Original Article

Synthetic anti-endotoxin peptides interfere with Gram-positive and Gram-negative bacteria, their adhesion and biofilm formation on titanium

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Abstract

Aims: Implant-associated infections arise from the formation of bacterial biofilms, which are difficult to be treated with conventional antibiotics. Therefore, there is a need for new implant functionalizations, which inhibit biofilm formation. The aim of the present study was to characterize the effect of synthetic peptides to assess their applicability for this purpose.

Methods and Results: Two synthetic anti-endotoxin peptides, Pep19-2.5 and Pep19-4LF (Aspidasept I and II) were tested against both Gram-positive (\textit{Staphylococcus aureus} and \textit{Streptococcus oralis}) and Gram-negative (\textit{Pseudomonas aeruginosa} and \textit{Aggregatibacter actinomycetemcomitans}) bacteria associated with implant infections. Their activity was evaluated against different states of biofilm formation on the implant material titanium using CFU, live/dead fluorescence staining and confocal microscopy. Both peptides inhibited planktonic bacteria growth, impacted initial bacterial adhesion, reduced biofilm volume and increased the proportion of dead cells. Additionally, cytotoxicity analyses showed that neither peptide harmed human gingival fibroblasts nor osteoblasts at lower concentrations.

Conclusion: A concentration-dependent antibacterial activity of both peptides against biofilms of four clinically relevant bacteria could be demonstrated.

Significance and Impact of the Study: The results of this study serve as a promising basis for the improvement of these peptides in order to finally achieve a peptide-equipped antibacterial implant surface.

Introduction

The use of medical implant devices as a therapeutic treatment has steadily increased in recent decades (Thevenot et al. 2008). However, the insertion of foreign materials into the human body increases the susceptibility to infections by pathogens (von Eiff et al. 2005). Implant-associated infections are a serious complication for patients after surgery and are the main reason for implant failure and implant loss (Choong et al. 2007; Ercan et al. 2011). The microbiological conditions of operating rooms, surgical equipment, or even the microbial habitat on the patient’s skin are the main reasons for early infections (An and Friedman 1996; Zilberman and Elsner 2008). However, even several years after implantation, bacteria may enter the blood stream through open wounds, for example in the oral cavity, and then cause late implant-associated infections (Zimmerli and Ochsner 2003). Once Gram-positive and/or Gram-negative bacteria have gained access to the human body they can take different morphological shapes. The single cell form of bacteria (planktonic) is most easily defined and is accessible to the
host’s natural defences or antibiotic therapy (Olson et al. 2002). Bacteria can also adhere to surfaces (living or abiotic-like implants) and subsequently change their activity—resulting in the production of extracellular polysaccharides, DNA and proteins to form a biofilm matrix (Davies 2003). Attached biofilms on medical implants are composed of different bacterial species (Donlan 2001) and are characterized by resistance to stressful environmental conditions, like heat, cold shock or pH alterations (Donlan and Costerton 2002). In addition, the extracellular matrix prevents diffusion of antibiotics and biocidal substances (Mah and O’Toole 2001). Minimal biofilm eradication concentrations of commonly used antibiotics are typically 10- to 1000-fold higher than those for planktonic bacteria of the same species (Prosser et al. 1987; Ceri et al. 1999). Moreover, during the last 20 years, it has become increasingly clear that the use of traditional antibiotics is not sufficient to eliminate such implant-associated bacterial infections (Fair and Tor 2014), since the dense biofilm matrix and the interplay of different species delays or gradually impairs antibiotic activity, and thus favours the development of resistance (Stewart 2015).

To combat implant-associated infections, there is a need for specifically equipped implant materials, which immediately inhibit biofilm formation. Possibilities for infection-resistant implant functionalizations are antimicrobial peptides (AMPs), which have excited increasing interest as alternatives to conventional antibiotics (Sang and Blecha 2008). These compounds are present in every living being and are part of the innate immune system (Zasloff 2002). Natural or synthetic AMP sequences with positively charged residues (arginine or lysine) and their amphipathic structure can express activity against a broad spectrum of both Gram-positive and Gram-negative bacteria and are not prone to resistance development, as they do not induce the production of extracellular polysaccharides (Mah and O’Toole 2001). Minimal biofilm eradication concentrations of commonly used antibiotics are typically 10- to 1000-fold higher than those for planktonic bacteria of the same species (Prosser et al. 1987; Ceri et al. 1999). Moreover, during the last 20 years, it has become increasingly clear that the use of traditional antibiotics is not sufficient to eliminate such implant-associated bacterial infections (Fair and Tor 2014), since the dense biofilm matrix and the interplay of different species delays or gradually impairs antibiotic activity, and thus favours the development of resistance (Stewart 2015).

