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On the Application of Gas Discharge Plasmas for the Immobilization of Bioactive Molecules for Biomedical and Bioengineering Applications

Frank Hempel et al.*
Leibniz Institute for Plasma Science and Technology e.V. (INP), Greifswald, Germany

1. Introduction

Biomedical and biotechnological applications of polymeric materials often require specific interactions of the substrate surface with the biochemical or biological milieu. However, the standard surface properties of polymers like polystyrene (PS), polycarbonate (PC), polypropylene (PP), fluorinated ethylene polypropylene (FEP) or cyclic olefin copolymers (COC) do not meet these requirements. Therefore, the creation of functional surfaces is a very important topic to meet the requirements of advanced applications in bioengineering.

For this purpose, gas-discharge plasmas offer some unique possibilities (Ohl & Schröder. 2008; Schröder et al., 2010a). They can lead to surface activation and functionalization, often not obtainable with conventional, solvent-based chemical methods. In addition, the superior chemical reactivity of plasmas allows surface activation of inert materials down to the nanoscale range including the creation of covalently bound functional groups in such small structures (Meyer-Plath et al., 2003). While it is possible to implant substances into the substrate and to etch surface structures by gas discharges, properly operated plasma processes might as well neither affect bulk materials characteristics nor produce undesirable substances and cause only minor thermal load to substrates (Schröder et al., 2011).

Plasma processes are especially suitable for the equipment of polymer surfaces with chemical functional groups. Plasma functionalizations as well as depositions of nanometer-thick coatings with chemical groups can be used to receive the required biological response, for instance to control the cell density, distribution, adhesion and differentiation (Wende et al., 2006). Furthermore, plasma based processes can be combined with lateral pattern generation. Common mask techniques are suitable to create

* Hartmut Steffen[1], Benedikt Busse[2], Birgit Finke[1], J. Barbara Nebe[3], Antje Quade[1], Henrike Rebl[3], Claudia Bergemann[3], Klaus-Dieter Weltmann[1] and Karsten Schröder[1].
1 Leibniz Institute for Plasma Science and Technology e.V. (INP), Greifswald, Germany,
2 zell-kontakt GmbH, Nörten-Hardenberg, Germany,
3 University of Rostock, Medical Faculty, Dept. of Cell Biology, Rostock, Germany.

lateral chemical structures with different dimensions and interface properties. Sometimes, e.g. for multi-spot arrays in consumables used in high throughput screening (HTS), micro structures are required if such surfaces should be applied. Plasma processes are especially suitable for the functionalization of fine morphological 3D-structures with complex shapes like for instance cavities and trenches in HTS platforms, holes in textiles and membranes, and pores in foams (Schröder et al., 2005). Another aspect is localized chemical (micro) pattern for lateral array structures. Applications of such micro-patterned polymer surfaces could be tissue-like cell culture systems as well as neuronal networks, bioartificial organs, implants, biosensors, DNA and protein biochips or further tools for pharmaceutical screening.

For the optimization of the plasma and patterning processes it is necessary to analyze the surfaces for their physicochemical properties and cell compatibility in an appropriate way. In particular different surface chemistries have to be analyzed in direct neighbourhood. Often highly resolved XPS analysis and imaging is applied for the chemical characterization of surface microstructures. Using XPS analysis, well-defined chemical functionalizations, good homogeneities, and sharp edges can be verified allowing a high density of such patterns.

Both, plasmas at atmospheric pressure and low pressure can be applied for surface modification. While atmospheric pressure plasmas offer advantages in terms of investment cost and process integrability, low pressure plasmas excel by their superior chemical selectivity. Therefore, in this article examples of low pressure plasma-assisted immobilization strategies will be given for cell-adhesion molecules for biomaterials surfaces and enzyme-carriers for white biotechnology.

2. Plasma enhanced micro structuring of cell-based RNA arrays

2.1 Background

The investigations and analyses of surface modifications to control cell adhesion are an important topic of biomaterials research. Chemical micro patterns consisting of cell-adhesive and cell-repulsive regions can be applied to additionally influence cell position, migration, proliferation, phenotype, membrane integrity and finally live and death (Chen et al., 1997; Ohl & Schröder, 1999). Gas-discharge plasma processes can be used to create functional groups or ultra thin functional coatings, which are covalently fixed to the substrate. They could be interesting for DNA- and protein chips in high-throughput-screening (HTS), too (Müller et al. 2003).

Prospective applications of chemically patterned polymer surfaces are cell-based RNA arrays. Cell-based arrays usually are made of polymers as substrate material. For this reason their surface is very heat sensitive and has to become chemically inert before it will come in contact with aqueous liquids. In addition, for bio-applications it must be biocompatible and so it needs a defined surface treatment for controlled modification of these material parameters. Plasma processing meets all these requirements (Ohl, 1999).

Plasma-assisted chemical surface functionalization is a frequently-used processing step for the preparation of chemical micro patterns on polymeric surfaces (Schröder et al., 2002). Plasma processes were also applied for the treatment of interior surfaces of small trench structures (Besch et al., 2008) and for the creation of localized chemical structures for a cell-based high content screening system (Steffen et al., 2007). In the present chapter we present a special pattern which consists of an arrangement with three different chemical zones for a cell-based array for reverse siRNA transfection.

The discovery of a ribonucleic interference (RNAi) (Fire et al., 1998) opened up new possibilities for functional genome research in particular by cell transfection with small interfering RNA (siRNA). In such a reverse transcription assay, the siRNA is immobilized in a gel-forming matrix like gelatine or alginate (Zlauddin & Sabatini, 2001). Arrays of similar gel spots with very small volumes have to be created on a carrier chip for high-throughput-screening (HTS) (Szili et al., 2004). To guarantee optimum transfection, the siRNA should be spotted in direct neighbourhood to the growing cells. For multiple analyses, the single cell spots have to be separated by barriers to prevent cell migration and siRNA cross-talk between the different assays. Many eukaryotic cells do not grow on untreated polymers which are typically applied as base materials for HTS labware such as polystyrene (PS), polycarbonate (PC), polypropylene (PP), cyclic olefin polymers- and copolymers (COP and COC), or poly(methyl methacrylate) (PMMA). For this reason, untreated polymers could act as barriers regions. But, γ- and e-beam sterilization methods introduce oxygen-containing functional groups (Fire et al., 1998) at the polymer's surface and lead to an undesired cell migration. Ideally, a small round spot of immobilized siRNA is surrounded by a cell layer in intimate contact. Obviously, specific surface structures of the chip have to be analyzed to verify the successful modification procedure.

On this account, a triple contrast has to be visualized for cell-based RNA arrays. A high local definition of chemical contrasts is required. For the development of such arrays, chemical surface properties have to be known with high spatial resolution. Obviously, quantifications of transfection effect need defined borderlines. Thus, the investigation of borders in chemical microstructures on polymer surfaces is of special importance with respect to accuracy of dimensions and geometry as well as the correlation with large-area treatments. The characterization of the quantitative chemical composition inside the micro pattern is important for the miniaturization of diagnostic tools (Schröder et al., 2009). The chemical structures can be visualized by fluorescence microscopy after labelling of functional groups with fluorescence dyes or by atomic force microscopy (AFM) by modification of the tips with specific molecules. Quantitative chemical analyses of such micro pattern can be performed with Auger electron spectroscopy (AES), time-of-flight secondary ion mass spectrometry (TOF-SIMS), and X-ray photoelectron spectroscopy (XPS). XPS is advantageous according to the absolute values without calibration (Schröder et al., 2004).

So, three surface zones with different physicochemical properties on optical clear substrates had to be produced: a cellophobic border (zone 1), cellophilic marrow area (zone 2) and a spotting zone (zone 3) for the siRNA.

2.2 Materials and methods

Cell array chambers (CAC) (Fig. 1) were applied as substrates for plasma modification. They were provided by zell-kontakt (Nörten-Hardenberg, Germany). These disposables contain four rectangular chambers, every chamber with the dimensions of a microscope slide (75 mm x 26 mm). They are injection-moulded from extremely clean polymers (PS, PC, COC) and have a very smooth surface necessary for pattern generation by mask processes.

Fig. 1 shows a medium-scaled array of 3x8 zones for siRNA immobilisation. The central analytical zones (zone 3) are surrounded by ring-shaped areas (zone 2) which support in this case the growth of adherence-dependent mammalian cells. The surrounding third zone (zone 1) fills the rest of the surface to prevent the cross-talk between different spots.

