J Mater Sci: Mater Med (2011) 22:1015–1026
DOI 10.1007/s10856-011-4287-6

Examination of the inflammatory response following implantation of titanium plates coated with phospholipids in rats

Alexandra Kochanowski · Andreas Hoene · Maciej Patrzyk · Uwe Walschus · Birgit Finke · Bérengère Luthringer · Frank Feyerabend · Regine Willumeit · Silke Lucke · Michael Schlosser

Received: 30 July 2010 / Accepted: 10 March 2011 / Published online: 1 April 2011

Abstract Implantation of biomaterials like titanium (Ti) causes inflammatory reactions possibly affecting implant functionality. Surface modifications could improve biocompatibility and functionality of implants. Biomembrane-derived phospholipids might be useful as implant coating due to their biomimetic properties. In vitro studies demonstrated beneficial effects for 2-oleoyl-1-palmitoyl-sn-glycero-3-phosphoethanolamin (POPE) as coating regarding interactions with cells and bacteria. Therefore, this in vivo study aimed at examining local inflammatory reactions after implantation of POPE-coated Ti plates. Ti implants with POPE attached non-covalently or covalent via octadecylphosphonic acid (OPA), with OPA alone and uncoated controls were simultaneously implanted intramuscularly in rats for 7, 14 and 56 days. The peri-implant tissue was quantitatively analyzed by immunohistochemistry for total macrophages, tissue macrophages, T cells, antigen-presenting cells and proliferating cells. Overall, both POPE-coated series were comparable to the controls. Furthermore, no differences were found between POPE coating on a covalently linked OPA monolayer and POPE coating dried from solution. Together with earlier in vitro results, this demonstrates the potential of phospholipids for implant surface modification.

Paper selected for publication from the 23rd European Conference on Biomaterials, Tampere, Finland, September 2010.

Alexandra Kochanowski and Andreas Hoene have contributed equally to this work.

A. Kochanowski · U. Walschus · S. Lucke · M. Schlosser (✉)
Department of Medical Biochemistry and Molecular Biology, Research Group of Predictive Diagnostics, Ernst Moritz Arndt University of Greifswald, Greifswalder Str. 11c, 17495 Karlsburg, Germany
e-mail: schlosse@uni-greifswald.de

A. Kochanowski · U. Walschus · S. Lucke · M. Schlosser
Institute of Pathophysiology, Ernst Moritz Arndt University of Greifswald, Karlsburg, Germany

A. Hoene · M. Patrzyk
Department of Surgery, Ernst Moritz Arndt University of Greifswald, Greifswald, Germany

B. Finke
Institute for Plasma Science and Technology (INP), Felix-Hausdorff-Straße 2, 17489 Greifswald, Germany

B. Luthringer · F. Feyerabend · R. Willumeit
Helmholtz-Zentrum Geesthacht, Institute for Materials Research, Geesthacht, Germany

1 Introduction

Biomaterials are in wide use for implants in regenerative medicine, for example for vascular grafts, catheters, joint replacements, pacemakers and others. By current concepts, biomaterials comprise 'substances that have been engineered to take a form which, alone or as part of a complex system, are used to direct, by control of interactions with components of living systems, the course of any therapeutic or diagnostic procedure, in human or veterinary medicine' [1]. The increasing life expectancy of older patients and the need for implants even in younger patients lead to a growing demand for more long-lasting implants. It is now well established that every implant is foreign to the host and causes a chronic inflammatory response usually for as long as it remains in the recipient's body. The last stage of these reactions is known as the foreign body reaction (FBR) and specifically characterized by the presence of a cellular layer consisting of macrophages, monocytes and foreign body giant cells which separates the

material surface from the granulation tissue, and later from the fibrous capsule, around the implant [2]. The nature and extent of this reaction depends on the material properties as well as on the intended application and the implantation site. Consequently, the current understanding of the biocompatibility of implant materials has evolved into a more sophisticated concept differing markedly from the early simplified idea of being inert and non-toxic. Rather, the focus of recent definitions of biocompatibility puts the emphasis on the ability of the biomaterial to perform its desired function without undesirable local or systemic effects in the host and with an appropriate beneficial cellular or tissue response in that specific situation [3].

One important feature of an implant that influences the initial cellular reaction at the tissue-material interface as well as the FBR, and thereby determining the integration of the implant into the tissue and its biodegradation, is the nature of its surface. Furthermore, the biocompatibility is not only influenced by the material properties but also by the host's response. Contrary to expectations, the primary reason for defective implantation is not a total failure of the implant but rather wear particles due to biodegradation and physical abrasion which provoke the host's macrophage reaction, leading to disturbed tissue remodelling due to inflammatory reactions [4]. Within this context, biodegradation and physical abrasion processes are mutually interlinked and potentiate each other. Particles from physical abrasion initiate and aggravate biodegradative processes which in turn further damage the implant material and especially its surface, consequently rendering it more prone to the effects of physical forces. Thus, the FBR may impact the biocompatibility of the implant and limit its durability [5]. Following resolution of the acute inflammatory phase, granulation tissue with presence of macrophages, fibroblasts and neovascularisation at the biomaterial interface is the precursor to fibrous capsule formation which interposes between the tissue and the implant thus leading to its mobilization and encapsulation [2]. Therefore, modulation of initial plasma protein adsorption and cell adhesion could reduce undesirable reactions regarding the biomaterial-mediated inflammatory response, thereby improving the biocompatibility and biofunctionality of the implant. A number of different surface modifications have been examined and demonstrated beneficial effects regarding the durability of titanium alloy based implants and the host response after their implantation. These modifications comprise for example highly adherent layers of nano-porous aluminium [6], tribological effects of diamond-like carbon [7], bioactive glass coatings [8] up to natural ceramics such as calcium phosphate [9] and hydroxyapatite [10], and can improve resistance to physical abrasion by providing more mechanical stability as well as to biodegradation by reducing implant-related inflammatory reactions.

Following the injury due to implantation, the cellular host response as directed by molecular interactions determines the extent of inflammation, wound healing and the foreign body response. The underlying molecular processes are affected by the properties of biomolecules such as proteins and lipids which are an integral part of cellular biomembranes which are able to interact with a variety of membrane-associated molecules. Therefore, biomembranes could serve as a model for molecular engineering of biocompatible and bioactive surfaces for implantable biomaterials. Phospholipids as the main component of the cell membrane have previously been shown to have many potential applications in the biomedical field used in self-assembled monolayers [11], as non adhesive coating for materials with blood contact [12, 13] and for diagnostic imaging and drug delivery due to their ability to form liposomes [14]. Properties such as their inherent resistance to biodegradation, to protein adsorption, to adhesion of cells and bacteria as well as to blood coagulation indicate the potential of phospholipids for use in the field of implant surface modification [11–15]. For example, phosphatidylcholine as the most abundant phospholipid in biological membranes has been shown to limit protein adsorption and subsequent cell adhesion in vitro when used as a supported film in the light of its ability to mimic the cell surface [15–21].

