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Poly(ether ester amide)s for tissue engineering

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Abstract

Poly(ether ester amide) (PEEA) copolymers based on poly(ethylene glycol) (PEG), 1,4-butanediol and dimethyl-7,12-diaza-6,13-dione-1,18-octadecanedioate were evaluated as scaffold materials for tissue engineering. A PEEA copolymer based on PEG with a molecular weight of 300 g/mol and 25 wt% of soft segments (300 PEEA 25/75) and the parent PEA polymer (0/100) sustain the adhesion and growth of endothelial cells. The in vivo degradation of melt-pressed PEEA and PEA discs subcutaneously implanted in the back of male Wistar rats was followed up to 14 weeks. Depending on the copolymer composition, a decrease in intrinsic viscosity of about 20–30% and mass loss up to 12% were measured. During the degradation process, erosion of the surface was observed by scanning electron microscopy and light microscopy. The thermal properties of the polymers during degradation were measured by differential scanning calorimetry. During the first 2 weeks, a broadening of the melting endotherm was observed, as well as an increase in the heat of fusion. Porous matrices of PEEAs and PEA could be prepared by molding mixtures of polymer and salt particles followed by leaching of the salt.

Keywords: Poly(ether ester amide)s; Tissue engineering; Cell adhesion; Degradation; Porous scaffolds

1. Introduction

Polymers based on poly(ethylene oxide) and poly(butylene terephthalate) (PEOT/PBT) are examples of segmented block copolymers of which the physical properties can be readily tuned by variation of the polymer composition. Materials with a broad spectrum of adequate mechanical properties, also in the swollen state, can be obtained [1]. Consequently, these materials have been extensively investigated for various biomedical purposes, such as tympanic membrane [2], bone filler [3], skin substitute [4] and more recently for the tissue engineering of bone [5,6] and cartilage [7]. The in vitro [1,8,9] and in vivo [10–12] degradation of PEOT/PBT has been extensively investigated. PEOT/PBT degradation is dependent on the copolymer composition and is characterized by a decrease in polymer soft segment (PEOT) content and molecular weight leading to sample fragmentation. A drawback is that the degradation after implantation is not complete or leads to insoluble products [13].

In an effort to obtain biocompatible polymers with varying hydrophilicity and adequate mechanical properties in the swollen state which can degrade completely, segmented poly(ether ester amide)s (PEEAs) may be an alternative. They are usually prepared by a two-step polycondensation using poly(ethylene glycol) (PEG) [14] or poly(tetramethylene glycol) [15] as polyethers. These materials are semicrystalline thermoplastic elastomers and undergo microphase separation [16,17]. Their physical properties can be modulated by either varying the ether/ester/amide ratio, or the nature and the length of the degradable ester blocks and the hydrophilic ether blocks [14–16]. To our knowledge, despite these interesting properties, the degradability and the biomedical applicability of segmented PEEAs have not been the subject of many studies. The biocompatibility of PEEAs based on poly(L-lactide) and PEG has been assessed by checking the viability of Caco-2 cells on polymer films [17]. These PEEAs are degradable in vitro and can be used as drug delivery carriers. Biodegradable PEEAs based on poly(ε-caprolactone) have been prepared and

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also studied as microspheres for the controlled release of drugs [18].

In our laboratory, segmented block copoly(ester amide)s containing polyether blocks (PEEA), based on PEG, 1,4-butanediol and dimethyl-7,12-diaza-6,13-dione-1,18-octadecanedioate, were developed for the controlled release of drugs [19,20]. Initial studies showed that several polymers in this series have adequate properties in the swollen state [21] and could be used in tissue engineering and other medical applications. In this paper, these materials have been evaluated with respect to cell adhesion and growth of endothelial cells, in vivo degradation and processability into porous scaffolds.

2. Materials and methods

2.1. Materials

PEGs of different molecular weights (PEG 300 and PEG 1000) supplied by Fluka (Switzerland), and 1,4-butanediol from Acros (Belgium) were used without further purification. All solvents used were analytical grade (Biosolve, the Netherlands).

2.2. Polymer synthesis

A detailed description of the synthesis of PEEAs based on PEG, 1,4-butanediol and dimethyl-7,12-diaza-6,13-dione-1,18-octadecanedioate (a diester–diamide monomer) (Fig. 1) has been published elsewhere [19,21]. The PEEAs were purified by dissolution in chloroform:methanol 1:1 (v/v) and precipitation in cold ether. Purified polymers were dried for 7 days under vacuum at room temperature and stored in vacuum-sealed bags at −21°C. The composition of the block copolymers is indicated as *a* PEEA *b*/*c*, in which *a* is the starting PEG molecular weight, *b* the wt% of soft segments and *c* the wt% of hard segments. The abbreviation PEG is used when referring to the starting material used for the synthesis, whereas PEO is used to refer to the repeating segment in the PEEA copolymers.

