Porous PEOT/PBT scaffolds for bone tissue engineering: Preparation, characterization, and *in vitro* bone marrow cell culturing

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Abstract: The preparation, characterization, and *in vitro* bone marrow cell culturing on porous PEOT/PBT copolymer scaffolds are described. These scaffolds are meant for use in bone tissue engineering. Previous research has shown that PEOT/PBT copolymers showed *in vivo* degradation, calcification, and bone bonding. Despite this, several of these copolymers do not support bone marrow cell growth *in vitro*. Surface modification, such as gas-plasma treatment, is needed to improve the *in vitro* cell attachment. Porous structures were prepared using a freeze-drying and a salt-leaching technique, the latter one resulting in highly porous interconnected structures of large pore size. Gas-plasma treatment with CO_2 generated a surface throughout the entire structure that enabled bone marrow cells to attach. The amount of DNA was determined as a measure for the amount of cells present on the scaffolds. No significant effect of pore size on the amount of DNA present was seen for scaffolds with pore sizes between 250–1000 μm. Light microscopy data showed cells in the center of the scaffolds, more cells were observed in the scaffolds of 425–500 μm and 500–710 μm pore size compared to the ones with 250–425 μm and 710–1000 μm pores. © 2002 Wiley Periodicals, Inc. J Biomed Mater Res 64A: 291–300, 2003

Key words: poly (ether ester) segmented block copolymer; gas-plasma treatment; salt leaching; freeze-drying; bone marrow cell culturing

INTRODUCTION

In the restoration of bone defects, the tissue engineering approach[1] mimics the natural process of bone repair. The system can be activated or assisted by (1) the delivery of cells capable of differentiating into osteoblasts; (2) inductive growth and differentiation factors; or (3) bioresorbable scaffolds, which enable cell attachment, migration, and proliferation. In many approaches two, sometimes even three, of these elements are combined.[2]

In the case of nonload-bearing bone tissue, a promising scaffold material for this purpose seems to be poly (ethylene oxide terephthalate)/poly (butylene terephthalate) (PEOT/PBT) block copolymers also known as Polyactive®. The composition of these copolymers is indicated as *a*PEOT*b*PBT*c*, with *a* the molecular weight of the poly (ethylene glycol) starting compound, *b* the mass percentage of the PEOT soft segments, and *c* the mass percentage of the PBT hard segments. Mechanical and physical properties of these materials can be tuned by varying the PBT (hard segment) content and PEO (soft segment) molecular weight of these copolymers.[3,4] As a result of differences in hydrophilicity, water uptake, and degradability, the composition of these copolymers also is expected to be of influence on the *in vitro* cell culturing of bone marrow cells on these materials.

Several subcutaneous and intra-bone (tibia) implantations of dense blocks and porous films in rats showed bone bonding, calcification, and degradation for PEOT/PBT copolymers with high PEO content (60 and 70 weight percent PEO containing soft segment).[5–8] However, these effects were not seen after implantation of porous blocks in goat[9] and human[10] ilea. We intend to prepare porous PEOT/PBT scaffolds on which a patient's own bone marrow cells can

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be cultured. These polymer/cell constructs will then be implanted at the site of the defect. For use in bone tissue engineering, the structures need to be highly porous with interconnected pores and a sufficiently large pore size to ensure tissue ingrowth and bone formation.[11]

There are several ways to prepare porous structures, each with their own level of control over pore size, porosity, and interconnectivity. Depending on the polymer system, each technique has its own advantages and disadvantages.[12] A technique that is applied widely is particulate leaching. Leachable particles, for instance sucrose, sodium chloride, or other salts, are mixed with a polymer solution. After solvent evaporation and leaching, porous membranes are obtained.[13] These polymer/salt composites can also be molded or extruded into various complex shapes.[14,15]

Another versatile technique is solid-liquid phase separation as described by Aubert et al.[16] in the preparation of microcellular polystyrene foams. By using solvents that can be freeze-dried, for example, cyclohexane, naphthalene, or 1,4-dioxane, and by use of different freezing temperatures and conditions, various pore sizes and pore structures can be obtained.[17–21]

We are developing those techniques that give us large flexibility in the preparation of our tissue-engineering scaffolds, in terms of pore size and porosity, because the optimal conditions and processing technique for a scaffold vary per material and tissue. Here, we describe the preparation and characterization of porous PEOT/PBT structures prepared by solid-liquid phase separation, followed by freeze-drying and by compression molding of polymer and salt particle mixtures, followed by salt leaching. These scaffolds were gas-plasma treated using a CO_2 gas-plasma to enable bone marrow cell attachment to these structures.[22] Scaffolds of varying pore size are evaluated to study the effect of pore size on the gas-plasma treatments and on the *in vitro* rat bone marrow cell culture.

MATERIALS AND METHODS

All solvents used were analytical grade and all chemicals were at least 99% pure unless otherwise mentioned. All were used as received.

Polymer scaffold preparation

Synthesis

PEOT/PBT multiblock copolymers were prepared by two-step polycondensation in the presence of titanium tetrabutoxide (Merck, Germany) as catalyst (0.1 wt %) as previously described,[3] with the exception that vitamin E (Sigma-Aldrich, Germany, approximately 95% pure) was used as antioxidant. Compositions were varied by changing the poly(ethylene glycol) (Fluka, Switzerland) molecular weight and the dimethyl terephthalate (Merck, Germany)/1,4-butanediol (Acros, Belgium) to polyethylene glycol ratio.

