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Identification of Antimicrobial Peptides and Immobilization Strategy Suitable for a Covalent Surface Coating with Biocompatible Properties

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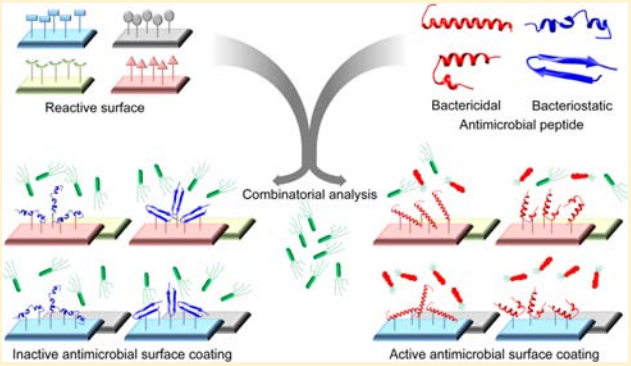
ABSTRACT: Bacterial accumulation on solid material displays a major source of biomaterial associated infections, cross contamination, and spreading. To overcome these problems, different investigations on surface modifications for the containment of bacterial adhesion have been done. The aim of this research is the development of a rapid and efficient screening procedure to identify and investigate biologically active peptides in an immobilized state in order to produce an antimicrobial surface coating. We figured out that the antimicrobial mode of action is the most important parameter because only peptides with pronounced membrane disruption abilities displayed meaningful activity in an immobilized state. In addition, we highlighted the influence of the coupling reaction chemistry on the activity and amount of the immobilized peptide. Thereupon we developed an optimized antimicrobial surface coating with unrestricted antimicrobial properties by adjusting the immobilization strategy in combination with lowering the necessary peptide amount. Moreover we demonstrated that this antimicrobial surface coating displayed no cytotoxic activity against a eukaryotic cell line and thereby indicates a promising biocompatibility. Furthermore, different antimicrobial peptides obtained either by chemical peptide synthesis or by recombinant DNA technology were used in this study and their activities as well as their potential applications were discussed.



INTRODUCTION

The control of bacterial populations and the protection against bacterial infections are of great interest in today's society. Because bacteria are able to accumulate on solid material, contaminated surfaces represent a significant reservoir. The control of adhesion of bacteria to surfaces is crucial in many applications, for example, in industrial tubes, food-processing equipment and institutions, educational facilities, hospital environment, and medical devices. In this connection, nosocomial infections are a major issue, and one of the most prominent routes of infection is the contamination of implant surfaces for surgery.[1−3] Once bacteria adhere to solid material, they may start to build up colonies resulting in biofilm formation. Bacteria within such a biofilm often present enhanced resistance against antimicrobial treatment, by up to 1000 times compared with the corresponding planktonic bacteria.[4] This may limit the use of an antibiotic mediated attendance. In addition, a previously treated biofilm tends to be populated again resulting in extended therapy.[2] Thus, preventive methods are desirable because they protect against surface-mediated contamination prior to biofilm formation.[3] This dramatically reduces biofilm formation, cross-contamination, and spreading of bacteria, thus decreasing the material deterioration while increasing the containment of nosocomial infections. Consequently, overall cost is reduced, and living conditions are improved.

In addition to traditional prevention methods, antimicrobial surface coatings have emerged as encouraging alternatives.[5,6] Different antimicrobial surface coatings have been developed incorporating antibiotics such as cefazolin,[7] minocycline/rifampin,[8] or vancomycin.[9] However, while highly effective, these modifications harbor the potential issue of antibiotic resistances occurring.[10] This would result in an initial colonization followed by a recruitment of additional bacteria, followed by a loss of bactericidal surface characteristics. On the other hand, chemical compounds, for example, silver,[11] quaternary ammonium compounds,[12] or salicylic acid,[13] as well as polymeric substances,[14] have also been used. Although the use of chemical modifications and metallic compounds are very effective they may be limited in their application due to toxic or incompatibility effects.[15] In order to develop highly effective antimicrobial coatings and to avoid these limitations, antimicrobial peptides (AMP) are discussed as promising class of bioactive substances with biocompatible properties.[16,17] They are a diverse class of biomolecules with many varying

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mechanisms of antimicrobial action, which are based on an interaction of a certain peptide with the corresponding bacteria. Since the discovery of their properties and high potential as promising therapeutic agents in the treatment of bacterial infections, they have become increasingly more important in the scientific context. One of these new scientific aspects is the use of antimicrobial peptides as functional molecules for an antimicrobial surface coating.[18]

A challenging aspect in the development of a peptide-based functional surface coating is the attachment of these biomolecules to a solid support in a functional state. There have already been several investigations on AMP coatings based on different immobilization strategies including passive adsorption procedures[19] or layer by layer depositions where the peptide is released into the surrounding media.[20,21] Alternatively, covalent immobilization strategies can be used. Thereby the peptide stays attached to the surface. Two major conjugation techniques, the selective and nonselective chemical ligation, are widely used. In this manner, the selective immobilization results in a covalent, mainly non-naturally occurring bond formation between the peptide and the surface. This can be achieved by the introduction of special reactive functionalities within the peptide sequence. Thereby, the route of reaction can be controlled.[18,22,23] On the other hand, nonselective immobilization can be used with naturally occurring peptides without additional chemical modifications. Using the naturally occurring amino, carboxyl, sulfhydryl, and hydroxyl functionalities within the peptide sequence, such a covalent attachment can be achieved, whereas the chemically activated surface provides the corresponding reactive modification for the immobilization including NHS, aldehyde, PDITC, or epoxy functionalities. These conjugations result in different orientations of the peptide at the surface as well as varying links between the peptide and the surface.[18,24] A further aspect is the amount of peptide immobilized to the surface, which influences the peptide density and the percentage of surface coverage.[25] In addition to the physical and chemical characteristics, the biological properties of an antimicrobial peptide are significant for the characteristics of a bactericidal surface coating, principally their mode of action, bacteria specificity, and degree of activity. In order to develop a peptide-based antimicrobial surface coating, all of these aspects have to be considered, and an appropriate strategy must be chosen. Beside peptide selection and immobilization strategy, additional requirements have to be met for large and small scale industrial applications. Not only are the peptides the key elements in an antimicrobial surface coating, they are also among the most expensive. The traditional in vitro solid phase synthesis of peptides is far better suited for small scale production. This method allows for almost any peptide sequence to be synthesized. In addition, non-natural amino acids or chemical modifications can be introduced into the peptide sequence. Moreover, extensive peptide libraries with thousands of molecules can be produced, making solid phase peptide synthesis a capable instrument in research and development facilities. Nevertheless, the method is limited by the length and amount of peptide synthesized as well as the rather high production cost. Therefore, large scale production is more easily attained by other methods. One method for high production of peptides is recombinant DNA technology in combination with industrial purification techniques. Still, the recombinant transcription and translation machinery of the expression system can only incorporate naturally occurring building blocks. These restrictions prevent the use of non-naturally abundant peptide modifications and therefore the use of selective chemical conjugation techniques for a large scale covalent modification. In order to fabricate a peptide based antimicrobial surface coating, these restrictions have to be considered and an appropriate development strategy must be chosen.

Here we present the development and characterization of an antimicrobial coating on planar surfaces based on covalent immobilization of naturally abundant, unmodified antimicrobial peptides suitable for recombinant expression and up-scaling procedures for intended large scale applications. In addition, we have characterized the antimicrobial mode of action for five peptides in comparison to the two well-known antibiotics ampicillin and polymyxin B. Furthermore, we have investigated the antimicrobial properties of the immobilized peptides in comparison to their behavior in solution. Consequently, we are able to recommend a rapid and efficient screening procedure to identify biologically active antimicrobial peptides in an immobilized state. Moreover, we have investigated the influence of chemical surface modifications, immobilization strategy, and amount of immobilized peptide on the nature of the antimicrobial surface coating. Finally, we have determined the cytotoxic properties of the antimicrobial peptide BMAP-27 in a soluble and immobilized state and conclude such an antimicrobial surface coating to be biocompatible. Therefore, our results pave the way for an efficient antimicrobial surface coating and deliver an optimized overall procedure featuring distinct antimicrobial characteristics with minimal consumption of antimicrobial peptide.

MATERIALS AND METHODS

Antimicrobial Peptides. The investigations were carried out with five antimicrobial peptides from different sources. The peptides buforin II, LL-37, and BMAP-27 were synthesized by standard solid phase peptide synthesis (Genscript) without further modifications and purified to at least 80%. The peptides melittin and protamine were extracted from natural sources. While melittin was extracted from honey bee venom and purified to at least 85% (Sigma-Aldrich), protamine was acquired from salmon as grade IV without histone impurities (Sigma-Aldrich). In addition the peptides BMAP-27 and protamine were produced by recombinant DNA technology in combination with an *Escherichia coli* based high yield expression system (International Laboratory for Biotechnology and Consulting GmbH, ILBC). All peptides were stored at −20 °C in a lyophilized state. Prior to use, they were solubilized in deionized water at a stock concentration of 2 mM followed by further individual dilution.

