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PEO-generated Surfaces Support Attachment and Growth of Cells *In Vitro* with No Additional Benefit for Micro-roughness in Sa (0.2-4 μm)

OLE JUNG[1*], RALF SMEETS[1*], ALEXANDER KOPP[2], DARIO PORCHETTA[2], PHILIPP HIESTER[2], MAX HEILAND[1], REINHARD E. FRIEDRICH[1], CLARISSA PRECHT[1], HENNING HANKEN[1], ALEXANDER GRÖBE[1*] and PHILIP HARTJEN[1]

[1]*Department of Oral and Maxillofacial Surgery, University Medical Center Hamburg-Eppendorf, Hamburg, Germany;* [2]*Meotec GmbH & Co. KG, Aachen, Germany*

Abstract. *Background/Aim: Plasma electrolytic oxidation (PEO), also known as micro-arc oxidation, is a promising electrochemical surface treatment technique for metals which has been used for the generation of various material surfaces and has been the focus of recent biomaterial research. It has been hypothesized that rough PEO surfaces should generally have properties that support cellular attachment and proliferation. However, this has not yet been demonstrated in systematically conducted studies. The present study investigated fibroblast cell proliferation and attachment to ground, electric discharge machining (EDM) and PEO-treated titanium surfaces differing in roughness and porosity. Materials and Methods: Three surface variants with 'smoother', 'medium-coarse' and 'rough' surface topographies were generated by PEO and EDM on specimens of titanium alloy (with 6 wt% aluminum and 4 wt% vanadium) for comparison with more smoothly ground specimens. The in vitro effects on cellular attachment and proliferation were determined in 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), 5-bromo-2'-deoxyuridine (BrdU) and live/dead staining assays with L929 fibroblasts cultivated directly on the metal specimens. Cytocompatibility was determined in accordance with DIN 10993-5/-12 regulations by extract assays. Results: Besides cytocompatibility, all PEO specimens exhibited similar biocompatibility and attachment properties, with vital, spindle-shaped adherent cells growing on the surface, regardless of their surface topology. There were no significant differences in cellular proliferation between the different surfaces and negative controls (cells growing in cell-culture plates). Discussion/Conclusion: With no differences in cellular proliferation and attachment between PEO surfaces with different roughness, we find no evidence to support the notion that rougher PEO surfaces are more favorable for cellular growth of fibroblasts in vitro.*

Biomaterials are becoming increasingly important in everyday medical practice and research. Various products with different specifications and applications are available today. The ideal bone replacement material has osteoinductive, osteoconductive and osteopromoting properties in addition to long-term stability and excellent biocompatibility. The surface characteristics of bone replacement materials and other biomaterials are crucial for their bioactive properties and are directly dependent on the manufacturing process and the material composition. Furthermore, the biocompatibility of biomaterial surfaces is of particular importance as the surface represents the interface between material and tissue/bone.

Plasma electrolytic oxidation (PEO), also known as micro-arc oxidation, is an electrochemical surface treatment technique for metals that allows the manufacture of biocompatible and bioactive titanium surfaces that are better suited for cellular attachment and growth than conventional titanium surfaces (1-3).

Numerous studies describe favorable bioactivity of rough and porous surfaces (4-15). Roughness and porosity are two interdependent surface characteristics of bioactive surfaces that result from the manufacturing process used. The superior properties of roughened surfaces ascribed to other manufacturing processes have also been attributed to PEO

*These Authors contributed equally to this study.

Correspondence to: Ole Jung MD, Department of Oral and Maxillofacial Surgery, University Medical Center Hamburg-Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany. Tel: +49 040741053259, Fax: +49 040741055467, e-mail: ol.jung@uke.de

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surfaces, but the characteristics underlying their bioactivity have not yet been verified in systematic investigations. Overall, it is not sufficiently clear whether and to what degree the bioactive effects are based on the roughness, coating or manufacturing method, physicochemical composition, or on all variables of PEO-treated surfaces.

