

Response of primary fibroblasts and osteoblasts to plasma treated polyetheretherketone (PEEK) surfaces

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Polyetheretherketone (PEEK) is a synthetic polymer with suitable biomechanical and stable chemical properties, which make it attractive for use as an endoprothetic material and for ligamentous replacement. However, chemical surface inertness does not account for a good interfacial biocompatibility, and PEEK requires a surface modification prior to its application in vivo.

In the course of this experimental study we analyzed the influence of plasma treatment of PEEK surfaces on the cell proliferation and differentiation of primary fibroblasts and osteoblasts. Further we examined the possibility of inducing microstructured cell growth on a surface with plasma-induced chemical micropatterning.

We were able to demonstrate that the surface treatment of PEEK with a low-temperature plasma has significant effects on the proliferation of fibroblasts. Depending on the surface treatment, the proliferation rate can either be stimulated or suppressed. The behavior of the osteoblasts was examined by evaluating differentiation parameters.

By detection of alkaline phosphatase, collagen I, and mineralized extracellular matrix as parameters for osteoblastic differentiation, the examined materials showed results comparable to commercially available polymer cell culture materials such as tissue culture polystyrene (TCPS). Further microstructured cell growth was produced successfully on micropatterned PEEK foils, which could be a future tool for bioartificial systems applying the methods of tissue engineering.

These results show that chemically inert materials such as PEEK may be modified specifically through the methods of plasma technology in order to improve biocompatibility.

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1. Introduction

With increasing average lifetime of the population, the need for biomaterials as tissue substitutes increases as well. Basic requirements of such materials consist of good mechanical properties and interfacial biocompatibility. Consequently, a material has to offer a good tissue tolerance (histocompatibility) without cytotoxic, carcinogenic, or mutagenic effects and, even more important, the ability to provoke a specific desired bioresponse in the target organism. The physiological interaction between the organism and the biomaterial is influenced by the surface characteristics of the latter. Roughness and surface chemistry in particular control the adsorption of proteins of the extracellular matrix

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0957-4530 ©2005 Springer Science + Business Media, Inc. (ECM) [1], which is responsible for a successful adhesion of endogenous cells to the biomaterial and therefore also for a stable bonding of the implant to the surrounding tissue [2].

The surface properties of a grafting material can be modified by a large number of techniques [3]. One of these techniques is the method of plasma treatment which has already been described as a highly innovative procedure [4]. Plasmas are ionized gases which can be produced in a closed reactor system containing a low pressure gas mixture by excitation with electromagnetic waves. By comparison to wet-chemical methods and film deposition, plasma modification of biomaterial surfaces is advantageous in respect of chemical flexibility and avoidance of potentially hazardous materials. The generated reactive particles interact with the surface of the biomaterial placed in the reactor and modify its physical and chemical surface properties [5]. Mechanical, electrical and optical properties of the material, which are relevant to their application, remain uninfluenced [6]. The method of plasma modification has been used for commercial purposes for a long time [7, 8]. Typical examples for plasma modified polymers used for cell culture experiments are Primaria[®] and TCPS. Both types of polymer surfaces generate good cell adhesion *in vitro* but are not able to fulfill the requirements for a prosthetic implant material. Until now mostly metal alloys are used for joint replacement in orthopaedic surgery. Metal alloys however provide a higher stiffness than human bone and can therefore cause a periprosthetic resorption of the bone through stress protection [9].

By using PEEK as an implant material, these disadvantages could be avoided, since PEEK has good mechanical [10] and chemical [11, 12] properties. Its elasticity is similar to that of human bone so that the phenomenon of stress protection has not to be expected after implantation. Since PEEK has a good combination of stiffness, tensile strength, distortion, abrasion, and fatigue resistance, it additionally seems to be suitable for syndesmoplasty [13]. However, PEEK is chemically inert and, due to its hydrophobic surface, neither allows protein absorption nor promotes cell adhesion [14]. For this reason it is necessary to

modify the primarily inert PEEK in regard to its surface characteristics so that the surface will enhance cell adhesion and biocompatibility.