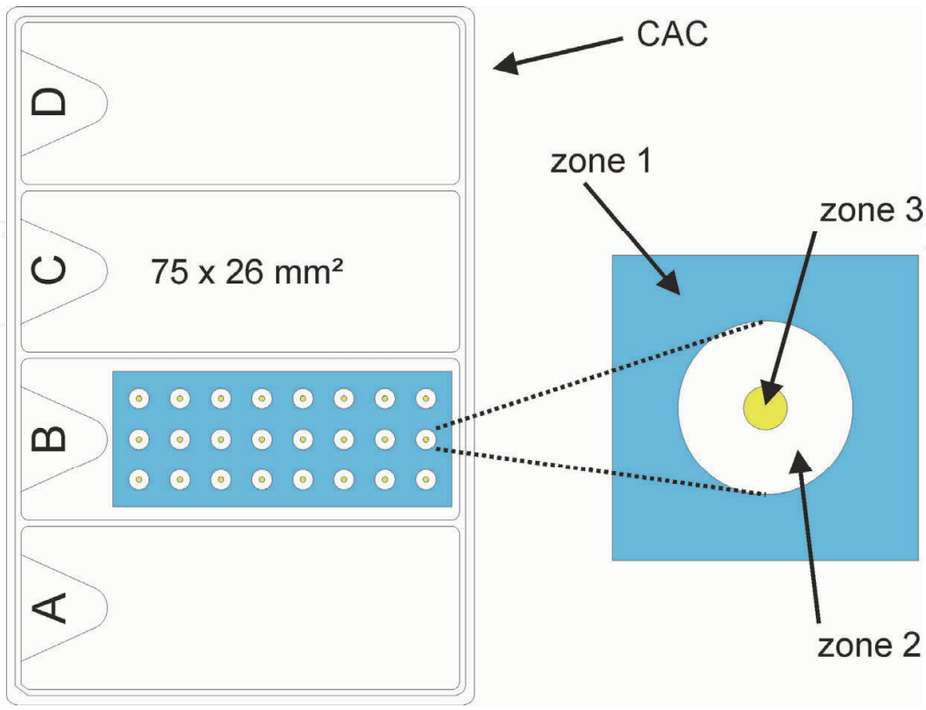

Fig. 1. Schematic view of a single element (right) and arrangement (left) of the cell-based RNA array in a cell array chamber (CAC) (Schröder et al., 2009).

Zone 1 prevents the cross-talk between the elements of the array. That way, many different experiments can be performed in interaction with one and the same cell culture on one array. For this purpose, surface properties of this zone have to suppress adhesion of cells reliably.

Zone 2 is the cell growth area. It needs a functionalization, which supports the growth of sensitive adherent cells. The contact angle should be between 40° and 60°. The attachment of more than 10-15 spread cells should be possible. This gives a lower limit of about 500 µm for the width.

The siRNA will be spotted onto zone 3. This zone should guarantee the immobilization of the siRNA and the interfacial interaction with the cells. The cells grow over the spotted area and absorb the siRNA. Zone 3 has to be remarkably more hydrophilic than zone 2 to keep the siRNA at this area.

All modified surfaces mentioned in this article can be generated with microwave plasmas. The plasma treatments were performed with a V55G plasma reactor (PLASMA-finish, Schwedt, Germany) (Fig. 2). The reactor consists of an aluminium process chamber which has the dimensions 40 x 45 x 34 cm^3 (width x depth x high). Microwaves (2.45 GHz) were coupled top down into the chamber. A parabolic reflector was used to spread microwave power over the whole area of a 200 mm in diameter quartz window in the top wall of the chamber. Thus, a relatively high lateral homogeneity of large area surface treatments can be achieved in certain cases (van Wachem, 1985).

NH_3 (40 sccm) was used as process gas for NH_3 plasma treatment at 20 Pa and 500 W. The distance to the microwave window was 5 cm and the treatment duration was 5 s.

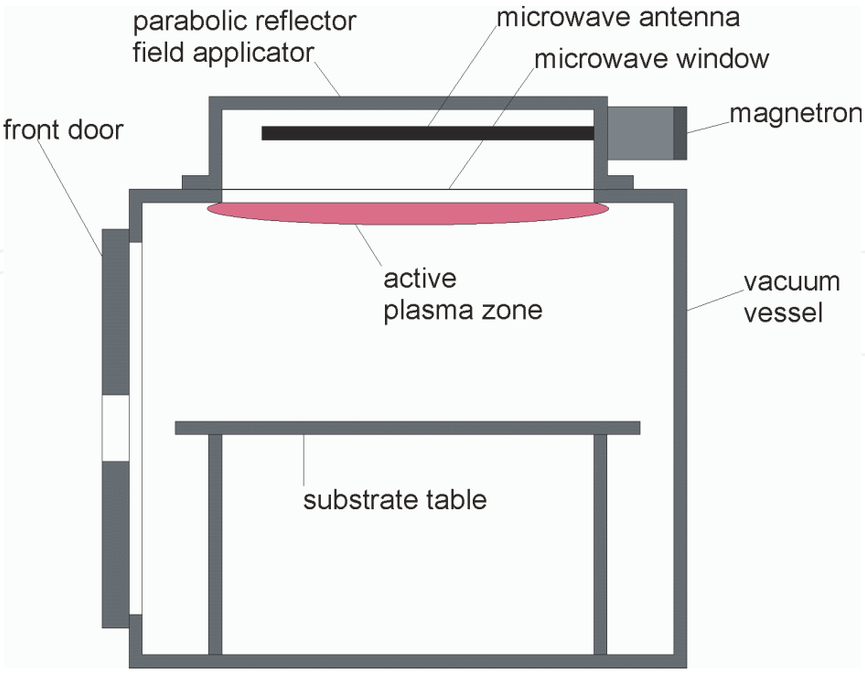

Fig. 2. Schematic view of the plasma reactor VG55 used for the three plasma modifications.

For the plasma polymerization, a mixture of allylamine and Ar (50 sccm) was used. The plasma was pulsed (0.1 s / 1.5 s, on/off) with a power of 500 W at a pressure of 25 Pa. The samples were placed 9 cm below the microwave window and treated for 288 s.
The fluorinated hydrocarbon surface was functionalized by pure NH_3 as well as H_2/NH_3 cw microwave plasma. In both cases the treatment time was varied between 1 and 60 s. The other conditions for the NH_3 plasma treatment were the same as mentioned above.
The chemical composition of the modified polymer surfaces was analyzed with high resolution XPS (Axis Ultra, DLD, Kratos, Manchester GB). For spectroscopic measurements, the spot size was 250 µm in diameter. All elements were quantified after measurement with low energy resolution (pass energy was (PE) of 80 eV) and calculated with atomic sensitivity factors given by a XPS-instrument-specific RSF data library. The C1s peak was measured with high energy resolution with a PE of 10 eV. Moreover, the chemical differences within the 3 zones were visualized with XPS images. The XPS measurements were evaluated with CasaXPS 2.3 (Casa Software Ltd) mainly (Fairley et al., 2005).
Contact angles were measured using the sessile drop method with ultra pure water at room temperature with a Digidrop contact angle meter (GBX Instrumentation Scientifique, Romance, France). At least five measurements were performed on different positions of the sample and averaged.
Cell culture experiments were carried out with Human Embryonic Kidney (HEK) cells in untreated and plasma modified CAC's. This cell line is a frequently used model. The HEK cells were seeded into the CAC's with a density of 200 cells/mm² and grown for 24 h (37 °C, 5 % CO_2). PC12HEK cells were cultured in RPMI 1640 Dulbecco's Modified Eagle's Medium (DMEM) with 7 % fetal calf serum (FCS). Microphotographic images were taken with an IX70 microscope (Olympus, Germany) to estimate cell density and morphology.

2.3 Results and discussion

The surface modification processes have to fulfil numerous requirements in such applications. Not any of the modifications may have a cytotoxic effect neither in direct nor in indirect contact with cells. This requires preventing solvent-based processes, which often leave small molecules (solvents or short-chain molecules). In contrast, gas-discharge plasma processes are advantageous to create functional groups or ultra thin functional coatings, which are covalently fixed to the substrate. This means, they can withstand rinsing procedures. Minimum coating thickness is mandatory to avoid interference with optical measurements in detailed optical or fluorescence cell observation in high content screening (HCS). A transparent organic coating with a thickness of about 100 nm can already interfere with optical and fluorescence signals at 400 nm wavelength. Cold processes are necessary to avoid thermal load in subsequent plasma processes with masks. Sufficient contrasts have to be created between different surface zones.

