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Porous polymer/hydroxyapatite scaffolds: characterization and biocompatibility investigations

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Abstract Poly-lactic-glycolic acid (PLGA) has been widely used as a scaffold material for bone tissue engineering applications. 3D sponge-like porous scaffolds have previously been generated through a solvent casting and salt leaching technique. In this study, polymer–ceramic composite scaffolds were created by immersing PLGA scaffolds in simulated body fluid, leading to the formation of a hydroxyapatite (HAP) coating. The presence of a HAP layer was confirmed using scanning electron microscopy, energy dispersive X-ray spectroscopy and Fourier transform infrared spectroscopy in attenuated total reflection mode. HAP-coated PLGA scaffolds were tested for their biocompatibility in vitro using human osteoblast cell cultures. Biocompatibility was assessed by standard tests for cell proliferation (MTT, WST), as well as fluorescence microscopy after standard cell vitality staining procedures. It was shown that PLGA–HAP composites support osteoblast growth and vitality, paving the way for applications as bone tissue engineering scaffolds.

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1 Introduction

Advanced tissue engineering strategies have involved the fabrication of artificial scaffolds acting as analogues of extracellular matrix and guiding cell adhesion, migration, proliferation, differentiation and tissue regeneration in three dimensions. Materials resembling the organic component of the bone extracellular matrix have been represented by degradable and resorbable polymers belonging to a family of aliphatic polyesters, namely polyglycolide, polylactides, poly-ε-caprolactone, and their copolymers. The main advantage of these materials is that they degrade hydrolytically, resulting in a breakdown of the polymer chain and the release of compounds naturally present in the body, which are finally metabolized.

In previous studies, we have used a particulate leaching technique to create porous scaffolds from poly-lactic-glycolic acid (PLGA), a copolymer of L-lactide and glycolide, and investigated their degradation behaviour [1, 2]. Results of cell experiments showed that a PLGA with a glycolide: L-lactide ratio of 85:15 had a favourable influence on the adhesion and proliferation of MG63 osteoblast-like cells [3].

Poly-lactic-glycolic acid scaffolds can be enhanced by the incorporation of ceramic components such as hydroxyapatite (HAP) to create composite scaffolds. HAP has been added to PLGA scaffolds to influence PLGA degradation [4], promote osteoblast attachment, proliferation and differentiation in vitro [5–7] and enhance bone regeneration in calvarial defects in rats in vivo [8, 9].

The first aim of this study was to manufacture PLGA–HAP composite scaffolds for bone tissue engineering by using a newly modified particulate leaching method with subsequent deposition of a HAP layer by incubation in simulated body fluid (SBF). Apatite coating of PLGA has been achieved before by immersion in SBF, which influenced osteoblastic differentiation of MC3T3-E1 pre-osteoblasts [10]. The second aim was to assess biocompatibility of PLGA–HAP composite scaffolds by standard tests for cell proliferation (MTT, WST), as well as fluorescence microscopy after standard cell vitality staining procedures. With a view to future clinical applications, it is preferable to use cells from human donors instead of osteoblastic cell lines. Therefore, human osteoblasts were used instead of the osteoblastic cell lines used in a previous study [3]. Other groups have conducted cell biological characterisation with murine [11] and human mesenchymal stem cells purchased from commercial sources [7] as well as murine osteoblasts [6], however, to our best knowledge, this is the first study conducted using osteoblasts from human donors.