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The synthetic compounds in the Pep19 series have been designed as a new class of synthetic AMPs with high dual efficiency against Gram-positive and Gram-negative bacteria (Gutsmann et al. 2010; Pfalzgraff et al. 2016). In this study, we focused on Pep19-2.5 and Pep19-4LF, which have already been shown to effectively neutralize pathogenicity factors of Gram-negative and Gram-positive bacteria (lipopolysaccharides and lipoproteins, respectively) and to reduce inflammatory immune host responses (Gutsmann et al. 2010; Heinbockel et al. 2013; Martin et al. 2015; Dupont et al. 2015; Martinez de Tejada et al. 2015; Martin et al. 2016; Pfalzgraff et al. 2016). Even if the two peptides have so far only been tested against planktonic bacterial growth (Gutsmann et al. 2010; Dupont et al. 2015), it has been suggested that they could be useful therapeutic agents for severe bacterial infections, particularly as their toxicity is low (Brandenburg et al. 2011; Pfalzgraff et al. 2016).

The aim of the present study was to characterize the effect of Pep19-2.5 and Pep19-4LF on bacterial adhesion and biofilm development on the implant material titanium, and to assess their applicability for the development of biofilm-resistant implant materials. For this purpose, four bacterial strains were used as model organisms that are involved in peri-implant infections associated with orthopaedic and dental biofilms: the Gram-positive Staphylococcus aureus (Dapunt et al. 2016) and Streptococcus oralis (Maruyama et al. 2014) as well as the Gram-negative Pseudomonas aeruginosa (Brouqui et al. 1995) and Aggregatibacter actinomycetemcomitans (Ong et al. 1992). Initially, the antibacterial activity of AMPs was determined for planktonic cultures. The determined MICs were then used to analyse the peptides’ effect on bacterial initial adhesion and biofilm formation on titanium. Additionally, the cytotoxic profile of the peptides was analysed using human gingival fibroblasts (HGFib) and osteoblasts.

Materials and methods

Peptide synthesis

Pep19-2.5 and Pep19-4LF were synthesized with an amidated C-terminus by solid-phase peptide synthesis in an automatic peptide synthesizer (Model 433 A; Applied Biosystems, Foster City, CA) at the Research Center Borstel—Leibniz Lung Center, according to the FastMoc synthesis protocol supplied by the manufacturer, including the removal of the N-terminal Fmoc group. The amide at the C-terminus was synthesized on an Fmoc-amide resin.
The peptides were deprotected and cleaved from the resin with 90% trifluoroacetic acid, 5% anisole, 2% thioanisole and 3% dithiothreitol for 3 h at room temperature. After cleavage, the solution was filtered and the soluble peptides were precipitated with ice cold diethyl ether, followed by centrifugation and extensive washing with cold ether. Peptides were purified by RP-HPLC at 214 nm, using an Aqua-C18 column (Phenomenex; Aschaffenburg, Germany). The elution was performed with a gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid. Purity levels of up to 98% were checked by matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry. The sequences are as follows——Pep19-2.5: GCKKYRR FRWKFGKFWFG; Pep19-4LF: GKKYRRFRWKFK GKLFLFG. The sequences are protected by patent 2108 372 A1 at the European Patent Office in 2015 for EU, USA and Japan.

**Bacterial strains and culture conditions**

The following strains were used: *Staph. aureus* (DSM 799, German Collection of Microorganisms and Cell Culture, Braunschweig, Germany), *Strep. oralis* (ATCC 9811, American Type Culture Collection, Manassas, VA), *A. actinomycetemcomitans* (MCCM 2474, Microbial Culture Collection Marburg, Marburg, Germany) and *P. aeruginosa* (ATCC BAA-47; American Type Culture Collection).

All bacteria were stored at −80°C as glycerol stock. Precultures from each bacterium were prepared 24 h before the experiment in tryptone soya broth (TSB; Oxoid, Hampshire, UK) supplemented with 10% yeast extract (Roth, Karlsruhe, Germany) or Shaedler broth (Oxoid), supplemented with 10 µg ml⁻¹ vitamin K (Roth), in the case of *A. actinomycetemcomitans*.

