Multiblock copolyesters as biomaterials: *in vitro* biocompatibility testing

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Cell adhesion, cell growth and cell activities of macrophages and fibroblasts, cultured on newly developed degradable multiblock-copolyesters were studied to examine the biocompatibility and the possible use of these polymers for medical applications. The biocompatibility and the biodegradability of the polymers were confirmed by subcutaneous implantation of polymer foils in rats.

The newly developed polymers, two polyesters (DegraPol/bsc43 and DegraPol/bsd43) and a polyesterether (DegraPol/bst41), were found to exhibit good cell compatibility; the cell-to-substrate interactions induced neither cytotoxic effects nor activation of macrophages.

The adhesion and growth of fibroblasts and macrophages were different among the substrate. Fibroblasts adhered on the polyesters to about 60% of control cell cultured on tissue culture polystyrene (TCPS) and proliferated in the same doubling time as on TCPS. On the polyetherester cells exhibited weak adhesion; however, they proliferated up to day 4 after plating at the same doubling time as on TCPS (of about 42 h), and then decreased their doubling time to 27 h. Macrophages attached to the polyesters to about 40–60% of TCPS but no significant change was seen in the doubling time of cells cultured on TCPS and the polyesters. Again on the polyetherester, macrophages exhibited relatively low adhesion (25% of TCPS) and high doubling time (about 100 h).

Fibroblasts produced high amounts (up to 500% of control cells) of collagen type I and type IV, and fibronectin. Macrophages responded to lipopolysaccharide treatment by the production of nitric oxide (NO) and tumour necrosis factor-α (TNF-α), indicating that the cell-to-polymer interactions allow fibroblasts and macrophages to maintain their phenotype.

All three test polymers exhibit favourable tissue compatibility. The formed capsule was just a few cell layers thick (< 30 μm). After 2 months implanted subcutaneously in rats, the molecular weight of the test polymers was reduced by > 20% depending on their chemical structure.

Taken collectively, the present data demonstrate that the newly developed multiblock copolyesters are biocompatible and biodegradable.

Abbreviations

BSA	Bovine serum albumin
C	DegraPol/bsc43 Poly{[α,ω-dihydroxy-poly((*R*)-3-hydroxybutyric acid-*co*-(*R*)-3-hydroxyvaleric acid)-*block*-ethyleneglycol-*block*-sebacic acid]-*co*-[α,ω-dihydroxy-(poly(ε-caprolactone)-*block*-diethyleneglycol-*block*-poly(ε-caprolactone))-*block*-sebacic acid]} with 43 wt % PHB-diol
D	DegraPol/bsd43 Poly{[α,ω-dihydroxy-poly((*R*)-3-hydroxybutyric acid-*co*-(*R*)-3-hydroxyvaleric acid)-*block*-ethyleneglycol-*block*-sebacic acid]-*co*-[α,ω-dihydroxy-poly (adipic acid-*alt*-(butanediol; diethyleneglycol; ethyleneglycol))-*block*-sebacic acid]} with 43 wt % PHB-diol
DCE	1,2-dichloroethane
Diorez-diol	α,ω-Dihydroxy-poly(adipic acid-*alt*-(1,2-ethanediol; 1,4-butanediol; 3-oxa-1,5-pentanediol)) (Diorez® 571)
Diglyme	Bis-(2-methoxyethyl)-ether
DSC	Differential scanning calorimetry
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
GPC	Gel permeation chromatography
HEPES	*N*-(2-Hydroxyethyl)-piperazine-*N*′-2-ethane sulphonic acid

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LPS	Lipopolysaccharide
MTT	Succinate dehydrogenase activity assay
NO	Nitric oxide
PBS	Phosphate-buffered saline
PCL-diol	α, ω-Dihydroxy-poly(ε-caprolactone)
PHB-diol	α, ω-Dihydroxy-poly[3-(*R*)-hydroxybutyric acid-*co*-3-(*R*)-hydroxyvaleric acid]
PTHF-diol	α,ω-Dihydroxy-poly(1-oxa-pentamethylene)
SC	Sebacoylchloride
SEM	Scanning electron microscopy
T	DegraPol/bst41 Poly{[α,ω-dihydroxy-poly((*R*)-3-hydroxybutyric acid-*co*-(*R*)-3-hydroxyvaleric acid)-*block*-ethyleneglycol-*block*-sebacic acid]-*co*-[α,ω-dihydroxy-poly(tetrahydrofurane)-*block*-sebacic acid]} with 43 wt % PHB-diol
TCPS	Tissue culture polystyrene
THF	Tetrahydrofurane
TNF-α	Tumour necrosis factor-α
VPO	Vapour pressure osmometry

1. Introduction

Degradable polymeric materials have widespread use in medicine and surgery [1]. Such materials are designed to degrade *in vivo* in a controlled manner over a predetermined implantation period such that degradation achieves, or helps to achieve, a particular function. Although the specific material requirements will differ according to the nature of the application, it is a fundamental requirement in each case that the device should display adequate biocompatibility. Some of the most important factors in determining *in vivo* biocompatibility have been recognized as being the cellular interactions which characterize the inflammatory response [1, 2].