2 Materials and methods

2.1 Production of HAP–PLGA composite scaffolds

2.1.1 Manufacture of porous PLGA Scaffolds

Poly-lactic-glycolic acid with a molar ratio of L-lactide to glycolide of 85:15 and a molecular mass $M_n = 50$ kDa, $M_w = 105$ kDa, was synthesised according to the method described previously [12]. The scaffolds were produced by the modification of a classical solvent casting/particulate leaching technique, which enabled us to prepare the scaffolds with opened pores homogeneously distributed throughout the entire volume of the scaffolds [2]. Sodium chloride particles (POCh, Gliwice, Poland) were sieved (Multiserw, Wadowice, Poland) to obtain particle fractions of defined size, e.g. 250–320 μm. The salt particles were mixed with 10% (w/v) copolymer solution in methylene chloride (POCh, Gliwice, Poland) in such a proportion as to obtain a salt volume fraction of 85%. The suspension of NaCl in the copolymer solution was stirred until most of the methylene chloride had evaporated, and the dense suspension was tightly packed into polypropylene syringes (diameter 12 mm, volume 5 ml). This procedure prevented sedimentation of the porogen and inhomogeneous distribution of pores, characteristic for typical solvent casting/particulate leaching methods on flat surfaces. Then the polymer/salt mixture was dried overnight in air, followed by vacuum treatment at a reduced pressure for 48 h. Afterwards the syringes with the rigid salt/polymer mixture were cut with a surgical blade into slices 2 mm in thickness and placed into 100 ml of ultra high purity water (UHQ-water of resistivity of 18.2 MΩcm, produced by Purelab UHQ-PS apparatus, Elga, U.K.). The water was changed several times until the conductivity of the water after washing was approximately 2 μS/cm, which took 36–48 h. The samples were then dried in a vacuum oven at 35°C for at least 24 h and stored in a desiccator prior to use. The scaffolds had the form of cylinders of diameter 12 mm and height 2 mm.

2.1.2 Deposition of hydroxyapatite on scaffolds

Simulated body fluid was prepared by dissolving reagent-grade $CaCl_2$, K_2HPO_4, NaCl, KCl, $MgCl_2$, $NaHCO_3$, Na_2SO_4 in ultra-high-purity water (UHQ-water, produced by Purelab UHQ-PS apparatus, Elga, UK), and pH 7.4 at 37°C was adjusted with Tris (hydroxymethyl aminomethane) and HCl. All chemicals came from POCh, Gliwice, Poland. The inorganic ion concentrations were the following: 426 mM Na^+, 15 mM K^+, 7.5 mM Ca^{2+}, 4.5 Mg^{2+}, 446.4 Cl^-, 12.6 HCO_3^-, 3 mM HPO_4^{2-}, 1.5 mM SO_4^{2-}, and were three times those of human blood plasma [13]. Ten individual scaffolds were immersed in 100 ml of SBF solution and placed in a vacuum oven (SPT-100, Poland) in order to force the solution into the pores of the scaffolds. Subsequently the samples were re-pressurised and stored in an incubator at 37°C for 6 days. The SBF solution was renewed every two days to ensure sufficient ion concentrations for mineral growth. Afterwards the samples were washed in UHQ-water, air and vacuum dried, packed in sterilization bags (Tyvdec^R Roll, ASP, Johnson & Johnson) and sterilized by the H_2O_2–plasma method (Sterrad 120, ASP, Johnson & Johnson).

2.1.3 Scaffold characterization

The porosity of the scaffolds, P, was calculated from the mass (weighed with a precision of 0.0001 g) and the dimensions of each scaffold (measured with a caliper with a precision of 0.1 mm). Next, the apparent density, ρ_S, of the scaffold was calculated by dividing the weight by the volume of the scaffold. The porosity was then determined according to the equation:

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

where ρ_p is the density of the copolymer (1.3 g/cm^3). The average porosity ± confidence interval ($\alpha = 0.95$) was

calculated from 24 measurements of the individual scaffolds.

The microstructure of the scaffolds was studied with the use of a scanning electron microscope (SEM) (JSM 5400, JEOL, Japan; accelerating voltage 15 kV) at magnifications of 50×, 500× and 2,000×. Before the analysis, the samples were sputter-coated with a thin carbon layer to make them conductive. Elemental composition of the samples was studied by energy dispersive X-ray (EDX) spectroscopy (Link AN 10000, UK). Infrared spectra were obtained by Fourier transform infrared spectroscopy in attenuated total reflection mode (FTIR-ATR) on a Digilab FTS 60v spectrometer from BioRad. The samples were analysed in the 600–1,800 cm^{-1} range with a 4 cm^{-1} resolution. For SEM, EDX and FTIR-ATR examinations three individual scaffolds of two experimental groups (e.g. pristine scaffolds and scaffolds after soaking in SBF) were evaluated.

