Published in final edited form as:

Macromol Biosci. 2011 November 10; 11(11): 1515–1525. doi:10.1002/mabi.201100124.

Self-Assembled Antimicrobial and biocompatible copolymer films on Titanium

Cornelia Pfaffenroth

Institute for Technical Chemistry, Braunschweig University of Technology, Hans-Sommer-Str. 10, D-38104 Braunschweig, Germany

Andreas Winkel

Department for Prothodontics, Hannover Medical School, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany

Wibke Dempwolf

Institute for Technical Chemistry, Braunschweig University of Technology, Hans-Sommer-Str. 10, D-38104 Braunschweig, Germany

Lara J. Gamble

National ESCA and Surface Analysis Center for Biomedical Problems, Departments of Bioengineering and Chemical Engineering, Box 357015, University of Washington, WA 98195, USA

David G. Castner

National ESCA and Surface Analysis Center for Biomedical Problems, Departments of Bioengineering and Chemical Engineering, Box 357015, University of Washington, WA 98195, USA

Meike Stiesch

Department for Prothodontics, Hannover Medical School, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany

Henning Menzel*

Institute for Technical Chemistry, Braunschweig University of Technology, Hans-Sommer-Str. 10, D-38104 Braunschweig, Germany

Abstract

Biofilm formation on biomedical devices such as dental implants can result in serious infections and finally in device failure. Polymer coatings which provide antimicrobial action to surfaces without compromising the compatibility with human tissue are of great interest. Copolymers of 4-vinyl-N-hexylpyridinium bromide and dimethyl(2-methacryloyloxyethyl) phosphonate are interesting candidates in this respect. These copolymers form ultrathin polycationic layers on titanium surfaces. As the copolymerization reaction is almost ideal statistical, copolymers with varying compositions can be synthesized and immobilized onto titanium surfaces for comprehensive screening concerning antimicrobial activity and biocompatibility. Copolymer films on titanium were characterized by contact angle measurements, ellipsometry and X-ray photoelectron spectroscopy. Antibacterial properties were assessed by investigation of adherence of *S. mutans* which represents a strain found in the human oral cavity. Biocompatibility was rated based on human gingival fibroblast adhesion, proliferation and cell morphology. Depending on polymer composition the coatings displayed a behavior ranging from biocompatibility equal to titanium but no antibacterial action to highly antimicrobial activity but poor biocompatibility. By

*h.menzel@tu-bs.de.

balancing these two opposing effects by tailoring chemical composition, copolymer coatings were fabricated, which were able to inhibit the growth of *S. mutans* on the surface significantly but still show a sufficient attachment of gingival fibroblasts.

Keywords

antimicrobial polymer coatings; biocompatibility; copolymerization; medical implants; cell adhesion

Introduction

Over the past few decades there has been a keen interest in antimicrobial modification of surfaces to prevent growth of microorganisms.^[1] Pathogenic bacteria can form biofilms on implant surfaces. In these biofilms the bacteria are embedded in an organic matrix of extracellular polysaccharides. Within they are perfectly protected, provided with nutrients and are able to strongly adhere to the surface. In case of biomedical devices such as dental implants biofilm formation can result in serious infections and finally in device failure.^[2] The weak point of dental implants is the passage from the bone into the oral cavity. If there is an imperfect attachment of the gingiva in this region, rapid adhesion of bacteria and biofilm formation is possible. Functional surfaces which selectively prevent biofilm formation but show a favourable impact on the adhesion of mammalian cells could be the key to long term success of such dental implants.

In nature host-defensive peptides like magainin or tachyplesin are known as macromolecules exhibiting antibacterial activity.^[3] These peptides target the lipid bilayer of bacteria cell membranes and act via permeabilization of cell membrane followed by aggregation and subsequent disruption.^[4] The ability of host-defense peptides to show selectivity against the membranes of microbes versus the host organism is of high interest for designing surfaces that inhibit bacteria growth but do not harm mammalian cells. This selective action is based on the fact that microbial membranes possess a more negatively charged outer surface whereas multicellular animals possess neutral and cholesterol rich membranes. Balance and spatial arrangement of hydrophobic and cationic amino acids within the host-defense peptide is therefore the key factor for this selectivity.^[5]

