Golden Standard Technologies:
Inspired by nature. Made by stimOS.

Mimicking Bone Technology
A Golden Standard Technology

Jennifer Knaus[1,2], Liangfei Tian[3], Tamara Schuhmacher[4], Christof Hauck[4], Dietmar Schaffarczyk*[2], Haohan Zou[5], Karina Klein[6], Brigitte von Rechenberg[6], Wolf-Dieter Müller[5], Andreas Schwitalla[5], Helmut Cölfen[1,2]

Comparative Animal Model I:

Bone Mimetic Implant Coating Facilitates Early Osseointegration

CONFIDENTIAL | PRE-RELEASE | CONFIDENTIAL

Author Affiliations:

1 Department of Chemistry, Physical Chemistry, University of Konstanz, Universitätsstraße 10, 78457 Konstanz, Germany
2 stimOS GmbH, Byk-Gulden-Straße 2, 78467 Konstanz, Germany
3 Department of Cemistry, Zhejiang University
4 Department of Biology, Cell Biology, University of Konstanz, Universitätsstraße 10, 78457 Konstanz, Germany
5 Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Dental Materials and Biomaterial Research, Department of Prosthodontics, Geriatric Dentistry and Craniomandibular Disorders, Aßmannshauser Str. 4-6, 14197 Berlin, Germany
6 Vetsuisse Faculty, Musculoskeletal Research Unit, Competence Center for Applied Biotechnology, University of Zurich,

* corresponding author: Dietmar Schaffarczyk – zyk@stimos.net

NF_2020MBT03

Table of content

Comparative Animal Model I 3
Abstract 3
Introduction 4
Material and Methods 5
In vivo: Animal model test results 7
References 11

Comparative Animal Model I

Bone Mimetic Implant Coating Facilitates Early Osseointegration

Jennifer Knaus[1,2], Liangfei Tian[3], Tamara Schuhmacher[4], Christof Hauck[4], Dietmar Schaffarczyk[2], Haohan Zou[5], Karina Klein[6], Brigitte von Rechenberg[6], Wolf-Dieter Müller[5], Andreas Schwitalla[5], Helmut Cölfen[1,2]

Author Affiliations:

1 Department of Chemistry, Physical Chemistry, University of Konstanz, Universitätsstraße 10, 78457 Konstanz, Germany
2 stimOS GmbH, Byk-Gulden-Straße 2, 78467 Konstanz, Germany
3 Department of Cemistry, University
4 Department of Biology, Cell Biology, University of Konstanz, Universitätsstraße 10, 78457 Konstanz, Germany
5 Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Dental Materials and Biomaterial Research, Department of Prosthodontics, Geriatric Dentistry and Craniomandibular Disorders, Aßmannshauser Str. 4-6, 14197 Berlin, Germany
6 Vetsuisse Faculty, Musculoskeletal Research Unit, Competence Center for Applied Biotechnology, University of Zurich, Winterthurerstrasse 204, 8057 Zurich, Switzerland

ABSTRACT

Osseointegration at the bone-implant interface is a crucial factor for the performance of implant materials, and this to improve long-term successes of bone and joint replacements. However, major implant materials like titanium and even more, polyetheretherketone (PEEK) often fail to connect to existing bone tissue before bacteria win the race to the surface, causing inflammation, and other adverse effects, often leading to revision or replacement. In this abstract, we show that surface modification of implant materials with a covalently anchored bone-mimetic coating enables excellent bulk implant properties, combined with the characteristic structure and functional properties of natural bone. The coating was achieved by covalently anchoring a 200-300 nm thick layer of gelatin to the surface of PEEK. This gelatin layer was then mineralised with calcium phosphate to create a bone-like composite as an interface site for proper bone integration. The resulting material displayed impressive increase in cellular adhesion and spreading in different in-vitro assays using NIH-3T3 fibroblast and MC3T3-E1 osteoblast cell lines, which also verified its biocompatibility. Animal studies utilising a sheep-model verified the fast connection of new bone to the implant surface within a 8 weeks timespan after insertion, demonstrating the high impact of our results on future bone-implant design and the vast potential in the clinical application of MBTg-PEEK.