The potential of phospholipids for the regulation of cell-material interactions has already been demonstrated in vitro [20, 21]. Among different lipids which were examined, the most promising results regarding the adherence and viability of the osteosarcoma cell line MG-63 were obtained with the phospholipid 2-oleoyl-1-palmitoyl-sn-glycero-3-phosphoethanolamine (POPE) for which a reduction of metabolic activity of macrophages was found. Furthermore, the bacterial growth on a POPE-coated surface was reduced by 51% [21]. As these in vitro results demonstrate the potential of POPE as a coating for medical implants, the present study aimed at examining the local inflammatory reactions to a POPE-based biomimetic surface coating in a longitudinal in vivo study. For this purpose, POPE-coated titanium (Ti) plates were implanted intramuscularly in rats for up to 56 days followed by a morphometric examination of the peri-implant tissue for different inflammatory cell populations. Ti plates coated with POPE attached via covalently bound octadecylphosphonic acid (OPA) were examined in comparison to plates with a non-covalent POPE coating as well as with plates which received the linker substance OPA alone and with uncoated control samples.

2 Materials and methods

2.1 Implants preparation and characterisation

2.1.1 Implants

Mirror-like-polished Ti plates (provided by DOT GmbH, Rostock, Germany) with a size of 5 × 5 × 1 mm were used and four types of implants designated as follows: (1) 2-oleoyl-1-palmitoyl-sn-glycero-3-phosphoethanolamin (POPE; Sigma-Aldrich-Chemie, Taufkirchen, Germany) as a non-covalent assembly of lipid bilayers on titanium plates (Ti–P implants), (2) POPE attached onto titanium samples via covalently bound octadecylphosphonic acid (OPA, Sigma-Aldrich-Chemie, Taufkirchen, Germany) (Ti–O/P implants) and (3) titanium plates surfaced with the linker molecule OPA alone (Ti–O implants). As controls, (4) titanium samples without any coating were implanted (Ti implants).

2.1.2 Implant coating with the phospholipid POPE

The POPE coating for the Ti–P samples was performed as described previously [20, 21]. Briefly, the Ti plates were ultrasonically cleaned in concentrated HELMANX-solution (Hellma GmbH & Co KG, Mülheim, Germany) and intensely rinsed in acetone (Merck, Darmstadt, Germany), double-distilled water (Millipore, Bilerica, Massachusetts, USA) and ethanol (Merck, Darmstadt, Germany). Afterwards the Ti plates were dried in a vacuum oven. For preparing non-covalently bound POPE coatings, the Ti plates were totally covered with a 1 mM POPE solution (chloroform:methanol 80:20% v/v) (Sigma–Aldrich-Chemie, Taufkirchen, Germany) and dried overnight at 40°C in a vaccum oven. This procedure led to the self-assembly of the lipid molecules [22] forming a stack of approximately 100 lipid bilayers.

For the coating of the Ti–O/P implants by formation of a POPE monolayer via the covalent bound linker molecule OPA, the uncoated Ti plates were preheated overnight achieving complete oxidation of the titanium surfaces [23, 24]. Subsequently, the specimens were coated with a solution of OPA dissolved in dry tetrahydrofurane and incubated overnight. Unbound OPA was removed using an ultrasonic bath. The Langmuir–Blodgett technique was then utilized for juxtaposition of the POPE lipid by immersing the substrate into the subphase, leading to the hydrophobic lipid tails facing the OPA coated implant surface (Fig. 1) which is comparable to the multilayer POPE coating regarding the phospholipid orientation. During the immersion process, the pressure in the lipid monolayer was kept stable at 0 mN/N by moving barriers which adjust the available area for the film according to the desired pressure. By monitoring the change in area it is possible to directly calculate the amount of transferred lipid. The POPE-coated Ti plates were stored in a small plastic box at room temperature until implantation.

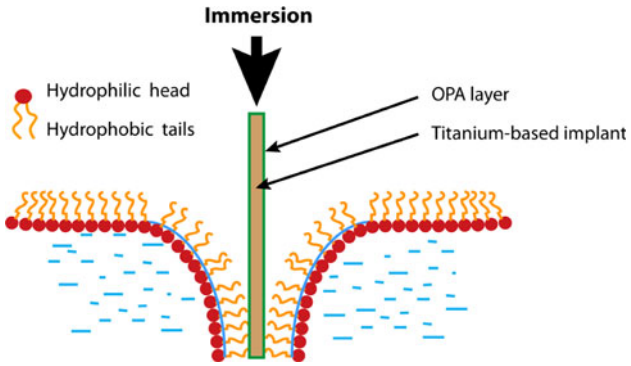

Fig. 1 Schematic representation of Langmuir–Blodgett film deposition. The phospholipids orient themselves with respect to their amphipathic character: their hydrophilic heads "adsorb" at the water/air interface while the hydrophobic tails extend out. A solid substrate (here the Ti–O implant) is dipped through the lipid monolayer resulting in lipid transfer onto the substrate

2.1.3 Implant surface characterisation

X ray photoelectron spectroscopy (XPS) measurements of the lipid-coated Ti surfaces were carried out using an AXIS Ultra DLD electron spectrometer (Kratos Analytical, Manchester, UK) equipped with a monochromatic Al K_α Xray source (1486 eV, 150 W), implemented charge neutralization and pass energy of 80 eV. The measurement conditions were described in detail previously [25]. Each surface composition value represents an average of three XPS measuring steps on the surface. Additionally, the modified Ti surfaces were analysed by contact angle measurements. The general wettability of the modified implant surfaces was measured by the sessile drop technique at 25°C using a goniometer-microscope (Carl Zeiss, Jena, Germany). Contact angles were obtained by calculating the means of ten static advancing and receding angles at four different sites of each sample surface.

2.2 In vitro cytotoxicity testing

Human osteoblasts were obtained from normal biopsy (University Clinic Hamburg-Eppendorf) and isolated following Gallagher's modified protocol [26]. Viability measurements were performed for all materials by application of 8×10^4 cells in 50 µl medium in agarose-coated wells to minimize adhesion to tissue culture plate (TCP). After 30 min to allow initial adhesion, 2 ml of DMEM were added, and the samples were incubated at 37°C in an atmosphere of 5% CO_2 and 100% humidity for 2 days. The

reference in this experiment were the cells seeded on TCP, and the experiments were done in a 3-fold redundant assay.

Following inoculation, viability of osteoblasts was measured with the MTT assay (cell proliferation kit MTT; Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. Briefly, 100 µl of the MTT-solution (5 mg/ml MTT in PBS) was added to cell supernatant. After an incubation period of 5 h, the cells were lysed and the formazan crystals solubilised by adding 1 ml solubilisation solution followed by incubation overnight in a humidified atmosphere (37°C, 5% CO_2). The solubilised formazan product was photometrically quantified using an ELISA reader (Tecan Sunrise, TECAN Deutschland GmbH, Crailsheim, Germany) at 570 nm with a reference wavelength of 655 nm. The results are given as mean of three samples for each implant series.

2.3 Animal experiments

2.3.1 Laboratory animals

Experiments were performed using fifteen male LEW.1A rats (age 100 days; weight 313 ± 14 g, mean ± SD) which were maintained at our animal facility under conventional housing conditions with water and food ad libitum. They were randomly divided into three groups ($n = 5$ per group), and each animal simultaneously received four different Ti implants. The rats were kept under compliance with the animal protection law of the Federal Republic of Germany of 1 January 1987 and the Guidelines for Keeping and Using Laboratory Animals (NIH Publication No.80-23, revised 1985).