2.2 Isolation and cultivation of osteoblasts

The study was approved by the ethics committee of the Christian-Albrechts-University of Kiel, Germany, (Approval No: AZ 402/07). Human osteoblasts were isolated from human iliac crest cancellous bone during reconstructive surgery procedures and cultured in 75 cm^2 cell culture flasks using an osteogenic medium consisting of Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin, 1 mM ascorbic acid and 100 nM dexamethasone. During passaging, cells were detached from 75 cm^2 cell culture flasks using 5 ml of a 0.05% trypsin solution in phosphate buffered saline (PBS). After 1:1 dilution of the cell suspension with DMEM containing 10% FCS and centrifugation at 3,200*g* for 3 min, cells were resuspended in DMEM containing 10% FCS, counted and reseeded at a density of 10^5 cells per 75 cm^2 cell culture flask. Cells were cultured in the same medium used for cell seeding in a humidified atmosphere with 5% CO_2 at 37°C. Medium change took place every 3 days. Seeding took place after two passages.

2.3 Assessment of cell vitality

Cell vitality was assessed by fluorescin diacetate (FDA) and propidium iodide (PI) staining. Staining was performed on cells cultured in eluate from scaffolds after 24 h incubation in cell culture medium. A total of 5×10^3 cells in cell culture medium with 10% FCS were seeded on 8-well objectives. After 1 day of culture, 200 µl eluate from scaffolds immersed in serum-free cell culture medium for 24 h was added to cells. After 24 h incubation at 37°C, cells were rinsed with PBS and immersed in an FDA solution made by diluting 30 µl × 1 mg FDA/ml acetone in 10 ml PBS. After incubation for 15 min at 37°C in the dark, the FDA solution was removed by suction and replaced with a PI solution made by diluting 500 µl × 1 mg/ml PI in 10 ml PBS. After incubation for 2 min at room temperature in the dark, cells were rinsed twice in PBS. While still immersed in PBS, cells were then subjected to fluorescence microscopy with excitation at 488 nm and detection at 530 nm (FDA, green) and 620 nm (PI, red).

2.4 Biocompatibility tests

2.4.1 MTT test

Osteoblasts were seeded in 96-well cell culture plates (Nunc, Germany) in 100 µl DMEM at a concentration of 5×10^3 cells/well. After 24 h culture in a humidified atmosphere with 5% CO_2 at 37°C, medium was removed and replaced with 100 µl eluate from scaffolds incubated in serum-free culture medium for 10 min, 1 h or 24 h. For each time interval, three samples were measured and an eight-fold measurement performed. Cells cultured in serum-free DMEM served as low controls. After 24 h incubation, proliferation was assessed with the aid of a MTT Cell Proliferation Kit (Roche Diagnostics, Mannheim, Germany, Cat. No. 11465007001). Calibration curves of $0.16–10 \times 10^3$ cells/well served as standards. Absorbance was measured at 450 nm.

2.4.2 WST test

After removal of eluate from scaffolds for the MTT test, the scaffolds, which had previously been incubated in serum-free culture medium for 10 min, 1 h or 24 h for the MTT test, were seeded in 24-well plates with 10^4 cells in 100 µl cell culture medium. After incubation for 1 h at 37°C to allow cell adhesion, 2,000 µl cell culture medium was added. After 7 days culture, proliferation was assessed with the aid of a Cell Proliferation Reagent WST-1 (Roche Diagnostics, Mannheim, Germany, Cat. No. 116446807001). Briefly, 200 µl WST-1 reagent was added to each well at a 1:10 ratio to cell culture medium. After 4 h incubation in a humidified atmosphere with 5% CO_2 at 37°C, 12 × 100 µl medium was transferred to 96-well plates and absorbance was measured at 450 nm. Cells cultured in wells without scaffolds at a density of 10^4 cells/well served as controls.

2.4.3 Statistics

The quantitative data are presented as mean and standard deviation. The statistical significance of the differences among the experimental groups was evaluated by a one-

way analysis of variance (ANOVA). Values where $P \leq 0.05$ were considered significant.