Expression, Isolation, and Purification of BMAP-27. The gene coding for BMAP-27 was amplified by PCR using primers BMAP27-forward (5′-ggtcgttttaaacgtttcg-3′) and BMAP27-reverse (5′-ttaacccagatgcagcagc-3′). The unpurified PCR product was mixed with linearized pET-SUMO vector (Invitrogen) and transformed by heat shock into *E. coli* DH5α cells (Invitrogen). Recombinant clones were verified by a commercial sequencing service (MWG Eurofins GmbH). Plasmid DNA from the successful clones was isolated and transformed into *E. coli* BL21 (DE3) strain (New England Biolabs) for the expression of the fusion construct.

For the expression of the SUMO−BMAP-27 fusion protein, cells were grown in LB medium at 37 °C to an optical density of about 0.5 at 600 nm and induced with 1 mM IPTG. After 2.5

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h of growth, cells were pelleted by centrifugation at 7000 rpm for 20 min at 4 °C. The supernatant was discarded, and the cell pellet was resuspended in lysis buffer (50 mM NaH_2PO_4, 150 mM NaCl, 10% glycerin, pH 8.0) in the presence of lysozyme (1 mg/mL). After 30 min incubation on ice, the suspension was sonicated 10 times for 10 s on ice (cycle 0.5, amplitude 100%). Cell debris was removed by centrifugation at 10000*g* for 20 min at 4 °C, and the supernatant was applied to Ni-NTA affinity column (His-Pur, Thermo Scientific). After 2 h incubation on ice, the bound SUMO−BMAP-27 was eluted by washing with elution buffer (lysis buffer + 250 mM imidazole). After purification by Ni-NTA affinity column, the eluted SUMO−BMAP-27 was cleaved with SUMO protease (Invitrogen). SUMO protease was used in a 1:500 molar ratio for 2 h at 37 °C in the presence of 150 mM NaCl. The cleaved material was used to purify BMAP-27 using Ni-NTA affinity column. The homogeneity of the obtained BMAP-27 in the flow through was approved by mass spectrometry and N-terminal sequence analysis.

Expression, Isolation, and Purification of Protamine. The expression, isolation, and purifiaction of protamine was accomplished in accordance with general guidelines.[26,27] Therefore, the corresponding gene coding for protamine was fused to a pelB leader sequence and cloned into the vector pET-9a. The plasmid was transformed into *E. coli* BL21 (DE3). Cells were grown in LB medium at 37 °C to an optical density of about 0.5 at 600 nm and induced with 1 mM IPTG. After 6 h of growth cells were pelleted by centrifugation at 7000 rpm for 20 min at 4 °C. Membrane fractions were extracted with deionized water, and protamine was purified using Biogel P10 column.

Peptide Identification by Mass Spectrometry and N-Terminal Sequencing. The identity and purity of the recombinantly expressed antimicrobial peptide BMAP-27 was evaluted by MALDI-TOF-MS analysis. Purified peptide samples were mixed with equal volumes of a water-saturated α-cyano-4-hydroxy cinnamic acid solution in 50% acetonitrile and 0.3% trifluoroacetic acid. Mass spectra were recorded in the reflectron mode of a Bruker microflex mass spectrometer. The peptide was identified by searches in SWISS-PROT. Searches were performed using the program MASCOT (www.matrixscience.com).

For N-terminal sequence analysis, MS/MS spectra were recorded using a LTQ XL mass spectrometer (Thermo Scientific) equipped with a MALDI ion source in a positive ionization mode. The ion of interest was selected in the ion trap. Fragments were generated in the collision induced dissociation cell by collision with nitrogen and analyzed. Instrument parameters were 27 normalized collision energy and 4.8 μJ laser energy. The collected data were analyzed using the same program as described above (MASCOT; www.matrixscience.com). The detected sequences were aligned with the theoretical peptide sequence of the corresponding peptide, and the accordance was calculated.

Minimal Inhibitory Concentration. The minimal inhibitory concentrations (MICs) of free soluble peptides were determined against Gram-negative *E. coli* Dh5α (Deutsche Sammlung von Mikroorganismen and Zellkulturen, DSMZ) by the use of a broth microdilution method, following the general guidelines of the CLSI[28] and EUCAST[29] with small adjustments according the investigation of cationic antimicrobial peptides.[30] Cells were cultured in Mueller−Hinton bouillon (MHB) to an optical density of 0.4 to 0.6 at 600 nm representing approximately 4×10^8 bacteria/mL. Afterward the bacterial cultures were diluted to about 1×10^5 bacteria/mL in sterile MHB. Next, a 2-fold serial dilution of the peptides was prepared in MHB starting at 128 μM to a final dilution of 0.5 μM. The peptide dilutions were mixed with diluted bacterial cultures in a 1:2 ratio and placed in a 96-well polypropylene microtiter plates (Starlab) resulting in final peptide concentrations ranging from 64 to 0.25 μM in combination with a final bacterial concentration of 5×10^4 bacteria/mL. Sterile MHB was used as a negative control, whereas MHB inoculated with bacteria served as a growth control. The microtiter plates were incubated for 18 h at 37 °C with constant shaking at 120 rpm in a humidity chamber. After incubation, the absorbance was measured at 600 nm. The lowest concentration showing no difference in the absorbance level of sample and negative control was determined as the MIC.

Flow Cytometry. The membrane disruption of bacteria was investigated by a live−dead staining in combination with an internal calibration and counting system measured by flow cytometry. For this purpose, SYBR Green I (Sigma-Aldrich) was used as total stain, while propidium iodide (Sigma-Aldrich) served as an indicator for membrane damage. Calibration and quantification of the measured events was achieved by PeakFlow orange flow cytometry reference beads (Life Technologies). Analysis was performed on a Cytomics FC 500 instrument (Beckman Coulter) equipped with an argon laser (488 nm) and the appropriate emission filter for SYBR Green I (525 nm BP) and propidium iodide (620 nm BP). All detectors were set on a logarithmic scale, and the gates were adjusted by preliminary experiments with living and isopropanol killed bacteria. Optical and electronic noise was eliminated by adjusting a threshold level on the forward scattering detector. The flow rates were kept in the range of roughly 300 events per second with total acquisition of 20000 events. Prior to sample analysis, the dyes, calibration particles, and sheath fluid were combined to create a master mix. After adding 50 μL of bacterial sample to 450 μL of master mix, final concentrations of 10 μM propidium iodide, 2×SYBR Green I, and 1.2×10^5 particles/mL were reached. Then the mixtures were incubated for 10 min in the dark at room temperature and analyzed accordingly. The respective number of events in each gate was recorded and evaluated using the CXP Analysis 2.2 software. The total numbers of living and killed bacteria were calculated. Any event that cannot be clearly defined as living or killed bacteria is assessed as ungated bacteria.

Peptide Immobilization. The antimicrobial peptides and the two antibiotics ampicillin and polymyxin B (Sigma-Aldrich) were immobilized on a solid support by their naturally occurring functional amino groups. In addition, reactive but unconjugated surfaces as well as 1 mM Tris, pH 8.0 (Applichem), deactivated surfaces were used as the negative controls (NC). As preactivated reactive surfaces epoxy, aldehyde, NHS, and PDITC functionalities were chosen (PolyAn). All these functionalities are suitable for the immobilization of amine-containing molecules without further modifications. The peptides were diluted in phosphate buffered saline (Applichem), pH 7.4, to a 140 μM coupling concentration. For liquid handling, a 16 well superstructure (Grace Bio-Laboratories) was attached to each surface. Each chamber was filled with 70 μL of the corresponding peptide solution and incubated in a humidity chamber at 4 °C for 16 h. Subsequently, the unconjugated excess peptide was removed,

and the surfaces were washed eight times with 200 μL of PBS each with gentle shaking at room temperature.

Peptide Quantification. For relative quantification of immobilized peptide, the amine-reactive fluorophore epicocconone as component of the FluoroProfile protein quantification kit (Sigma-Aldrich) was used. The modified surfaces carrying the immobilized peptides were inserted into a liquid flow handling apparatus filled with PBS. Afterward, the reactive fluorescent dye epicocconone was diluted according to the manufacturer's instructions and applied to the top of the surfaces to be analyzed. The incubation was carried out by a gentle repetitive steady stream of the reactive fluorophore across the surface for 1 h at room temperature in the dark. Finally, the surfaces were washed for 15 min with deionized water by a steady flow, removed from the flow chamber, and dried by a stream of nitrogen. The fluorescence of each surface was measured using the Axon GenePix 4200A (Molecular Devices) laser scanner with excitation at 532 nm and a standard 575 nm green emission filter. The settings were as follows: 50% laser power, 500 PMT, and 10 μm resolution. The fluorescence was quantified using GenePix Pro 6.1 software. Afterward the relative amount was calculated by dividing the fluorescence intensity of each individual sample by the fluorescence intensity of the corresponding 140 μM coupling concentration in a NHS mediated reaction chemistry. The curve profiles were calculated by different model equations, and the corresponding matching parameters were compared using OriginPro 8.1 G (OriginLab) software. The final fitting was realized by an asymptotic assumption model due to the minimal error coefficient.

