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Evaluation of free or anchored antimicrobial peptides as candidates for the prevention of orthopaedic device-related infections.

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3 **Evaluation of free or anchored antimicrobial peptides as candidates for the prevention of**
4 **orthopaedic device-related infections.**
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33 **Short title:** α -helical AMPs for the prevention of orthopaedic infections.
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Abstract

The prevention of implant-associated infection, one the most feared complications in orthopaedic surgery, remains a major clinical challenge and urges development of effective methods to prevent bacterial colonization of implanted devices. Alpha-helical antimicrobial peptides (AMPs) may be promising candidates in this respect due to their potent and broad-spectrum antimicrobial activity, their low tendency to elicit resistance and possible retention of efficacy in the immobilized state.

The aim of this study was to evaluate the potential of five different helical AMPs, the cathelicidins BMAP-27 and BMAP-28, their (1-18) fragments and the rationally designed, artificial P19(9/G7) peptide, for the prevention of orthopaedic implant infections. Peptides were effective at micromolar concentrations against 22 *Staphylococcus* and *Streptococcus* isolates from orthopaedic infections, while only BMAP-28 and to a lesser extent BMAP-27 were active against *Enterococcus faecalis*. Peptides in solution showed activities comparable to those of cefazolin and linezolid, on a molar basis, and also a variable capacity to neutralize bacterial lipopolysaccharide, while devoid of adverse effects on MG-63 osteoblast cells at concentrations corresponding to the MIC. The (1-18) BMAP fragments and P19(9/G7) were selected for further examination, based on better selectivity indices, and showed effectiveness in the presence of hyaluronic acid and in synovial fluid, while human serum affected their activity to variable extents, with BMAP-27(1-18) best retaining activity. This peptide was immobilized on streptavidin-resin beads and retained activity against reference *S. epidermidis* and *S. aureus* strains, with negligible toxicity towards osteoblasts, underlining its potential for the development of infection-resistant biomaterials for orthopaedic application.

Keywords: α -helical antimicrobial peptide, prosthetic joint pathogens, *Staphylococcus*, human serum, hyaluronic acid, peptide immobilization.

Abbreviations: ALP, alkaline phosphatase; AMP, antimicrobial peptide; BHI, Brain-Heart Infusion; CFU, colony-forming unit; CLSI, Clinical and Laboratory Standards Institute; DEG, diethylene glycol; DMEM, Dulbecco's Modified Eagle's Medium; DMF, N,N-dimethylformamide; DXM, dexamethasone; FBS, foetal bovine serum; HA, hyaluronic acid; HBSS, Hanks' Balanced Salt Solution; HPLC, high performance liquid chromatography; HS, human serum; HSA, human serum albumin; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MBC, minimum bactericidal concentration; MH, Mueller-Hinton; MIC, minimum inhibitory concentration; MRSA, methicillin-resistant *Staphylococcus aureus*; MRSE, methicillin-resistant *Staphylococcus*

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3 *epidermidis*; NO, nitric oxide; OM, osteogenic medium; PBS, phosphate buffered saline; RPMI,
4 Roswell Park Memorial Institute; SF, synovial fluid; SD, standard deviation; TFA, trifluoroacetic
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Introduction

Implant-associated infection is a major challenge in orthopaedic surgery. Although it occurs in only a small proportion of cases, infection of prosthetic joints is a feared and devastating complication of arthroplasty procedures and is associated with substantial morbidity and a huge economic burden [1-3]. The incidence of infection can be much higher following trauma surgery, such as fracture fixation [4]. More than half of infections are caused by Gram-positive cocci, the most common being coagulase-negative *Staphylococcus* spp. and *Staphylococcus aureus*, followed by *Streptococcus* and *Enterococcus* spp. A relevant proportion of these is polymicrobial, often involving methicillin-resistant *S. aureus* (MRSA). Gram-negative bacilli, anaerobes and fungi are less frequent causative agents [1, 3]. Treatment of infections associated with orthopaedic devices is challenging as bacteria tend to grow in biofilms, which reduces antibiotic efficacy, added to which is the growing incidence of antibiotic resistance also in orthopaedic settings [1, 3, 5]. The prevention of initial bacterial colonization of the implant surface thus remains a priority.

In this respect, natural antimicrobial peptides (AMPs) may represent a valuable reservoir of lead compounds for the development of novel anti-infective agents. AMPs comprise structurally diverse molecules endowed with potent microbicidal properties, acting as relevant components of the immune system in a wide range of living organisms [6]. Unlike conventional antibiotics, which act on specific bacterial targets, the mode of action of AMPs in most cases relies on their capacity to selectively perturb bacterial membranes, owing to their cationic and amphipathic nature [6, 7]. This mechanism accounts for their broad spectrum of activity and efficacy also against antibiotic-resistant strains, as well as for their low propensity to promote bacterial resistance [7]. Additionally, in several instances AMPs have exhibited immunomodulatory properties due to their ability to interact with host cells and modulate their functions, without damaging them [8, 9]. Several natural or artificial AMPs are currently under preclinical or clinical development as novel anti-infectives [6, 9, 10]. Moreover, an emerging approach focuses on exploiting these molecules for the fabrication of infection-resistant biomaterials for medical use [10-12].

Among the diverse structural classes of AMPs, linear peptides adopting an α -helical conformation appear to possess the most robust antimicrobial activities, with lower susceptibility to medium conditions [13, 14]. In addition to broad-spectrum activity, they can bind and neutralize proinflammatory bacterial components such as lipopolysaccharide (LPS) [9, 15]. Given their abundance in nature and their relatively easy chemical production with respect to other structural classes, native peptide sequences have been exploited as templates for numerous synthetic helical AMPs with optimized structural parameters to enhance antimicrobial potency and selectivity [16, 17]. As their killing mechanism does not require internalization by target bacteria, α -helical AMPs

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3 may retain activity upon immobilization, which may render them suitable for the development of
4 antimicrobial surfaces [18-20].

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6 In the present study five cationic α -helical AMPs, *i.e.*, two bovine members of the
7 cathelicidin AMP family [21], two shorter derivatives [22] and a rationally designed artificial AMP
8 [17], have been evaluated for potential applications in the prevention of orthopaedic device-related
9 infections. Antimicrobial efficacy of peptide candidates has been assayed against bacterial isolates
10 from clinical cases of orthopaedic infection, in comparison with two conventional antibiotics. The
11 peptides were also evaluated for LPS-neutralizing capacity and for potential effects on osteoblast
12 viability and differentiation. Based on their more favourable selectivity indices the study was then
13 narrowed to the three shorter peptide candidates, that were examined for antimicrobial efficacy
14 under conditions more closely resembling those encountered *in vivo* in arthroplasty settings. The
15 peptide with the best activity overall was then tethered to resin beads via streptavidin-biotin
16 technology to obtain a proof-of-concept demonstration of antimicrobial efficacy upon
17 immobilization.
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Materials and methods

Media and reagents

Derivatized polyethylene glycol–polystyrene (PEG-PS) resins, coupling reagents for peptide synthesis and 9-fluorenylmethoxy carbonyl (Fmoc)-amino acids were purchased from Applied Biosystems/Thermo Fisher Scientific (Waltham, MA, USA), Novabiochem (Laufelfingel, Switzerland) and ChemImpex (Wood Dale, IL, USA). Peptide synthesis-grade N,N-dimethylformamide (DMF), dichloromethane, piperidine and HPLC-grade acetonitrile were from Biosolve (Valkenswaard, The Netherlands). Trifluoroacetic acid (TFA), trifluoroethanol and N-methylmorpholine were from Acros Chimica (Beerse, Belgium). The biotinylation reagents D(+)-Biotin and O-(N-Biotinyl-3-aminopropyl)-O'-(N-glutaryl-3-aminopropyl)-diethyleneglycol (N-Biotinyl-NH-PEG₂-COOH) were obtained from Calbiochem (La Jolla, CA, USA) and Novabiochem, respectively. High-performance Streptavidin-Sepharose™ resin was purchased from GE Healthcare Life Sciences (Little Chalfont, Buckinghamshire, UK).

Dehydrated media for microbiological assays were obtained from Difco laboratories (Detroit, MI, USA) and Oxoid/Thermo Fisher Scientific. Cefazolin sodium salt, linezolid, lipopolysaccharide (LPS) from *E. coli* O111:B4, dexamethasone (DXM), hyaluronic acid sodium salt (HA) and normal human serum (HS) were from Sigma-Aldrich (St. Louis, MO, USA). Media and supplements for cell culture were from Sigma-Aldrich with the exception of foetal bovine serum (FBS) (Euroclone, Pero, Italy). Unless otherwise specified, FBS and HS were inactivated at 56°C for 30 min prior to use. Synovial fluid (SF) samples were obtained with informed consent at Udine University Hospital (Udine, Italy); samples were centrifuged, aliquoted and stored at -80°C.

