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Hydrophobicity as a design criterion for polymer scaffolds in bone tissue engineering

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Abstract

Porous polymeric scaffolds play a key role in most tissue-engineering strategies. A series of non-degrading porous scaffolds was prepared, based on bulk-copolymerisation of 1-vinyl-2-pyrrolidinone (NVP) and *n*-butyl methacrylate (BMA), followed by a particulate-leaching step to generate porosity. Biocompatibility of these scaffolds was evaluated in vitro and in vivo. Furthermore, the scaffold materials were studied using the so-called demineralised bone matrix (DBM) as an evaluation system in vivo. The DBM, which is essentially a part of a rat femoral bone after processing with mineral acid, provides a suitable environment for ectopic bone formation, provided that the cavity of the DBM is filled with bone marrow prior to subcutaneous implantation in the thoracic region of rats. Various scaffold materials, differing with respect to composition and, hence, hydrophilicity, were introduced into the centre of DBMs. The ends were closed with rat bone marrow, and ectopic bone formation was monitored after 4, 6, and 8 weeks, both through X-ray microradiography and histology. The 50:50 scaffold particles were found to readily accommodate formation of bone tissue within their pores, whereas this was much less the case for the more hydrophilic 70:30 counterpart scaffolds. New healthy bone tissue was encountered inside the pores of the 50:50 scaffold material, not only at the periphery of the constructs but also in the center. Active osteoblast cells were found at the bone-biomaterial interfaces. These data indicate that the hydrophobicity of the biomaterial is, most likely, an important design criterion for polymeric scaffolds which should promote the healing of bone defects. Furthermore, it is argued that stable, non-degrading porous biomaterials, like those used in this study, provide an important tool to expand our comprehension of the role of biomaterials in scaffold-based tissue engineering approaches.

Keywords: Tissue engineering; Scaffolds; Biocompatibility in vivo; Demineralised bone matrix

1. Introduction

Porous polymeric scaffolds play a pivotal role in tissue engineering of bone and cartilage [1]. To repair a bone or cartilage defect, one of the ideal scenarios can be summarised as follows: the patient's own osteoblasts, chondrocytes, or mesenchymal stem cells are harvested, expanded (in vitro), and seeded onto and in a scaffold (in vitro). The scaffold is then used to fill the defect cavity. In situ, the scaffold degrades slowly, as tissue growth proceeds towards complete filling of the defect

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[2]. In an alternative scenario, a scaffold without cells is implanted directly into the defect cavity to serve as guidance for cell and tissue growth [3].

The last years have seen continuous refinement and improvement of tissue engineering strategies, but a number of tough practical problems persists [4]. This may have to do, in part, with the truly multidisciplinary nature of this field, which integrates knowledge from (i) polymer material properties; (ii) micro- and macro-structure of scaffolds; (iii) biomechanics; (iv) cell biology; (v) biocompatibility and host defence reactions; (vi) and surgery. Four practical problems can be identified:

1. The rate of degradation in vivo is often difficult to control. This holds particularly true for poly (α-hydroxy acids) such as poly (lactic acid) and poly (glycolic acid), which show burst-degradation [5].
2. Efficiency of cell seeding is usually low. In many cases cells are found close to the surface of the scaffold, but not in the interior [6].
3. The breakdown products formed during degradation of the scaffold may be cytotoxic. They may also invoke a local inflammatory response. For example, degradation of poly (lactic acid) can result in acidic building blocks that may elicit a local inflammatory response. This risk is especially high when there is little vascularisation, i.e., slow drainage of waste products from the implant site [7,8].
4. Cells could dedifferentiate when seeded onto scaffolds. Chondrocytes, for example, have a well-known tendency to dedifferentiate after being seeded into polymeric scaffold structures in vitro [9].

It is clear that our fundamental understanding of the role of the scaffold biomaterial is still rather limited. It has been argued already that it is necessary to improve the biomaterials in such a way that the scaffolds accommodate cell proliferation and deposition of extracellular matrix throughout their entire volume. Surface modification, for instance with the adhesive protein fibronectin, represents a promising strategy [10]. Furthermore, in depth investigations are necessary to find out how de-differentiation of seeded cells can be prevented (complication # 4).

A possible way to expand our understanding of scaffold materials may be to dissect the various factors that determine its ultimate success. One approach is to study scaffolds which do not decompose. Evaluation of the performance of such scaffolds, both in vitro and in vivo, may shed new light on the importance of the choice of the material. Such information can easily be obscured when degradable scaffolds are used, for example when decomposition products are cytotoxic for cells inside, or in the proximity of, the scaffold.

In this paper, we report the results of a study on a series of new porous polymeric scaffolds, which have in common that the biomaterial is stable, i.e., no degradation occurs. We prepared scaffolds from 1-vinyl-pyrrollidinone (NVP, a hydrophilic reactive monomer) and *n*-butyl-methacrylate (BMA, a hydrophobic reactive monomer) [11–13]. Our choice of NVP–BMA copolymers was based on our previous experience with biocompatibility of these materials [14]. Two series of scaffolds were studied, one is relatively hydrophilic, with composition NVP:BMA = 70:30 (mole:mole), and one is more hydrophobic, with composition NVP:BMA = 50:50 (mole:mole). These scaffolds were compared on the basis of in vitro and in vivo experiments on biocompatibility and ectopic bone formation experiments [15].