With this study, we aimed to evaluate the bioactivity and surface characteristics of PEO-treated titanium surfaces of different roughness *in vitro* by measuring influences on cellular adhesion, proliferation, morphology and overall viability of cells cultured on PEO surfaces compared with untreated controls.

Materials and Methods

Materials and surface treatment. For the experiments, the commercially available alpha/beta alloy Ti6Al4V (grade 4 and 5) was used. The alloy contains titanium with 6 wt% aluminum and 4 wt% vanadium. Disc-shaped specimens of 18 mm diameter and 1 mm thickness were cut from a rod using electrical discharge machining (ADM) (AQ537L; Sodick, Duesseldorf, Germany). After polishing with silicon carbide paper (600 grit), the specimens were washed in ethanol for 10 min and in 15% HF/30% HNO_3 for 15 s in order to remove oxide layers resulting from cutting and polishing. Finally, all samples were washed with distilled water for 15 min in an ultrasonic bath and dried on a sterile blanket at room temperature.

Plasma electrolytic oxidation. PEO was achieved using a pulsed rectifier set (M-PEO A1; Meotec, Aachen, Germany). Different electrolytic compositions were prepared for the coating process (Table I). Positive and negative pulsed directed currents of 2 A and 3 A with voltages from 0 V to 500 V were applied by two feed cables. The electrochemical cell consisted of a titanium specimen as anode and platinum mesh as cathode. Pulse frequency was set to 20 Hz and discs were treated for up to 15 min. After processing, all samples were rinsed with distilled water in an ultrasonic bath for 15 min and dried on a sterile blanket at room temperature.

Physical and chemical characterization of the surfaces. The coated surfaces were imaged by scanning electron microscopy (SEM) (XL30 CP; Philips, Amsterdam, the Netherlands) and energy-dispersive X-ray spectroscopy (EDX). 3-D structures and roughness of coated surfaces were characterized using a non-contact optical 3-D profilometer (ZygoLOT ZeGage; AMETEK GmbH, Wiesbaden, Germany).

Experimental groups. Five samples displaying different surface compositions and roughness were prepared (Table I). Untreated samples were used as reference or control group. RM-A, a polyurethane film containing 0.1% zinc diethyldithiocarbamate (Hatano Research Institute, Food and Drug Safety Center, Japan), was used as a positive control reference material. Cells directly seeded onto the surface of the well-plates were used as negative control.

Cell culture. Cell culture was accomplished with L929 fibroblasts (LGC Standards, Wesel, Germany) in minimum essential medium (Life Technologies, Carlsbad, USA; further referred to as cell culture medium) with 10% fetal calf serum (Gibco, Darmstadt, Germany), 1% Penstrep (Gibco, Darmstadt, Germany) and 4 mmol L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) at 37˚C, with 21% O_2, 5% CO_2 and 95% humidity (innova CO-48-230; New Brunswick Scientific, Enflied, CT, USA) hereafter referred to as cell culture condtions. Cell passaging was accomplished at 70-80% cell confluence. For the experiments, cells between the 5th and 10th passage were used.

In vitro characterization of the test samples. Experiments and settings were applied as described in detail in our previous work (16), except that the assay volume was 1 ml in the direct test scheme. If applicable, all tests (direct, extract-based and live/dead staining) were carried out in accordance with DIN ISO 10993-5/-12 regulations.

Specimen sterilization. All samples were sterilized by immersion in 2-propanol for 5 min within a laminar floor hood (Microflow Biological Safety Cabinet). Using sterile gloves, the samples were dried on a sterile blanket and placed in sterile 12-well plates.