Being much more inexpensive and less laborious to prepare, synthetic polymers showing antimicrobial activity are a promising approach to create antimicrobial surfaces. Polymers assigned to this class are membrane disrupting cationic macromolecules most commonly with quarternary ammonium or phosphonium groups.^[6-8] So far most studies with these polymers report on investigations regarding their effectiveness against a wide range of bacteria. Biocompatibility testing is less comprehensive and has been carried out with different assays. For example Fischer et al. carried out hemolysis experiments with poly(vinyl pyridinium bromide) solutions observing no hemolytic effects up to a concentration of 10 mg/ml during an incubation period of 1 hour.^[9] On the other hand Allison et al. investigated the hemolytic behavior of copolymers of 4-vinyl-N-hexylpyridinium bromide and hydroxyethylmethacrylate as well as polyethylene glycol methyl ether methacrylate (PEGMA).^[10] Applying comparable conditions to Fischer et al. during hemolysis studies Allison et al. observed that an increase in 4-vinyl-N-hexylpyridinium bromide content leads to a blood response switching from almost no hemolysis to complete hemolysis at a critical comonomer content. Concerning the biocompatibility of polymers containing quarternary ammonium groups on living cells Fischer et al. measured poly(vinyl pyridinium bromide) standards for their effect on mouse fibroblasts with respect to membrane damage and affect on metabolic activity.^[9] In brief

none of the polymers tested induced significant membrane damage, but an increase in the average number of cationic charges per monomer unit in partially quarternized polymers was correlated with an increase in cytotoxic effects concerning metabolic activity. Stratton et al. studied copolymers of N-hexylvinylpyridinium bromide and poly(ethylene glycol) methyl ether methacrylate (PEGMA 1100) with respect to their biocompatibility towards human intestinal epithelial Caco-2 cells.^[11] It was found that the homopolymer of PEGMA 1100 as well as the copolymer containing 10 % N-hexylvinylpyridinium bromide caused no detectable cell death. However the copolymers containing 50 % and more N-hexylvinylpyridinium bromide were less biocompatible. Recently Stratton et al. developed a promising approach to correlate the results of red blood cell hemolysis with the results of in vitro cell viability testing for copolymers of N-hexylpyridinium bromide and PEGMA.^[12] However, a comprehensive understanding of which properties render antimicrobial polymers compatible with mammalian cells is not yet available.

Classical schemes to render surfaces antimicrobial by immobilization of quarternary ammonium compounds involve several modification steps or binding via silane groups that bear the risk of being hydrolyzed under physiological conditions.^[13] For example Tiller et al. first coated glass slides with aminopropyltrimethoxysilane and subsequently reacted them with acryloyl chloride. The acryl groups at the surface were then used for a graft polymerization with 4-vinylpyridine. Final reaction step was the conversion of surface bond polyvinylpyridine into the corresponding quarternary pyridinium compound using alkyl bromides.^[6]

In comparison Sambhy et al. report the synthesis of a composite material of silver bromide embedded in the cationic polymer poly(4-vinylpyridine)-*co*-(4-vinyl-N-hexylpyridinium bromide) as organic component. In that case immobilization of the composite was limited to electrostatic interactions of the cationic pyridinium groups with a negatively charged glass surface.^[14] Introduction of methoxysilane groups by alkylation of the polyvinylpyridine partially with 1-bromopropyltrimethoxy silane resulted in polymers which attach to several substrate materials like glass, silicon, stainless steel, copper, gold and Parylene-C. The films are not only bound to the surface via reaction of the silane group with hydroxyl groups at the surface but also crosslinked via silane condensation.^[13] The films do not show any antibacterial activity unless silver nanoparticles are introduced^[13], which are known to show antibacterial activity.^[15]

Copolymers with (4-vinylbenzyl)phosphonic acid diethylester have been described in an earlier study to self assemble on metall oxides as found on most metall surfaces in particular on titanium.^[16] The binding is a simple sequence of coating from a polymer solution, drying the polymer films and with that binding the polymer coils in contact with the surface and removing superfluous material by washing. This represents a simple and effective method for applying thin films on titanium and other metaloxide and ceramic surfaces. In order to introduce antibacterial activity a copolymer bearing phosphonate groups, which are able to bind to titanium surfaces, and quarternary ammonium groups, which can inhibit bacteria growth, have to be combined. We herein describe a copolymer system of 4-vinyl-N-hexylpyridinium bromide and dimethyl(2-methacryloyloxyethyl) phosphonate (P(HBVP-*co*-DMMEP)). This copolymer should be able to bind to titanium by means of the phosphonic ester group and to show antibacterial activity. The layers formed by the copolymer on titanium were examined by contact angle measurements, ellipsometry and XPS. The antibacterial activity was tested via bacterial adhesion of *S. mutans*. Furthermore, the compatibility of the polymer films with gingival fibroblasts was investigated using an LDH assay to check if the copolymer coatings provide an antimicrobial effect without compromising the compatibility with human gingival fibroblasts.

Experimental Part

Materials and Instrumentation

Dimethyl(2-hydroxyethyl)phosphonate, triethylamine and 4-vinylpyridine were purchased from Acros Organics. 2,6-Di-tert-butyl-4-methyl phenol and 1-bromohexane were obtained from Aldrich. Methacryloyl chloride was purchased from Alfa-Aesar. All solvents were dried using standard procedures.^[17] Triethylamine and 4-vinylpyridine were dried by distillation from CaH₂ prior to use.