Tissue injury following implantation of a medical device initiates tissue repair and regeneration processes. Cells such as fibroblasts, macrophages, and endothelial cells migrate into the implantation site (inflammatory site) and proliferate [2]. Activated macrophages produce cytokines, notably interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-10 (IL-10), and tumour necrosis factor-α (TNF-α) [3, 4], cytokines involved in the regulation of the inflammatory and the wound healing process. In addition to these cytokines, activated macrophages produce nitric oxide (NO). Macrophages are also involved in the biodegradation of foreign materials (for a review see [5]). The size, shape, chemical and physical properties, and the degradability of the polymers are important factors, determining the type, intensity, and duration of the inflammatory response and wound healing process. The biocompatibility of a test material can be examined either *in vivo* via implantation or injection of the test material, or *in vitro* using appropriate cells that play an important role during wound healing and regeneration processes.

Linear homo-, diblock-, and triblock-polyesters of lactic and glycolic acids have been prepared as polymeric materials for medical applications [6–11]. To our knowledge, however, investigations on multiblock-copolyesters have not been reported. These systems have the advantage that the softening temperature and the mechanical properties, as well as the degradability rates can be adjusted to requirement. This can be achieved by varying the nature, the length, and the mass fraction of the different blocks. The systems described in this paper are built up from a crystallizing "hard segment", a telechelic diol-terminated block of [{3-(*R*)-hydroxybutyrate}-co-{3-(*R*)-valerate}] (PHB-diol), and an amorphous "soft segment" of dihydroxy-poly(adipic acid-*alt*-(1,2-ethanediol; 1,4-butanediol; 3-oxa-1,5-pentanediol)) (Diorez® 571) or a crystallizable polyester-diol of ε-caprolactone (PCL-diol), or an amorphous dihydroxy-poly(1-oxa-pentamethylene) (PTHF-diol). Details of the polymer synthesis are described elsewhere [12].

In the present investigation, the biocompatibility as well as the biodegradability of these newly developed copolymers were determined *in vitro* using macrophages, and fibroblasts, and in *in vivo* studies.

2. Materials and methods

2.1. Preparation of polymer samples

2.1.1. Materials

1,2-Dichloroethane (DCE), 1,4-dioxane, *n*-hexane, petrolether, sebacoylchloride (SC), pyridine, and 2-dimethylaminopyridine were obtained from Fluka; dihydroxy-poly(1-oxa-pentamethylene) (PTHF-diol) ($M_w = 650$) was obtained from Polyscience Inc.; dihydroxy-poly(-caprolactone) (PCL-diol) ($M_w = 1200$) was obtained from Aldrich; dihydroxy-poly(adipic acid-*alt*-(1,2-ethanediol; 1,4-butanediol; 3-oxa-1,5-pentanediol)) (Diorez® 571) ($M_w = 1000$) was obtained from MacPherson Polymers. All solvents and chemicals were purchased in the best quality available. The solvents and chemicals were used as received, except for SC, which was distilled twice prior to use.

2.1.2. Methods

All glass-transition temperatures and melting points were determined by differential scanning calorimetry (DSC) measurements (Mettler DSC 30) at a heating rate of $10\,^{\circ}C\,min^{-1}$.

Advancing contact angles for water in air were measured with a Ramé–Hart 100-00 goniometer.

Molecular weights and molecular weight distributions were estimated by gel permeation chromatography (GPC) on a Knauer high-temperature GPC with a PL-gel column (mixed D, 5 μl, 7.5×600 mm, THF as solvent, flow $1.0\,ml\,min^{-1}$, 5 MPa, 45 °C, injection 100 μl, sample $5\,mg\,ml^{-1}$) equipped with a Knauer high-temperature refractometer and a viscometer detector (Viscotec, type H 502 B). The number-average molecular weight was measured by vapour pressure osmometry (VPO) on a Corona Wescan (type 232 A) apparatus with chloroform as solvent at 25 °C.

The water content was determined by Karl-Fischer titration with a Metrohm 684 KF Coulometer.

TABLE I Composition and some properties of the polymers tested

Polymer abbreviation and trade name	Type of soft segment	Composition (mmol) Diol	PHB	SC	M_w	dn/dc (ml g^{-1})
C DegraPol/bsc43	PCL-diol	4.24	2.17	6.57	29 000	0.068
D DegraPol/bsd43	Diorez-diol	10.03	4.36	14.49	40 000	0.071
T DegraPol/bst41	PTHF-diol	7.71	2.18	9.92	42 000	0.084

2.1.3. Preparation of the Block-copolyesters

2.1.3.1. Hard and soft segments. The usefulness of block-copolymers rests largely on the fact that blocks of different composition, constitution, or configuration that constitute the macromolecule are mutually incompatible and therefore tend to segregate into separate domains, often of just two types. One of these domains is typically designed to be, at the temperature of usage, crystalline or glassy (amorphous clearly below T_g), and hence of high modulus, and imparts mechanical stiffness to the material – it is customarily termed "hard segment". The other domain is then designed to be "soft" (amorphous clearly above T_g) and so retains good molecular flexibility in the material; these terms are unsatisfactory but in common use and we adopt them here lacking better definitions.