2.3.2 Implantation procedure and tissue sampling

Intramuscular implantation of the coated and non-coated Ti plates into the neck musculature was performed under sterile conditions following general anaesthesia by application of Rompun® (Bayer, Leverkusen, Germany) and Ketamin® (Sanofi-Ceva, Duesseldorf, Germany). Four implants, one from each series as described above (Ti–P, Ti–O/P, Ti–O, Ti), were simultaneously placed in a quadrangular formation into small intramuscular pockets of each animal at a distance of at least 2 cm.

For fixation of the intramuscular pockets containing the implants, a nonresorbable synthetic polypropylene suture (PROLENE®, Ethicon Endo-Surgery, Inc., Hamburg, Germany) was used. After 7, 14 and 56 days, respectively, five randomly selected animals were euthanized. After opening the implantation site, the tissue samples containing the implants were carefully dissected and excised. The tissue samples containing the titanium implants were frozen using laboratory freezer spray New Envi-Ro-Tech™ (Thermo Electron Corporation, Pittsburgh, USA). After careful removal of the titanium plates from the frozen tissue utilizing tweezers, the cavity was filled with the embedding medium Shandon Cryomatrix™ (Thermo Electron Corporation, Pittsburgh, USA). Subsequently, the tissue samples were immediately shock frozen in liquid nitrogen and stored at −80°C until further histological analysis.

2.4 Morphological examination

2.4.1 Immunohistochemistry

The immunohistochemical staining was performed using frozen tissue sections with a thickness of 5 µm prepared using a Cryotome 2800 Frigocut N (Reichert-Jung, Nussloch, Germany). Total monocytes and macrophages were stained with the monoclonal anti-CD68 antibody ED1 (MorphoSys AbD Serotec GmbH, Duesseldorf, Germany). Tissue macrophages were detected with the monoclonal anti-CD163 antibody ED2 (MorphoSys AbD Serotec GmbH, Duesseldorf, Germany). Furthermore, T lymphocytes were detected using the monoclonal anti-TCR antibody R73 and MHC class II positive cells were stained using the monoclonal antibody OX6 (both from MorphoSys AbD Serotec GmbH, Duesseldorf, Germany). Cell nuclei undergoing division were detected with the monoclonal anti-Ki-67 antibody MIB-5 (DakoCytomation GmbH, Hamburg, Germany) as a marker for proliferating cells. After evaluation of optimum working dilutions, the staining was performed according to the respective manufacturer's protocols for all antibodies. To detect bound primary antibodies, the alkaline phosphatase anti-alkaline phosphatase method (APAAP; DakoCytomation GmbH, Hamburg, Germany) was used according to the manufacturer's protocol. As control for unspecific bindings of antibodies, tissue samples were treated to the same staining protocol except for omission of the primary antibody and/or the secondary antibody, respectively. Eosin-stained cryosections were counterstained with a nuclear stain (Haematoxylin) following established standard procedure. To avoid overlapping of nuclei staining with MIB-5-positive cells, the counterstaining was omitted for the MIB-5-samples. Exemplary images of the stained tissue samples are given in Fig. 2 for total monocytes and macrophages (ED1; Fig. 2a), tissue macrophages (ED2; Fig. 2b), MHC class II positive cells (OX6; Fig. 2c), and proliferating cells (MIB-5; Fig. 2d).

2.4.2 Image analysis procedure

The equipment for the digital image analysis consisted of a light microscope (Nikon Eclipse E400, Nikon GmbH,

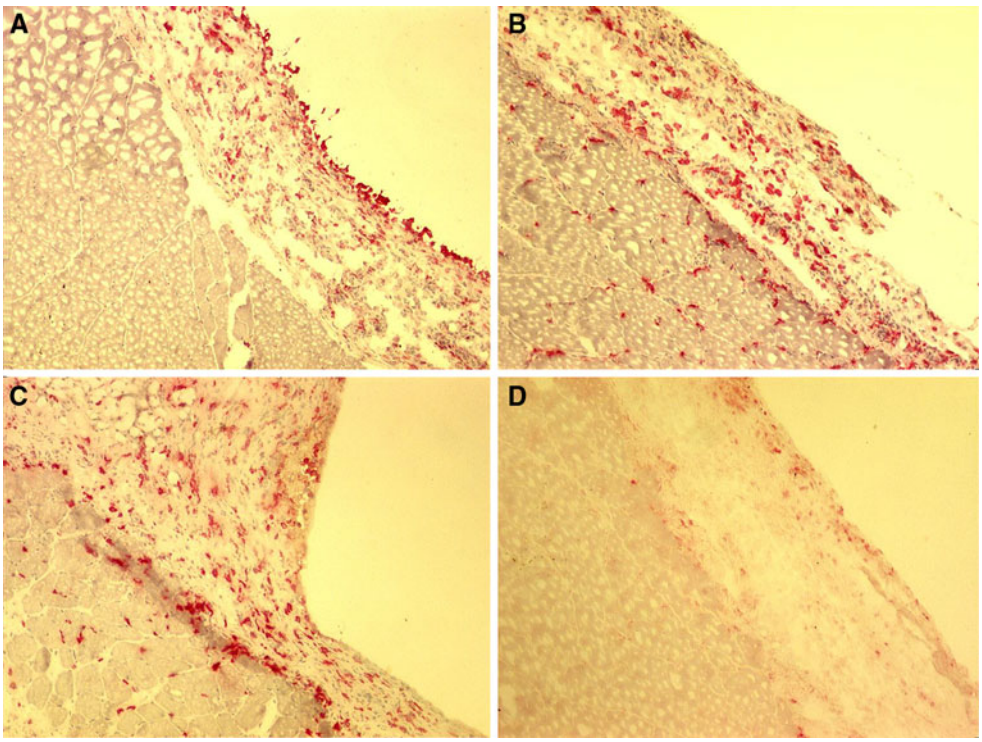

Fig. 2 Four exemplary images illustrating the immunohistochemical staining. **a** ED1 staining (total monocytes and macrophages) for an implant sample with POPE linked via OPA (Ti–O/P) on experimental day 7. **b** ED2 staining (tissue macrophages) for an implant sample with non-covalently linked POPE on experimental day 14. **c** OX6 staining (MHC class II positive antigen-presenting cells) for an implant sample with non-covalently linked POPE on experimental day 7. **d** MIB-5 staining (proliferating cells) for an implant sample with non-covalently linked POPE on experimental day 7

Duesseldorf, Germany), a compact digital colour camera (Hitachi HV-1020M, CCD-chip 768 × 576 pixel, Japan) and a personal computer (Windows XP). Two independent investigators evaluated the stained tissue samples using the image analysis program ImageJ v1.38 (U. S. National Institutes of Health, Bethesda, Maryland, USA) with the plugins Grid and CellCounter. The image analysis procedure was performed as described previously [27]. The results are the mean of the counts obtained by both investigators.

2.5 Statistical data analysis

The cell counts are expressed as positive cells per μm^2. For statistical comparison, the non-parametric Mann–Whitney test was used to compare the results for the contact angle measurement, and the non-parametric Kruskal–Wallis test for the comparison of the osteoblast viability results. The non-parametric paired Friedman-test was used to test for significant differences between the results for the four different implants on each experimental day for five rats. The differences for each implant series on different experimental days were analyzed using the Kruskal–Wallis test. The results were considered significant for p values less than 0.05. Statistical analyses were performed using the software system GraphPad Prism version 4.02 (GraphPad Software, Inc., San Diego, CA, USA).