INTRODUCTION

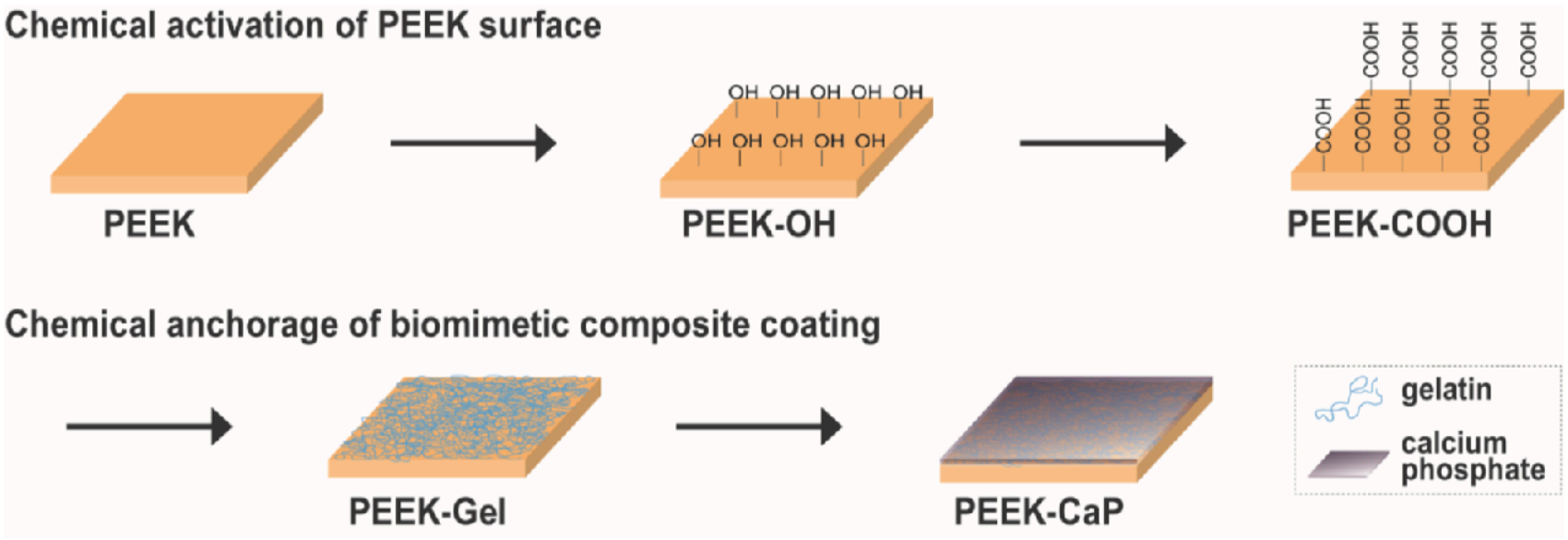
In modern medicine, the use of artificial bone and joint implants is rising and expected to further grow within the next two decades.[1-2] In 2017 over 400,000 artificial bone and joint replacement surgeries were reported within the german endoprosthesis register and range among the 20 most performed operations in clinics. With an ageing global population and the desire for longer, more active lifestyle, the demand for these implants is expected to continue grow. At the same time, and unfortunately, over 45 000 revision surgeries were necessary within the same reported year. [3-4] Nowadays, the general life expectancy of a bone implant is estimated to be around 15 years.[3] The deciding factors for the long term success of an implant are ranging from the implant material and design, the experience of the surgeon, operation technique and -duration, postoperative complications, and up to the health of the patient within the type of postoperative rehabilitation care.[3-4] This development is not only detrimental to the patient's health but also poses a considerable financial burden on the health care system. The high revision rate highlights that the challenge of improving long-term implant stability has not yet been adequately addressed. The major causes of revision interventions are aseptic loosening of implants from the surrounding bone tissue, dislocations and infections following endoprosthetic replacements. [3-10] Within this category, three major causes are discussed to be responsible for aseptic loosening, including excessive wear between articular surfaces and stress shielding of the bone. Another cause is the development of soft tissue at the bone-implant interface due to relative micromotion of the prosthesis due to poor osseointegration during the early healing stages. [9, 11-13]

It shows that not only the bulk properties of the implant materials are critical determinants in the biological performance of medical devices, but also that bone-implant interface plays a crucial role.[14] This interface is essential for the development of new implant materials with improved biocompatibility and osseointegration aiming to enhance the stability of the implant in the host in short time frames and reduce implant failures. Significant advancements have been achieved in the development of new biocompatible materials, by using surface modificating techniques, such as electrospray deposition. [15-17] Although many of these modifications demonstrated improvements in the biological performance of the implant materials, the resulting coatings possess several inherent disadvantageous properties. For example, delamination or diffusion of particles from the surface has been reported for coatings with a non-covalent character after long-term use. More so, such coatings neglect the biomimicry to their biological surrounding in terms of chemical composition and structure, such as titanium coatings, which can corrode and therefore reduce the implant´s biocompatibility.