2.1.3.2. Hard segment – PHB-diol. Following Hirt *et al.* [13], telechelic blocks of the "hard segment" were obtained by glycolysis of commercially available, high-molecular-weight PHB, i.e. poly(3-(*R*)-hydroxybutyric acid-*co*-3-(*R*)-hydroxyvaleric acid) with somewhat less than 5% w/w of valeric acid (Zeneca, Biopol®). The bacterial PHB was dissolved in diglyme (bis-(2-methoxyethyl)-ether) at 140 °C, ethylene glycol in excess and some dibutyltin dilaurate as catalyst were added, and the mixture was stirred at this temperature until the desired molecular weight ($M_w = 2400$) of the telechelic α,ω-dihydroxy-oligo((*R*)-3-hydroxybutyric acid-*co*-3-(*R*)-hydroxyvaleric acid) (PHB-diol) was obtained. The product was purified by column chromatography and dried at 80 °C.

2.1.3.3. "Soft segments". Three types of commercially available soft segments were employed: (i) α,ω-dihydroxy-poly(adipic acid-*alt*-(1,2-ethanediol; 1,4-butanediol; 3-oxa-1,5-pentanediol)) (MacPherson, Diorez® 571) of molecular weight $M_n = 1000$, hereafter termed Diorez-diol; (ii) α,ω-dihydroxy-poly(1-oxa-pentamethylene) (PolySciences, PTHF-650) of molecular weight $M_n = 650$, called PTHF-diol below; and (iii) α,ω-dihydroxy-poly(ε-caprolactone) (Aldrich, PCL-1200) of molecular weight $M_n = 1200$, named PCL-diol in the following. The soft segments were used as received.

2.1.3.4. Synthesis of the Block-copolymers. Equimolar amounts of the PHB-diol and a soft segment were dissolved in DCE and the solution dried azeotropically by refluxing under nitrogen over activated molecular sieve (Aldrich, 0.5 nm) until the water content fell below 5 p.p.m. The reaction mixture was cooled to 4 °C and the stoichiometric quantities of sebacoyl dichloride (doubly distilled) and of pyridine (< 15 p.p.m. H_2O) as well as 0.1% w/w of 2-dimethylaminopyridine were added. Stirring at 4 °C under nitrogen for an extended time yielded the desired molecular weight polymer. The products were purified by addition of some DCE, washing with a large excess of water and subsequent drying at 50 °C at reduced pressure. The polymer, redissolved in hot dioxane, was precipitated in distilled water and then dried at 50 °C and < 1 kPa.

The resulting polymers all contained approximately 40% w/w of PHB-diol and had a molecular weight of approximately 30 000–40 000. Compositions and molecular weights are given in Table I.

2.1.4. Preparation of the films

The polymer was heated to 140 °C and the resulting melt pressed between metallic, Teflon-coated plates (140 °C, 25 MPa, 1 min, and then ambient temperature, 25 MPa, 10 min). The film was washed with hexane and petrol ether.

2.2. *In vitro* testing of the biocompatibility

2.2.1. Cell culture

Cells were cultured in polystyrene flasks (Falcon, Inotech Dottikon, Switzerland) in a humidified atmosphere at 5% CO_2. Macrophages (murine macrophage cell line, J774) were maintained in Dulbecco's modified Eagle's medium (DMEM medium) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and 50 μg/ml gentamycin. Fibroblasts (mouse fibroblast cell line, 3T3) were cultured in RPMI-1640 medium supplemented with 2 g/l $NaHCO_3$, 25 mM HEPES, 10% FCS, 2 mM L-glutamine, and 50 μg/ml gentamycin.

2.2.2. Determination of cell adhesion and cell growth

For the determination of the cell adhesion and cell growth, discs of 14 mm diameter and 100 μm thickness of each of the three test polymers were

prepared and placed in the bottom of each well of a 24-well tissue culture plate. Fibroblasts (3T3) and macrophages (J774) were trypsinized using 0.05% trypsin and 0.02% EDTA for 5 min, centrifuged at 200 *g* for 10 min and resuspended in the same culture medium. Single-cell suspensions were added to the polymers at a density of 2×10^4 cells per well in 1 ml of their respective culture medium and were allowed to attach at 37 °C. After 24 h the culture medium was replaced with the same culture medium. The cell density on the polymers was determined 1, 2, 4 and 8 days after cell seeding, using the MTT test. All determinations were carried out in duplicates. For each experimental value, three independent experiments were carried out. As positive control, cells were platted onto tissue culture 24-well plates (NUNC, Roskilde, Denmark).

2.2.3. MTT assay

The number of attached and viable cells was determined with MTT assay as already described [14]. In brief, 10 µl of a 5 mg/ml MTT tetrazolium solution in PBS was added to the cells, and incubated for 1 h at 37 °C. The dye was then removed and cells were lysed by addition of 200 µl of isopropanol with 5% formic acid and 200 µl 10% sodium dodecyl sulphate (SDS). Plates were read at 550 nm on a multiscan bichromatic ELISA Reader (Dynatech, MR 5000).