Unless otherwise mentioned, the resulting polymers were dissolved in chloroform (Biosolve Ltd., The Netherlands) or chloroform/hexafluoroisopropanol (Acros, Belgium) mixtures (approximately a 10% solution) and precipitated in a ten-fold excess of technical grade ethanol. The copolymer composition was determined by ^{1}H-NMR (Varian Inova 300 MHz). All copolymers had compositions close to the intended ones. The composition is indicated as *a*PEOT*b*PBT*c*, where *a* is the starting poly (ethylene glycol) molecular weight, *b* the weight percentage of PEOT soft segments, and *c* the weight percentage of PBT hard segments. It should be noted that terephthalic ester units are present in both the soft and the hard segments.[3]

Film preparation

Films (75–100-μm thick) were prepared by casting of 10% (w/w) polymer solutions in chloroform or chloroform/hexafluoroisopropanol mixtures on a glass plate using a casting knife. All films were placed in ethanol (overnight) to remove any residual hexafluoroisopropanol and/or chloroform. Films were dried in a vacuum oven under a small N_2 current for 5 days.

Porous structures prepared by freeze-drying

Typically, 5–20% of copolymer solutions were prepared in 1,4-dioxane (Merck, Germany), solutions were heated until a clear solution was obtained (usually between 80 and 110°C). Solutions were poured into polyethylene vials and frozen at different temperatures: −196°C (liquid nitrogen), −78°C (acetone/CO_2), −28°C (freezer), and +7 or +10°C (refrigerator). Subsequent freeze-drying (5 days, 20 mbar) yielded white foams.

Porous structures prepared by compression molding of polymer/salt mixtures followed by salt leaching

Copolymer granulate was cryogenically ground using an IKA Labortechnik (Germany) A10 grinder. Polymer powder and sodium chloride (Merck, Germany) was sieved using Endecotts (United Kingdom) test sieves of 250-, 425-, 500-, 710-, 1000-, and 1180-μm mesh size. The desired salt volume fractions were calculated using a salt density of 2.165 gr/cm^3. The powders were mixed and subsequently compression molded in a laboratory hot press (THB 008, Fontijne Holland BV, The Netherlands) into the desired shapes. Powders were heated at 180°C for 3 min and subsequently pressed for 1 min at 2.9 MPa. Samples were leached with milliQ water for 48 h and dried under reduced pressure in a vacuum oven.

Characterization of porous structures

Porosity was determined by measurement of scaffold mass and dimensions (volume) in the dry state. The porosity was calculated from the densities of the solid materials, 1000PEOT70PBT30: $\rho = 1.188 \pm 0.011$ g/cm^3, 300PEOT55PBT30: $\rho = 1.2437 \pm 0.0029$ g/cm^3. Pore sizes were measured from scanning electron micrographs at a magnification of 100 times. The average diameter of at least 20 pores (± SD) was determined. For salt leached samples, the pore sizes were found to be in accordance with the sizes of the salt crystals used. Compression moduli were determined at room temperature using a Zwick Z020 tensile tester. Moduli were measured at 10% strain at a strain rate of 2 mm/min with a 0.1*N* preload. The scaffolds had a diameter of 17 mm and a height of 8 mm. Results are averages of at least three measurements (± SD).

Water uptake

Polymer films were weighed at regular intervals. For every polymer, three samples were swollen in milliQ water (shaking bath, 37°C). All results are averages of triplicate measurements (± SD).

CO_2 gas-plasma treatment

Both solution cast films (treated on both sides) and porous structures were treated for 30 min. Discharge power was 49 W. CO_2 gas-plasma pressure: 0.06–0.07 mbar. A gas flow of 10 cm^3/min was used. Samples were treated with a predelay of 2 min and a post-delay of 2 min.[22] After treatment samples were rinsed using demineralized water, followed by ethanol (p.a.). Samples were dried in a vacuum oven overnight at room temperature.

Bone marrow cell culturing

Goat bone marrow cell culturing on copolymer films

Samples with a diameter of 10 mm were cut from the copolymer films. For preliminary screening experiments, goat bone marrow stromal cells, passage 3, were used (GBMC-P3). The cells were seeded with a density of 10,000 cells/cm^2, on discs in the presence of 3 mL of minimal essential medium (Life Technologies, The Netherlands) containing:[23] 15% fetal bovine serum (Life Technologies, The Netherlands), 100 units/mL penicillin, 100 μg/mL streptomycin (Life Technologies), 2 m*M* L-glutamine (Life Technologies), 0.2 m*M* ascorbic acid 2-phosphate (Life Technologies, The Netherlands), 10 m*M* β-glycerophosphate (Sigma, The Netherlands), 10^{-8} *M* dexamethasone (Sigma).

Rat bone marrow cell culturing

Bone marrow stromal cells were isolated from seven femora of four male Wistar rats. The femora were cut on both sides, and the marrow was flushed out using 5 mL of medium per femur. The collected cells were resuspended with a 21G needle and cultured at 37°C, 5% CO_2, for 7 days, with periodic medium changes every other day. After 7 days, cells were confluent and were washed with phosphate-buffered saline (PBS; Life Technologies) and treated with trypsin/EDTA (Sigma). The collected cell suspension was cultured for another week, yielding 69×10^6 rat bone marrow cells.