MATERIAL AND METHODS

Inspired by the complex hierarchical structure of bone, we developed a systematic approach to engineer the interface between the implant surface and the surrounding (hard) bony tissue, by creating a covalently bonded, biomimetic coating. This design enables the combination of the characteristic structural and functional properties of natural mineralised hard tissues with desirable bulk properties of established implant materials by masking the direct surface with a bone-mimetic coating. As proof of concept, we chose the implantable high-performance polymer polyetheretherketone (PEEK) as a first approach, for being considered bioinert and inherently not possessing any bioactive properties. [18] By means of first generation coating, the PEEK-implants were coated with titanium when used as endosseous implant. The surface coating was obtained through chemical activation of a PEEK surface in a first step, on which gelatin was covalently attached as a thin layer (Fig. 1). This 3D gelatin layer was subsequently mineralised to obtain a protein-calcium phosphate nanocomposite.

Fig 1. Schematic representation of the chemical activation and biomimetic coating of the PEEK implant surface



After ensuring the successful mineralisation of MBTg-PEEK aswell as ensuring its durability, it was investigated if the superior surface characteristics indeed mediate enhanced cell attachment and spreading. As a first step, the general cytotoxicity and biocompatibility of the biomimetic surface coating were evaluated by testing two cell lines, a fibroblast (NIH3T3) and an osteoblast (MC3T3-E1) line regarding their growth behaviour, viability and phenotype. Fibroblasts and osteoblasts represent two subsets of cell types, which mediate the first contact between bone and implant during the crucial ingrowth phase.

To assess the general cytotoxicity of the bone implant coating, the materials were evaluated for their indirect and contact toxicity on the cells. The indirect influence was assessed by exposing both, osteoblasts and fibroblasts, to culture medium, which was incubated with MBTg-PEEK and evaluated for their survival with a FACS based life-death staining. The overall apoptotic rate did not differ significantly from the control in both cell lines, implying that no cytotoxic substances or nanoparticles have dissolved from the material. (Extended Data Fig. 9a-b)

The assay showed no significant difference regarding lethality between PEEK and MBTg-PEEK for both, fibro- and osteoblasts. Similar results were observed in a direct toxicity testing, where both cell types were incubated

together with PEEK or MBTg-PEEK coating. (Extended Data Fig. 9c-d). Surprisingly, both cell lines showed a significantly increased metabolic activity of about 1.5 to 3-fold when incubated on MBTg-PEEK compared to unmodified PEEK (Extended Data Fig. 9e-f) This divergence most probably results from elevated proliferation on the mineralised material. Thus, these results attest excellent biocompatibility with no release of cytotoxic components and a compatible surface structure verifying the overall cytotolerance of MBTg-PEEK towards cells relevant in the implant bone-interface.

To determine the proliferation difference in PEEK and MBTg-PEEK, both cell lines were allowed to adhere, propagate and proliferate on the respective coating for one day. After this, the cells were enzymatically removed from the surface and quantified. While the fibroblasts showed a significantly increased cell density when incubated on MBTg-PEEK (Fig. 2a), this tendency could not be seen for osteoblasts despite an apparent increase in their metabolic activity (Extended Data Fig. 9e).