2. Materials and methods

2.1. Scaffold preparation

All solvents and starting reagents were of the highest purity available, or purified as specified. 1-Vinyl-2-pyrrolidone (NVP) and *n*-butyl-methacrylate (BMA) were purchased from Acros Organics ('s-Hertogenbosch, The Netherlands). Prior to synthesis, the monomers were purified by distillation under reduced pressure. Purity was checked through nuclear magnetic resonance (NMR) spectroscopy. The radical initiator 2,2′-azobis(2-methylpropionitrile) (AIBN) was purchased from Aldrich (Aldrich Chemical Co. Inc., Milwaukee, WI, USA), and used as received.

All materials were prepared according to the following procedure. NVP and BMA were mixed in the appropriate ratios, such that approx. 10 g of monomer mixture was obtained. AIBN was added to a concentration of 0.2 mole% of total monomer. The mixtures were homogenised in an ultrasonic bath for 5 min until the AIBN was dissolved completely. Then the monomer mixture was transferred into poly-tetrafluoroethylene tubes (inner diameter = 8 mm, wall thickness = 1 mm, length = 20 cm), which were closed by a glass stopper on one end. The tubes were immersed in a thermostated oil bath, which was interfaced with a time-temperature control system as described previously [16,17]. The polymerisations afforded hard opaque rods, which could easily be removed from the Teflon® tubes. The top and bottom ends (approx. 1 cm) were cut off and discarded. The copolymer rods were machined into pieces (approx. 500 mg each), which were dissolved in chloroform at a concentration of 10% by weight. Under continuous mechanical stirring at room temperature (fume hood) the pieces completely dissolved in 2 days.

Sodium chloride crystals (MERCK) were separated through sieving (Analysensieb, DIN-ISO 3310/1,

Retsch, Germany) such that three fractions were obtained: 200–300 μm, 300–425 μm, and greater than 425 μm. The salt crystals were added to the copolymer-solvent solution such that ratios salt (g):copolymer (g):$CHCl_3$ (mL) = 9:1:10 and 8:2:10 were obtained. Resulting viscous slurries were poured into a glass beaker and stirred continuously with a spatula. Meanwhile the chloroform was allowed to evaporate (fume hood), which gradually increased the viscosity of the slurry. The material was then cast into a Teflon® mould that consisted of four cylindrical holes with diameter 20 mm, and depth 4 mm. Some experience in determining the right time of casting was required. Casting too early (viscosity too low) resulted in precipitation of the salt crystals to the bottom, leaving an inhomogeneous disk. Casting too late (viscosity too high) would make it impossible to cast the slurry into the moulds. Remnants of chloroform were allowed to evaporate for 2 days at room temperature in a fume hood, followed by vacuum drying in a dessicator for 2 h. After evaporation of the chloroform, the copolymer-salt disks were removed from the moulds and wetted in nuclease-free sterile water for 30 min. A biopsy punch was used to cut the scaffolds (diameter 4 mm, height 4 mm). The salt was leached out completely by immersing the scaffolds in nuclease-free sterile water on a shaker for 1–2 days. During this time period, the water was changed three times a day. The scaffolds were air-dried in a laminar airflow chamber for 48 h and sterilised using ethylene oxide (EtO) gas following a standard protocol. The samples were degassed for a minimum of 48 h in air, and for 3 days in a dessicator under vacuum. It is our experience that treatment with EtO gas is the preferred method of sterilisation for polymeric biomaterials.

2.2. Material characterisation

Purity of the scaffold copolymers was determined through ^1H nuclear magnetic resonance (NMR) spectroscopy. All scaffolds dissolved completely in deuterated chloroform. Spectra were recorded at 400 MHz on a Varian Unity-Plus spectrometer.

The glass transition temperatures (Tg) of the copolymers were measured on a Perkin-Elmer DSC (differential scanning calorimetry) at a heating rate of 10 centigrades/min.

Scaffold morphologies were examined by scanning electron microscopy (SEM), using a Personal SEM (R.J. Lee Instruments Ltd., USA). Samples were prepared by cutting the scaffolds in half perpendicular to their longitudinal axis. They were mounted on aluminium stubs and gold-coated using a sputter coater (BioRad SC500) set at 20 mA for a total of 2 min. The instrument was set to 15 kV and the samples were oriented at an angle such that the inside of the scaffolds could be viewed.

2.3. Biocompatibility in vitro

Biocompatibility in vitro was studied by MTT-assay using mouse fibroblasts (3T3 cells, subclone CCL-92 from the American type culture collection) and direct contact methods using isolated rat calvarial bone cells and rat bone marrow cells. In the MTT-experiment, cell viability was estimated after cell culturing in scaffold extract compared to latex extract (1610 mg latex (cytotoxic) in 8 mL medium) or medium. Prior to extraction in medium the latex was sterilized by immersing the latex in ethanol 70% during 30 min. Then the latex was dried in an air flow cabinet. Extracts were prepared by extracting 10 scaffolds (porosity 80%, pore size 200–300 μm) at 37 °C for 4 days in 8 mL culture medium using a shaker. Culture medium was Dulbecco's Modification of Eagle's medium/F12 nutrient mix (1:1) with L-Alanyl-L-Glutamine supplemented with pyridoxine (Gibco, UK), 10% Fetal Calf Serum and 1% penicillin/streptomycin/amphotericin. 3T3 cells were seeded in 96-well plates (Costar®, Corning, USA) at a density of approx. 1000 cells/well in culture medium at 37 °C and 5% CO_2 in an incubator overnight. The undiluted and diluted (10x) scaffold extracts were added to the cells. After 3 days the extracts were replaced with culture medium containing (4,5-dimethylthiazol-2yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (600 μg MTT/mL culture medium). Cells were cultured for another 4 h. Subsequently, the medium was discarded and the precipitated formazan was dissolved in DMSO (60 μL/well). The plates were gently shaken for 1–2 min. The absorbance at 550 nm was measured on a microplate reader. The raw data from the MTT experiments were statistically treated in a non-parametric test. For the number of pair wise comparisons the Bonferroni correction was used [18]. An α-level of 0.00416 was considered as significantly different.

