Analysis of the aging behaviour of plasma polymer-coated titanium surfaces and correlation with the response of osteoblastic cells

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Abstract: The rapid acceptance of osteoblastic cells just after implantation is a crucial factor for implant performance also after previous storage of the implant on air. It is shown that the change of surface chemical properties of thin plasma polymerized allylamine coatings on titanium alloys has no significant influence on cell adhesion and spreading during one year of storage.

Keywords: plasma polymerized allylamine, microwave excitation, aging, human osteoblasts.

1. Introduction
Plasma treatments with nitrogen-containing gases can be successfully applied for the surface modification of different cell-contacting polymer materials\textsuperscript{[1]}. Similarly, cell adhesion characteristics of metallic implant materials like titanium suffer from plasma polymer coatings with nitrogen-containing films in vitro and in vivo\textsuperscript{[1]-3}.

The knowledge of long-term stability of such plasma polymer coatings is essential for storage stability of coated implants like hip and knee joints. Therefore, aging studies of plasma polymer coatings on titanium surfaces are important to detect the surface chemistry changes over a long time period. For this purpose, results of physicochemical surface diagnostics have to be correlated with biological experiments. Cell adhesion and spreading were measured in adequate tissue culture experiments. The objective of this paper was to determine surface chemical characteristics of thin plasma polymerized allylamine (PPAAm) coatings on titanium alloys for one year after preparation and to correlate these data with adhesion and spreading of MG-63 cells.

2. Materials and Methods
Substrates: Polished titanium alloy discs (Ti-6Al-4V_P) (cp, grade 2, R\textsubscript{s} 0.19\,\mu m, 1 cm diameter) were applied for plasma treatment, physicochemical analysis and in vitro cell culture.

Deposition process: A commercial microwave plasma reactor (2.45 GHz; V55G, Plasma Finish, Schwedt, Germany) was used for preparation. Substrates were downstream positioned in 9 cm distance to the microwave coupling window. Discs made from Titanium alloy were first polished (Ti-6Al-4V\textsubscript{P}) and then cleaned and activated by a continuous wave (cw) low pressure oxygen plasma (500 W, 50Pa, 100 sccm O\textsubscript{2} / 25sccm Ar) for 60s. Subsequently, they were coated with 50-100 nm thick layers of plasma polymerized allylamine (PPAAm-Ti) using pulsed low pressure microwave plasma with a duty cycle (DC) of 0.15 and a total pulse length of 2 s. DC is the ratio of plasma on time (t\textsubscript{on}) divided by the overall pulse duration t\textsubscript{on} + t\textsubscript{off}. A liquid handling system allows an exact dosing of the precursor allylamine.

XPS: X-ray photoelectron spectra were measured with an Axis Ultra spectrometer (Kratos, Manchester, UK). Irradiation by monochromatic Al K\textsubscript{a} line at 1486 eV (150 W) was used for determination of the elemental surface composition and chemical binding properties. Spectra were recorded with pass energy of 80 eV for the estimation of the chemical elemental composition and of 10 eV for highly resolved C1s and N1s peaks\textsuperscript{[4]}.

Chemical derivatization with 4-trifluoromethyl- benzaldehyde (TFBA) was used for labeling of amino groups in XPS measurements (CD-XPS) in a saturated gas phase at 40° C for 24 h. Three fluorine atoms mark one amino group.

FT-IR: PPAAm coatings were investigated by the diamond ATR- or IRRAS-unit of a FT-IR spectrometer (Spectrum One, Perkin-Elmer, Rodgau-Jügesheim, Germany). For this purpose, Au was sputtered on polystyrene wafers to improve the sensitivity of the FT-IR measurement. The PPAAm coating was carried out in direct neighbourhood to the Ti alloy samples (PPAAm-Au).

Surface free energy measurements: The polar and dispersive part of surface free energy was calculated from measurements of contact angles with different liquids. Water, ethylene glycol, and methylene iodide contact angles were determined with the help of the contact angle measuring system OCA 30 (Data Physics Instruments GmbH, Filderstadt, Germany) by the sessile drop method.

In vitro cell culture: Human osteoblasts (MG-63 cells) were cultured under serum-free conditions to avoid masking of the PPAAm-Ti surfaces with adsorbed proteins\textsuperscript{[5]}. Adhesion and cell cycle phases were investigated by flow cytometry by using the equipment FACS Calibur (BD Biosciences, Germany). Spreading and actin cy-
toskeleton were visualized by confocal microscopy.

3. Results and Discussion

The aging of PPAAm-Ti was investigated by physico-chemical analysis and cell culture experiments for one year.

XPS and FT-IR measurements as well as the determination of surface free energy demonstrated the oxidation of the PPAAm films by subsiding post plasma processes initiated by the reaction of surface free radicals with atmospheric oxygen after sample storage on air.