The structure was prepared by a bottom-up method. This method requires the compatibility of subsequent plasma steps among each other and the selection of adequate masks. Several materials were tested for the mask. These experiments advised us to use materials with reduced stiffness. Finally, biaxial oriented polypropylene (BOPP) was the material chosen. This material was able to have a tight contact with the PS surface because this flexible material could huddle against PS (Schröder et al., 2009).

Several versions of surface combinations were tested. The surface combinations which where under investigation are listed in Fig. 3. Because of these large number of surface combinations the structures can be generated on several substrates and customized for specific cell lines and assays.

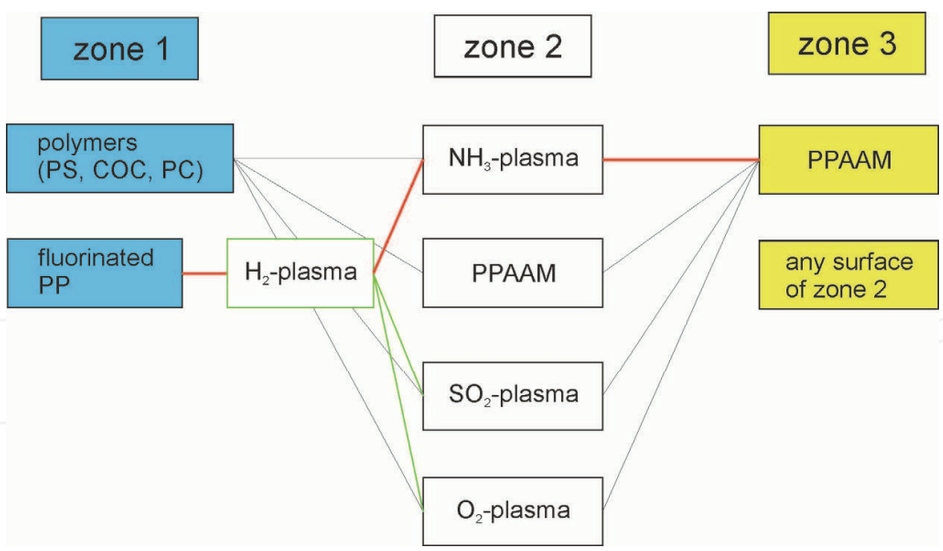

Fig. 3. Possible surface combinations for zones 1 to 3.

In principle for zone 1 untreated polymer surfaces can be used. A rather simple version comprised untreated polymer (e.g. PS) for zone 1, NH_2-funtionalized polymer for zone 2, and an allylamine plasma polymer (PPAAm) for zone 3. Two plasma processes and two different masks were applied to generate this structure. The production process is schematic shown in Fig. 4.

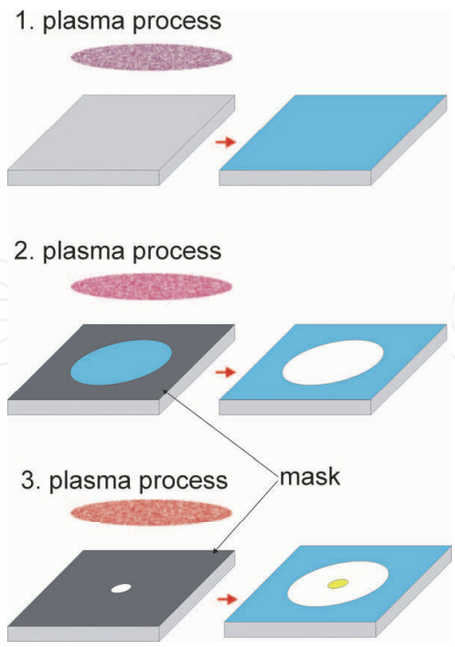

Fig. 4. Steps for the generation of the chemically structured surface.

In case of untreated surface for zone 1 the production starts with the generation (e.g. NH_2-funtionalization) of zone 2. The parts of the CAC which should not be functionalized were protected by a mask. The next plasma process is the deposition of allylamine plasma polymer in zone 3 using another mask. In case of a clean PS for zone 1 the cell- repulsive properties are sufficient. Unfortunately, the practical usefulness of this first version of micro pattern is limited, because subsequent γ- or e-beam sterilisation will introduce oxygen-containing functional groups reducing cell-repulsive properties of zone 1.
Therefore, cell-repulsive materials or modifications have to be used for zone 1, which do not lose their properties during sterilisation. Poly(ethylene oxide) (PEO) or PEO-like surfaces would be the best choice, because they are already highly oxidized and prevent protein and cell adhesion very well. But it is not recommended to modify their surfaces using subsequent plasma processing steps. Therefore, we tried to apply fluorinated polymer surfaces for zone 1. Plasma polymerized fluorinated hydrocarbons are convenient for forming inert hydrophobic surfaces. Moreover, this surface minimizes the cell adhesion, too. The water contact angle was above 115°, which is considerable higher than the contact angle of untreated PS (90°). A fundamental advantage of the application of a plasma polymer for zone 1 is the large number of possible substrates e.g. glass with excellent optical properties.
It is known that plasma assisted functionalization of fluorocarbons with amino groups leads to better cell culture supports than standard tissue culture polystyrene (TCPS). Nevertheless, the functionalization of this layer with NH_2-groups is a technological problem. While PS surfaces can be functionalized with superior cell adhesion characteristics using ammonia plasma for several seconds, it takes remarkable longer treatment times to obtain similar effects on fluorinated surfaces. A pure ammonia plasma treatment reduces the water contact angle of the fluoropolymer to about 60° after long treatments times of more than 60 s. To avoid overheating of the polymer substrate, hydrogen plasma activation was applied before ammonia plasma treatment. A 60 s hydrogen plasma pre-treatment

allows a water contact angle lower than 60° after an ammonia plasma treatment of less than 30 s. This reduces the treatment time by a factor of three and water contact angles lower 60° can be obtained which are useful for cell culture.

For zone 3 any surface of zone 2 is possible. However, a good choice is a thin plasma polymerized allylamine (PPAAm) layer. A PPAAm layer with a thickness of some nm only is not closed. That means the surface is rough in the nanometer range. According to the Wenzel equation the contact angle of a hydrophilic surface will be reduced (Marmur et al., 2003).

A structure version comprised a fluorinated hydrocarbon layer for zone 1, NH_2-funtionalized fluorinated hydrocarbon for zone 2, and a thin PPAAm for zone 3 will be described in more detail. Three low-pressure plasma processes and two different masks were applied to generate this structure (Fig. 4).

Fig. 5 shows a XPS line scan over one single element of the assay. The figure clearly demonstrates the contrasting chemical properties between the different zones, the fluorinated hydrocarbon for zone 1, NH_2-funtionalized hydrocarbon for zone 2, and a thin allylamine plasma polymer for zone 3.

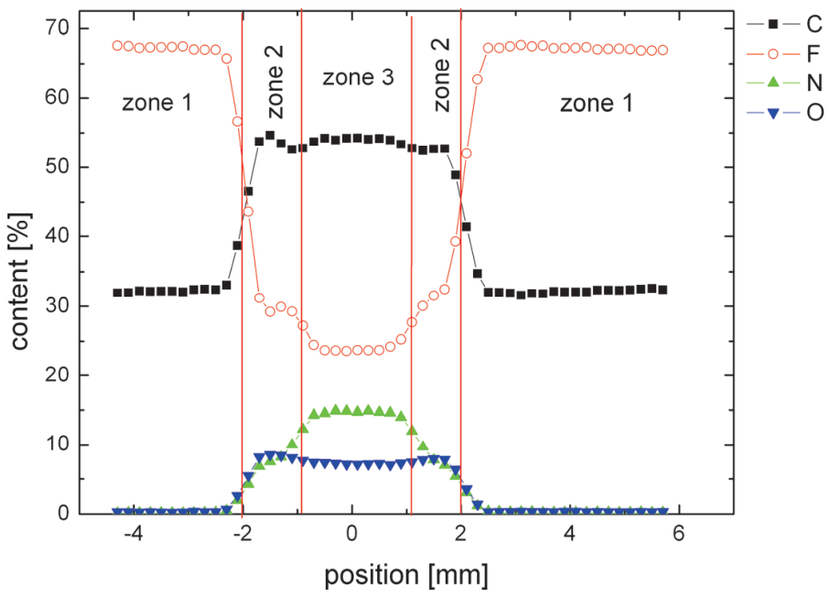

Fig. 5. Line scan across a single element of the cell-based RNA array (spectra with 250 µm spot size).