Cell seeding and growth on porous scaffolds

Scaffolds were washed with: distilled water, 100% ethanol, distilled water, 70% ethanol/water, 3X sterile PBS containing 100 units/mL penicillin and 100 μg/mL streptomycin. The scaffolds were stored in PBS containing 100 units/mL penicillin and 100 μg/mL streptomycin until cell culturing (1.5 days) and seeded with 2×150 μL of cell suspension (~2×10^5 cells per scaffold). Cell suspensions were injected into the scaffolds. Scaffolds were incubated at 37°C for 3 h, after which 2 mL of cell culture medium was added. Cells were cultured at 37°C, 5% CO_2, for 10 days, with periodic medium changes every other day. Samples were analyzed at days 1, 3, 7 (methylene blue staining), and 10 (alkaline phosphatase staining). The amount of cells seeded was kept constant at approximately 2×10^5 cells per scaffold because the estimated internal surface area was in the same order of magnitude for all the scaffolds: between 13 cm^2 (250- μm pores) and 3.3 cm^2 (1000-μm pores), resulting in a seeding density in the range of 1.5×10^4–6×10^4 cells/cm^2.

Analysis

Methylene blue staining

Samples were analyzed at days 1, 3, and 7. Scaffolds were washed with warm PBS containing 100 units/mL penicillin and 100 μg/mL streptomycin and subsequently fixed with glutaraldehyde (Merck, Germany, 1.5% solution in 0.14*M* cacodylic acid buffer, pH = 7.35). Then the samples were washed with water and stained for 30 s using a 1% methylene blue solution in 0.1*M* borax buffer (pH = 8.5). Scaffolds were subsequently washed with demineralized water until the water was clear. Samples were stored in a refrigerator until further analysis, at which time they were evaluated using a Nikon SM2-10A stereomicroscope (1X objective). Digital photographs were taken using a Sony progressive 3 CCD camera. To obtain a qualitative comparison between the different polymers (concerning the amount of cells present on the film surfaces), the intensity of blue staining of cells on the film surfaces was evaluated. Higher magnifications showed blue staining of the cells. The samples were divided in 4 categories: 1) no cells (–), 2) few cells (±), 3) cells (+), and 4) many cells at the surface (++/+++). The distribution of cells within the scaffolds was examined using diagonally cut cross-sections of the scaffolds.

DNA assay

Scaffolds were washed with PBS containing 100 units/mL penicillin and 100 μg/mL streptomycin at 37°C and stored in a freezer (–80°C) until further analysis. Scaffolds were cut in at least four pieces and incubated at 56°C overnight in 0.5 mL of lysis medium to lyse all cells.

The lysis medium consisted of 26.47 mg iodoacetamide (Sigma), 1.60 mg of pepstatin A (Sigma), and 150 mg of proteinase K (Sigma) in 150 mL of Tris/EDTA buffer.

The next day, 250 μL of these suspensions were mixed with 250 μL of Rnase solution. The RNase solution was prepared from 60 μL RNase (Sigma, 86 Kunitz units/mg) and 100 μL of heparin (Leopharma,The Netherlands, 5.000 IE/mL) in 25 mL of PBS and incubated at 37°C for 45 min to remove the single-stranded RNA and DNA. Various dilutions were prepared with PBS. Dilutions were mixed with CyQUANT® dye. After 15 min, fluorescence was measured in 96-well plates using a PerkinElmer Luminescence Spectrometer LS 50 B (excitation at 480 nm, slit width 2.5 nm, emission at 520 nm, slit width 7.5 nm). The measured fluorescence intensities were correlated to the amount of DNA using a calibration curve made by using DNA (Sigma) dilutions of known concentration.

Data shown are the result of triplicate measurements (± SD). Six scaffolds without cells were used as blanks. Results were analyzed using one-way ANOVA, followed by a Tukey's Honestly Significant Difference Post-Hoc Test. Differences were considered statistically different when $p < 0.05$. ANOVA calculations were performed using SPSS software for Windows (version 10.0, SPSS).

Alkaline phosphatase staining

Samples were analyzed at day 10. Scaffolds were washed with PBS containing 100 units/mL penicillin and 100 μg/mL streptomycin of 37°C and subsequently fixed with paraformaldehyde (4% solution in Sørensen buffer [phosphate buffer]) for 3.5 h.

Samples were washed with water (3X) and stained for 35 min using a Fast Blue RR salt (4-benzoylamino-2,5-dimethoxybenzene-diazonium chloride hemi[zinc chloride], Sigma) in naphthol As-B-1 stock solution (1 g /L). Besides the cultured scaffolds, three blank scaffolds also were stained (pore size 500–710 μm). Samples were investigated using a Nikon SM2-10A stereomicroscope (1X objective). Digital photographs were taken using a Sony progressive 3 CCD camera.

Scanning electron microscopy (SEM)

Porous scaffolds and films containing bone marrow cells were fixed (as with methylene blue staining) and stored in 70% ethanol. Samples were dehydrated using an ethanol/water gradient. Dehydrated samples were dried using a Balzers CPD 030 critical point dryer before coating. Samples were coated with Au/Pd in a Polaron E5600 sputter coater. Pictures were taken with a Hitachi FE-SEM S-800 (6 kV).