2.3. Preparation of polymer films

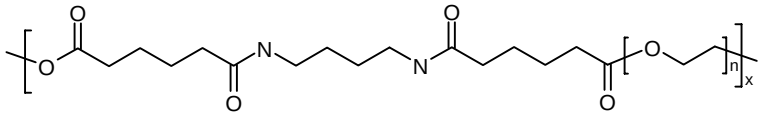
Films of purified PEEAs were prepared by compression molding (laboratory press THB008, Fontijne, The Netherlands). The molding temperatures were 180°C for PEA, 170°C for 300 PEEA 25/75, 160°C for 1000 PEEA 31/69 and 145°C for 300 PEEA 56/44. The thickness of the specimens was 400–600 μm. Discs of 10 mm diameter were punched from these films for in vivo degradation experiments.

2.4. Polymer characterization

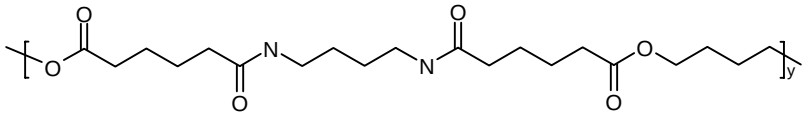
The intrinsic viscosities (η) of the (non)degraded PEEA melt-pressed films were determined by single point measurements [22,23] at 25°C using an Ubbelohde OC viscometer. Polymer solutions (chloroform/methanol; 1:1 v/v) were prepared at a concentration of approximately 0.3 g/dl.

The polymer composition was determined by proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) using a Varian Inova 300 MHz (USA) and polymer solutions in deuterated dimethylsulfoxide (Sigma).

The thermal properties of the melt-pressed copolymers before and after subcutaneous implantation for a specific time period were evaluated by differential scanning calorimetry (DSC) with a Perkin–Elmer Pyris 1 (USA) at a heating rate of 10°C/min. The copolymer samples (5–10 mg) were placed in stainless-steel pans and were heated from −100°C to 250°C. The glass transition temperatures were taken as the midpoint of the heat capacity change. Cyclohexane, indium, gallium and tin were used as standards for temperature calibration.



PEO-containing soft segment



Hard segment

Fig. 1. Chemical structure of segmented PEEA block copolymers. The soft segments are derived from PEG and dimethyl-7,12-diaza-6,13-dione-1,18-octadecanedioate and the hard segments from 1,4-butanediol and dimethyl-7,12-diaza-6,13-dione-1,18-octadecanedioate.

The mass loss was defined as

$$\text{Mass loss} = \frac{m_0 - m}{m_0} 100, \quad (1)$$

where m_0 is the initial specimen weight and m the weight of the degraded specimen after drying for 10 days under reduced pressure at room temperature.

Contact angles of copolymer films in demineralized water were determined using the captive bubble technique. Measurements were done using a Contact Angle System OCA 15 plus from Dataphysics. Results are averages of at least 3 measurements.

2.5. Adhesion and growth of human umbilical vein endothelial cells (HUVEC)

HUVEC (passage 2) were cultured on circular polymer films. The cells were seeded at a density of 40,000 cells/cm^2 in 3 ml culture medium. The culture medium consisted of 50 vol% M199 (with Hank's solution; Gibco, Life Technologies), 50 vol% RPMI 1640 (with 25 mM HEPES; Gibco, Life Technologies), in which 100 μg/ml penicillin-G, 100 μg/ml streptomycin (Gibco, Life Technologies) and 2 mM Glutamax-I (Gibco, Life Technologies) are added. Prior to use in cell culture, the culture medium was supplemented with filter-sterilized pooled human serum (20 vol%). Cultured films were quantitatively analyzed after 6 h, 1, 3 and 6 days. Tissue culture polystyrene (TCPS) was used as positive control.

2.6. In vivo degradation

Melt-pressed 300 PEEA 25/75, 1000 PEEA 31/69 and PEA discs were implanted subcutaneously in the back of young male Wistar rats (150–170 g) along the dorsomedial line. Prior to implantation, the melt-pressed polymer discs (diameter: 10 mm, thickness: 0.4–0.6 mm) of known mass were sterilized by immersion in ethanol 70% and washed with sterile phosphate buffered saline (PBS) (Life Technologies). Four subcutaneous pockets were formed in the back of each rat and the polymer samples ($n = 6$) were randomly implanted. After insertion of the samples, the wounds were closed with Vicryl® sutures. Five rats were killed at 1, 2, 4, 8 and 14 weeks after implantation. Characterization of the degraded materials was done by means of mass loss, intrinsic viscosity, composition (^{1}H-NMR), thermal properties (DSC) and surface morphology (SEM). The surrounding tissues were excised for histological analysis. After explantation, the samples were fixated in a 4% paraformaldehyde solution (Sigma). Prior to embedding, the samples were dehydrated through a series of isopropanol/water solutions with increasing isopropanol concentrations (70–100%). Subsequently, samples were embedded in glycol methacrylate (Sigma). Coupes (5 μm) were then cut with a microtome HM 355S (Microtom, Germany) and stained with a hematoxilin–eosin staining agent (Sigma). Histological sections were evaluated by light microscopy.

