

Gas-Discharge Plasma-Assisted Functionalization of Titanium Implant Surfaces

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Abstract. A crucial factor for in-growth of metallic implants in the bone stock is the rapid cellular acceptance whilst prevention of bacterial adhesion on the surface. Such contradictorily adhesion events could be triggered by surface properties. There already exists fundamental knowledge about the influence of physicochemical surface properties like roughness, titanium dioxide modifications, cleanness, and (mainly ceramic) coatings on cell and microbial behavior *in vitro* and *in vivo*.

The titanium surface can be equipped with antimicrobial properties by plasma-based copper implantation, which allows the release and generation of small concentrations of copper ions during contact with water-based biological liquids.

Additionally, the titanium surface was equipped with amino groups by the deposition of an ultrathin plasma polymer. This coating on the one hand does not significantly reduce the generation of copper ions, and on the other hand improves the adhesion and spreading of osteoblast cells.

The process development was accompanied by physicochemical surface analyses like XPS, FTIR, contact angle, SEM, and AFM. Very thin modified layers were created, which are resistant to hydrolysis and delamination. These titanium surface functionalizations were found to have either an antimicrobial activity or cell-adhesive properties. Intramuscular implantation of titanium samples coated with the cell-adhesive plasma polymer in rats revealed a reduced inflammation reaction compared to uncoated titanium.

Introduction

Gas discharge plasma processes are well-known for their high potential to optimize surface properties regarding specific applications, including biomedical ones [1-6]. Such application include bone contacting implants, which can be equipped with rough surfaces and hydroxylapatite coating to improve healing [7]. But, these surface modifications do not meet all needs of hip, knee, and dental implants made from titanium (Ti). There is no rapid cellular acceptance of the Ti or hydroxylapatite implant surface. However, osteoblast cell adhesion is a crucial factor for the ingrowth in the bone. This is especially valid for titanium implants which have to bear considerable mechanical strain. Thus, the physico-chemical properties of the implant surface like roughness, purity or a thin coating, affect the cellular response *in vitro* and *in vivo*. Additionally, implant surfaces should prevent bacterial adhesion and infection. Of course, a surface which exhibit similarities to the extracellular matrix (ECM) accelerates the initial osteoblast adhesion and this way keeps bacteria away from the implant surface. Typically, immobilized proteins and peptides are used for the improvement of the interface. Cell fixation to surfaces is basically mediated via integrins to an extracellular matrix and has an important influence on many cell functions. But actually, the matrix substance hyaluronan [8, 9] seems to take a key role in initial interface reactions. Thus, a surface functionalization process was developed based on a positively charged implant surface to interact with the negative charge of hyaluronan which is due to the carboxyl group of the glucuronic acid [10, 11]. An amino groups carrying surface would present the desired properties, because these groups are protonated in aqueous solutions at physiological pH values and therefore generate positive charges.

In addition, hip and knee implants have to be replaced as a result of microbial infections sometimes. Bacterial colonization has to be avoided at the implant surface in every case. Systemic antibiotics therapy as well as implant coating with antibiotics is disputed. Thus, silver or copper-based coatings could be advantageous. Copper is known to be toxic against prokaryotic bacteria, fungi, viruses, and algae, while eukaryotic tissues, especially skin tissues [12] are reported to withstand contact with this metal. Of course, a direct contact of the metal to the living cells has to be avoided. But there are different possibilities to include copper in surfaces. Here, copper was implanted in the titanium surface and protected by an ultra thin coating. For this purpose, allylamine was plasma polymerized (PPAAm) on a Cu-containing Ti surface to accelerate the initial adhesion of osteoblasts, to prevent the direct contact of osteoblasts with the Cu, while preserving an antimicrobial activity of the Cu ions. A very thin, adherent, cross-linked, pinhole- and additive-free PPAAm layer could be deposited, which is resistant to hydrolysis and delamination and equipped with a sufficient density of positively charged amino groups. This article will give a short overview on material aspects as well as influences on bacteria and bone cells *in vitro* and inflammation *in vivo*.

Materials and Methods

Plasma treatments, physicochemical surface analyses, as well as *in vitro* and *in vivo* investigations were performed with polished titanium discs (1-3 cm diameter or 5x5x1 mm³, respectively) with a defined roughness of $R_a=0.19 \mu\text{m}$. These samples are denoted as TiP.

Plasma Immersion Ion Implantation (PIII) was performed in radio frequency (RF) plasma in an ultra high vacuum (UHV) reactor. The applied pulse voltage was 5 kV and the working pressure was about 2 Pa. The pulses for the PIII had a short rise time in the range of 200 ns, a repetition rate of 50 Hz and pulse length of 15 ms. These parameters induced a mean implantation current of about 35 mA. The temperature of the sample attains about 450 °C. These samples are named Cu PIII-TiP.