RESULTS AND DISCUSSION

Improvement of cell attachment

Based on the literature, the PEOT/PBT multiblock copolymer with a 70 to 30 soft-to-hard segment ratio and a PEO molecular weight of 1000 (1000PEOT70PBT30) holds promise as a scaffold material for bone tissue engineering. Besides degradation, calcification, and bone bonding,[5] *in vivo* studies on porous 1000PEOT70PBT30 also have shown bone ingrowth.[8] In contrast to our expectation, however, screening experiments using goat bone marrow cells did not show any cell attachment to this 1000PEOT70PBT30 *in vitro*.

As studies have shown, the hydrophilicity of polymers is an important factor in cell adhesion and growth.[24,25] We therefore varied the copolymer compositions to adjust the hydrophilicity. As indicated in Table I, it is possible to vary the physical properties of the tissue engineering scaffolds within a wide range by adjusting the copolymer composition. The water uptake is very much dependent on the PEO molecular

TABLE I
PEOT/PBT Block Copolymer Films: Composition, Water Uptake, and Results of Goat Bone Cell Attachment Experiments

Copolymer	Soft-to-Hard Segment Ratio[a]	PEO Content (%)[a]	Water Uptake (m%)	Bone Cell Attachment	
				Untreated	30 min CO_2 Plasma
4000PEOT70PBT30	80/20	77.5	212	–[b]	+
1000PEOT30PBT70	30/70	27	32	–	+++
1000PEOT40PBT60	41/59	37	38	–	
1000PEOT55PBT45	55/45	49	46	–	
1000PEOT60PBT40	63/37	54	52	–	
1000PEOT70PBT30	77/23	65	74	–	+/++
300PEOT55PBT45	52/48	36	5	+	+/++
300PEOT70PBT30	67/33	44	6	±	+++

[a]Experimental from NMR.
[b]–, no cell attachment; ±, some round, not well-attached cells at the surface; +, cell attachment; ++ and +++, surface covered with cells.

weight and the soft-to-hard segment ratio. To screen for the viability of these materials as scaffold materials for bone tissue engineering, previously frozen goat bone marrow cells were cultured on these materials. Goat bone marrow cells only attached to the relatively hydrophobic 300PEOT55PBT45 and somewhat to the 300PEOT70PBT30 but not to the 1000PEOT70PBT30. Interestingly enough, cells did also not attach to the other two copolymers with low PEO contents: 1000PEOT30PBT70 and 1000PEOT40PBT60. Despite their low PEO content, these polymers have a considerable water uptake of 32 and 38 mass percent, respectively, this in comparison to the 300PEOT55PBT45 and the 300PEOT70PBT30, which have a much lower water uptake, even though they have a comparable PEO content. Phase separation is likely to play an important role here.[3]

An effective way of making PEOT/PBT copolymers more suitable for bone marrow cell attachment is by a 30 min gas-plasma treatment with CO_2.[22,26] From an assessment of goat bone marrow cell attachment to PEOT/PBT films (cells were stained using methylene blue, data not shown), it could be seen that the amount of cells present on the copolymer surfaces increased substantially after gas-plasma treatment, as qualitatively indicated in Table I. Details of these techniques, such as changes in surface chemistry and morphology and other ways of improving cell attachment to these materials, will be discussed in a forthcoming work.[27] All gas-plasma modified PEOT/PBT copolymers tested showed an improvement in goat bone marrow cell attachment, including the 1000PEOT70PBT30 copolymer.

Preparation of porous structures

An important step towards a tissue-engineered polymer/cell construct is the preparation of a suitable three-dimensional matrix for *in vitro* cell culturing. We are aiming for methods that enable us to prepare highly porous scaffolds with large pores and good mechanical properties from these hydrophilic copolymers.

A well-known method to prepare porous polymeric scaffolds is by solid-liquid phase separation of a solvent that can easily be removed, for instance by freeze-drying. The resulting porosity of the structures is governed by the polymer concentration of the solution. The obtained pore sizes are mostly governed by the temperature at which the polymer solution is frozen. In correspondence with literature, smaller pores are obtained at lower freezing temperatures, a result of the faster rate of nucleation of the solvent, leading to more and hence smaller crystals.[16,21,28] The effect of freezing temperature of a 1,4-dioxane solution on the pore size of 1000PEOT70PBT30 is shown in Figure 1. In this figure, the pore size of the pores are shown, as they were observed in SEM pictures (Fig. 2).

Based on *in vivo* studies it was concluded by Radder et al.[8] that 300-μm pores were suboptimal for bone ingrowth, probably because of the combination of press-fit implantation and extensive swelling *in situ*. To obtain pores larger than 300 μm, we froze 1,4-dioxane solutions at 10°C, a temperature close to the melting point of the solvent (11°C). As indicated in Table II, pores well over 300 μm are obtained; even pores as large as 1.5 mm were observed.

To our knowledge, these are the largest pores reported for porous structures prepared by solid-liquid phase separation.[12] The polymer concentration did not seem to have an effect on the obtained pore size. Even though highly porous structures were obtained with porosities ranging from 85 to 95%, most of the pores have a closed cell structure (Fig. 3), in accordance with previous observations.[21] Pore interconnectivity is one of the important parameters for tissue engineering because it enables tissue ingrowth and vascularization, making the obtained structures not very suitable as tissue engineering scaffolds.