Ti90/Al6/V4 (Goodfellow) foil was cut into discs with 13 mm in diameter using a water jet cutting system. Afterwards the discs were sanded with 800, 1200 and 2500 grit silicon carbide paper and polished with colloidal silica (type MasterMet and MasterMet 2, Buehler GmbH, Germany) on a ChemoMet Polishing Cloth. Subsequently the discs were rinsed and sonicated twice in dichloromethane, acetone, methanol and Millipore water 10 minutes each, dried in a stream of nitrogen and stored under vacuum at 120 °C. Before use the substrates were sonicated in HPLC-grade solvents, namely twice in dichloromethane, acetone, methanol and twice in Millipore water 10 minutes each and dried in a stream of nitrogen.

¹H NMR, ¹³C NMR and ³¹P NMR spectra were recorded on a Bruker AM400 instrument. Elemental Analysis was performed using a Thermo Quest CE Instruments Flash EA 1112. Molecular weight analysis was carried out using SEC in aqueous solution containing 0.05 wt.-% NaN₃. PL aquagel-OH 8 µm mixed columns, 40°C and a flow rate of 0.5 mL / min. were applied. Detection was performed using a Wyatt Technology Dawn DSP light scattering detector and a Shodex RI-101 refractive index detector using ASTRA software, Wyatt.

The cells used for biocompatibility testing were Human Gingival Fibroblasts (HGFIB, Cat. No.: 121 0412) purchased from Provitro GmbH, Berlin, Germany. The cells were cultured in Dulbecco's modified Eagle's medium (FG0435, Biochrom AG, Berlin, Germany) supplemented with 10 vol.-% fetal bovine serum (P270521, PAN-BIOTECH GmbH, Aidenbach, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin (A2212, Biochrom AG, Berlin, Germany). Cells were cultured at 37 °C in a 5 % CO₂, 95 % humidified air atmosphere.

For the evaluation of bacterial adhesion on different implant coatings a *Streptococcus mutans* strain isolated from carious dentine (DSM 20523^T, DSMZ, Braunschweig, Germany) was purchased, aliquoted and stored in glycerol stocks until usage. At least one day prior to experiments bacteria were inoculated in tryptic soy broth medium (TSB; 30 g Tryptone Soya Broth (CM0129T, Oxoid, Cambridge, England), 3 g yeast extract (2363, Roth, Karlsruhe, Germany), pH 7.1 – 7.3 adjusted with 37 % HCl (J.T. Baker, Deventer, Netherlands)) and incubated at 37 °C under rotation (500 rpm) until reaching late stationary phase.

Synthesis

Monomer synthesis of dimethyl(2-methacryloyloxyethyl) phosphonate DMMEP was carried out according to Bressy-Brondino et al.^[18] Free radical copolymerization of DMMEP with 4-vinylpyridine (VP) was carried out using 2 molar monomer solutions in THF. Thirteen different ratios of the monomers were tested. 2 mol-% AIBN was used as initiator. The mixture was allowed to react for 16 hours at 60 °C. Polymerization was terminated by precipitation in diethyl ether resulting in a white polymer. The product was filtered and dried overnight under reduced pressure at room temperature. The polymers were characterized by GPC, NMR and elemental analysis. The composition was calculated using the C/N ratio from elemental analysis.

N-alkylation was achieved by heating the copolymers in nitromethane adding 1.5 equivalents of 1-bromohexane with respect to pyridine units at 72 °C for 68 hours. The polymers were then isolated by precipitation in diethyl ether and dried in vacuum for 24 hours. The degree of N-alkylation was determined from the ¹H-NMR peak ratios.

Preparation of Polymer Layers and Coating Characterization

A solution of the polymer (10 mg/ml) in methanol was spin coated onto the titanium discs at a speed of 2000 rpm for 30 seconds. The discs were heated in an oven at 120 °C for 18 h and then sonicated six times in HPLC-grade methanol 10 minutes each in order to remove unbound polymer.

Water contact angle measurements were performed after washing of polymer coated surfaces using the tilting plate method applying a tilt angle of 45°. A minimum of five measurements on different spots were recorded for each substrate.

Polymer film thicknesses were determined using a Multiskop® (Optrel, Germany) in the null ellipsometer mode. Each titanium disc was measured as reference prior to the polymer coating process for determination of substrate refractive index (RI) and extinction coefficient. Polymer layer thicknesses were based on a single film model relating values to RI of the polymer films to find the thickness (assumed RI of 1.5). Five ellipsometric measurements were made at different spots for each sample.

XPS measurements were performed with a Kratos AXIS Ultra DLD instrument using a monochromatic Al K X-ray source. Compositional survey was acquired using a pass energy of 80 eV. Spectra were taken at a takeoff angle of 90° with respect to the sample surface plane. Three spots on two replicates of each sample were analyzed. Data processing was carried out employing the Vision2 software.