**Planktonic susceptibility assay**

Planktonic bacteria susceptibility testing was modified from a previously published protocol (Dupont et al. 2015). Bacterial precultures were harvested, washed two times with Tris-HCl buffer (50 mol m⁻³, pH 7.5) and diluted in the same buffer to a final concentration of 2 × 10⁵ cells per ml. Peptides were dissolved in deionized water (1 mg ml⁻¹) and serially diluted to the following concentrations: 200, 100, 50, 25, 12.5, 6.25, 3.125 and 0.625 µg ml⁻¹. Bacteria were incubated with the peptides for 1 h under agitation at 500 rev min⁻¹ and at 37°C in aerobic conditions for *Staph. aureus* and *P. aeruginosa* and in 5% CO₂ for *Strep. oralis* and *A. actinomycetemcomitans*. Bacterial cultures without peptide served as positive controls. Afterwards bacterial growth was measured by counting colony forming units (CFU per ml) after plating on appropriate agar plates and incubation under suitable conditions: *Staph. aureus* and *P. aeruginosa* were incubated aerobically on TSB agar plates for 24 h, whereas *Strep. oralis* and *A. actinomycetemcomitans* were incubated on TSB or Columbia blood (Oxoid) agar plates for 3 days or 1 week, respectively, in 5% CO₂ atmosphere. The lowest peptide concentration, which prevented residual colony formation, was defined as minimal inhibitory concentration (MIC).

**Initial bacterial adhesion assay**

To assess the effect of peptides against bacterial initial adhesion on titanium surfaces, bacterial precultures were harvested, washed two times with Tris-HCl buffer (50 mol m⁻³, pH 7.5) and vortexed for 5 min before adjusting the optical density at 600 nm (OD₆₀₀) to 0.5, 0.7, 0.2 and 1.0 for *Staph. aureus*, *Strep. oralis*, *P. aeruginosa* and *A. actinomycetemcomitans*, respectively, using a photometer (Biophotometer; Eppendof AG, Hamburg, Germany). Prior to each experiment, peptide powder was dissolved in deionized water (1 mg ml⁻¹) and diluted in the bacterial suspension to the following final concentrations: 200, 100, 50, 25 and 12.5 µg ml⁻¹. The bacteria-peptide solutions were immediately added to titanium discs (grade 4, 12 mm in diameter, finished with 45 µm diamond abrasive polishing wheels) and incubated for 5 h under agitation with 150 rev min⁻¹, at 37°C aerobically or 5% CO₂ for *A. actinomycetemcomitans* in a humidity chamber. As a positive control, bacterial solutions without peptides were used.

**Biofilm formation assay**

To analyse the effect of peptides against biofilm formation, precultures were harvested and resuspended in TSB supplemented with 50 mol m⁻³ glucose for *Staph. aureus* and *Strep. oralis*, whereas Shaedler broth + vitamin K (10 mg ml⁻¹) was used for *A. actinomycetemcomitans* and *P. aeruginosa*. The OD₆₀₀ was adjusted to 0.001 for *Staph. aureus* and *P. aeruginosa*, to 0.03 for *Strep. oralis* and to 0.3 for *A. actinomycetemcomitans* using a photometer (Biophotometer; Eppendorf AG). As in adhesion experiments, peptides were dissolved in deionized water (1 mg ml⁻¹) before each experiment and mixed with bacterial suspensions to final concentrations of 200, 100, 50, 25 and 12.5 µg ml⁻¹. Bacterial solutions with or without peptides (positive control) were immediately incubated on titanium discs (grade 4, 12 mm in diameter, finished with 45 µm diamond abrasive polishing wheels) and biofilm was grown statically for 24 h without agitation, at 37°C under aerobic conditions for *Staph. aureus* and *Strep. oralis* or anaerobic conditions for *A. actinomycetemcomitans* or in 5% CO₂ atmosphere for *P. aeruginosa*.
Quantification with confocal laser scanning microscope

The initial adhered bacterial cells and the biofilm formed on titanium discs were fluorescently stained using the LIVE/DEAD® BacLightTM Bacterial Viability Kit (Life Technologies, Darmstadt, Germany). The two DNA-binding fluorescent dyes were applied as a 1 : 1000 dilution in phosphate-buffered saline. SYTO®9 is able to cross membranes and stains all bacteria, whereas propidium iodide (PI) only penetrates bacteria with damaged membranes. When both dyes are present, PI binds onto nucleic acids more strongly than SYTO®9; so that SYTO®9 is displaced by PI (Stocks 2004). Therefore, viable bacteria emit a green fluorescence signal and dead bacteria are stained in red. Afterwards bacteria were fixed with 2.5% glutaraldehyde. Confocal laser scanning microscopy (Leica TCS SP2; Leica Microsystems, Mannheim, Germany) with a 488 nm laser and an emission range of 500–550 nm for SYTO®9 and 675–750 nm for PI was used to observe the fluorescently labelled cells on the titanium discs at five positions, each. For initial adhesion, a single image was taken with 63× magnification, an image area of 190 × 190 μm² and 1024 × 1024 pixel² resolution. The number of adhered bacteria per image and the respective live/dead distribution (relative amount of green and red cells) were calculated using ImageJ 1.48v (Wayne Rasband, National Institutes of Health). For biofilm formation, z-stacks with a step size of 5 μm were taken with 40× magnification, an image area of 300 × 300 μm² and 1024 × 1024 pixel² resolution. The biofilm volume per image and the respective live/dead distribution (relative volume of green and red cells) were calculated using the Imaris® ×64 6.2.1 software package (Bitplane AG, Zurich, Switzerland). Colocalizing fluorescence was assigned as red value and subtracted from the amount of green fluorescence.