The fluorinated hydrocarbon layer consists mainly of C and F. The oxygen and nitrogen content is lower then 0.3 %. The ratio F/C is about 2.0 suggesting a teflon like structure. This assumption will be approved by the analysis of the highly resolved C1s peak (Fig. 6a). The CF_2 component was adjusted to 291.7 eV. The C 1s peak shows mainly CF_2 chain groups and CF_3 end groups. A small part of C is bonded in cross links (CF-CF).

By the H_2/NH_2--functionalization, the fluorine content was essentially reduced to about 30 % and moreover nitrogen was incorporated into the surfaces. The highly resolved C 1s peak shows drastically changes of the bond relations at the surface (Fig. 6b). There is still the bond group which belongs to the fluorinated plasma polymer. However, the peak structure is dominated by aliphatic (C-C/C-H), N and O containing groups. Nitrogen is bonded to C as amines (C-N) and amides (N-C=O).

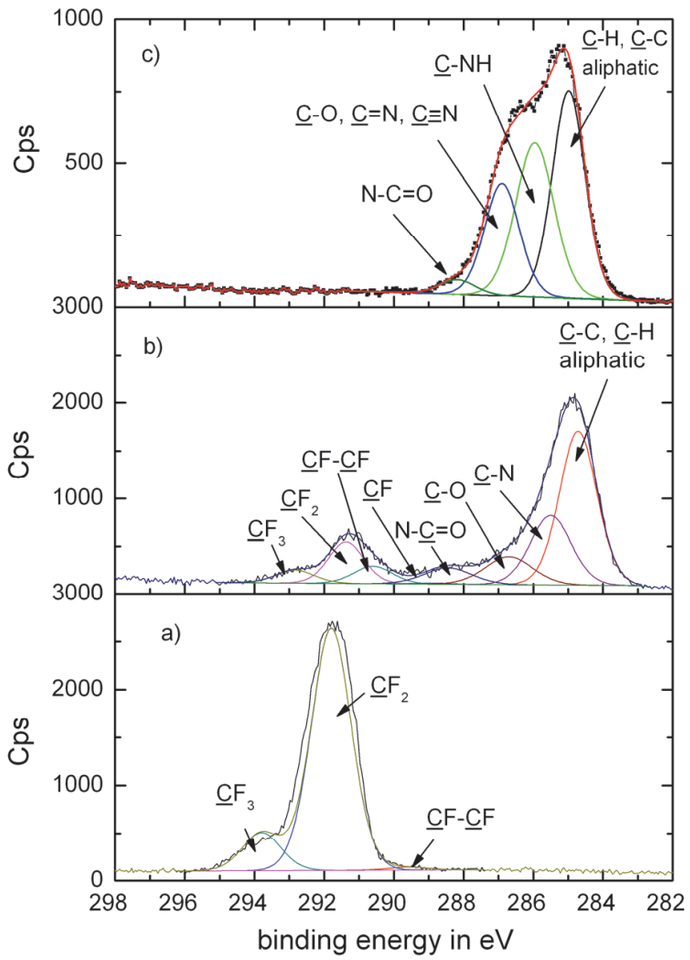

Fig. 6. Highly resolved C 1s peaks for a) the fluorinated plasma polymer, b) the H_2/NH_2 functionalized fluorinated plasma polymer and c) PPAAm.

The plasma polymer in zone 3 yields a further reduction of the fluorine content and the nitrogen content increases to 15 %. Because of the large amount of fluorine we can conclude that this layer is not really closed. The C 1s peak is dominated by aliphatic and N containing groups (Fig. 6c)). The C-C/C-H peak was adjusted to 285 eV. The other components of the C 1s peak can be assigned to: amines (C-N), imines and nitriles (C=N, C≡N) and amides (N-C=O). Nitriles have been found in FTIR measurements, too. The C-O bond which occurs most likely can not be separated from imines and nitriles (Finke et al., 2011).

The allylamine plasma polymer is quiet rough and has got a gel-like structure. These properties are important for the immobilization of the siRNA. The contact angle for water on zone 3 is remarkable lower than on zone 2. On zone 2 the contact angle for water is about 60°. This is well qualified for cell culture. After coating with an allylamine plasma polymer, the water contact angle was reduced to 25°. This is still reasonable for cell growth. On the other hand, the difference of contact angles is sufficient for retaining spotted siRNA on zone 3. About 10 nl of the solved siRNA will be spotted at one single element of the array. The diameter of the drop will be mainly determined by the contact angle for water of the

spotting zone 3. It is about 600 µm for the allylamine plasma polymer surface. Hence, the diameter of zone 3 should not be less than about 800 µm. This requirement was met by the here investigated arrays.
Zone 2 and zone 3 contain about 10 % oxygen. This concomitant oxygen functionalization cannot be avoided. One very possible reason for this oxygen contamination is the reaction of metastable carbon surface radicals with molecular oxygen upon contact with air. Moreover, zone 2 and 3 contain a large amount of fluorine. Because of this reason, a reliable quantification of amino group density by derivatisation with TFBA was not possible (Schröder et al., 2009).
The sharpness of the borders between the three zones is another feature which might influence the function of the assay. However, Fig. 5 displays the borders wrong. The reason is the spot size of 250 µm which was used during the measurements. This means, that about 68 % of the signal was generated from an area with the diameter of 250 µm (Gauss function with a width of 250 µm). Actually, a three times greater diameter has to be considered. In case of the Axis ultra, the spot size can be reduced to 15 µm to increase the spatial resolution. Unfortunately, this is often not practicable, because the intensity of the signal will be reduced remarkable and the resolution is still not high enough. On the other hand, the spatial resolution of the XPS device is much better in the imaging mode than in spectroscopy mode. In case of the Axis Ultra the spatial resolution can reach less than 2 µm. With the Axis Ultra stitched images (up to 10x10 single images) can be taken. By this technique a whole single element can be pictured in one XPS image. The edges can be analyzed by line scans from such XPS images. With this method the width of the transitions between the several zones were estimated to be between 30 µm and 70 µm. These values are in the order of the thickness of the masks (50 µm) (Steffen et al., 2011).
Cell culture tests with microstructures obtained by this version of processing and modification revealed a vey good pattern reproduction. Fig. 7 shows a typical example. Actually, cells growing on zone 2 could detect the borderline between zone 1 and zone 2.
A special effect was observed at this borderline, namely a concentration of cells near the borderline compared to the inner regions of zone 2. The forces for this accumulation are not yet clear. Perhaps there is a migration of cells from the cell-repulsive to cell-adhesive areas where they are captured. This hypothesis is supported by the observation that cells are able to build up clusters on zone 1. This accumulation of cells influences cell morphology. It makes additional cells available for the assay. But it could be seen that cell morphology was partially influenced. This indicates different cell function which could falsify the results of the assay. Also, the high cell density will complicate the analyses. So, it is suggested to restrict the analysis to the inner parts of the adhesion zones.

2.4 Summary

Arrays of chemical micro patterns in CAC's were tested for the purpose of cell-based siRNA assays. Basically, the micro patterns consist of three surface zones with different adhesion properties. A central spot-like region with very good wetting properties and sufficient cell adhesion is surrounded by a ring-like area exhibiting excellent cell adhesion. These regions are embedded in a background area which is highly repulsive for cells. A version of such a structure comprises a plasma polymerized allylamine coating, ammonia plasma functionalized fluorinated hydrocarbon and a fluorinated hydrocarbon layer. Results of surface analysis and cell culture tests are reported.