3 Results

3.1 Characterisation of PLGA-HAP composite scaffolds

Figures 1 and 2 show the gross morphology and microstructure of the scaffolds registered by a digital camera and scanning electron microscope, respectively. The scaffolds have interconnected pores of a size close to the size of porogen particles (250–320 μm). The porosity of the scaffolds was 84 ± 1%. Under higher magnifications, e.g. 500× and 2,000×, round cauliflower-like deposits on the pore walls are visible on the scaffolds submitted to incubation in SBF. The deposits were observed both on the surface of the scaffolds as well in the cross sections.

Fig. 1 Images of poly-lactic-glycolic acid (PLGA) scaffold (*left*) and poly-lactic-glycolic acid–hydroxyapatite (PLGA–HAP) composite scaffold (*right*). Scaffolds are cylindrical, 12 mm in diameter and 2 mm in height

Energy dispersive X-ray spectroscopy analysis shows that on PLGA scaffolds only oxygen and carbon were detected (Fig. 3a), while on PLGA scaffolds after contact with SBF calcium and phosphorus were present (Fig. 3b). The Ca/P ratio was 1.7, which is similar to that of hydroxyapatite.

Figure 4 presents FTIR-ATR spectra of PLGA scaffolds without and after soaking in SBF. In the spectrum of unsoaked scaffolds, the following bands are visible: at 1,750 cm^{-1} from stretching vibrations of C=O, bands in the range of 1,050–1,250 cm^{-1} from C–O and C–O–C stretching vibrations and bands in the range 1,300–1,500 cm^{-1} attributed to deformational vibrations of CH_2 and CH_3. Those bands are characteristic for aliphatic polyesters [14]. In the spectrum of the scaffolds after soaking in SBF, apart from the bands attributed to PLGA, additional bands in the range of 950–1,050 cm^{-1} from stretching vibration of PO_4^{3-}, and at 600 cm^{-1} from deformation vibration of O–P–O are visible. Those bands are typical of low-crystalline hydroxyapatite, similar to apatite present in bone tissue [15].

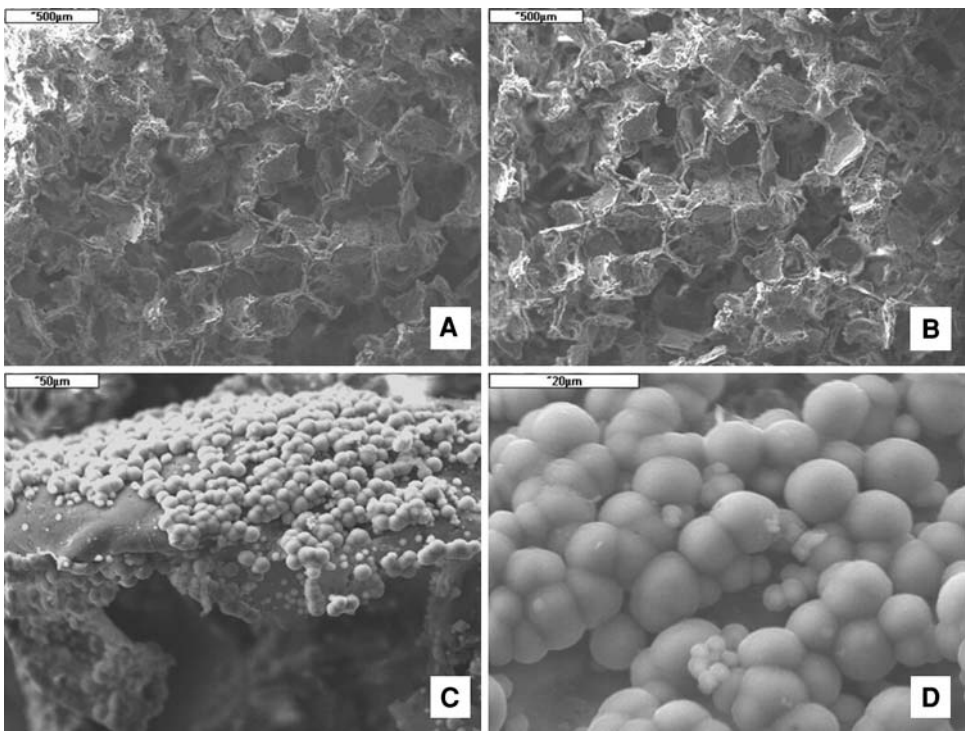

Fig. 2 SEM microphotographs of PLGA scaffold (**a**) and PLGA–HAP composite scaffolds (**b–d**). Original magnification 50× (**a**, **b**), 500× (**c**), and 2,000× (**d**)