2.7. Preparation of PEEA porous scaffolds

Porous scaffolds were prepared by molding mixtures of ground polymer and salt particles followed by salt leaching. The copolymer particles (250–500 μm) were mixed with sodium chloride (sieved to 500–710 μm, 90 vol%). The mixtures were compression molded using a hot press (laboratory press THB 008, Fontijne, The Netherlands). Samples were heated to 10°C above the melting point at 20 Pa for 3 min and then pressed at 3 MPa for 1 min. Subsequently, the salt was leached out in demineralized water (48 h). The materials were dried in a vacuum oven for 48 h at room temperature. The densities and porosities were determined from mass and volume measurements of the materials in duplicate. The density of the non-porous materials was 1.10 g/cm^3 for 300 PEEA 56/44, 1.22 g/cm^3 for 1000 PEEA 31/69 and 1.33 g/cm^3 for PEA.

2.8. Scanning electron microscopy (SEM)

A Leo 1550 field emission SEM (Germany) was used. Freeze-fractured samples of the porous structures were cut and coated with Au/Pd using a Polaron E5600 sputter coater. No coating was necessary when high magnifications (200× and higher) were used.

3. Results and discussion

A previous study showed that PEEAs with short PEO lengths and/or low PEO contents were the most suitable candidates for use in medical applications, as they possess good mechanical properties in the swollen state [21]. PEEAs prepared with PEG 300 or PEG 1000 and containing at most 28 wt% of PEO (Table 1) have therefore been evaluated with respect to endothelial cell adhesion and growth, in vivo degradation and processability into porous scaffolds.

3.1. HUVEC adhesion and growth

For use in medical applications requiring mechanical strength, copolymers with relatively low PEO contents should be chosen as these retain adequate mechanical properties in the swollen state [21]. Three hundred PEEA 25/75 and the parent polymer PEA were chosen to evaluate their capacity to sustain cell adhesion and growth. HUVECs, which form the inner layer of blood vessels, were used to explore the potential of PEEA polymers in the engineering of vascular tissue.

Table 1
Characteristics of the purified poly(ether ester amide)s used in this study

Composition[a]	PEO content[a] (wt%)	η[b] (dl/g)	Water uptake[c] (%)	Contact angle ± 2°	E_{dry}[d] (MPa)	$E_{swollen}$[d] (MPa)
0/100 (PEA)	0	0.60	6	37	427	295
300 PEEA 25/75	12	0.64	10	35	326	131
1000 PEEA 31/69	24	0.69	24	33	245	108
300 PEEA 56/44	28	0.35	34	n.d.[e]	153	68

[a] As determined by ^{1}H-NMR.
[b] Solvent: $CHCl_3$/MeOH (1:1 v/v) at 25°C.
[c] At equilibrium [21].
[d] *E*-modulus in the dry and water-swollen state [21].
[e] Not determined.

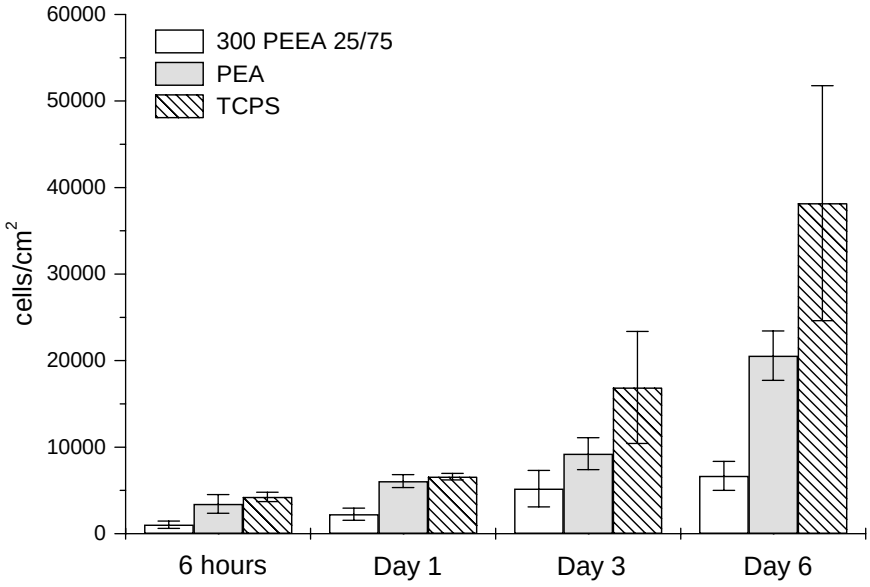


Fig. 2. HUVEC adhesion and growth on 300 PEEA 25/75, PEA and TCPS.