2.2.4. Determination of NO production

To measure the amount of nitrite in the culture, 50 µl aliquots of culture supernatant were mixed with 200 µl of the Griess reagent [15]. The absorbance was read at 540 nm after 20 min of reaction and the NO_2^- concentration was determined with reference to a standard curve using concentrations from 1 to 100 µM sodium nitrite in culture medium. As positive control, NO was measured in supernatants of lipopolysaccharide (LPS) stimulated cells (10 µg LPS/ml).

2.2.5. Quantification of TNF-α production

TNF-α levels in the supernatant of cells cultured on the test polymers or of LPS-treated cells (as positive control for cell activation) were determined in a TNF-α specific bioassay using the WEHI cell line as previously described [16]. WEHI 164 clone 13 fibrosarcoma cells at a concentration of 2×10^4 cells per 100 µl were incubated with serially diluted samples in 96-well flat-bottom microtitre plate for 48 h at 37 °C, 5% CO_2. Then 10 µl of a 5 mg/ml MTT tetrazolium solution in PBS was added in the plate, which was further incubated for 1 h. The dye was then removed and cells were lysed by addition of 200 µl of isopropanol with 5% formic acid. Plates were read at 550 nm on a multiscan bichromatic ELISA Reader (Dynatech, MR 5000).

2.2.6. Determination of the concentration of fibronectin, collagen type I and IV on cultured fibroblasts

For the determination of the effect of the cell–substrate interaction on the production of collagen type I and IV and fibronectin by cultured fibroblasts, cells (1×10^5 cell/polymer samples) were cultured for 4 days on test polymers and then solubilized for 1 h at 4 °C in buffer containing 20 mM Tris, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1 M Urea and 0.5% Triton X-100, pH 7.2, and sonicated for 30 min. Protein concentrations were determined according to Bradford [17] using bovine serum albumin (BSA) as standard. The content of fibronectin, collagen type I and IV in cell solubilizates was determined in an ELISA as described earlier [18]. 100 µl of 300 µg protein/ml were incubated in the 96-well microtitre plates for 1 h at 37 °C, or overnight at 4 °C. After three washing steps in PBS, non-specific binding sites were blocked in PBS containing 2% BSA for 1 h at room temperature. After another three washing steps with PBS, polyclonal antibodies against collagen type I (rabbit anti-mouse collagen type I, Inotech, Dottikon, Switzerland), fibronectin (rabbit anti-human fibronectin, Inotech, Dottikon, Switzerland), or collagen type IV (rabbit anti-mouse collagen type IV, Inotech, Dottikon, Switzerland) were added (dilution 1 : 100) in 100 µl PBS containing 2% BSA for 2 h at room temperature. The microtitre plates were then washed and the second, alkaline phosphatase conjugated antibody (diluted 1 : 1000), was added in 100 µl PBS containing 2% bovine serum albumin for 2 h at room temperature. After three washing steps, 100 µl "substrate" (2 mg/ml *p*-nitrophenyl-phosphate (Fluka, Switzerland) in 0.1 M glycine buffer (pH 10.4) containing 1 mM $MgCl_2$ and 1 mM $ZnCl_2$) were added and the absorption at 405 nm was measured in an ELISA reader. All washing steps were carried out with PBS at room temperature. Background values measured in the absence of primary antibodies were subtracted from the experimental values.

2.2.7. Determination of the protein adsorption on test polymers

For the measurement of the adsorption of serum proteins on test polymers, discs of the polymers of 5 mm diameter and 100 µm thickness were prepared and placed in the bottom of each well of a 96-well ELISA plate (Nunc, Roskilde, Denmark). The polymers were then incubated with normal mouse serum (1 : 500 diluted) (Bio-Science productions, Emmenbrücke, Switzerland) for 24 h at 4 °C. The amount of albumin and γ-globulin was quantified in ELISA tests using specific antibodies. After three washing steps in PBS, non-specific binding sites were blocked in PBS containing 2% BSA for 1 h at room temperature. After another three washing steps with PBS, polyclonal antibodies against albumin (peroxidase-conjugated sheep anti-mouse albumin antibodies Inotech, Dottikon, Switzerland) or immunoglobulins (peroxidase-conjugated goat anti-mouse immunoglobulins antibodies, Dako, Zurich, Switzerland), were added (dilution 1 : 100) in 100 µl PBS containing 2% BSA for 2 h at room temperature. After three washing steps, 100 µl "substrate" (0.5 mg 2,2-azino-di-3-ethylbenzothiazoline-6-sulphonic acid per ml 100 mM Na-acetate, 50 mM Na-phosphate and 9×10^{-3}% H_2O_2) were

added and the absorption at 405 nm was measured in an ELISA reader. All washing steps were carried out with PBS at room temperature. Background values measured in the absence of antibodies were subtracted from the experimental values.