There have been several investigations reporting on the surface modification of PEEK including the use of plasma treatment in order to provide a substrate surface promoting better conditions for cell attachment and proliferation [9, 14–16]. However, there are no data available reflecting the proliferation and differentiation of primary cells of the skeletomotor system after plasma modification of PEEK. In order to study these effects an experimental *in vitro* investigation was performed.

2. Materials and methods

2.1. Study design

In the course of this experimental study the influence of surface modification of PEEK on the proliferation and differentiation of primary fibroblasts and osteoblasts was analyzed. The examination of the cell proliferation was performed by cultivating human preputial cells on plasma-activated as well as plasma-passivated PEEK (PEEK foils (250 µm thick) were supplied by Reichelt Chemie, Heidelberg, Germany). After 24, 48, and 72 h, the cell proliferation by means of a cell count, the cell morphology as well as the DNA content of the cells were analyzed and compared to the cell behavior on TCPS. To quantify the cytotoxicity, the LDH concentration of the cell media was determined after 24, 48, and 72 h.

In order to analyze cell differentiation, primary osteoblasts from calvaria of newborn CD-1 mice were cultivated on plasma-activated PEEK up to 30 d. The specific cell differentiation of the osteoblasts was evaluated by typical histological staining procedures, and again compared to cells grown on TCPS.

The goal of a separate series of experiments was to find out if a microstructured cell growth in cell-typical dimensions could be induced on biomaterial surfaces with chemical micropatterning. In this case primary osteoblasts were grown on plasma treated micropatterned PEEK.

2.2. Plasma modification

Two plasma process steps were applied: Firstly, the whole surface was modified by a process which generates functional groups capable of bonding biomolecules and promotes cell adhesion. For this purpose, a microwave plasma in ammonia/argon = 1/4 was applied at a power of 500 W and a pressure of 0.1 mbar in a specially designed UHV reactor system [17] for 77 s.

Secondly, patterning was performed by covering the surface with a laser-cut metallic mask [18] exhibiting characteristic dimensions down to 30 µm and partially removing the functional groups by a soft plasma etching process. For that purpose, the samples were treated in a downstream microwave plasma in hydrogen/argon = 1/3 for 1000 s.

Both processes were also used alone in order to assess the influence of plasma activation and passivation on cell attachment and proliferation. A more detailed description of the plasma processes has recently been introduced [19]. The chemistry of the surface modifications was analyzed by contact angle (H_2O , sessile drop) and X-ray photoelectron spectroscopy (XPS) measurements.

2.3. Culturing of primary fibroblasts and osteoblasts

The primary fibroblasts were isolated from human foreskin. The foreskin was prepared under sterile conditions by cutting it into small pieces. After digestion with collagenase (Boehringer Mannheim Biochemicals, Mannheim, Germany), the fibroblasts cell pellets were suspended in DMEM containing 10% fetal calf serum (FCS) and 1% penicillin-streptomycin (PS). After 24 h the cells were replated and placed on plasma treated PEEK and TCPS at an initial density of $1.5 \times 10^4 \text{ cm}^{-2}$.

Cell proliferation was microscopically determined by using Neubauer's counting chamber. In addition to the cell count the proliferation of the primary fibroblasts was addressed by measurement of the DNA concentration of the cells. For this, cells were detached from the substrate surface at each time point by trypsination, counted and exposed to a specific dye reagent. The DNA content of the cells is proportional related to the coloration and was photometrically measured at 625 nm. In order to assess cytotoxic effects of the modified substrate the activity of LDH released from the cytosol of damaged cells into the supernatant was measured by a colorimetric assay (Cytotoxicity Detection Kit, Boehringer Mannheim Biochemicals, Mannheim, Germany). The amount of color formed in the assay is proportional to the number of lysed cells.