The Griess and PrestoBlue® reagents were from Molecular Probes/Thermo Fisher Scientific and Invitrogen/Thermo Fisher Scientific, respectively. The CytoTox 96® Non-Radioactive Cytotoxicity Assay kit was from Promega (Madison, WI, USA). All other reagents, including p-Nitrophenyl Phosphate Liquid Substrate System, were from Sigma-Aldrich. Buffers were prepared in double glass-distilled water.

Peptide synthesis and characterization

Peptides (Table 1) were synthesized in the solid phase using the Fmoc-chemistry. Difficult coupling steps were handled as described previously [14, 23]. BMAP-27 and BMAP-27(1-18) were biotinylated by coupling 5 equivalents of D(+)-Biotin to the N-terminus of resin-bound peptides in the presence of equimolar 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoro-borate (TBTU) and 1H-hydroxybenzotriazole (HOBt) in DMF containing 0.6 N N-methylmorpholine for 4

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3 h at room temperature. BMAP-27(1-18) was alternatively biotinylated with N-Biotinyl-NH-PEG₂-
4 COOH using the same procedure. The biotinylated peptides are referred to as biot-B27, biot-B27(1-
5 18) and biot-DEG-B27(1-18) in the following sections. After cleavage and deprotection, the
6 peptides were HPLC-purified and confirmed by mass spectrometry using a Q-STAR hybrid
7 quadrupole time-of-flight mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON,
8 Canada) equipped with an electrospray ion source. Peptide concentrations were determined in
9 aqueous solution by measuring the absorbance at 257 nm (Phe residues) for BMAP-27 and BMAP-
10 27(1-18), and at 280 nm (Tyr and Trp residues) for BMAP-28, BMAP-28(1-18) and P19(9/G7) [14,
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19 *Peptide immobilization on Streptavidin-Sepharose resin*

20 250 µL of 1 mM solutions of the biotinylated peptides [*i.e.*, biot-B17, biot-B27(1-18) and biot-
21 DEG-B27(1-18)] in PBS were mixed with an equal volume of high performance Streptavidin-
22 Sepharose resin (previously washed free of the storage buffer) resuspended in PBS, and allowed to
23 react overnight at 4°C under gentle agitation. One resin sample was coupled in parallel with D(+)-
24 Biotin for use as a reference control. Samples were then allowed to re-equilibrate at room
25 temperature, centrifuged and washed extensively with PBS until the absorbance at 257 nm of the
26 resin supernatants reached the baseline level. Resin samples were then resuspended in PBS and
27 either tested immediately, or stored at 4°C for up to 1 month before use.
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34 The amount of immobilized peptide was estimated after peptide elution from a resin aliquot by 15
35 min treatment with 0.05% (v/v) TFA and lyophilization. The lyophilized eluates were redissolved in
36 water and assayed using the Bradford reagent with reference to standard curves generated by serial
37 dilutions of the corresponding soluble peptides.
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43 *Bacteria and culture conditions*

44 The reference strains were *Staphylococcus epidermidis* ATCC 12228 and ATCC 35984,
45 *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa*
46 ATCC 27853. Clinical bacterial isolates were collected from hip revision and other orthopaedic
47 surgery at the Valdoltra Orthopaedic Hospital (Ankaran, Slovenia) and the Udine University
48 Hospital (Udine, Italy), and included 8 strains of *Staphylococcus epidermidis*, 8 strains of
49 *Staphylococcus aureus*, 1 strain each of *Staphylococcus capitis*, *Staphylococcus hominis* and
50 *Staphylococcus caprae*, 2 strains of *Streptococcus agalactiae*, 1 group G *Streptococcus* and 2
51 isolates of *Enterococcus faecalis*. Bacteria were maintained on Mueller-Hinton (MH) agar plates.
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58 One isolate of *S. epidermidis* from the Udine hospital was methicillin-resistant as assessed
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3 according to the guidelines and interpretative tables of the Clinical and Laboratory Standards
4 Institute (CLSI). Bacteria were cultured in either liquid Brain Heart Infusion (BHI)
5 (*Staphylococcus*, *Streptococcus* and *Enterococcus* strains) or MH broth (*E. coli* and *P. aeruginosa*)
6 for 18 h, 1:50-diluted in fresh medium and allowed to grow in an orbital shaker at 37°C. Mid-log
7 phase bacteria were harvested after 10 min centrifugation at $1000 \times g$ and resuspended in MH broth
8 to an optimal density before use in antimicrobial assays. Bacterial density was assessed by turbidity
9 at 600 nm, with reference to previously determined standards. For biofilm experiments, the bacterial
10 inoculum was prepared essentially according to Pompilio *et al.* [24], by direct suspension of
11 colonies grown overnight on MH plates in liquid medium.
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20 *Antimicrobial assays*

21 The minimum inhibitory concentration (MIC) of the peptides in solution was determined by a broth
22 microdilution assay in 96-well microtiter plates, using MH broth with logarithmic-phase
23 microorganisms at 2.5×10^5 CFU/mL, as previously reported [14], following CLSI guidelines. The
24 clinically used antibiotics cefazolin and linezolid were tested in parallel for comparison. The
25 minimum bactericidal concentration (MBC) was determined by plating aliquots from wells showing
26 no visible growth on solid medium to allow colony counts.
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33 *Assays for inhibition of biofilm formation*

34 50 μ L of a *S. epidermidis* ATCC 35984 suspension (2×10^6 CFU/mL in MH broth), prepared as
35 described above, was dispensed into flat-bottomed 96-well microtiter plates containing 50 μ L in
36 MH of each test agent, at $2 \times$ the final dose. Plates were then incubated for 24 h at 37°C, followed
37 by removal of non-adherent bacteria by aspiration and rinsing with PBS. Fresh MH broth was then
38 added and adherent cells were quantified using the PrestoBlue cell viability reagent according to the
39 manufacturer's specifications.
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46 *Bacterial growth kinetics analysis*

47 Serial dilutions of peptides, at $2 \times$ the final concentration, were prepared in 50 μ L PBS alone or in
48 PBS supplemented either with 1 or 6 mg/mL hyaluronic acid (HA) (leading to 0.5 and 3 mg/mL
49 final HA concentrations, respectively), or with 50% human serum (HS, 25% final concentration), or
50 with 40% synovial fluid (SF, 20% final concentration) in U-bottom 96-well plates. 50 μ L of
51 adjusted suspensions of *S. epidermidis* ATCC 35984 or *S. aureus* ATCC 25923 in MH broth were
52 added to each well to achieve the final density of 1×10^7 CFU/mL. Plates were then sealed with
53 optically-clear plastic films to avoid evaporation, and bacterial growth was monitored at 600 nm for
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3 6 h at 37°C, with 10-sec shaking steps at 15-min intervals. Bacteria grown in each medium in the
4 absence of peptides served as growth controls. In some experiments peptide dilutions in the selected
5 media were pre-incubated for 3 h at 37°C prior to the addition of bacteria. Data are reported either
6 as growth curves, or as the calculated percent growth inhibition at 6 h with respect to bacteria
7 incubated in corresponding media in the absence of peptides.
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11 12 13 *Antibacterial activity of immobilized peptides*

14 10 µL aliquots of peptide-functionalized Streptavidin-Sepharose resins (5% v/v final resin
15 concentration), or the corresponding supernatants, were diluted in PBS to a final volume of 100 µL
16 in microcentrifuge tubes and supplemented with 100 µL of a 2×10^7 CFU/mL suspension of *S.*
17 *epidermidis* ATCC 35984 or *S. aureus* ATCC 25923 in MH broth. Bacterial viability was assessed
18 after 1 h incubation at 37°C on a rotating wheel by the PrestoBlue assay and CFU counts.
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24 25 *Cell culture and stimulation*

26 The murine macrophage-like RAW 264.7 and the human osteoblast-like MG-63 cell lines were
27 obtained from ATCC (Manassas, VA, USA) and maintained in RPMI-1640 and DMEM medium,
28 respectively, in a humidified incubator at 37°C and 5% CO₂ atmosphere. Both media were
29 supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL
30 streptomycin (complete media).
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34 To analyze the effects on LPS-induced nitric oxide (NO) production, RAW 264.7 cells were seeded
35 in 24-well plates at a density of 9×10^5 cells /well, cultured overnight and treated for 24 h with 100
36 ng/mL LPS in the absence and presence of peptides at the indicated concentrations, in complete
37 RPMI.
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41 MG-63 cells used for cell viability and cytotoxicity assays were seeded in 96-well plates at a
42 density of 7500/well and grown for 24 h in complete DMEM before being incubated with
43 increasing peptide concentrations in complete medium, or, alternatively, in DMEM supplemented
44 with 10% (v/v) HS, or in HBSS supplemented with 10% HS in the absence and presence of 0.5
45 mg/mL HA. Peptide-functionalized resins were tested at 5% (v/v) in complete medium. Incubation
46 times were 1 h and 24 h for lactate dehydrogenase (LDH) release and cell viability assays,
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53 For osteoblast differentiation experiments, MG-63 cells were seeded in 24-well plates at a density
54 of 3×10^5 /well in complete medium, grown to 80% confluence and incubated for 7 days in serum-
55 free DMEM supplemented with 10 mM β-glycerophosphate and 50 µg/mL L-ascorbic acid
56 (osteogenic medium, OM) in the absence or presence of 2 µM peptides, of 100 nM dexamethasone
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3 (DXM, positive control) or a combination of peptides and DXM. During the incubation period
4 medium was replaced twice with fresh OM supplemented with the stimuli.
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8 *Nitric oxide (NO) determination*