LDH activity assay

A modified LDH activity assay as described elsewhere^[19] was used to evaluate biocompatibility of the polymer coatings to primary human gingival fibroblasts. This approach includes the evaluation of calibration plots relating the number of gingival fibroblast cells present on distinct titanium surfaces to the optical density in the LDH activity assay after trypsinization of attached cells. For adhesion/proliferation experiments surface coated titanium discs were placed in 24-well cell culture plates under sterile conditions. Cells were seeded at a density of 1.5×10^4 cells/ml/well. After an incubation period of 24 and 72 hours respectively viability of the cells attached to the titanium disc surfaces was determined by washing them with 1 ml HBSS (L2035; Biochrom AG, Berlin, Germany) once and covering them with another layer of 1 ml HBSS. Subsequently 250 µl of 10 % Triton X-100 solution (93416, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were added and cells were incubated at 37 °C for 30 minutes. 100 µl of lysed cell solution were then transferred into 96-well plates and combined with 100 µl of the staining solution (Cytotoxicity Detection Kit, 11 644 793 001, Roche Diagnostics GmbH, Mannheim, Germany – ratio 1:46). Plates were incubated in the darkness for 10 minutes until the staining reaction was stopped by adding 50 µl 1 N HCl. Cell counts were then quantified by measurement of optical densities using an ELISA reader (Infinite F200, Tecan Group Ltd., Männedorf, Switzerland) at 490 nm. The cell number and viability was determined relative to that of a control series containing non-coated titanium substrates which were incubated for 24 and 72 hours respectively.

Statistical analysis was performed using the two-tailed Wilcoxon test for paired non-normally distributed data with a confidence level of 0.05. All values reported are given as the mean values. The standard deviation is specified as well.

Cell morphology of the fibroblasts attached to the polymer coated titanium discs was analyzed by SEM. For that purpose samples were rinsed with PBS (L1825, Biochrom AG, Berlin, Germany) and fixed in 2.5 % glutaraldehyde diluted in 0.1 M cacodylate buffer for 2 hours. After rinsing with 0.1 M cacodylate buffer samples were dehydrated in graded ethanol solutions before applying critical point drying. SEM samples were mounted on stubs and sputter coated (POLARON Sputter Coater SC7500, Ringmer, England) with a thin layer of gold and examined in a SEM 505 (Philips, Eindhoven, Netherlands) at 5 kV.

Antibacterial Testing

S. mutans was used for all experiments regarding the bacterial adhesion. After overnight culture *S. mutans* was centrifuged at 6000 rpm and 4 °C for 15 minutes. The resulting pellet was washed twice, each with 20 ml of 50 mM Tris HCl buffer (pH 7.5), and finally resuspended in 10 ml of the same buffer. After mixing thoroughly the optical density at 600 nm was measured and adjusted by dilution to a final value of 1.2 (which equates to 6×10^7 cfu/ml). In a static cultivation system 2 ml of the bacterial suspension were seeded on three replicates of each titanium sample coated with the polymer as well as a bare titanium sample as reference placed together in 35 mm culture dishes. The bacterial cells were allowed to adhere onto the sample surfaces by incubation in a wet chamber under gentle rotation for 1 hour at 37 °C. Afterwards unattached cells were removed by rinsing the samples six times with distilled water. The adhered bacteria were fixed with 2.5 % glutaraldehyde (3778, Roth, Germany; 1:10 dilution with PBS) for 30 min at 4 °C. For further storage at 4 °C the fixation solution was replaced by 3 ml PBS (L1825, Biochrom, Germany). Adhered bacteria were stained with 1% Acridine Orange (0249, Roth, Germany; 1:10 dilution with 50 % Ethanol) and incubated in absence of light for 30 min at room temperature. Subsequently, samples were rinsed several times with distilled water to remove excess dye, then covered with PBS and analyzed by confocal laser scanning microscopy (CLSM, Leica Upright) at 63-fold magnification.

Results and Discussion

Polymer Synthesis and Characterization

A series of copolymers of 4-vinylpyridine VP and dimethyl(2-methacryloyloxyethyl) phosphonate DMMEP was prepared by free radical polymerization in order to determine copolymerization parameters and to study the influence of the copolymer composition on antimicrobial activity as well as on biocompatibility. The resulting polymers were characterized by NMR and elemental analysis. The prepolymers of those polymers used for biological testings (Table 1) were also characterized by SEC. SEC measurements were performed in aqueous solution containing 0.05 wt.-% NaN_3 as eluent. All polymers have a monomodal distribution and show a range of molecular weights of about 32 – 42 500 g/mol relative to pullulan standards.