Calvarial bone cell attachment and differentiation in direct contact to scaffolds was examined. Calvarial cells were obtained from calvarial bones of 17–19 days old Dark-Agouti (DA) rat embryos. The calvarial bone was carefully dissected from adhering tissues and washed twice in cold Phosphate-buffered saline (PBS) solution at room temperature. Subsequently the calvarial bones were cut into small pieces and digested in trypsin-EDTA solution (16–24 calvarial bones in 45 mL digestion solution) in a 7% CO_2 humidified incubator for 20 min at 37 °C. Cell pellets were formed by centrifuging the supernatant at 1500 rpm for 10 min (4 °C). Then the cell pellet was suspended in culture medium (Dulbecco's Modified Eagle's Medium (Biological Industries, Kibbutz Beit Haemek, Israel) supplemented with 10% fetal calf serum) to a concentration of 50,000 cells/mL. Scaffolds (porosity 80%, pore size greater than 425 μm) were rinsed with PBS and degassed in a sterile filter unit (25944-500, 0.22 μm Nylon, Corning, USA)

before use. Subsequently in each 60-mm culture dish (Falcon) one single scaffold and 4 mL of cell containing medium was dispersed. Culture dishes without scaffold material were used as control groups. The cells were allowed to attach onto the scaffold for 3–4 days. The medium was replaced by culture medium and changed each 4–6 days. After 25–30 days the scaffolds were harvested, fixed and stained with Alizarin-red for histological examination.

Rat bone marrow cell attachment and differentiation in direct contact with scaffolds was also studied. Eight 70:30 (NVP:BMA) and eight 50:50 scaffolds were used (porosity 80%, pore size $>425\,\mu m$). Scaffolds were rinsed with sterile PBS and degassed in a filter (25944-500, 0.22 μm Nylon, Corning, USA). Bone marrow cells were obtained by puncturing the femurs removed from 2-month-old DA rats with a 1.2 mm blunt needle. Cells of one femur were dispersed in each 60-mm dish containing one single scaffold. Cells were allowed to attach for 30 min. Then 4 mL of culture medium (Dulbecco's Modified Eagle's Medium + 4,5 g/L D-Glucose (Biological Industries, Kibbutz Beit Haemek, Israel) supplemented with 10% fetal calf serum + L-Glutamine + penicillin/streptomycin + 1–1.2 mM Ca^{2+}) was added. The medium was changed every 2–3 days. Subsequently the cells were cultured in a humidified incubator at 37 °C, 7% CO_2, and prepared for histological examination at 9 and 23 days follow up respectively.

2.4. Biocompatibility in vivo

Biocompatibility in vivo was studied by implanting scaffolds subcutaneously in rats and by incorporating scaffolds in ectopic bone formation in rats using the demineralised bone matrix cylinder model.

In the first in vivo study scaffolds were implanted subcutaneously in rats. The Maastricht University Committee for animal experiments approved the rat experiment protocols. The experiments were conducted following the national and European guidelines for animal experiments. A total of 8 Lewis rats (age 10–12 weeks) was used. In each rat a 50:50 scaffold and a 70:30 (NVP:BMA) scaffold were implanted. All scaffolds had a porosity of 80% and pore sizes of 200–300 μm. The rats were fasted overnight. General anaesthesia was induced by subcutaneous injection of ketamin hydrochloride (100 μL/100 g) and xylazin (50 μL/100 g). The back was shaved, disinfected with 2% iodine solution and sterilely wrapped. Under rigorous aseptic conditions subcutaneous pouches were made in the back of the rat, in which the materials were implanted. The incisions were closed with Polysorb® 4.0 or closed with clips. The animals were examined daily for signs of wound infection, behaviour abnormalities or illness. After 7 or 84 days the rats were sacrificed. The implants were located and harvested with the surrounding tissue. After examination for signs of infection the explants were photographed and prepared for further evaluation. The specimens were fixed in 10% formalin solution during 3–5 days at 4 °C. Excessive fixative was rinsed with tap water for 1 h. Samples were then gradually dehydrated in an ethanol series at room temperature, and embedded in 2-hydroxyethyl methacrylate (Technovit® 7100, Heraeus Kulzer GmbH&Co., Darmstadt, Germany). The resin was left to harden, according to the manufacturer's instructions. Blocks were cut along the midsagittal plane into 4-μm thick sections by means of a microtome (Leica RM 2065) and stained with Gill's haematoxylin-eosin. Sections were evaluated using light microscopy.

In the second in vivo study scaffolds participated in ectopic bone formation in rats, induced by the implantation of demineralised bone matrix (DBM) cylinders containing rat bone marrow cells and scaffold parts (vide infra). In previous studies ectopic bone formation in young rats was seen when bone marrow cells were inserted into the DBM model [15]. Three-month-old DA rats were sacrificed and their femurs were harvested. Soft tissue and metaphyses were carefully removed in such a way that only cortical bone was left. Then diaphysic cylinders of approx. 7 mm length were prepared. Subsequently the bone cylinders were treated with 0.6 M HCl for 24–48 h. The resulting DBMs were rinsed with distilled water and kept in 70% alcohol. Prior to implantation, alcohol was washed out with sterile PBS. Scaffolds, 50:50 and 70:30 (NVP:BMA; porosity 80% and 90%, pore size greater than 425 μm) were cut into pieces of approx. $1 \times 1 \times 3$ mm, because the original scaffold dimensions were larger than the dimensions of the DBM cylinders. Fresh bone marrow cells were obtained from femurs of two-month-old DA rats using a 1200 μm blunt needle. Scaffold particles were placed in the centre of the DBM cylinders and the fresh bone marrow cells were immediately transferred into the DBM cylinders. DBM cylinders with fresh bone marrow cells, but without scaffold particles served as a positive control for bone formation. DBM cylinders with scaffold particles, but without fresh bone marrow cells served as negative controls.