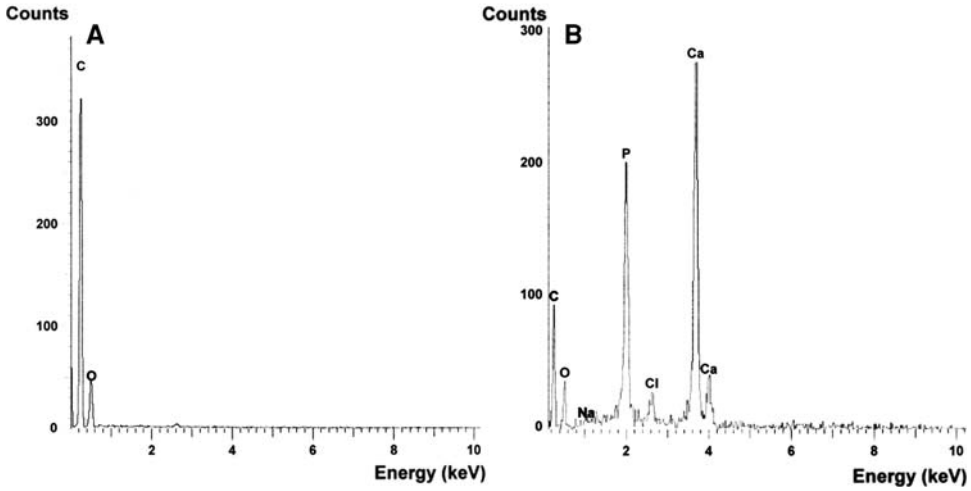

Fig. 3 Energy dispersive X-ray (EDX) spectroscopic analysis of PLGA scaffold (**a**) and PLGA–HAP composite scaffold (**b**)

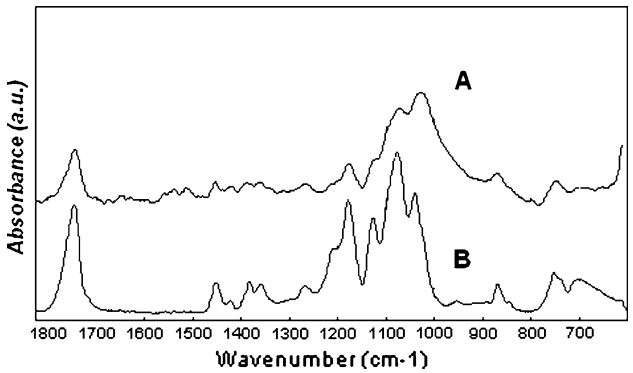

Fig. 4 Fourier transform infrared spectra in attenuated total reflection mode (FTIR-ATR) of PLGA scaffold (A) and PLGA–HAP composite scaffold (B)

3.2 Assessment of cell vitality

Representative images of human osteoblasts cultured in eluate from scaffolds can be seen in Fig. 5. The pronounced green colour of cells cultured in eluate from PLGA–HAP composite scaffolds due to FDA staining demonstrated their vitality, while the absence of a red colour despite PI staining indicated that no cells died as a result of eluate.