The thin film coat of plasma polymerized allyl amine (PPAAm) was deposited in the microwave (2.45 GHz) plasma reactor V55G (Plasma Finish, Schwedt, Germany), in a two step process without breaking the vacuum. At first, all samples were decontaminated and activated by a continuous wave oxygen plasma (500 W, 50 Pa, 100 sccm O₂ / 25 sccm Ar) and secondly the samples were coated with the thin film (about 10 – 100 nm thick) of PPAAm (500 W, 50 Pa, 50 sccm allylamine /

50 sccm Ar, different pulse regimes). Two different duty cycles (ratio of t_{on} divided by the overall pulse duration $t_{\text{on}} + t_{\text{off}}$) and pulse lengths were used. These pulse / pause conditions were applied for different coatings in the *in vivo* investigations: Film 1 – 0.30 s on / 1.7 s off; Film 2 – 0.1 s on / 0.7 s off. The overall plasma-on time was 144 s in all cases and the total processing times were 960 s and 1152 s. This way coated samples are abbreviated with PPAAm-TiP or PPAAm-Cu PIII-TiP, respectively.

The stability of the coating was checked by a treatment in an ultrasonic water bath (T570H, Elma, Singen, Germany) in ultrapure water for 10 min.

The elemental chemical surface composition and chemical binding properties of the surfaces were determined by XPS (Axis Ultra spectrometer, Kratos, Manchester, UK) using the monochromatic Al K_{α} line at 1486 eV (150 W), implemented charge neutralization and a pass energy of 80 eV for estimating the chemical elemental composition or of 10 eV for highly resolved peaks. The C-C/C-H component of the C 1s peak was adjusted to 285.0 eV [13]. A labelling technique was utilized to determine amino groups on surfaces.

To investigate antibacterial effects, 10 μl of a solution (10^6 bacteria/ml) of the methicillin-resistant *Staphylococcus aureus* strain (MRSA) “Northern German Epidemic Strain” (ST 247; culture collection of Friedrich-Loeffler-Institute of Medical Microbiology, University of Greifswald, reference strain) was disposed on treated and TiP samples and dried under laminar flow conditions for 2 h. Thereafter, bacteria were rinsed with 0.5 ml solution of NaCl (0.9 % in H_2O) and plated on Mueller-Hinton II agar plates. The number of colonies formed was counted after incubation of plates at 37 °C for 24 h. In order to verify the identity of staphylococci the colonies were characterized by the Staphaurex Plus-Test (Remel Europ. Ltd., Dartford, UK).

Human osteoblasts (MG63 cells) were cultured under serum-free conditions. Adhesion and cell cycle phases were investigated by flow cytometry by using the equipment FACS Calibur (BD Biosciences, Germany). Spreading and actin cytoskeleton were visualized by confocal microscopy (LSM 410, Zeiss, Germany).

In vivo biocompatibility was studied after i.m. implantation of TiP and PPAAm-TiP samples in male LEWIS rats (age ca. 100 days). The tissue reaction was analyzed after 7, 14, and 56 days for each explanted sample by haemalaun-eosin staining of frozen sections and scoring by two independent investigators (rating 0 - no reaction to 3 - severe reaction).

Results and Discussion

Plasma treatment is an ideal tool to improve biomaterials surfaces, because it allows creating nanometer-thin surface or modifications while effectively suppressing influence of surface contaminations. Hence, contamination related adhesion problems can be reduced, which can be very serious problems for Ti coating processes. Further, the amount of potentially harmful foreign substances, which could be released e.g. during implantation failure, is kept at a minimum in general.

The problem of release control can be further reduced by use of PIII, because this technique allows subsurface modifications. For the here reported topic, the interface of TiP to Cu is generated some 10 nm below the surface.

Table 1: Elemental composition of untreated and different plasma treated titanium surfaces determined by XPS

	Ti [%]	Cu [%]	C [%]	O [%]	N [%]
TiP	15.7+/-0.3	0	36.2+/-0.7	41.8+/-0.2	2.2+/-0.2
Cu PIII-TiP	14.9+/-0.1	2.4+/-0.3	46.9+/-0.3	34.7+/-0.4	1.3+/-0.1
PPAAm-Cu PIII-TiP	0	0	72.7+/-0.6	2.1+/-0.1	19.5+/-0.1

Comparing untreated TiP and Cu PIII-TiP with XPS (Tab. 1), titanium dioxide (TiO_2) is present at both surfaces. The analysis of untreated TiP additionally demonstrates the complex surface structure of the uppermost about 5 nm of TiP: TiP is not only covered by a TiO_2 layer of some nm, but additionally carbon is found in conspicuous amounts and the O/Ti ratio is much higher than two, as expected for TiO_2 . PIII etches the residual carbon by the oxygen plasma applied, i.e. no carbon is found on a Cu PIII-TiP sample analyzed immediately after the plasma process without breaking the ultra high vacuum. But, a considerable amount of carbon is detected again after storage in air (see Table 1). Only 2.4 % Cu are detected in the uppermost surface of the Cu PIII-TiP samples after air contact. Argon ion sputter etching of the surfaces showed that the concentration of Cu increases some nanometers below the surface.