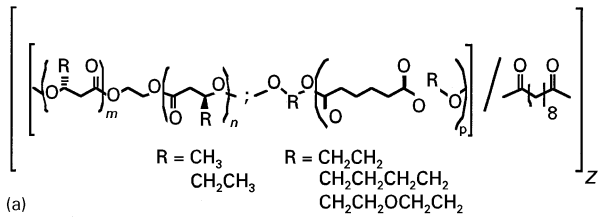
2.2.8. Subcutaneous implantation of the polymers

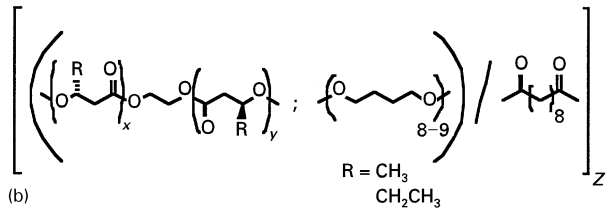
All samples were sterilized with ethylene oxide at ambient temperature and degassed in the usual manner. The tissue compatibility and biodegradation of the polymers (D, T, C) were examined *in vivo* by subcutaneous implantation of polymer films in three SIV-Zur rats (male, 250–360 g (BW), four samples per rat) for 2 months. Specimens were then explanted and the formation of the biological encapsulation was measured (histology, haematoxylin and eosin, Masson). For the determination of molecular weights of the polymers, polymeric samples were cut out of the biological capsule, rinsed three times with a cleaning solution (1% Triton X-100 in distilled water), washed with distilled water and dried under vacuum at ambient temperature for 48 h. The samples were finally dissolved in THF and the molecular weight distribution determined by GPC.

3. Results

One polyetherester (T) and two polyesters (D, C) were prepared (chemical structures are sketched in Fig. 1). Some physical properties of the polymers were measured (Table I) but no significant difference was observed between the polyester (C, D) and polyetherester (T).

For the determination of the biocompatibility of the newly developed polymers, cell morphology, cell attachment, cell proliferation, cell functions, and cell activation of mouse macrophages (J774) and mouse fibroblasts (3T3), cultured on the polymers were measured. In addition, the tissue compatibility and biodegradability of the polymers were examined by subcutaneous implantation of polymer foils in rats.





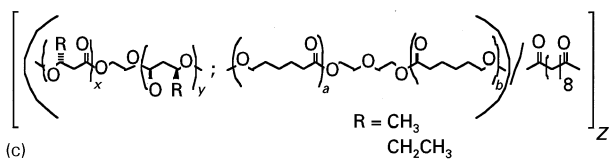


Figure 1 Synthetized polymers. (a) D (DegraPol/bsd43); (b) T (DegraPol/bst41); (c) C (DegraPol/bsc43).

TABLE II NO and TNF-α production by control and LPS-stimulated macrophages

Substrate abbreviation and trade name	Untreated		LPS-treated	
	NO	TNF-α	NO	TNF-α
TCPS	<1	<2	21	120
C DegraPol/bsc43	<1	<2	18	100
D DegraPol/bsd43	<1	<2	12	100
T DegraPol/bst41	<1	<2	14	115

For the determination of cell activation of macrophages, cells were cultured on polymer discs (2×10^4 cells/cm^2) in the absence and presence of 10 μg LPS/ml. Concentrations of TNF-α (U ml^{-1}) were measured 6 h and of NO (μM) 2 days after LPS treatment. The given values are the averages from a representative experiment carried out in triplicate. The standard deviation was between 4–7%. The detection limits for NO and TNF-α were 1 μM and 2 U ml^{-1}, respectively.

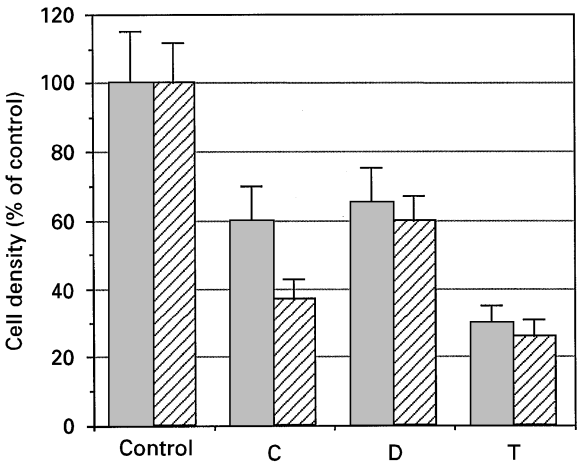


Figure 2 Cell adhesion of fibroblasts (□) and of macrophages (▨). For the determination of cell adhesion of macrophages and fibroblasts, single cell suspensions were added to the polymers at a density of 2×10^4 cells/polymer (discs of 14 mm diameter) and allowed to attach at 37 °C. The number of attached and viable cells was determined 24 h after cell seeding using MTT test. Values given represent the mean, the error bars indicate the standard deviation estimated.

3.1. Cell attachment

The number of attached, viable cells 24 h after cell seeding was taken as an indicator for cell attachment. Compared to control cells cultured on TCPS, fibroblasts attached on the polyesters C and D to about 60%. On the polyetherester T fibroblasts exhibited weak cell attachment (30% of TCPS) (Fig. 2).

Cell-to-polymer interactions also affected macrophage attachment. D exibited relatively high macrophage attachment (70% of TCPS). On the T, C polymers macrophages attached at 25–35% of TCPS (Fig. 2).