Primary osteoblastic cells were obtained from newborn CD-1 mice by sequential digestion of the calvaria in a solution of 0.1% clostridial collagenase Ia (Sigma Chemical Co., St. Louis, MO) and 0.2% dispase (Boehringer Mannheim Biochemicals, Mannheim, Germany). The cells were then cultured in α -MEM containing 10% fetal bovine serum (FBS), and 1% PS. After 48 h the cells were replated and placed on plasma activated PEEK and TCPS at an initial density of $1.5 \times 10^4 \text{ cm}^{-2}$.

The medium was changed after the first and the third day. Thereafter the medium was supplemented with 5 mM β -glycerolphosphate, and $100 \mu\text{g ml}^{-1}$ ascorbic acid (mineralization medium) and replaced every other day. Cytotoxicity analyses were repeatedly performed as described before. Cultures were maintained up to 30 days at 37°C under 95% air and 5% CO_2 atmosphere. The cells were then fixed with 3.7% formaldehyde in phosphate-buffered saline solution (PBS) for 10 min at room temperature and washed in PBS. Analyses for alkaline phosphatase and collagen (van Gieson staining), and for presence of a mineralized matrix (von Kossa staining) were performed according to standard protocols [20].

In a separate experiment primary osteoblasts were seeded on to microstructured PEEK foils. The foils were prepared by applying two different steps of plasma treatment as described above. After two hours the cells were fixed with 3.7% formaldehyde in PBS for 10 min at room temperature, washed in PBS and stained with a Giemsa dye.

2.4. Statistical analysis

The distribution of the parameters was summarized by reporting mean \pm standard error of the mean. Comparisons between the several materials were evaluated by using the Mann-Whitney rank sum test. A statistical level of $\alpha = 0.05$ was considered as significant. All statistical analyses were performed with the software program "Sigma Stat[®]/Sigma Plot[®]".

3. Results

3.1. Surface properties of the modified materials

Chemical transitions in the polymer surface are the result of complex heterogenic reactions at the interface between the surface and the processing gas. Atoms or side groups of the polymer can be substituted directly by reactions of the surface with chemically reactive substances generated in the plasma.

The plasma treatment of the applied PEEK foils included two steps. First, amino groups were introduced in the PEEK surface by using an ammonia/argon plasma (plasma functionalization). This created a surface with around 5% nitrogen containing functional groups, 50% of them being amino groups (Fig. 1). Indeed, the plasma does not only accomplish a direct substitution, but ad-

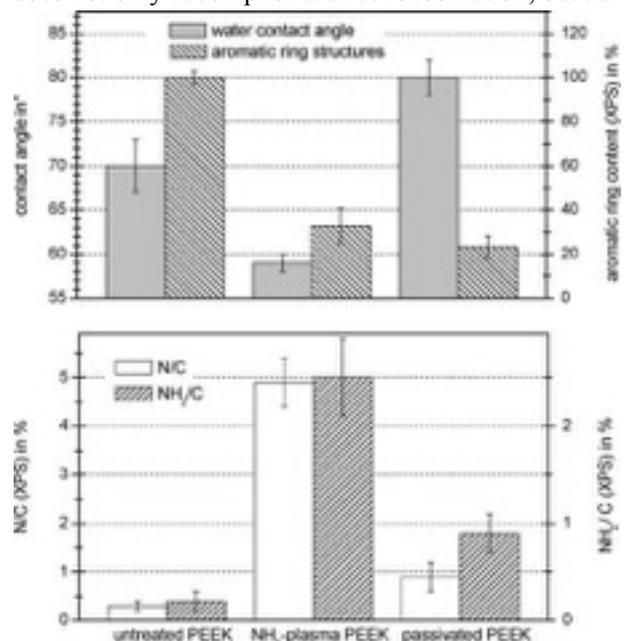


Figure 1 Results of physicochemical surface analysis by contact angle and XPS measurements for untreated and plasma-modified PEEK.

ditionally, changes polymer chains, exemplified by the reduction of aromatic ring content.