Furthermore, as endothelium is practically ubiquitous in the body, the use of HUVECs can be considered as a relevant in vitro model for the development of other engineered tissues [24]. TCPS was used as a positive control. As can be seen in Fig. 2, HUVECs adhere to and grow on both polymer surfaces. However, the surfaces do not perform as well as TCPS. Although both surfaces had similar contact angles (Table 1), HUVECs seem to perform better on PEA than on 300 PEEA 25/75. It seems therefore important to use PEEA polymers containing low contents of PEO for tissue engineering application. Nevertheless, these results imply that PEEA copolymers and PEA may be used for vascular tissue engineering. Further improvement of cell attachment and growth may possibly be achieved by use of surfaces modified by gas plasma treatment. Gas plasma treatment of segmented copolymer based on PEO and poly(butylene terephthalate) improved the adhesion and growth of bone marrow cells [6].

3.2. *In vivo degradation*

Three hundred PEEA 25/75, 1000 PEEA 31/69, and the parent poly(ester amide) PEA, were implanted subcutaneously in rats and their properties during in vivo degradation were followed up to 14 weeks. Table 2 summarizes the characteristics of the degraded samples.

No change in polymer composition could be detected by ^{1}H-NMR during the 14 weeks of the study (Table 2). The changes in intrinsic viscosity [η] and mass loss during the implantation period are presented in Figs. 3A and B, respectively. The intrinsic viscosity of the polymers decreased slowly over 14 weeks (Fig. 3A). Despite the decrease in (η), the polymers were still mechanically stable and the samples were not brittle. Although the three polymers showed similar degradation profiles, the in vivo degradation rate of 1000 PEEA 31/69, which is the polymer containing the most PEO, is slightly higher than that of 300 PEEA 25/75 and PEA.

Table 2
Composition, intrinsic viscosity (η) and thermal properties of PEEAs during in vivo degradation

Copolymer	Time (weeks)	Composition[a]	η[b] (dl/g)	$T_{g\,soft}$ (°C)	Melting range (°C)	$T_{m\,hard\,max}$ (°C)	ΔH_{hard} (J/g)
PEA	0	0/100 (0)	0.60	−20	110–170	147	33.3
	1	0/100 (0)	0.58	−22	80–170	144[c]	49.5
	2	0/100 (0)	0.55	−22	80–170	147[c]	47.2
	4	0/100 (0)	0.53	−24	75–165	143[c]	54.0
	8	0/100 (0)	0.51	−26	75–165	146[c]	50.9
	14	0/100 (0)	0.49	−19	85–170	150[c]	53.3
300 PEEA 25/75	0	25/75 (12)	0.64	−34	95–150	143[c]	39.5
	1	25/75 (12)	0.67	−35	80–145	142[c]	47.8
	2	25/75 (12)	0.60	−33	85–145	143[c]	57.3
	4	25/75 (12)	0.58	−32	85–145	142[c]	52.2
	8	25/75 (12)	0.56	−39	80–140	137[c]	54.0
	14	25/75 (12)	0.54	−29	90–150	146[c]	54.1
1000 PEEA 31/69	0	31/69 (24)	0.68	−45	100–155	139	43.9
	1	31/69 (24)	0.69	−47	70–145	134	55.5
	2	31/69 (24)	0.64	−47	80–150	138	52.7
	4	31/69 (24)	0.59	−47	90–145	137	45.0
	8	31/69 (24)	0.54	−47	85–145	136	46.6
	14	31/69 (24)	0.53	−46	90–150	140	51.8

[a] Soft/hard segment ratio (PEO content, wt%).
[b] Solvent: $CHCl_3$/MeOH (1:1 v/v) at 25°C.
[c] Shoulder at approximately 95°C.

After 14 weeks in the body, only little mass loss was observed ranging from 7 to 12 wt% for 300 PEEA 25/75, as seen in Fig. 3B. The mass loss at 1 week is relatively high in comparison with the overall mass loss. This can be explained by the leaching of low molecular weight compounds shortly after the implantation. The small increase in [η] at 1 week for 300 PEEA 25/75 and 1000 PEEA 31/69 seems to confirm this hypothesis. The low mass loss and the slow decrease in intrinsic viscosity point towards a bulk degradation process (Fig. 3).

An important outcome of this study is the in vivo degradation of the parent polymer PEA. PEA exhibits mass loss and a decrease in intrinsic viscosity. This implies that degradation also occurs in the ester–amide units, which constitute the hard segments of the PEEAs. Therefore, for the design of degradable segmented copolymers, the use of ester–amide monomer seems a good alternative to dimethyl terephthalate previously used in the synthesis of PEOT/PBT.

