Received: 8 August 2019 | Revised: 1 November 2019 | Accepted: 9 November 2019

DOI: 10.1002/jbm.b.34522

ORIGINAL RESEARCH REPORT

Poly (hexamethylene biguanide), adsorbed onto Ti-Al-V alloys, kills slime-producing *Staphylococci* and *Pseudomonas aeruginosa* without inhibiting SaOs-2 cell differentiation

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Abstract

Antimicrobial coating of implant material with poly(hexamethylene biguanide) hydrochloride (PHMB) may be an eligible method for preventing implant-associated infections. In the present study, an antibacterial effective amount of PHMB is adsorbed on the surface of titanium alloy after simple chemical pretreatment. Either oxidation with 5% H_2O_2 for 24 hr or processing for 2 hr in 5 M NaOH provides the base for the subsequent formation of a relatively stable self-assembled PHMB layer. Compared with an untreated control group, adsorbed PHMB produces no adverse effects on SaOs-2 cells within 48 hr cell culture, but promotes the initial attachment and spreading of the osteoblasts within 15 min. Specimens were inoculated with slime-producing bacteria to simulate a perioperative infection. Adsorbed PHMB reacts bactericidally against *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Pseudomonas aeruginosa* after surface contact. Adhered SaOs-2 cells differentiate and produce alkaline phosphatase and deposit calcium within 4 days in a mineralization medium on PHMB-coated Ti6Al4V surfaces, which have been precontaminated with *S. epidermidis*. The presented procedures provide a simple method for generating biocompatibly and antimicrobially effective implant surfaces that may be clinically important.

KEYWORDS

contact killing surface, PHMB adsorption, SaOs-2 cell differentiation, surface modification, titanium alloys

1 | INTRODUCTION

Whereas biomaterial implants and devices are becoming more popular in surgical care, the frequency of implant-associated infections rises (Zimmerli, Trampuz, & Ochsner, 2004). Three major strategies have been pursued to preserve implants from becoming a reservoir of infection: (a) surface properties of devices that thwart bacterial adherence; (b) surface impregnation with immobilized antimicrobial agents, and (c), eventually, devices containing covalently tethered antibiotics in their surface (Francolini, Vuotto, Piozzi, & Donelli, 2017). Contact-killing surfaces facilitate physical interactions between surface-linked antimicrobial agents and bacterial membranes. Commonly used molecules include quaternary ammonium compounds, antibiotics, antimicrobial peptides, and nonleaching physically adsorbed polycationic molecules (Ferreira & Zumbuehl, 2009). Poly(hexamethylene biguanide) hydrochloride (PHMB), another

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positively charged polymer, has a broad-spectrum antimicrobial efficacy against Gram-positive and -negative bacteria, fungi, and yeast (Kramer, Eberlein, Müller, Dissemond, & Assadian, 2019). Preliminary results for PHMB adsorbed onto Ti6Al4V-surfaces shows antimicrobial properties against *Staphylococcus aureus* that was while inhibiting neither proliferation nor growth behavior of osteoblasts (MG-63 cells) but only 3–4 μg PHMB were adsorbed onto the generated OH-groups of the Ti6Al4V specimen after H_2O_2-oxidation (Müller et al., 2014). Therefore, in the present study it should be verified that alkaline treatment increases PHMB adsorption due to extra introduced anionic —OH groups.

Device-associated infections are typically caused by biofilm-producing bacteria. The infecting and slime-producing microorganisms are either derived from a temporary bacteremia or introduced during implantation of a medical device. *S. epidermidis* generally exhibited the highest prevalence, closely followed by *S. aureus* and *P. aeruginosa*, but these results pertain only to infections associated to knee or hip arthroprosthesis (Arciola, Campoccia, Speziale, Montanaro, & Costerton, 2012; Montanaro et al., 2011). An effective contact bacteria-killing capacity resulting in a complete elimination of biofilm-producing bacteria is an absolute prerequisite, that is, for a subsequent pathogen-free attachment and proliferation/differentiation of human osteoblasts on antimicrobial implant materials. In the present study, a 6 hr-adherence drop technique simulates bacterial perioperative contaminations by slime-producing *S. aureus*, *S. epidermidis*, and *P. aeruginosa* on Ti6Al4V specimens. The effect on adhesion and differentiation of osteoblasts (SaOs-2) was assessed before and after PHMB coating by detecting both enzymatic activity of alkaline phosphatase and deposition of calcium apatite. The same parameters were utilized after previous antimicrobial activity.

2 | MATERIALS AND METHODS

2.1 | Test specimen

Ti6Al4V test samples (11 mm in diameter, 2 mm thickness) were manually polished to mirror finish by the manufacturer (DOT,Germany). Ti6Al4V discs were ultrasonically cleaned for 30 min each in a 2% (v/v) Hellmanex III solution (Hellma, Germany), acetone and sterile deionized water. Prior to surface treatment procedures the Ti6Al4V discs were disinfected with 80% (v/v) ethanol.

For the measurement of surface roughness the Veeco Dektak 3ST surface profiler (USA) was used (0.08 mm/s velocity with a 30 mg stylus force). The surface parameters were: R_a = 0.43 ± 0.20 μm, R_{max} = 0.11 ± 0.06 μm, R_q = 0.52 ± 0.22 μm, R_t = 2.41 ± 0.34, and R_z = 0.42 ± 0.34 μm.

2.2 | Ti6Al4V surface treatment procedures

2.2.1 | H_2O_2 treatment procedure

Each Ti6Al4V disc was placed into a cavity of a 24-well cell culture plate with 1.0 ml 5% (v/v) H_2O_2 (Sigma 95321). The oxidation procedure was carried out at room temperature protected from light for 24 hr, followed by rinsing with 4 × 1.5 ml sterile water, each for 3 min.

2.2.2 | Alkaline treatment procedure

To assess the most effective NaOH-concentration, each Ti6Al4V disc was placed into a cavity of a 24-well cell culture plate with 2.0 ml of 0.5–10 M NaOH. Alkaline treatment was done at 60°C for 4 hr. To assess the optimal operation time, treatment of Ti6Al4V discs in 5 M NaOH was carried out for 10 min – 6 hr. The standardized alkaline processing of each Ti6Al4V disc was for 2 hr at 60°C in 2.0 ml 5 M NaOH, followed by rinsing with 4 × 2.5 ml sterile water, each for 5 min.

2.3 | Poly(hexamethylene biguanide) hydrochloride (PHMB)

Polihexanid solution (Fagron, Lot 15C20-B06-309679) containing 20% (w/v) PHMB (Arch Biocides Ltd., UK, Lot 14GR274618) in water was used as the antibacterial agent.

2.4 | Determination of PHMB

Aqueous PHMB concentrations were quantified spectrophotometrically using the maximum absorption in the range of between 236 and 238 nm (De Paula, Netto, & Mattoso, 2011). Aliquots of 0.25 ml containing 0–30 μg/ml PHMB were transferred in a 96-well quartz microplate (Hellma). A calibration curve was established by measurement of the absorption at 236 nm (Microplate reader PowerWave XS, Biotek Instruments).

PHMB adsorption procedure was done in 24-well cell culture plates. The cavities of the plate were precoated with aqueous PHMB solution for 24 hr. Each Ti6Al4V specimen was transferred into 1.0 ml of 30 μg/ml PHMB. After 2–24 hr of incubation at room temperature, the residual amount of PHMB was quantified in the supernatant. The amount of adsorbed PHMB on each Ti6Al4V disc equals to the difference of applied and residual PHMB concentration.

2.5 | XPS surface analysis

X-ray photoelectron spectroscopy (XPS) was applied for the determination of chemical elements at the Ti6Al4V surface and characterization of chemical functional groups/bonds with AXIS ULTRA (Kratos) using the Al-K_α line at 1486 eV (150 W), implemented charge neutralization and a pass energy of 80 eV for estimating the chemical elemental composition or 10 eV for highly resolved C1s spectra. XPS spectra were generally recorded for 1s electrons from C, N, O, and 2p from Ti. The C1s peak (C—C and C—H components) was adjusted to 285.0 eV. The peak fitting was done with CasaXPS software version 2.2; Gauss–Lorentz (30% Lorentz) distribution, Shirley baseline, and a fixed FWHM (full width at half maximum) were used. The FWHM for C1s high resolution spectra was between 0.9 and 1.1 eV. Additional

chemical components of the C1s peak were assigned to amines (—C—NH) and secondary carboxyls (O—C⍰O) at 285.7 ± 0.1 eV; hydroxyls, ethers, imines, and nitriles (—C—O, —C⍰N, —C⍰N) at 286.6 ± 0.2 eV; aldehydes, ketones, and amides (—C⍰O, —N—C⍰O) at 288.0 ± 0.3 eV; esters and acids (O—C⍰O) at 289.2 ± 0.2 eV. The FWHM for O1s high resolution spectra was between 1.1 and 1.5 eV. The O1s spectra were fitted to (1) at 530 eV (main peak) assigned to O1s electrons in TiO_2, Ti—OH, (2) at 531.2 ± 0.2 eV to O⍰C, and (3) at 532.1 ± 0.2 eV to adsorbed H_2O and O—C (Textor, Sittig, Franchinger, Tosatti, & Brunette, 2001). The FWHM of Ti2p high resolution spectra was between 1 and 2.5 eV. The two dominating peaks for $Ti2p^{3/2}$ at 458.9 ± 0.2 eV and Ti $2p^{1/2}$ at 464.5 ± 0.2 eV for Ti^{4+} were found in Ti2p spectra. The oxidation states of Ti(0), Ti(II), and Ti(III) were also included (Textor et al., 2001). All values are given in at.-% and ratios thereof.