The second processing step was applied to the material equipped with functional groups (plasma passivation). Atomic hydrogen produced in the gas phase etches oxygen and nitrogen functional groups. It created a very low energetic surface (passivated), which is characterised by reduced O/C and N/C ratios (Fig. 1).

3.2. Primary fibroblasts

Primary fibroblasts were spread onto plasma activated (NH₃-modified) PEEK and TCPS at a surface density of 1.5×10^4 cells cm⁻². After 24 h a confluent cell layer could be microscopically observed on both materials. No morphological differences were seen between the different materials. The toxicity analysis performed by determining the LDH concentration was repeatedly negative in all cases. These results (not shown) were confirmed in three independent series of experiments.

In a second experimental setup the cells were again placed on plasma-activated (NH₃-modified) PEEK and TCPS at the same density. Every 24 h cell pellets were produced after trypsination and centrifugation and were preserved at -80 °C for the time being. After completion of this experiment, the DNA concentration of the cell pellets was analyzed. The cells attached to the plasma treated PEEK showed a significantly higher DNA amount than the controls (Fig. 2).

In a further experimental setup primary fibroblasts were distributed onto plasma-passivated (H₂-modified) PEEK and the DNA concentration was determined after 24, 48 and 72 h. In this case the DNA concentration was significantly lower than in the control group (Fig. 3).

3.3. Primary osteoblasts

To analyze the biocompatibility of the plasma treated PEEK in comparison to TCPS we incubated the

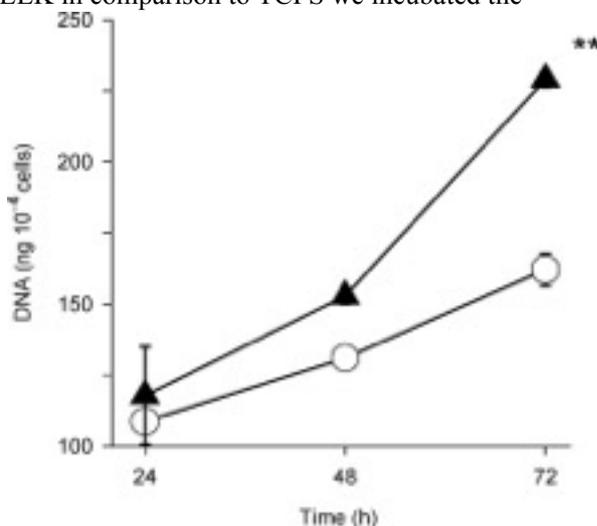


Figure 2 DNA content (ng/dl) of primary fibroblasts related to the cell count on NH₃ plasma-modified PEEK (vs. TCPS (°)); ** = $p < 0.001$ (Mann-Whitney rank sum test).

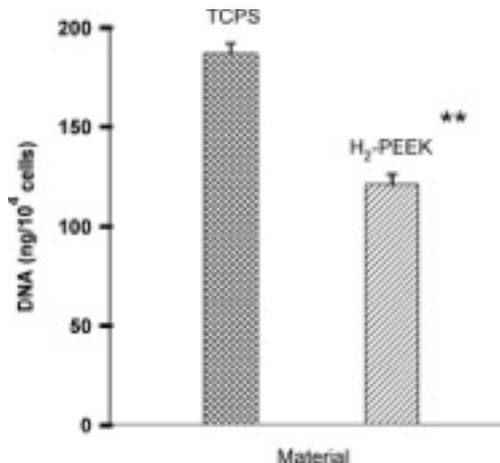


Figure 3 DNA content (ng/dl) of primary fibroblasts related to the cell count ($t = 72$ h) on H₂ plasma passivated PEEK (2000 s) vs. TCPS; ** = $p < 0.001$ (Mann-Whitney rank sum test).

samples with primary osteoblasts from mouse calvaria in α -MEM culture medium under standardized conditions. In the presence of the different materials the behavior of the osteoblasts was analyzed by morphological and biochemical means. Within 3–4 days a confluent cell-layer was observed in the presence of each material. Microscopically no morphological differences were seen in comparison to the control materials (Fig. 4). The osteoblasts continued to proliferate, forming a multilayer of cells and finally produced an extracellular matrix. The toxicity analysis performed by determining the LDH concentrations was repeatedly negative. These results were confirmed in three independent series of experiments.