To obtain a well-interconnected structure with large pores, a salt-leaching approach was followed. PEOT/PBT granules were cryogenically ground to a powder and subsequently sieved using standard test sieves to obtain powders of known particle size. As leachable porogen sodium chloride was selected, which was also sieved to fractions of known sizes. By compression molding, these polymer powder/salt mixtures can be processed into devices of different sizes and shapes. By varying the polymer powder to salt ratio and the salt size, it was possible to obtain porous structures with porosities ranging from 75 to 90% and pore sizes of 250 to 1000 μm. Not all polymer/salt mixtures, however, yield coherent structures that stay stable during salt leaching. In Table III, the stability

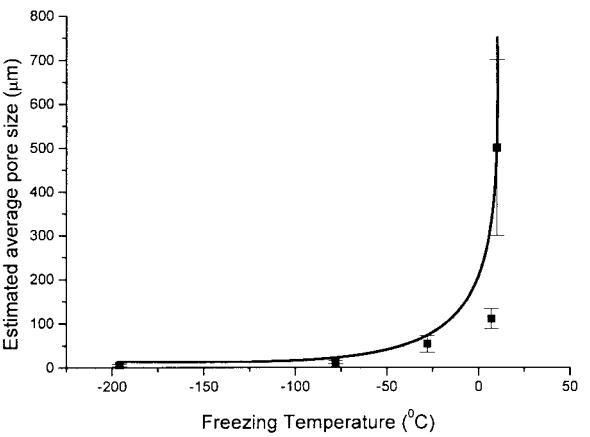

Figure 1. Estimated average pore size (± SD) as obtained from SEM pictures of 1000PEOT70PBT30 of 91% porosity.

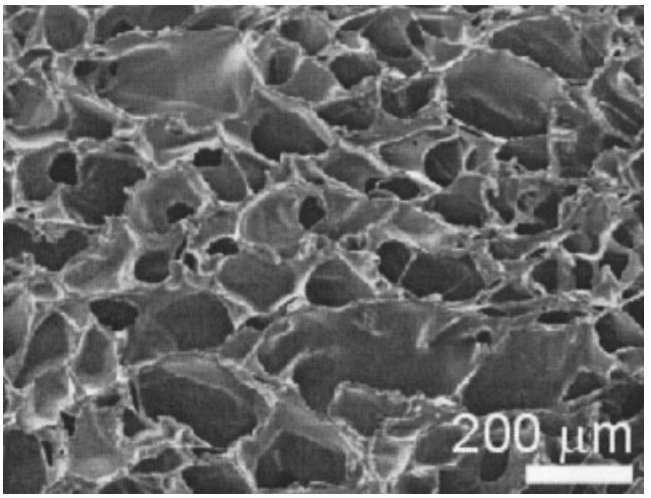

Figure 2. SEM of a 92% porous 1000PEOT70PBT30 scaffold prepared by freeze-drying (frozen at +7°C, pore size: 167 ± 78 μm).

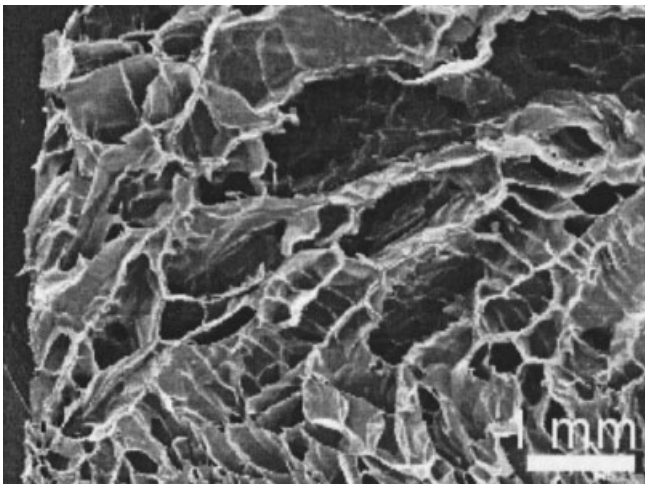

Figure 3. 5% solution of 1000PEOT70PBT30 frozen at +10°C; most pores show a closed cell structure.

during leaching of 1000PEOT70PBT30 structures is indicated, defining the processing window of this technique.

The actual porosity was determined by measuring the relative density compared to the density of solid compression molded 1000PEOT70PBT30 (ρ = 1.188 g/cm^3). The obtained porosity is in almost every case higher than that based on the calculated value. Remaining small air bubbles formed during compression molding within the polymer fraction might be an explanation for this. Nevertheless, there is a clear linear relationship, as indicated in Figure 4, giving good control over the porosity in a range of 75–90%.

Interconnectivity of the obtained structures is much better than was the case for the scaffolds prepared by solid-liquid phase separation, as shown in Figure 5. The obtained pores are cubic, formed after the shape of the salt crystals used.

Even though both preparation techniques give porous structures of high porosity (up to 90–95%), the latter described technique of compression molding and leaching shows a better control over pore size and interconnected pores. Therefore, the scaffolds prepared by this technique were thoroughly characterized in terms of mechanical properties and were used in bone marrow cell culturing experiments.