3 Results

3.1 Implant surface characterisation

3.1.1 XPS analysis

In the results of the XPS analysis (Fig. 3), the C 1s signal indicates that the Ti substrate surface is contaminated by different hydrocarbons, characterized by C–O–, C=O– and ester bonds. The FWHM is at 1.2 eV. For the Ti–O samples, the FWHM (1.0 eV) as well as the C–O- and the C=O-bonds were reduced in comparison to the uncoated Ti samples. The POPE layer caused a broadening of the C–C-bond (FWHM = 1.1 eV) as well as an enhancement of the C–O-, the C=O– and the ester bonds. The phosphorus signal (Fig. 3, right part) demonstrated that the uncoated Ti sample surface did not contain any phosphorous traces. The Ti–O and the Ti–O/P samples exhibited the characteristic phosphorous spectra P 2p3/2 at 133 eV

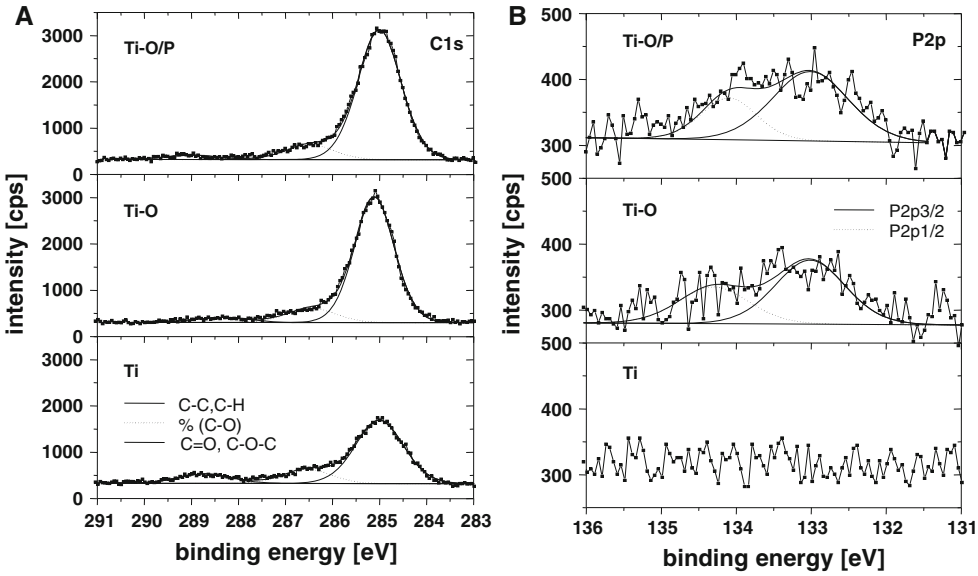

Fig. 3 Surface analysis by XPS C 1s (**a**) and P 2p (**b**) high resolution spectra for titanium plates coated covalently with POPE via OPA linking (Ti–O/P), with the linker substance OPA alone (Ti–O) as well as for uncoated titanium plates used as controls (Ti)

and P 2p1/2 at about 134 eV with a distance of about 1 eV between the bands. The ratio of the intensities P 2p3/2/P 2p1/2 was nearly 2:1.

From these data and from the spectra for Ti and O, the amount of the corresponding elements in the surface layer of the samples was calculated. As seen in Fig. 4, in comparison to the Ti sample the decrease of the Ti and O ratio concomitant with a C and P ratio increase indicate a successful coating for the Ti–O and the Ti–O/P samples. Furthermore, XPS analysis confirmed the finding from the contact angle measurement that the POPE lipid monolayer on the surface of the Ti–O/P samples is not completely homogenous. As the penetration depth of this analytical method is only a few nm, an examination of the thick multilamellar POPE coating on the Ti–P samples would not provide meaningful results as the signal from the metal surface would not be detectable.

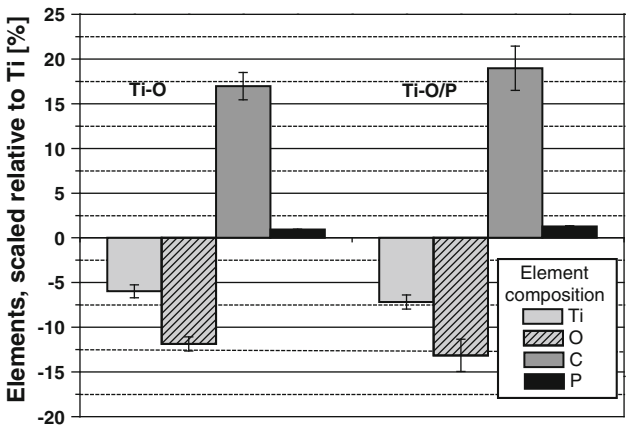

Fig. 4 Evaluation of the elemental composition of the Ti–O and Ti–O/P treated surfaces compared to the non-treated surface, by XPS experiments (percentage of elements scaled relative to pure titanium based implant). With the apposition of the POPE monolayer on the OPA-pretreated surface, a decrease of Ti/O ratio concomitant with a C/P ratio increase is observed, highlighting an effective coating of the titanium surface

3.1.2 Wettability

The Ti samples exhibited a contact angle of 62.6° which was significantly lower ($P < 0.0001$) than for the Ti–O implants which had a contact angle of 102.6° due to the hydrophobicity from the OPA alkyl chains (Table 1). In comparison, the Ti–P implants had a contact angle of 34.6°, significantly lower ($P < 0.0001$) than both the Ti and the Ti–O samples, resulting from the surface hydrophilicity produced by the phospholipid head groups. In contrast, the contact angle measured for the Ti–O/P samples was 95.9°, significantly higher ($P < 0.0001$) than for the Ti and the Ti–P samples and comparable with the Ti–O samples, indicating an incomplete coverage of the OPA layer with the POPE lipid.

3.2 In vitro cytotoxicity testing

Cell viability measurements demonstrated that all four sample series were well tolerated by osteoblasts as no pronounced differences were observed for the cell viability (Table 1). After 2 days, cells on Ti surface exhibit a viability of 79.6% relative to the reference culture which was grown on TCP. Likewise, Ti–O (82.6%), Ti–P (72.8%) and Ti–O/P (79.1%) modified surfaces had no distinct effect on cell viability as the four implant series did not differ significantly from each other ($P = 0.1570$).

Table 1 Wettability, determined by contact angle measurement, and viability of surface-grown osteoblasts, given relative to cells grown on tissue culture plates as reference, for titanium plates coated non-covalently with POPE (Ti–P), covalently with POPE via OPA linking (Ti–O/P), with the linker substance OPA alone (Ti–O) as well as for uncoated titanium plates used as controls (Ti)

	Ti–O	Ti–P	Ti–O/P	Ti
Contact angle (°)	102.6 ± 1.5	34.7 ± 2.7	96.0 ± 1.6	62.0 ± 4.4
Osteoblast viability (%)	82.6 ± 3.1	72.8 ± 1.4	79.1 ± 11.7	79.6 ± 0.5

Data are given as mean ± standard deviation

3.3 Morphological examination

3.3.1 Total monocytes/macrophages (ED1)

For the number of total monocytes and macrophages, a high individual variability in the early phase on experimental day 7 was found (Fig. 5). Comparing the four implant types, both POPE coatings (Ti–O/P; Ti–P) were comparable with the uncoated titanium sample on day 56. There were no significant differences between the implants on any experimental day. The Ti–P implants caused the most consistent decline of monocytes and macrophages from day 7 to day 56 in the surrounding tissue which narrowly failed to be significant ($P = 0.0549$). None of the other implants demonstrated a significant change between day 7 and day 56. However, a general decrease in the number of ED1 positive macrophages was observed for all implants.