During the follow-up a strong expression of alkaline phosphatase could be demonstrated after 21 days (Fig. 5). In addition, it was also possible to detect strong collagen production (Fig. 6). Further, the von Kossa staining revealed the production of a bone specific extracellular matrix after 30 days by means of the black color of calcium deposits (Fig. 7). Overall no significant differences were noticed in comparison to the control materials. These findings confirmed that the cells maintained their osteoblastic differentiation in the presence of the different materials, and especially that the plasma treated PEEK is compatible with cell attachment on the surface of the foils.

In order to demonstrate the effect of the chemical micropatterning of the biomaterial surface on the topography of the cell layer, primary osteoblasts were applied onto micropatterned PEEK. After two hours a giemsa staining technique was applied and the distribution of the cells according to the micropatterned, plasma-treated PEEK was demonstrated (Fig. 8).

4. Discussion

Metal alloys are still most commonly used as a material for joint replacement in orthopedic surgery. However, resorption of the adjacent bone frequently occurs due to stress protection resulting from the relatively high stiffness of the prosthesis. In order to address this, polymers such as polyetheretherketone (PEEK) have been considered as alternative materials

for bone and joint replacement [9, 14, 21]. For example, the elasticity of PEEK is similar to that of human bone so that the phenomenon of stress protection experienced with the use of other materials is not to be expected [9]. In addition, PEEK seems to be suitable for syndesmoplasty due to its tensile strength and abrasion resistance [13]. Because of these good mechanical properties, PEEK

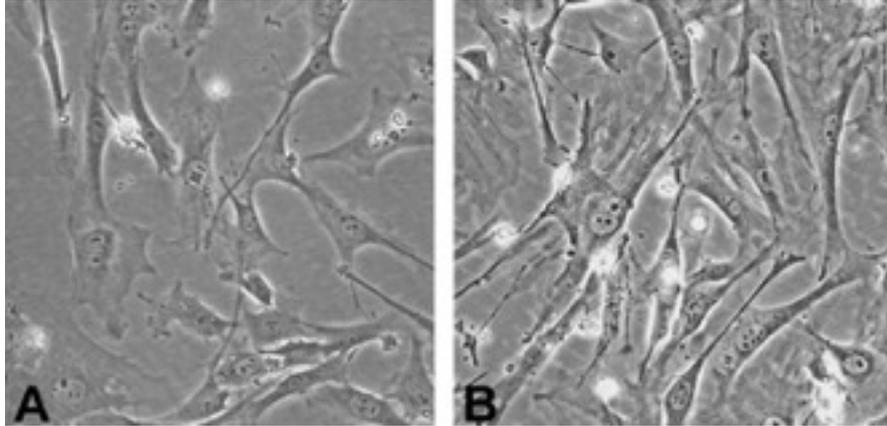


Figure 4 Lightmicroscopy presenting the shape of primary osteoblasts on NH₃ plasma activated PEEK (A) vs. TCPS (B) (initial cell density 10⁴ cells cm⁻², magnification: 300×).