2.6 | Wettability

The water contact angel (WCA) of untreated and treated Ti6Al4V surfaces was measured by the sessile drop method (1 µl deionized water, 25°C, 45% relative humidity) utilizing an OCA40 Micro and the SCA software (Dataphysics).

2.7 | Cell culture

Cell culture was carried out at 37°C in a humidified atmosphere of 5% CO_2 in 95% air. Human osteoblast-like SaOs-2 cells (ATCC HTB-85, CLS Cell Line Service) were placed in a 1:1 mix of DMEM and Ham's F-12 (PAN Biotech) supplemented with 5% fetal bovine serum (FBS) (Gibco) and 2 mM L-glutamine (basal medium). SaOs-2 cells were detached using 0.05% trypsin/0.02 mM EDTA at 80% confluency and adjusted to 2×10^6 cells/ml in basal medium. To prevent drying of medium, each Ti6Al4V disc was placed on a socket of a cellulose filter (12–13 mm in diameter, 0.8–1 mm in thickness), which was saturated by sterile water. Amounts of 50 µl (1×10^5 cells) SaOs-2 cells in basal medium were seeded on the whole surface of the specimen. After 60 min cell culture, each specimen with adhered cells was placed into a 24-well cell culture plate with 1.0 ml of fresh basal medium.

2.8 | Cell adhesion experiments

Each specimen was placed into a cavity of a 24-well cell culture plate. SaOs-2 cells were seeded in 50 µl (1×10^5 cells) basal medium without phenol red and evenly plated on the surface of the Ti6Al4V disc. After 15, 30, and 60 min contact, each specimen was fully covered with 1.0 ml basal medium without phenol red. Nonadhering cells (100 µl) were determined by ATP assay with 100 µl BacTiter- Glo™ reagent (Promega) twofold for each specimen in a 96-well plate with the TriStar LB 941 (Berthold Technologies). For calculation, amounts of 200 µl of analogous lysed SaOs-2 cells (10^3–10^5 cells/ml) served to establish a calibration curve.

2.9 | Cell viability

Viability of SaOs-2 cells was evaluated with the tetrazolium salt (XTT) reagent which indicates metabolically active cells. After cell culture, specimens were washed with basal medium without phenol red and transferred into a new 24-well cell culture plate containing 1.5 ml of tetrazolium salt (XTT) reagent (0.33 mg/ml XTT containing 1.27 µg phenazine methosulfate). Specimens were carefully withdrawn after 3 hr cell culture. The amount of formazan product was measured in the corresponding 24-well plate using an ELISA reader at 450 nm.

2.10 | Osteogenic cell differentiation

Specimens with cultured SaOs-2 cells were exposed to osteogenic supplements of 50 µg/ml ascorbic acid, 10 mM Na-β-D-glycerophosphate, and 20 nM dexamethasone in basal medium in order to induce biomineralization (Att et al., 2009). SaOs-2 cells were incubated for 3 days without osteogenic supplements and subsequently for 4 days in the mineralization medium that was supplemented with antibiotic-antimycotic (Sigma). The mineralization medium was changed every other day.

2.11 | Alkaline phosphatase (ALP) activity and mineralization assay

Osteoblastic cells cultivated for a total of 7 days were washed with 2×1.5 ml PBS, each time for 1 min, and then each specimen with cultured cells was transferred into a new 24-well cell culture plate containing 1.0 ml ice-cold 70% (v/v) ethanol. After fixing cells for 1 hr at 4°C, Ti6Al4V discs were dried on sterile cellulose tissue for 2 min without touching the cells. For histochemical analysis of the ALP activity, fixed cells were incubated with 0.1 M Tris-buffer, pH 8.4, containing 0.9 mM naphthol AS-MX phosphate (Sigma), 1.8 mM Fast Red TR salt (Sigma), 25 mM NaCl, and 4 mM $MgCl_2$ for 30 min at 37°C. Positive stained areas of ALP were evaluated stereo-microscopically. For colorimetric measurement of ALP activity, fixed cells were incubated for 15 min at 37°C with 0.5 ml substrate solution per specimen using SigmaFast™ p-nitrophenyl phosphate tablets (Sigma). Enzymatic reaction was stopped when adding 125 µl 3 M NaOH, and ALP activity was evaluated with regard to the amount of released p-nitrophenol, measuring aliquots of 250 µl in 96-well plates at 405 nm. P-nitrophenyl phosphate (PNP, Sigma) in 0.1 M NaOH served to establish a calibration curve for the calculation of PNP-equivalents. For mineralization assay, fixed cells were incubated for 30 min in 40 mM Alizarin Red S (AR-S) solution, pH 4.1–4.3 (Sigma). AR-S-positive areas were evaluated stereo-microscopically. For colorimetric measurement of calcium deposition, AR-S-stained cultures were washed with PBS and incubated with 2 ml of 10% (w/v) cetylpyridiniumchloride (Sigma) in 10 mM phosphate buffer, pH 7.0, while shaking for 1 hr. The amounts of eluted dye were calculated applying an established calibration curve of AR-S in the eluent at 562 nm. Corresponding Ca^{2+}-equivalents were deduced from the assumption that one molecule AR-S binds ≈2 molecules of Ca^{2+}

(Puchtler, Meloan, & Terry, 1969). For analysis, both PNP-equivalents of ALP and AR-S-staining for Ca^{2+}-equivalents were normalized to XTT activity.

2.12 | Cell Actin filament staining

After fixing SaOs-2 cells for 10 min with 1.5% (w/v) PBS-buffered paraformaldehyde, pH 7.2, the cultures were washed with PBS and cells were permeabilized with 0.5% (w/v) Triton™ X-100 for 10 min. SaOs-2 cell actin filaments were stained with 5 μg/ml Alexa Fluor®488 conjugated phalloidin (Molecular Probes) in PBS for 1 hr. All procedures were carried out protected from light at room temperature. Specimens were washed again and examined with the LSM510 Exciter (Carl-Zeiss) by confocal laser scanning microscopy.

2.13 | Microorganisms, media, and bacterial multiplication

The slime-producing Gram-positive *S. aureus* (ATCC 29213) and *S. epidermidis* (ATCC 35984) as well as the Gram-negative bacterium *P. aeruginosa* (ATCC 27853) were used as test microorganisms. Second bacterial subculture was harvested with 1.5 ml PBS; then it was rinsed first with 1 ml 1% Tween 80 (Sigma), to disrupt preexisting cell clusters, and afterward with 3 × 1 ml PBS. Each washing step was followed by centrifugation for 1 min at 13,000 rpm. The resuspended pellet was moved at least 10 times forward and backward through a syringe fitted with a 23-gauge hallow needle to further singularize bacterial cells. A final suspension of 1–3 × 10^8 colony forming units (cfu)/ml results after adjusting the optical density (OD) at 620 nm to 0.16–0.18 for *S. aureus* and *P. aeruginosa*, and 0.30–0.35 for *S. epidermidis*, respectively. Bacteria and SaOs-2 cells were propagated in a co-culture medium consisting of 90% basal medium and 10% tryptic soy broth (TSB).

2.14 | Microbicidal test

For 6 hr-adherence drop technique, Ti6Al4V discs were arranged into a Petri dish, inoculated with 50 μl bacterial suspensions containing 5 × 10^3 cfu and evenly spread on the Ti6Al4V surface. The Petri dish was placed in the incubator at 37°C using a humidified chamber for 6 hr. For quantification, recovery of adhered bacteria was performed mechanically. For that, each contaminated specimen was treated in 1.0 ml inactivator on sterile glass beads (2.9–3.5 ± 0.3 mm in diameter) on an orbital shaker (400 rpm) for 2 min to dislodge bacteria. Inactivation was for 5 min. Subsequently, serial dilutions were prepared in inactivator and 0.1 ml of each dilution was spread in duplicate on TSA plates. The cfu of bacteria were counted after 48 hr of incubation at 37°C.

Aqueous PHMB (<20 μg/ml) was effectively inactivated by 1.2% (w/v) egg yolk phosphatidylcholine (Lipofundin® MCT 20%), but inactivator does not inhibit bacterial growth of all tested bacteria, as assessed in separate experiments.