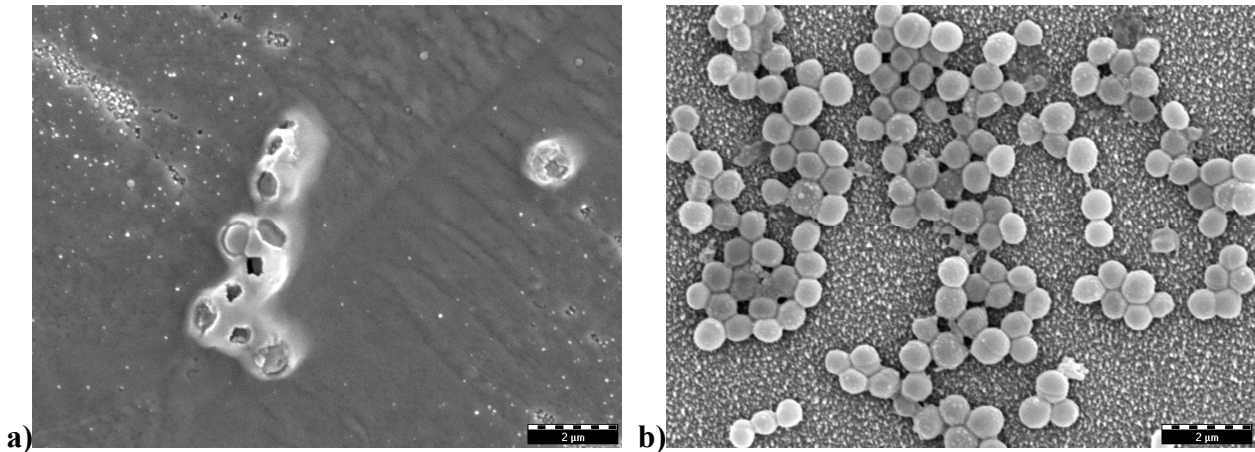


Figure 1: SEM pictures of methicillin-resistant *Staphylococcus aureus* (MRSA) cultivated on a) Cu PIII-TiP and b) PPAAm-Cu PIII-TiP.

How does the plasmachemical surface modification influence bacterial colonization? Apparently, TiP has no antibacterial effect. 1810 colony forming units (CFU) were found on TiP, a value comparable to polyethylene (PE), which is applied as general reference in our experiments. In contrast, only 30 CFU were found on Cu PIII-TiP. Interestingly, the Cu implanted in the TiP caused a disruption of cellular integrity (Fig. 1a). Coating with PPAAm reduced the antimicrobial activity of Cu PIII-TiP. 114 CFU were estimated on samples coated with PPAAm (about 10 nm) and the cell structure of the bacteria remained unchanged. Thus, PPAAm coating reduces antimicrobial activity of the surface, but it does not completely disable the antimicrobial effect.

An amino-functional coating like this should improve osteoblast cell adhesion and thus compensate for stress by copper ions. Therefore, it is worthwhile to characterize coating properties in more detail. Basically, a wide variety of such coatings can be prepared. But in the present case coatings selected for high stability in aqueous environments have to be chosen, ultimately. The investigation of the PPAAm-Cu PIII-TiP by XPS showed neither Ti nor Cu from the underlying substrate (see Table 1). Hence it is free from pinholes and thicker than the analysis depth of XPS, which is about 10 nm in such polymer-like layers. Independent thickness determination by profilometry revealed a thickness of about 60 nm. XPS revealed C, O, and N, content of the coating. The element ratio of the monomer ($\text{N/C} = 33.3\%$) was nearly kept by the pulsed plasma polymerization. The amount of hydrogen in the coating is not detectable by XPS, but it is generally accepted, that during plasma polymerization of allylamine, hydrogen is abstracted from the monomer [14]. Oxygen is not a constituent of the monomer. Its existence is most probably a consequence of free radicals in the coating, which can react with oxygen after air contact. The $-\text{NH}_2/\text{C}$ ratio of 2-3 %, which is relatively low compared to the maximum value of 18 % reported for a special RF-plasma [15]. This is a consequence of cross-linking of the precursors and formation of different amines, amides, and nitriles, as demonstrated by FTIR analyses [16]. Therefore, some oxygen is found in the coating. The amount of oxygen increases after sonication in water (see

Table 2). Oxidation increases the density of carbonyls, amides, and some carboxyls, while no nitrates, nitrites, or nitroso compounds were detected. Additionally, there is a small loss of nitrogen, which could be attributed to hydrolysis of imines or dissolution of short chain molecules (leachables). Nevertheless, no titanium could be detected for both films (see Table 2) after sonication. This means, there is neither delamination of the coating from titanium surface nor a massive reduction of layer thickness. All in all, surface properties of PPAAm-Cu PIII-TiP and PPAAm-TiP seem to be similar to a large extent.