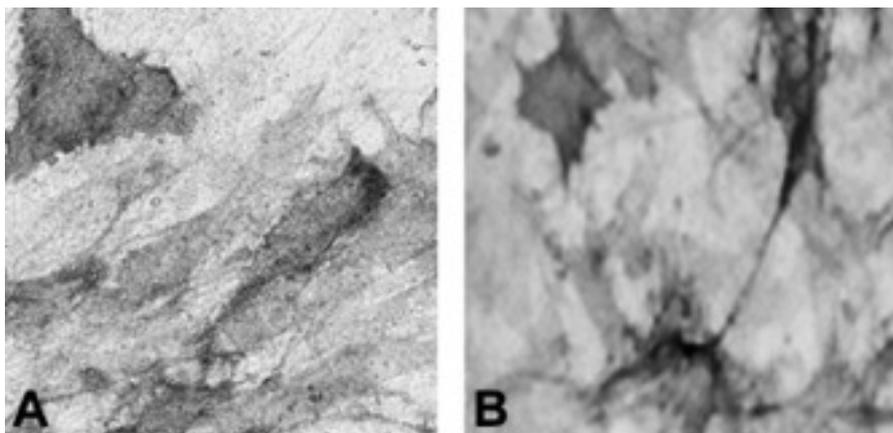


Figure 5 Expression of alkaline phosphatase by primary osteoblasts on NH₃ plasma-modified PEEK (A) vs. TCPS (B) after 21 d of culturing (magnification: 400×).

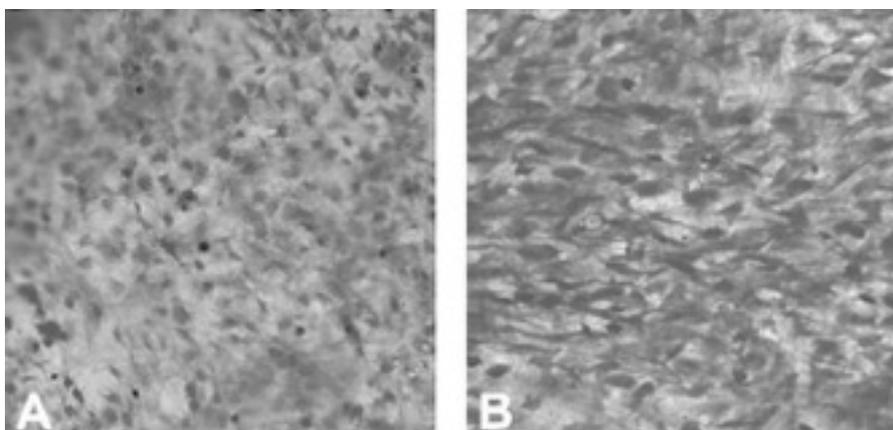


Figure 6 Detection of collagen on NH₃ plasma activated PEEK (A) vs. TCPS (B) after 21 d of culturing (magnification: 200×).

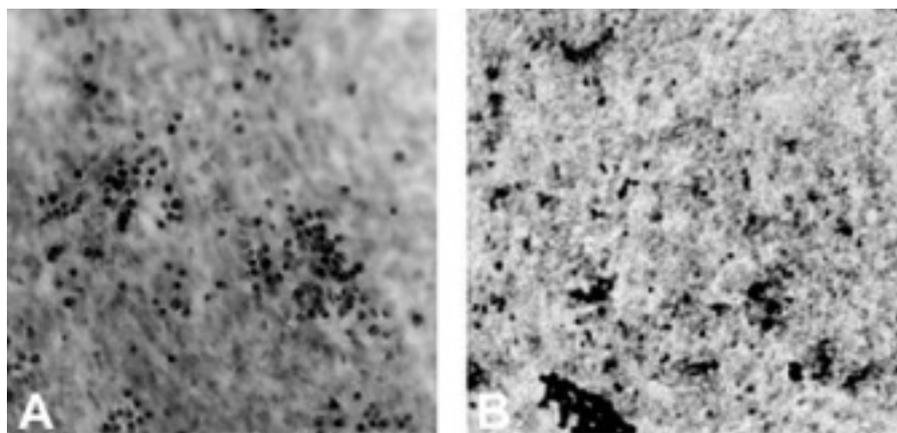


Figure 7 Detection of mineralisation through typical “bone nodules” on NH₃ plasma activated PEEK (A) vs. TCPS (B) after 30 d of culturing

(magnification: 200×).

was chosen as a polymer substrate for this experimental investigation.