Extract and direct tests. All experiments were repeated twice. For the extract tests, four specimens from each group were extracted in 1.88 ml/cm^2 cell culture medium and under cell culture conditions for 72 h in 12-well plates. The resulting extracts were centrifuged at 21,952 × *g* for 15 min. 96-Well plates (Sarstedt, Nürmbrecht, Germany) were seeded with 1×10^4 L-929 cells/well in 100 μl cell culture medium and incubated under cell culture condition 24 h before the end of the extraction process. Subsequently, the cell culture medium was discarded and 100 μl extract of each sample was equally divided among four wells. Assays were carried out after 24 h incubation with extract under cell culture conditions.

Direct assays were accomplished by directly seeding 5×10^4 fibroblasts onto the surface of the test specimens in 12-well plates. After 24 h incubation, the test specimens were washed in PBS at 37˚C to remove residues and assays were carried out as described below.

XTT assay. Cell Proliferation Kit II (XTT) (Roche Diagnostics, Mannheim, Germany) was used according to the manufacturer's instructions. The included electron-coupling reagent was briefly mixed with XTT labeling reagent (1:50 dilution) and added to the wells. After 4 h, the absorbance was measured by an enzyme-linked immunosorbent assay (ELISA) plate reader with filters for 450 nm and 650 nm (reference wavelength).

Bromodeoxyuridine (BrdU) assay. Cell Proliferation ELISA, BrdU (colorimetric) test kit (Roche Diagnostics) was used according to the manufacturer's instructions. A recommended background check was conducted.

Briefly, the cells were labeled with BrdU for 2 h under cell culture conditions and subsequently fixed for 30 min at room temperature with FixDenat reagent from the kit. Following anti-BrdU-POD incubation at room temperature for 1 h, wells were washed three times with incubation times of 5 min with PBS. Tetramethyl-benzidine substrate was then added and the wells incubated for 20 min at room temperature. The reaction was stopped by the addition of 25 μl 1 M H_2SO_4 to each well. Subsequently, absorbance of wells was determined by an ELISA plate reader with filters for 450 nm and 690 nm (reference wavelength).

Table I. *Overview of the test materials with corresponding electrolytic composition and roughness.*

Material	Surface	Electrolyte composition	Roughness (Sa) in µm
A	Untreated	–	0.19
B	PEO	Phosphate, ethylenediaminetetra-acetic acid, ammonium hydroxide	0.2
C	PEO		2.0
D	PEO		3.99

PEO: Plasma electrolytic oxidation.

Live/dead cell staining. Cell adhesion and morphology can be directly visualized by live/dead staining, visible as green (living) and red (dead) fluorescence of cells.

12-Well plates (Sarstedt) were seeded with 240,000 fibroblasts in 1 ml cell medium and incubated for 24 h under cell culture conditions. Then, 60 µl propidium iodide (PI) stock solution (50 µg/ml in PBS) and 500 µl fresh fluorescein diacetate (FDA) working solution (20 µg/ml in PBS from 5 mg/ml FDA in acetone stock solution) was added to the prepared 12-well plates. After 3 min incubation at room temperature, the specimens were rinsed in Dulbecco's phosphate buffered saline (DPBS, Life Technologies, Carlsbad, USA) at 37˚C and the surface immediately examined with an upright fluorescence microscope (Nikon ECLIPSE Ti-S/L100; Nikon GmbH, Düsseldorf, Germany). Red and green fluorescence was accomplished by use of appropriate filters.

Data evaluation and statistical analysis. All results were transferred to and encoded in Microsoft Excel 2010 (Microsoft, Redmond, Washington, USA) and GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Statistical analysis was performed using SPSS 21 (IBM, New York, NY, USA).

The mean absorbance of blank controls (cell culture medium without cells) was calculated and used as the baseline. After subtracting the baseline, the mean absorbance and standard deviation were calculated from the corresponding replicates for each test material and control. The mean absorbance of test materials was normalized against that of the negative controls.

Differences were analyzed using one-way ANOVA with appropriate *post hoc* Scheffé or Games-Howell procedures. *p*-Values of 0.05 or less were considered statistically significant. Deviations of 30% in viability were considered cytotoxic according to EN ISO 10993-5.