Two-month-old DA rats were fasted overnight. General anaesthesia was induced by ketalar (Malgene 1000 Rhone Merieux, Lyon, France) and xylazine (Rampun Bayer, Leverkusen, Germany). The upper thoracic region was shaved, disinfected with iodine solution, and sterily wrapped. A 1-cm incision was made over the midline of the chest and subcutaneous tissue plains dissected laterally to form pouches in which the cylinders were implanted. Wounds were closed with silk 3.0 and washed again with iodine solution. Animals were sacrificed after 4, 6 or 8 weeks. The DBM cylinders were harvested and treated with 10% buffered formalin solution. Subsequently they were analyzed microradiographically using a Hewlett Packard Faxitron Cabinet. Specimens were

exposed for 5 s at 25 KeV using Kodak Ektaspeed E safety film [19,20]. After microradiographic analysis, the cylinders were demineralised in EDTA and stained with haematoxylin-eosin. The sections were evaluated for bone apposition and bone ingrowth onto the scaffolds.

3. Results and discussion

Synthesis of the copolymers proceeded without difficulties. Our NMR spectra of both copolymers, measured directly after their synthesis, confirmed the presence of unreacted NVP (approx. 1% of the original amount of NVP), while unreacted BMA could not be detected (data not shown). It is important to recall that NVP is known to be much less reactive as compared to methacrylates [21]. Consequently, BMA is consumed faster than NVP during copolymerization. It is also known that diffusion limitation towards the end of the reaction leaves unreacted NVP, as well as relatively short NVP homo-oligomers within the material. Different mixtures of NVP and BMA yield copolymers with markedly different hydrophilicities, also after complete release of extractables. Water uptake for the 70:30 (NVP:BMA) copolymer was found to be 150%; for the 50:50 (NVP:BMA) copolymer this was approx. 50% [22]. Fig. 1 shows a representative example of these spectra, which confirmed the identity and purity of our materials. It should be noted that the presence of extractable oligomers is difficult to establish from the NMR spectra. Monomers, on the other hand, can easily be detected, even if their concentration is as low as 0.05%. In particular, we focused on the spectral region δ 7.2–4.6. The presence of unreacted monomer results in signals in this region (i.e., $=CH_2$ protons of NVP as a four-line pattern centered at δ 7.10 and $=CH_2$ protons of BMA at δ 6.10 and 5.54). Even at large vertical expansion (inset in Fig. 1), these peaks could not be detected. This made us conclude that all unreacted BMA, NVP and NVP homo-oligomers were effectively removed. Differential scanning calorimetry experiments showed clear glass transitions, with Tg = 50 °C for the 50:50 material and 58 °C for 70:30 (NVP:BMA).

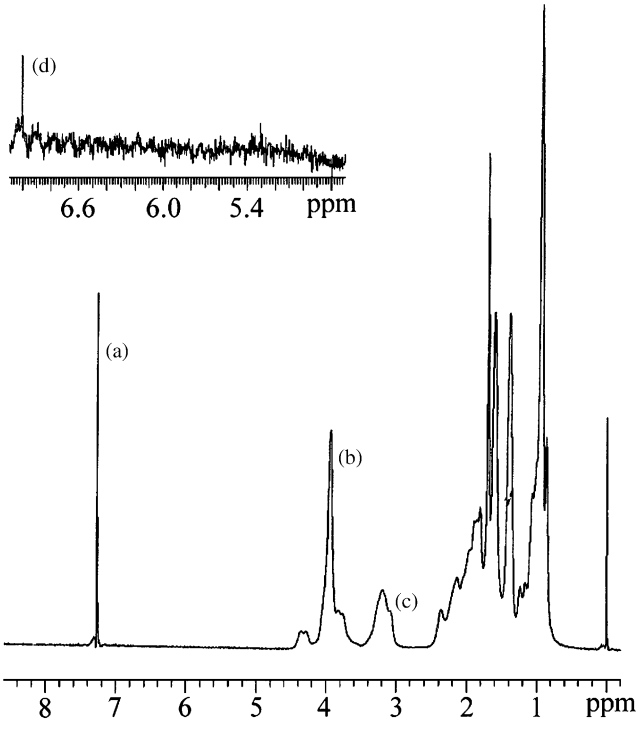

Fig. 1. ^{1}H NMR spectrum of the 50:50 scaffold material, dissolved in deuterated $CDCl_3$. Peak a corresponds with traces of $CHCl_3$ in the solvent, peak b corresponds with the O–CH_2- protons of the BMA blocks; peak c corresponds with the N–CH_2- protons within the pyrrolidone ring of NVP blocks. Overlapping signals, arising from all other protons in the copolymer, are seen in the spectral region δ 2.6–0.8. The inset shows the spectral region δ 7.1–4.8, after 40 × vertical expansion. No resonances due to unreacted BMA or NVP are found. Peak d is the ^{13}C satellite of the protons in $CHCl_3$.

