Novel antibacterial strategies to combat biomaterial-associated infection
Riool, M.

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Controlled release of LL-37-derived synthetic antimicrobial and anti-biofilm peptides SAAP-145 and SAAP-276 prevents experimental biomaterial-associated Staphylococcus aureus infection

Martijn Riool⁵, Anna de Breij⁵, Leonie de Boer, Paulus H. S. Kwakman, Robert A. Cordfunke, Or Cohen, Nermina Malanovic, Noam Emanuel, Karl Lohner, Jan W. Drijfhout, Peter H. Nibbering*, Sebastian A. J. Zaat*

⁵, *Authors contributed equally

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ABSTRACT

The aim of the present study was to develop implant coatings releasing novel antimicrobial agents to prevent biomaterial-associated infections. The LL-37-derived Synthetic Antimicrobial and Anti-biofilm Peptides SAAP-145 and SAAP-276 exhibit potent bactericidal and anti-biofilm activities against clinical multidrug-resistant *Staphylococcus aureus* strains by rapid membrane permeabilization, without inducing resistance. Injection of SAAP-145, but not SAAP-276, along subcutaneous implants in mice reduces numbers of CFU of *S. aureus* on implants by approximately 2-logs, but do not reduce numbers in tissue. SAAP-145 and SAAP-276 are incorporated in a Polymer-Lipid Encapsulation matrix coating, providing a constant release of 0.6% daily up to 30 days after an initial burst release of >50%. In a murine model for biomaterial-associated infection, SAAP-145-PLEX and SAAP-276-PLEX coatings significantly reduce the number of culture positive implants and show ≥3.5- and ≥1.5-log lower numbers of CFU of *S. aureus* on implants and in tissues, respectively. Interestingly, these coatings are also highly effective against a MDR *S. aureus*, reducing the numbers of CFU on the implants with ≥2-log, and the SAAP-276-PLEX additionally reduces the numbers of CFU in the tissue with 1-log. Together, peptide-releasing PLEX coatings hold promise for further development as an alternative to coatings releasing conventional antibiotics to prevent infection.
INTRODUCTION

Implanted medical devices, such as orthopedic prostheses, catheters and fracture fixation devices are prone to bacterial colonization leading to biomaterial-associated infection (BAI). Up to 80% of clinical infections are caused by bacteria able to form a biofilm, i.e. an aggregate of microorganisms embedded within a self-produced matrix of extracellular substances. It is known that biofilm-encased bacteria can be 10- to 1,000-times more tolerant to conventional antibiotics than their planktonic counterparts. This may be explained by difficulties in reaching the bacteria due to the extracellular polymeric substances forming a penetration barrier, the slow growth and low metabolic activity of the bacteria residing within the biofilm, and the presence of persister cells. Biofilms formed by Staphylococcus aureus and Staphylococcus epidermidis have been recognized as the most frequent cause of BAIs. The emergence of multidrug-resistant (MDR) staphylococci further complicates the treatment of BAIs by conventional antibiotics.

Obviously, there is a very pressing need for the development of novel agents and strategies, e.g. efficient coatings and delivery systems, to replace or complement current antibiotics for the treatment of BAIs. Antimicrobial peptides (AMPs) are considered promising treatment alternatives owing to their broad range activity and their mode(s) of action being different from those of conventional antibiotics. The cationic and amphipathic nature of many AMPs contribute to binding to the anionic bacterial cell surface and insertion into the cytoplasmic membrane, resulting in membrane perturbation and killing of the target microorganism. Thus, unlike antibiotics, AMPs do not act on a specific target but rather attack the cell membrane and are therefore thought to be less likely to induce resistance.