Results

Surface characterization. We manufactured three different rough PEO surfaces on TiAl6V4. Results from SEM/EDX and profilometry are demonstrated in Figure 1.

As expected, peaks of titanium, aluminum and vanadium were visualized on all test samples. Residues of copper were considered to be a result of EDM. The surface roughness of the test and control samples differed from 0.2-4 µm. The porosity of the surfaces increases with higher roughness.

In vitro tests. Regardless of the surface treatment method and roughness, all test materials were covered with large numbers of green FDA-positive vital cells in the live/dead staining assays. Suggesting improved adhesion, the cell morphology on all test samples was predominantly spindle-shaped (Figure 2). No dead cells were observed on the test materials.

Cell viability and proliferation results were on the same level or slightly higher than that of the negative controls in all direct as well as indirect XTT assays (Figure 2). The indirect BrdU assay showed significant differences between the untreated sample and PEO-treated samples. However, the results did not exceed the cytotoxic range.

Discussion

The present study aimed to evaluate the influence of PEO-treated titanium surfaces with different roughness *in vitro* by measuring possible influences on cellular adhesion, proliferation, morphology and overall viability of cells cultured on PEO-treated surfaces and of untreated controls. When applicable, cell-based assays were applied according to DIN ISO 10993-5/-12 regulations.

In a first step, we successfully manufactured titanium test samples with three PEO-treated surfaces of different roughness. Further test results revealed no significant differences in terms of cellular adhesion, proliferation and morphology between the different rough surfaces and the negative controls. However, cells seeded onto PEO-treated surfaces showed better attachment and there were more spindle-shaped cells than on the untreated materials. All indirect cytocompatibility results were within the non-toxic range for the PEO-treated and untreated test samples. We conclude that an increase in surface area and roughness of PEO-treated materials does not automatically improve cellular performance.

The indicated negligible differences in cell behavior between rough and smooth surfaces were also identified by Ramaglia *et al*., who applied an acid etching procedure for surface roughening (12). Various other studies investigated a positive influence of material roughness on cellular adhesion, proliferation and differentiation (3-6, 9-11, 13, 15, 17). Although the results were mostly better for rougher surfaces, no comparisons bewteen different rough surfaces of the same