PEEAs and the parent polymer PEA are semicrystalline polymers with a glass transition at low temperature [21]. Before implantation, PEEAs and PEA exhibit a melting endotherm at approximately 145–150°C (corresponding to the maximum of the endotherm). The DSC thermograms of the (non)degraded samples are presented in Fig. 4. After 1 week in the body, a broadening of the melting transitions (extending over 60–80°C) is observed. The maximum of the melting temperature remained, however, almost unchanged. The thermal transition visible at approximately 50°C disappeared in time. After an initial increase due to annealing of the samples at 37°C, the heat of fusion ΔH_{hard} reaches a constant value, which is similar for all polymers (Table 2). The glass transition temperature corresponding to the PEO-containing segments is relatively unchanged up to 14 weeks. Although the effect of annealing is noted, the results suggest that the thermal properties of the polymers have not substantially been modified during in vivo degradation (Fig. 4).

The degradation behavior of 1000 PEEA 31/69 in vivo is similar to the degradation in PBS at 37°C. In both situations, after 8 weeks, no composition change is noted and a decrease in (η) of approximately 20% is measured [19]. Based on the similar degradation behavior of the polymers in vitro and in vivo, one can conclude that in vivo degradation also takes place via hydrolysis in the bulk, probably involving random scission of ester bonds. A major difference between the results of the in vitro and in vivo studies is the change in polymer structures during implantation. As seen in the SEM pictures in Fig. 5, the surfaces of the three polymers became rougher in time. Cross sections of the samples did not show the same pitted structure. Therefore, one can draw the conclusion that the mass loss after the initial release of low molecular weight compounds mostly originates from the sample surface. The observed patterns are probably caused by the activity of cells at the surface. As shown by the histological analysis (Fig. 6), pores and cracks are infiltrated by cells from the first week of implantation.

After 1 and 14 weeks of implantation, cross sections of the polymer samples and the surrounding tissues were

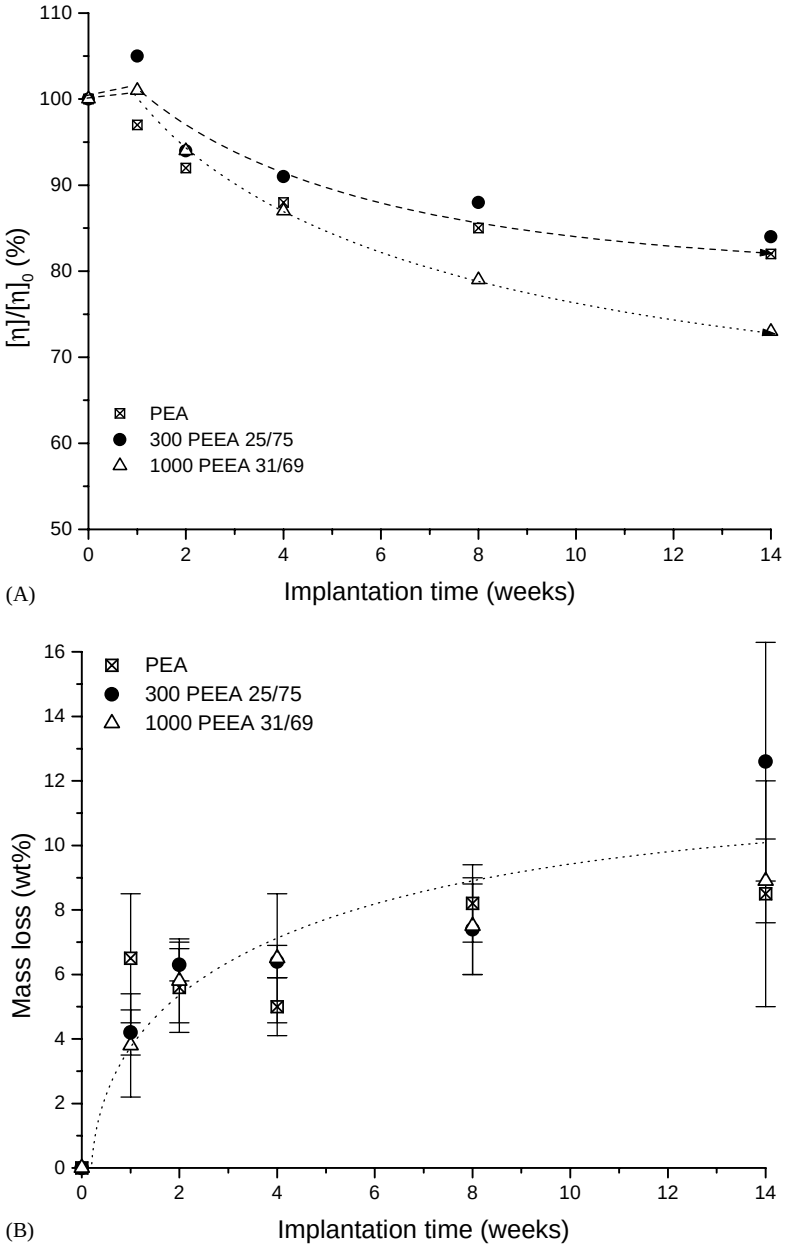


Fig. 3. Relative intrinsic viscosity (A) and mass loss (B) as a function of degradation time in vivo for (⊠) PEA, (●) 300 PEEA 25/75 and (△) 1000 PEEA 31/69. The lines drawn in the graphs are guides for the eye.

studied by optical microscopy (Fig. 6). The gaps between the polymer surface and tissue observed in a few pictures (A1, A14, B14 and C1) are artifacts due to the processing of the samples for the histological analyses.