LL-37 is one of the principal human AMPs that plays an important role in protection against infections by exerting broad range antimicrobial activity and by neutralizing microbial endotoxins like lipopolysaccharide and lipoteichoic acid. Moreover, this peptide displays anti-biofilm activities against (antibiotic-resistant) S. aureus. LL-37, which is generated by enzymatic degradation of hCAP18, is produced by a variety of human cells, including mucosal epithelial cells, keratinocytes, mast cells and multiple immune cells, e.g. granulocytes, monocytes and macrophage subsets, dendritic cells, and certain T-lymphocytes. The peptide recently been evaluated in a phase I/II study in patients with venous leg ulcer as a treatment for wound healing. As the antimicrobial activities of LL-37 are highly sensitive to environmental conditions, research has focused on the development of synthetic peptides based on the amino acid sequence of LL-37. Earlier, we developed
an LL-37 synthetic derivative, designated as OP-145 or P60.4ac, with improved antimicrobial activity compared to LL-37\textsuperscript{23}. As OP-145’s activity was reduced in the presence of human plasma, we recently developed novel LL-37-derived synthetic antimicrobial and anti-biofilm peptides (SAAPs) with high efficacy against MDR bacteria under physiological conditions, superior to that of most published (pre-)clinical phase peptides\textsuperscript{24}. One of these peptides, SAAP-148, was selected for development as an agent for topical treatment of bacterial skin infections\textsuperscript{24}. For the present study we selected two other equally potent peptides, i.e. SAAP-145 and SAAP-276, for further development as effective agents against BAI caused by (MDR) \textit{S. aureus}.

Our study revealed that the selected synthetic antimicrobial and anti-biofilm peptides SAAP-145 and SAAP-276 are highly effective against \textit{S. aureus}, including MDR clinical strains, can prevent biofilm formation, do not induce resistance, and rapidly permeabilize the membrane of \textit{S. aureus}, resulting in killing within minutes. A Polymer-Lipid Encapsulation matriX (PLEX) coating allowing controlled release of SAAP-145 and SAAP-276 applied on titanium implants is effective against (MDR) \textit{S. aureus} in a mouse model of biomaterial-associated infection, and is therefore a promising alternative for coatings releasing conventional antibiotics.

**MATERIALS AND METHODS**

**BACTERIA**

Table 1 shows the clinical \textit{S. aureus} strains used in this study and their antibiotic resistance profiles. \textit{S. aureus} JAR060131 and the MDR \textit{S. aureus} LUH15101 strains were used in the murine biomaterial-associated infection studies (see below). Strain LUH15101 is resistant to doxycycline (MIC 32 µg/ml) and was used to assess the relative potency of the SAAPs to this antibiotic\textsuperscript{25}. Prior to each experiment, bacteria from frozen stocks were grown overnight at 37 °C on sheep blood agar plates (BioMerieux). Susceptibility to antibiotics was determined by disc diffusion and/or E-tests according to CLSI recommendations\textsuperscript{26}.

**PEPTIDE SYNTHESIS**

Peptides SAAP-145 (acetyl-LKRLYKRLAKLIKRLYRYLKKPVR-amide) and SAAP-276 (acetyl-LKRVWKAVFKLLLKRYWRQLKKPVR-amide) were prepared by 9H-fluorenlymethyloxycarbonyl (Fmoc)-chemistry as described previously\textsuperscript{27}. Purity of the final products was >95% (RP-HPLC, detection at 214 nm). Integrity of the peptides was determined with MALDI-TOF (Microflex,
SAAP-145 and SAAP-276 coatings against biomaterial infection

Bruker). For peptide release experiments, SAAP-145 and SAAP-276 were labelled with the fluorophore nitrobenzoxadiazole (NBD) by coupling NBD-glycine as an additional N-terminal residue.

**IMPLANT MANUFACTURE AND PLEX COATING PROCEDURE**

Solid medical grade (ISO 5832/11) titanium implants (10 × 4 × 1 mm, with a slit to allow cutting the implants after explantation) were machined at AO Research Institute (Davos, Switzerland) and anodized in the final processing step at KKS Ultraschall AG (Steinen, Switzerland). A previously described biodegradable Polymer-Lipid Encapsulation MatriX technology (PLEX) coating solution consisting of poly lactic-co-glycolic acid (PLGA; Purac), dipalmitoyl phosphatidyl choline (DPPC; Lipoid), distearoyl phosphatidyl choline (DSPC; Lipoid) and cholesterol (Dishman) with incorporation of SAAP-145, SAAP-276 or doxycycline (Hovione), or without active component, was prepared to coat the implants (Table 2). The coating without active component is further referred to as placebo-PLEX coating. The implants were coated by spraying the coating solution on the implant surface to create a thin coating. Residual solvent was evaporated by incubation at 45°C for 2 h,