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10 The amount of NO released by LPS- and peptide-treated RAW 264.7 cells was estimated by
11 spectrophotometric quantification of the stable NO metabolite nitrite in the cell culture supernatants
12 using the Griess reagent. Data were expressed as percent of nitrite produced in response to LPS in
13 the absence of peptides.
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18 *Cytotoxicity and cell viability assays*

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20 Membrane damage to MG-63 cells was evaluated as leakage of the cytosolic enzyme lactate
21 dehydrogenase (LDH). LDH activity was quantified in both cell-free supernatants and cell lysates
22 using the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit. Data were calculated as percentage
23 of total cellular LDH activity. Cell viability was assessed using the resazurin-based PrestoBlue
24 metabolic dye according to the manufacturer's instructions.
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30 *Measurement of alkaline phosphatase (ALP) activity*

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32 MG-63 cells, incubated in OM with peptides and DXM as described above, were harvested by
33 trypsinization, resuspended in water and sonicated. Lysate proteins were quantified by the Bradford
34 assay. ALP activity was assessed spectrophotometrically from the transformation of p-nitrophenyl
35 phosphate to p-nitrophenol at 37°C. Briefly, 10 µg protein aliquots of cell lysates were diluted in
36 water in microtiter plates to a final volume of 50 µL, mixed with 150 µL of p-Nitrophenyl
37 Phosphate Liquid Substrate System and incubated for 80 min at 37°C. Absorbance was read at 405
38 nm and compared to a p-nitrophenol standard curve. Data were calculated as nmoles/mg/min and
39 expressed as fold-increase with respect to basal ALP activity, or as percent of ALP activity induced
40 by DXM alone.
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Results

Design and selection of candidate peptides

The peptides used in this study (Table 1) were selected on the basis of their reported broad antimicrobial activity spectra that covered Gram-positive cocci [17, 22, 23, 25]. BMAP-27 and BMAP-28 are amphipathic α -helical bovine cathelicidins that were previously shown to exert potent bactericidal activity based on rapid membrane permeabilization [22, 25]. The two shorter derivatives, BMAP-27(1-18) and BMAP-28(1-18) (hereafter referred to as B27(1-18) and B28(1-18)), were synthetically more accessible and showed improved selectivity with respect to prokaryotic cells [22]. The peptide P19(9/G7) (hereafter P19) was rationally designed based on a consensus sequence derived from a panel of over 100 of natural helical AMPs of invertebrate, anuran and mammalian origin [17, 23]. This peptide is based on an amidated, 18-residue sequence, like the BMAP fragments, with an added C-terminal Tyr residue to improve quantification accuracy.

Antimicrobial activity against orthopaedic pathogens

The MIC and MBC values of the peptides towards representative reference strains of Gram-positive *S. epidermidis* and *S. aureus* and Gram-negative *E. coli* and *P. aeruginosa* are reported in Table 2. Based on the frequent presence of Gram-positive microorganisms among the causative agents of orthopaedic device-related infections [1, 3], the peptides were assayed against a panel of Gram-positive clinical isolates from such infections, including *Staphylococcus*, *Streptococcus* and *Enterococcus* species (Table 3). All peptides proved effective at low micromolar concentrations against most strains, with MBC values very close to the MICs, with the exception of *E. faecalis*, which was susceptible only to BMAP-28 and to a lesser extent to BMAP-27. On a molar basis, their potency was comparable to cefazolin and linezolid, two conventional antibiotics used in orthopaedic practice [26, 27]. Considering *S. aureus* and *S. epidermidis* species, susceptibility of clinical isolates and corresponding reference strains to the AMPs was similar. Among the peptides, the rationally designed P19 was overall somewhat less effective against *S. aureus*, while its activity against the other *Staphylococcus* strains was comparable to that of the BMAP peptides. In general, peptides were highly active against *S. epidermidis*, whereas somewhat higher MIC values were observed against *S. aureus* isolates. In addition, all peptides prevented the formation of *S. epidermidis* ATCC 35984 biofilm at their MIC values, as shown in Figure 1a. Cefazolin and linezolid, tested in parallel, resulted in complete biofilm inhibition at 16 μ M (4-fold MIC) and 6 μ M (MIC), respectively (Figure 1b and data not shown).

Neutralization of bacterial lipopolysaccharide

Peptides were next evaluated for their ability to neutralize the effects of bacterial lipopolysaccharide (LPS), an activity common to several amphipathic helical AMPs [15] that could be advantageous in the context of orthopaedic biomaterials [28-30]. This was tested by inhibition of LPS-induced nitric oxide (NO) release from RAW 264.7 macrophage-like cells co-stimulated with peptides and LPS. BMAP-27 and -28 completely abolished the NO response at concentrations as low as 0.5 - 1 μ M (Fig. 2), consistent with previous reports on their direct LPS-binding properties [31-33]. Among the three shorter peptides, B28(1-18) virtually abolished the LPS-induced response at 2 μ M, while B27(1-18) and P19 respectively caused 80% and 66% inhibition at 8 μ M (Fig. 2). These results could not be ascribed to cytotoxic effects on the RAW cells, as cell viability was not impaired under the experimental conditions used (resazurin-based metabolic assays, data not shown).

Effects on osteoblast viability and differentiation

To assess their safety for orthopaedic applications, the effects of peptides on osteoblast cells were investigated using the human osteosarcoma-derived MG-63 cell line, as a well-established *in vitro* osteoblast model [34, 35]. Potential short- and long-term effects were assessed in terms of membrane damage, cell viability and cell differentiation. Figure 3a shows the percentage of lactate dehydrogenase (LDH) release by cells incubated with the peptides for 1 h in standard cell culture medium, as an indicator of impaired plasma membrane integrity. None of the peptides caused detectable LDH release at concentrations in each peptide's MIC range (cf. Fig. 3a and Table 3). The shorter BMAP derivatives were safe up to 10 μ M, while the parent peptides caused 38-54% LDH release at this concentration. P19 did not cause detectable effects up to at least 20 μ M (Fig. 3a). Remarkably, the toxicity of full-length and truncated BMAPs was clearly reduced when using human rather than standard foetal bovine serum in the cell culture medium (Fig. 3c), indicating that the range of safe concentrations under conditions relevant to *in vivo* settings could actually be wider. To mimic the joint environment, peptides were assayed in Hanks' Balanced Salt Solution (HBSS) supplemented with human serum (HS) and hyaluronic acid (HA) at 0.5 mg/mL, a concentration reflecting those found in periprosthetic synovial fluid [36]. Under these conditions, peptides were safe up to 10 μ M with the exception of BMAP-27, which caused approximately 40% and 68% LDH release at 10 and 20 μ M, respectively (Fig. 3d). The three shorter peptides, and in particular P19 and B27(1-18), showed lower overall toxicity. This was also confirmed in terms of cell viability after 24 h incubation with 1.25 - 10 μ M peptides in standard culture medium, which indicated the impairment of cell viability only on treatment with the full-length BMAPs at the

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3 highest tested concentration (Fig. 3b). Long-term effects on osteoblast cells were also addressed by
4 measuring alkaline phosphatase (ALP) activity as a differentiation marker for these cells [37].
5 Peptides were evaluated in comparison with dexamethasone (DXM) as a reference osteogenic
6 stimulus [37]. MG-63 cells incubated for 7 days with 2 μ M peptides in osteogenic medium showed
7 comparable ALP activity as control cells except after treatment with BMAP-27, which caused an
8 approximately 50% reduction in activity (Fig. 3e). A 2.7-fold increase in ALP activity was detected
9 in DXM-treated cells (Fig. 3e). The three shorter peptides were further investigated in combination
10 with the latter stimulus, showing no significant effects on the DXM-induced response (Fig. 3f),
11 suggesting they would not be detrimental to the osseointegration processes.
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20 *Antimicrobial activity in the presence of hyaluronic acid, synovial fluid and blood serum*