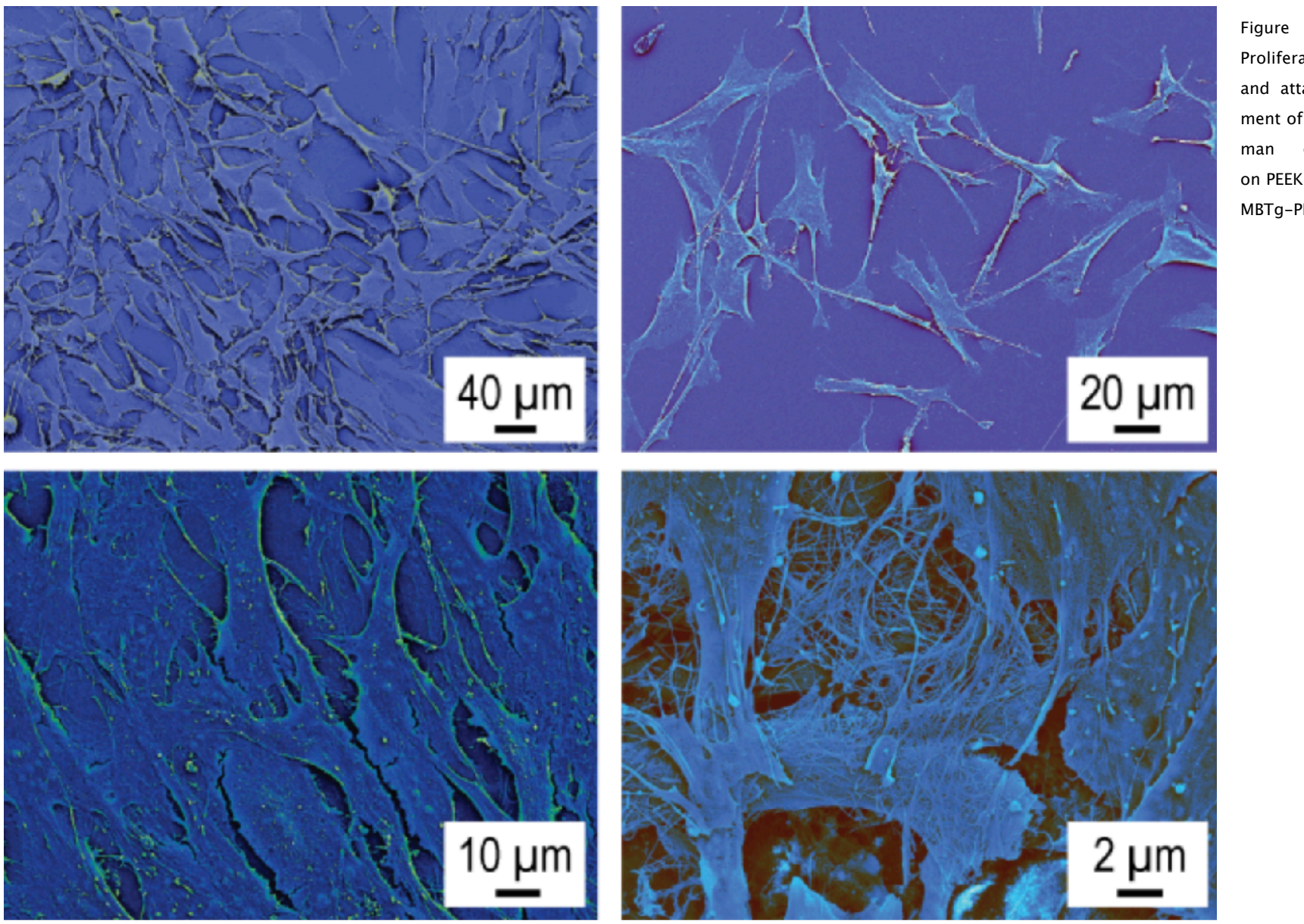


Figure 2. Proliferation and attachment of human cells on PEEK and MBTg-PEEK

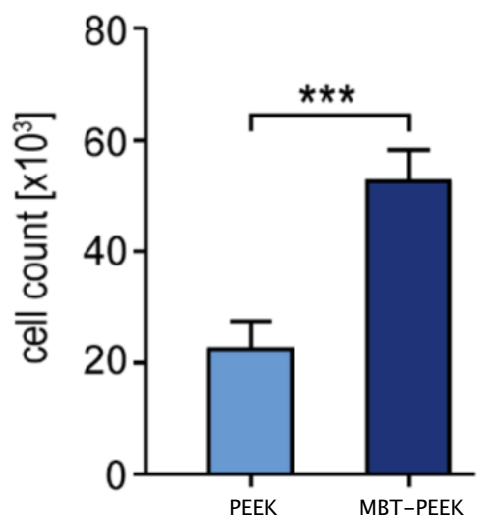


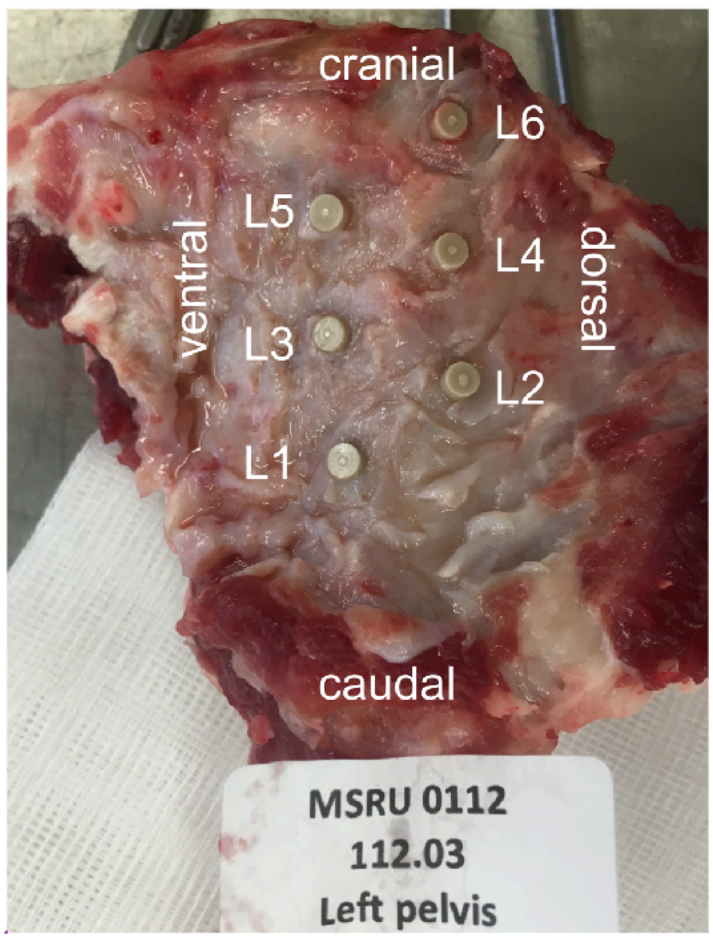
Figure 2a (above) SEM micrographs displaying cell spreading of osteoblasts on PEEK-CaP (top) or PEEK (bottom) respectively after 24 h of cell incubation. Images are displayed in false colours for better visualisation.