TABLE II
Average Pore Sizes (±SD) for 1000PEOT70PBT30 in 1,4-Dioxane, Frozen at 10°C, as Estimated From SEM

Volume % 1,4-Dioxane	Average Pore Size (mm)
85	0.6 ± 0.3
90	0.5 ± 0.2
95	0.6 ± 0.3

Mechanical properties of scaffolds prepared by polymer powder/salt mixing

Although these materials are not intended in load-bearing applications, mechanical properties like scaffold stiffness are of interest. Scaffolds should maintain their structure and stiffness after long periods of cell culture and should maintain their structure for subsequent implantation. Depending on culture medium and culturing time and the place of implantation, very soft or more rigid scaffolds might be required with good handling characteristics up to the time of insertion into the bone defect.

The mechanical properties, especially the compression modulus, of porous materials are mainly governed by porosity. Various empirical relationships relate properties like tensile strength, compressive modulus and flexural modulus to the relative density or porosity.[29] From experimental and theoretical studies on cancellous bone, it was concluded that the compression modulus of a porous structure (C_{foam}) is re-

TABLE III
Stability of Scaffolds During the Leaching Process

Intended Porosity	Actual Porosity*	250–425 μm	425–500 μm	500–710 μm	710–1000 μm
60%	74.3 ± 2.0	n.p.	++	++	+
70%	79.1 ± 1.3	++	++	++	++
80%	84.8 ± 2.2	++	++	++	+
90%	91.6 ± 1.1	±	−	±	−−

Scaffolds were prepared by polymer powder mixing/salt leaching, ++ denotes no fragmentation, + single polymer fragment comes off, ± several polymer fragments come off, − many polymer fragments come off, −− complete scaffold disintegration.

* = average of all the scaffolds of all pore sizes, determined by means of density.

n.p. = not prepared.

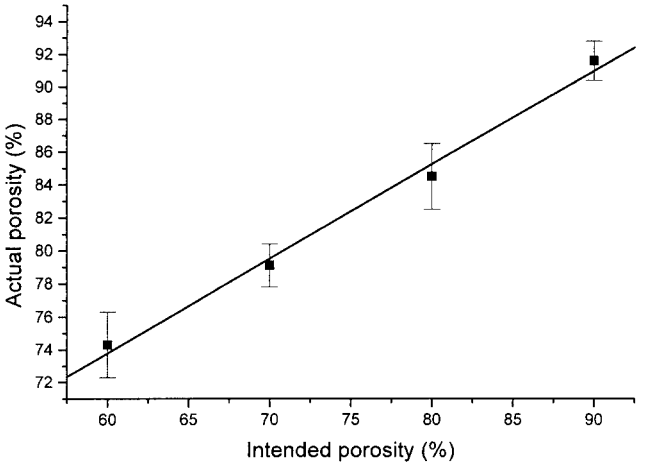

Figure 4. Actual porosity as a function of the salt volume fraction. Values are an average of the porosities of at least 10 scaffolds of various pore size (± SD).

lated to the relative density (ρ_{foam}/ρ_{solid}) according to the following formula[29]:

$$C_{foam} = C_{solid} * \left(\frac{\rho_{foam}}{\rho_{solid}}\right)^{x} \quad (1)$$

where x is an exponent with a value ranging between 2 and 3. The same relation is also valid for polymeric foams.[30] Experimentally, the exponent can easily be obtained by preparation of log-log plots of the relative compression modulus versus the relative density. As shown in Figure 6, such relations fit the observed data very well. From our data on 1000PEOT70PBT30 scaffolds of varying porosity an exponent of 3.2 ± 0.2 was obtained, in good correspondence with the previously mentioned value of 2–3, as often seen in literature.

Parallel studies on the scaffolds prepared by freeze-drying showed that these porous scaffolds also obey this exponential decrease in modulus with increasing

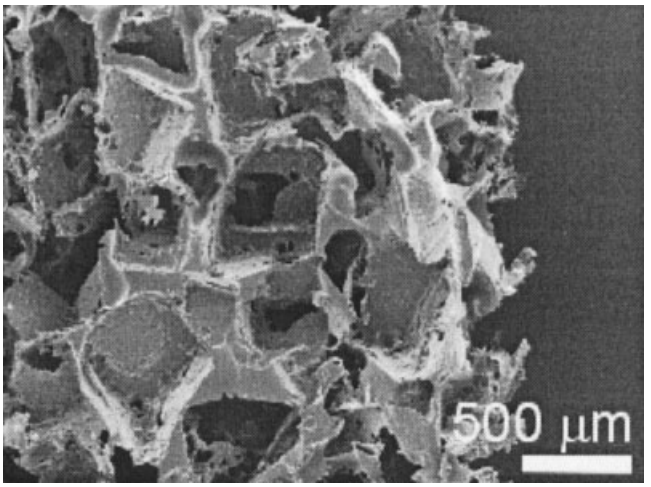

Figure 5. SEM of a 1000PEOT70PBT30 scaffold of 85.1 ± 3% porosity prepared with sodium chloride particles of 500–710 µm.