3.3.2 Tissue macrophages (ED2)

For the tissue macrophages, no significant differences between the four implant types were found on any day (Fig. 6). The number of ED2 positive cells declined significantly for the Ti–P implants from day 7 to day 56 ($P = 0.0464$). In comparison to the uncoated titanium plates, both POPE-coated implants (Ti–P; Ti–O/P) demonstrated comparable long-term effects on surrounding tissue macrophages. The Ti–O and Ti–P implants demonstrated a general decrease for the tissue macrophages over the course of the study similar to the total macrophages, while a slight but non-significant increasing trend was found for the Ti–O/P and Ti implants.

3.3.3 Total T cells (R73)

The number of T lymphocytes was markedly lower than the number of ED1 and ED2 positive cells (Fig. 7) and

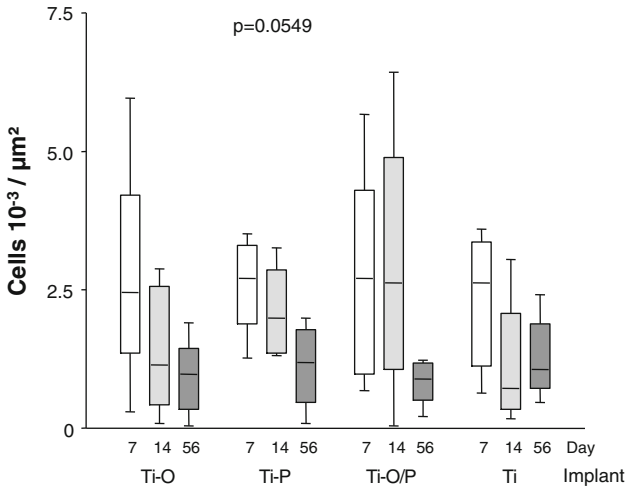

Fig. 5 Time course for the number of total monocytes and macrophages (ED1) in the peri-implant tissue of titanium plates coated non-covalently with POPE (Ti–P), covalently with POPE via OPA linking (Ti–O/P), with the linker substance OPA alone (Ti–O) as well as for uncoated titanium plates used as controls (Ti) after 7 days (*white boxes*), 14 days (*light grey boxes*) and 56 days (*dark grey boxes*) post implantation. Boxes represent the median and interquartile range for five individual animals, the whiskers indicate the lowest and highest individual values. The *P*-value given refers to the decrease from day 7–56 (Kruskal–Wallis non-parametric ANOVA)

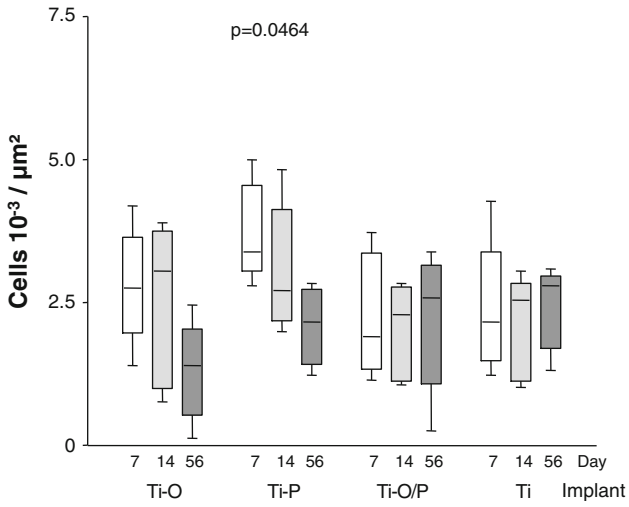

Fig. 6 Time course for the number of tissue macrophages (ED2) in the peri-implant tissue of titanium plates coated non-covalently with POPE (Ti–P), covalently with POPE via OPA linking (Ti–O/P), with the linker substance OPA alone (Ti–O) as well as for uncoated titanium plates used as controls (Ti) after 7 days (*white boxes*), 14 days (*light grey boxes*) and 56 days (*dark grey boxes*) post implantation. Boxes represent the median and interquartile range for five individual animals, the whiskers indicate the lowest and highest individual values. The *P*-value given refers to the decrease from day 7 to 56 (Kruskal–Wallis non-parametric ANOVA)

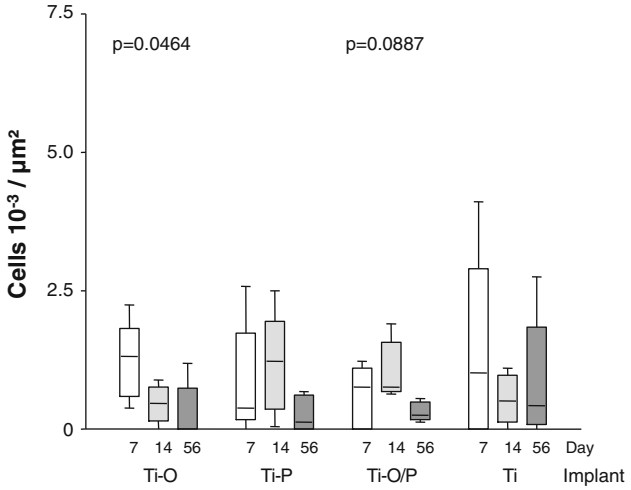

Fig. 7 Time course for the number of T lymphocytes (R73) in the peri-implant tissue of titanium plates coated non-covalently with POPE (Ti–P), covalently with POPE via OPA linking (Ti–O/P), with the linker substance OPA alone (Ti–O) as well as for uncoated titanium plates used as controls (Ti) after 7 days (*white boxes*), 14 days (*light grey boxes*) and 56 days (*dark grey boxes*) post implantation. Boxes represent the median and interquartile range for five individual animals, the whiskers indicate the lowest and highest individual values. The *P*-values given refer to the decrease from day 7 to 56 (Kruskal–Wallis non-parametric ANOVA)