Depolarizing Properties of Soluble Peptides. To determine the depolarizing properties of soluble antimicrobial peptides a serial peptide dilution was prepared, incubated with *E. coli*, and analyzed by flow cytometry. For this, a 2-fold serial dilution of the peptides was prepared in MHB starting at 128 μM to a final dilution of 0.5 μM. Afterward the bacterial population was adjusted to 2×10^6 bacteria/mL in MHB by flow cytometry measurement and was mixed with the peptide solution in a 1:2 ratio resulting in final peptide concentrations ranging from 64 to 0.25 μM and a bacterial concentration of 1×10^6 bacteria/mL. Afterward the samples were incubated for 2 h with gentle shaking at room temperature. After the incubation, the bacteria were analyzed according the general flow cytometry setup and the lowest peptide concentration where at least 80% of the bacterial population displayed depolarized membranes was determined and summarized.

Antimicrobial Surface Properties and Release Control Measurements. To exclude a release of antimicrobial peptide from the surface by remaining adhesive immobilized peptides, a release control experiment was carried out. For this purpose, the peptide modified surfaces were incubated for 2 h in 70 μL of MHB with gentle shaking. Meanwhile the bacterial population was adjusted to 2×10^6 bacteria/mL in MHB by flow cytometry measurement and calibration. Afterward the supernatant of the surface was mixed with bacteria in a 1:2 ratio to reach a concentration of 1×10^6 bacteria/mL and was incubated without peptide modified surface at room temperature for another 2 h with gentle shaking. To evaluate the antimicrobial characteristics of the prepared surfaces, the remaining liquid was removed, 70 μL of freshly adjusted bacteria with a concentration of 1×10^6 bacteria/mL was added on top of the peptide modified surfaces, and the surfaces were incubated for 2 h at room temperature by gentle shaking. Afterward, all samples were stained and measured according to the general flow cytometry setup. The amount of released peptide was estimated by the number of bacteria killed in the supernatant fraction, whereas the antimicrobial properties of the peptide-modified surfaces were determined directly by the bacteria killed within the surface-treated samples. Untreated or inactivated surfaces were summarized as NC. In addition, all bacteria samples were diluted logarithmically in MHB, and the samples covering the dilution factors ranging from 10^2 to 10^6 were plated in triplicate on MHB agar plates to assess the surviving bacteria by colony counts. The agar plates were incubated at 37 °C for at least 16 h, the resulting colonies were counted, and the initial amount of colony forming units was calculated. Samples in which the amount of surviving bacteria was too low to count at the lowest dilution were indicated as less than 1×10^3 bacteria/mL.

Analysis of Bacterial Adhesion. To evaluate the adhesion of bacteria to the surfaces by peptide-mediated interactions, the surfaces were treated as described above. Afterward the attached bacteria were stained and analyzed by fluorescence microscopy. For this, the surfaces were rinsed four times with MHB, and 40 μL of 2× SYBR Green I (Sigma-Aldrich) solution was filled into each chamber of the attached superstructure. Thereupon, all samples were incubated for 20 min under light exclusion at room temperature. After removal of the superstructure, the surface was slightly cleaned and a coverslip was applied. Afterward, the samples were investigated by fluorescence microscopy with a 480 nm excitation and 515 nm emission filter.

Cytotoxicity of Dissolved and Immobilized Peptide. To investigate biocompatibility of the peptide-modified surfaces, the cytotoxic potential was determined. In order to compare the antibacterial and cytotoxic properties of the peptide BMAP-27, its characteristics were analyzed in a dissolved and immobilized state to the human histiocytic lymphoma model cell line U937 (DSMZ). Cells were inoculated at a cell concentration of 1×10^5 cells/mL and were cultured in 15 mL of RPMI 1640 medium supplemented with 2 mM glutamine and 10% FCS (Biochrom) for 3 days at 37 °C in T75 culture flasks (TPP) at constant ventilation with 5% CO_2 in a humidity chamber. Bacterial contamination was prevented by supplementing the cell culture with 10 U/mL penicillin and 100 μg/mL streptomycin (Applichem). Subsequent recultivation was carried out without any addition of antibiotics. The analysis of the cytotoxic properties was carried out in analogy to the antimicrobial experiments. The analyses were accomplished according to the general flow cytometry setup with small adjustments on account of the eukaryotic cell line.

RESULTS

Isolation and Purification of Recombinant BMAP-27 and Protamine. BMAP-27 was expressed by recombinant DNA technology by the use of the SUMO expression system and further purified by Ni-NTA affinity columns. The corresponding analysis by mass spectrometry displays only one predominant detected ion with a m/z value of 3283 (Figure 1).

In addition to the MALDI-TOF-MS analysis the recombinantly expressed, isolated, and purified peptides were analyzed by N-terminal sequencing. The analysis of the BMAP-27 samples revealed an N-terminal sequence of the amino acids Gly-Arg-Phe-Lys-Arg-Phe, while the sequence of the protamine samples displayed an amino acid sequence of Met-Pro-Arg-Arg-

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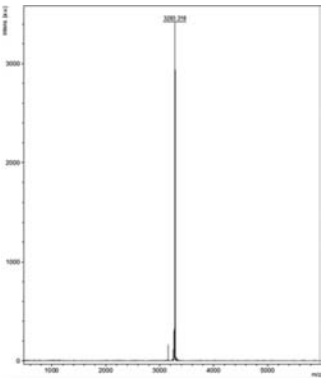

Figure 1. Mass spectrometry analysis of recombinant expressed and purified BMAP-27. The recombinantly expressed and purified peptide BMAP-27 was analyzed by MALDI-TOF-MS. The analysis displays only one predominant ion with an m/z value of 3238.

Arg-Arg-Ser. Furthermore, no additional sequences could be detected within the purified peptide samples. The alignment of the detected N-terminal sequences with the theoretical peptide sequences of BMAP-27[31] and protamine[32] revealed a complete accordance.

Determination of MIC and Membrane Disruption Abilities of Dissolved Peptides. For the initial characterization of the peptides, the MIC and membrane disruption abilities were analyzed and compared with those of the two antibiotics ampicillin and polymyxin B. The MIC values indicated a general activity against *E. coli* for all the peptides investigated (Table 1).

Table 1. Summary of Peptide-Mediated Antimicrobial Properties against *E. coli*[a]

AMP	MIC [μM]	≥80% depolarized *E. coli* [μM]
LL-37	4	8
BMAP-27	1	2
BMAP-27, recombinant	1	
melittin	8	32
polymyxin B	<0.25	1
buforin II	2	>64
protamine	8	>64
protamine, recombinant	8	
ampicillin	<0.25	>64

[a]Displayed are the MIC value and depolarization concentration of five different antimicrobial peptides as well as two antibiotics. The peptide concentration for the depolarization of greater or equal 80% of the entire bacterial population was identified by flow cytometry measurements. Recombinantly expressed peptides were only analyzed according their MIC value. Each entry is a summary of a concentration dependent test series (n = 4).

Among the tested peptides, the MIC values ranged from 1 to 8 μM with BMAP-27 displaying the highest antimicrobial activity. In contrast, the tested antibiotics displayed MIC values below 0.25 μM. In comparison to the MIC value, BMAP-27 displayed the lowest effective concentration for membrane disruption of at least 80% of *E. coli* with 2 μM (Figure 2), whereas LL-37 and melittin featured concentrations of 8 and 32 μM, respectively. On the other hand, buforin II and protamine showed MIC values of 2 and 8 μM, respectively, and generated no membrane disruption at the concentrations investigated. In addition, the recombinantly expressed peptides BMAP-27 and

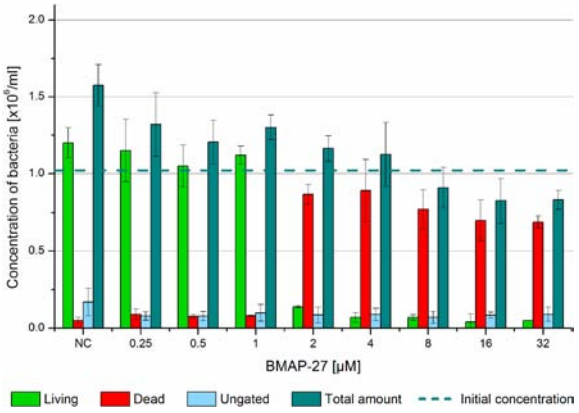

Figure 2. Concentration-dependent membrane depolarization of *E. coli* by BMAP-27. The membrane integrity was examined by a live–dead staining in combination with flow cytometry measurements. The bacteria display a strong depolarization due to a peptide interaction down to a concentration of 2 μM. Below this concentration, no meaningful depolarization can be detected (n = 3).

protamine featured MIC values of 1 and 8 μM, respectively, and thereby in the same order of magnitude as their synthetic counterparts. The tested antibiotics displayed different behavior concerning their membrane disruption abilities, while polymyxin B indicated a membrane disruption down to a concentration of 1 μM, ampicillin treatment resulted in totally unaffected bacterial membranes at the examined concentrations.