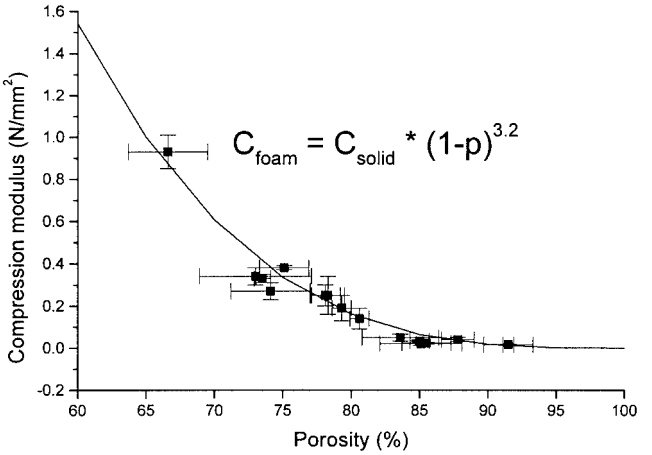

Figure 6. Dependence of compression modulus on scaffold porosity (p) in the case of 1000PEOT70PBT30 foams. 1 – p equals the relative density as mentioned in formula 1. Moduli are the average of three measurements (± SD).

porosity, albeit with a somewhat smaller exponent of 2.2 (1000PEOT70PBT30) or 2.8 (300PEOT55PBT45), depending on the copolymer tested. The obtained relation allows us to accurately predict the compression modulus of a scaffold of a given porosity, enabling us to tune the mechanical properties to a specific need.

In vitro rat bone marrow cell culture on porous scaffolds

After the initial screening with goat bone marrow cells, further experiments were performed using rat bone marrow cells. Future *in vivo* experiments will be conducted using the rat as a small animal model. To study the effect of scaffold pore size on gas-plasma treatment and *in vitro* cell culturing, scaffolds of 84.5 ± 2.0% porosity of different pore sizes were used. Scaffolds of 4 × 4 × 4 mm^3 were first treated using a 30-min CO_2 gas-plasma to ensure cell attachment. Methylene blue staining (data not shown) and SEM on scaffold cross sections showed that at day 1 of the cell culturing experiments rat bone marrow cells (obtained from rat femora) attached throughout the entire scaffold, also in the center, showing that the gas plasma is able to reach the center of the scaffold. Figure 7 shows an SEM picture of bone marrow cells spread on the surface of a 1000PEOT70PBT30 scaffold.These results also indirectly show the interconnectivity of pores prepared by the powder mixing/salt leaching technique.

To study the effect of pore size on *in vitro* rat bone marrow cell culture, scaffolds (porosity 84.5 ± 2.0%) with four different pore sizes were prepared: 250–425, 425–500, 500–710, 710–1000 µm. Rat bone marrow cells were cultured during 7 days, with media refreshments every other day. The amount of DNA, a direct indica-

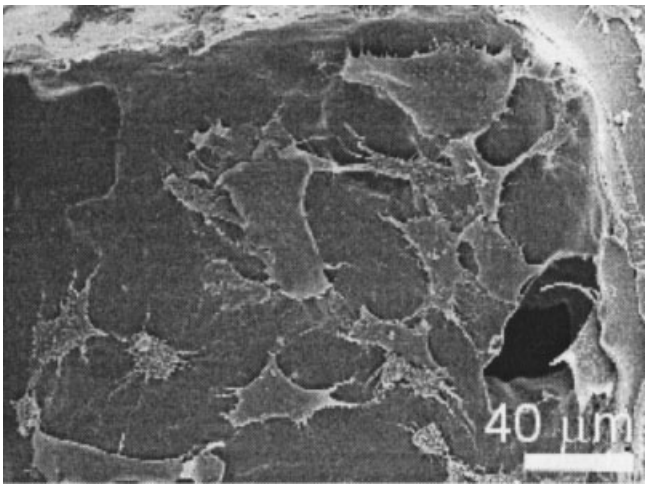

Figure 7. Rat bone marrow cells attached to the surface of a 1000PEOT70PBT30 scaffold of 85% porosity and pore size of 250–425 μm.

tion for the amount of cells present in the samples, was then determined. The scaffolds were evaluated at 1, 4, and 7 days as indicated in Figure 8.

The data show a statistically significant increase in the amount of DNA (and hence in cell numbers) present on the scaffolds after 4 and 7 days. As a reference, a non gas-plasma–treated scaffold of pore size 500–710 μm also was included in the study, clearly showing the beneficial effect of the gas-plasma treatment. After 7 days of culture, only 443 ± 548 ng of DNA is present on these scaffolds, significantly less than the 9694 ± 1579 ng present on the same scaffolds that were gas-plasma treated. The same trend was also observed by light microscopy evaluation of methylene blue-stained samples: in time there is a large increase in the amount of cells present on these scaffolds.

Over the culture period, no significant differences between the amount of DNA present on the different scaffolds could be observed. On the days of evaluation (1, 4, and 7) all the scaffolds, within the margins of error, have similar amounts of DNA. These data show that for the examined pore ranges, there is no significant effect of pore size on the amount of cells on these scaffolds. Both goat (Table I) and rat (Fig. 8), bone marrow cells show improved cell attachment and growth on CO_2 gas-plasma treated PEOT/PBT surfaces.