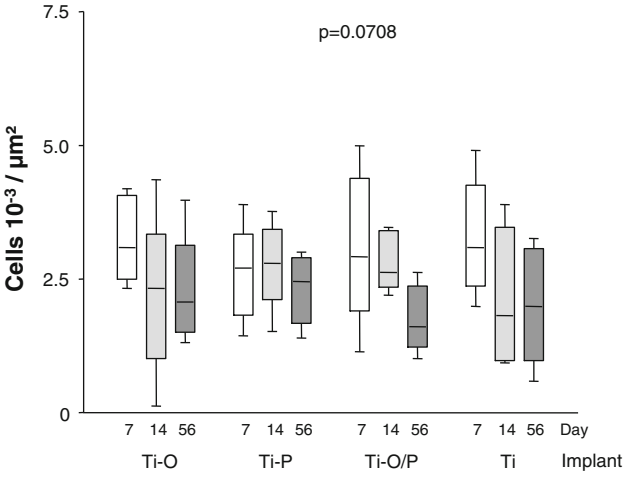

Fig. 8 Time course for the number of MHC class II positive antigen-presenting cells (OX6) in the peri-implant tissue of titanium plates coated non-covalently with POPE (Ti–P), covalently with POPE via OPA linking (Ti–O/P), with the linker substance OPA alone (Ti–O) as well as for uncoated titanium plates used as controls (Ti) after 7 days (*white boxes*), 14 days (*light grey boxes*) and 56 days (*dark grey boxes*) post implantation. Boxes represent the median and interquartile range for five individual animals, the whiskers indicate the lowest and highest individual values. The *P*-value given refers to the decrease from day 7 to 56 (Kruskal–Wallis non-parametric ANOVA)

generally declined for all implants over the course of the study. This decline between day 7 and day 56 was significant for the Ti–O implants ($P = 0.0464$) and narrowly failed to be significant ($P = 0.0887$) for the Ti–O/P implants. No significant differences between covalently (Ti–O/P) and non-covalently (Ti–P) attached POPE were found throughout the study course. Furthermore, no significant differences between the implant types were detected on any experimental day. Especially on day 56, the T cell response for all three coated implant series (Ti–O, Ti–P, Ti–O/P) was comparable to the controls (uncoated titanium plates). Furthermore, the statistical dispersion on day 56 was generally lower than on days 7 and 14, indicating a lower level of individual variability in the late phase.

3.3.4 MHC class II positive cells (OX6)

No significant differences between the four implant series were found for the number of MHC class II positive cells on any day (Fig. 8). The number of OX6 positive cells showed a general decline from day 7 to day 56 in all groups which narrowly missed statistical significance ($P = 0.0708$) for the implants with the covalently linked POPE (Ti–O/P).

3.3.5 Proliferating cells (MIB5)

Compared to the other four cell types investigated, the number of proliferating cells was very low for all implant series on all days (Fig. 9). On no experimental day significant differences between the implants were observed. Similarly, no significant differences between the experimental days were found although a trend for a decrease from day 7 to day 56 was observed for all implants. A noteworthy observation was the high variability of the number of proliferating cells for the Ti–O implants on day 7. Similar to the total macrophages and the T cells, the individual variability was lower on day 56 compared to day 7 and 14. For the Ti–O samples, the statistical dispersion on days 7 and 56 was markedly higher compared to the other three implant series, indicating an increased proliferation rate in the peri-implant tissue of the Ti–O implants for some individual animals. On days 7 and 14, both POPE-coated implant series (Ti–P and Ti–O/P) had a higher median than the controls for which only some individual animals had a detectable number of proliferating cells on these days.

4 Discussion

The implantation of a medical implant causes an inflammatory response in the peri-implant tissue with its last stage called the Foreign Body Response (FBR) which, with the intensity depending on the material properties, decreases over time even for non-biodegradable materials but usually persists for as long as the implant remains in the

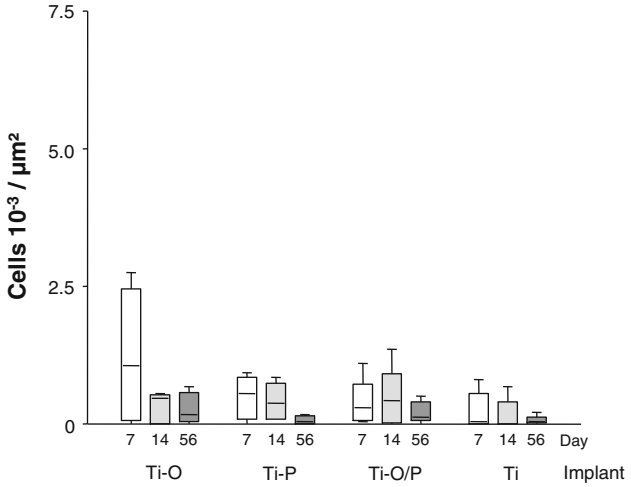

Fig. 9 Time course for the number of proliferating cells (MIB5) in the peri-implant tissue of titanium plates coated non-covalently with POPE (Ti–P), covalently with POPE via OPA linking (Ti–O/P), with the linker substance OPA alone (Ti–O) as well as for uncoated titanium plates used as controls (Ti) after 7 days (*white boxes*), 14 days (*light grey boxes*) and 56 days (*dark grey boxes*) post implantation. Boxes represent the median and interquartile range for five individual animals, the whiskers indicate the lowest and highest individual values

body [2, 28]. These inflammatory reactions include the accumulation of macrophages, and their fusion into foreign body giant cells, as well as T and B lymphocytes, and lead to formation of a fibrous capsule as well as immobilization and potential biodegradation of the implant. Furthermore, complement activation [29, 30], the expression of adhesion receptors and their products, phagocytic cell activity, release of cytokines [31, 32], hypersensitivity [33] as well as basophilic leukocyte and mast cell reactions [34] are part of the host response causing biodegradative processes and chronic inflammation [32]. Therefore, the inflammatory response of the body is a major reason for bioincompatibility and possible implant failure [2, 28]. Accordingly, the examination of the body's response to implanted materials is necessary to improve the biocompatibility and stability of implants. Among the complex individual short- and long-term reactions mentioned above, phagocytic cells and particularly activated macrophages play a central role in their ability to phagocytize a foreign material and to subsequently cause attraction of neutrophils, monocytes, T- and B-lymphocytes by secretion of cytokines [28, 35].

For the interactions between cells as well as between cells and the surface of biomaterials, the phospholipid bilayer of the cell membrane plays an important role by influencing cellular reactions, protein interactions and the distribution and function of membrane proteins. Furthermore, it has recently been demonstrated that an anionic phospholipid like phosphatidylserine can induce fast mineralisation and integration of titanium implants [36, 37], demonstrating in principle the suitability of phospholipids as implant coatings. However, the development of a phospholipid-coated implant is complicated by the huge diversity of phospholipids in membranes varying between different species or even within the same organism [20]. Furthermore, the exact biological role of the different phospholipids has not been conclusively established so far. Consequently, only few in vivo studies regarding the examination of phospholipids-based coatings and the respective FBR have been performed up to now [38, 39].

In the current study, particular emphasis was placed on the morphometric examination of the short- and long-term local inflammatory reactions following intramuscular implantation of titanium plates coated non-covalently and covalently via the linker compound octadecylphosphonic acid (OPA) with the phospholipid 2-oleoyl-1-palmitoyl-sn-glycero-3-phosphoethanolamin (POPE), one of the major components in the membrane of eukaryotic cells. Based on experiences from previous studies in which we used varying time points [27, 40], day 7 after implantation was chosen for examination of the acute inflammatory phase, day 14 for the transition from the acute to the chronic stage and day 56 being representative for the chronic inflammatory phase.

Similar to the examination of other implant coatings, a physico-chemical characterization of the modified material surface is crucial. This is however not trivial for lipid-based coatings as lipids are liquid crystalline structures. Thus, many of the methods used for surface characterisation in other studies, for example scratch or peeling tests, cannot be used. Additionally, the single POPE monolayer attached to the OPA layer on the Ti–O/P samples has a film thickness of only 4–5 nm, requiring sophisticated techniques for detection and analysis of these layers. However, POPE layers coated on mirror polished titanium with or without pre-incubation in water were already characterised by scanning electron microscopy (SEM), element analysis by energy dispersive X-ray analysis (EDX) and atomic-force microscopy (AFM) [41, 42]. These studies indicate that the multilamellar POPE-coating results in a complete coverage of the surface with various film thickness and morphology. Furthermore, the existence of single POPE bilayers was demonstrated by AFM [43].