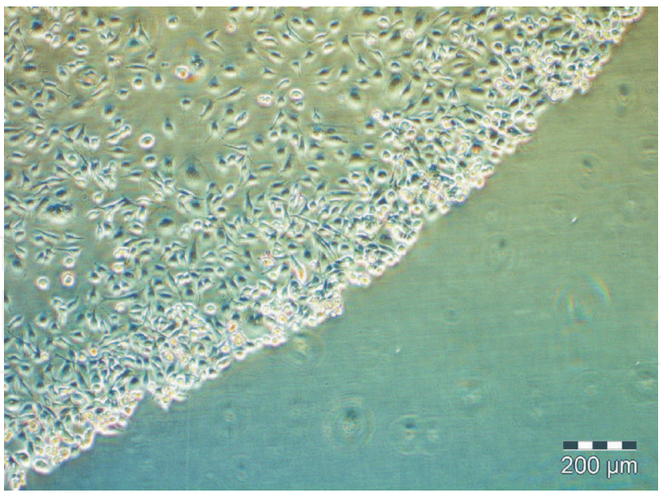

Fig. 7. Transition between zone 2 (NH_2-funtionalized polymer, left) and zone 1 (untreated polymer, right). HEK cells growing on zone 2, only.

3. Plasma-based bioequivalent coatings of biomaterial surfaces for an improved cell physiology

3.1 Background

The biologization of medical devices and in particular of implants is increasingly in the focus of research and development all over the world. For optimal adaptation of implants to the human surrounding tissue, the main focus of investigations is on molecular regeneration technologies and on improvements in material- and nanotechnology. The combination of these two technologies is the key for the realization of innovative implants.

Reliable first adhesion and spreading of cells on surfaces plays an important role for the success of implant applications. Typically, the adhesion of biomolecules on untreated biomaterial surfaces, e.g. synthetic polymers, ceramics or metals is only poor. A successful bioactivation of such surfaces can be achieved by different coupling strategies as for instance wet-chemical application of silane chemistry to bind amino group carrying silanes (Puleo, 1997). But properties of this silane-linker as their deliquescence and their tendency to polymerize and to form island-like domains gave rise to search for other coupling strategies (Falconnet et al., 2006).

Plasma polymerization is a convenient alternative method to prepare polymeric-like thin coatings not only on metals or ceramics (Morra et al., 2003) but also on inert synthetic polymer substrates, e.g. poly-ether-ether-ketone (PEEK) (Schröder et al., 2010b) or polylactid (PLA). PLA is a suitable biodegradable polymer for the improvement of the mechanical strength of calcium phosphate scaffolds (Ma et al., 2001). Low osteoconductivity of PLA surfaces is known but can be eliminated by plasma treatment (Wang et al., 2005). Due to plasma processes the number of hydroxyl groups on biomaterial surfaces can be increased and reactive functional amino- or carboxyl groups can be deposited (Schröder et al., 2010b, 2010c). Plasma polymerized allylamine (PPAAm) coatings induce strong cell-adhesive effects (Nebe et al., 2010; Nebe et al., 2007; Finke et al., 2007). Decisive for plasma applications on biomaterial surfaces is if cell physiology can be stimulated by the deposited functional groups of its own or, if additional immobilization of biorelevant molecules is necessary.

3.2 Improvement of osteoblast adhesion: comparision of a plasma polymer nanolayer vs. collagen-coating

Basically, cell adhesion to surfaces is mediated via integrins to the extracellular matrix (ECM) and has considerable influence on many cell functions. Thus, the properties of the artificial surface affect the cellular response *in vivo* and coatings which exhibit similarities of this surface to the extracellular matrix are in widespread use. Typically, immobilized proteins and peptides are used for the improvement of the interface (Rammelt et al., 2006; Schuler et al. 2006; Rychly and Nebe, 2009). Immobilized proteins may contain RGD sequences (arginine-glycin-aspartat) which are binding sites for cellular integrin receptors. Also our own adhesion experiments with human bone cells demonstrate the adhesive effect of collagen (COL) coated surfaces (Fig. 8).

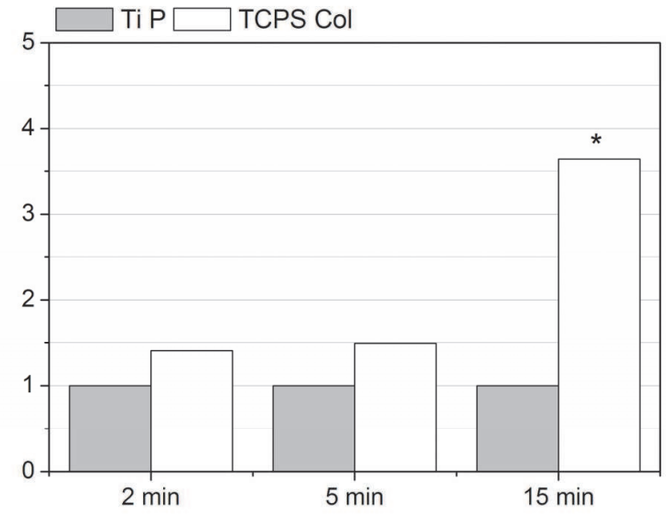

Fig. 8. Cell adherence in the initial phase is significantly enhanced on a COL-coated tissue culture polystyrene surface (TCPS Col) vs. pure polished titanium (Ti P). Human MG-63 osteoblastic cells, $1x10^4$/Ø 30mm, adherent cells related to Ti_P=1, flow-cytometry, BD FACSCalibur, n = 6, T-test, *p<0.005.

In contrast to conventional methods, plasma polymers offer a greater versatility for binding biomolecules using different immobilization chemistries. Unfortunately, knowledge of the bonding mechanisms between the TiO_2-surface and deposited polymers is rather poor up to now (Possart, 1998). For plasma polymers it has been shown that swelling and dissolution of unbound material may be a problem in aqueous environments (Chu et al., 2006; Friedrich et al., 2003).

Therefore we studied the characteristics of an extremely thin, homogeneous plasma polymer film on titanium oxide, which is also sufficiently adherent in aqueous medium (Fig. 9). The polymer deposition process based on pulsed low pressure micro wave discharge plasmas has some advantages over radio frequency excitation (Hamerli et al., 2003). Allylamine is a well-suited molecular precursor for film deposition since it gives good retention of amino groups (Finke et al., 2006; Förch et al., 2005; Tang et al., 1998; Kühn et al., 2001). Films were characterized by advanced surface analytical techniques, such as high resolution scanning XPS, attenuated total reflectance Fourier transform infrared spectroscopy (ATR FT-IR), water contact angle measurements and zeta-potential measurements. More information on these

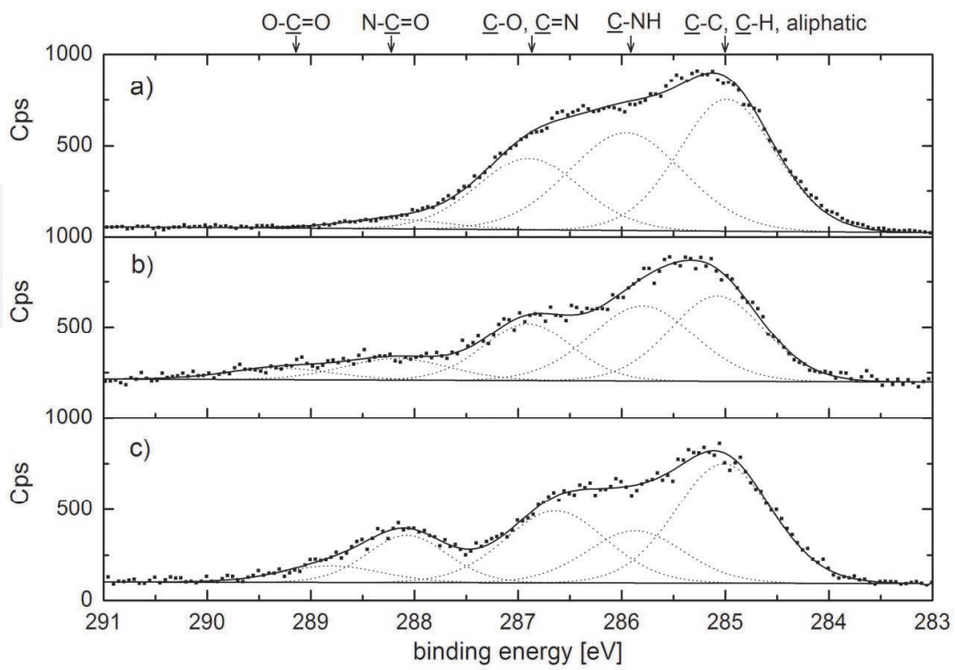

Fig. 9. High-resolution XPS-analysis of C1s spectra of PPAAm (a), the immobilization of polyethylene glycol diacid (PEG DA, b) in the first and the following coupling of collagen I (COL, c) in the second step.