Measurement of peptide aggregation

Dynamic light scattering (DLS) measurements were performed to obtain the hydrodynamic diameters of the peptides and their distributions, using a Zetasizer Nano ZSP (Malvern Instruments Ltd., Enigma Business Park, Malvern, UK) and the associated software Zetasizer Software ver. 7.11. Samples were prepared by dissolving peptides in distilled water to five concentrations (200, 100, 50, 25 and 12.5 μg ml⁻¹). The preprogrammed RI (1-450) and absorption (0-001) for proteins were used. The measurement temperature was set at 37°C and the samples were equilibrated for 120 s to reach the desired temperature. The scattered light was detected at an angle of 173° (backscattering). Per sample, three measurements were performed, consisting of at least 14 subruns (each 10 s). For data processing the general-purpose analysis model was used.

Cytotoxicity assay

For cytotoxicity experiments, isolated cells from peri-implant tissues within the oral cavity were used: HGFiB (Provitro GmbH, Berlin, Germany) were cultured in Dulbecco’s modified Eagle medium (Biochrom AG, Berlin, Germany) supplemented with 10% foetal bovine serum (Panbiotech GmbH, Aidenbach, Germany), 100 U ml⁻¹ penicillin and 100 mg ml⁻¹ streptomycin (Biochrom AG). Human osteoblasts (NHOst) were cultured in osteoblast growth medium (Lonza, Cologne, Germany) containing foetal bovine serum and ascorbic acid. Both cell types were subcultured according to provider protocol and maintained at 37°C in a 5% CO₂, 95% humidified air atmosphere.

To analyse the effect of antibacterial peptides on membrane integrity and cell metabolism of tissue cells, HGFiB or NHOst were seeded in a volume of 200 μl with a density of 3000 cells per well in 96-well plates. After achieving 80% confluence, the medium was exchanged to stimulate cells with different concentrations of Pep19-2.5 or Pep19-4LF respectively. Therefore, peptides were dissolved in deionized water (1 mg ml⁻¹) and serially diluted in corresponding culture medium to concentrations of 200, 100, 50, 25 and 12-5 μg ml⁻¹. As a control, cell culture medium was diluted with the respective volume of deionized water. Undiluted cell culture medium without peptides served as nontoxic reference. Medium with 1% Triton X-100 (Fluka, Buchs, Swiss) served as a reference for maximum cytotoxicity. After 24 h stimulation, the different samples were analysed according to the manufacturer’s protocols.

For the analysis of cell metabolism, the activity of mitochondrial enzymes was estimated by measuring the conversion of resazurin into a fluorescent end product by living cells. Briefly, 20 μl of staining solution (Promega GmbH, Mannheim, Germany) were added to the remaining cells and media in the 96-well plates. After 4 h incubation at 37°C, fluorescence was measured with an excitation at 540 and emission at 595 nm. Fluorescence values were normalized to the nontoxic reference (100% metabolic activity).

Membrane integrity was analysed by measuring the amount of leaked lactate dehydrogenase (LDH) using the enzymatic reduction of the tetrazolium salt INT to formazan. Briefly, 100 μl of supernatant is mixed with 100 μl of the staining solution (Roche Diagnostics, Rotkreuz, Swiss). After 5–10 min incubation at RT, the reaction was stopped with 50 μl 1 mol l⁻¹ HCl and absorption was measured at 492 nm with a reference at
650 nm. Absorption values were normalized to the maximum cytotoxicity reference (100% LDH activity).

**Statistical analysis**

All experiments were repeated three times in duplicates. Statistical analysis was performed by GraphPad Prism 8.1.0 software (GraphPad Software Inc., La Jolla, CA). For number of adhering bacteria and biofilm volume, the Shapiro–Wilk test was used to test for Gaussian distribution. For data, which passed normality test, significant differences to the control were analysed using ordinary one-way ANOVA with Dunnett’s multiple comparison test. Data, which did not pass normality test, were analysed using Kruskal–Wallis test with Dunn’s multiple comparison test. Live/Dead distribution was analysed using two-way ANOVA with Dunnett’s multiple comparison test. A value of *P* < 0.05 was defined as significant difference to the control.