21 Given their compatibility with osteoblast cells, the (1-18) BMAP derivatives and P19 were
22 subjected to further investigation. Their antimicrobial efficacy against *S. epidermidis* ATCC 35984
23 and *S. aureus* ATCC 25923 was assayed in the presence of HA (as it could interact electrostatically
24 with the cationic peptides due to its negative charge), used at 0.5 and 3 mg/mL to mimic
25 periprosthetic and normal synovial fluid concentrations, respectively [36, 38]. Figure 4 shows
26 growth kinetics analyses in the presence of increasing peptide concentrations, expressed as percent
27 growth inhibition after 6 h incubation in each media. HA *per se* did not significantly affect bacterial
28 growth (data not shown). Remarkably, all of the peptides retained full activity against both
29 *Staphylococcus* species under these conditions. Furthermore, full peptide efficacy was retained even
30 after 3 h pre-incubation of peptides with HA (6 mg/mL) before the addition of the bacterial
31 suspension (data not shown), suggesting that peptides should retain efficacy in the joint
32 environment, where HA is a relevant component. This was further supported by growth kinetics
33 analysis of *S. aureus* ATCC 25923 incubated with the peptides in the presence of a clinical sample
34 of synovial fluid (SF, 20%) (Fig. 5). A limited number of SF samples were available, showing a
35 rather variable intrinsic inhibition of bacterial growth (data not shown); the one that was chosen for
36 the reported experiment allowed an appreciable *S. aureus* growth within the 6 h experimental time
37 frame. Peptides, tested at 2 μ M in its presence instead completely prevented bacterial growth (Fig.
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51 The applicative potential of the peptides was next assessed by antimicrobial assays
52 performed in the presence of human serum (HS), based on reported evidence of inhibitory effects of
53 serum components on AMP activity [14, 39-41]. Figure 6 shows the percentage of growth
54 inhibition of *S. epidermidis* and *S. aureus* incubated for 6 h with increasing peptide concentrations
55 in the absence and presence of commercial pooled human serum at 25%. HS *per se* did not impair
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3 growth of either strain (data not shown), while affecting the peptides' inhibitory activity to variable
4 extents. Complete inhibition of *S. epidermidis* growth in the presence of HS was achieved at the
5 same B27(1-18) concentrations as those effective in its absence, and only slightly higher B28(1-18)
6 and P19 concentrations (Fig. 6a, b). In the case of *S. aureus*, B27(1-18) and P19 showed a two-fold
7 increase and B28(1-18) a four-fold increase in the concentrations necessary to obtain complete
8 bacterial inhibition (Fig. 6d, e). Notably, when peptides were pre-incubated with 50% HS for 3 h
9 prior to addition of the bacterial suspension, all of the peptides were similarly affected, with the
10 concentration required for complete inhibition of *S. aureus* growth increasing to 64 μ M (Fig. 6f).
11 With respect to *S. epidermidis* growth inhibition under these conditions, B27(1-18) clearly retained
12 the best activity [complete inhibition at 2-4 μ M vs >8 μ M for B28(1-18) and P19] (Fig. 6c). When
13 B27(1-18) efficacy against *S. epidermidis* was tested in the presence of heat-inactivated HS (as in
14 the experiments described thus far) or in the presence of HS not subjected to heat inactivation,
15 results were overlapping (data not shown).
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26 *Antimicrobial efficacy in the immobilized state*

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28 Considering the overall results obtained thus far, the functional analysis of peptide
29 candidates narrowed the choice to B27(1-18) for further evaluation of efficacy upon
30 immobilization. Its parental BMAP-27 was previously reported to retain activity when covalently
31 linked to a solid surface [19], so it was tested in parallel for comparison. Peptides were N-terminally
32 biotinylated and conjugated to a commercial agarose resin functionalized with streptavidin. B27(1-
33 18) was also derivatized with a biotinylated diethylene glycol (DEG) spacer to favour peptide
34 mobility. In solution, the biotinylated derivatives showed comparable MIC values to the
35 corresponding non-biotinylated peptides against reference *S. epidermidis* and *S. aureus* strains (data
36 not shown), indicating that this modification did not alter activity. Peptide-functionalized resin
37 samples were incubated with *S. epidermidis* ATCC 35984 and *S. aureus* ATCC 25923 suspensions
38 for 1 h under agitation. Antibacterial effects were evaluated by the resazurin-based metabolic assay
39 and colony counts. As reported in Figure 7a and c, total bacterial inactivation was achieved upon
40 exposure to BMAP-27 and B27(1-18)-derivatized resins, while with the DEG-B27(1-18)-
41 functionalized sample 65% and 33% inhibition of *S. epidermidis* and *S. aureus* was respectively
42 observed. Consistent with this, CFU counts revealed complete killing in the case of immobilized
43 BMAP-27 and B27(1-18), and a partial effect with DEG-B27(1-18) (Fig. 7b, d). It is important to
44 note that the control resin, derivatized with biotin only, and the supernatants from peptide-
45 derivatized resin samples did not affect bacterial viability (Fig. 7a, c). To verify the stability of the
46 functionalized resins, their activity against *S. epidermidis* ATCC 35984 was also measured after 1
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3 month storage at 4°C and, as shown in Figure 7e and f, it was fully retained, indicating that the
4 peptide-resin coupling is quite stable. This was also supported by the lack of activity of supernatants
5 from 1 month-old resin samples (Fig. 7e). Finally, LDH release assays on MG-63 cells incubated
6 for 1 h with 5% resin suspensions did not reveal any impairment of cell membrane integrity (Table
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8 4) supporting compatibility of functionalized resins with osteoblast cells.
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Discussion

Surface modifications to render implants refractory to bacterial colonization are among the main strategies currently pursued to prevent orthopaedic implant-associated infection [42, 43]. The antimicrobial peptides of innate immunity are receiving increasing attention as novel anti-infective agents also in the field of medical devices [8-11]. The aim of the present study was to identify a peptide candidate active against relevant orthopaedic implant pathogens under conditions resembling *in vivo* settings, and suitable to be immobilized onto solid supports in view of future development of peptide-based infection-resistant biomaterials for orthopaedic applications.

Five α -helical peptide candidates were compared for their *in vitro* efficacy against clinically relevant Gram-positive bacterial strains isolated from orthopaedic infections. In keeping with previous reports, the BMAP peptides displayed similar low micromolar MIC values against most of the tested strains under standard conditions [14, 25, 44-46]. The comparable, or only slightly decreased potency of the (1-18) BMAP fragments confirms that antimicrobial activity is mainly mediated by the N-terminal segment predicted to adopt the amphipathic helical conformation [22, 25, 33]. In this regard, peptide modifications on parental or truncated BMAPs not subverting the overall helical fold were shown to be irrelevant to peptide activity [33, 39, 47, 48], supporting a relatively non-specific mode of peptide interaction with bacterial membranes that accounts for broad-spectrum permeabilizing effects.

While showing comparable efficacy to the BMAPs on other tested bacterial species, P19 displayed a clearly decreased activity against *S. aureus* strains. This suggests a partially different mode of action. Indeed, peptides of the P19 series have been proposed to act on *Staphylococcus* strains by a mechanism dependent on interference with functional processes at membrane level rather than on direct permeabilization [49].

With the exception of BMAP-28, the peptides under investigation showed lower efficacy against the clinical isolates of *E. faecalis* with P19 being the least effective. Concerning the BMAPs and derivatives, despite the limited number of strains tested, the observed MICs and the differences in potency are on the whole in line with a previous study assaying their activity against ten *E. faecalis* isolates, including vancomycin-resistant strains [25].

In general, the peptides under study showed a comparable efficacy to conventional antibiotics on a molar basis, and moreover proved effective against a methicillin-resistant clinical isolate of *S. epidermidis* (MRSE). This adds to previous reports on the efficacy of BMAPs and their derivatives, as well as of P19, on antibiotic-resistant Gram-positives, including MRSA [23, 25, 44, 50]. Moreover, peptides prevented *S. epidermidis* biofilm formation at microbicidal concentrations, with an all-or-nothing effect overall, likely due to rapid killing of bacteria before their