<table>
<thead>
<tr>
<th>Strain</th>
<th>Isolated from</th>
<th>GEN</th>
<th>RIF</th>
<th>OXA</th>
<th>CPF</th>
<th>MOX</th>
<th>CLI</th>
<th>ERY</th>
<th>DOX</th>
<th>TET</th>
<th>CTR</th>
</tr>
</thead>
<tbody>
<tr>
<td>JAR060131</td>
<td>Infected hip prosthesis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LUH14616 (MRSA)</td>
<td>Blood (catheter)</td>
<td>&gt;16</td>
<td>&gt;4</td>
<td>&gt;4</td>
<td></td>
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<td>&gt;16</td>
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<tr>
<td>LUH15094 (MRSA)</td>
<td>Unknown</td>
<td>0.5</td>
<td>&gt;4</td>
<td>&gt;8</td>
<td>&gt;8</td>
<td>&gt;8</td>
<td>6</td>
<td>&gt;16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LUH15095</td>
<td>Nose swab</td>
<td>0.5</td>
<td></td>
<td>&gt;8</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&gt;32</td>
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<td>LUH15096</td>
<td>Blood</td>
<td>0.5</td>
<td></td>
<td>&gt;8</td>
<td>2</td>
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<td>&gt;32</td>
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<tr>
<td>LUH15101 (MRSA)</td>
<td>Exema</td>
<td></td>
<td>&gt;4</td>
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Table 1. Clinical *S. aureus* strains used in this study and their antibiotic resistance profiles. Susceptible to all (green boxes) or intermediate/resistant to at least one (red boxes) of the following antibiotics per class: GEN, gentamicin (aminoglycosides); RIF, rifampicin (ansamycins); OXA, oxacillin (β-lactams); CPF, ciprofloxacin, MOX, moxifloxacin (fluoroquinolones); CLI, clindamycin (lincosamides); ERY, erythromycin (macrolides); DOX, doxycycline, TET, tetracycin (tetracyclines); CTR, ceftriaxone (cephalosporins). Gray boxes are shown if the susceptibility to agents in this class is not assessed.

<table>
<thead>
<tr>
<th>PLEX coating composition in % (w/w)</th>
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</thead>
<tbody>
<tr>
<td>Placebo</td>
</tr>
<tr>
<td>PLGA</td>
</tr>
<tr>
<td>DPPC</td>
</tr>
<tr>
<td>DSPC</td>
</tr>
<tr>
<td>Cholesterol</td>
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<tr>
<td>Active component</td>
</tr>
</tbody>
</table>

Table 2. PLEX coating composition.
followed by an overnight incubation at room temperature under vacuum. This procedure was repeated 4 times, resulting in a 5 layer coating. The final weight of the coating was calculated from the weights of the implants before and after coating. As the doxycycline and peptide concentration in the coating formulations were 10% and 11% (w/w), respectively, we calculated that the average coating used for the *in vivo* experiment with *S. aureus* JAR060131 contained 589 ± 118 µg of SAAP-145 or 666 ± 90 µg of SAAP-276. For the *in vivo* experiment with doxycycline-resistant MDR *S. aureus* LUH15101 the average coating contained 348 ± 81 µg of doxycycline, 554 ± 125 µg of SAAP-145 or 493 ± 175 µg of SAAP-276 per implant (n = 18 implants per group).

**IN VITRO ANTIMICROBIAL ACTIVITY**

*S. aureus* were cultured to mid-logarithmic growth phase in tryptic soy broth (Oxoid) at 37 °C and 200 rpm, pelleted, washed once with phosphate-buffered saline (PBS; pH 7.4), resuspended and diluted in PBS to 5 × 10^6 colony forming units (CFU)/ml, based on the optical density of the suspension at 600 nm (OD 600). Twenty microliter of this bacterial suspension were added to 80 µl peptide [final concentrations of 0.8 – 204.8 µM] in PBS without or with a final concentration of 50% (v/v) pooled human plasma (Sanquin) in polypropylene flat bottom microtiter plates (Greiner). As non-treated control, bacteria were incubated without peptides in PBS or PBS with 50% plasma. After 2 hours of incubation at 37 °C and 200 rpm, the numbers of viable bacteria were determined by quantitative culture29. Antimicrobial activity is expressed as the 99.9% lethal concentration (LC99.9), i.e. the lowest peptide concentration that killed ≥99.9% of bacteria relative to the non-treated control after 2 h of incubation. For time-kill experiments, 1×10^6 CFU/ml *S. aureus* JAR060131 were incubated with 1.6 µM peptide in PBS in polypropylene tubes (Micronics) in a total volume of 750 µl. As untreated control, bacteria were incubated in PBS. After incubation at 37 °C and 200 rpm for 0.5 – 5 min, a 50 µl sample was taken from the incubation and added to 50 µl of a 0.05% sodium polyanethole sulfonate (SPS; Sigma) solution (v/v) to neutralize peptide activity30, after which the number of viable bacteria was determined.