**Results**

**Activity of peptides against planktonic bacteria**

As a basis for the assessment of anti-biofilm properties, the efficiency of both peptides against the four bacterial species grown in planktonic cultures was determined by CFU method. The MIC was defined as lowest concentration of peptides that prevented any residual colony formation. As shown in Fig. 1, both peptides caused intense reduction of CFU per ml compared to the control—even at very low concentrations. The MIC was 1-25 μg ml⁻¹ for most of bacteria/peptide combinations tested (Fig. 1a–d). Except, for Strep. oralis and A. actinomycetemcomitans treated with Pep19-2.5 the MIC was 0-125 μg ml⁻¹ (Fig. 1b,d). Therefore, Pep19-2.5 showed slightly greater activity against Strep. oralis and A. actinomycetemcomitans, whereas Pep19-4LF was slightly more effective against P. aeruginosa. The peptides were equally active against Staph. aureus.

**Activity of peptides against the initial adhesion of bacteria to titanium surfaces**

Inhibition of initial bacterial adhesion is an essential step in preventing implant-associated infections and subsequent biofilm formation, a state which is extremely resistant to the host immune defence and classical antibiotic treatment (Veerachamy et al. 2014). The effect of the peptides Pep19-2.5 and Pep19-4LF on initial adhesion was analysed using live/dead fluorescent staining and confocal microscopy, as this method allows bacterial visualization and reliable quantification also in case of low amounts (Doll et al. 2016). As the evaluated peptides are analysed for their potential application as implant functionalization where higher concentrations will be applied, concentrations starting from 10-fold MIC were used already for this initial biofilm experiments.

For most of the combinations investigated, the number of adherent bacteria was reduced upon peptide treatment (Fig. 2). The reduction increased with increasing concentrations of both peptides and was highest at 100 or 200 μg ml⁻¹. Reductions at these concentrations were significant for Pep19-2.5 against all strains but Staph. aureus and for Pep19-4LF against A. actinomycetemcomitans and Staph. aureus (Figs 2 and 3). For some combinations, there was a distinct reduction even at concentrations of 12.5 or 25 μg ml⁻¹; this was significant for Staph. aureus and A. actinomycetemcomitans with both peptides (Fig. 2). The only case in which the visible effect was not confirmed by a real reduction in bacterial numbers was Strep. oralis treated with Pep19-4LF (Figs 2 and 3). However, the aggregation of bacteria, which can be seen in Fig. 3, also indicates a certain impairment of the bacterial cells.

Beside reduced amounts of initially adhering bacteria, both peptides remarkably increased the proportions of cells with impaired membrane, defined as dead cells, by up to 50%, especially at low concentrations (Fig. 2). This increase was significant for both peptides against Staph. aureus, Strep. oralis and P. aeruginosa.

In summary, both peptides impaired the initial adhesion of Gram-positive and Gram-negative bacteria on titanium surfaces. The effect was greatest on Staph. aureus and A. actinomycetemcomitans. Pep19-2.5 seemed to have a slightly greater impact on lowering the number of attached cells, whereas Pep19-4LF increased the proportion of dead cells to a greater extend. There was no distinct difference between Gram-positive and Gram-negative strains in their susceptibility towards both peptides.

**Activity of peptides against bacterial biofilm formation on titanium surfaces**

The ability of bacteria to form biofilms on implant surfaces is a very important virulence factor and a critical issue for medical treatments (Donlan 2001). Therefore, we tested the effect of the peptides on bacterial biofilm formation using also live/dead fluorescent staining and confocal microscopy.

Quantification of biofilm volume after treatment with peptides Pep19-2.5 and Pep19-4LF for 24 h showed a reduction for all bacterial strains tested compared to the respective controls. For all combinations, the reduction in biofilm volume was greater at higher peptide
concentrations and was greatest at 100 or 200 µg ml⁻¹, as shown in Figs 4 and 5. At these concentrations, the reduction was statistically significant for *P. aeruginosa* and *A. actinomycetemcomitans* treated with either of the peptides and for *Staph. aureus* treated with Pep19-2.5 (--- black dotted line) or Pep19-4LF (—— grey solid line). Results are depicted as mean ± SD.

The proportion of bacterial cells with impaired membrane (dead cells) increased up to 80% after treatment with Pep19-4LF, which was statistically significant for all strains at least at 100 and 200 µg ml⁻¹ compared to the untreated control (Fig. 4). Treatment with Pep19-2.5 only increased the proportion of dead cells up to 40–60% for *P. aeruginosa* and *A. actinomycetemcomitans*, and was significantly different from the control at 25 and/or 50 µg ml⁻¹ (Fig. 4). Pep19-2.5 had almost no effect on the viability of Gram-positive bacteria, (Figs 4 and 5).