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3 deposition/attachment to the surface of microplate wells. At variance with the AMPs, both cefazolin
4 and linezolid displayed a gradual inhibition of biofilm formation, consistent with a mode of action
5 not directly targeting the bacterial membrane [51, 52]. Furthermore, while linezolid was effective at
6 preventing biofilm formation also at sub-MIC concentrations, cefazolin showed complete inhibition
7 only at 4-fold its MIC. Comparing the efficacy of the peptides to cefazolin, the antibiotic of choice
8 in perioperative orthopaedic prophylaxis [26], the selected AMPs appear to be suitable candidates
9 for the prevention of bacterial colonization of implanted devices.
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15 A distinctive feature of cationic AMPs is their capacity to neutralize proinflammatory
16 bacterial components such as lipopolysaccharide (LPS, endotoxin) [15]. This property may be
17 advantageous for applications in the field of orthopaedic biomaterials since endotoxin
18 contamination may be responsible for adverse tissue reactions and can negatively affect the
19 osseointegration process [28-30, 53]. Indeed, the murine cathelicidin CRAMP, another α -helical
20 cathelicidin that is paralogous to BMAPs, has been shown to inhibit LPS- and flagellin-induced
21 osteoclastogenesis by direct neutralization of these bacterial components, and accordingly has been
22 proposed to act as a protector of bone resorption induced by bacterial infection in mice [54]. In the
23 present study, in comparison to the complete LPS-neutralizing activity of the full length BMAPs,
24 the three shorter peptides displayed a lower but appreciable effect. With respect to (1-18) BMAP
25 peptides, this residual but still appreciable LPS-neutralizing effect, as already reported by Lee *et al.*
26 [33], is in line with that also reported for the rabbit paralog CAP18, and sheep BMAP ortholog
27 SMAP-29 [55]. These latter peptides have been proposed to bear LPS-binding sites, located both at
28 the N- and C-terminal ends, that function cooperatively [55]. The lack of the equivalent to one of
29 these sites in the (1-18) BMAP fragments could explain their somewhat lower LPS-binding
30 capacity compared to the parental molecules. Nevertheless, the residual activity might still be
31 relevant in an *in-vivo* setting, where LPS levels are expected to be significantly lower than those
32 normally used in *in-vitro* assays [56].
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46 One of the major drawbacks in the clinical development of AMPs are cytotoxic effects on
47 host cells [8, 9], that offsets several beneficial activities on host cell functions, in addition to their
48 direct microbicidal properties [8, 9]. In this respect the human cathelicidin LL-37, while exerting
49 some toxic effects on osteoblasts [57], has been shown to promote bone regeneration *in vivo* by
50 distinct mechanisms involving stimulation of other cell types [58-60]. In addition, LL-37 has been
51 shown to inhibit osteoclastogenesis *in vitro* [61]. Moreover, the toxic effects of this peptide may be
52 counteracted *in vivo* by endogenous factors [62, 63]. Another class of human AMPs, the beta-
53 defensins, are reported to display stimulatory effects on osteoblast proliferation and differentiation
54 [64]. In the present study, all candidate peptides proved safe to osteoblast cells in their MIC value
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3 range, with BMAP fragments and P19 clearly displaying a wider selectivity index. This was
4 observed both in standard media and in the presence of human serum and hyaluronic acid as
5 relevant factors in view of an orthopaedic application. Our data confirm the improved selectivity of
6 BMAP truncated analogs against prokaryotic vs mammalian cells with respect to the parent peptides
7 [22, 33]. Moreover, these fragments as well as P19 did not affect osteoblast differentiation *per se*,
8 nor impaired the effect of a recognized osteogenic stimulus [37], which bolsters their suitability for
9 applications on orthopaedic devices.
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14 Although in contrast with several reports on AMP activity being inhibited by anionic
15 polysaccharides [65-68], in the present study the activity of B27(1-18), B28(1-18) and P19 against
16 *S. epidermidis* and *S. aureus* was fully retained in the presence of HA at concentrations
17 representative of those found in periprosthetic and normal synovial fluid [36, 38]. The potential for
18 peptide efficacy in the skeletal joint environment was further supported by potent activity in the
19 presence of a clinical synovial fluid sample, selected among a limited number of available samples
20 due to its compatibility with *S. aureus* growth. Notably, expression of several AMPs was reported
21 in both healthy and inflamed synovial membranes and joint fluid [69-71], and this may reasonably
22 contribute to the inhibitory effects of joint fluid on microbial growth [72]. In an applicative
23 perspective, the presence of endogenous AMPs could be advantageous since they may synergize
24 with exogenously introduced AMPs.
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29 Influence of blood components, *e.g.*, serum albumin and lipoproteins [41, 73, 74], on AMP
30 activity represents a crucial issue to be addressed in view of potential therapeutic applications. In
31 fact, both BMAP-27 and BMAP-28 have been shown in previous studies to retain antimicrobial
32 properties in the presence of heat-inactivated bovine serum, although at higher peptide
33 concentrations [14, 39]. In our growth kinetics assays, the AMPs were effective against both
34 *Staphylococcus* species in serum coinubation experiments, at concentrations comparable or only
35 slightly higher than those in serum-free medium (see Fig. 6b, e). Peptides showed a more evident
36 decrease in potency upon pre-incubation with serum (Fig. 6c, f), but it was interesting to note that
37 while a decrease in activity against *S. aureus* was observed for all the tested AMPs, B27(1-18) was
38 the least affected in the case of *S. epidermidis*. The inhibitory effect of serum on peptide activity
39 likely depends on peptide sequestration by serum components, as supported by overlapping activity
40 of B27(1-18) against *S. epidermidis* in the presence of active and heat-inactivated HS, suggesting a
41 minimal contribution of enzymatic degradation. In this respect, the different behaviour of B27(1-18)
42 towards two *Staphylococcus* species was rather unexpected, since peptide binding to serum proteins
43 would be expected to comparably affect peptide activity regardless of the target microorganism. It
44 is widely accepted that membrane-active AMPs exert their microbicidal action through both
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3 electrostatic and hydrophobic interactions with bacterial membrane surfaces [6, 7], while binding to
4 serum seems preponderantly of a hydrophobic nature [74-76]. As B27(1-18) is the least
5 hydrophobic and most cationic of the three shorter AMPs (see Table 1), hydrophobic interactions
6 with serum factors may be relatively weaker and electrostatic attractions with the bacterial surface
7 play a more dominant role. One could thus reasonably speculate that in the presence of a
8 sufficiently anionic bacterial surface, peptide-bacteria interaction would be favoured even in the
9 presence of serum.
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12 According to published reports *S. epidermidis* has a more anionic surface than *S. aureus* [77,
13 78], which might explain the generally higher activity observed for the three short AMPs against *S.*
14 *epidermidis* strains as compared to *S. aureus* (Table 2 and 3). Moreover, for the same reason, *S.*
15 *epidermidis* could be less prone to shielding by serum, and this is particularly evident for the less
16 hydrophobic and more cationic B27(1-18). The competition of serum and bacterial surface for this
17 AMP is evidently finely balanced, as the less anionic *S. aureus* has a more serum-sensitive
18 susceptibility to this peptide. A similar hypothesis has been proposed by Huang *et al.* [73], to
19 explain the different susceptibilities of selected bacterial species to a cationic α -helical AMP in the
20 presence of human serum albumin (HSA). Notably, in this latter study *S. epidermidis* was the only
21 microorganism whose susceptibility to the peptide was not affected by HSA [73], again indicating
22 competitive binding to AMPs of this structural class.
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25 Due to its better performance under *in vivo*-mimicking conditions, B27(1-18) was the best
26 candidate for further studies with immobilized peptide. Like the parent BMAP-27, reported to
27 maintain activity on surface tethering [19], B27(1-18) was bactericidal to both *S. epidermidis* and *S.*
28 *aureus* when bound to resin beads. The streptavidin/biotin-based anchoring method, that approaches
29 the strength of a covalent bond [79], was selected as being a facile and straightforward procedure,
30 suitable for obtaining a proof-of-concept demonstration of peptide efficacy on immobilization [80].
31 In our study, peptides were tethered via the N-terminus as this facilitated the biotinylation
32 procedure, also given the reported efficacy of several N-terminally immobilized α -helical AMPs
33 [11, 18, 20]. In our hands, immobilization of biotinylated peptides on streptavidin-functionalized
34 resin beads led to a highly stable conjugate, as bactericidal activity from free peptide was not
35 detected in the resin supernatants, even after a month of storage.
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38 Comparison between full length surface-linked BMAP-27, surface-linked B27(1-18) and
39 also a derivative of the latter with a diethylene glycole spacer [DEG-B27(1-18)] indicated similar
40 activity of BMAP-27 and B27(1-18) on both tested strains, whereas DEG-B27(1-18) showed a
41 somewhat lower efficacy. This however may be due to a lower peptide-loading on the resin, as
42 assessed by peptide quantification upon elution from resin samples. For the DEG-modified peptide,
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3 resin loading was assessed to be only 25% that of unmodified B27(1-18) (data not shown). In fact, a
4 five-fold increase in DEG-B27(1-18) resin (from 5 to 25%) led to complete inhibition of *S.*
5 *epidermidis* and an almost 80% reduction of *S. aureus* viability (data not shown). The reason for
6 including a flexible spacer is that it could be relevant to the efficacy of surface-immobilized AMPs
7 [11, 12], but in our system activity appeared to be retained also in its absence, in keeping with the
8 findings of Rapsch *et al.* on directly-immobilized α -helical AMPs, including BMAP-27 [19]. In that
9 study, immobilized BMAP-27 was significantly less cytotoxic than the soluble counterpart, and was
10 proposed to act via bacterial membrane depolarization [19]. Notably, in our study also, none of the
11 anchored peptides induced appreciable membrane damage to osteoblast cells. In view of a potential
12 application of these peptides in the prevention of orthopaedic device-related infections, surface-
13 immobilized full-length BMAP-27 and the synthetically more accessible B27(1-18) fragment
14 appear to have equivalent antimicrobial efficacy and host-cell compatibility, so that issues related to
15 chemical synthesis procedures and production costs would obviously play in favour of the shorter
16 analog.
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30 **Conclusions**

31 Collectively, the results of this study highlight the potential of the α -helical peptide B27(1-
32 18) for the prevention of orthopaedic implant-related infections. It effectively killed bacterial
33 species responsible for orthopaedic infections and prevented staphylococcal biofilm formation.
34 Moreover it attenuated an LPS-induced inflammatory response and was devoid of adverse effects
35 on osteoblast cells. Its antimicrobial efficacy was comparable to that of cefazolin, widely used in
36 orthopaedic perioperative prophylaxis, and was retained in the presence of relevant biological fluids
37 and components. The proof-of-concept demonstration of its efficacy upon immobilization, obtained
38 with B27(1-18)-functionalized resin beads, encourages further efforts aimed at tethering this peptide
39 onto supports relevant for orthopaedic medical devices, such as titanium or ceramic, in view of the
40 development of peptide-based infection-resistant biomaterials for prostheses.
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Table 1. Peptide sequences and molecular characteristics.