Quantification of Immobilized Peptide. BMAP-27 was immobilized and its relative concentration on the surface was determined by a fluorescence based method. The relative amount of immobilized peptide was analyzed and correlated to the initial dissolved peptide concentration prior to immobilization. The different surface modifications were compared and the relative peptide amount on each surface and at each coupling concentration was estimated. Measurements revealed differences in the relative amount of immobilized BMAP-27 on different surfaces. With increasing coupling concentrations, the amount of immobilized peptide increased following an asymptotic curve profile with an approximation to an upper limit that varies for the individual surfaces under investigation.

The amount of peptide immobilized differed noticeably depending on the surface chemistry applied. Upon increase of coupling concentrations, all modified surfaces displayed strong increase of immobilized peptide reaching a saturation plateau at high peptide concentrations. The NHS and aldehyde modifications exhibited a relative peptide amount of 1.0 and 0.95, respectively, in a mostly saturated state. In addition, 90% of their capacity was reached at BMAP-27 concentrations of 14.7 μM for NHS and 25.4 μM for aldehyde. The epoxy and PDITC modifications featured relative immobilized peptide amounts of 2.3 and 2.7 while 90% of their respective capacity was reached at coupling concentrations of 47.1 and 41.1 μM (Figure 3).

Antimicrobial Surface Properties and Release Control Measurements. To investigate the antimicrobial properties of the previously characterized soluble antimicrobial peptides in an immobilized state, they were attached on solid planar surfaces. The antimicrobial properties highlighted considerable differences of the generated, peptide modified surfaces, depending on the peptide and the immobilization strategy (Table 2). The two immobilized antibiotics displayed contrary

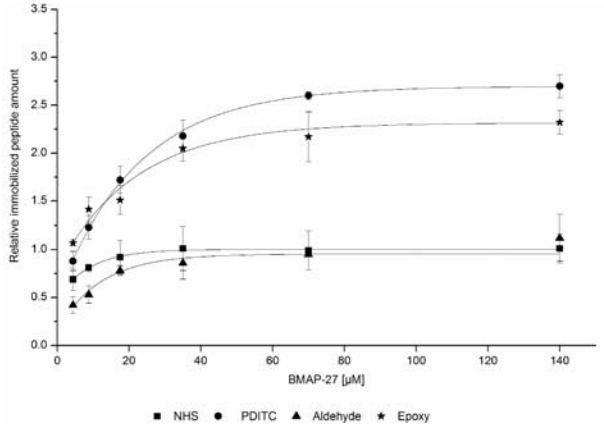

Figure 3. Relative peptide quantification of immobilized BMAP-27 by fluorescent epicocconone staining. The data display an asymptotic curve profile with an approximation to an upper limit. The relative peptide amounts vary depending on the reaction chemistry. The NHS and aldehyde mediated coupling of BMAP-27 result in lower peptide amounts compared with conjugation via epoxy and PDITC reaction (n = 4).

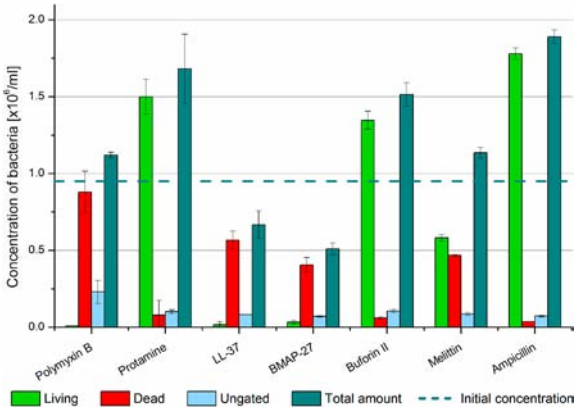

Figure 4. Membrane depolarization of *E. coli* by tethered antimicrobial peptides using a NHS reaction chemistry and a peptide coupling concentration of 140 μM. The peptides exhibit contrary membrane disruption potential in an immobilized state. While LL-37, BMAP-27, and polymyxin B modified surfaces result in bacterial population with highly depolarized membranes, protamine, buforin II, and ampicillin display no meaningful antimicrobial effect. Melittin shows an intermediate activity with a depolarization of 49% (n = 4).

antimicrobial behavior in an immobilized state using NHS coupling chemistry (Figure 4). While immobilized polymyxin B featured almost complete depolarization of all bacterial membranes indicated by a shift of bacterial fluorescence in combination with a reduction of living bacteria by more than 3 orders of magnitude, ampicillin exhibited no depolarization and no reduction of living bacteria. Thus, the latter showed an antimicrobial profile similar to the untreated or inactivated surface controls. In addition, flow cytometry measurements revealed a significant difference in the total count of bacteria. While the bacteria interacted with the polymyxin B modified surface displayed with a concentration of 1.1×10^6 bacteria/mL only a slight difference from the initial concentration of 0.95×10^6 bacteria/mL, the bacteria interacted with the ampicillin treated surface exhibited a concentration of 1.9×10^6 bacteria/mL and thereby approximately two times the amount of the initial concentration. BMAP-27 and LL-37 displayed a reduction of living bacteria by more than 3 orders of magnitude in combination with membrane depolarization of more than 90% of the bacterial cells. Melittin displayed a reduction of 1 order of magnitude and a depolarization of 49% of the bacterial population. In addition, the surfaces treated with BMAP-27 or LL-37 indicated a reduction of overall bacteria to 45% and 63%, respectively, while the melittin modified surface displayed a slight increase in overall bacteria count to 1.1×10^6 bacteria/mL. In contrast, protamine and buforin II showed neither membrane disruption abilities nor any growth inhibition in an immobilized state. Furthermore, the overall amount of bacteria increased to 1.5×10^6 and 1.7×10^6 bacteria/mL, respectively. For aldehyde modified surfaces, the results fell into the same range (Table 2). Here, complete depolarization was observed for LL-37, BMAP-27, and polymyxin B modified surfaces. Additionally, the amount of living bacteria was also reduced by more than 3 orders of magnitude. Furthermore, melittin-modified surfaces displayed only a small reduction of living bacteria by about 1 order of magnitude and a depolarization of approximately 54%. Moreover, the antimicrobial properties of protamine and buforin II displayed neither membrane disruption nor any growth inhibition characteristics immobilized on an aldehyde modified surface. In comparison to these two surface modifications, peptides immobilized by PDITC or epoxy modification displayed rather contrary results. In general, the antimicrobial properties were less pronounced, which is reflected in a reduced growth inhibition by the immobilized

Table 2. Summary of Antimicrobial Properties of Peptides Immobilized by Various Reaction Chemistries at a Constant Peptide Coupling Concentration of 140 μM[a]

	surviving bacteria [log cfu/mL]				amount of depolarized *E. coli* [%]			
AMP	NHS	aldehyde	PDITC	epoxy	NHS	aldehyde	PDITC	epoxy
LL-37	<3	<3	5.9	5.9	97.0	95.0	13.0	20.0
BMAP-27	<3	<3	4.4	5.0	93.0	96.0	48.0	45.0
melittin	5.1	5.1	6.2	5.3	49.0	54.0	5.0	17.0
polymyxin B	<3	<3	4.9	4.6	>98	95.0	53.0	61.0
buforin II	5.9	6.3	6.3	6.2	11.0	8.0	7.0	8.0
protamine	6.3	6.1	6.5	6.0	11.0	11.0	9.0	7.0
ampicillin	6.4	6.2	6.0	5.8	6.0	9.0	11.0	10.0
NC	6.3	6.2	6.5	6.3	6.0	11.0	8.0	8.0

[a]Displayed are the number of colony forming units and the amount of depolarized *E. coli* after an interaction with five different antimicrobial peptides as well as two antibiotics in an immobilized state. The amount of depolarized *E. coli* after interaction with the corresponding surfaces was determined by flow cytometry measurements (n = 4).