Starting with a N/C ratio of 27.2±0.3% and an O/C ratio of 5.6±0.4% of the coatings measured after less than one hour of air contact, an exponential changes were observed for N/C (decrease to 19 %) and O/C (increase to 17 %) during the storage for one year. In contrast, poly(allylamine) [-HC-(CH2-NH2)n]n features a N/C ratio of 33.3 % and no oxygen content. XPS signals from the Ti-6Al-4V_P were found not at all. This showed, that the thickness and conformity were qualified to correlate all XPS to the coating and no signal to the substrate. The aging process is characterized by a loss of about 70 - 80 % of primary amino groups within the first 15 days of storage (Fig. 1), whereas the nitrogen content N/C remains nearly stable, and shows only an exponentially loss of about 8 % over one year.

Fig. 1: NH2/C ratios of PPAAm-Ti as a function of storage duration measured by CD-XPS.

Fig. 2 demonstrates the conversion of chemical functional groups from a freshly prepared PPAAm-Ti coating to an aged one. Primary amino groups (CF3 groups after derivatization with TFBA) have been lost under enhancing of amide N-C=O and carboxyl O-C=O bonds. The oxidation process causes predominantly an oxidation of carbon atoms with an attached amino group leading to the formation of amides.

This assumption can be verified by FT-IR IRRAS investigations. These studies demonstrated a high retention of the structural properties of the monomer allylamine for the deposition method used here. Basic features of the monomer structure are dominant as the stretching vibrations of the aliphatic C-H groups, ν-CH3 at 2980 - 2880 cm⁻¹, the deformation vibration, δ-CH3 at 1465 - 1375 cm⁻¹, and deformation vibrations of amines, δ-NH at 1650 - 1510 cm⁻¹. Also ν-NH stretching vibrations are clearly visible between 3380 - 3200 cm⁻¹. But typical plasma effects are also observable as e.g. significantly broadening, disappearing or arising bands. Amino groups were partially transformed into amide, imine or nitrile functional groups by the plasma process.

Fig. 2: Oxidation of carbon-based functional chemical groups is displayed by changed contents of nitrogen-containing peaks in the C1s region measured with high energy resolution after deposition and after storage of PPAAm-Ti for one year. Formation of amides (N-C=O) and carboxyls (O-C=O) is demonstrated under a loss of primary amino groups. CF3-groups are a consequence of derivatization with TFBA.

The oxidized carbon-based functional groups are displayed by changed contents of nitrogen-containing peaks in the C1s region measured with high energy resolution after deposition and after storage of PPAAm-Ti for one year. The formation of amides (N-C=O) and carboxyls (O-C=O) is demonstrated under a loss of primary amino groups. CF3-groups are a consequence of derivatization with TFBA.

An indication for that is the band between 2300 - 2200 cm⁻¹ associated with stretching vibrations of nitrile and ethine groups, ν-CN, ν-CC. FT-IR spectra are compared in Fig. 3 after preparation and ageing on air for 30 days. Characteristic changes due to ageing are: the
formation of O-H vibrations near 3700-3200 cm\(^{-1}\) by water absorption and the formation of acid amides at 1700-1680 cm\(^{-1}\) [6]. Furthermore a degradation of nitrile and ethine groups, \(\nu-CN\) and \(\nu-CC\) at about 2200-2150 cm\(^{-1}\) was found [7].

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The initial water contact angle increases from 47\(^\circ\) to 57\(^\circ\) within 7 days and decreases over the further storage time achieving its minimum at about 37\(^\circ\) by an increasing number of hydrophilic groups as for instance amides and carboxylic groups within the first half year. The results could be confirmed by a threefold replication of these investigations.

Strongly correlated to the determined physico-chemical surface properties, studies on the attachment (adhesion and spreading) of human osteoblastic cells MG-63 were carried out in the same time frame.

Initial cell adhesion experiments reveal a significant, more than 2.3-fold increase of adherent cells on the bio-functionalized PPAAm-Ti surface compared to uncoated Ti-6Al-4V\(_P\). The initial cell adhesion is constantly increased over the whole storage period of one year and results in a cell adhesion of 88 -96 \% of adherent cells after 5 min and 80 -96 \% after 10 min.

Concerning cell spreading the surface modification with PPAAm boosts significantly the initial (30, 60 min) as well as the long term spreading (24 h) of the osteoblasts over the time period of one year (Fig. 5). This is even more pronounced in the initial cell spreading phase at 30 min where a nearly 2.2 - 2-fold increase can be observed whereas a 1.6-fold increase can be found after 24 h. This underlines the superior effect of PPAAm in the first hours of cell attachment and spreading and its stability throughout one year storage on air (Fig. 6).

4. Conclusion
A plasma polymer coating with PPAAm was deposited on a titanium alloy to enhance osteoblast cell adhesion by a positive surface charge induced by surface amino groups. This coating exhibits a number of chemical changes during ageing in air for one year. First of all, the density of amino groups was drastically reduced.

Tissue culture experiments with MG-63 human osteoblastic cells demonstrate a considerable enhanced adhesion and spreading on a plasma-coated titanium alloy (PPAAm-Ti) compared to the uncoated polished Ti-6Al-4V\(_P\) substrate. This effect was independent of the storage duration and all measurements regarding cell behaviour resulted in highly significant differences over the whole time period of one year.
Summarizing chemical and biological results, cell adhesion does not correlate with amino group density. This implies, that other functional groups contribute to osteoblastic cell adhesion, proliferation, and growth as well.

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