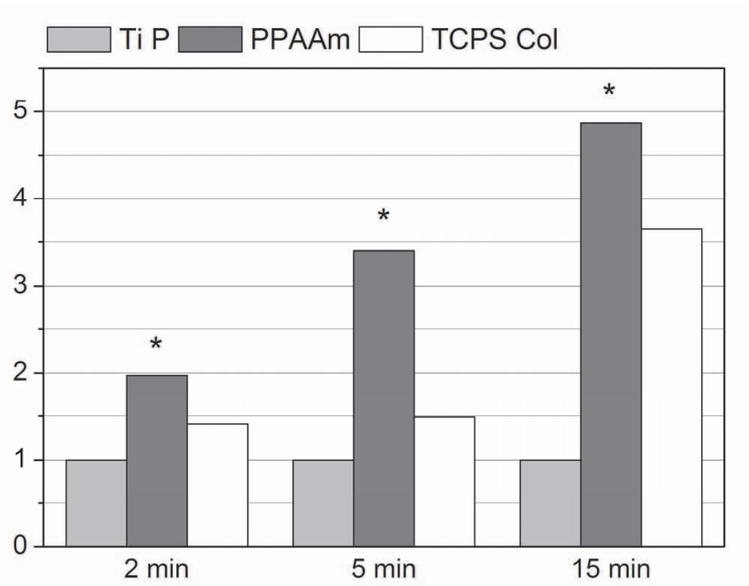

Fig. 10. Comparison of the initial osteoblast adhesion on titanium biofunctionalized with PPAAm vs. a COL-treated surface. Note that already after 2 min cell adhesion is impressively improved on PPAAm which is significant, but not for cell adhesion on COL (details see Nebe et al., 2007). Human MG-63 cells, adherent cells related to Ti_P=1, flow cytometric approach, T-test paired, *p<0.01.

experiments can be found elsewhere (Finke et al., 2007; Nebe et al., 2007; Rebl et al., 2010a). Furthermore, covalent coupling reactions of the plasma generated amino groups with collagen I (COL) were carried out to compare the cell adhesion between solely surface charges received by PPAAm and additionally immobilized COL. For the immobilization of collagen I the bifunctional spacer polyethylene glycol diacid (PEG DA) catalyzed by carbodiimide, was used. Collagen I is a main component of the extracellular matrix whose usefulness for the improvement of implant surfaces was already shown (e.g. Yang et al., 2009).

Although many studies apply the technique of the bioactivation of material surfaces with immobilized proteins and peptides, our results indicated that bioequivalent functionalization of titanium with the positively charged amino-groups (PPAAm) is sufficiently enough to significantly improve initial steps of the osteoblast's contact to the titanium surface (Fig. 10). Not only short time adhesion was impressively improved but also cell functions, e.g. spreading characterized by the time-dependent increase of the cell area as well as the organization of the actin cytoskeleton (Fig. 11) (Finke et al., 2007; Nebe et al., 2007). The induced development of the actin cytoskeleton on PPAAm was accompanied by longer actin filaments and a higher number of stress fibres per cell.

Finally, this PPAAm nanolayer was able to stimulate the cell occupation of the biomaterial surface by cells.

In our studies MG-63 osteoblastic cells (human osteoblastic cell line, ATCC, CRL-1427) were used and cultured in serum-free Dulbecco's modified Eagle medium (DMEM, Invitrogen) to investigate cell behavior on PPAAm deposited, polished titanium substrates (roughness average 0.19 µm, Lüthen et al., 2005) in comparison to untreated titanium substrates (grade 2) and to collagen I (COL) coated tissue culture polystyrene (TCPS). The improvement of adhesion and spreading of osteoblasts on plasma polymer deposited thin films of PPAAm was more increased as found on COL coated surfaces or on Ti-PPAAm additional immobilized with COL via the spacer PEG-DA (Finke et al., 2007).

To engineer the direction of a cell response to implant surfaces, it is essential to gain clear insights in how cell adhesion mechanisms contribute to cell-material interactions at the interface of plasma modified surfaces. Earlier in our cell studies we recognized hyaluronan (HA) - a large linear glycosaminoglycan with carboxyl groups in the molecule - as to be responsible for osteoblast attachment in the first phase of the material surface occupation (Nebe and Lüthen, 2008; Nebe et al., 2007). Because of the net negative charge of HA the strong adhesion capacity of the positively charged PPAAm is explainable based on electrostatic power. Cells get a growth advantage due to this very early strong adhesion. Although these plasma-based surfaces are non-protein coated surfaces without any ligands for integrin receptors cell's development is pushed as lot of our experiments could demonstrate: the spreading was increased, the formation of adhesion components (paxillin, vinculin) was pronounced (Finke et al., 2007; Nebe et al., 2007) and an increase in long term adhesion of living cells could be observed, accompanied by enhanced cell migration (Rebl et al., 2010a; Rebl et al. 2010b).

The cell physiology of cells on plasma polymerized allylamine is altered in a positive manner - signalling molecules in the downstream signal cascade become stimulated intracellularly or are faster in their phosphorylation process (Fig. 12).

Further interdisciplinary research is required to find out new possibilities of low temperature plasmas to optimize titanium implants because plasma processes are preeminently suitable for the goal-directed finishing of their surfaces.