Table 2: Elemental ratios O/C and N/C for PPAAm-TiP surfaces prepared by different duty cycles determined by XPS before and after sonication in distilled water.

Pulse on/off	O/C as deposited [%]	O/C sonicated [%]	N/C as deposited [%]	N/C sonicated [%]
0.3 s on/1.7 s off-film 1	4.3+/-0.2	7.7+/-1.4	26.4+/-0.4	23.1+/-1.2
0.1 s on/0.7 s off-film 2	4.3+/-0.2	7.5+/-0.7	30.6+/-0.5	25.7+/-0.8

Significant differences in N/C between film 1 and film2 ($p=0.027$). Nearly significant differences in $N/C_{\text{sonicated}}$ and O/C between film 1 and film 2 ($p=0.06$).

PPAAm-TiP is definitely advantageous compared to TiP concerning the initial cellular effects, i.e. adhesion and spreading [14]. A six-fold higher cell area was found on PPAAm-TiP compared to TiP after 30 min. *In vivo* investigations show a relatively strong tissue reaction (score 2.1) after 7 days in comparison with untreated control samples (score 0.7). In the further course of the study, the inflammatory response decreased for the PPAAm-TiP samples and was lower than for the uncoated TiP controls after day 56 (see Fig.2). In this case, the score for PPAAm-TiP samples is with 1.1 definitely not higher than on the TiP samples with a score of 1.5. Overall, the inflammatory response seems to be mainly associated with the activity of macrophages (data not shown, manuscript in preparation).

In summary, the PPAAm layer seems to have no long-term negative influence on the *in vivo* performance compared to TiP.

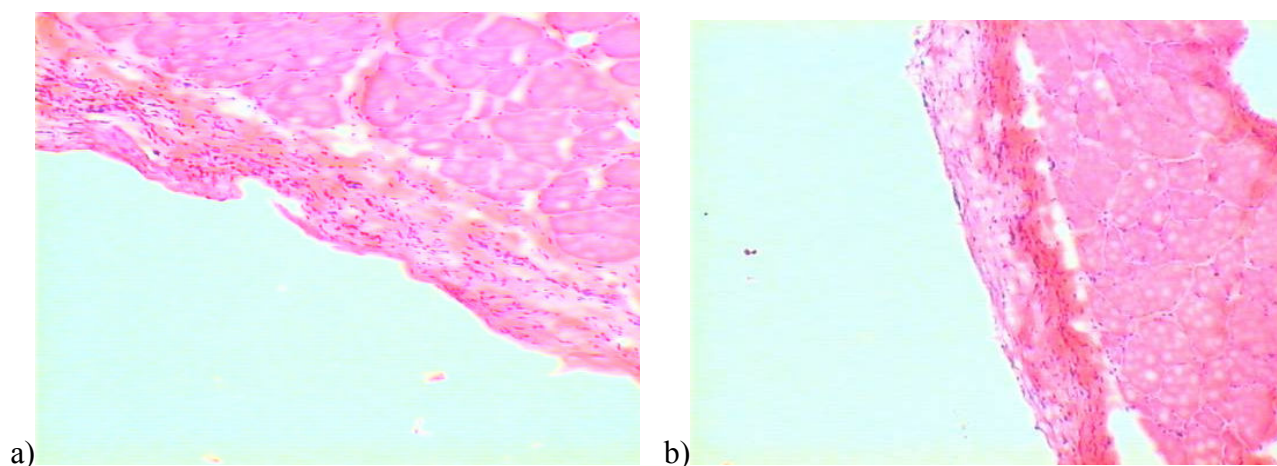


Figure 2: Section of tissue in direct neighborhood to a) TiP and b) PPAAm-TiP after 56 days of implantation.

Summary and Outlook

The combination of suppressed adhesion of bacteria and improved adhesion of eukaryotic cells to titanium implants is an actual challenge of materials surface modification research. Plasma-based copper implantation into titanium surface leads to an antimicrobial effect. The coating of such surfaces with an amino-functional plasma polymer is known to improve bone cell adhesion. Intramuscular implantation of test samples in rats revealed a reduced inflammation reaction compared to uncoated titanium. The next efforts will be focused on the optimization of the combination of both properties, namely antimicrobial and cell-adhesive effects.

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