A typical NMR of a copolymer is shown in Figure 1. As the NMR integrals required for determination of the copolymer composition were not perfectly separated, the copolymer compositions were determined via elemental analysis by means of the C/N ratios. By plotting copolymer compositions against monomer feed composition the correspondent copolymerization diagram was constructed (Figure 2). As can be seen from this plot the copolymerization is almost ideal statistical, since the data points are lying close to the diagonal line. The plot is only slightly s-shaped. Hence, any user-defined copolymer composition can be adjusted well-directed. Determination of copolymerization parameters according to the method of Kelen and Tüdös result in $r_1 = 0.856$ for DMMEP and $r_2 = 0.657$ indicating that the copolymers show a slight tendency to add the monomers not perfectly statistical but in an alternating order.

Pyridine groups readily react under mild conditions with n-haloalkanes via nucleophilic displacement to form N-alkylpyridinium halides. The precursor copolymers were N-alkylated with 1-bromohexane to form N-hexylpyridinium bromide which should enable ideal penetration of bacterial cell membranes.^[6] The degree of N-alkylation determined by ¹H NMR spectroscopy indicates that the quaternization reaction went to near completion.

Polymer Layers

As titanium and its alloys are commonly used as implants for dental replacements^[20] we have chosen a titanium alloy (90% Ti, 6% Al and 4% V) as substrate material. The surface of titanium metal readily oxidizes but the native oxide layer passivates the metal surface. Alkanephosphonic acids have been reported to self-assemble on the native oxide surface of titanium. Adden et al.^[16] reported the fabrication of a strongly surface-bound film of phosphonate containing copolymers on the titanium oxide surface. The P(HBVP-co-DMMEP) copolymers prepared here were applied to titanium samples using the protocol developed by Adden et al.^[16] The polymer overcoats were deposited onto titanium discs by spin coating from methanol solution and annealed at 120°C for 18 hrs. Excess polymer was subsequently removed by thorough solvent washing with sonication leaving a monolayer of polymer coils at the surface.

Surface wettability of the films was investigated by determination of advancing and receding contact angles employing the tilted plate method.^[21] Contact angle determination for uncoated titanium substrates directly after washing and cleaning showed values of about $\theta_{adv} = 33^\circ$ and $\theta_{rec} = 22^\circ$ changing to $\theta_{adv} = 84^\circ$ and $\theta_{rec} = 64^\circ$ after aging of the substrates. The contact angles of the copolymer coatings vary in the range of 58° to 72° for θ_{adv} and in the range of 40° to 50° for θ_{rec} , depending on copolymer composition (Table 2). The contact angle of P(DMMEP) homopolymer displays values of 51° for θ_{adv} and 34° for θ_{rec} which are in good agreement with the values reported previously.^[22] The water contact angle measurements clearly indicate the presence of surface-anchored polymer coatings. Moreover plotting of the contact angles over 4-vinyl-N-hexylpyridinium bromide (VP-HB) content of the copolymers reveals a defined correlation (Figure 3): An increase in VP-HB content results in a decrease in contact angle values and therefore in a decrease in surface hydrophobicity. Thus it can be concluded that the influence of cationic charge density due to the pyridinium groups within the copolymer overbalances the influence of hydrophobic hexyl side chains with regard to surface wettability. The homopolymer P(DMMEP) is more hydrophilic than any of the copolymers.

Ellipsometry was used to determine the thickness of the copolymer coatings on titanium substrates. In general the layer thickness obtained by the grafting onto method is a function of the bulk radius of gyration (R_g) of the polymer applied. As there is a linear relationship between layer thickness and R_g and the R_g values are a function of the average molecular weight (M_w) of the polymer a similar correlation between layer thickness and M_w can be observed.^[22,24] The copolymers of N-hexylpyridinium bromide and DMMEP applied for surface coating here do not differ significantly or even systematically in molecular weight. Thus no differences in resultant layer thicknesses are expected. As can be seen from Table 2 the layer thickness does indeed not depend on the molecular weight of the polymer but a defined correlation between the film thickness and the phosphonate content of the applied polymer is observed (Figure 4). This can be explained by two approaches. First the polymers investigated in previous studies^[22] have been homopolymers of the same monomer but showing distinct variations in molecular weight. In the present study copolymers with comparable molecular weight but different compositions have been used. Therefore in the case of copolymers showing different compositions it might be assumed that a comparable molecular weight does not necessarily result in a comparable bulk radius of gyration. Thus different copolymer compositions will result in different layer thicknesses. Even more

important might also be the assumption that the comonomers selectively interact with the substrate surface. Thus a deviation from coil shape is expected whereas the dimension of this deviation will depend on the copolymer composition. Furthermore, an increase in polymer phosphonate content implies an increase in the number of binding sites between polymer chain and substrate surface. In addition aggregation phenomena might have to be taken into account, in particular for the copolymer with the highest DMMEP content. In this case the layer thickness is significantly higher than expected. Typically a thickness between 2 and 10 nm is observed for polymer monolayers.^[23,24] However, upon aggregation of polymer coils higher values would result. It has to be noted that an increase in DMMEP content not only results in an increase of the layer thickness but in higher variations, as can be estimated from the standard deviation. This might indicate a less homogeneous distribution of the polymer coils on the substrate surface caused by aggregation phenomena.