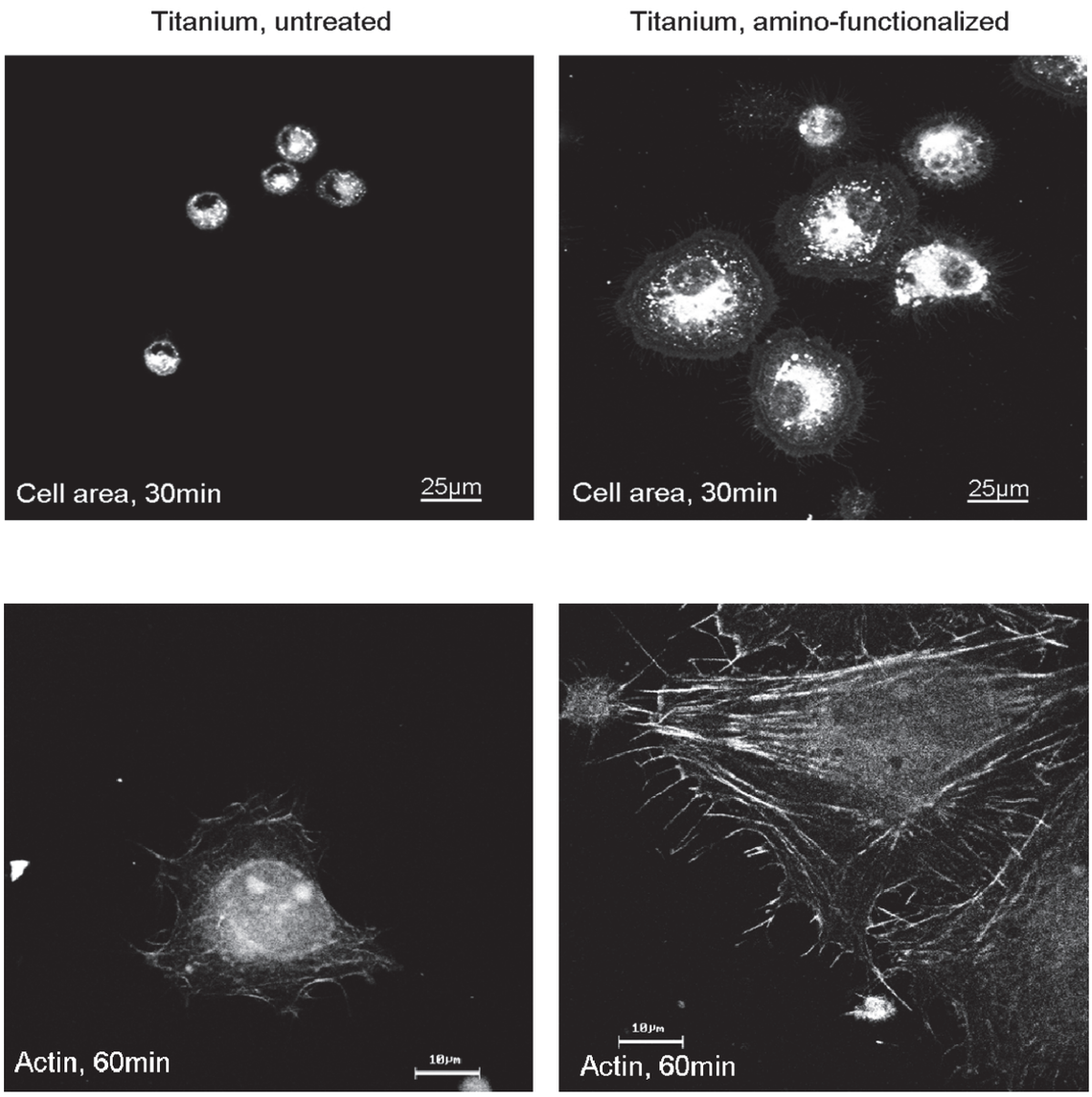

Fig. 11. Positive charges due to the deposition of plasma polymerized allylamine (PPAAm) induce cell spreading (above) as well as the development of the actin cytoskeleton (below). The cell area is increased 6 fold already after 30 min and many of the actin filaments are impressively formed as stress fibres after 60 min of cell culture.
Human MG-63 osteoblasts; cell staining: PKH-26 for spreading, phallacidine BODYPI for actin; confocal microscopy, LSM 410, Carl Zeiss, 63x oil immersion objectives 1.25 oil/0.17.

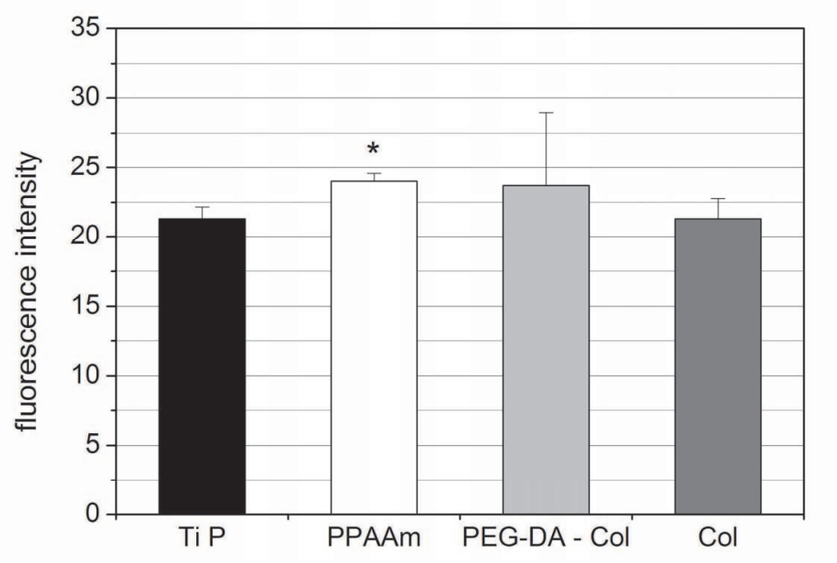

Fig. 12. The plasma deposition of PPAAm on titanium stimulate the cell physiology compared to the pure polished titanium surface (Ti P) to the same extent as an additional COL immobilization via the spacer PEG DA. The phosphorylated signal protein IkappaB (p-IκB-a), responsible for the activation of NFkappaB in the downstream signalling process in cells, is significantly enhanced in human osteoblasts due to PPAAm. Human MG-63, Bio-Plex analysis, BioRad, n=3, T-test, *$p < 0.05$.

3.3 Surface functionalization by plasma deposition of functional groups for regulation of stem cell behavior

Surface functionalization for bioengineering applications may have several different goals. By the plasma-based deposition of functional groups like -OH, -COOH, -CHO, Epoxide or -NH_2 the surface-bound of spacer molecule for cell immobilization, containing hydrocarbon chains, cyclic compounds or ethylene oxide chains may be improved. In addition the covalent fixation of bioactive molecules and the immobilization by self-assembly (by van-der Waals interactions, hydrogen bridges or ionic interaction, hydrophobic-hydrophobic interaction and thiols on gold) determines the bioactivity of the surface and the surface-bound of bioactive molecules as well. These effects can be used to achieve the immobilization of proteins, DNA, sugars and fatty acids or even bacteria. On the other hand the repulsion of biomolecules can be utilized for instance for antifouling applications. Similiar immobilization strategies are used as well for the covalent collagen immobilization for stem cell adhesion. The adhesive and cell stimulatory properties of plasma modified surfaces were investigated using human stem cells important for orthopedic and dental implantology and are described here.

The gas discharge-based processing of the substrate material first applied an ultrathin plasma polymer allylamine (PPAAm) coating on the plasma-activated titanium surface to modify the water contact angle from a typical value of 77° ± 3° of pure titanium to 48° ± 3°. Then, a spacer substance, as for example polyethylene glycol diacid (PEG-DA), was deposited on the PPAAm to achieve a covalent immobilization of collagen I (COL). The chemistry and length of the spacer determines the physico-chemical properties of the surface and so the efficiency of the collagen coupling.

In Fig. 13 the bonding components in the C1s peak of PPAAm- and PPAAc-prepared surfaces are demonstrated.

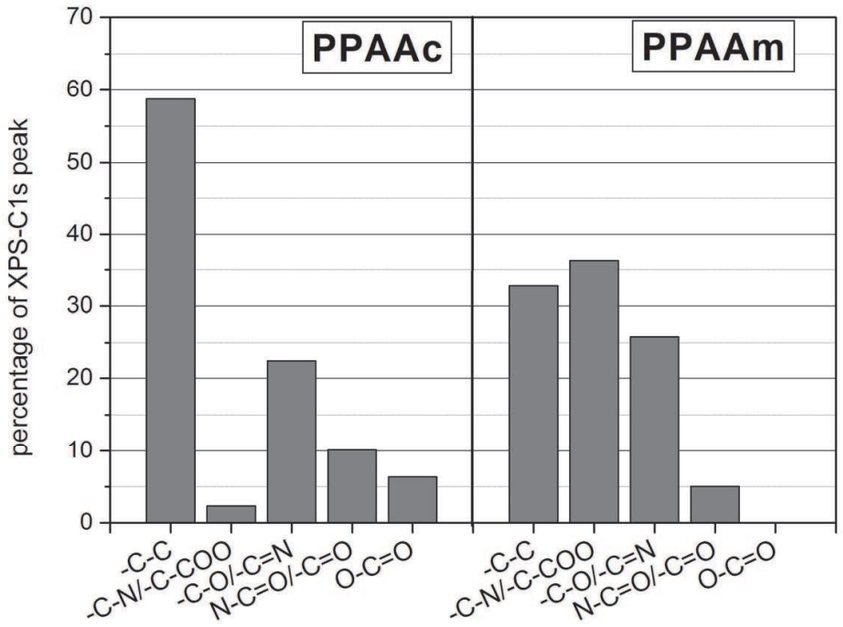

Fig. 13. Percentages of bonding components in C1s peak after film preparation on polished titanium for PPAAm (right) and PPAAc (left).

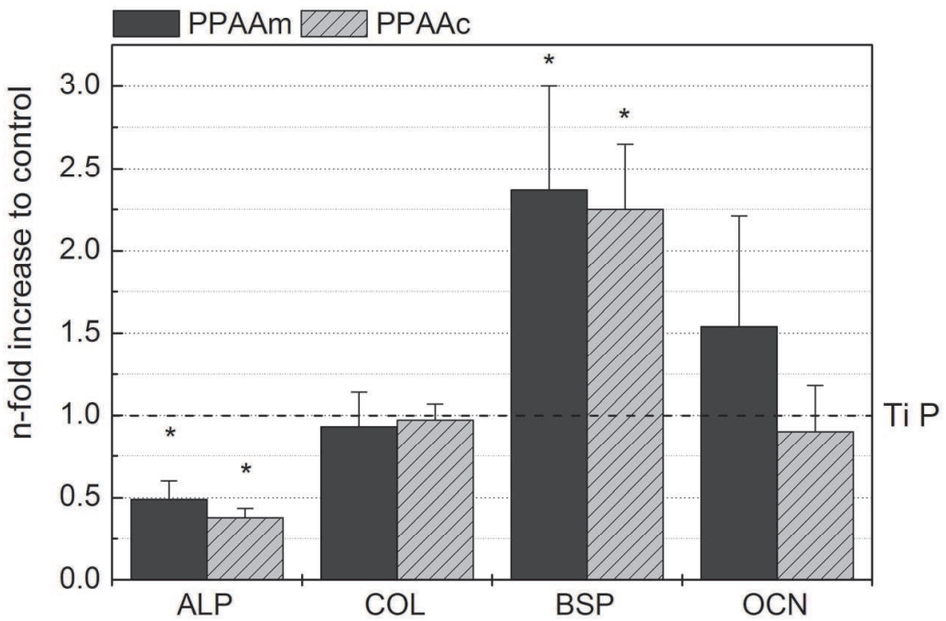

Fig. 14. mRNA expression for the osteogenic marker proteins ALP, COL, BSP, and OCN in stem cells hMSC on PPAAm and PPAAc in osteogenic medium for 3 days, compared to cells on Ti P (as control). Note the increase of BSP and OCN on PPAAm and PPAAc in osteogenic medium (details see Schröder et al., 2010c). The influence of the medium seems to be dominant. Cells: hMSC (Lonza), quantitative real-time RT-PCR, ABI PRISM 7500, Applied Biosystems, calculated by the comparative ΔΔCT-method and normalized to Ti_P (control), n=3 (3 measurements each), U-test, *p < 0.1.