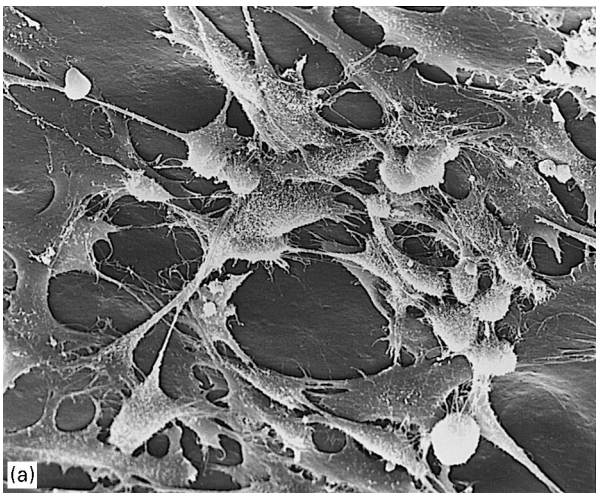
3.2. Cell morphology

Twenty-four hours after cell seeding, both cell types exhibited minimal spreading on polyester and polyetherester polymers, forming cell aggregates instead, in marked contrast to the flattened appearance of control cells. The morphological difference decreased as the cultures aged. At day 8, no difference was observed between cells cultured on test polymers or on TCPS. Furthermore, no sign of cell damage or cell death was observed by light microscopy and scanning electron microscopy (SEM) (Fig. 3).

3.3. Cell growth

The growth kinetics of fibroblasts and macrophages cultured on the test polymers or on TCPS are shown in Fig. 4. The proliferation rates of fibroblasts, as well as the shape of the growth curves are different for the different substrates (Fig. 4a). Twenty-four hours after plating, fibroblasts proliferated on C and D with similar doubling rate as on TCPS of about 42 h. On the polyetherester T fibroblasts proliferated up to day 4 after plating at the same doubling rate as on TCPS, and then increased their proliferation from day 4 to day 8 with doubling rate of about 27 h.

Proliferation of macrophages was also affected by cell–substrate interaction. There was no change in the doubling rate of macrophages cultured on TCPS and the polyesters C and D (about 65 h). In contrast to the polyesters, cells cultured on the polyetherester T proliferated at lower rate, with doubling rate of about 100 h (Fig. 4b).



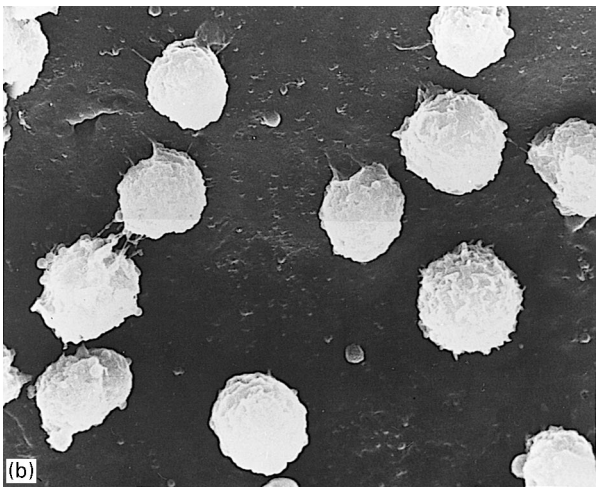
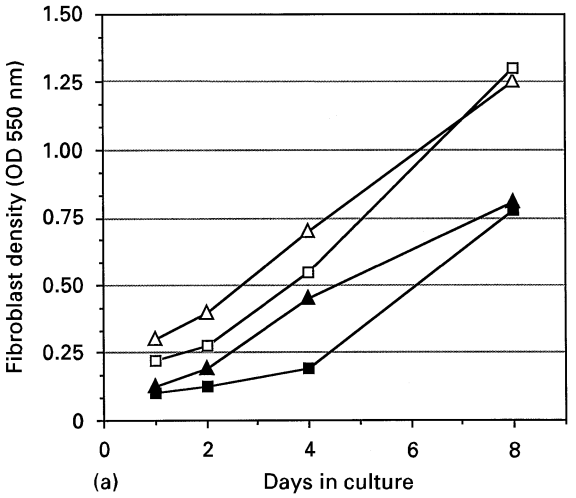


Figure 3 Raster electron micrographs of fibroblasts (a) and macrophages (b) cultured on polyester C for 4 days. The scale bars represent 100 μm and 10 μm in A and B, respectively.