XPS measurements were performed on polymer-coated titanium oxide surfaces to prove the chemical identity of the attached macromolecules. Polished and solvent cleaned Ti90/Al6/V4 discs were measured as reference. It is well known that exposed surfaces of titanium are spontaneously covered by a 3–6 nm layer of titanium oxide.^[25] Thus expected elements of XPS measurements on native polished titanium discs are Ti and O. Assuming that titanium is mostly present in the Ti^{IV}-form within the titanium oxide layer^[25] it can be seen from Table 3 that there is a discrepancy between the measured (3:1) and the expected (2:1) ratio of oxygen to titanium. Possible reasons for this discrepancy are adsorption of atmospheric oxygen and water on the surface of oxidized titanium.^[25] Beside Ti and O of the native titanium oxide layer contamination with C and N were detected as well, originating from traces of organic contaminants.^[25,26] The extent of these contaminants is within acceptable limits so that no interference with subsequent modification steps has to be expected. After deposition of copolymer films the XPS spectra are dominated by a significant increase in carbon and nitrogen signals as well as the appearance of phosphorus and bromine signals due to the presence of the polymer. Signals from the underlying substrate are still visible but less distinct than in case of the non-coated reference substrates. Comparison of the substrates bearing copolymers of different compositions shows that an increase in phosphonate content results in an increase in XPS phosphorus signal. It can also be observed that an increase in copolymer VP-HB content causes an increase in XPS nitrogen signals (Table 3). Furthermore a slight decrease in Ti signal with increasing DMMEP content in the polymer coating indicates an increasing film thickness with increasing phosphonate content. As XPS has a limited penetration depth this finding confirms the data obtained by ellipsometric measurements. Beside the elemental compositions for the different polymer films the nitrogen to phosphorus ratios calculated from XPS findings are also of importance. The ratios show a decrease with increasing polymer phosphonate content. Nitrogen to phosphorus ratios of the bulk polymer determined via elemental analysis not only show the same trend but even the absolute values are in good agreement (Table 4). Although XPS analysis confirmed the presence of particular polymer coatings on the surface the bromine contents detected via XPS is much lower than the expected values. This finding can be easily explained as the conditions present during XPS measurements lead to a degradation of bromine. Therefore the bromine content could not be used for quantification of N-alkylation degree within the copolymer coatings. However, the carbon to nitrogen ratio can be used as an alternative tool to determine the degree of N-alkylation. As can be seen from Table 5 degrees of N-alkylation between 77.9% and 97.3% have been calculated using XPS data. Overall XPS values indicate successful binding of the phosphonate containing copolymers to titanium oxide surfaces. However, for polymer E a significant deviation was found between the degree of N-alkylation determined by H-NMR studies (almost complete) and XPS (only 11 mol-%). Similar results have been observed with other copolymers having a low content of pyridinium groups (data not shown). The exact reason for this behaviour is still unknown and subject of ongoing research.

Gingival Fibroblast adhesion, proliferation and cell morphology

To assess the compatibility of the copolymers with gingival fibroblasts the number of cells strongly attached to the coated titanium surfaces is calculated from the overall LDH activity relative to that of a control series with bare titanium substrates. The number of attached cells per mm² on non-coated titanium discs is set at 100 % and the number of adherent fibroblasts on polymer coated discs is calculated accordingly (Figure 5). After 24 hours of incubation the number of cells attached to the surfaces modified with polymers A to C is slightly enhanced compared to reference titanium, but there are no differences among the polymer coatings themselves. Comparison of the cell adhesion after 72 hours of incubation with that after 24 hours allows drawing conclusions regarding the cell proliferation rate. As the number of adherent fibroblasts is slightly lower on the modified surfaces compared to reference titanium after 72 hours it can be suggested that cell proliferation is most efficient on reference titanium. However, these differences are not statistically significant. Overall cell adhesion as well as cell proliferation indicate good biocompatibility for polymer coatings A to C and short term cytotoxic effects can be excluded. As can be seen from figure 5 coating D and E as well as the homopolymer showed a significantly different behavior. After 24 hours of incubation the number of adherent cells on substrates coated with polymer D is about 30 % lower than on reference titanium and even about 50 % lower after 72 hours of incubation. In case of polymer coating E the number of adherent cells is about 40 % lower than on reference titanium after an incubation period of 24 and 72 hours as well. As for polymer coating E the reduction of adherent cells is the same at both points in time the cell proliferation rate is comparable to reference titanium even though the absolute number of cells is decreased. Therefore, the initial cell adhesion is the parameter which is negatively influenced by the polymer coating, but cell toxicity in terms of a reduction in proliferation rate is not detected. On substrates coated with p(DMMEP) almost no cell adhesion is observed. This finding is in line with the investigation of Adden et. al. Screening different polymer surfaces for their ability to promote osteoblast adhesion p(DMMEP) was identified as a polymer coating showing very bad cell adhesion.^[23] Overall variation of DMMEP content within a range of 20 % to 40 % did not affect fibroblast adhesion and proliferation resulting in polymer coatings that are comparable to titanium reference. Increasing the DMMEP content further, a continuous decrease in the number of adherent cells can be observed whereas cell proliferation is only slightly or even not affected.