After 1 week of implantation, all polymer samples are encapsulated by fibrous tissue (T), and macrophage-like cells were present on the polymer surfaces (arrowheads). At larger distances from the polymer surfaces also fat cells were observed. In accordance with the cell growth experiments, the polymers did not cause any toxic reactions and no significant adverse tissue reaction was notable. At 1 week of implantation, the surface and bulk of PEA (Fig. 6, A1) and 300 PEEA 25/75 (Fig. 6, B1) appeared relatively intact. On the surface of 1000 PEEA 31/69, cracks are visible, in which tissue ingrowth and cells are observed (Fig. 6, C1).

In agreement with the patterns observed by SEM, the histological pictures show erosion of the surface after 14 weeks in comparison with samples implanted for 1 week. At 14 weeks of implantation, the PEA surface is eroded, while the bulk does not show any changes (Fig. 6, A14). Numerous macrophage-like cells infiltrate the erosion pits. The implants of 300 PEEA 25/75 look similar to

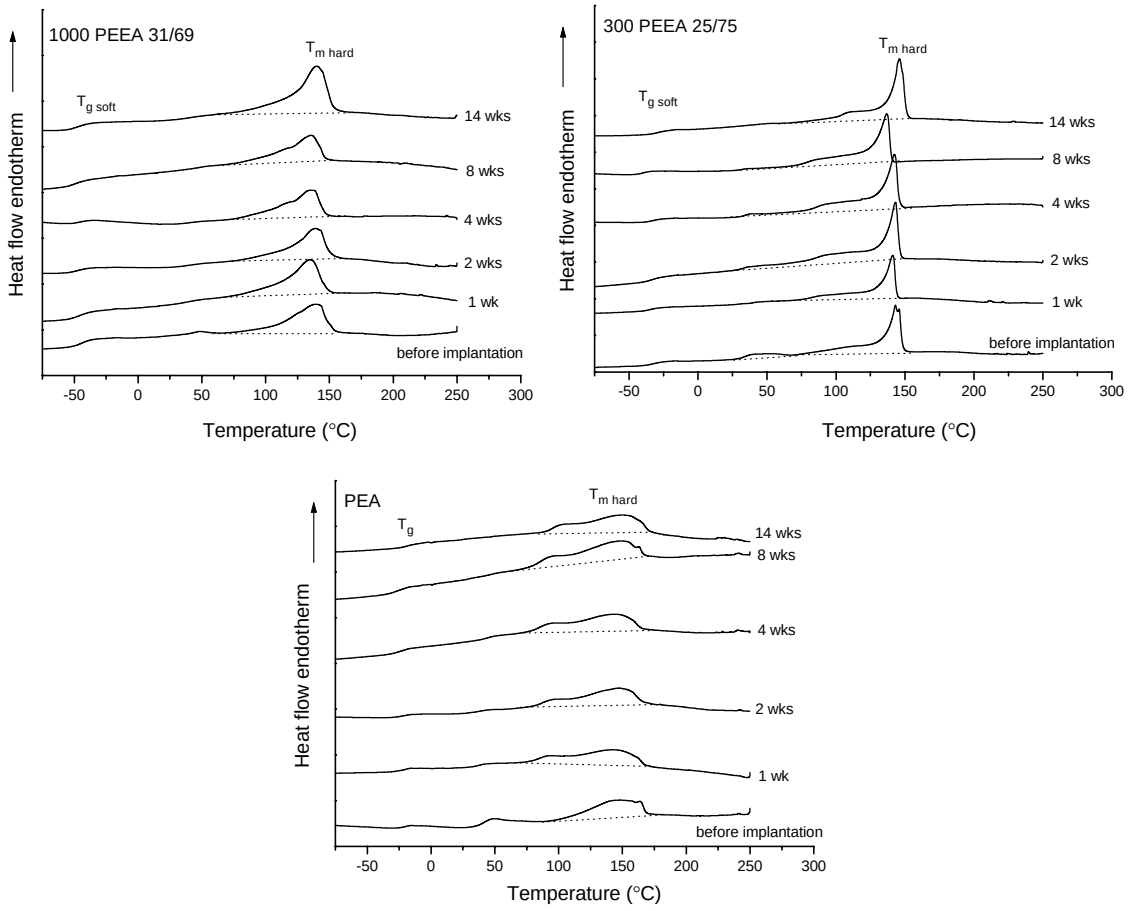


Fig. 4. DSC thermograms of PEEA and PEA polymers before and after implantation. The dotted lines enclose the area used for the calculation of the heat of fusion ΔH_{hard}.