Fig. 2 shows SEM micrographs of scaffolds that were prepared from the 70:30 (NVP:BMA) copolymer and sodium chloride in the mass:mass ratio 1:9 respectively (vide supra). Hence, these structures have approx. 10% of their volume filled with copolymer, and 90% with pores. This resulted in an open pore structure in all cases. The highly porous nature resulted in clear interconnectivity of the pores. This is an essential feature, as interconnectivity is mandatory to facilitate ingrowth of cells.

Fig. 3 summarizes the raw data of the MTT cytotoxicity tests. Apparently, the different undiluted extracts increase in toxicity in the order: negative control < 50:50 < 70:30 < latex. All differences are statistically different ($p \leqslant 0.001$). In all cases cytotoxicity decreases upon 10-fold dilution. Then, cytotoxicity changes as follows: negative control $\approx$ 50:50 $\approx$ 70:30 < latex. The p-values are: 50:50 vs. negative control: $p = 0.050$ (ns); 70:30 vs. negative control: $p = 0.065$ (ns); 50:50 vs. 70:30: $p = 0.878$ (ns); 50:50 vs. latex: $p = 0.000$ (s) and 70:30 vs. latex: $p = 0.000$ (s).

The MTT experiments prompted us to study the scaffold materials in direct contact with cells. Rat calvarial bone cells and rat bone marrow cells were used. Fig. 4 shows calvarial bone cells after 30-day incubation with 50:50 and 70:30 (NVP:BMA) materials. Cells were stained with Alizarin-red. It could be concluded that the cells proliferated in contact with both materials. Interestingly, some bone nodules were also observed. The presence of these bone nodules, including their mineralization, underlines the biocompatibility of both materials.

Furthermore, we evaluated the behaviour of primary rat bone marrow cells cultured in close contact with 70:30 (NVP:BMA) and 50:50 scaffolds for up to 23

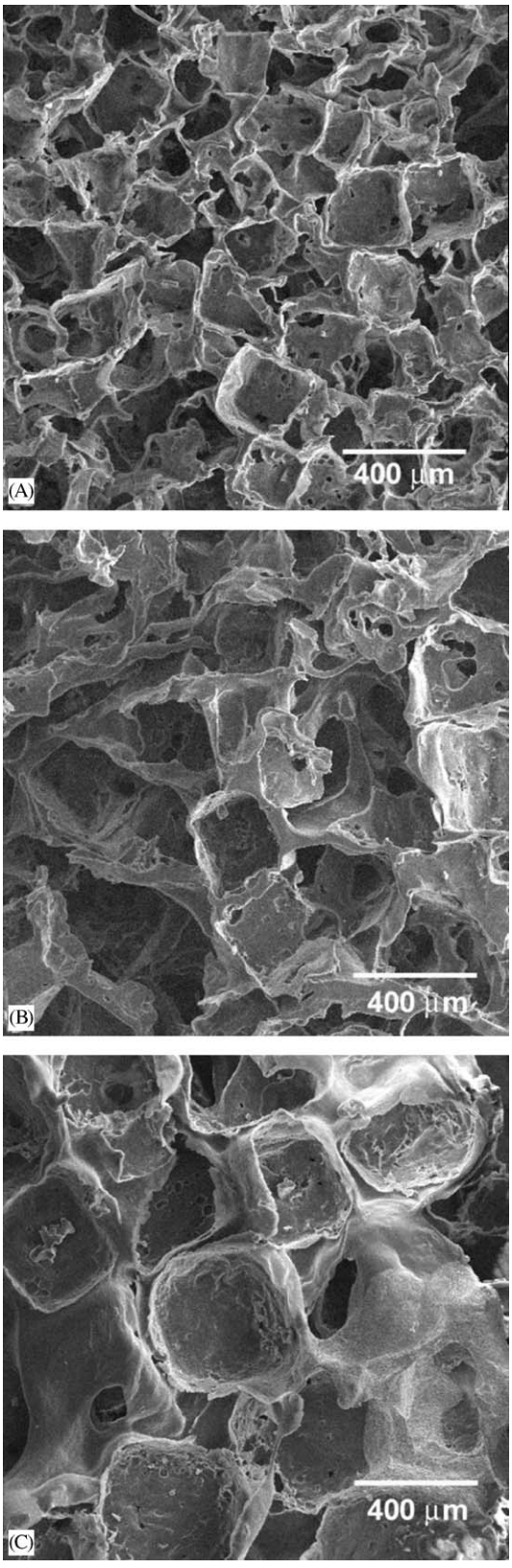

Fig. 2. Representative SEM micrographs of dry 70:30 (NVP:BMA) scaffolds with porosity 90%. These were fabricated through solvent casting and particulate leaching, as described in the Materials & Methods section. Control over the pore size was obtained by varying the size of the salt crystals. (A) Scaffold prepared with salt particle sizes in the range: 200–300 µm. (B) Scaffold prepared with salt particle sizes in the range 300–425 µm. (C) Scaffold prepared with salt particles >425 µm.