Therefore, the distinct adhesion properties of fibroblasts and osteoblasts were investigated. In a first approach, the cells were incubated on the respective material for 6 h. After this time, non-adhered cells were removed, and the number of adherent ones was determined. These tests indicated that fibroblasts have significantly better adhesion to MBTg-PEEK (Fig. 2b / left)

than to the unmodified PEEK. To check if protein adsorption to the MBTg-PEEK have a major influence on fibroblast adherence, this assay was performed in the presence and absence of serum in the culture medium. The results of this paper showed no significant difference in the cell attachment to the surfaces with or without proteins present in the culture medium. This adherence assay test was also performed for the osteoblastic cell line, however, no results could be obtained with this assay setup. However, during the experimental procedure, it became apparent, that osteoblast cells adhere firmly to the MBTg-PEEK surface, and the detachment process through trypsination was incomplete. Therefore in order to further assess the osteoblast adherence, the remaining cells on the implant material were stained with crystal violet after the cell removal through trypsination. After an exposition of the osteoblasts to a surplus of trypsin and elongated incubation periods, it became apparent that osteoblasts remained firmly attached on MBTg-PEEK coating even after the excessive detachment treatment. In contrast, PEEK coating alone did not provide such potent attachment interface.

Further analysis of the spreading and morphology of fibroblasts osteoblast cells by SEM (Fig. 2a) proved our observation from the crystal violet staining experiments. Osteoblasts, which were grown on MBTg-PEEK were flat, densely grown and spread over the whole surface, suggesting that the cells had firmly adhered to the surface. On the pure PEEK-surface were less total surface coverage and less protruded cells implying a deficient attachment behaviour. This diverging attachment quality could be attributed to the hydrophobic surface characteristic of PEEK, which is less prone to support cell spreading when compared to the hydrophilic MBTg-PEEK coating. Remarkably, we found strong extracellular matrix formation after 24 h incubation in the osteoblast culture when incubated on a MBTg-PEEK surface, indicating an early differentiation stage.[19-20] Excretion of extracellular matrix proteins is the first crucial step preceding strong cell adherence and proliferation for complete implant ingrowth. This is the first step to a fast bone formation on the MBTg-PEEK surface.

IN VIVO: ANIMAL MODEL TEST RESULTS



This was indeed confirmed with animal tests using threaded cylindrical PEEK implants (diameter: 3.55 mm, length: 8 mm) with the CaP surface.These implants were inserted in an animal experiment in which the osseointegration of three other PEEK implants with different surface modifications compared to titanium and an HA-filled PEEK was investigated, the results of which will be published elsewhere.In brief, one implant of each group was inserted into each pelvic bone of three sheep, whereas three implants were inserted dorsally and three ventrally to the linea glutea in the right (R) and left (L) pelvis, where position 1 was the most caudal and position 6 the most cranial (Fig.3). After eight weeks, the animals were sacrificed and the samples were histomorphometrically analyzed (Fig. 4).

Figure 3. Macroscopic aspect of the implant sites after sacrifice with visible cover caps of PEEK after 8 weeks in-vivo, showing no signs of infection or implant loosening.

For the MBTg-PEEK implants, we revealed a mean BIC value of 80 ± 16 % within the cortical bone and 74 ± 8 % within the cancellous bone. These values were significantly different from those of the titanium implants (cortical bone: 41 ± 23 %, p=0.036; cancellous bone: 31 ± 12 %, p=0.0001) and of the HA-filled PEEK implants (cortical bone: 50 ± 21 %, p=0.243; cancellous bone: 37 ± 24 %, p=0.001).

Histologically, the new bone (dark blue coloration) could be clearly distinguished from the old bone (lighter blue coloration). Especially in the area of the cortical bone a direct contact between the thread flanks of the implant and the old bone was shown, whereby only the gaps such as in the area of the threads between old bone and implant surface were filled with new bone. This suggests that no significant resorption of the old local bone has taken place in the course of bone remodeling during osseointegration. Accordingly, the implants showed the highest proportion of old cancellous bone (27 ± 5 %) compared to the control implants (titanium: 20 ± 4 %, HA-PEEK: 24 ± 3 %). Only isolated resorption lacunae within the old bone were observed, which occurred primarily in the periphery of the implants. In addition, new peripheral bone formation could be determined around the covering caps of the implants.