The $\log_{10}$ microbicidal reduction value for each contact time was calculated according to the formula:

$$\log_{10}\text{microbicidal reduction value} = \log_{10} n_c - \log_{10} n_p$$

where, n_c is the number of cfu of viable cells in the inoculum after contact with the control surface and n_d is the number of cfu of viable cells in the inoculum after contact with the prepared surface. All tests of antibacterial efficacy were performed at least in triplicate.

2.15 | Live/dead imaging of bacteria

For the live-dead staining of attached bacteria, the LIVE/DEAD™ BacLight ™ bacterial viability kit for microscopy (Molecular Probes) was used as described by the manufacturer. Test specimens with adhered bacteria were washed twice with 1.5 ml PBS for 1 min and then transferred into 1 ml isotonic saline containing 3 μl staining reagent. After 30 min, labeled bacteria were examined with LSM510 Exciter (Carl-Zeiss) by confocal laser scanning microscopy.

2.16 | Combined assay of microbicidal activity, cytotoxicity, and osteogenic differentiation

S. epidermidis were set to a density of 10^5 cfu/ml in co-culture medium. Amounts of 50 μl bacterial suspensions (5 × 10^3 bacterial cells) were applied on the whole surface of the specimens. To prevent drying of medium, each inoculated Ti6Al4V disc was placed on a socket of a cellulose filter (12–13 mm in diameter; 0.8–1 mm mm in thickness) which was saturated by sterile water. After 6 hr of culture, the present medium was carefully removed. Thereafter, amounts of 50 μl (1 × 10^5 cells) SaOs-2 cells in co-culture medium were seeded on the whole bacterial inoculated surface. After 1 h cell culture, each test setup with cultured cells was placed into a new 24-well cell culture plate with 1.0 ml basal medium. Culture was for 3 days in basal medium and 4 days in mineralization medium changing the mineralization medium every other day (see Section 2.10). To assess bacterial growth, untreated specimens were inoculated with *S. epidermidis* and cultivated essentially as described but without SaOs-2 cells. Presence and viability of SaOs-2 cells was proved with tetrazolium salt (XTT) reagent (Section 2.9), ALP activity tests, and mineralization assays (see Section 2.11).

2.17 | Confocal laser scanning microscopy

ALSM 510 Exciter (Carl-Zeiss) equipped with an Ar-laser for 488 nm, a band pass filter (505–530 nm), a long pass filter (>560 nm), and the objectives Zeiss EC Plan-Neofluar 10×/0.3 and a Zeiss Plan-Apochromat 63×/1.40 for immersion oil was used for inverted confocal laser scanning microscopy. ZEN 2008 light edition software (Carl-Zeiss) was used for post-acquisition processing of images.

2.18 | Stereo microscopy

The stereo microscope SMZ18 (Nikon) was equipped with the 1.6× SHR Plan Apo WD30 objective (Nikon). Image acquisition was carried out with 13.5 fold magnification. NIS-Elements software 4.0 (Nikon) was applied for postacquisition processing of images.

2.19 | Statistics

Comparisons between two different groups were performed with Mann–Whitney's *U* test, and between more than two groups with a nonparametric one-way analysis of variance ANOVA using a Kruskal–Wallis test, followed by a post hoc test of Dunn's comparison. The software version 5.04 of GraphPad Prism was used for the statistical analysis. The p values <0.05 were considered statistically significant.

3 | RESULTS

3.1 | PHMB adsorption

Treatment of Ti6Al4V specimens with aqueous NaOH solution improves adsorption of PHMB on its surface. Effective amounts of PHMB were adsorbed after performing the procedure at 60°C in 5 M NaOH (Figure 1a) for 2 hr (Figure 1b), which was applied as the standard operation procedure for preparing the surface for PHMB adsorption. Applying this standard alkaline treatment, the amount of adsorbed PHMB on the surface is individually adjustable by the incubation time of Ti6Al4V discs in PHMB coating solution (Figure 1c). The range of adsorbed PHMB on control Ti6Al4V discs was 0.1–0.2 μg/disc. After processing in 5 M NaOH for 2 hr at 60°C the amount of adsorbed PHMB at the Ti6Al4V surface was increased around 100 times to 20.9 ± 0.6 μg per disc after 24 hr contact with 30 μg/ml PHMB. Adsorption of PHMB takes 48 hr to reach the equilibrium concentration of 22–25 μg PHMB per disc (Figure 1c).

3.2 | Surface characterization: XPS analysis

In Table 1 are shown the results of the XPS analysis of the elements C, N, O, and Ti present on the surface of the untreated control and different processed Ti6Al4V specimens. The alkaline treatment produces a significant increase of the O- and Ti-content on the surface and additionally 5% Na is indicated. However the oxidation process with H_2O_2 led to a virtually constant O-content, but the Ti content is significantly reduced, Na is naturally absent. Compared with the untreated control surface (33 at.-% C), the NaOH treatment reduces significantly (25 at.-% C) the amounts of hydrocarbons on the surface but not that of oxidation with H_2O_2 (36 at.-% C). However, PHMB adsorption was evident after oxidation with H_2O_2, which is shown by a significant increase of N and C of ≥10%, respectively. But the most effective adsorption of PHMB is demonstrated after alkaline processing of Ti6Al4V discs. More than 18% N and C has been proven on the PHMB-modified surface compared to the H_2O_2 treatment. After adsorption of PHMB, further reduction of the O- and Ti-content is evident on both activated surfaces and Na is clearly reduced on the alkaline processed surface. The WCA shows a significantly sharp decline after the utilized activation procedures with H_2O_2 or NaOH, subsequently followed by an increase of the WCA values after they were coated with PHMB. The XPS spectra of the Ti6Al4V surfaces after treatment with sodium hydroxide or H_2O_2 were compared with the control. The results of peak fitting of the high resolution spectra of C1s, O1s, and Ti2p are shown in Figure 2 and that of the corresponding fitted high resolution spectra after PHMB adsorption in Figure 3.

In the C1s high resolution spectrum, the control surface is contaminated with aliphatic hydrocarbons (75%) and C-compounds of other functional groups (Figure 2a). NaOH treatment reduces the aliphatic C-counts at 285 eV (Figure 2d), whereas H_2O_2 reduces it to a lesser extent (Figure 2g). Especially striking are the secondary and primary carboxylic groups at 285.7 and 289.2 eV for both procedures (Figure 2d,g). The control surface shows in the O1s spectrum the TiO_2 main peak at 530.3 eV and additionally two further peaks that are attributed to acidic —OH groups (Ti—OH and/or OC) at 531.3 eV and basic —OH groups (O—C and/or adsorbed H_2O) at 532.5 eV. In strong contrast to the control and H_2O_2 procedure, the NaOH treatment increases the TiO_2 counts considerably, but in parallel, the basic and acidic —OH groups decrease (Figure 2e). After H_2O_2 oxidation the TiO_2 counts were strongly reduced and the basic and acidic —OH

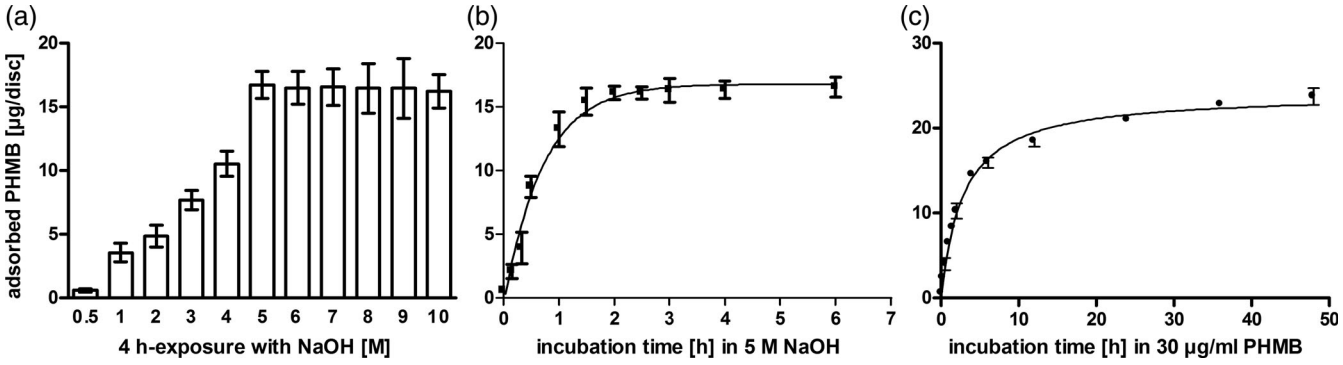

FIGURE 1 Adsorbed amounts of PHMB on the surface of Ti6Al4V discs. (a) after 4 hr treatment with 0.5–10 M NaOH (n = 16); (b) after treatment with 5 M NaOH for 10 min-6 hr (n = 4); and (c) after treatment with 5 M NaOH for 2 hr and incubation in 30 μg/ml PHMB for 0–48 hr (n = 4)

TABLE 1 Elemental composition (at.-%) of untreated and differently treated Ti6Al4V surfaces before and after PHMB-adsorption as determined by XPS and the corresponding water contact angle (WCA) [°]; n (number of analyzed samples)