The results from the LDH-assay are supported by microscopic evaluation using SEM. Polymer coatings A to C show no visible effect on amount and morphology of attached gingival fibroblasts in comparison to uncoated titanium. Even with increasing portion of DMMEP in the copolymer (polymer D, Figure 6) cells demonstrate only sporadic changes in morphology, whereas LDH assay reveals slight reduction in the number of attached cells. First throughout morphological changes can be observed for coating E after 24 hours of adherence. Cells appear more rounded and less fibroblastoid, in some cases even giving the impression of less surface coverage despite only moderate cell reduction. This effect is compensated upon longer incubation and adhered fibroblasts reconstitute normal spindle-shaped appearance. Corresponding to the LDH-assay SEM shows for P(DMMEP) a serious loss of attached cells accompanied with substantial morphology changes after 24 hours already. After 72 hours only single fibroblasts can be detected on this type of surface coating.

Antimicrobial activity

Results of antibacterial testing show that with increasing content of DMMEP within the copolymer, the response to the substrate surfaces switches from a uniform bacterial spreading to almost no bacterial adherence. Coatings with copolymers A to C containing up to 41 mol-% DMMEP show no significant impact on bacterial adherence as shown

exemplary for *S. mutans*. Like on titanium control the single bacteria or short chains of agglomerated bacteria are uniformly spread at a comparable density over the whole surface (Figure 7). First effects on the pathogen distribution are observed for copolymer D containing 60 mol-% of DMMEP. Bacterial cells show pattern formation on this surface, partially forming complex structures. This can be an indication for increasing bacterial stress as described previously.^[27,28] However, distinct changes in the amount of adhered bacteria do not result from this aggregation. However, a further increase in DMMEP content to 76 mol-% for copolymer coating E decreases the number of adhered bacteria significantly. Only few bacteria remain present on the surface.

In the assay used here any bacteria which are killed and therefore do not attach to the surface are not detected in the assay. We also used live/dead staining (SYTO®9 and propidium iodide) to investigate the effect of the surface coating on the bacteria (data not shown). For *S. Mutans* the staining is not very effective, so that some dead bacteria may be falsely identified as living. Indeed most of the bacteria which in spite of the coating can be found on the surface seem to be still alive in this assay; however their number is significantly reduced. On the other hand the gingiva fibroblasts are still attaching to the coated surfaces and are proliferating. Therefore we conclude some antibacterial effect which efficiently reduces biofilm formation, even if a bactericidal action cannot unambiguously proven by the live / dead staining.

The increased efficiency of the copolymers against bacteria adhesion with increasing the amount of the more hydrophilic DMMEP is in line with observations by Sellenet et al. who investigated copolymers of 4-vinyl-N-hexylpyridinium bromide (HBVP) with hydroxyethylmethacrylate and oligoethyleneglycol methacrylate as hydrophilic monomers in solution. In all cases the introduction of the hydrophilic monomers improved the antibacterial effect of the copolymers compared to PHBVP homopolymer and in particular compositions with low amounts of HBVP showed strong effects.^[29]

Conclusion

To create polymers possessing antibacterial properties as well as biocompatibility and being able to bind to titanium surfaces a copolymer series of 4-vinyl-N-hexylpyridinium bromide and dimethyl(2-methacryloyloxyethyl) phosphonate was prepared by copolymerization of 4-VP and DMMEP followed by subsequent N-alkylation. Determination of the copolymerization parameters ($r_{\text{DMMEP}} = 0.856$ and $r_{4\text{-VP}} = 0.657$) characterized the copolymerization reaction to be almost ideal statistical with a slight tendency to add the monomers in an alternating order. Surface analysis indicates that the polymers are forming ultrathin polycationic layers on titanium surfaces. The layer thickness is a function of the copolymer composition or, more precisely, of the phosphonate content as evidenced by ellipsometry and XPS measurements.