In summary, both peptides impaired the formation of biofilms of all strains tested. The effect was greatest for *A. actinomycetemcomitans*. As regards initial adhesion, Pep19-2.5 more strongly reduced biofilm volume, whereas Pep19-4LF had a greater impact on the live/dead distribution (Fig. 5). Both peptides were more effective against the biofilm of Gram-negative than Gram-positive bacteria.

**Analysis of peptide aggregation**

It was also investigated whether peptide aggregation influences antibacterial activity. For this purpose, DLS...
Figure 2  Concentration-dependent influence of peptides on the initial adhesion of bacteria after 5 h of incubation. Numbers of adherent bacteria per image of 190 × 190 µm² on titanium discs are shown as Tukey boxplots and the corresponding relative live/dead distribution as mean ± SD. Proportions of living bacteria are shown in green/lower part, proportions of dead bacteria are shown in red/upper part. * indicates significant differences from the control with \( P < 0.05 \). [Colour figure can be viewed at wileyonlinelibrary.com]
was used to measure any peptide aggregation above the critical micellar concentration. The hydrodynamic diameter—the diameter of a sphere that diffuses in liquid at the same speed as the measured particles (Stetefeld et al. 2016)—was calculated for peptide particles dissolved in distilled water. The particle sizes of peptides at concentrations of 12 \( \mu \text{g mL}^{-1} \) and 25 \( \mu \text{g mL}^{-1} \) were not measurable by the DLS device, as the concentrations were too low. At concentrations of 50, 100, 200 \( \mu \text{g mL}^{-1} \) of either peptide, the DLS signal did not change with concentration (Fig. S1).

### Cytotoxic effects of peptides against human cells

In order to evaluate the clinical potential of both peptides, their cytotoxicity to human cells must be investigated. Cytotoxicity was measured using normal human osteoblasts or HGFib, which are both essential cell types of the peri-implant tissue and responsible for firm wound closure (Kaga et al. 2017; Miao et al. 2017).

Cellular metabolic activity was analysed by CellTiter-Blue assay, which is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product.
**Figure 4** Concentration-dependent influence of peptides on biofilm formation of bacteria after 24 h of incubation. Biofilm volumes (\(\mu m^3\)) per image of 300 x 300 \(\mu m^2\) on titanium discs are shown as Tukey boxplots and the corresponding live/dead distributions as mean ± SD. Proportions of living bacteria are shown in green/lower part, proportions of dead bacteria are shown in red/upper part. * indicates significant differences to the control with \(P < 0.05\). [Colour figure can be viewed at wileyonlinelibrary.com]
product (resorufin). Cellular membrane damage was quantified by measurement of extracellular LDH activity, which is released from lysed cells. For fibroblasts, reduced metabolic activity and increased membrane damage were detected at concentrations of 100 or 200 µg ml⁻¹ for Pep19-4LF and Pep19-2.5, respectively, and were significant (P < 0.05) at these concentrations (Fig. 6a,c). For osteoblasts, metabolic activity and membrane integrity started to decrease significantly at 50 or 100 µg ml⁻¹ for Pep19-4LF or Pep19-2.5 respectively (Fig. 6b,d). For both cell lines, Pep19-4LF exhibited cytotoxicity at lower concentrations than Pep19-2.5, whereas osteoblasts in general appeared more sensitive to the treatment than fibroblasts. In any case, concentrations up to 25 µg ml⁻¹ for Pep19-4LF and 50 µg ml⁻¹ for Pep19-2.5 did not influence cellular viability or membrane integrity of fibroblasts or osteoblasts.

Discussion

Bacterial attachment to medical devices such as implant surfaces and subsequent biofilm formation can cause severe problems with the functions of these materials in the human body (Donlan 2001). Bacterial embedment in the
Biofilm matrix of extracellular polymeric substances, reduced growth as well as changes in gene expression patterns increase resistance to traditional antibiotics by up to 1000-fold (Ceri et al. 1999; Davies 2003). To prevent matrix encapsulation, there is a need for novel implant materials, which immediately inhibit biofilm formation. Antimicrobial peptides have recently emerged as potential implant functionalization with a broad spectrum of activity and simultaneously reduce resistance development in different types of bacteria, fungi and viruses (Zasloff 2002; Jenssen et al. 2006; Ma et al. 2012). In previous studies, Pep19-2.5 and Pep19-4LF exhibited intense anti-inflammatory activity against bacterial toxins. They strongly inhibited toxin-induced cytokine (tumour necrosis factor-α and interleukin-6) production by relevant immune cells, such as mononuclear cells and macrophages. They act by binding and neutralizing lipopolysaccharide from Gram-negative and lipoproteins/-peptides from Gram-positive bacteria, whether these were present as constituents of the bacteria or in the isolated form (Gutsmann et al. 2010; Heinbockel et al. 2013; Martin et al. 2015; Dupont et al. 2015; Pfalzgraff et al. 2016; Martin et al. 2016).