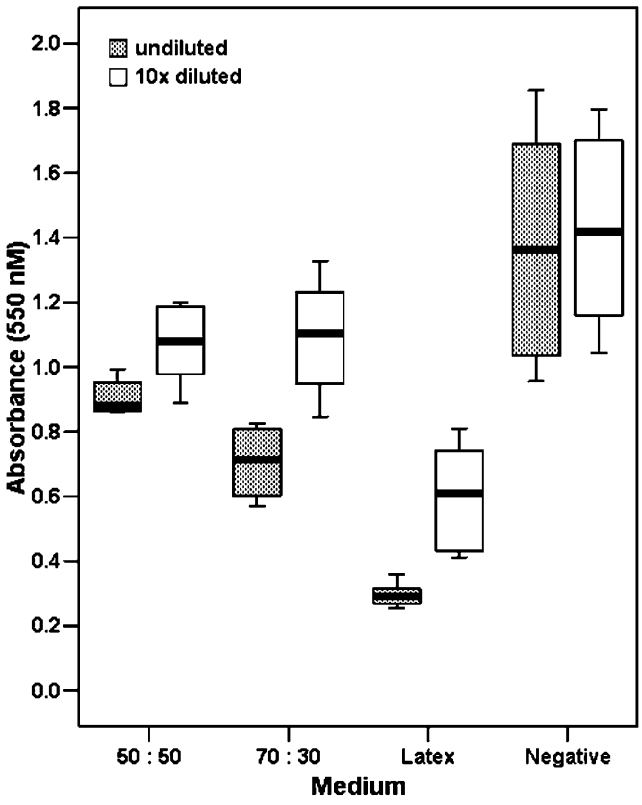

Fig. 3. Mean absorbance as measured in the MTT test with fibroblast cells, incubated in undiluted extracts, and 10-fold diluted extracts. Extracts from latex rubber were used as a positive control. Error bars designate means plus/minus standard deviation for $n = 8$, except for undiluted 50:50 ($n = 7$), and undiluted 70:30 ($n = 6$).

days. The scaffolds had no negative influence on cell morphology, cell viability and proliferation. Nevertheless, cell differentiation was not seen after 9 and 23 days. Fig. 5 shows a representative micrograph of rat bone marrow cells cultured in close contact with a 70:30 (NVP:BMA) scaffold. No bone nodules and/or mineralization were observed. Instead, de-differentiation of the bone marrow cells towards fibroblast-like cells seemed to have occurred.

Based on the combined data of our in vitro biocompatibility tests, we decided to do a series of in vivo experiments. In the first series, scaffolds were implanted subcutaneously on the back of 8 rats. Each animal received 2 scaffolds, i.e., one 50:50 and one 70:30 (NVP:BMA) scaffold. All scaffolds had a porosity of 80% and pore sizes of 200–300 µm. Follow-up was 7 days (4 animals) or 84 days (4 animals). Fig. 6 shows micrographs taken from samples which were harvested 1 week after subcutaneous scaffold implantation. The 50:50 scaffold group (Fig. 6A and B) was comparable to the 70:30 (NVP:BMA) scaffold series (Fig. 6C and D). In all animals, both implants were surrounded by a thin capsule containing proliferating fibroblasts, collagen fibres, newly formed capillary sprouts and some inflammatory cells. From this capsule, endothelial

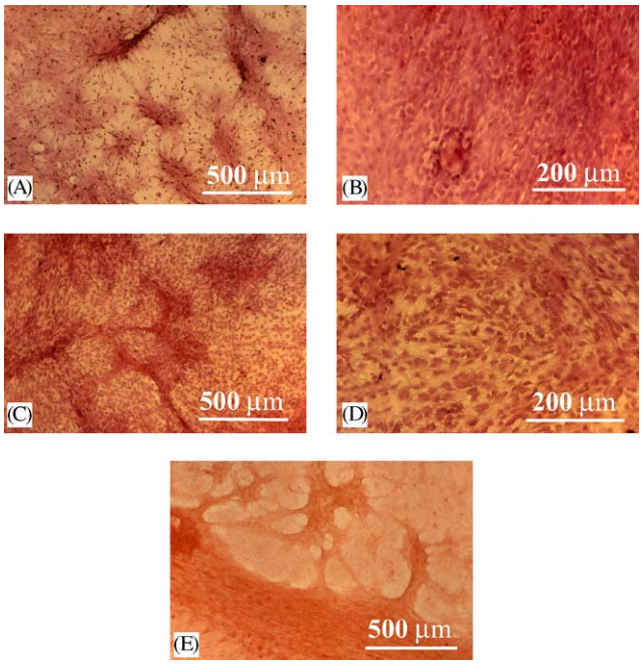

Fig. 4. Alizarin-red-stained histological sections of rat calvarial bone cells in contact with our scaffolds. (A) Bone nodules formed when calvarial bone cells were cultured in close contact with a 50:50 scaffold for 25–30 days. (B) Bone tissue, formed under the same conditions as for A. (C) Bone nodules, formed when calvarial bone cells were cultures in close contact with a 70:30 scaffold for 25–30 days. (D) Cubical cells, formed under the same conditions as for C. (E) Cell attachment onto the 50:50 scaffold.

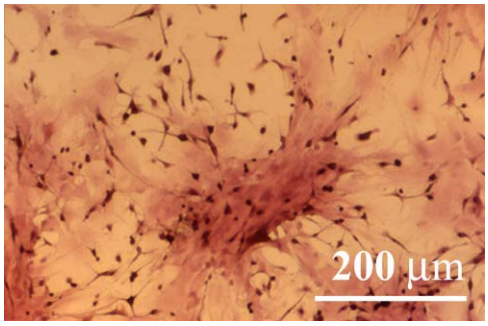

Fig. 5. Alizarin-red-stained histological section of rat bone marrow cells cultured in contact with a 70:30 scaffold for 23 days. Fibroblast-like cells are present. No bone nodules are seen.