The lack of toxicity of PEEK was already demonstrated in 1990 [22]. In this study mouse fibroblast cell lines were used and the toxicity analysis was performed by determining the LDH concentration. The growth behavior of cell lines of the skeletomotor system on untreated PEEK has been compared to a number of potential implant materials such as polyethersulfone (PES), ultra high molecular weight polyethylene, titanium as well as cobalt—chromium—molybdenum enamels [23]. In this case no toxic effects were seen for untreated PEEK, but also no stimulating effect concerning the cell proliferation in comparison to the other materials. In addition, it has recently been proven that PEEK shows neither cytotoxicity nor mutagenicity *in vitro* [24].

Difficulties exist concerning the biocompatibility of untreated PEEK, because PEEK is chemically inert and, due to its low amount of hydrophilic groups, only

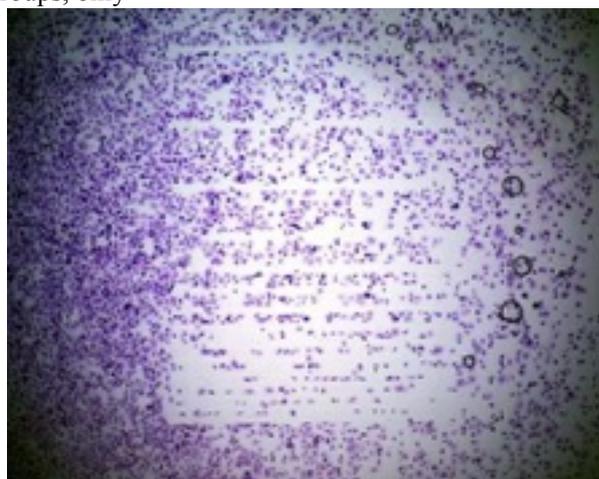


Figure 8 Targeted cell formation of primary osteoblasts 2 h after culturing on plasma-modified micropatterned PEEK (giemsa staining, magnification: 25×).

provides cell adhesion to a limited degree. Attempts to improve the biocompatibility of PEEK have

already been reported. In these cases PEEK was either treated with a N₂/O₂ plasma and a layer of carbon containing calcium phosphate or with fibronectin connected to a chemically modified PEEK surface [14, 15]. These studies report on different cell lines, which most commonly offer the advantage of an easy and unlimited handling. However, major limitation of cell lines is their inability to differentiate and therefore reflect the physiological behavior of primary cells. Studies addressing this background have not been reported so far.

We therefore performed a first series of cell culture studies concerning the behaviour of primary fibroblasts and osteoblasts after surface modification of PEEK. The aim of this study was to analyze, if the biocompatibility of PEEK can be improved through surface modification by plasma treatment. These results were compared to TCPS, a polymer which provides excellent conditions for the attachment and proliferation of cells *in vitro*. In addition, the advantages of an extended use of plasma treatment in the production of biomaterials with structured surfaces was analyzed. As fibroblasts are easy to grow *in vitro* they were used to study the proliferation of primary cells on the plasma treated material. However, only osteoblasts provide information about bone specific biocompatibility. In order to address this fact, primary osteoblasts obtained from mouse calvaria were applied to the surface of the material.

Primary Fibroblasts and osteoblasts have already been investigated in a number of experiments analyzing the biocompatibility of different substrates [9, 23, 25–29]. Both cell types can provide useful information concerning adhesion, proliferation, differentiation, and apoptosis. Osteoblast differentiation and function can be tested by analysis of alkaline phosphatase and collagen, two major products typically expressed by osteoblasts. Enzyme labeling for AP and van Gieson staining for collagen are easy and established methods and additionally allow expression analysis in the direct proximity of the probes [30].