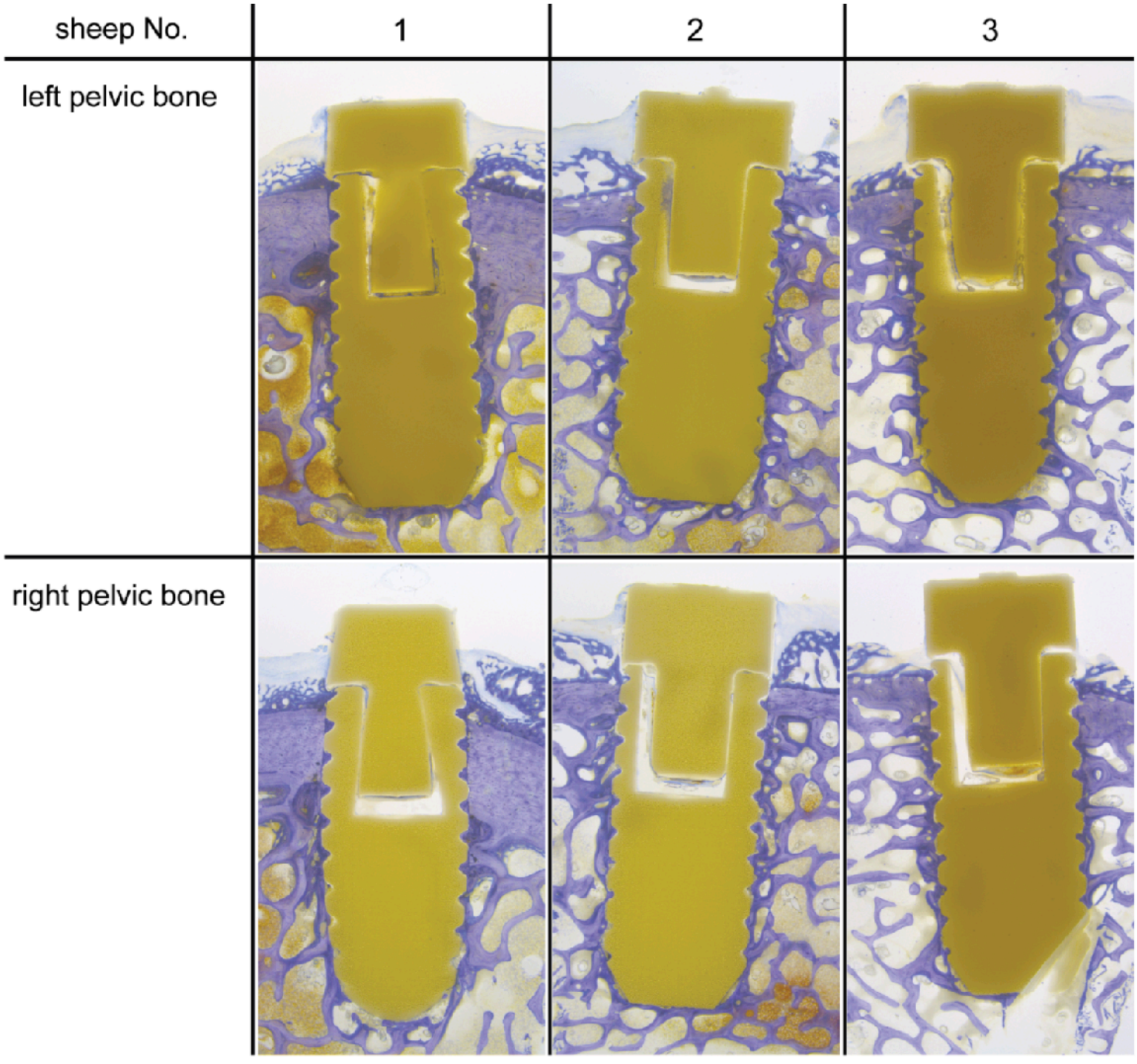


Figure 4. Histological longitudinal sections of the six MBTg-PEEK implants with the bone-implant contact within the cortical bone (BICcor) and the bone-implant contact within the cancellous bone (BICcan) after 8 weeks in-vivo.