Sample (n = 4)	Ti	Al	V	C	O	N	Na	WCA (°)
Untreated	17.2 ± 0.8	2.2 ± 0.4	0.3 ± 0.1	33.0 ± 0.7	45.8 ± 1.3	1.4 ± 0.5	n.d	59.3 ± 10.2
H_2O_2	14.1 ± 0.6[a]	2.4 ± 0.4	n.d.	35.9 ± 2.1	46.2 ± 2.3	0.9 ± 0.3	n.d.	19.8 ± 9.7[a]
H_2O_2 + PHMB	9.6 ± 1.8[a]	0.3 ± 0.1	n.d.	48.4 ± 2.5[a]	29.2 ± 1.9[a]	12.7 ± 1.3[a]	n.d.	46.5 ± 10.2
NaOH	19.4 ± 0.4[a]	n.d.	n.d.	24.7 ± 1.2[a]	50.5 ± 0.9[a]	n.d.	5.4 ± 0.4	10.8 ± 5.4[a]
NaOH+PHMB	11.2 ± 1.4[a]	n.d.	n.d.	42.9 ± 0.9[a]	27.4 ± 1.5[a]	18.1 ± 1.3[a]	0.5 ± 0.2	37.0 ± 4.5[a]

Abbreviation: n.d. = not detectable.

[a]$p < .05$ for Ti, C, O, N, and WCA compared to untreated group.

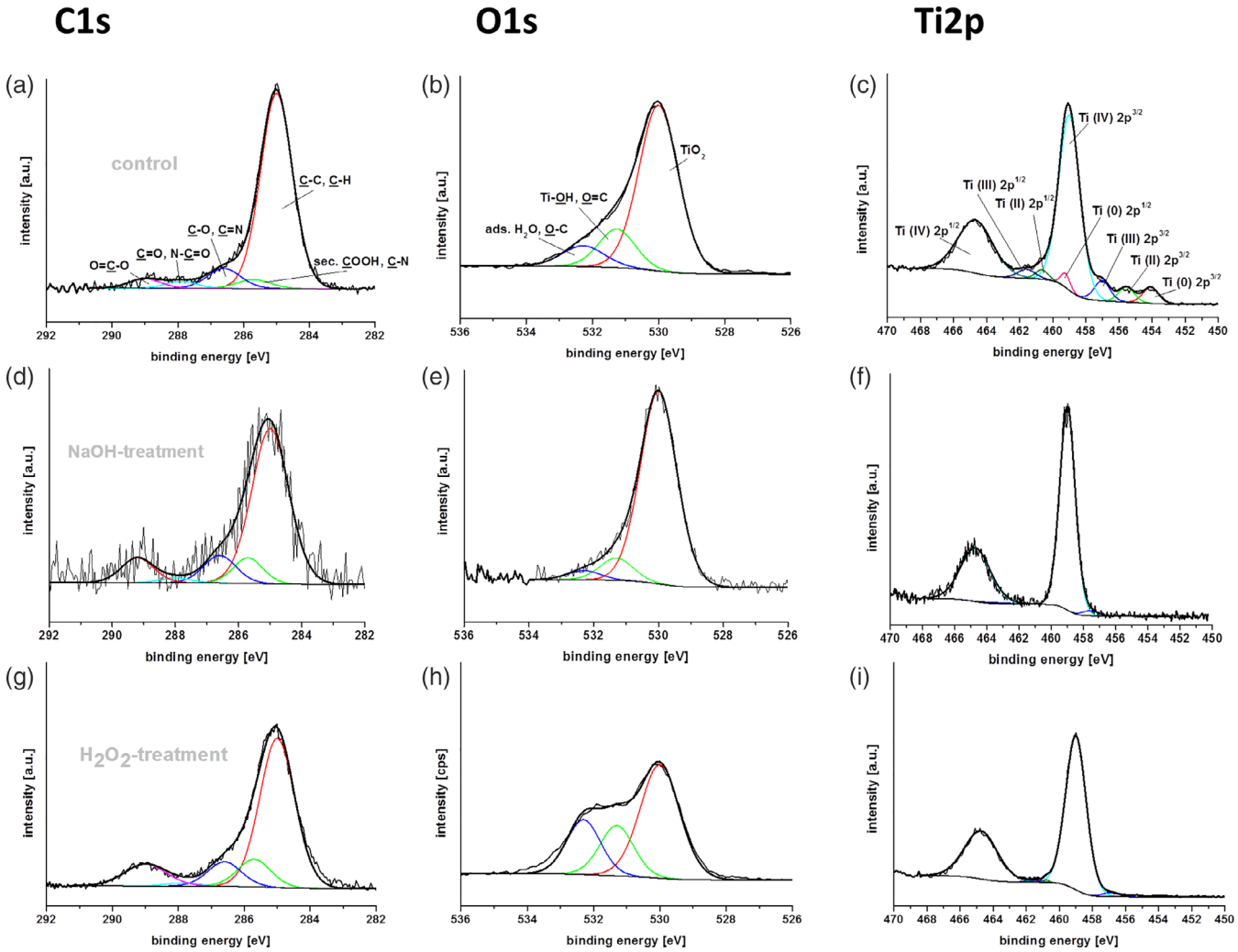

FIGURE 2 XPS high-resolution spectra of C1s (a,d,g), XPS spectra of O1s (b,e,h), and Ti2p (c,f,i) of untreated control (a–c), H_2O_2-treated (g-i), and NaOH-processed (d-f) surface of Ti6Al4V specimen

groups increased (Figure 2h). The Ti2p spectrum of the native control exhibits titanium in all its possible oxidation states of Ti (0), Ti (II), Ti (III), and Ti (IV) (Figure 2c), which is the result of a very thin titanium oxide layer. Processing of Ti6Al4V with NaOH or H_2O_2 increased the thickness of the oxide layer at the surface shown by the presence of only Ti(III) and Ti(IV) (Figure 2f,i). PHMB coating produces no changes in the spectra of C1s, O1s and Ti2p of the control surface compared to that without PHMB (Figure 2a–c and Figure 3a–c). PHMB adsorption after treatment with either H_2O_2 or NaOH increases the intensity of all bonds fitted in the C1s spectrum (Figure 3d,g) compared to the control (Figure 3a). Specifically N-containing bonds (—C—N, —C⍰N, N—C⍰O) but also the C-bonds of primary and secondary —COOH groups are increasing, which results in a sharp rise to overall 50% functional groups after H_2O_2 treatment and to even 70% after PHMB