3.3 Biocompatibility tests

MTT and WST test results can be seen in Fig. 6. The MTT test showed that proliferation in eluate from PLGA–HAP

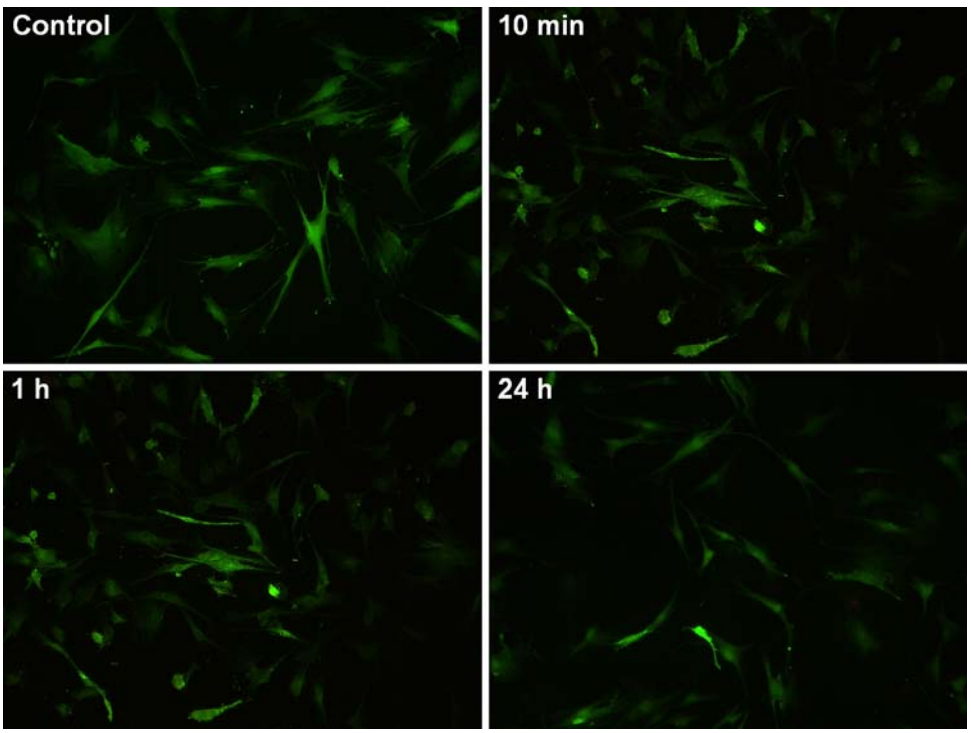

Fig. 5 Fluorescence microscopy images of human osteoblasts cultured in eluate from PLGA–HAP composite scaffolds after 10 min, 1 h and 24 h incubation in serum-free cell culture medium. Staining was performed using fluorescin diacetate (FDA) and propidium iodide (PI). Green colour due to staining with FDA indicates living cells. Lack of red colour despite staining with PI indicates absence of dead cells. Only green, living cells were observed for all samples

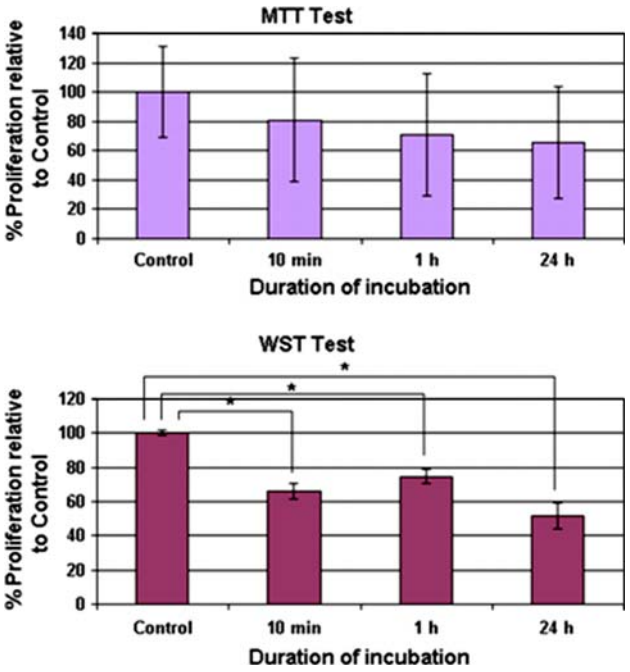

Fig. 6 *Top*: MTT test of human osteoblasts cultured in eluate from PLGA–HAP composite scaffolds incubated for 10 min, 1 h or 24 h in serum-free cell culture medium. Proliferation is shown relative to controls (osteoblasts cultured in normal serum-free cell culture medium). No significant differences among experimental groups were found by ANOVA ($P \leq 0.05$). *Bottom*: WST test with human osteoblasts cultured on PLGA–HAP composite scaffolds. Proliferation is shown relative to controls (cell culture polystyrene). Error bars show standard deviation. *Asterisks* indicate a statistically significant difference according to ANOVA: * $P \leq 0.05$