To show the bone apposition during the healing process, the animals were injected calcein green after 2 weeks, xylenol orange after 4 weeks and oxytetracyclin after 8 weeks (72 h before sacrifice) subcutaneously. Accordingly, fluorescence microscopy revealed the largest bone apposition after 2 weeks, which occurred mainly in the area of the threads (Fig. 5). The following orange colored areas (fluorescence labelling after 4 weeks with xylenol orange) showed a growth direction of the bone towards the implant surfaces. Only a few blue stained areas were visible directly on the implant surface, which indicated that osseointegration was largely complete after 8 weeks. Only in the area of extraosseous bone formation below the periosteum large blue-colored areas were visible indicating that bone formation was still active in those regions at the time of sacrifice. This was the same in all groups, whereas at the titanium implants blue stained new bone formation also at the bone-implant interface seemed to be more pronounced and accordingly still active after 8 weeks.

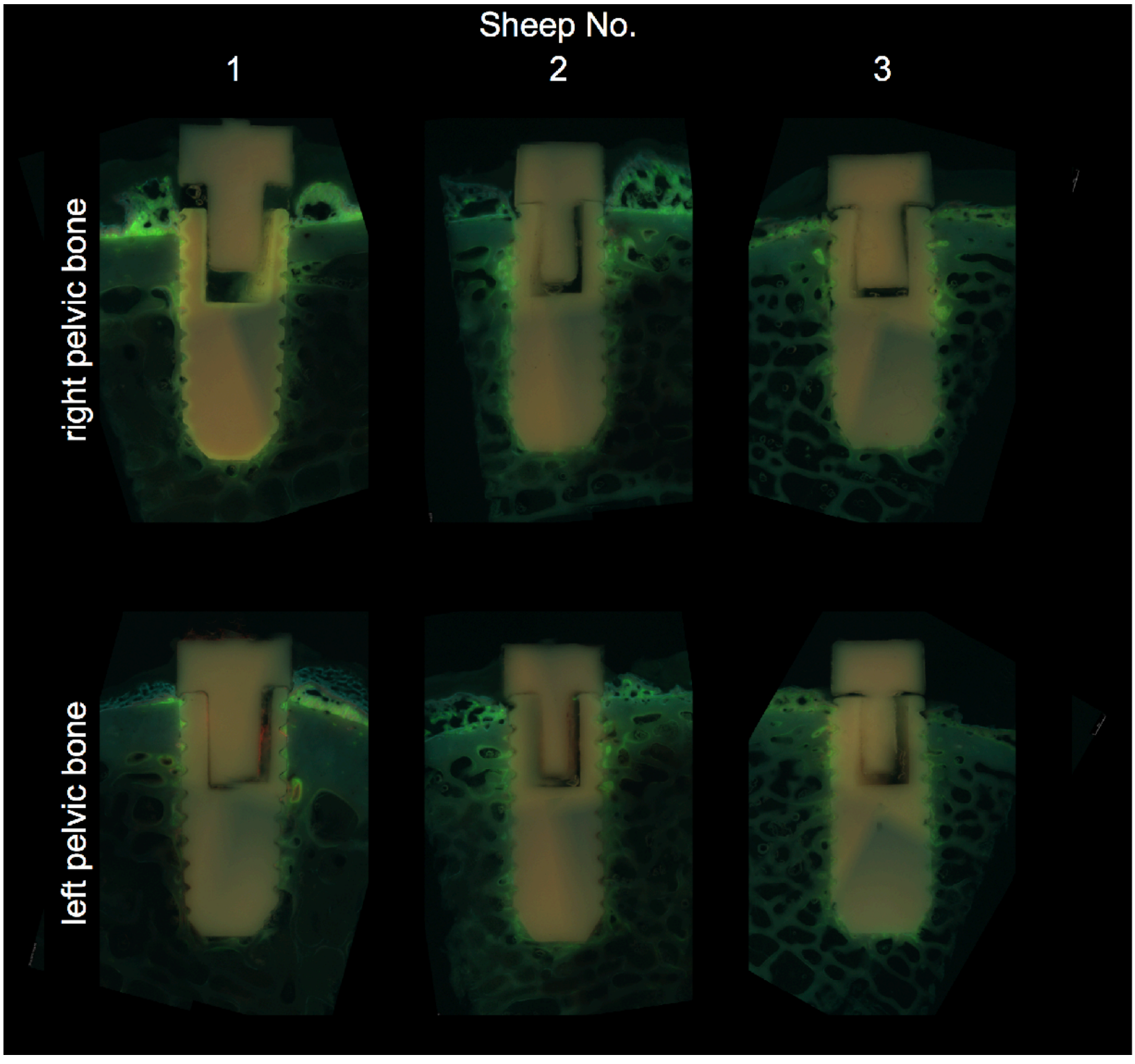


Figure 5. Fluorescence images of the samples like in Fig.4. Due to the calcein green stained areas, the highest rate of bone formation could be observed after 2 weeks. Afterwards bone was formed towards the implant surfaces mainly in the areas of the threads after 4 weeks (xylenol orange) and slightly after 8 weeks (oxytetracyclin (blue)). Due to the oxytetracyclin staining new extraossary bone formation seemed to be still active after 8 weeks.