**PREVENTION OF BIOFILM FORMATION**

*S. aureus* JAR060131 were cultured to mid-logarithmic growth phase in tryptic soy broth (TSB; Oxoid) at 37 °C and 200 rpm, pelleted, washed once with PBS and resuspended and diluted in the biofilm-culture medium BM231 to 2 × 10^8 CFU/ml, based on the OD_{600}. Polypropylene flat bottom microtiter plates were incubated overnight with 20% (v/v) pooled human
plasma (Sanquin) in PBS at 4 °C, and washed with sterile water\textsuperscript{32}. Fifty microliters of the bacterial suspension were added to 50 μl of peptide solution (final peptide concentrations 1.6 – 12.8 μM) in biofilm-culture medium in wells of the plasma-coated microtiter plate. As untreated control, bacteria were incubated in biofilm-culture medium without peptides. After 24 h of stationary incubation at 37 °C in a humidified atmosphere, planktonic bacteria were removed by 4 washes with PBS and biofilms were stained with 1% (w/v) crystal violet (Sigma-Aldrich) for 15 min. After 4 washes with water and solubilization of the remaining crystal violet with 150 μl of 96% ethanol, 100 μl were transferred to a polystyrene flat bottom microtiter plate (Greiner) and the optical density at 595 nm (OD\textsubscript{595}) was determined as a measure of biofilm quantity.

**RESISTANCE DEVELOPMENT**

The resistance development assay was adapted from Habets and Brockhurst\textsuperscript{33}. *S. aureus* JAR060131 was cultured overnight in RPMI 1640 medium supplemented with 20 mM Hepes and L-glutamine but not sodium bicarbonate (Sigma), further referred to as modified RPMI, at 37 °C and 200 rpm. Modified RMPI supports the growth of the bacteria without affecting the bactericidal activity of the antimicrobial peptides\textsuperscript{34}. In wells of flat bottom polystyrene (for peptides) or polystyrene (for antibiotics) microtiter plates, 2 μl of the overnight bacterial suspension were added to 100 μl serial dilutions of SAAP-145, SAAP-276, doxycycline (Sigma) or rifampicin (Sigma) in modified RPMI. All incubations were performed in quadruplicate. The plates were sealed with breathseals (Greiner) and incubated for 20 h at 37 °C and 150 rpm. After incubation, plates were visually inspected for growth and the minimal inhibitory concentration (MIC\textsubscript{RPMI}), i.e. the lowest concentration without visible growth, was determined. For each individual incubation series, 2 μl of the culture with 0.5-fold MIC\textsubscript{RPMI} were added to a new concentration series, ranging from 0.25- to 8-fold MIC\textsubscript{RPMI} and incubated as described above. This was repeated for 22 passages.

**INTERACTION WITH MEMBRANE IMICS**

Lipid films were prepared using 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-rac-glycerol (POPG; Avanti Polar Lipids) and 1,2-dipamitoyl-sn-glycero-3-phospho-rac-glycerol (DPPG; Avanti Polar Lipids) dissolved in chloroform/methanol (9:1 v/v), evaporated under a stream of nitrogen and incubated overnight under vacuum. After addition of PBS, with or without peptide (in a lipid-to peptide molar ratio of 25:1 for differential scanning calorimetry (DSC) experiments), formation of lipid vesicles was achieved by intermittent
vigorous vortexing at a temperature above the phase transition temperature of the phospholipids. DSC measurements were performed using a Microcal VP-DSC high-sensitivity differential scanning calorimeter (Microcal). Scans of 1 mg/ml DPPG lipid concentration were recorded at a constant rate of 30 °C/h and data were analyzed using Microcal’s Origin software. Calorimetric enthalpies were calculated by integration of the peak areas after baseline correction and normalization to the mass of the phospholipid. The phase transition temperature was defined as the temperature at the peak maximum. For leakage experiments, large unilamellar POPG vesicles were obtained by several cycles of extrusion of the hydrated liposomes through a polycarbonate filter (Millipore-Isopore) of 0.1 µm pore size following a protocol described previously. Leakage of the aqueous content of the 8-aminonaphthalene-1,3,6-trisulfonic acid/p-xylene-bis-pyridinium bromide (ANTS/DPX; Molecular Probes)-loaded POPG vesicles upon incubation with SAAP-145 or SAAP-276 (with final concentrations of 0.0625 – 8 µM, corresponding to lipid-to-peptide molar ratios from 800:1 to 6.25:1) was determined as described. Fluorescence emission was recorded as a function of time using the SPEX Fluoro Max-3 spectrofluorimeter combined with Datamax software. The percentage of leakage was calculated relative to the positive control (1% Triton-X).