However, analysis of the protein and cell adhesion on PEEK surfaces are only possible to a restricted degree since PEEK has a significant inherent auto-

fluorescence, and therefore immuno-fluorescence methods for characterization of surface proteins are not applicable [23]. With regard to these characteristic properties of PEEK we decided to perform histological stainings by which the cell differentiation of osteoblasts on PEEK can be visualized microscopically. Since it is known that a stimulating effect on the cell proliferation may not only be evaluated by cell count but also through protein and DNA analysis [9] we measured the DNA content of the cells by a colorimetric assay.

After cultivating primary human fibroblasts on plasma-activated (NH₃-modified) PEEK foils we were able to show that the proliferation rate determined by analyzing the DNA concentrations was significantly higher compared to TCPS. A confluent cell layer was observed on both substrates. Differences in cell morphology and spreading behavior were not observed. The toxicity testing by analyzing the LDH concentrations was repeatedly negative in all cases. We were also able to show that the surface passivation of PEEK by H₂ plasma causes a suppression of cell growth compared to the control material by means of the DNA content of the cells. Again, there was no evidence of a toxic component caused by the plasma treatment.

As the stability of implant interfaces is predominantly provided by ECM proteins and the production of mineralised bone matrix by the osteoblasts attached to the implant surface we concentrated on analyzing the differentiation parameters of primary osteoblasts. The differentiation of osteoblasts *in vitro* is known to reflect the culture conditions and enables the evaluation of growth behavior and generation times. Variations of the surface characteristics not only lead to differences concerning the affinity of the cells to the substrate but also lead to differences in differentiation [31].

After cultivating the osteoblasts on NH₃ plasma treated PEEK and TCPS no toxic components were found. LDH concentrations always remained below the detection limit. Morphological criteria were the same on both substrates. The differentiation of the osteoblasts was demonstrated by using specific staining techniques and dyeing the alkaline phosphatase, collagen type I, and bone nodules. After cultivating the osteoblasts for 30 days on plasma-activated PEEK foils, all specific differentiation parameters seen in the synthesis of mineralized bone matrix *in vivo* could be observed.

These data show that the plasma-guided surface modification of PEEK provides excellent conditions for the cell specific differentiation of primary osteoblasts. We further did not observe any differences compared to cell culture materials such as TCPS. These results indicate that PEEK can be converted into a material with a high biocompatibility by using the plasma technique. In relation to the good cell-surface interaction of plasma treated PEEK *in vitro* as well as its good elastic qualities demonstrated in different experiments [13, 14] PEEK can be concluded as a potential implant material, which fulfills all criteria of an orthopaedic biomaterial.

We further observed a microstructured growth of osteoblasts in cellular dimensions on plasma-induced micropatterned TCPS films. The local differences in cell adhesion are presumably due to different concentrations of ECM protein adsorbed at the polymer surface [32]. The plasma etching causes regions of high protein affinity (hydrophilic) and regions with low protein affinity (hydrophobic). The chemical micropatterning of polymer biomaterials through plasma-induced surface modification is a very suitable procedure to produce areas with high and very low concentrations of functional groups in microscopic dimensions [33] and regulate cell adhesion and cell growth.

In summary it can be said that no toxic effects of PEEK were found and that its biocompatibility may be improved by surface modification using plasma treatment. Considering the findings of other authors it can be assumed that the improved biocompatibility is probably due to the increased adsorption of ECM proteins and increased cell adhesion [14]. In the course of this experimental work we were able to demonstrate that the surface modification of PEEK with NH₃-plasma has a reproducible stimulating effect on primary fibroblasts and osteoblasts.

5. Conclusion

The biocompatibility of a plasma-treated polymer (PEEK) was demonstrated by osteoblast-specific biocompatibility testing consisting of long-term cell culture with primary mouse osteoblasts. Evidence for the good biocompatibility was derived from light microscopy (cell morphology) as well as histology (mineralization, expression of alkaline phosphatase and collagen). Further, a reproducible stimulation and suppression of cell proliferation could be achieved by the methods of plasma modification. These results indicate that chemical inert material such as PEEK can be converted to a suitable graft for bone and joint replacement providing good conditions for cell attachment, proliferation and differentiation.

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