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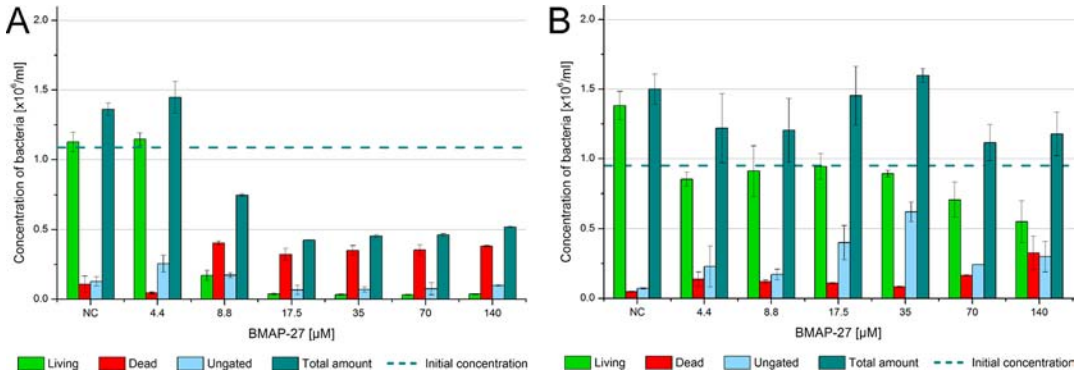

Figure 5. Coupling concentration dependent membrane depolarization of *E. coli* by tethered BMAP-27 using a NHS (A) and PDITC (B) reaction chemistry. Down to a coupling concentration of 17.5 μM in combination with a NHS reaction chemistry, the surfaces exhibit more than 90% membrane disruption. Upon further lowering the concentration to 8.8 μM, the depolarizing effect becomes less pronounced and vanishes at a concentration of 4.4 μM, where no meaningful difference from the NC can be detected (n = 4). With PDITC immobilization, only a coupling concentration of 140 μM displays minor membrane depolarizing characteristics, which disappear with decreasing concentrations. From a concentration of 35 μM, only negligible antimicrobial properties can be detected (n = 4).

peptides BMAP-27, LL-37, and melittin in combination with decreased membrane disruption abilities. For PDITC immobilization, the antimicrobial activity of immobilized melittin vanished entirely. In a similar fashion, immobilization of BMAP-27 either by PDITC or by epoxy immobilization strategy showed minor antimicrobial potential. Here, a reduction of living bacteria by 1 order and 1.6 orders of magnitude was observed, respectively. In addition, depolarization of approximately 50% of the bacterial population was detected. In contrast, protamine and buforin II featured no antimicrobial activity in an immobilized state by any of the immobilization strategies tested (Table 2).

To further investigate the influence of the relative amount of attached peptide and the immobilization strategy used on the corresponding antimicrobial characteristics of the resulting surface, BMAP-27 was immobilized on NHS (Figure 5A) and PDITC (Figure 5B) modified surfaces. Here, different coupling concentrations were used, and the resulting surfaces were analyzed according their membrane disruption and growth inhibition abilities. In accordance with the previously conducted experiments, BMAP-27 showed a very similar behavior at a coupling concentration of 140 μM on a NHS modified surface. In detail, membrane disruption of more than 90% of the bacterial population and a reduction of viable colony forming units by more than 3 orders of magnitude were observed. As coupling concentrations decreased to 17.5 μM, no significant differences compared with the 140 μM coupling concentration were detected. Within this range of concentrations, the antimicrobial properties of the modified surface remained unchanged. Further reduction of the coupling concentration to 8.8 μM revealed a significant drop in depolarization to approximately 50% of the bacteria. The remaining 50% were divided equally between living and ungated bacteria in flow cytometry. The amount of viable colony forming units after the 2 h incubation time was 5×10^4 bacteria/mL and represents a reduction of more than 1 order of magnitude compared with the negative control. Further lowering the coupling concentration to 4.4 μM resulted in a modified surface with no detectable antimicrobial properties. This surface displayed no detectable depolarized bacteria and only a minute amount of ungated. In contrast to the NHS modified surface, BMAP-27 showed only minor antimicrobial activity after immobilization on PDITC surfaces. Thus, viable colony forming units were reduced by 1.6 orders of magnitude at a coupling concentration of 140 μM. Depolarization of bacteria at this concentration was detectable in only 47% of the bacterial population. Decreasing the coupling concentrations even further led to a complete loss of any antimicrobial properties. At coupling concentrations below 70 μM, no antimicrobial abilities were found as the results were equal to the negative controls (Table 3).

Table 3. Coupling-Concentration-Dependent Influence on the BMAP-27 Amount and Surviving Bacteria

	NHS		PDITC		
coupling concentration [μM]	relative amount regarding NHS	surviving bacteria [log cfu/mL]	relative amount regarding NHS	relative amount regarding PDITC	surviving bacteria [log cfu/mL]
NC		6.1			6.1
4.4	0.69	6.3	0.88	0.33	5.8
8.8	0.81	4.7	1.23	0.46	6.0
17.5	0.92	<3	1.72	0.64	5.7
35	1.01	<3	2.18	0.81	5.7
70	0.99	<3	2.6	0.96	5.5
140	1.00	<3	2.7	1.00	4.4

In order to exclude a release of antimicrobial peptide from the surface by remaining adhesive immobilized peptides or by hydrolysis of unstable bond formation, a release control experiment was carried out. After cleaning, the peptide modified surfaces were covered by MHB, which was afterward evaluated for its antimicrobial properties. The results indicated no detectable antimicrobial characteristics in the supernatant fraction of all tested antimicrobial surfaces, and only completely viable bacterial populations could be detected.

Based on the decrease of the countable bacteria after incubation on different surfaces, the surfaces were stained and analyzed for remaining bacteria to account for peptide-mediated attraction. However, neither light microscopy nor fluorescence imaging revealed any traces of bacteria on the surfaces.

Cytotoxicity. The cytotoxicity of soluble BMAP-27 was analyzed using the model cell line U937. In this context, the direct damaging of the cell membrane was analyzed after 2 h incubations. The results revealed that the antimicrobial peptide BMAP-27 harbors cytotoxic potential. Flow cytometry

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measurements in combination with a live−dead staining showed the influence of the peptide concentration on the membrane disruption (Figure 6). Thereby, a disruption of 50%

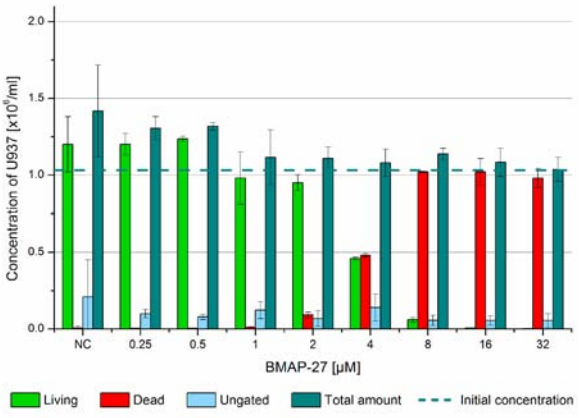

Figure 6. Concentration-dependent membrane depolarization of U937 by soluble BMAP-27. The cells display a strong membrane depolarization down to a concentration of 8 μM. At a concentration of 4 μM, the effect decreases and only 50% cells with damaged membranes can be detected. Upon further lowering of the peptide concentration, the cytotoxic properties disappear (n = 3).

of the cells was attained using a 4 μM peptide solution. This concentration falls into close proximity of the minimal inhibitory concentration against *E. coli*. With increasing peptide concentrations, the membrane disruption abilities became more pronounced until more than 95% of the cell population displayed substantial membrane damage at a concentration of 16 μM. In contrast, no cytotoxic activity was detected for concentrations below 2 μM.

Although cytotoxic effects of BMAP-27 in a soluble state were observed, immobilized BMAP-27 using a NHS coupling mechanism displayed no cytotoxicity whatsoever. These results were not only attained in single cell type cultures but more importantly in mixed cultures where only a selective reduction of the bacteria was achieved without harming the cell line U937. In fact, viability remained high for U937 after three days of incubation, while the bacteria were severely hampered in their spreading using a coupling concentration of 17.5 μM of BMAP-27. This finding continued up to a coupling concentration of 140 μM. Moreover, the generated antimicrobial surface coating was capable of the selective disruption of the bacterial membrane even in a mixed culture experiment. After an incubation of 2 h (Figure 7A), almost all bacteria in a mixed culture displayed a considerable disruption of the cell membrane, while the membrane of U937 remained unaffected. Below a coupling concentration of 17.5 μM, the bacterial membrane became less affected by the antimicrobial surface coating leading to completely unaffected bacterial membranes at coupling concentrations of 4.4 μM. The bacteria and cells were left on the surfaces and incubated for a prolonged time of 3 days. Afterward, the total number and membrane integrity was determined once more (Figure 7B). With coupling concentrations above 17.5 μM, the bacterial population displayed no measurable increase in cell number, while the concentration of U937 cells had tripled. Furthermore, U937 exhibited unaffected membrane potentials as a negligible amount of cells with depolarized membranes were detected. On the other hand, at coupling concentrations below 17.5 μM, the bacterial population featured a substantial increase in living bacteria with unaffected membrane permeability. The high number of bacterial cells prevented a detection of U937. Untreated or inactivated surfaces acted in a similar way to the treated surfaces with concentrations below 17.5 μM.