In contrast to *in vivo* results, where it was concluded that 300 μm was a suboptimal pore size,[8] in our *in vitro* study, in which pore sizes were varied from 250 to 1000 μm, no significant effect of pore size was seen. During *in vitro* cell culture, there is no decrease in pore size as as result of swelling and press-fit implantation.

Methylene blue staining of scaffold cross-sections showed cell attachment in the center of the scaffolds. Although not quantitative, the methylene blue staining suggested the presence of more cells in the scaffolds with pore sizes of 425–500 μm and 500–710 μm. The presence of bone marrow cells in the center of these scaffolds indirectly shows the interconnectivity of the pores, allowing cells to attach in the center of the scaffolds.

Rat bone marrow cell differentiation into the osteogenic cell lineage was verified by staining the cells for alkaline phosphatase activity, an enzyme not present in marrow cells. One of the first signs of differentiation is the presence of alkaline phosphatase. Active cells color purple whereas inactive cells stay yellowish (color of the staining medium). Figure 9 shows the alkaline phosphatase active cells present on a 1000PEOT70PBT30 scaffold (710–1000 μm) after 10 days of culture, showing the ability of the rat bone marrow cells to differentiate into the osteogenic lineage on the surface of the gas-plasma treated scaffolds.[23,31–33]

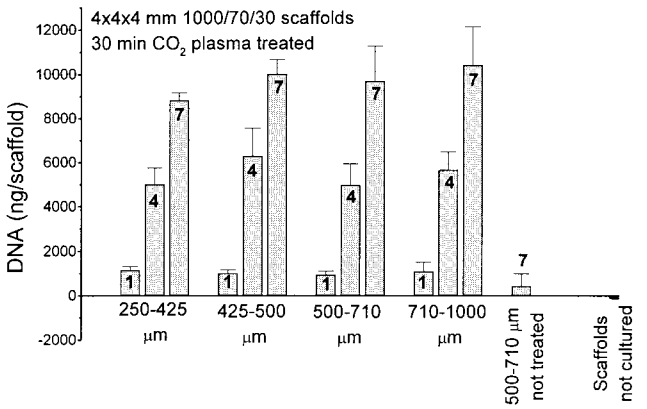

Figure 8. DNA amount (ng) present on 1000PEOT70PBT30 scaffolds (4 × 4 × 4 mm) of varying pore size (porosity 84.5 ± 2%) after rat bone marrow cell culturing. DNA amounts are the result of triplicate measurements (± SD).

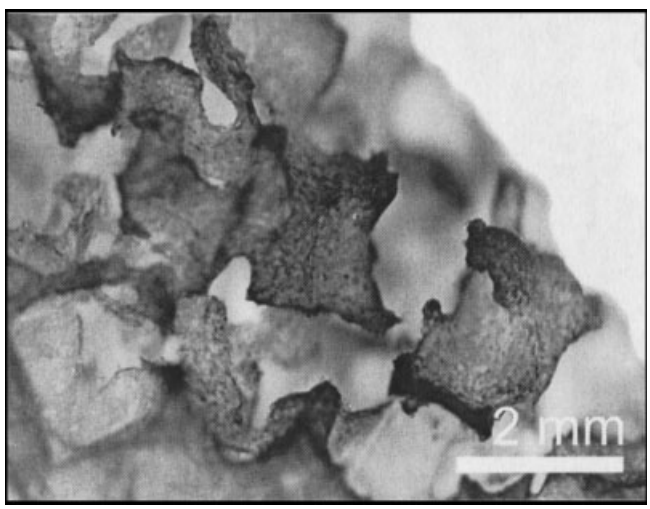

Figure 9. Alkaline phosphatase stained 1000PEOT70PBT30 scaffold of 710–1000 μm after 10 days of rat bone marrow cell culturing. Cells containing alkaline phosphatase (differentiated) color purple.

CONCLUSIONS

The preparation of porous structures by means of compression molding of polymer powder/salt mixtures followed by salt leaching yields porous structures suitable as scaffold material in *in vitro* bone marrow cell culturing. Variation of polymer/salt ratio and the salt size gives good control over porosity and pore size respectively, enabling us to prepare scaffolds with pore sizes over 300 μm and porosities over 75%.

Because during cell culturing, rat bone marrow cells were able to attach in the center of the scaffolds, two points could be proven: 1) the structures prepared by powder mixing/salt leaching contain interconnected pores and 2) gas plasma treatments using a CO_2 gas-plasma for 30 min are effective for the improvement of bone marrow cell attachment to PEOT/PBT porous scaffolds. With gas-plasma treatments, it is possible to surface modify porous scaffolds both on the outside and the inside.

In the *in vitro* rat bone marrow cell culturing, no significant effect of pore size, in the range of 250 to 1000 μm, on the amount of cells present on the scaffolds was observed. The large increase in the amount of DNA present on the scaffolds over the culture period of 7 days shows the beneficial effect of gas-plasma treatments on porous PEOT/PBT scaffolds. Methylene blue-stained scaffold cross-sections showed cells in the center of the scaffolds, suggesting more cells in the center of the scaffolds of 425–500 and 500–710 μm pore size.

The control over the scaffold preparation, the good cell attachment (after gas-plasma treatment), and the previously reported *in vivo* degradability[5] make 1000PEOT70PBT30 a good candidate as a tissue engineering scaffold material.

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