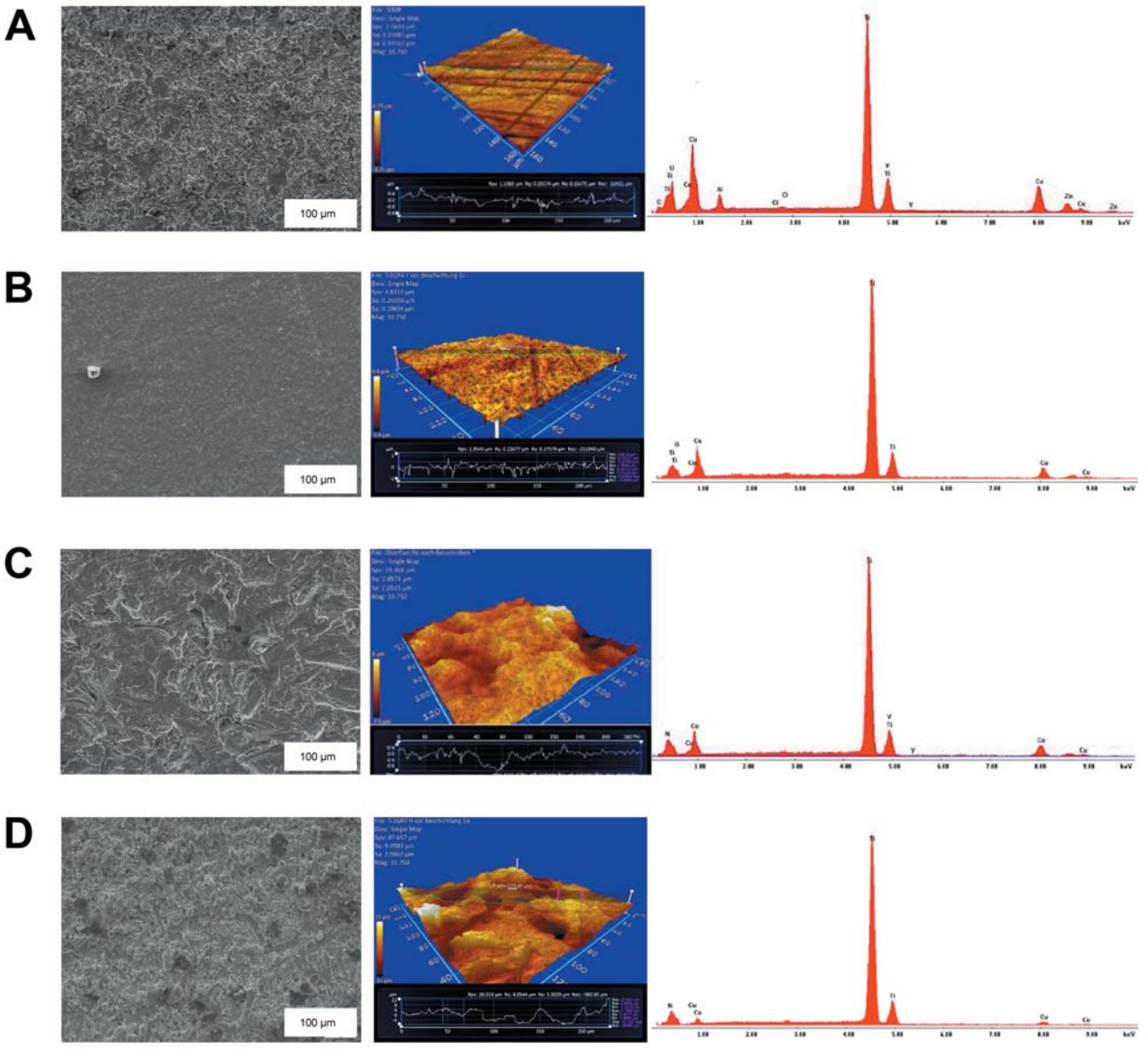

Figure 1. *Physical surface characterization of untreated materials (A) and materials after (B-D; as described in Table I) plasma electrolytic oxidation (PEO) coating. Scanning electron microscopy (left) and energy dispersive X-ray spectroscopy (right) images demonstrate the surface topography and composition. Profilometry (middle) gives an exact value for the achieved roughness.*

origin were made. In our opinion, surface roughness does not automatically result in better cell performance and should be investigated individually for each individual coating technique.

The methods applied in the present study represent only fundamental techniques. Further studies should also investigate cellular differentiation by more specific methods (*e.g.* immunohistochemistry, immunofluorescence). Thereby, an additional osteoblastic cell line should be implemented. Similarly to our study, a suitable control (*e.g.* untreated or uncoated sample) should be always part of a comparative study.

Conclusion

In an effort to manufacture different rough PEO samples for identifying the impact of surface roughness on cellular adhesion, proliferation and morphology, the compared compared plasma electrolytic oxidized surfaces to