In comparison, the detection of the asymmetric bilayer consisting of the covalently bound OPA with the POPE monolayer is significantly more difficult. Direct visualisation of the layers by AFM or thickness studies by neutron and X-ray reflectometry are the focus of ongoing work. However, the combination of contact angle measurement, XPS analysis, and in vitro cytotoxicity testing on human osteoblasts as presented here principally demonstrates the suitability as implant coating.

The examination of the lipid coating after implantation would be interesting as well but would require the use of radioactive labelled lipids which is a complex technique and beyond the scope of the study. With other methods, it would not be possible to distinguish between lipids from the coatings and lipids which are physiologically present in the host body and to analyze the fate of the coated lipids to see whether they are removed or replaced by native lipids. As such remodelling of a biomimetic surface by the organism would be desirable, this possibly beneficial aspect will be addressed in future studies.

Previous in vitro studies demonstrated beneficial effects of POPE coatings, which has the capacity to enable membrane fusion and cell division, regarding cell growth and adherence as well as TNFα release [21]. In these studies, a coating with POPE did not influence the metabolic activity of macrophages in vitro and resulted in a reduction of bacterial growth by 51% and an increase of the viability of adherent cells by 116% in comparison to uncoated titanium samples, indicating its potential for in vivo applications [21]. This is confirmed by the results from in vitro experiments in the present study regarding the viability of osteoblast cells (Table 1).

Overall as presented in the current study, no significant POPE-induced inflammatory reactions in comparison to the controls were found over the study period. Furthermore, no significant differences were observed in vivo between covalent or non-covalent POPE coating. In principal, the POPE layer on top of the covalently linked OPA might lead to a more stable phospholipid coating, thus avoiding or delaying elutriation of the POPE layer compared to non-covalently linked POPE which is attached only via its hydrophobic and charge effects [21]. This might attenuate a possible increase in cell attachment by the higher stability of the covalent POPE coating, which could explain the observed absence of a significant difference between the reactions against samples with covalently single asymmetric bilayer (Ti–O/P) and multiple symmetric but non-covalently (Ti–P) linked POPE bilayers.

Despite the fact that no significant differences were found between the different implants, there are a number of notable observations regarding the time course. First, only for the implants with the non-covalent POPE coating (Ti–P) the decline for the total macrophages and for the tissue macrophages from day 7 to day 56 was significant. Furthermore, this significant decline for the tissue macrophage response for the Ti–P implants is in contrast to the Ti control implants and the implants with the POPE coating covalently linked via OPA (Ti–O/P). Both these series have a comparable increasing trend until the end of the study which is however not significant. Regarding this, the number of tissue macrophages is slightly higher for the Ti–P implants than for the Ti–O/P implants and the controls on day 7 while it is slightly lower for the Ti–P implants on day 56. Second, the number of T lymphocytes declines already on day 14 for the Ti–O implants and the controls while the POPE-coated implants (Ti–P and Ti–O/P) seem to have a delayed decline as both series have comparable numbers between day 7 and day 14. As it is unlikely that these observations result from the differences in the contact angles of the different implants, they might rather be explained by the fact that the presence of lipid bilayers induces a certain softness of the surface and hence a mechanical signal.

Taken together, these observations indicate on the one hand that POPE as implant coating indeed has a distinct effect on at least some aspects of the inflammatory response. On the other hand the results for the total and tissue macrophage response suggest that the non-covalent coating might be preferable over the covalent coating approach. Further examination of other immune and inflammatory cells could help to address these two points. Also noteworthy is the time course for the number of proliferating cells. On the one hand, a coating with the linker substance OPA alone seems to induce a higher proliferation rate in the peri-implant tissue of some animals compared to the other three implant series. This effect, which could be due to the chemical nature of OPA, is however prevented by an additional POPE layer over the OPA (Ti–O vs. Ti–O/P). On the other hand, in the early study phase on days 7 and 14 both POPE-coated implant series (Ti–P and Ti–O/P) also seem to induce a slightly higher proliferation rate than the controls for which only some animals had a detectable number of proliferating cells on these days.

Further efforts should be undertaken to increase the Langmuir–Blodgett transfer efficiency in order to achieve a real effectiveness of a covalent monolayer POPE coating comparable to the "pure" POPE coating. Moreover, in future studies utilisation of radio-labelled phospholipids should also contribute to demonstrate the value of this monolayer and its fate and behaviour in vivo.

5 Conclusions

In summary, the results of the present study show that the concept of coating an implant material using a biomembrane mimic based on the phospholipid POPE does not have a negative influence on the short- and long-term inflammatory reactions in the peri-implant tissue. Despite the fact that inbred animals of identical age and sex were used, there was however a broad level of individual variability particularly in the early phase especially for the number of total macrophages, T cells and proliferating cells. In the late study phase, the variability of the cell numbers was generally found to be lower. This indicates

that the inflammatory response in the late chronic phase is more uniform than in the early acute phase. Taking into account the data obtained by Willumeit et al. in different in vitro studies [20, 21] which demonstrated beneficial effects on attachment of cells and bacteria, it can be concluded that phospholipid-based coatings are a promising approach to achieve long-lasting integration and biofunctionality of various implants used in clinical applications. In further studies it would be interesting to examine the effect of phospholipid bilayers on the interaction with proteins, such as fibronectin and vitronectin, which influence cells adherence as well as to investigate additional inflammatory and immune cell populations like mast cells, neutrophil granulocytes or natural killer (NK) cells. Additionally, a quantitative evaluation of the degree of fibrosis and connective tissue formation could help to further reveal how phospholipid-based coatings influence the tissue reactions after implantation.

Acknowledgments We would like to thank Kirsten Tornow for excellent technical assistance. In addition we acknowledge the valuable contribution of Dr. Anna Schuster and Jessica Rutz. The study was supported by the Federal State of Mecklenburg-Vorpommern and the Helmholtz Association of German Research Centers (Grant No. VH-MV1).