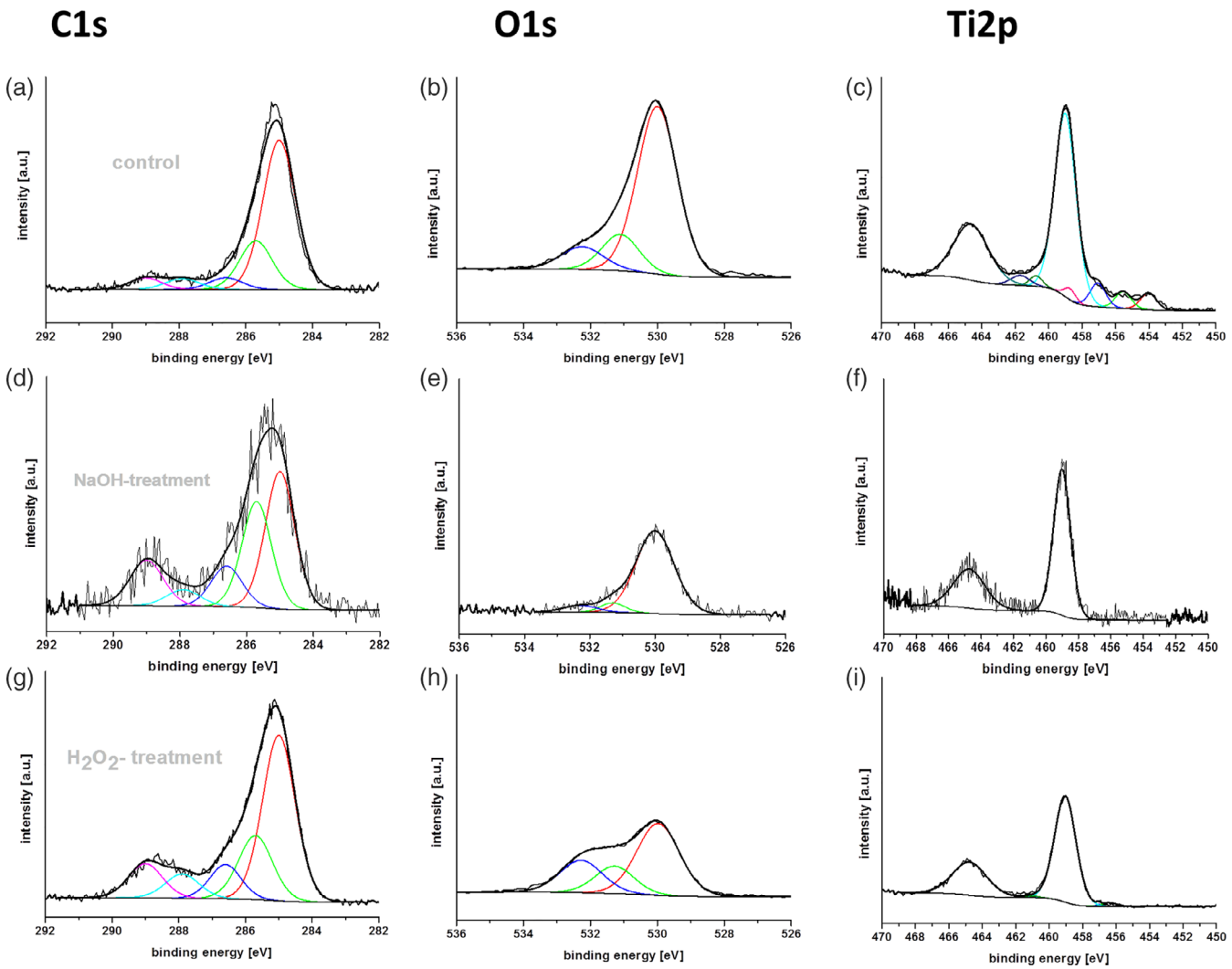

FIGURE 3 XPS high-resolution spectra of C1s (a,d,g), XPS spectra of O1s (B,E,H), and Ti2p (c,f,i) of untreated control (a–c), H_2O_2-treated (g-i), and NaOH-processed (d-f) surface of Ti6Al4V specimen after PHMB coating. For peak identification see Figure 2a–c

coating on the NaOH processed surface. Simultaneously, PHMB adsorption after processing Ti6Al4V discs with either H_2O_2 or NaOH decreases the counts of the O1s spectra (Figure 3e,h) compared to the control coated with PHMB (Figure 3b). Additionally, after PHMB coating of the treated surfaces the counts of oxidation states of Ti (0), Ti (II), and Ti (III) in the Ti2p spectrum decrease (Figure 3f,i) due to increased PHMB adsorption. Especially, the oxidation states of Ti (0), Ti (II), and Ti (III) in the Ti2p spectrum are not present after PHMB coating of the NaOH treated surface.

3.3 | Osteoblast adhesion and proliferation

Adhesion experiments of SaOs-2 cells to control and different treated Ti6Al4V discs were performed for 15, 30, and 60 min. For the H_2O_2-processed specimen, the surface was coated with 2.5 ± 0.2 µg PHMB/disc and after NaOH-treatment there were adsorbed 20.2 ± 1.9 µg PHMB. - For each contact time, the attachment of osteoblasts was increased after H_2O_2 or NaOH treatment alone and after PHMB coating of the specimens, SaOs-2 cell adherence was not influenced quantitatively in the presence of PHMB. Differences were present only after 15 min of contact. Approximately 83% or 92% of initially applied osteoblasts adhered on the surface of the specimen, which have been processed either with H_2O_2 or NaOH and even 85% or 91% after PHMB adsorption, respectively. In parallel, 76% of seeded cells adhered on the control surface. However, all investigated test groups were statistically not different from each other within the 15, 30, and 60 min setup, and even after 60 min 92–99% of initially applied SaOs-2 cell were attached onto all investigated surfaces. The quantitative results of SaOs-2 cell adherence to native and modified Ti6Al4V surfaces were markedly supported by images of osteoblasts after actin staining using confocal laser scanning microscopy (Figure 4). The number of osteoblasts attached to both the H_2O_2-treated Ti6Al4V surface (Figure 4b) and the PHMB coated specimen (Figure 4c) after 15 min incubation was substantially greater than those attached to the native Ti6Al4V surface (Figure 4a). It parallels the appearance of SaOs-2 cells on both the NaOH-processed surface

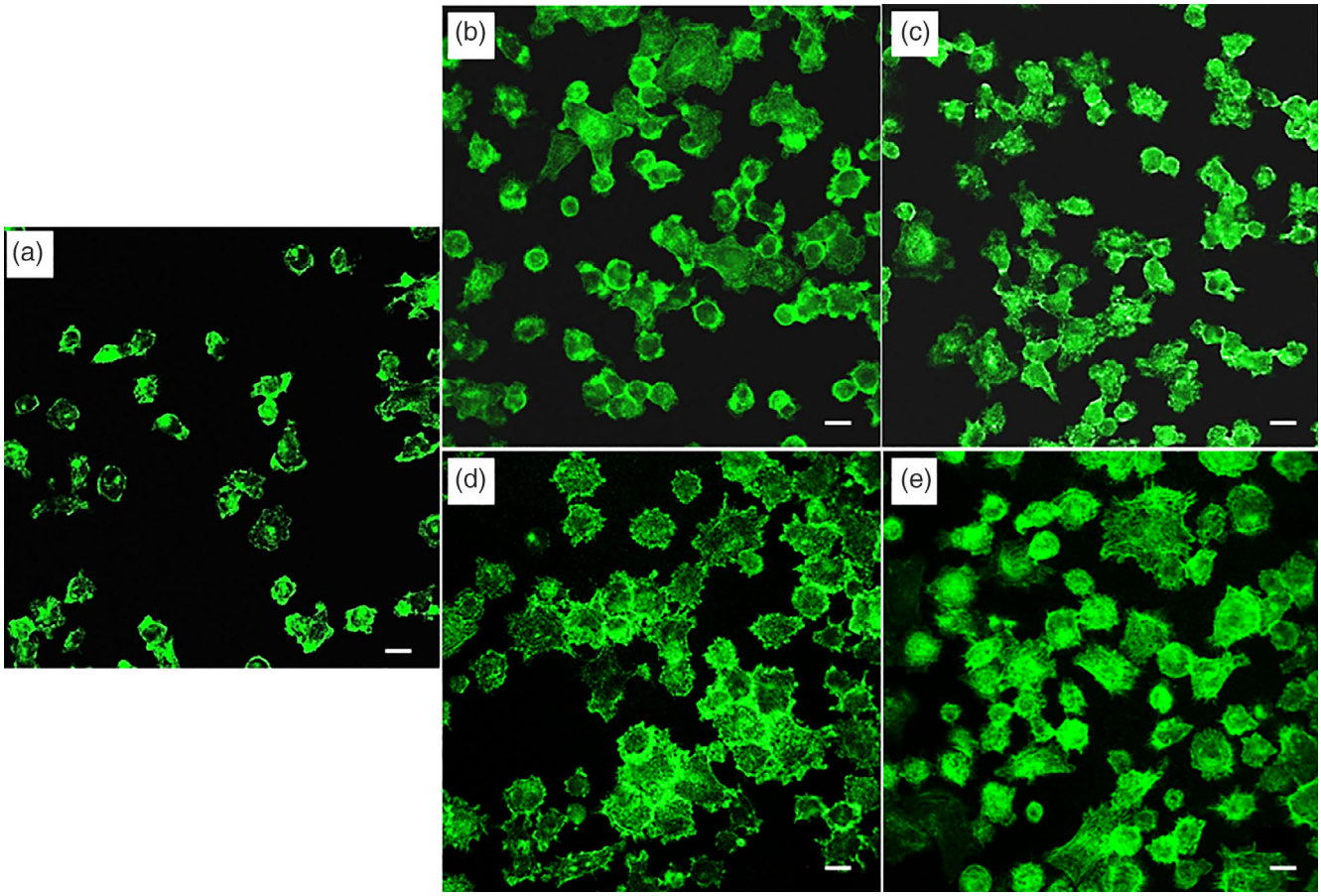

FIGURE 4 Initial spread and cytoskeletal arrangement of SaOs-2 cells 15 min after seeding onto untreated (a), H_2O_2- (b), NaOH-treated (d), and PHMB coated specimen of H_2O_2- (c) and NaOH-processed (e) Ti6Al4V surfaces. Representative images of cells stained with Alexa Fluor® 488 conjugated phalloidin for actin filaments (green) are presented at 63-fold magnification (bar 10 μm)

TABLE 2 Viability of SaOs-2 cells after 48 hr of cell culture applying 1×10^5 cells directly onto NaOH-treated, and PHMB coated specimen of NaOH-treated Ti6Al4V surfaces with 4–21 μg of adsorbed PHMB as a result of incubation time of NaOH-processed Ti6Al4V discs in 30 μg/ml aqueous PHMB

Incubation time (hr)	Adsorbed PHMB (μg/disc)	SaOs-2 cell viability (%)
0	-	117 ± 4
0.5	4.03 ± 0.75	114 ± 8
1	6.17 ± 0.48	114 ± 9
1.5	8.13 ± 0.57	112 ± 8
2	10.06 ± 0.90	106 ± 8
4	14.20 ± 0.43	102 ± 8
6	16.08 ± 0.61	99 ± 10
24	21.06 ± 0.58	92 ± 12[a]

Note. Cell growth and proliferation were estimated by XTT-assay (n = 6).
[a]p <0 .05 significantly different from NaOH-treated surface.