CONCLUSION

In conclusion, we developed a covalently bonded 3D continuous biomimetic surface layer on an inert implant material using a cost-effective wet-chemical approach. This nano-layer exhibits an excellent combination of surface free energy and mechanical stability, which are the essential criteria for long-term use. Most importantly, MBTg-PEEK showed a high cytotolerance for bone constituting and ingrowth relevant cell lines. When testing for their growth behaviour, these cells display superior attachment, proliferation and differentiation on mineralized PEEK compared to the unmodified structure.

The observed collagen deposition within 24h marking early bone formation shows that this implant functionalization actively promotes bone formation and connection to the existing bone tissue. After a short period of 8 weeks in-vivo, MBTg-PEEK implants showed significantly higher bone implant contact than both, titanium as the gold standard and a HA-filled PEEK material, which is advertised by the industry as being particularly osseointegrative. This successful combination of the desirable mechanical properties of PEEK implants with the bone-mimetic osseointegrative surface structure constitutes a so far unsurpassed implant material and paves the way for the preparation of biomimetic implants for long-term use.

Additionally, the osseointegration of conventional implant materials like titanium can be enhanced using this bioinspired coating.

REFERENCES

[1] Kurtz, S.; Ong, K.; Lau, E.; Mowat, F.; Halpern, M., JBJS 2007, 89 (4), 780-785.

[2] Wolford ML, P. K., Bercovitz A Hospitalization for Total Hip Replacement Among Inpatients Aged 45 and Over: United States, 2000-2010; Hyattsville, MD: National Center for Health Statistics: 2015.

[3] Steinbrück, A., Endoprothesenregister Deutschland (EPRD) – Jahresbericht 2017. 2018.

[4] Weißer, M.; Zerwes, U.; Krupka, S.; Schönfelder, T.; Klein, S.; Bleß, H.-H., Status of Healthcare. In White Paper on Joint Replacement, Springer, Berlin, Heidelberg: 2018; pp 41-89.

[5] Dalury, D. F.; Pomeroy, D. L.; Gorab, R. S.; Adams, M. J., The Journal of Arthroplasty 2013, 28 (8, Supplement), 120-121.

[6] Schroer, W. C.; Berend, K. R.; Lombardi, A. V.; Barnes, C. L.; Bolognesi, M. P.; Berend, M. E.; Ritter, M. A.; Nunley, R. M., The Journal of Arthroplasty 2013, 28 (8, Supplement), 116-119.

[7] Clohisy, J. C.; Calvert, G.; Tull, F.; McDonald, D.; Maloney, W. J., Clinical Orthopaedics and Related Research® 2004, 429, 188-192.

[8] Sakka, S.; Baroudi, K.; Nassani, M. Z., Journal of Investigative and Clinical Dentistry 2012, 3 (4), 258-261.

[9] Sundfeldt, M.; V Carlsson, L.; B Johansson, C.; Thomsen, P.; Gretzer, C., Acta Orthopaedica 2006, 77 (2), 177-197.

[10] Preininger, B.; Haschke, F.; Perka, C., Der Orthopäde 2014, 43 (1), 54-63.

[11] Goodman, S. B., Acta Orthopaedica Scandinavica 1994, 65 (sup258), 1-43.

[12] Szmukler-Moncler, S.; Salama, H.; Reingewirtz, Y.; Dubruille, J. H., Journal of Biomedical Materials Research 1998, 43 (2), 192-203.

[13] Wooley, P. H.; Schwarz, E. M., Gene Therapy 2004, 11 (4), 402-407.

[14] Puleo, D.; Nanci, A., Biomaterials 1999, 20 (23-24), 2311-2321.

[15] A.S.Hoffman, Macromol. Symp. 1996, 101, 443-454.

[16] Goodman, S. B.; Yao, Z.; Keeney, M.; Yang, F., Biomaterials 2013, 34 (13), 3174-3183.

[17] Schliephake, H.; Scharnweber, D.; Dard, M.; Sewing, A.; Aref, A.; Roessler, S., Journal of Biomedical Materials Research Part B: Applied Biomaterials 2005, 73 (1), 88-96.

[18] Kurtz, S. M., PEEK Biomaterials Handbook. Elsevier: 2011.

[19] Owen, T. A.; Aronow, M.; Shalhoub, V.; Barone, L. M.; Wilming, L.; Tassinari, M. S.; Kennedy, M. B.; Pockwinse, S.; Lian, J. B.; Stein, G. S., Journal of cellular physiology 1990, 143 (3), 420-430.

[20] Quarles, L. D.; Yohay, D. A.; Lever, L. W.; Caton, R.; Wenstrup, R. J., Journal of Bone and Mineral Research 1992, 7 (6), 683-692.