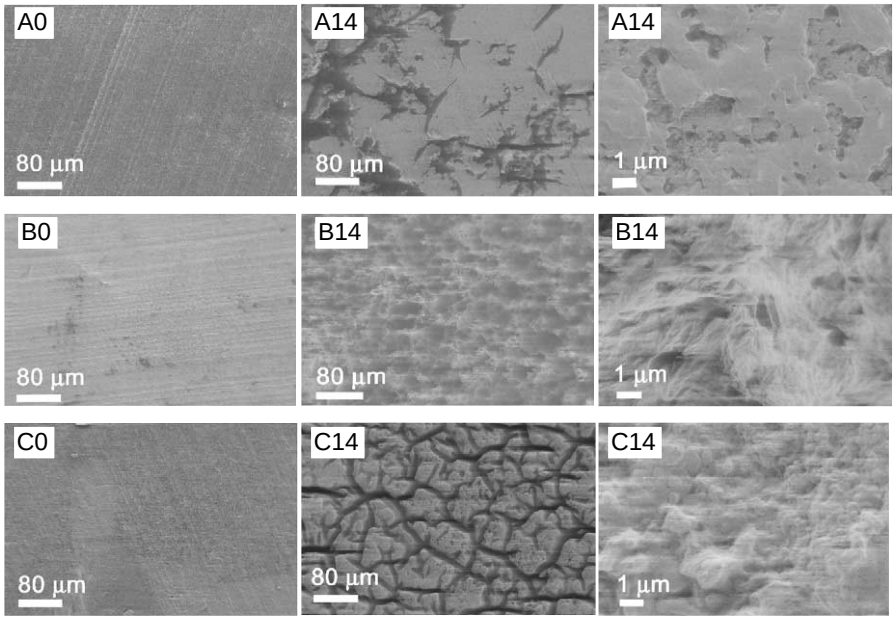


Fig. 5. Surface morphology observed by SEM for PEA (A), 300 PEEA 25/75 (B) and 1000 PEEA 31/69 (C) before implantation (left) and after 14 weeks of implantation (center and right).

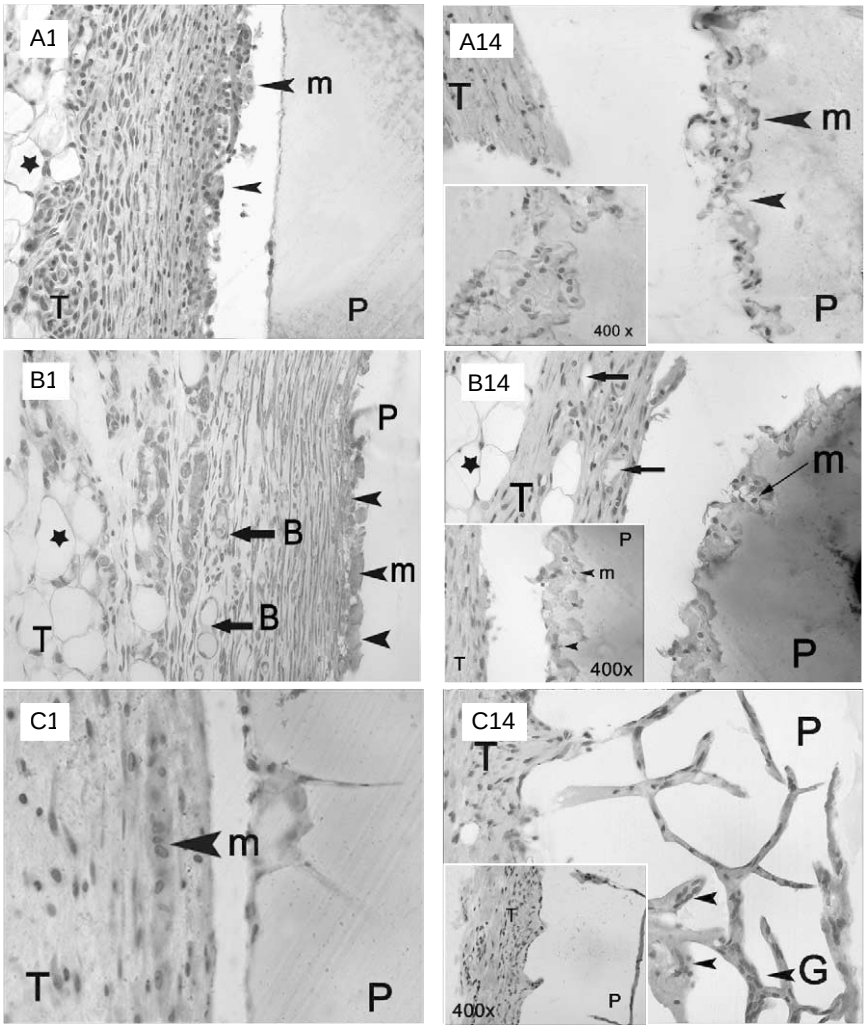


Fig. 6. Histological section of (A) PEA, (B) 300 PEEA 25/75 and (C) 1000 PEEA 31/69 after 1 week and 14 weeks of implantation. Unless otherwise mentioned, magnification: 200 ×. (P) polymer, (T) fibrous tissue, (B) blood vessel, (m) macrophage-like cell, (G) giant cells, (★): fat tissue.