cells, fibroblasts and inflammatory cells penetrated into the porous cavities at the periphery of the scaffold. Very few giant cells (multinucleated macrophages) were observed at the border of the scaffold. A striking difference between the 50:50 and 70:30 (NVP:BMA) scaffolds was seen 84 days after subcutaneous scaffold implantation. The 50:50 scaffolds (Fig. 7A and B) were invaded with endothelial cells, fibroblasts and blood cells. The endothelial cells formed a large cavernous vascular network filled with blood. Furthermore, a dense network was present throughout the

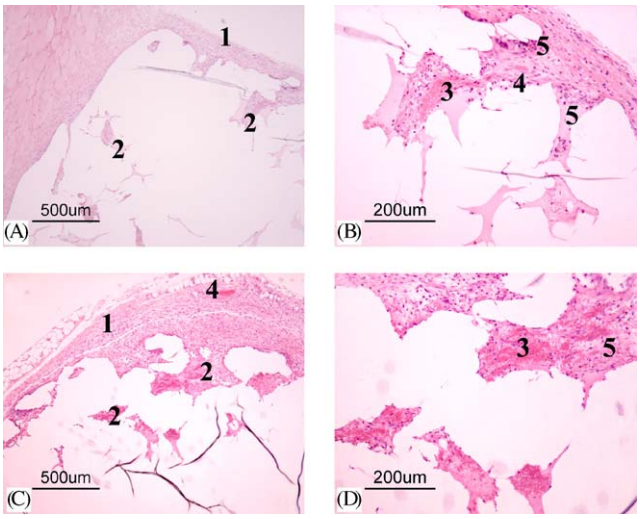

Fig. 6. Haematoxylin/eosin-stained sections of specimens which were harvested 1 week after subcutaneous implantation in rats. (A, B) 50:50 scaffold shown at different magnifications. (C, D) 70:30 scaffold shown at different magnifications. All scaffolds are surrounded by fibrous tissue (1). Note fibroblast infiltration (2) and erythrocytes (3) in the peripheral pores of both scaffolds. Also, formation of blood capillaries (4) and some multinuclear cells (5) are seen.

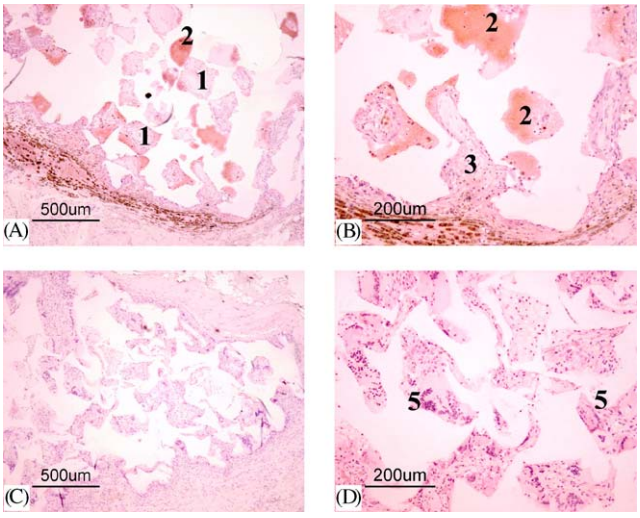

Fig. 7. Haematoxylin/eosin-stained sections of specimens which were harvested 84 days after subcutaneous implantation in rats. (A, B) 50:50 scaffolds shown at different magnifications. Note that the pores retained their rectangular shape (1). The pores are invaded by erythrocytes (2), endothelial cells and fibroblasts (3). Note the iron-containing macrophages (4) between the dermis and the 50:50 scaffold. (C, D) 70:30 scaffolds shown at different magnifications. The pores in these scaffolds lost their rectangular shape, due to the softer nature of this material. The cavities are filled with fibrous tissue and many giant cells (5).

scaffold consisting of fibroblasts, collagen and newly formed capillaries. Also, some macrophages were observed. The pores maintained their rectangular or square form, which corresponded to the dimensions of the salt crystals used in the preparation of the scaffold. The capsule around the scaffold contained

many iron-containing macrophages, which are remnants from phagocytised red blood cells. This reflected the biocompatibility of the 50:50 scaffolds, as well as the fact that the supply of nutrients, and the efflux of waste products from these cells was realised. Only very few giant cells were seen in the 50:50 group. In contrast to the 50:50 group, a severe foreign body reaction was observed in and around the 70:30 (NVP:BMA) scaffolds (Fig. 7C and D). Giant cells were observed between a network of fibroblasts, collagen and newly formed capillaries. These observations revealed that only 50:50 scaffolds were biocompatible in vivo.

The second series of in vivo experiments were based on the work of Nimni et al. [15], and modifications made by Bahar et al. [19]. These workers showed that ectopic bone formation could be induced in a so-called demineralised bone matrix (DBM). Four different scaffold materials were tested in this way: 50:50 with 80% porosity, 50:50 with 90% porosity, 70:30 (NVP:BMA) with 80% porosity, and 70:30 (NVP:BMA) with 90% porosity. We then monitored the process of ectopic bone formation as closely as possible, using X-ray microradiography and histological analysis. DBMs were harvested 4, 6, or 8 weeks postimplantation. Fig. 8 shows a series of representative X-ray micrographs. White areas (enhanced X-ray absorption) reveal mineralization. Clearly, mineralization occurred fast in the control DBM, which was entirely filled with bone marrow. In the 50:50 series, mineralization occurred after 6 weeks, predominantly in the centre. After 8 weeks, mineralization was seen throughout the entire volume of the DBM. In the 70:30 (NVP:BMA) series, there was much less mineralization. After 6 weeks and after 8 weeks post-implantation, some enhanced contrast was observed at the ends of the DBM cavity, but certainly not in the centre. We concluded from this that the 50:50 material had significantly better osteoconductivity, as compared to the 70:30 counterpart. Fig. 9 shows micrographs from our histological analysis of scaffolds which were explanted after 8 weeks, and which were subsequently removed from the DBM. Fig. 9A, C and E show cross-sections of one of the 50:50 scaffolds; Fig. 9B and D show sections of one of the 70:30 (NVP:BMA) scaffolds. The pores in the 50:50 scaffold were clearly visible as rectangles, which revealed that the scaffold did not deform (compare: Fig. 7A and B). On the other hand, the pores in the 70:30 (NVP:BMA) scaffold completely lost their rectangular shape. Fig. 9C and E provide a detailed image of the pores in the 50:50 material. Newly formed bone was deposited directly at the interface of the polymer with very active osteoblasts present at these sites. Fig. 9D shows, an image of the pores in the 70:30 material