(Figure 4d) and the PHMB coated specimen (Figure 4e) after the same contact time. On processed surfaces (Figure 4b,d) as well as after PHMB coating (Figure 4c,e) much more actin filaments of cells were stained compared with the control (Figure 4a). Additionally, SaOs-2 cells were enlarged with a clear stretch of lamellipodia-like projections and a cytoskeleton within their cytoplasm (Figure 4b–e), whereas a majority of cells on the native surfaces still showed a round shape with no initiation of an elongation processes (Figure 4a). Compared with the untreated surface, cell growth and proliferation of osteoblast-like cells is not inhibited within 48 hr of cell culture on Ti6Al4V discs, which had been treated with NaOH alone or after coating the NaOH-processed surface with PHMB. However, in the presence of >20 μg of adsorbed PHMB, there was a significant reduction of the dehydrogenase activity (XTT) compared with the Ti6Al4V surface treated with NaOH alone (Table 2).

3.4 | Co-culture medium

The planktonic test microorganisms *S. aureus* and *P. aeruginosa* grow in basal medium, but *S. epidermidis* did not tolerate basal medium alone. In co-culture medium (90% basal medium/10% TSB) all organisms multiply, and also SaOs-2 cells grow without any cytotoxic effect tested in Neutral Red-, MTT- and XTT-assay.

TABLE 3 Log_{10} cfu of initial inoculum [*S. aureus* (ATCC 29213), *S. epidermidis* (ATC35984), and *P. aeruginosa* (ATCC 27853)], the resulting bacterial regrowth on the control and bacterial reduction after 6 hr of contact of attached bacteria with the surface of Ti6Al4V discs treated with H_2O_2 (Ti-H_2O_2), NaOH (Ti-NaOH) and after coating with PHMB (Ti-H_2O_2 + PHMB, Ti-NaOH+PHMB) of H_2O_2- or NaOH-processed specimen, respectively, each for $n = 6$

Test microorganisms	Initial inoculum	Bacterial regrowth on the control after 6 hr	Bacterial reduction after 6 hr			
			Ti-H_2O_2	Ti-H_2O_2 + PHMB	Ti-NaOH	Ti-NaOH + PHMB
S. aureus	3.68 ± 0.04	6.55 ± 0.35	−2.70 ± 0.21[a]	6.13 ± 1.27	−2,48 ± 1.33[a]	6.13 ± 0.27
S. epidermidis	3.45 ± 0.19	4.06 ± 0.41	−0.36 ± 0.08[a]	3.84 ± 0.34	−0.49 ± 0.17[a]	3.89 ± 0.36
P. aeruginosa	3.80 ± 0.05	5.91 ± 0.17	−1.87 ± 0.10[a]	5.60 ± 0.26	−1.97 ± 0.06[a]	5.78 ± 0.08

[a]Negative values indicate additionally regrowth of that of the control.

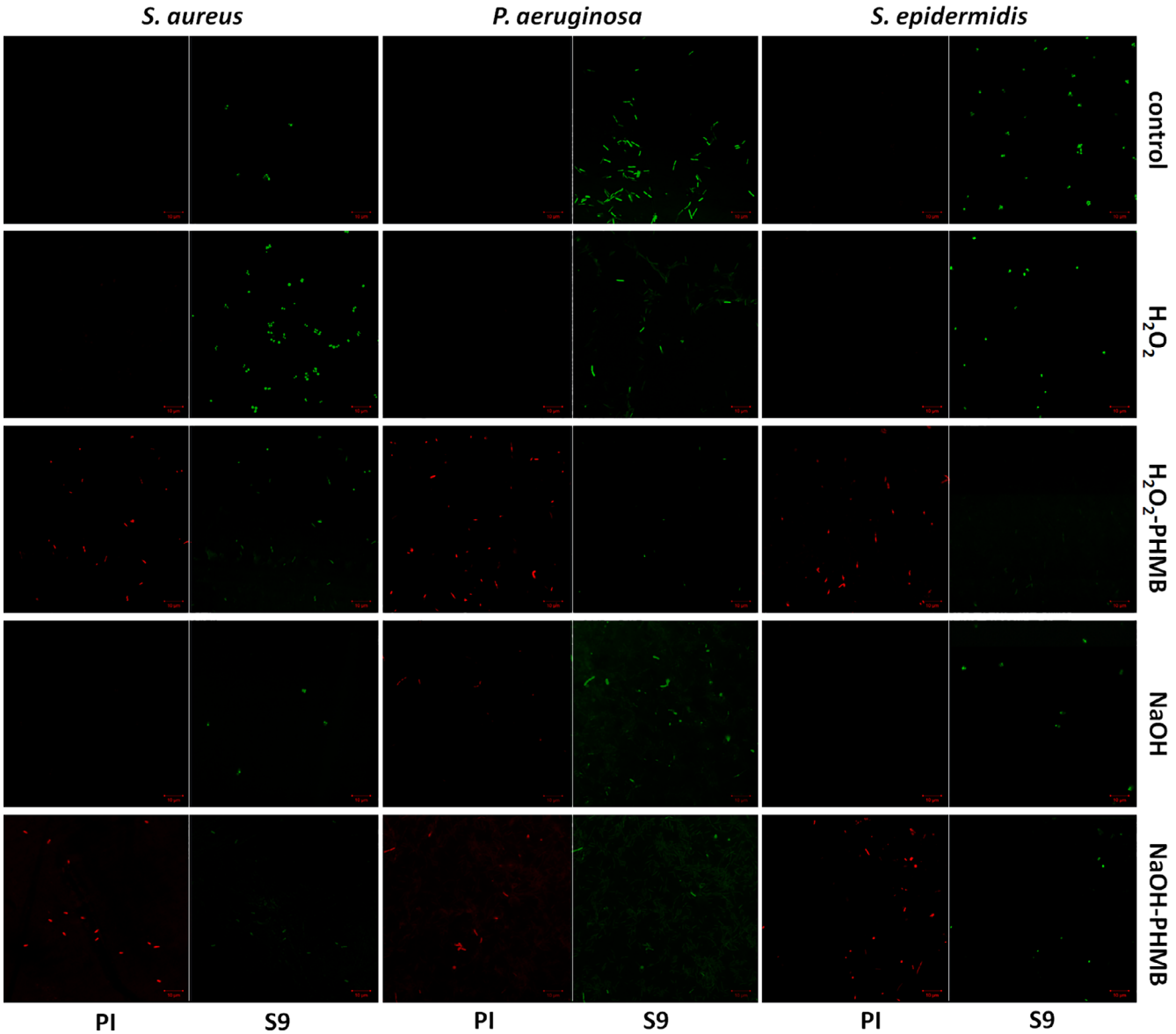

FIGURE 5 Microfluorography of adhered *S. aureus* (both left columns), *P. aeruginosa* (both middle columns), and *S. epidermidis* (both right columns) on native control (first panel sequence), H_2O_2-treated specimen (second panel sequence), after PHMB-coating of H_2O_2-processed Ti6Al4V discs (third panel sequence), NaOH-treated specimen (fourth panel sequence), and after PHMB-coating of NaOH-processed Ti6Al4V discs (fifth panel sequence). Viable cells (green) are stained with SYTO9 (S9) and damaged/dead cells (red) with propidium iodide (PI), respectively. Micrographs are presented at 63-fold magnification (10 μm bar)

3.5 | Microbicidal test

Contact-killing of *S. aureus* and *S. epidermidis* was already effective after 1–3 hr at 37°C, but that of *P. aeruginosa* needs 3–5 hr of incubation. Additionally, initial biofilm formation begins after 5 hr of incubation (Christensen, Baldassarri, & Simpson, 1995) and thus, the long-term efficacy of PHMB is brought to bear. To ensure complete killing of bacteria, a 6 hr incubation period was chosen. The microbicidal effect on slime-producing test bacteria using the 6 hr-adherence drop technique for bacterial attachment to the surface of Ti6Al4V discs is summarized in Table 3. The regrowth of test microorganisms is inhibited by neither the untreated control nor the H_2O_2- or NaOH-processed surface, but there is an increase of initial inoculum after 6 hr of incubation. A bacterial reduction is obvious only on specimen coated with PHMB (Table 3). The $\log_{10}$ reduction of all tested microorganisms after 6 hr is lower or equal than those of their regrowth in the control, reaching >99.99% killing of test microorganisms. After 6 hr of contact, no bacteria are present, which is qualitatively supported by confocal laser microfluorographs of live-dead stained microorganisms. Only dead bacteria are present after 6 hr contact of *S. aureus*, *P. aeruginosa*, and *S. epidermidis* with the PHMB-coated surface of H_2O_2- or NaOH-processed Ti6Al4V specimen (Figure 5).

