absorption in the inner part of the films suggests that the degradation was more advanced inside the implant than in the outer parts, which were tightly bound up with the surrounding tissue. It may explain why the degraded central part of the films was removed during preparation of the tissue slices. The PDS sutures absorbed considerably more stain than on week 4, implying that their degradation was more advanced.

Twenty two *weeks* after implantation, all the scaffolds changed colour to light-blue, while fragmentation of the films and sutures was apparent. The degraded films and sutures were stained in dark-blue and black, demonstrating the accumulation of large amounts of acidic degradation products in the implant site. Interestingly, a fibrous capsule around the suture was quite thick, with the circular area

enriched with degradation products and inflammatory cells (mainly macrophages) stained in violet.

Thirty *weeks* after implantation, the scaffolds were still visible in the tissue, but their degradation was more pronounced in comparison with the previous series. The films were almost completely degraded, with only individual polymeric traces of polymer, having the shape of flakes, visible in the implantation site. The PDS sutures were totally resorbed.

4 Discussion

Copolymer of L-lactide and glycolide, PLGA 85:15, synthesized with the use of zirconium compound, was processed in order to produce two materials of different microstructure, e.g. non-porous films and porous scaffolds. Thanks to an applied manufacturing procedure (modification of the classical solvent casting-particulate leaching method), the scaffolds were characterized by an interconnected porous structure. The films were relatively smooth, with a roughness of a few nanometers. Both forms of PLGA materials were submitted to degradation in vitro and in vivo.

4.1 Mechanism of scaffold degradation

The PLGA scaffolds were dimensionally stable for relatively long period of time in vitro (23 weeks in PBS); dimensional stability for a certain period of time is an important prerequisite for materials evaluated for tissue engineering. The molecular weight of the scaffolds remained initially constant (up to week 6), followed by a linear decrease up to week 26. Mass loss in the scaffolds, due to the release of degradation products, was detected after 16 weeks of incubation in PBS. Afterwards, the scaffolds started to crumble and collapse. Microscopically, the morphology of the pores changed and the size of the pores decreased. Our findings are consistent with previous results of Wu and Ding [22] on the degradation of scaffolds produced from the copolymer of DL-lactide and glycolide (85/15), synthesized with the use of a tin component as a polymerization initiator. For comparison, these studies showed dramatic mass loss after 16 weeks of incubation in PBS of the scaffolds having porosity similar to ours, but being produced with a copolymer having a much higher molecular weight (M_n = 253 kDa, M_w = 577 kDa) [22]. On the other hand, in the other study of Lu et al. [5], the mass of the PLGA scaffolds having porosity and molecular weight similar to those in this study, remained constant throughout the degradation up to week 20 in PBS [5]. The differences observed can be explained, at least partly, by different chemical composition (L-lactide versus DL-lactide), chain structure (segmental, block, random) or the microstructure of the scaffolds.

Degradation of PLGA scaffolds in vivo was observed on week 12 after implantation as faint blue stains of some parts of the scaffolds, due to reaction with acidophilic histological stain (MGG). On week 22 the scaffolds changed the colour to light-blue, while on week 30 blue and some dark-blue staining of the scaffolds were visible. This change of colour was caused by the presence of acidic degradation by-products, namely lactic and glycolic acids. Interestingly, no preferable accumulation of the stain was detected on the surface or in the inner parts of the scaffold walls, in contrast to films, which degraded heterogeneously.

4.2 Mechanism of films degradation

The films degraded relatively quickly: after 5 weeks in vitro they became opaque, and after 7 weeks they started to disintegrate. Loss of transparency of PLGA films has been attributed to water absorption [9] or an increase in the crystallinity of the degraded material [21]. In our previous study we found that the change of colour of initially amorphous PLGA films from transparent to white after 5 weeks of incubation in PBS coincided with increase in copolymer crystallinity [21]. Indeed, becoming whitish and opaque corresponded to a huge decrease in molecular weight due to chain-scission of polyester bonds in a hydrolysis reaction, enabling, as a consequence, easier mobility of shorter chains and their arrangement in crystalline structures.

The degradation was more advanced inside the films than on their surface and in the near-surface region, as observed in the cross sections of the films under SEM. Moreover, AFM examinations provided evidence that observed protuberances were produced during excessive drying of the degraded films, which caused the collapse of more-degraded inner regions, thus creating hill-like topographical structures. Note that wave-like structures typical of the films before degradation were still visible, and the roughness between the protuberances was not altered. All these results show that the films degrade through an 'in bulk' mechanism, so just why they became brittle with negligible strength after a relatively short period in PBS. Heterogeneous degradation was widely reported in the literature for large-size PDLLA and PLLA devices [7, 10] and for PLGA 85:15 films having the thickness of 100 μm [9]. In the case of our PLGA films (thickness of 180 ± 14 μm), more advanced degradation in bulk was observed also in vivo after 12 weeks of implantation. On week 30 the films were almost resorbed, and only a few polymeric traces were visible. It must be pointed out that degraded by-products of the films were stained in dark-blue

and black, suggesting that the accumulation of acidic degradation products in the tissue was high.

And last but not least, accelerated degradation of PLGA films in comparison with scaffolds might be caused by the presence of residual monomers, which are known to speed up degradation of aliphatic polyesters. However, this hypothesis may be ruled out due to the precautions taken to remove unreacted monomers from the copolymer. Furthermore, ^{1}H-NMR analysis of PLGA films and scaffolds before incubation in PBS did not show the presence of the signals originating from glycolide and L-lactide monomers.

For comparison purposes, the histological pictures of PDS sutures used to sew muscles above the PLGA implants were also presented. The results clearly show that this polymer (also belonging to a group of resorbable aliphatic polyesters) undergoes hydrolytic degradation in bulk, but without the hollow residual structures as in the PLGA films. Indeed, tiny particles evenly distributed were observed in the place of implantation on week 22, and they provoked intensive inflammation. However, by week 30 after implantation, the sutures were totally resorbed and not visible in the implantation site.

5 Conclusions

In vitro and in vivo degradation behaviour of PLGA porous scaffolds and non-porous films was studied up to 30 weeks. It was shown that in both conditions the films degrade faster than the scaffolds. Moreover, the films degrade heterogeneously, the degradation being more advanced inside the films than on the surface and in the near-surface region. The degradation of the scaffolds was more uniform because the thickness of the walls in the porous scaffolds was smaller, which enabled faster diffusion of the soluble degradation products to the environment, thus reducing autocatalytic hydrolysis. In addition, our experiment shows that PLGA synthesized with the use of zirconium compound degrades according to a mechanism similar to that described for PLGA polymers synthesized with other, more typical polymerization initiators.

Acknowledgements The authors thank Dr. P. Dobrzyński for providing polymer samples and MSc M. Żołnierek for her help in implantation. This study was supported by the research project of the Polish Ministry of Science (3 T08D 019 28).

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