DISCUSSION

The recombinantly expressed and purified antimicrobial peptides BMAP-27 and protamine were detected and identified by mass spectrometry analysis. Here the detected m/z values directly match the theoretical molecular weights of the corresponding peptides and thereby indicate the correct expression of the peptides, as well as the complete digestion of the fusion protein. Furthermore, these results were confirmed by N-terminal sequence analysis of the expressed and purified peptides. The complete accordance of the theoretical peptide sequences and the detected N-terminal sequences verifies the identity of the expressed antimicrobial peptides BMAP-27 and protamine. Moreover the absence of additional dominant biomolecules within the isolated peptide fractions indicates the successful purification of the recombinantly expressed antimicrobial peptides.

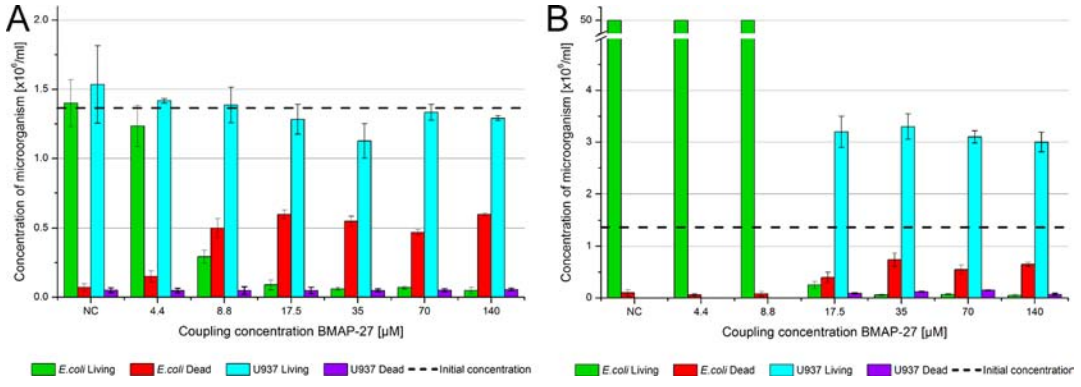

Figure 7. Coupling concentration-dependent membrane depolarization of *E. coli* and U937 after 2 h (A) and 3 days (B) by tethered BMAP-27 using a NHS reaction chemistry. After 2 h, the antimicrobial surfaces display a reduction of bacteria with unaffected membranes at coupling concentrations of 17.5 μM or higher, while the membrane potential of U937 remains unaffected in all samples. After 3 days, the number of bacteria displays no meaningful increase, while the amount of U937 has tripled. Below a concentration of 17.5 μM, the antibacterial effect becomes less pronounced, and after 3 days the bacterial concentration increased drastically (n = 4).

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All the investigated peptides displayed antimicrobial activity in a soluble state either by membrane disruption or by growth inhibition. Furthermore, the recombinantly expressed peptides BMAP-27 and protamine displayed almost identical MIC values compared with their synthetic counterparts, thereby indicating the functional production of antimicrobial peptides by an *E. coli* based expression system, as well as further confirming the purity of the isolated peptide fractions. While all peptides displayed MIC values at low molecular concentrations, the membrane disruption abilities differed considerably. The antimicrobial peptides BMAP-27, LL-37, and melittin were therefore more related to the function of the antibiotic polymyxin B, which displayed reduction of living bacteria in combination with distinct membrane disruption abilities. On the other hand the peptides protamine and buforin II were more related to the mode of action of the antibiotic ampicillin, which displayed a quite strong inhibition of bacterial growth in combination with no detectable membrane damage. Upon tethering of these peptides to a solid support, only the peptides that have similar action to polymyxin B kept their antimicrobial activity. This implies that only peptides that display membrane disruption abilities as their main mode of action are capable of acting as potential functional molecules for an antimicrobial surface coating by covalent attachment. In addition, the activity in an immobilized state correlated with the in-solution experiments but with an overall decreased antimicrobial effect. This is mainly caused by a decrease in degree of freedom for the peptides in an immobilized state and consequently a lack of mobility. Our results point out the significance of the mode of action, which was also demonstrated earlier.[33] Thereby it was possible to differentiate between two major modes of action and confirm that only peptides with pronounced membrane disruption abilities kept their antimicrobial activity in a tethered state. All these findings correlate with widely accepted models for the mode of action of antimicrobial peptides, which were already described as bactericidal or bacteriostatic. While the bactericidal mode of action is characterized by the direct interaction between the membrane and the peptide, which results in a stable pore formation and the loss of essential cell internal compounds,[34] the bacteriostatic mode of action is characterized by the inhibition of key elements within the metabolism of the bacteria without pore formation. Thus, it is essential for the antimicrobial peptide to cross the bacterial membrane. Therefore, bacteriostatic peptides display no inhibition of bacterial growth in an immobilized state and are inept as functional molecules for a covalent antimicrobial surface coating.[35,36] Moreover the used live−dead staining in combination with a flow cytometry measurement proved to be an efficient method for the fast and easy categorization of antimicrobial peptides according their membrane disruption abilities and therefore for the estimation of potential candidates for a functional covalent antimicrobial surface coating.

Since all applied reaction chemistries are capable of reaction with primary amines at pH 7−9, the antimicrobial peptides were immobilized by their N-terminal amine or by internal amine containing lysine residues. This results in a random orientation of the peptides on all surfaces investigated and leads to multiple cross-links between the surface and the peptide. Nevertheless this reaction pattern is identical for all immobilization strategies investigated. Furthermore, the immobilized peptides that have similar action to polymyxin B displayed antimicrobial activity even in an inconsistent orientation. These findings support previous investigations on the antimicrobial activity of peptides in an immobilized state.[23] Here it was also concluded that tethering conserved the activity spectra of the soluble peptides with reduced activity. In addition it could be deduced that the peptide orientation is less important than the properties of the peptide and the surface including spacer length and peptide amount.[23]

In addition to the influence of the general mode of action, our results indicate not only the reduction of living bacteria through the interaction with the immobilized bactericidal acting peptides but also a reduction of countable bacteria with damaged membranes by about 50% through the interaction of a BMAP-27 modified surface at high coupling concentrations using a NHS mediated reaction chemistry. In comparison to that, the soluble BMAP-27 reduced the number of bacteria with damaged membranes only to a minor extent. This observation may be due to an interaction with the surface in combination with an adhesion. Furthermore, even a lytic event can be suspected, which could lead to an alteration of the antimicrobial surface by the adsorption of biomolecules especially phosphoglycerides from bacterial membranes or proteins, which may be released upon cell lysis. This may influence the performance of the antimicrobial surface coating by shielding the antimicrobial peptides from living bacteria, which would result in the loss of activity.[37] However, an adsorption of bacteria could not be proven by surface analyses using fluorescence microscopy. Since the durability and reusability of such antimicrobial surface coatings are of great interest, further experiments need to be conducted in order to examine the bactericidal interactions more closely.

Furthermore, our findings indicate the difference between various immobilization strategies. Though all coupling procedures are based on the covalent linkage between an amino group within the peptide sequence and the surface, the results vary. While aldehyde and NHS immobilization strategies led to stronger acting antimicrobial surfaces, the use of PDITC and epoxy coupling strategies displayed only reduced overall activity. In combination with that the relative immobilized amount of peptide on an epoxy or PDITC modified surface were about 2.3 and 2.7 times the amount of peptide immobilized by a NHS or aldehyde modification. While the relative amount of peptide increased by the use of a PDITC or epoxy modifications the overall antimicrobial abilities decreased, compared with the NHS and aldehyde modified surfaces. These findings become more pronounced by the investigation of the peptide coupling concentration in combination with the relative amount of immobilized BMAP-27 on either a NHS or PDITC modified surface. The NHS based surface coating displayed a significant drop of antimicrobial activity at a coupling concentration of 8.8 μM, whereas all concentrations above indicated a strong inhibition of bacterial growth. By comparison of these finding with the immobilized amount of peptide, it becomes obvious that the activity substantially increases by increasing peptide amounts above 81% saturation of the corresponding surface. In comparison, a 70% saturated surface equal to a 4.4 μM coupling concentration displayed hardly any antimicrobial activity.