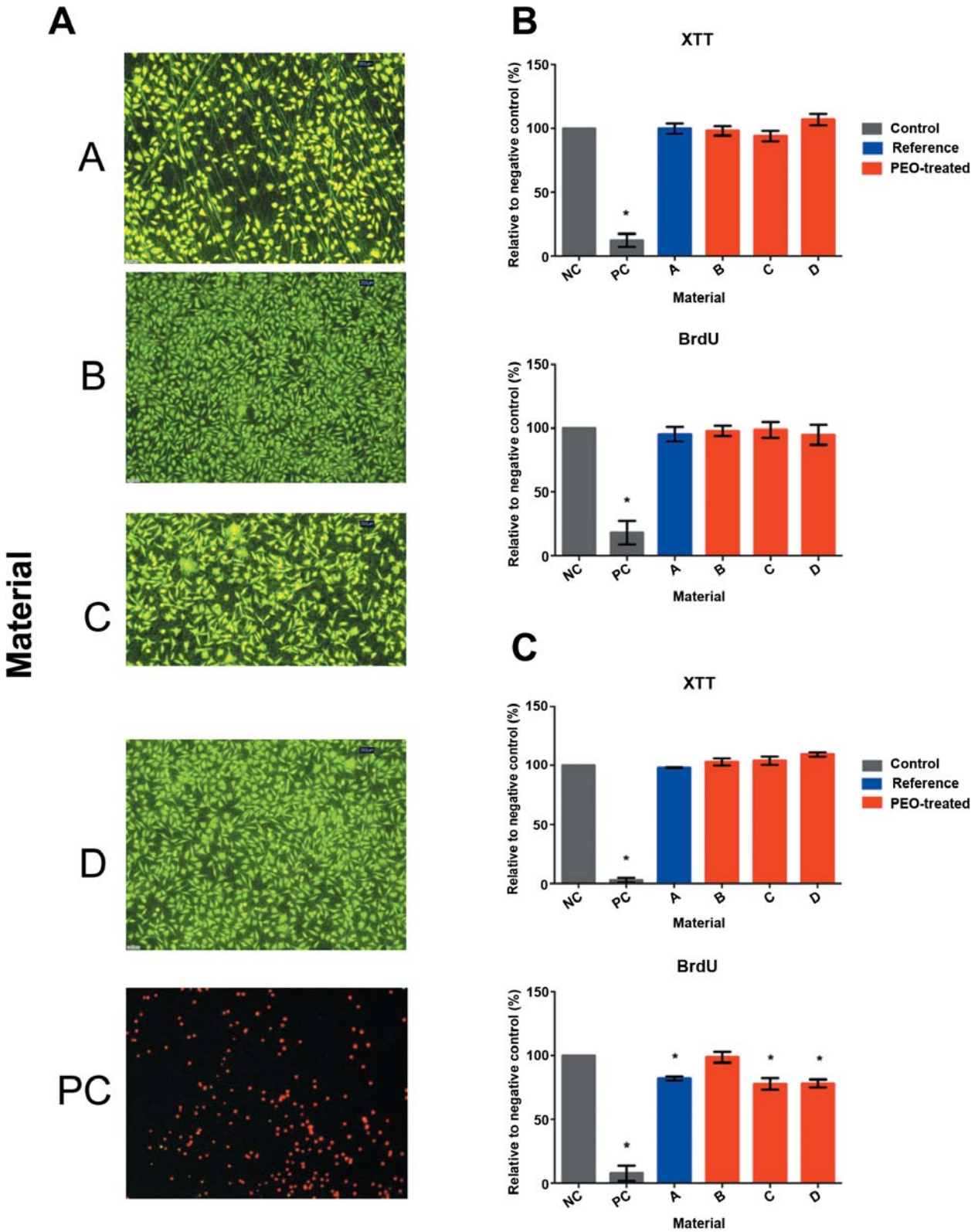

Figure 2. *In vitro results for the test materials A: Live (green)/dead (red) staining images; B: proliferative assay; C: cytocompatibility assay showing relative cell viability. *Significantly different from the negative control samples at p≤0.05. Materials: A: untreated; B-D: after plasma electrolytic oxidation (PEO) coating; PC: positive control.*

conventional smoother grinded titanium samples. Thereby, no differences between the different PEO samples could be detected. However, PEO alone seems to improve cellular attachment. Overall, the PEO coating technique demonstrated attractive features for the further use on biomedical products.

Conflicts of Interest

AK, PH and DP are employed by the Meotec GmbH & Co. KG, a company that is specialized in electrochemical surface treatment of metals. Meotec manufactured the test materials and performed the PEO-coating technology in this study.

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