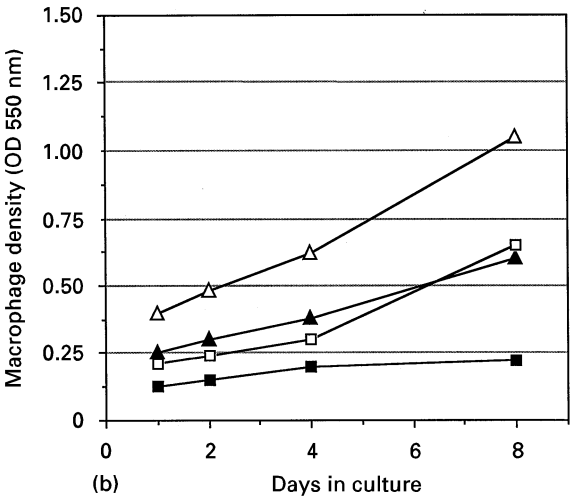


Figure 4 Cell growth of fibroblasts (a) and of macrophages (b). For the determination of cell growth of cultured macrophages and fibroblasts, single cell suspensions were added to the polymers at a density of 2×10^4 cells/polymer (discs of 14 mm diameter) and allowed to attach at 37 °C. The cell density on the polymers was determined 24 h, 2, 4 and 8 days after cell seeding using MTT test. (△) control; (▲) C; (□) D; (■) T.

3.4. Cell function

In addition to the cell attachment and cell growth, it is important that the attached cells can maintain their functions. Activated fibroblasts produce various extracellular matrix proteins, such as collagen and

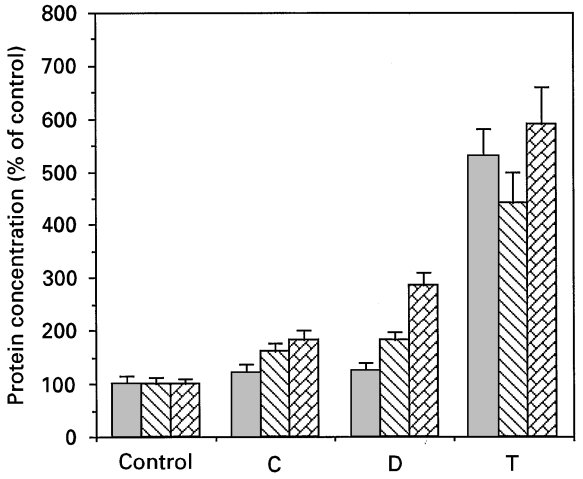


Figure 5 Extracellular matrix proteins production by fibroblasts. The amount of extracellular matrix components fibronectin (▨), collagen type I (▩) and IV (▧) were immunochemically determined 4 days after cell seeding on the test polymers. Values given represent the mean, the error bars indicate the standard deviation estimated.

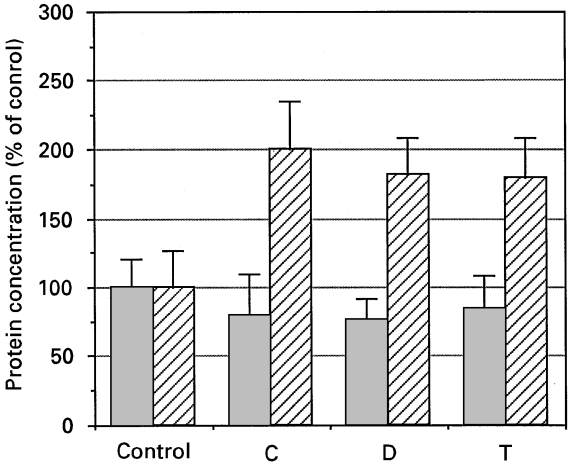


Figure 6 Serum protein adsorption to polymers. The adsorption of serum proteins albumin (▩) and immunoglobulin G (IgG) (▨) were immunochemically measured using specific antibodies. Values given represent the mean, the error bars indicate the standard deviation estimated.

fibronectin. For measuring the cell function of cultured fibroblasts, the production of the extracellular matrix proteins collagen type I, type IV, and fibronectin was determined in ELISA tests using specific antibodies. The average amount of extracellular matrix proteins produced was found to be two to three times higher on polyetherester than on polyesters. Cells cultured on polyetherester produced five times more extracellular proteins than cells cultured on TCPS (Fig. 5).

During inflammation, activated macrophages produce NO and several cytokines, such as TNF-α. None of the test polymers seems to activate cultured macrophages as measured by bioassay (TNF-α) and with the Griess reaction (NO) (Table II). These observations underscore the biocompatibility of the test polymers.

3.5. Cell activation

In a further experiment, the cell response of macrophages to lipopolysaccharide (LPS) treatment, a known activator of macrophages, was tested. Table II shows that cells cultured on all tested polymers responded to LPS treatment by the production of NO and TNF-α, indicating that macrophages were able to maintain their phenotype.

3.6. Protein adsorption

Protein adsorption prior to cell contact with the substrate obviously has an important effect on subsequent cell adhesion, cell growth, and cell functions. Discs of the test polymers were incubated with mouse serum for 24 h and the amount of albumin and γ-globulin was quantified in ELISA tests using specific antibodies. Compared with ELISA plates (very high protein adsorption) all tested polymers showed a high γ-globulin and albumin adsorption (Fig. 6).