This indicates that a threshold concentration on the surface must be reached to reveal the antimicrobial characteristics of a peptide based antimicrobial surface coating. Therefore, the relative amount of peptide and thus the overall surface coverage seems to be the most important aspect. Further, the area of interaction between peptide and the bacteria might influence

the overall performance of the peptide-modified surfaces. This becomes apparent by the reduced antimicrobial activity of immobilized BMAP-27 on a PDITC modified surface. This combination resulted in surfaces with minor antimicrobial characteristics even at a 140 μM coupling concentration. Since the antimicrobial surface properties of a NHS conjugation became significantly pronounced at a surface area coverage of 81%, the PDITC modified surface hardly displayed any antimicrobial activity even in a saturated state, although the total amount of peptide immobilized was 2.7 times higher. Similarly these observations correspond to the results of LL-37, melittin, and BMAP-27 on PDITC and epoxy surfaces, which hardly displayed any reduction of viable colony forming units and barely depolarized the bacterial membrane compared with the NHS and aldehyde activated surfaces. Only the peptide BMAP-27 displayed minor antimicrobial features on PDITC and epoxy modified surfaces, which may be related to its major overall antimicrobial properties compared with melittin and LL-37. Therefore, these results cannot solely be explained by the overall surface coverage or the peptide amount of the corresponding surface. Since the applied coupling reaction chemistries differ regarding their reactivities and stability these differences may justify the different activities of one and the same peptide immobilized on different surfaces. Because the covalent attachment of amine containing molecules to an aldehyde results in an unstable Schiff base formation, there is a possibility of hydrolytic cleavage and an associated release of the peptide from the surface. Likewise NHS modifications may hydrolyze prior to conjugation, resulting in anionic carboxylic acid attached to the surface. This may cause an adsorption by electrostatic interactions of the cationic antimicrobial peptides, which could be released afterward. In order to exclude a release of peptide from the surface by remaining adhesive immobilized peptides or by hydrolysis of unstable bond formation, the antimicrobial properties of all supernatant fractions were analyzed. However, no antimicrobial properties could be detected in any supernatant investigated, indicating that the antimicrobial peptides remained in an immobilized state during the experiments and an electrostatic adsorption or stability of the conjugation is not responsible for the different activities. This is further supported because only the bactericidal antimicrobial peptides melittin, LL-37, and BMAP-27 displayed an inhibition of bacterial growth after tethering to the surface, while protamine and buforin II did not reduce the amount of living bacteria after immobilization. In addition, the antimicrobial peptide BMAP-27 did not exhibit any cytotoxic properties while immobilized to NHS modified surfaces, as it would have been the case in a soluble state or upon a release from the surface. Therefore it can be concluded that the different activities of one and the same peptide immobilized on different surfaces cannot be attributed to any kind of released peptide. Therefore, the coupling chemistry itself results in different activity levels of the immobilized peptide. This may due to a different orientation of the peptide after immobilization. Since all functionalities react with primary amines within the peptide sequence and are capable of a reaction at pH 7−9, the resulting points of conjugation are only dependent on the protonated or deprotonated state of the amines and thereby identical at all reaction chemistries. A difference between these immobilization strategies is the resulting junction between the peptide and the surface. The immobilization by a NHS coupling reaction chemistry results in peptide bond formation, followed by an alkane chain between the peptide and the surface, whereas the conjugation by a PDITC reaction forms a thiourea bond, followed by a phenyl ring. Due to the hydrophobic nature of phenyls, the interaction interface might be altered, compared with the NHS coupling procedure.

By comparison of these results, it becomes obvious that the selection of the coupling reaction chemistry is an important parameter for the development of a peptide based antimicrobial surface coating. Further it becomes evident that the selection of the coupling reaction chemistry leads to an optimization of the antimicrobial surface coating by minimizing the coupling concentration of peptide and still maintaining the unrestricted antimicrobial activity.

While the activity of the peptide in an immobilized state displays the most crucial criteria for the development of a covalent antimicrobial surface coating, other general requirements are also very significant, particularly regarding potential applications. One major feature is the biocompatibility of the peptide modified surface, especially when an antimicrobial peptide with pronounced cytotoxic properties is used. For this reason, peptide based antimicrobial surface coatings were tested for their cytotoxic properties toward eukaryotic cell lines.[22,38] In our experiments, we used the cell toxic antimicrobial peptide BMAP-27, which displayed distinct membrane disruption abilities against *E. coli* and U937. The concentration dependent membrane disruption revealed an antimicrobial and cytotoxic activity down to a concentration of about 2 and 4 μM, respectively, and thereby in the same order of magnitude. Upon tethering of the antimicrobial peptide BMAP-27 to a solid support, the antimicrobial and cytotoxic behavior changed. While the antimicrobial activity remained unaffected above a coupling concentration of about 17.5 μM, the cytotoxic abilities against the eukaryotic cell line U937 totally disappeared at any coupling concentration. These findings were also confirmed by mixed culture experiments, where only the viability of *E. coli* was reduced, while the viability of U937 remained unaffected in short- and long-time experiments. Previous results indicate that immobilized antimicrobial peptides tend to kill bacteria by causing destruction of the bacterial cell permeability. Because tethered peptides are limited in their mobility, the disturbance of the membrane was postulated by a primarily electrostatic driven mechanism.[39] Since bacteria[40,41] and U937[42] display negatively charged cell surfaces and became depolarized by soluble BMAP-27, the selective membrane disruption activity of immobilized peptide cannot be solely explained by a charge dependent mechanism. In addition morphological differences between bacteria and eukaryotes might be the reason for the different interactions. The U937 cells display about 10 times the median size of an average *E. coli* cell and thereby only have a reduced interaction interface between the round shaped cells and the planar surface. This might explain the missing cytotoxic interactions of immobilized BMAP-27. Otherwise eukaryotes possess much thicker cell walls and thereby may prevent the interaction between the peptide and the target site within the membrane through the restricted mobility of the peptide.

CONCLUSION

Here we have presented the development of a peptide-based covalent antimicrobial surface coating on planar surfaces. For this purpose, we investigated the antimicrobial characteristics of different peptides toward *E. coli* and categorized them as bacteriostatic and bactericidal peptides. We further investigated the antimicrobial properties of these peptides in an immobilized state and concluded that only bactericidal peptides maintain

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their activity after attachment onto a solid support. In this respect, we found that BMAP-27 is the most promising candidate for an antimicrobial surface coating because it displayed the most pronounced antimicrobial activity in an immobilized state with reduction of living bacteria by more than 3 orders of magnitude. Since the membrane disruption abilities of the antimicrobial peptides are crucial for the activity in an immobilized state, the live−dead staining in combination with a flow cytometry measurement proves to be an efficient method for the detection of such bactericidal peptides. Therefore, this technique may be used in future experiments for the fast and cost efficient identification of promising peptides for an antimicrobial surface coating.

Furthermore, the impact of the immobilization strategy to the overall antimicrobial characteristics was highlighted. Although the immobilization by a PDITC reaction chemistry displayed 2.7 times the amount of immobilized BMAP-27 compared with a NHS mediated reaction, the immobilization by NHS chemistry resulted in surfaces with more pronounced antimicrobial properties. Thereupon it could be concluded that the chemical surface modification and thereby immobilization strategy displayed major influence on the antimicrobial properties, while the overall peptide amount was less important. Nevertheless it could be demonstrated that an immobilization strategy dependent threshold concentration of tethered peptide must be reached to reveal the antimicrobial properties of the covalent surface coating. By the selection of the chemical functionality on the surface and the variation of the peptide coupling concentration, an optimization of the antimicrobial surface coating could be achieved by minimizing the coupling concentration of peptide and still maintaining the unrestricted antimicrobial activity. In addition to these antimicrobial properties, the reduction of cytotoxicity of BMAP-27 by covalent attachment to the surface could be demonstrated and thereby the potential biocompatibility indicated. Furthermore, the investigated antimicrobial peptides BMAP-27 and protamine could be produced by recombinant DNA technology in combination with an *E. coli* based expression system. In addition these peptides displayed no meaningful difference in their antimicrobial activity compared with their synthetic counterparts. This proves the potential production of functional antimicrobial peptides by recombinant DNA technology and thereby facilitates the development of a cost-effective, large scale production of these peptides for intended industrial applications.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Hetrick, E. M., and Schoenfisch, M. H. (2006) Reducing implant-related infections: Active release strategies. *Chem. Soc. Rev. 35*, 780−789.

(2) Costerton, J. W., Stewart, P. S., and Greenberg, E. P. (1999) Bacterial biofilms: A common cause of persistent infections. *Science 284*, 1318−1322.

(3) Darouiche, R. O. (2001) Device-associated infections: A macroproblem that starts with microadherence. *Clin. Infect. Dis. 33*, 1567−1572.

(4) Coenye, T., and Nelis, H. J. (2010) In vitro and in vivo model systems to study microbial biofilm formation. *J. Microbiol. Methods 83*, 89−105.

(5) Matl, F. D., Obermeier, A., Repmann, S., Friess, W., Stemberger, A., and Kuehn, K. D. (2008) New anti-infective coatings of medical implants. *Antimicrob. Agents Chemother. 52*, 1957−1963.

(6) Matl, F. D., Zlotnyk, J., Obermeier, A., Friess, W., Vogt, S., Buchner, H., Schnabelrauch, H., Stemberger, A., and Kuhn, K. D. (2009) New anti-infective coatings of surgical sutures based on a combination of antiseptics and fatty acids. *J. Biomater. Sci. Polym. Ed 20*, 1439−1449.