composite scaffolds was similar to proliferation in control eluate for all three incubation times (10 min, 1 h, 24 h). No statistically significant differences were found. WST test results show that proliferation on scaffolds incubated for 10 min was approximately 65% of that on polystyrene controls, while proliferation on scaffolds incubated for 1 and 24 h amounted to approximately 75% and 50% of control values, respectively. Proliferation on controls was significantly higher than on all sample groups (incubation for 10 min, 1 h and 24 h), however no differences were seen between the sample groups.

4 Discussion

The aims of this study were to manufacture PLGA–HAP composite scaffolds for bone tissue engineering by using a newly modified particulate leaching method with subsequent deposition of a HAP layer by immersion in SBF and to test the biocompatibility of coated scaffolds using standard tests for cell proliferation (MTT, WST), as well as fluorescence microscopy after standard cell vitality staining procedures.

The results of the SEM investigations (Fig. 2) and EDX and FTIR-ATR spectroscopy examinations (Figs. 3, 4) show that it is feasible to produce PLGA scaffolds enriched in hydroxyapatite deposits. The method proposed in this study requires only a 6-day incubation in SBF, contrarily to a typical biomimetic procedure, which requires at least two weeks of material contact with aqueous solution [15]. For this reason the risk of hydrolytic degradation of PLGA is diminished.

Cell vitality staining (Fig. 5) and the MTT test (Fig. 6) indicated that osteoblasts cultured in eluate from HAP-coated PLGA scaffolds after 10 min, 1 h and 24 h incubation showed good vitality and comparable proliferation to controls. In the MTT test, cells on cell culture polystyrene are cultured in eluate from scaffolds incubated in serum-free cell culture medium for 10 min, 1 h and 24 h. The fact that values after 10 min, 1 h and 24 h are similar suggest that only low, if any amounts of toxic substances are released between 10 min and 24 h. Since proliferation values for all coated samples were slightly lower than controls, it is possible that the coating releases small amounts of harmful substances within 10 min of the start of incubation. The WST test results (Fig. 6) showed lower proliferation of osteoblasts on scaffolds compared to polystyrene controls. This may be explained by the fact that osteoblasts seeded on the rough three-dimensional scaffolds (see Figs. 1, 2) do not proliferate as quickly as on the flat polystyrene surfaces which served as controls. In the WST test, samples were incubated in serum-free cell culture medium for 10 min, 1 h and 24 h, which was then removed immediately before cell seeding took place. The low differences between the samples incubated for 10 min, 1 h and 24 h appear to indicate that incubation for more than 10 min has little effect on proliferation and support the findings of the MTT test, which suggest that any toxic substances leached from the scaffolds are released within the first 10 min of incubation.

The results of the cell vitality staining and MTT and WST tests suggest that the PLGA scaffolds mineralised by immersion in SBF are biocompatible for osteoblasts. Warnke et al. [16] used the combination of MTT test, WST test and cell vitality staining to determine the biocompatibility of scaffolds for osteoblasts in previous work. Kohn et al. [17] found that mineralisation of PLGA by immersion in SBF improved murine cell adhesion up to 2 h after cell seeding, suggesting good biocompatibility.

5 Conclusion and Outlook

It can be concluded that 6-day immersion in SBF with a concentration of ions three times higher than that of human plasma is a suitable method to coat PLGA scaffolds with

HAP. On the basis of the results of the cell vitality staining and the biocompatibility tests (MTT, WST) using human osteoblasts, it can be concluded that the PLGA–HAP composites thus produced show acceptable biocompatibility in vitro. Further work will concentrate on in vivo use and also the possibility of incorporating proteins into the mineralised layer. Co-precipitation of protein during mineralisation after immersion in SBF is possible [18, 19]. Rai et al. [20] found that immersion of platelet-rich plasma-coated scaffolds in SBF aided protein retention.

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