Even if the antibacterial properties of both peptides had already been addressed in a planktonic susceptibility assay (Gutsmann et al. 2010; Dupont et al. 2015), in this study, we analysed for the first time the effect of these peptides against biofilm formation on the implant material titanium. For this purpose, bacterial strains associated with implant infections in different human body habitats, and with different cell wall types were used: *Staph. aureus* and *P. aeruginosa* belong to the hosts commensal microbiome, but are also among the major pathogens associated with infections of medical devices, especially of orthopaedic implants (Brouqui et al. 1995; Pihl et al. 2010; Rohacek et al. 2010; Dapunt et al. 2016). *S. oralis* and *A. actinomycetcomitans* are constituents of the oral microflora and can be involved in the development of periodontitis and dental peri-implantitis (Zambon 1985; Ong et al. 1992; Dorkhan et al. 2012; Maruyama et al. 2014).

According to the present results, both peptides were highly active against planktonic bacteria in suspension even at low concentrations, with maximal MIC values of 1.25 μg ml⁻¹. It could be noted that the measured MICs were strikingly lower than those determined in previous studies on Pep19-2.5 and similar peptides (Gutsmann et al. 2010; Dupont et al. 2015), which, however, were performed with other bacterial species and partially with other methods.

![Figure 6](image-url) Concentration-dependent influence of peptides on human cells. Human gingival fibroblast (a, c) and human osteoblasts (b, d) were treated with Pep19-2.5 (–– black dotted line) or Pep19-4LF (— grey solid line) or respective volume of deionized water as control (–– black solid line) for 24 h. Graphs show metabolic activity (a, b) quantified by CellTiter-Blue assay and membrane damage (c, d) quantified by extracellular lactate dehydrogenase activity relative to nontoxic (a, b)/maximum cytotoxicity (c, d) references.
With regard to a prospective application as implant functionalization, in the next step AMPs were analysed for their effectiveness against initial bacterial adhesion and biofilm formation on titanium. For initial adhesion, even the lowest concentration tested was able to reduce the number of attached cells, as well as to increase the proportion of dead cells compared to the control. For biofilm formation, decreased biofilm volume was detected at increasing peptide concentrations. The number of dead cells was also distinctly enlarged.

The ability to eradicate bacterial biofilms of both Gram-positive and Gram-negative bacteria has recently been shown for other antibacterial peptides (de la Fuente-Nunez et al. 2012; Hirt and Gorr 2013; Wang et al. 2015). Cationic AMPs consist of short amino acid sequences with amphipathic properties that allow them to interact with the negatively charged bacterial membrane (Jenssen et al. 2006). In general, it is assumed that they disrupt the membrane by pore formation through the ‘barrel-stave’, ‘carpet’ or ‘toroidal-pore’ mechanisms, which lead to cell death (Shai 2002). Although the sequences and structures of these peptides are highly diverse, membrane disruption by the AMPs and subsequent bacterial cell death through pore formation have recently been explained on the basis of topological and electrostatic interactions between the membrane-bound peptides and lipids (Paterson et al. 2017).

According to the results of this study, even though both peptides damaged the bacterial membrane, their mode of action seems to differ: Pep19-2.5 more strongly reduced the amount of bacteria on the surface, whereas Pep19-4LF gave a greater increase in the proportion of cells with disrupted membranes (designated as dead). Both amphiphilic peptides consist of 19–20 amino acids supplemented with positively charged arginine (R) or lysine (K) residues, a polar, hydrophilic N-terminus and hydrophobic amino acids localized near the C-terminal end (Gutsmann et al. 2010; Pfalzgraf et al. 2016). However, the terminal tryptophan (W) residues in Pep19-2.5 were replaced with the more hydrophobic amino acid leucine (L) in Pep19-4LF (Wolffenden 2007; Pfalzgraf et al. 2016). Increasing the hydrophobicity of the amphiphatic peptide would also magnify their interaction with the hydrophobic core of the phospholipid bilayer and subsequently increase their activity (Eisenberg et al. 1984; Avrahami and Shai 2002). This may explain the increase in the proportion of bacteria with damaged membrane with Pep19-4LF. Furthermore, the reduction in bacterial attachment and biofilm formation was observed with both peptides, but especially with Pep19-2.5. The presence of a cysteine in position 2 at the N-terminus of Pep19-2.5 could have an impact on the effectiveness, as well as the CG motif at the N-terminal end, which is known to be relevant for the high antimicrobial activity of other peptides of the Pep19-series (Gutsmann et al. 2010). The activity of both peptides may also be attributed to an additional impact on the expression of bacterial genes involved in bacterial adhesion and biofilm formation (Overhage et al. 2008; de la Fuente-Nunez et al. 2012). According to the results of this study, the not yet analysed peptide’s mode of action would be of further interest to shed more light on the different behaviour of the analysed peptides Pep19-2.5 and Pep19-4LF.