(7) Kamal, G. D., Pfaller, M. A., Rempe, L. E., and Jebson, P. J. (1991) Reduced intravascular catheter infection by antibiotic bonding. A prospective, randomized, controlled trial. *JAMA, J. Am. Med. Assoc. 265*, 2364−2368.

(8) Darouiche, R. O., Raad, I. I., Heard, S. O., Thornby, J. I., Wenker, O. C., Gabrielli, A., Berg, J., Khardori, N., Hanna, H., Hachem, R., Harris, R. L., and Mayhall, G. (1999) A comparison of two antimicrobial-impregnated central venous catheters. Catheter Study Group. *N. Engl. J. Med. 340*, 1−8.

(9) Thornton, J., Todd, N. J., and Webster, N. R. (1996) Central venous line sepsis in the intensive care unit. A study comparing antibiotic coated catheters with plain catheters. *Anaesthesia 51*, 1018−1020.

(10) Campoccia, D., Montanaro, L., Speziale, P., and Arciola, C. R. (2010) Antibiotic-loaded biomaterials and the risks for the spread of antibiotic resistance following their prophylactic and therapeutic clinical use. *Biomaterials 31*, 6363−6377.

(11) Jones, J. R., Ehrenfried, L. M., Saravanapavan, P., and Hench, L. L. (2006) Controlling ion release from bioactive glass foam scaffolds with antibacterial properties. *J. Mater. Sci. Mater. Med. 17*, 989−996.

(12) Ravikumar, T., Murata, H., Koepsel, R. R., and Russell, A. J. (2006) Surface-active antifungal polyquaternary amine. *Biomacromolecules 7*, 2762−2769.

(13) Bryers, J. D., Jarvis, R. A., Lebo, J., Prudencio, A., Kyriakides, T. R., and Uhrich, K. (2006) Biodegradation of poly(anhydride-esters) into non-steroidal anti-inflammatory drugs and their effect on *Pseudomonas aeruginosa* biofilms in vitro and on the foreign-body response in vivo. *Biomaterials 27*, 5039−5048.

(14) Siedenbiedel, F., and Tiller, J. C. (2012) Antimicrobial polymers in solution and on surfaces: Overview and functional principles. *Polymers 4*, 46−71.

(15) Ramstedt, M., Ekstrand-Hammarstrom, B., Shchukarev, A. V., Bucht, A., Osterlund, L., Welch, M., and Huck, W. T. (2009) Bacterial and mammalian cell response to poly(3-sulfopropyl methacrylate) brushes loaded with silver halide salts. *Biomaterials 30*, 1524−1531.

(16) Gordon, Y. J., Romanowski, E. G., and McDermott, A. M. (2005) A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs. *Curr. Eye Res. 30*, 505−515.

(17) Costa, F., Carvalho, I. F., Montelaro, R. C., Gomes, P., and Martins, M. C. L. (2011) Covalent immobilization of antimicrobial peptides (AMPs) onto biomaterial surfaces. *Acta Biomater. 7*, 1431−1440.

(18) Onaizi, S. A., and Leong, S. S. (2010) Tethering antimicrobial peptides: Current status and potential challenges. *Biotechnol. Adv. 29*, 67−74.

(19) Soliman, W., Bhattacharjee, S., and Kaur, K. (2010) Adsorption of an antimicrobial peptide on self-assembled monolayers by molecular dynamics simulation. *J. Phys. Chem. B 114*, 11292−11302.

(20) Shukla, A., Fleming, K. E., Chuang, H. F., Chau, T. M., Loose, C. R., Stephanopoulos, G. N., and Hammond, P. T. (2010) Controlling the release of peptide antimicrobial agents from surfaces. *Biomaterials 31*, 2348−2357.
(21) Etienne, O., Picart, C., Taddei, C., Haikel, Y., Dimarcq, J. L., Schaaf, P., Voegel, J. C., Ogier, J. A., and Egles, C. (2004) Multilayer polyelectrolyte films functionalized by insertion of defensin: A new approach to protection of implants from bacterial colonization. *Antimicrob. Agents Chemother. 48*, 3662−3669.
(22) Gao, G., Lange, D., Hilpert, K., Kindrachuk, J., Zou, Y., Cheng, J. T., Kazemzadeh-Narbat, M., Yu, K., Wang, R., Straus, S. K., Brooks, D. E., Chew, B. H., Hancock, R. E., and Kizhakkedathu, J. N. (2011) The biocompatibility and biofilm resistance of implant coatings based on hydrophilic polymer brushes conjugated with antimicrobial peptides. *Biomaterials 32*, 3899−3909.
(23) Bagheri, M., Beyermann, M., and Dathe, M. (2009) Immobilization reduces the activity of surface-bound cationic antimicrobial peptides with no influence upon the activity spectrum. *Antimicrob. Agents Chemother. 53*, 1132−1141.
(24) Chen, R., Cole, N., Willcox, M. D., Park, J., Rasul, R., Carter, E., and Kumar, N. (2009) Synthesis, characterization and in vitro activity of a surface-attached antimicrobial cationic peptide. *Biofouling 25*, 517−524.
(25) Appendini, P., and Hotchkiss, J. H. (2001) Surface modification of poly(styrene) by the attachment of an antimicrobial peptide. *J. Appl. Polym. Sci. 81*, 609−616.
(26) Tadros, M. (2003) World-wide Patent WO 2003/106485 A1.
(27) Tadros, M. (2009) U.S. Patent US 7544483 B2.
(28) Clinical and Laboratory Standards Institute. (2006) Performance standards for antimicrobial susceptibility testing; sixteenth informational supplement. CLSI document M100-S16CLSI, Wayne, PA.
(29) European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) (2003) Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. *Clinical Microbiology and Infection 9*, ix−xv.
(30) Wiegand, I., Hilpert, K., and Hancock, R. E. (2008) Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat. Protoc. 3*, 163−175.
(31) Skerlavaj, B., Gennaro, R., Bagella, L., Merluzzi, L., Risso, A., and Zanetti, M. (1996) Biological characterization of two novel cathelicidin-derived peptides and identification of structural requirements for their antimicrobial and cell lytic activities. *J. Biol. Chem. 271*, 28375−28381.
(32) Ando, T., Yamasaki, M., and Suzuki, K. (1973) Protamines. Isolation, characterization, structure and function. *Mol. Biol. Biochem. Biophys. 12*, 1−114.
(33) Bagheri, M., Beyermann, M., and Dathe, M. (2012) Mode of action of cationic antimicrobial peptides defines the tethering position and the efficacy of biocidal surfaces. *Bioconjugate Chem. 23*, 66−74.
(34) Brogden, K. A. (2005) Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria? *Nat. Rev. Microbiol. 3*, 238−250.
(35) Gennaro, R., and Zanetti, M. (2000) Structural features and biological activities of the cathelicidin-derived antimicrobial peptides. *Biopolymers 55*, 31−49.
(36) Park, C. B., Yi, K. S., Matsuzaki, K., Kim, M. S., and Kim, S. C. (2000) Structure-activity analysis of buforin II, a histone H2A-derived antimicrobial peptide: the proline hinge is responsible for the cell-penetrating ability of buforin II. *Proc. Natl. Acad. Sci. U. S. A. 97*, 8245−8250.
(37) Avalos Vizcarra, I., Emge, P., Miermeister, P., Chabria, M., Konradi, R., Vogel, V., and Moller, J. (2013) Fluorescence-based in situ assay to probe the viability and growth kinetics of surface-adhering and suspended recombinant bacteria. *Biointerphases 8*, 22.
(38) Li, P., Poon, Y. F., Li, W., Zhu, H. Y., Yeap, S. H., Cao, Y., Qi, X., Zhou, C., Lamrani, M., Beuerman, R. W., Kang, E. T., Mu, Y., Li, C. M., Chang, M. W., Leong, S. S., and Chan-Park, M. B. (2011) A polycationic antimicrobial and biocompatible hydrogel with microbe membrane suctioning ability. *Nat. Mater. 10*, 149−156.
(39) Hilpert, K., Elliott, M., Jenssen, H., Kindrachuk, J., Fjell, C. D., Korner, J., Winkler, D. F., Weaver, L. L., Henklein, P., Ulrich, A. S., Chiang, S. H., Farmer, S. W., Pante, N., Volkmer, R., and Hancock, R. E. (2009) Screening and characterization of surface-tethered cationic peptides for antimicrobial activity. *Chem. Biol. 16*, 58−69.
(40) Magnusson, K. E. (1989) Physicochemical properties of bacterial surfaces. *Biochem. Soc. Trans. 17*, 454−458.
(41) Hong, Y., and Brown, D. G. (2008) Electrostatic behavior of the charge-regulated bacterial cell surface. *Langmuir 24*, 5003−5009.
(42) Utsugi, T., Schroit, A. J., Connor, J., Bucana, C. D., and Fidler, I. J. (1991) Elevated expression of phosphatidylserine in the outer membrane leaflet of human tumor cells and recognition by activated human blood monocytes. *Cancer Res. 51*, 3062−3066.