3.6 | Combined assay of microbicidal activity and osteogenic differentiation

For simulation of a perioperative implant contamination, the combined assay of bactericidal effectiveness and osteogenic differentiation was applied. The PHMB-coated surfaces of H_2O_2 (HP)- and NaOH (NP)-processed Ti6Al4V surfaces were contaminated with 10^3 cfu of *S. epidermidis*. Immediately after 6 hr of incubation at 37°C, 10^5 SaOs-2 cells were seeded on the bacterial inoculated surface. To demonstrate unimpaired biofilm formation, bacterial contaminated untreated Ti6Al4V discs were processed without SaOs-2 cells in parallel. HP- and NP-Ti6Al4V discs and also untreated control specimen with 10^5 SaOs-2 cells served to proof simultaneously unaffected cell growth and osteogenic differentiation of osteoblasts. The culture was performed first for 3 days in a basal medium and subsequently for 4 days in a mineralization medium. Already after 24 hr, bacterial biofilm formation was present on artificially contaminated controls but not for specimen coated with PHMB. Bacterial growth and viability on the untreated Ti6Al4V surface was not reduced during culture in basal medium. Additionally, the antibiotic and antifungal supplements in mineralization medium did not inactivate *S. epidermidis* in the produced biofilm during 4 day-culture and refreshing antimicrobial agents by daily changing the medium. On the PHMB-coated surface bacteria were absent and only SaOs-2 cell grew. Without a bacterial contaminated surface, SaOs-2 cell proliferation (XTT assay) on HP and NP specimen showed a slight increase compared with untreated controls. SaOs-2 cell produce alkaline phosphatase (Figure 6a) and deposit calcium apatite (Figure 6b) on both the bacterial contaminated and non-inoculated HP- and NP-Ti6Al4V surfaces after 4 day-culture in mineralization medium. Activity of ALP and calcium apatite deposition

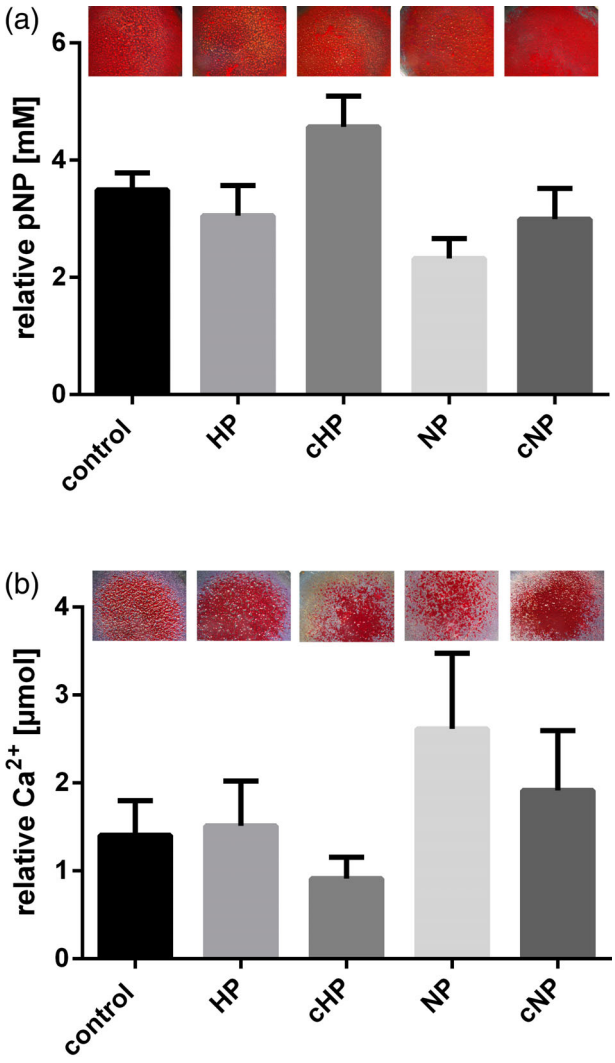

FIGURE 6 (a) Colorimetrically quantified alkaline phosphatase activity (ALP) as relative pNP-equivalents and (b) quantified deposited Ca^2+-ions, calculated relative to the cell viability. Top panels in (a) show representative stereomicroscopic images (0.75-fold magnification) of histochemical stained ALP and in (b) of Alizarin Red S-stained calcium deposition of differentiated SaOs-2 cells after 4 days culture in mineralization medium on the native control, the PHMB-coated surfaces of H_2O_2 (HP) or NaOH (NP) processed Ti6Al4V discs and on PHMB-coated specimen after precontamination with *S. epidermidis* (cHP, cNP). Data are shown as mean ± SEM (n = 8)

is not affected by PHMB-coating. In parallel, there is no inhibition of these osteogenic markers after antimicrobial efficacy of PHMB-coating toward *S. epidermidis*. SaOs-2 cell growth and their osteogenic differentiation on the contaminated and noninoculated HP- and NP-Ti6Al4V discs were comparable with that of the noninoculated control (Figure 6a,b) because apparent minor variation of means of eight separate experiments are not significant different.

4 | DISCUSSION

The simple chemical treatment procedure for Ti6Al4V with 5% H_2O_2 enables the adsorption of the antiseptic agent PHMB in

antimicrobially effective and noncytotoxic amounts. The resulting titanium oxide layer equipped with surface-bound water and surface hydroxyl groups facilitates molecular adsorption (Healy & Ducheyne, 1992). Alkali treatment has also been used for surface modification of Ti6Al4V to provide bioactive properties of the implant material (Kim, Miyaji, Kokubo, & Nakamura, 1996; Su, Komasa, Sekino, Nishizaki, & Okazaki, 2016; Wang, Wang, & Zhou, 2003; Wen, de Wijin, Cui, & de Groot, 1998). The resulting sodium titanate gel layer was heat-treated to produce a dense nano-porous oxide layer which promotes cell adhesion of rat bone marrow mesenchymal stem cells (Su et al. 2016) and the production of a uniform bone-like apatite layer on the specimen surface in a simulated body fluid (Kim et al., 1996). Apatite deposition was also evident without heat-treatment of NaOH-processed specimen containing still surface-bound water (Wang et al., 2003; Wen et al., 1998). In another study, PHMB and other tested antiseptics showed greater antimicrobial activity due to its internalization into a hydrophilic, porous hydrogel compared to being adsorbed by nonporous materials (Forbes et al., 2014). In the present study, the introduced negatively charged —OH groups and the water film mediate also the interaction of the alkali-processed Ti6Al4V discs with PHMB (Figure 1). The applied alkaline treatment with 5 M NaOH for 2 hr at 60°C did not completely reduce the hydrocarbon contamination, which was shown in another study for 10 M NaOH after 30 min (Su et al., 2016). However, the water contact angle of the surface decreased from 60° (native) to 10° after 5 M NaOH treatment of Ti6Al4V specimen (Table 1). PHMB adsorption is not prevented by the presence of these aliphatic contaminations, but the desired amounts of between 1 and 25 μg PHMB/disc can be adjusted by the incubation time in 30 μg/ml PHMB coating solution (Figure 1c, Table 2). This was not evident on H_2O_2 processed Ti6Al4V specimens because a 24 hr oxidation of Ti6Al4V specimen with 5% H_2O_2 produces an effective surface for coating of Ti6Al4V discs with only 4 μg PHMB using similar PHMB coating conditions for 24 hr (Müller et al., 2014). The most effective adsorption of PHMB takes place after an alkaline treatment of Ti6Al4V discs; this is much less the case after H_2O_2 processing. The elemental composition of these two modified surfaces show a significant difference for N (at.-%) indicating that the amount of adsorbed PHMB oligomers of ca. 20 μg cover the whole NaOH-treated surface (Table 1). This is supported by C1s high resolution spectra (Figure 3d,g). Especially, 70% of N-containing functional groups are presented by PHMB coating after NaOH treatment (Figure 3d) and only 50% of these were indicated on H_2O_2-processed specimen (Figure 3g). Additionally, the oxidation states of Ti(0), Ti(II), and Ti(III) in the Ti2p high resolution spectrum are not present after PHMB coating of the NaOH treated surface (Figure 3F). Whole coverage of the surface with PHMB did not influence the adherence or initial spreading of SaOs-2 cells (Figure 4), but the presence of amounts of 20 μg PHMB decreases significantly the viability of SaOs-2 cells after 48 hr of cell culture (Table 2). To exclude any inhibitory effect produced by the PHMB-loaded surface with 20 μg of PHMB, only half-maximally coated specimen containing 8–12 μg PHMB/disc were used for tests of antimicrobial efficacy and osteogenic differentiation.