Peptide	Sequence ^a	MW	Length	q ^b	%H ^c
BMAP-27	GRFKRFRKKFKKLFKKLSPVIPLLHL	3225	26	+12	42
BMAP-28	GGLRSLGRKILRAWKKYGPIHVIIRI	3074	27	+8	48
BMAP-27(1-18)	GRFKRFRKKFKKLFKKLS	2342	18	+11	33
BMAP-28(1-18)	GGLRSLGRKILRAWKKYG	2058	18	+7	39
P19(9/G7)	GLLKKIGKKAKKALKKLGY	2085	19	+9	42

^a The C-terminus of all peptides is amidated

^b q, net charge

^c %H, percent hydrophobic residues (sum of A, F, I, L, Y and W residues divided by number of residues)

Table 2. Antimicrobial activities against reference strains.

Organism	BMAP-27	BMAP-28	B27(1-18)	B28(1-18)	P19
<i>S. epidermidis</i> ATCC 12228	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)
<i>S. epidermidis</i> ATCC 35984	2 (2)	2 (2)	2 (2)	2 (2)	4 (4)
<i>S. aureus</i> ATCC 25923	2 (4)	2 (2)	4 (8)	2 (4)	16 (32)
<i>E. coli</i> ATCC 25922	1 (1)	2 (2)	2 (2)	1 (1)	2 (2)
<i>P. aeruginosa</i> ATCC 27853	2 (4)	4 (16)	2 (4)	2 (4)	2 (2)

^a Determined in MH broth

^b Data are means of at least three independent experiments

Table 3. Antimicrobial activities against bacterial isolates obtained from clinical cases of orthopaedic infections.

Organism (no. of isolates)	BMAP-27	BMAP-28	B27(1-18)	B28(1-18)	P19	cefazolin	linezolid
	MIC range (μM) ^{a,b}						
<i>S. epidermidis</i> (8)	0.5-2 (1 ^c)	1-2 (2 ^c)	0.5-4 (1 ^c)	1-2 (1 ^c)	0.5-32 (1 ^c)	0.5-16 (>16 ^c)	<0.4->95 (3 ^c)
<i>S. aureus</i> (8)	2-4	1-4	2-16	2-8	16->64	0.5-2	6
other <i>Staphylococcus</i> spp. (3)	0.5-1	1-2	0.5-1	0.5-1	0.5-1	0.5-4	3
<i>E. faecalis</i> (2)	8-32	4	>64	64	>128	>16	6
<i>Streptococcus</i> spp. (3)	1-2	0.5-4	1-2	1-2	0.5-2	0.25	1.5-3
MBC range (μM) ^{a,b}							
<i>S. epidermidis</i> (8)	1-2 (1 ^c)	1-2 (2 ^c)	0.5-4 (1 ^c)	0.5-4 (1 ^c)	0.5-64 (0.5 ^c)	0.5-16 (>16 ^c)	n.d.
<i>S. aureus</i> (8)	2-4	1-4	2-16	2-8	16->64	1-4	n.d.
other <i>Staphylococcus</i> spp. (3)	0.5-1	1-2	0.5-1	0.5-1	0.5-1	1->16	n.d.
<i>E. faecalis</i> (2)	8->32	4	>64	>64	>128	>16	n.d.
<i>Streptococcus</i> spp. (3)	1-2	2-4	1-2	1-2	0.5-2	1	n.d.
MIC ₅₀ (MIC ₉₀) (μM) ^{a,b,d}							
<i>S. epidermidis</i> (8)	1 (1)	2 (2)	1 (1)	1 (1)	1 (2)	2 (8)	3 (6)
<i>S. aureus</i> (8)	2 (2)	2 (2)	4 (8)	4 (4)	16 (32)	1 (2)	6 (6)

^a Determined in MH broth

^b Data are means of at least three independent experiments

^c Methicillin-resistant strain

^d MIC₅₀ and MIC₉₀: concentrations that inhibited 50% and 90%, respectively, of the strains

n.d., not determinable

Table 4. Effects of immobilized peptides on cell membrane integrity in osteoblast cells.

treatment ^a	% LDH release ^b
control	3.6 ± 0.8
resin-biotin	1.7 ± 2.1
resin-B27	4.9 ± 1.9
resin-B27(1-18)	3.0 ± 0.3
resin-DEG-B27(1-18)	2.7 ± 0.3

^a 1 h exposure of MG-63 cells to 5% resins in complete medium

^b Data were calculated as percentage of total cellular LDH activity, and are the means ± SD of three separate experiments

Figure captions

Figure 1. Inhibition of *S. epidermidis* biofilm formation. *S. epidermidis* ATCC 35984 (1×10^6 CFU/mL in MH broth) was seeded into flat-bottom 96-well polystyrene microtiter plates in the absence and presence of the indicated concentrations of peptides (a) or antibiotics (b). Adherent bacterial cells were quantified after 24 h incubation by the Prestoblu metabolic assay. Results are expressed as percent viability compared to untreated bacteria, and are the means \pm SD of three independent experiments performed at least in triplicate.

Figure 2. Lipopolysaccharide (LPS) neutralization. RAW 264.7 cells were stimulated with 100 ng/mL *E. coli* O111:B4 LPS in complete RPMI, in the absence and presence of peptides at the indicated concentrations. Nitrite values were determined in the culture supernatants after 24 h incubation using the Griess reagent. Data were calculated as percent of nitrite produced in response to LPS in the absence of peptides, and are the means \pm SD of three independent experiments.

Figure 3. Effects on osteoblast viability and differentiation. a, c, d) MG-63 osteoblast cells were incubated in the absence and presence of the indicated peptide concentrations under the following medium conditions: a) DMEM supplemented with 10% FBS (complete medium); c) DMEM supplemented with 10% human serum (HS); d) HBSS supplemented with 10% HS and 0.5 mg/mL hyaluronic acid (HA). LDH activity was measured in the culture supernatants after 1 h incubation and expressed as percentage of total cellular LDH activity. b) Cells were incubated with peptides for 24 h in complete medium. Cell viability was evaluated by the Prestoblu metabolic assay and is reported as percentage of untreated cells. e, f) Cells were incubated in osteogenic medium (OM) in the absence and presence of 2 μ M peptides or 100 nM dexamethasone (DXM) (e), or a combination of peptides and DXM (f). Alkaline phosphatase (ALP) activity was measured in cell lysates after 7 days of incubation, and expressed as fold-increase with respect to basal ALP activity (e) or as percent of ALP activity induced by DXM alone (f). Data are the means \pm SD of three independent experiments.