those of PEA. No change in the middle of the sample is noted, whereas surface erosion of the polymer is evident (Fig. 6, B14). The edge of the sample is completely eroded into a rounded shape, while macrophage-like cells infiltrate the pits. Polymer fragments (indicated by arrows) can be observed in between connective tissue. The cracks already present on the surface of 1000 PEEA 31/69 after 1 week seem to have enlarged after 14 weeks in vivo (Fig. 6, C14). At higher magnification (400 ×), macrophage-like cells and giant cells are clearly visible in those cracks.

Several mechanisms can be involved in PEEA degradation. It appears from the sample analyses that hydrolysis occurs in the polymer bulk. Naturally, such hydrolysis also takes place at the surface of the polymer. Based on the pitted polymer surfaces observed after in vivo degradation, which did not occur after in vitro degradation, and on the presence of macrophage-like cells, one can concluded that cellular activity is playing an essential role in the in vivo degradation of PEEA copolymers. Specific activated cells such as macrophages and foreign-body giant cells release oxygen radicals and superoxide anion radicals, which can combine with protons to form hydroperoxide radicals [25,26]. Several investigations have suggested that in vivo degradation of segmented poly(ether urethane) elastomers involves oxidation of the aliphatic ether groups in these polymers by oxygen radicals [25,27] and phagocyte-derived oxidants [28]. Therefore, the polymer erosion can be caused by oxidation of the PEO segment present in the amorphous domains. Activated cells can also release enzymes, which might be involved in the polymer degradation.

3.3. Porous scaffolds

To be used as scaffolds in tissue engineering, PEEAs need to be processed into porous devices. Porous

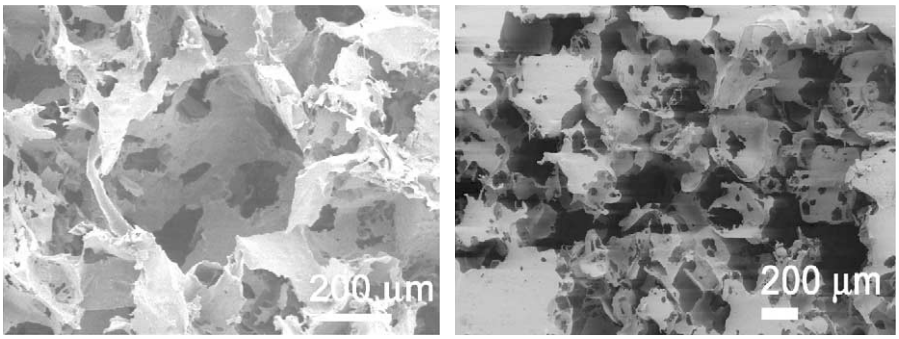


Fig. 7. Porous structures obtained from 300 PEEA 56/44 (left, porosity: 90%.) and PEA (right, porosity: 92%.).

structures have been prepared by mixing sodium chloride and ground polymer particles, followed by melt-pressing and subsequent salt leaching [6]. The characteristics of the obtained porous structure, size and porosity, are widely adjustable by variation of the size and amount of the salt particles added. Salt particles of 500–750 µm were used and scaffolds with a porosity of 90% were prepared. Fig. 7 shows porous structures made of 300 PEEA 56/44 and PEA. Similar structures with a porosity of 90% were obtained with 1000 PEEA 31/69. These highly porous devices could be handled with ease and were mechanically stable. It was not possible to obtain stable structures with PEEAs prepared with PEG 4000. These copolymers are very hydrophilic with water-uptakes up to 350 wt% and loose their mechanical properties upon swelling [21]. As a consequence, during the leaching of the salt particles the structures were not stable.

4. Conclusions

PEEA copolymers based on PEG, 1,4-butanediol and dimethyl-7,12-diaza-6,13-dione-1,18-octadecanedioate (a diester-diamide monomer) possess good mechanical properties and are suitable for use in medical devices, especially PEEAs with short PEO length and/or low PEO content. PEEA copolymers and PEA sustain endothelial cell adhesion and growth. The growth rate of HUVECs is higher when the PEO content in the copolymer decreases. PEEAs degrade in vivo, although the degradation rate is low. The parent poly(ester amide) PEA also undergoes in vivo degradation. This result shows that the ester–amide units that constitute the hard segments in the block copolymers can be degraded. Analyses of the polymer samples reveal that degradation occurs in the bulk but also at the surface of the polymers. As histology shows numerous cells infiltrating the polymer pits, the surface erosion likely involves cellular activity. The PEEA copolymers did not induce an adverse tissue reaction. Furthermore, it is shown that porous scaffolds can be readily prepared from PEEA materials.

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