Test microorganisms were embedded in a co-culture medium to simulate a perioperative transfer of bacteria by means of a smear infection in the presence of serum-containing fluids. The application of a co-culture medium (90% basal medium/10% TSB) was essential because *S. epidermidis* did not tolerate the cell culture medium alone. In a co-culture medium, all test microorganisms multiply and produce biofilm; furthermore SaOs-2 cells grow without any cytotoxic effect that may arise by TSB. The co-culture medium allows for the simultaneous growth of bacteria and tissue cells on the same biomaterial surface; therefore it is important to investigate the race for the surface between bacteria and tissue cells (Subbiahdoss, Kuijer, Grijpma, van der Mei, & Busscher, 2009) and whether osteoblasts (SaOs-2 cells) were able to grow on the PHMB-coated surface after microbicidal efficacy in a single experiment. For 6 hr-adherence technique, the antibacterial surface of Ti6Al4V discs was contaminated with a bacterial inoculum of 10^3 cfu to simulate perioperative bacterial contamination. For effective counting, applied amounts of bacteria were 1 $\log_{10}$ greater than those of 10^2 cfu of attached *S. aureus* producing already foreign body infections in an animal model (Zimmerli, Waldvogel, Vaudaux, & Nydegger, 1982). A bacterial reduction is evident only on PHMB-coated specimen, but not on Ti6Al4V surfaces that were either oxidized with H_2O_2 or treated with NaOH (Table 3). Performing the 6 hr-adherence drop technique, an effective antimicrobial impact against all three applied test bacteria was shown, combined with good reproducibility (Table 3). In the absence of leachable microbicidal agents, bacteria must come in close contact with the antimicrobial surface; this is realized after a 6 hr incubation applying this inoculation method. In the present study, there was no difference in antimicrobial efficacy of the PHMB-coated surface against Gram-positive *Staphylococci* or the Gram-negative strain of *Pseudomonas*; this indicates an effective surface contact between test bacteria and the immobilized active agent. Practically no bacteria were present after 6 hr of contact-killing by PHMB-coated Ti6Al4V specimens.

PHMB is not only adsorbed in a simple monolayer to different substrates (Blackburn, Harvey, Kettle, Payne, & Russell, 2006; Müller et al., 2014). The increasing PHMB adsorption on the hydroxylated TiO_2 surface and also that to cellulose are attributed to a self-assembly aggregation and a multilayer stacking of the PHMB molecule through electrostatic interactions with counterions and hydrogen bonding of biguanide groups. The self-assembly process of PHMB may eventually produce nano-objects (Müller et al., 2014) containing high-density cationic charges such as that described for PHMB molecules in solution (Zaki, Troisi, & Carbone, 2016) that additionally enhances the microbicidal efficacy of adsorbed PHMB.

In the present study, experiments to determine microbicidal effects and osteogenic differentiation were performed in comparable culture media to establish a common base for a combined assay. At first, bacterial inoculation of untreated (control for biofilm production) and PHMB-coated Ti6Al4V discs with *S. epidermidis* was conducted in a co-culture medium. After antimicrobial efficacy testing for 6 hr, SaOs-2 cells were seeded directly on the inoculated and non-inoculated specimen and cultured for 3 days in basal medium. Non-contaminated specimens with adsorbed PHMB were used as controls

for SaOs-2- cell growth and differentiation. Osteogenic differentiation of osteoblasts takes place for 4 days in a mineralization medium. Bacterial regrowth of each culture medium was evident only in the untreated control. The viability of *S. epidermidis* in the produced biofilm was not inhibited during the culture in basal medium, which was shown before to be not tolerated by planktonic microorganisms. Moreover, the mineralization medium supplemented with antibiotic and antifungals did not influence in any way the biofilm formation; this indicates that embedded microorganisms were protected from attacks of antibiotics by the matrix constituents of their biofilm. Already after 24 hr of cultivation, SaOs-2 cells adhere and grow on the PHMB-coated surface without any evidence of bacterial regrowth; this verifies the antibacterial effectiveness of the PHMB-coated Ti6Al4V surface under practical conditions. Immediately, after terminating the combined assay of microbicidal activity and osteogenic differentiation, viability of SaOs-2 cells was evaluated with XTT assay to generate a reference value for normalizing ALP activity and calcium deposition of the same specimen. Normalizing the ALP activity regarding the total number of cells, which correlates with XTT assay, eliminates the effect of proliferation on this parameter (Conserva, Menini, Ravera, & Pera, 2013). Cell cultures were not lysed for quantitation of protein or DNA as reference values and specimen can further be used for histochemical staining or determining ALP activity. After XTT assay, the osteogenic differentiation of SaOs-2 cells was determined by separately processing each kind of specimen in a duplicate for four different parameters: (a) AR-S staining of calcium deposition; (b) histochemical staining of ALP; (c) colorimetric measurement of ALP followed by AR-S staining for Ca^{2+}; and (d) AR-S staining for Ca^{2+} followed by the elution of the dye.

In the present study, SaOs-2 cell produce ALP and deposit calcium on both the bacterial contaminated and noninoculated PHMB-coated Ti6Al4V surface already after a 4 days culture in a mineralization medium (Figure 6a,b). In the investigations on the effect of bio-glass on growth and mineralization of bone-related SaOs-2 cells, which were incubated for 3 days in cell culture medium and subsequently for 5 days in a mineralization medium, ALP activity and an AR-S stained matrix were already present (Wang et al., 2014). There was no significant difference in ALP activity of osteoblasts cultured on PHMB-coated surfaces alone and after microbicidal efficacy of that Ti6Al4V discs compared with the untreated control without bacterial inoculation in our study (Figure 6a). In addition, no significantly greater amount of calcium was deposited by osteoblasts after the antimicrobial effect of adsorbed PHMB against *S. epidermidis* compared with that of the untreated noncontaminated control and PHMB-coated specimens, respectively (Figure 6b). Thus, adsorbed PHMB alone and the PHMB-coated surfaces after contact-killing of bacteria did not inhibit or stimulate SaOs-2 cell differentiation for production of osteogenic markers, such as ALP and calcium deposition. Values did not differ significantly from that of the untreated control, which is the result of the great variance of individual parameters. This variation may arise from the XTT assay, which was initially performed before estimating ALP activity and the AR-S staining of calcium. However, the presented results support the good compatibility of PHMB for tissue cells combined with a concomitant antimicrobial efficacy against a wide range of bacteria, which was also proven in other studies (Llorens, Calderon, del Valle, & Puiggali, 2015; Zhi et al., 2017). Therefore, both adsorbed and immobilized PHMB seems to be more tolerated by tissue cells than PHMB in solution; this opens up a wide field to use biocompatible and antimicrobial coating for medical and other relevant products.

5 | CONCLUSIONS

Simple chemical processing of a Ti6Al4V alloy with either 5% H_2O_2 or 5 M NaOH increases the adsorption of effective amounts of the antiseptic agent PHMB. The self-created multi-layered PHMB-film did not prevent the attachment or the spreading of osteoblast-like SaOs-2 cells, but it produces bactericidal efficacy against relevant device-associated slime producing bacteria. *S. aureus*, *P. aeruginosa*, and *S. epidermidis* were killed after surface contact with adsorbed PHMB. The applicability of these simple chemical treatments for an increased adsorption of other potentially antibacterial substances should be investigated to extend the spectrum of antimicrobial coatings. Both chemical procedures for the treatment of Ti-Al-V alloys, the oxidation with H_2O_2 and processing with NaOH can be easily performed without any special equipment. The presented procedures provide a promising alternative to other costly surface coating procedures including the one which operates only on flat materials. The presented procedures of oxidation with H_2O_2 and treatment with NaOH are suited for geometric nonuniform implant materials, fo example, screws, producing a homogenous activation of the surface in combination with an introduction of additional —OH groups and a hydration film. The final product is more passivated due to the susceptibility of further oxidation. Additionally, the implant material is disinfected during chemical processing and the subsequent storage in an antiseptically effective PHMB solution permits its instant use without further disinfection procedures. Possibly unwanted transmitted bacteria from the environment or after bacteremia will be killed after contact with the PHMB-coated implant material and osteoblasts proliferation is not inhibited on that previously bacterial contaminated surface. Adverse effects of residual bacterial components on the differentiation of SaOs-2 cells (ALP activity, calcium deposition) were not perceived. The PHMB-coated Ti6Al4V specimen containing different amounts of adsorbed PHMB should be investigated *in vivo* to verify the presented *in vitro* data.

ACKNOWLEDGMENTS

The authors thank Anne Brandenburg (Institute of Hygiene and Environmental Medicine, University Medicine Greifswald), Dagmar Jasinski, and Uwe Lindemann (INP, Greifswald) for competent technical assistance, and Reinhard G. Müller (University of Texas at Austin, USA) for proofreading the English manuscript. This work was supported by the Ministry of Economics, Employment, and Health Mecklenburg-Vorpommern in Germany (grant No. TBI-V-1-059-VBW-020).

COMPETING INTERESTS

The authors declare no potential conflict of interest.

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How to cite this article: Hornschuh M, Zwicker P, Schmidt T, Finke B, Kramer A, Müller G. Poly (hexamethylene biguanide), adsorbed onto Ti-Al-V alloys, kills slime-producing *Staphylococci* and *Pseudomonas aeruginosa* without inhibiting SaOs-2 cell differentiation. *J Biomed Mater Res*. 2020;108B: 1801–1813. https://doi.org/10.1002/jbm.b.34522