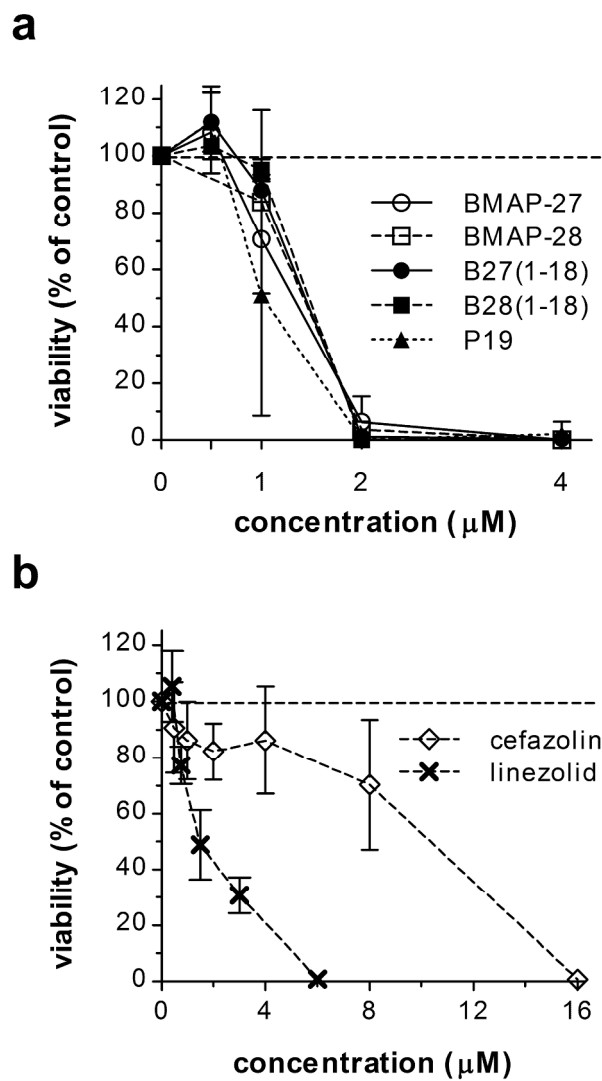
Figure 4. Peptide effects on bacterial growth in the presence of hyaluronic acid (HA). *S. epidermidis* ATCC 35984 (a-c) and *S. aureus* ATCC 25923 (d-f) (1×10^7 CFU/mL in 50% MH broth) were incubated with the indicated concentrations of B27(1-18) (a, d), B28(1-18) (b, e) or P19 (c, f) in the absence (circles, solid lines) and presence of 0.5 mg/mL (squares, dashed lines) and 3 mg/mL (triangles, dotted lines) HA. Bacterial growth was monitored for 6 h by optical density (OD)

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3 at 600 nm. Data were calculated as percentage of growth inhibition at the 6 h time point with
4 respect to bacteria incubated in corresponding media in the absence of peptides. The means \pm SD of
5 three independent experiments are reported.
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10 **Figure 5. Effects on *S. aureus* growth in the presence of synovial fluid (SF).** *S. aureus* ATCC
11 25923 was incubated at 1×10^7 CFU/mL in 50% MH broth in the absence (closed symbols) and
12 presence (open symbols) of 20% synovial fluid and of 2 μ M of the indicated peptides. OD readings
13 at 600 nm (OD₆₀₀) at 30 min intervals are reported.
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18 **Figure 6. Effects on bacterial growth in the presence of human serum (HS).** *S. epidermidis*
19 ATCC 35984 (a-c) and *S. aureus* ATCC 25923 (d-f) (1×10^7 CFU/mL) were grown in 50% MH
20 broth in the absence (a, d) and presence (b, c, e, f) of 25% human serum (HS) and of peptides at the
21 indicated concentrations. Bacteria were added immediately after the preparation of peptide dilutions
22 in PBS (a, d) or HS-containing PBS (b, e) or, alternatively, were dispensed to the wells after 3 h
23 pre-incubation of the peptides at 37°C in the presence of HS (c, f). Bacterial growth was monitored
24 for 6 h by OD₆₀₀ determinations at 30 min intervals. Data were calculated as percentage of growth
25 inhibition at 6 h with respect to corresponding controls incubated in the absence of peptides, and are
26 the means \pm SD of three independent experiments.
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35 **Figure 7. Antimicrobial efficacy of biotinylated peptides immobilized on Streptavidin-**
36 **Sepharose resin.** Streptavidin-Sepharose resin beads functionalized with biotinylated derivatives of
37 BMAP-27 and B27(1-18) as described in the method section were assayed for antimicrobial
38 efficacy either immediately after completion of the coupling procedure (a-d) or upon 1 month
39 storage at 4°C (e, f). *S. epidermidis* ATCC 35984 (a, b, e, f) and *S. aureus* ATCC 25923 (c, d) ($1 \times$
40 10^7 CFU/mL in 50% MH broth) were incubated for 1 h with 5% of the indicated resins (closed bars)
41 or their respective supernatants (open bars) under gentle agitation. a, c, e) Bacterial viability was
42 determined by the Prestoblue metabolic assay and expressed as percent relative to bacteria
43 incubated with the control resin functionalized with biotin only, or the corresponding supernatant. b,
44 d, f) Bacterial killing in resin-treated samples was determined based on colony counts, and
45 expressed relative to the control resin. The means \pm SD of three independent experiments are
46 reported.
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Figure 1. Inhibition of *S. epidermidis* biofilm formation.

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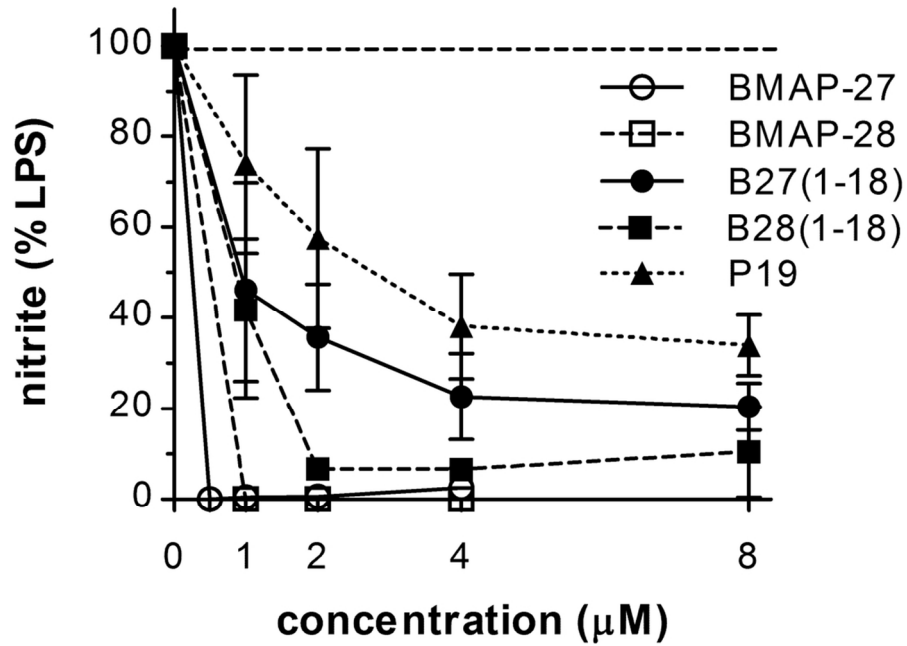


Figure 2. Lipopolysaccharide (LPS) neutralization.

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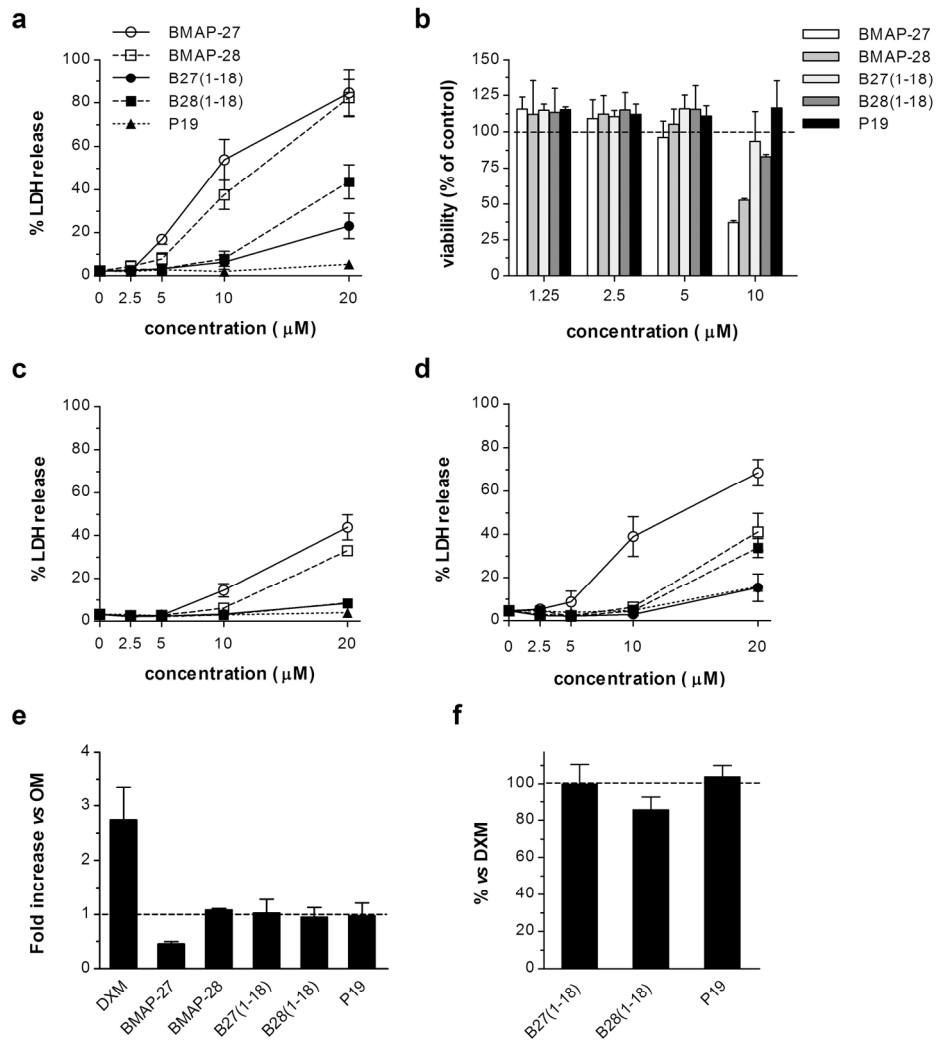


Figure 3. Effects on osteoblast viability and differentiation.