References

1. Williams DF. On the nature of biomaterials. Biomaterials. 2009;30:5897–909.
2. Anderson JM, Rodriguez A, Chang DT. Foreign body reaction to biomaterials. Semin Immunol. 2008;20:86–100.
3. Williams DF. On the mechanisms of biocompatibility. Biomaterials. 2008;29:2941–53.
4. Zreiqat H, Kumar RK, Markovic B, Zicat B, Howlett CR. Macrophages at the skeletal tissue-device interface of loosened prosthetic devices express bone-related genes and their products. J Biomed Mater Res A. 2003;65:109–17.
5. Allemann F, Mizuno S, Eid K, Yates KE, Zaleske D, Glowacki J. Effects of hyaluronan on engineered articular cartilage extracellular matrix gene expression in 3-dimensional collagen scaffolds. J Biomed Mater Res. 2001;55:13–9.
6. Briggs EP, Walpole AR, Wilshaw PR, Karlsson M, Pålsgård E. Formation of highly adherent nano-porous alumina on Ti-based substrates: a novel bone implant coating. J Mater Sci Mater Med. 2004;15:1021–9.
7. Roy RK, Lee KR. Biomedical applications of diamond-like carbon coatings: a review. J Biomed Mater Res B Appl Biomater. 2007;83:72–84.
8. Moritz N, Vedel E, Ylänen H, Jokinen M, Hupa M, Yli-Urpo A. Characterisation of bioactive glass coatings on titanium substrates produced using a CO_2 laser. J Mater Sci Mater Med. 2004;15:787–94.
9. Chen JS, Juang HY, Hon MH. Calcium phosphate coating on titanium substrate by a modified electrocrystallization process. J Mater Sci Mater Med. 1998;9:297–300.
10. Osborn JF. Biological behavior of the hydroxyapatite ceramic coating on the femur shaft of a titanium endoprosthesis—initial histologic evaluation of a human explant. Biomed Technol (Berl). 1987;32:177–83.
11. Trojanowicz M. Miniaturized biochemical sensing devices based on planar bilayer lipid membranes. Fresenius J Anal Chem. 2001;371:246–60.
12. Ishihara K, Oshida H, Endo Y, Ueda T, Watanabe A, Nakabayashi N. Hemocompatibility of human whole blood on polymers with a phospholipid polar group and its mechanism. J Biomed Mater Res. 1992;26:1543–52.
13. Choi J, Konno T, Matsuno R, Takai M, Ishihara K. Surface immobilization of biocompatible phospholipid polymer multilayered hydrogel on titanium alloy. Colloids Surf B Biointerfaces. 2008;67:216–23.
14. Rudolph AS. Biomaterial biotechnology using self-assembled lipid microstructures. J Cell Biochem. 1994;56:183–7.
15. Murphy EF, Keddie JL, Lu JR, Brewer J, Russell J. The reduced adsorption of lysozyme at the phosphorylcholine incorporated polymer/aqueous solution interface studied by spectroscopic ellipsometry. Biomaterials. 1999;20:1501–11.
16. Andersson AS, Glasmästar K, Sutherland D, Lidberg U, Kasemo B. Cell adhesion on supported lipid bilayers. J Biomed Mater Res A. 2003;64:622–9.
17. Chapman D. Biocompatible surfaces based upon the phospholipid asymmetry of biomembranes. Biochem Soc Trans. 1993;21:258–62.
18. Hayward JA, Chapman D. Biomembrane surfaces as models for polymer design: the potential for haemocompatibility. Biomaterials. 1984;5:135–42.
19. Hayward JA, Johnston DS, Chapman D. Polymeric phospholipids as new biomaterials. Ann N Y Acad Sci. 1985;446:267–81.
20. Willumeit R, Schuster A, Iliev P, Linser S, Feyerabend F. Phospholipids as implant coatings. J Mater Sci Mater Med. 2007;18:367–80.
21. Willumeit R, Schossig M, Clemens H, Feyerabend F. In vitro interactions of human chondrocytes and mesenchymal stem cells, and of mouse macrophages with phospholipid-covered metallic implant materials. Eur Cell Mater. 2007;13:11–25.
22. Willumeit R, Kamusewitz H, Schossig M, Schröder J, Clemens H. Implant surface modification by biological multi-layer systems. Mater Res Soc Symp Proc. 2003;734:B8.6.1–6.
23. Adden N, Gamble LJ, Castner DG, Hoffmann A, Gross G, Menzel H. Phosphonic acid monolayers for binding of bioactive molecules to titanium surfaces. Langmuir. 2006;22:8197–204.
24. Silverman BM, Wieghaus KA, Schwartz J. Comparative properties of siloxane vs phosphonate monolayers on a key titanium alloy. Langmuir. 2005;21:225–8.
25. Finke B, Luethen F, Schroeder K, Mueller PD, Bergemann C, Frant M, Ohl A, Nebe BJ. The effect of positively charged plasma polymerization on initial osteoblastic focal adhesion on titanium surfaces. Biomaterials. 2007;28:4521–34.
26. Gallagher JA. Human osteoblast culture. Methods Mol Med. 2003;80:3–18.
27. Walschus U, Hoene A, Neumann HG, Wilhelm L, Lucke S, Lüthen F, Rychly J, Schlosser M. Morphometric immunohistochemical examination of the inflammatory tissue reaction after implantation of calcium phosphate-coated titanium plates in rats. Acta Biomater. 2009;5:776–84.
28. Tang L, Eaton J. Inflammatory responses to biomaterials. Am J Clin Pathol. 1995;103:466–71.
29. Brett T. The laboratory assessment of biocompatibility: the role of complement activation testing. Med Dev Technol. 1992;3:326–30.
30. Mollnes T. Complement and biocompatibility. Vox Sang. 1998;74:303–7.
31. Ward RA. Phagocytic cell function as an index of biocompatibility. Nephrol Dial Transplant. 1994;9:46–56.
32. Pereira BJ, Dinarello CA. Production of cytokines and inhibitory proteins in patients on dialysis. Nephrol Dial Transplant. 1994;9:60–71.

33. Grammer LC, Patterson R. IgE against ethylene-oxide-altered human serum albumin (ETO-HSA) as an etiologic agent in allergic reactions of hemodialysis patients. Artif Organs. 1987;11:97–9.
34. Tang L, Jennings TA, Eaton JW. Mast cells mediate acute inflammatory responses to implanted biomaterials. Proc Natl Acad Sci USA. 1998;95:8841–6.
35. Anderson JM. Biomaterial biocompatibility and the macrophage. Biomaterials. 1984;5:5–10.
36. Bosetti M, Santin M, Lloyd AW, Denyer SP, Sabbatini M, Cannas M. Cell behaviour on phospholipids-coated surfaces. J Mater Sci Mater Med. 2007;18:611–7.
37. Santin M, Rhys-Williams W, O'Reilly J, Davies MC, Shakesheff K, Love WG, Lloyd AW, Denyer SP. Calcium-binding phospholipids as a coating material for implant osteointegration. J R Soc Interface. 2006;3:277–81.
38. Wilson JT, Cui W, Sun XL, Tucker-Burden C, Weber CJ, Chaikof EL. In vivo biocompatibility and stability of a substrate-supported polymerizable membrane-mimetic film. Biomaterials. 2007;28:609–17.
39. De Souza R, Zahedi P, Allen CJ, Piquette-Miller M. Biocompatibility of injectable chitosan-phospholipid implant systems. Biomaterials. 2009;30:3818–24.
40. Hoene A, Walschus U, Patrzyk M, Finke B, Lucke S, Nebe B, Schröder K, Ohl A, Schlosser M. In vivo investigation of the inflammatory response against allylamine plasma coated titanium implants in a rat model. Acta Biomater. 2010;6:676–83.
41. Willumeit R, Feyerabend F, Kamusewitz H, Schossig M, Clemens H. Biological multi-layer systems as implant surface modification. Matwiss Werkst. 2003;34:1084–93.
42. Pressl D, Teichert C, Hlawacek G, Clemens H, Iliev PP, Schuster A, Feyerabend F, Willumeit R. Characterization of phospholipid bilayers on Ti-6Al-4V and Ti-6Al-7Nb. Adv Eng Mater. 2008;10:B47–52.
43. Pressl D. Surface modification of titanium alloys by phosphlipid membrane systems. Diploma Thesis, Institute of Physics, University of Leoben, 2004.