Different surface functional groups are reported to induce various differentiation of stem cells manifesting in discret gene expression. According to a human osteoblast differentiation model, alkaline phosphatase (ALP) and collagen 1 (COL) mRNA´s are early differentiation markers in the osteoblast lineage and decline again during osteoblast maturation. On the other hand, bone sialo protein (BSP) and osteocalcin (OCN) mRNA´s are expressed at very low levels in the early osteoblast differentiation stage, but transcription is enhanced during later differentiation stages (Billiard et al., 2003). In our studies (Schröder et al., 2010d) we

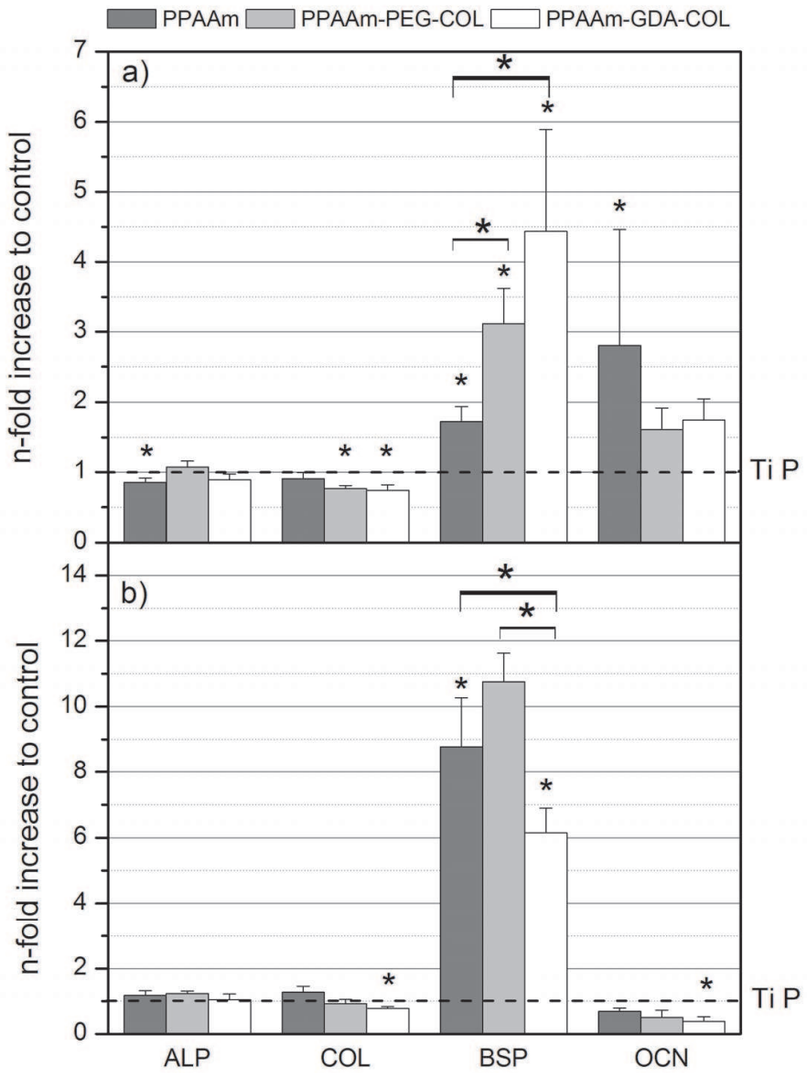

Fig. 15. mRNA expression for the osteogenic marker proteins ALP, COL, BSP, and OCN in stem cells hMSC on PPAAm, PPAAm-PEG-COL and PPAAm-GDA-COL after 14 days of culture in basal (A) and osteogenic medium (B), compared to cells on Ti P (control). Note the huge increase of BSP as late stage diffentiation marker after 14 days of culture. Cells: hMSC (Lonza), quantitative real-time RT-PCR, ABI PRISM 7500, Applied Biosystems, calculated by the comparative ΔΔCT-method and normalized to Ti P (control), n=3 (3 measurements each), U-test, *p < 0.1.

investigated the influence of positivly and negatively charged plasma deposited polymers e.g. PPAAm and plasma polymerized acrylic acid (PPAAc). We could observe that BSP late-stage differentiation-related mRNA expression was enhanced in human stem cells (hMSC, Lonza) cultivated on PPAAm and PPAAc in osteogenic medium for three days relatively to Ti_P, whereas OCN is upregulated on PPAAm only (Fig. 13). Concerted with the increase of late-stage differentiation markers the downregulation of ALP and COL is understandable as a sign of maturation. These results show that both positively and negatively charged surfaces facilitate osteogenic differentiation controlled by the osteogenic medium used. The osteogenic medium may have the dominant effect on cell behavior in these approaches, but additional charges at the surfaces due to functional groups suppose differentation. Apart from that the spreading of hMSCs on PPAAm surfaces in basal cell culture medium without osteogenic factors was significantly enhanced, but reduced on PPAAc (data not demonstrated). The positively charged PPAAm on Ti surfaces can promote differentiation of hMSC´s independently of osteogenic supplements in the culture media, but not a PPAAc deposition.

The positive influence of PPAAm surfaces on the differentiation of hMSC could be further improved by covalent immobilization of COL through spacers e.g. polyethylene glycol diacid (PEG) and glutardialdehyde (GDA). After 14 days of culture on these surfaces hMSC´s showed a significantly enhanced BSP mRNA expression under basal culture conditions (Fig. 15A). Here a significant impact due to covalent coupling of COL could be observed for both coupling methods, which reached its peak for PPAAm-GDA-COL. But a significant influence was detectable also for PPAAm alone.The increase of BSP mRNA expression on PPAAm surfaces relativly to non treated Ti P was further developed using osteogenic medium (Fig. 15B). Osteogene stimulation obliterated differences resulting from COL coupling. Thus PPAAm treatment of Ti seems to be sufficant for surface optimation in respect of diffentiation of hMSC´s under osteogenic conditions.

4. Acknowledgments

We would like to thank U. Kellner, U. Lindemann, G. Friedrichs (INP), I. Mardi (Zell-Kontakt) and A. Peters, J. Wetzel (University of Rostock) for the excellent technical accomplishment of the experiments. We appreciate technical support of G. Fulda of the Electron Microscopic Center of the Medical Faculty of the University of Rostock. This study was supported by the Federal State of Mecklenburg-Vorpommern and the Helmholtz Association of German Research Centres (UR 0402210), by the Federal Ministry of Education and Research (Campus PlasmaMed, grant No. 13N9779 and 13N11188) and by AIF (FKZ: KF0086501UL4).

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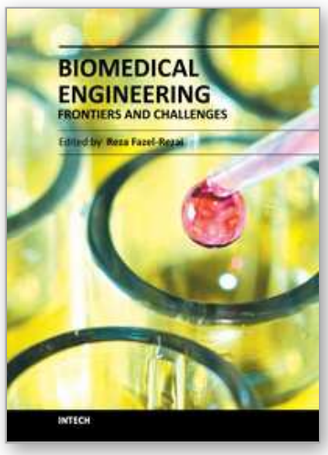

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Edited by Prof. Reza Fazel

ISBN 978-953-307-309-5
Hard cover, 374 pages
Publisher InTech
Published online 01, August, 2011
Published in print edition August, 2011

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