The two peptides had greater effects on adhesion and biofilm formation at high concentrations and were mostly significant at 100 and 200 µg ml⁻¹. Other studies also showed that random mixtures of phenylalanine and lysine and the antibacterial peptide GL13K disrupted biofilms of methicillin-resistant Staph. aureus and P. aeruginosa biofilm at high concentrations of 200 µg ml⁻¹ (Hirt and Gorr 2013; Stern et al. 2016).

It was striking that the greatest proportion of dead cells was found with the lowest concentrations of peptides. This could perhaps be due to simple aggregation of peptides at higher concentrations, which would reduce the overall effective surface. Therefore, aggregation was measured with DLS. Contrary to expectation, there was no evidence for increased aggregation at higher peptide concentrations. Thus, this observation cannot serve as an explanation for the effect on the live/dead distribution. Probably, aggregation may only occur in direct contact with a phospholipid membrane or does not at all influence the peptides mechanism. The unravelling of both hypotheses would require more sophisticated analyses and could be addressed in future studies.

The results of this study show that Gram-negative bacteria were more susceptible to the tested peptides than Gram-positive bacteria (especially for biofilm formation). It is known that antimicrobial substances may exhibit differential activity between these types of bacteria (Lambert 2002). Although not always, Gram-negative bacteria are often more susceptible to antimicrobial therapy because their cell wall is thinner and contains fewer peptidoglycans (Lambert 2002; Epand et al. 2016). It may be that cationic AMPs are more active against Gram-negative bacteria as they may be inserted across the outer membrane of Gram-negative bacteria with the ‘self-promoted uptake’ mechanism (Sawyer et al. 1988; Hancock and Rozek 2002).

Besides antibacterial activity, cytotoxicity is an important issue in the development and use of AMPs in the clinic. Cationic AMPs are presumed to be highly selective to the negatively charged bacterial membrane made of anionic lipids. In contrast, the mammalian cell membrane has no net charge, due to their composition of zwitterionic lipids and cholesterol (Zasloff 2002; Bacalum and
Radu 2015). Our study indicated that human osteoblasts and fibroblasts are tolerant to direct interaction with both peptides at lower concentrations (up to 50 µg ml⁻¹). Pep19-4LF was more cytotoxic than Pep19-2.5 in both cell types. Although both peptides have the same proportion of positively charged residues, Pep19-4LF has increased hydrophobicity in the C-terminus, which might affect its cytotoxicity towards human cells (Li et al. 2006).

Our results showed that both peptides impair bacterial adhesion and biofilm formation also at low concentrations. However, for a prospective clinical application there is a need for further improvement of the peptides activity, in particular to achieve a broader therapeutic window with significant antibacterial activity while maintaining cytocompatibility with the surrounding tissue. The clarification of the peptides mode of action may therefore provide helpful suggestions.

In conclusion, we have reported in this study for the first time the anti-biofilm activity of antimicrobial Pep19-2.5 and Pep19-4LF against a variety of both Gram-positive and Gram-negative bacterial strains. We detected very low MICs against planktonic cultures as well as reduced initial bacterial adhesion and biofilm formation on titanium surfaces, with a simultaneous increase in the proportion of dead bacteria. In contrast, the peptides showed no cytotoxic effects on relevant human cells up to 50 µg ml⁻¹. These results serve as a promising basis for prospective clinical applications of improved versions of these peptides in order to finally achieve a peptide-equipped antibacterial implant surface, which decreases the risk of implant-associated infections in both orthopaedics and dentistry.

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Conflict of Interest

The authors declare no conflict of interest. Klaus Brandenburg provided the study with the synthetic peptides Pep19-2.5 and Pep19-4LF (Patent-No: PCT/EP2009/002565) in his function as a chief scientific officer of the commercial company Brandenburg Antiinfektiva GmbH. This study neither was a contract work for this or another company nor was there any exertion of influence on the scientific procedure or outcome.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Concentration-dependent distribution of hydrodynamic diameters of Pep19-2.5 and Pep19-4LF particles.