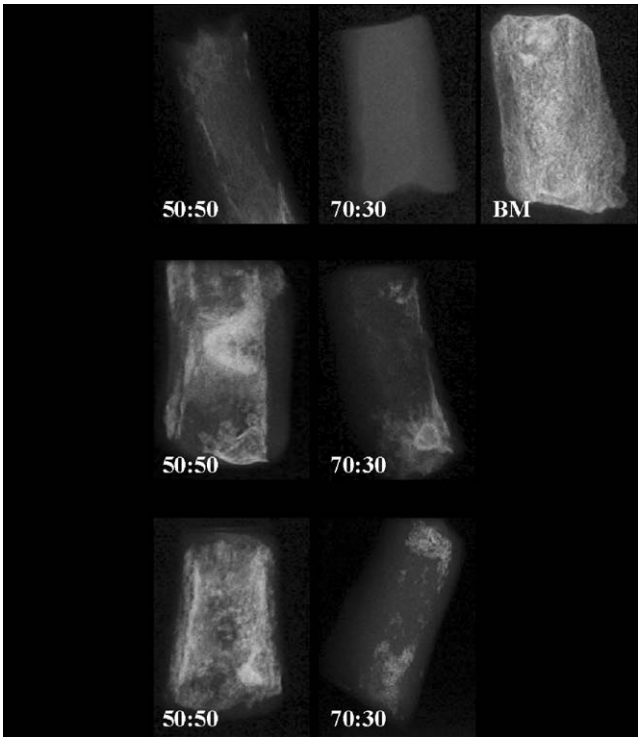

Fig. 8. X-ray micrographs of explanted DBM cylinders, 4, 6 and 8 weeks post-implantation. White areas (enhanced X-ray absorption) correspond with mineralisation; see Materials and Methods section for further experimental details. DBM cylinders were filled with 50:50 scaffold parts and bone marrow cells (50:50); 70:30 scaffold parts and bone marrow cells (70:30), or with bone marrow cells alone (BM).

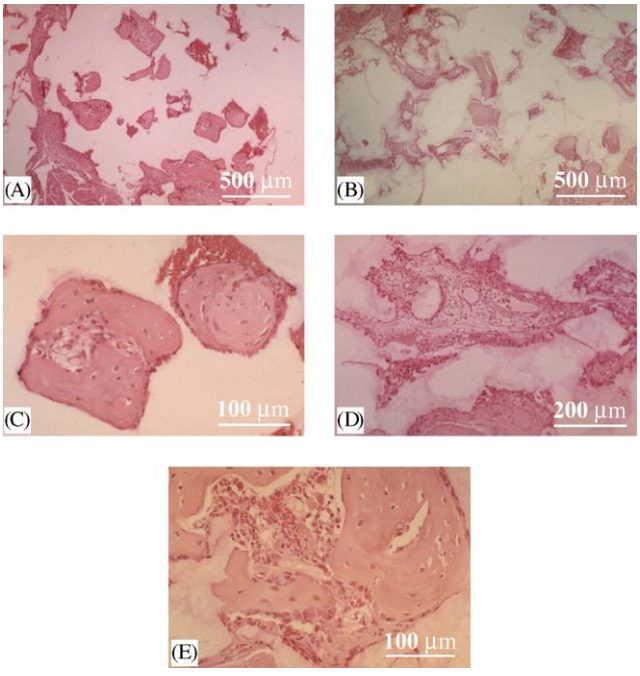

Fig. 9. Cross sections of the scaffolds, explanted after 8 weeks. Scaffolds were carefully removed form the DBMs, followed by histological work-up, cutting and staining: (A) representative section of the 50:50 scaffold, (B) representative section of the 70:30 scaffold, showing severe deformation of the pores in this material, (C) detailed image of the pores in the 50:50 material, (D) detailed image of the pores in the 70:30 material. (E) Newly formed bone in 50:50 scaffold.

(NVP:BMA); these were filled with fibrous tissue and many multinucleated giant cells.

4. Concluding remarks

Tissue engineering on the basis of degradable polymeric scaffolds still poses many technical problems that are only partly understood. This study reveals that the physico-chemical properties of the scaffold biomaterials play an important role by themselves. Comparison of two stable scaffold materials, i.e., 70:30 (NVP:BMA) and the more hydrophobic 50:50 counterpart showed that the latter is less cytotoxic, more biocompatible, and more osteoconductive in vivo. A possible explanation for this difference may lie in the fact that the 50:50 material has a more favourable balance between hydrophobic and hydrophilic properties. Therefore the 50:50 material is likely to be associated with better adherence of cells, and more pronounced adsorption of proteins its surface.

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