Comparison of the results obtained for the adhesion of gingival fibroblasts and of *S. mutans* clearly reveals two opposing trends with respect to the composition of the copolymer layer. Antimicrobial effect of the surface is enhanced by an increase in the content of DMMEP within the copolymer whereas compatibility with human gingival fibroblasts is improved with decreasing content of DMMEP. A coating with P(HBVP-co-DMMEP) 24:76 shows a significant antimicrobial effect and is sufficiently biocompatible at the same time. To the best of our knowledge this is the first report on a copolymer, which can be applied in easy coating, drying, washing process to titanium implants and which shows antibacterial effect without compromising the compatibility with fibroblasts attachment.

Acknowledgments

The authors acknowledge financial support from the *DFG* within the *SFB 599 "Biomedical Technology"*

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Figure 1.
NMR of Poly(4-vinylpyridine-*co*-dimethyl(2-methacryloyloxyethyl) phosphonate) in CDCl₃.

Figure 2.
Copolymerization diagram of 4-Vinylpyridine with Dimethyl(2-methacryloyloxyethyl) phosphonate (DMMEP).

Figure 3.
Contact angle as function of copolymer composition for P(HBVP-co-DMMEP).

Figure 4. Layer thickness dependency as function of copolymer composition for P(HBVP-co-DMMEP).

Figure 5. Number of gingival fibroblasts adherent to polymer coated surfaces after 24 and 72 hours of incubation relative to titanium as reference.

Figure 6. SEM pictures of human gingival fibroblasts on titanium and a subset of polymer coatings after 24 and 72 hours of incubation.

Figure 7. Distribution of initial bacteria attachment for polymer coatings and control. (A) Blank titanium substrate. (B) P(HBVP-co-DMMEP) 40:60. (C) P(HBVP-co-DMMEP) 24:76. (D) polyDMMEP

Table 1

Composition of copolymers prior to N-alkylation used for further experiments as determined by elemental analysis.

Polymer	Ratio VP	Ratio DMMEP
P(VP-co-DMMEP) A	0.79	0.21
P(VP-co-DMMEP) B	0.68	0.32
P(VP-co-DMMEP) C	0.59	0.41
P(VP-co-DMMEP) D	0.40	0.60
P(VP-co-DMMEP) E	0.24	0.76

Table 2

Molecular mass and coating characteristics of P(HBVP-co-DMMEP).

Polymer	<u>Molecular weight (Mn)^{a)}</u>	<u>Layer thickness</u>	<u>Contact angle</u>	
	g/mol	nm	adv in °	rec in °
A	----- ^{b)}	3.1 ± 0.1	63 ± 1	39 ± 1
B	41 400	3.6 ± 0.2	65 ± 1	40 ± 1
C	32 000	3.5 ± 0.2	67 ± 1	40 ± 2
D	42 500	7.4 ± 0.4	70 ± 3	47 ± 3
E	39 700	10.9 ± 2.9	74 ± 1	49 ± 1
<i>polyDMMEP</i>	2.3×10^6	19.4 ± 4.9	51 ± 2	35 ± 4

^{a)} Mn was determined by GPC of copolymers prior to N-alkylation;^{b)} not determined as polymer was insoluble in water

Table 3

XPS determined elemental compositions of copolymer films and polished Ti90/Al6/V4 substrate

Polymer	Atomic Concentration [%]					
	Ti 2p	O 1s	C 1s	P 2p	N 1s	Br 3d
<i>substrate</i>	18.4 ± 0.3	54.4 ± 0.2	26.5 ± 0.5	-----	0.7 ± 0.1	-----
A	11.0 ± 0.1	39.2 ± 0.4	45.5 ± 0.2	0.9	3.1 ± 0.1	0.3
B	10.7 ± 1.2	40.2 ± 0.7	44.7 ± 1.9	1.2 ± 0.1	2.9 ± 0.1	0.3
C	10.3 ± 0.6	39.6 ± 1.6	46.4 ± 2.5	1.4 ± 0.1	2.7 ± 0.1	0.3
D	4.7 ± 0.4	32.3 ± 1.0	57.2 ± 0.9	2.7 ± 0.3	2.3 ± 0.1	-----
E	4.3 ± 0.3	33.6 ± 1.1	55.9 ± 1.8	3.2 ± 0.2	1.7 ± 0.2	-----

Table 4

Nitrogen to phosphorus ratios (P/N) of copolymer films.

Polymer	N/P (XPS)	N/P (EA)
A	3.38	3.76
B	2.38	2.13
C	1.96	1.44
D	0.85	0.67
E	0.53	0.28

Table 5

Degree of N-alkylation determined via carbon to nitrogen ratios (C/N) of copolymer films as determined by XPS.

Polymer	C/N (theory) ^{a)}	C/N (XPS)	degree of N-alkylation ^{b)} [%]
A	15.13	14.90	96.2
B	16.76	15.40	77.9
C	18.56	17.51	82.5
D	25.00	24.78	96.3
E	38.33	33.0	11.0

^{a)} values assuming complete N-alkylation;

^{b)} based on XPS data