3.7. *In vivo* biocompatibility and biodegradability studies

In addition to the *in vitro* tests, the biocompatibility and biodegradability of the polymers was examined *in vivo* by subcutaneous implantation of polymer films in rats. Specimens were explanted after 2 months and the thickness of the biological capsule was measured. All explanted and cleaned polymer films were slightly opaque and brittle. The capsule thickness was less than 30 μm, except D for which 60 μm were obtained (Table III). No significant difference of the specific orientation of the fibroblasts was detected. After 2 months *in vivo*, the molecular weight measurements of the polymers showed a significant decrease. For the polyesters (D, C) the reduction in the molecular weight was about 40%, whereas for the polyetherester (T) it was only 20%. The difference most probably derives from the higher rates in hydrolysis that ester bonds exhibit, in general, compared to ether bonds. Contact angles could no longer be measured, because the explanted pieces were too small.

TABLE III Contact angle with water of the native films and the fibrocapsule thickness measured after 2 months *in vivo* implanted subcutaneously in rats

Polymer	Contact angle of water in air (°)	Capsule thickness (μm)
C DegraPol/bsc43	62±1	29±7
D DegraPol/bsd43	64±3	57±4
T DegraPol/bst41	60±2	17±10

4. Discussion

Cell–substrate interactions play an important role in the development, differentiation and regeneration of multicellular organisms (for a review see [19]). The cellular interaction, in particular the inflammatory response and the cytotoxicity of the prospective

degradation products, are the most important factors determining the biocompatibility of biomaterials and therefore must be considered in the development of degradable biocompatible polymers. When a foreign substance, such as polymers, comes in contact with the organism, the system initiates its host-defence mechanisms involving the inflammatory response. The infiltration of macrophages and fibroblasts and the formation of a mature vascularized fibrous capsule are typical for the tissue response. Macrophages play a major role in all these kinds of inflammation (for a review see [2]). Therefore, in the present study both fibroblasts and macrophages were used to characterize the biocompatibility of the polyesters and polyetherester polymers. In addition, results obtained *in vitro* were compared with results obtained by *in vivo* implantation studies.

The results obtained in the present study indicate that the newly developed degradable polyesters and polyetherester exhibit good biocompatible properties. They fulfilled one of the important parameters of biocompatibility, namely, they failed to activate macrophages and did not induce cytotoxic effects. In addition, *in vitro* observations indicate that:

(i) fibroblasts cultured on the test polymers preserved their phenotype; they produced high amounts of extracellular matrix proteins;
(ii) macrophages were not activated when cultured on the test polymers and they maintained their ability to respond to bacterial toxins such as lipopolysaccharide;
(iii) no sign of cell damage or cell death were seen in either cell types.

Although all polymers studied are biocompatibile, different cell behaviour to the polyesters and the polyetherester was observed (cell adhesion, cell growth). It has been widely accepted that cell–substrate interactions take place via cell adhesion molecules [20]. The cell–substrate interaction of fibroblasts, found in connective tissues, occurs via cell receptors on the cell membrane of fibroblasts and proteins of the extracellular matrix. The integrin family is the most widely known group of cell receptors for extracellular matrix proteins [21]. Also in the cell culture system, cells such as macrophages and fibroblasts seem to adhere to the surface of culture dishes through adsorbed cell adhesion molecules either already found in the serum medium or secreted from the cells during the culture [22].

Cell adhesion has been found to be correlated with the substrate contact angle [23, 24]. However, in the present study no correlation was found between the contact angle with water and cell adhesion of fibroblast or macrophages. Possibly, the cells attached to the polymers are not in direct contact with the substrate surface, but in indirect contact through the plasma proteins adsorbed on the substrates or through substances produced by the cells themselves. It follows that the difference in the growth rates among the substrates may result from the difference in protein adsorption onto the polymers or from the difference in the amount of proteins synthetized by the cells on the substrates during proliferation.

We determined the adsorption of serum proteins albumin and immunoglobulin G in order to test if the difference in the protein adsorption may be responsible for the difference in the cell adhesion to polyester and to polyetherester. Again, as for the comparison between cell adhesion and contact angle, no obvious correlation was found between protein adsorption and cell adhesion. Therefore, other factors than contact angle and protein adsorption must be responsible for the different cell adhesion and cell growth on the polyesters compared to the polyetherester. Indeed, both cell adhesion and cell growth paralleled the amount of extracellular matrix proteins produced by fibroblasts during cell culture. Cells with low cell adhesion and cell growth (cultured on polyetherester) produced higher amounts of extracellular matrix proteins than cells with relatively high cell adhesion and cell growth.

The *in vivo* tests confirmed the good biocompatibility already found through *in vitro* tests. The capsule formed was very small, only a few layers of fibroblast cells (in two of three cases $< 30\ \mu m$). The test polymers proved to be subcutaneously biodegradable in rats. The average molecular weight of the pure polyesters was reduced by 40%, whereas that of the polyetheresters only by 20%. These results were expected since ether bonds are more stable against hydrolytic degradation than polyester bonds. Increasing the number of hydrolytic unstable bonds, therefore, increases the degradation rate.

5. Conclusions

In summary, based on the *in vitro* investigations, the newly developed polyesters fulfil one of the important parameters of biocompatibility, namely, they fail to activate macrophages and do not induce cytotoxic effects. In addition, cells cultured on the test polymers showed good levels of cell adhesion, cell growth, and maintainance of function. These results were confirmed by results obtained *in vivo*.

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