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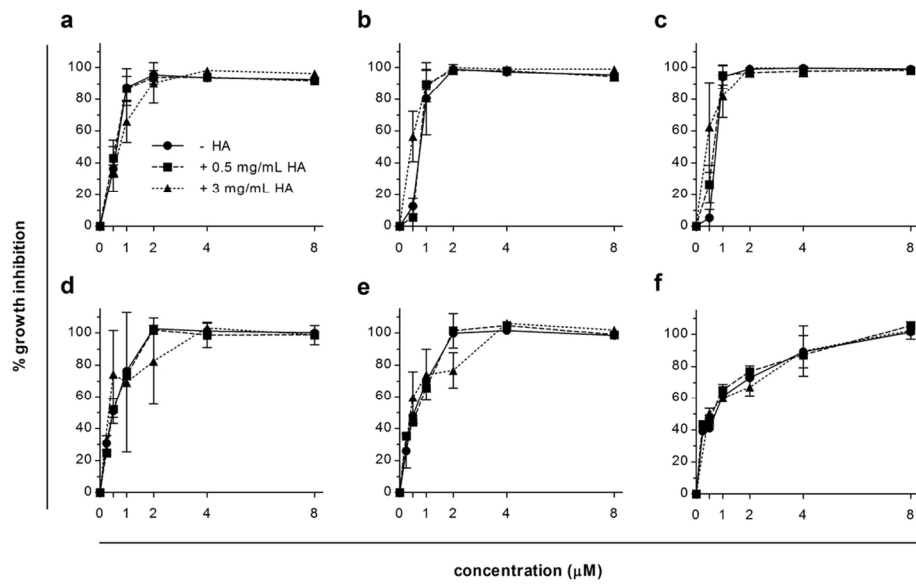


Figure 4. Peptide effects on bacterial growth in the presence of hyaluronic acid (HA).

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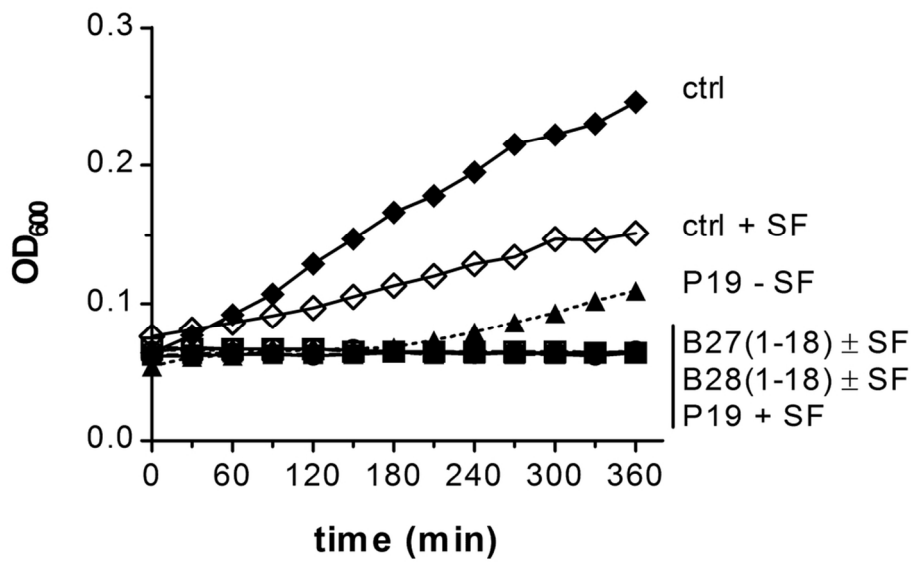


Figure 5. Effects on *S. aureus* growth in the presence of synovial fluid (SF).

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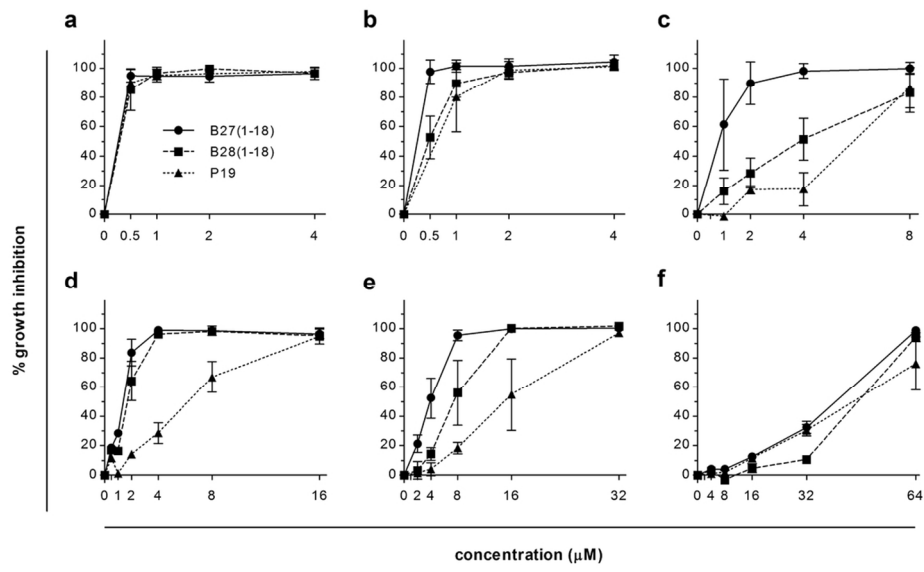


Figure 6. Effects on bacterial growth in the presence of human serum (HS).

107x64mm (300 x 300 DPI)

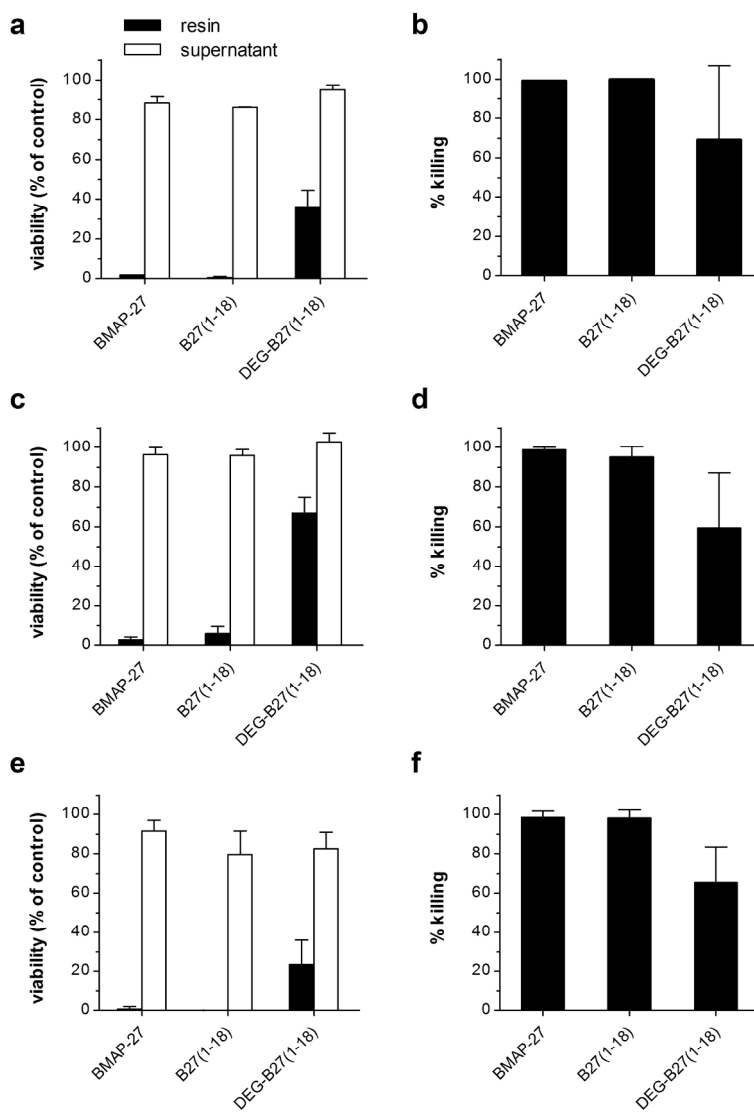


Figure 7. Antimicrobial efficacy of biotinylated peptides immobilized on Streptavidin-Sepharose resin.

176x256mm (300 x 300 DPI)