MEMBRANE PERMEABILIZATION IN LIVE BACTERIA

A mid-logarithmic growth phase culture of S. aureus JAR060131 was diluted in PBS to 1×10⁶ CFU/ml. One hundred eighty microliters of this bacterial suspension were added to 20 μl of propidium iodide (PI; Sigma-Aldrich; final concentration of 1 μg/ml) in polypropylene tubes. After 10 min of incubation on ice in the dark, PI fluorescence of the bacterial cells was measured over time using the BD Accuri C6 706 flow cytometer. Approximately 20 s after the start of the measurements, 20 μl of peptide (final concentration of 1.6 μM) were added and samples were measured up to a period of 5 min.

RELEASE OF PEPTIDE FROM THE PLEX COATING IN VITRO

For release experiments, SAAP-145-PLEX- and SAAP-276-PLEX coatings containing 2% (w/v) NBD-labelled peptide, were applied to tri-calcium phosphate (TCP) granules as described previously. The average coating of the TCP granules contained 11.0 µg of SAAP-145 or 11.2 µg of SAAP-276 per mg of TCP. In order to quantify the amount of peptide released from the coating, 100 mg of PLEX-peptide-coated TCP granules were incubated in 1 ml of distilled water at 37 °C. The supernatants were collected and replaced with fresh water.
after the first hour of incubation and daily thereafter for a period of 30 days. At each time-point, the amount of released peptide in the water was quantified by measuring the level of NBD-fluorescence using a spectrophotometer (BMG LABTECH). Peptide release from the coated TCP granules was assumed to be similar to its release from coated titanium implants.

MOUSE SUBCUTANEOUS BIOMATERIAL-ASSOCIATED INFECTION MODEL

The mouse studies were approved by the Animal Ethical Committee of the Academic Medical Center at the University of Amsterdam, the Netherlands. Specific pathogen-free C57BL/6J OlaHsd immune competent female mice (Harlan) aged 7 to 9 weeks and weighing 17 to 20 g were used. Mice were housed individually for the short duration of the experiments and were provided with sterile food and water ad libitum. The subcutaneous biomaterial-associated infection experiments were performed in a mouse model as described previously\(^29\). Groups of 9 mice with 2 titanium implants each (\(n = 18\) implants per group) were used in the experiments. The efficacy of the peptides was first assessed in the model by delivering the peptides by injection along the implant. Two implants were placed subcutaneously at the back of each mouse. Immediately after implantation, 25 µl of a suspension containing \(10^6\) CFU of \(S.\) aureus JAR060131 were injected along the implants. One hour later, 50 µl of 4.4 mM SAAP-145 or SAAP-276 in PBS or 50 µl of PBS (negative control) were injected with a repetitive injector along the titanium implants. The dose of the peptides was derived from earlier studies with the related peptide OP-145\(^23\). In a second setup of the model the peptides were delivered by controlled release from PLEX coatings, in two experiments. In the first experiment, mice carrying titanium implants with SAAP-145- or SAAP-276-PLEX coatings, or with a placebo-PLEX coating or no coating as controls, were challenged with \(S.\) aureus JAR060131. In a second experiment, mice carrying titanium implants with SAAP-145-, SAAP-276- or doxycycline-PLEX coatings, or with no coating as control, were challenged with doxycycline-resistant MDR \(S.\) aureus LUH15101. One day after implantation, standardized biopsies (Ø 12 mm) containing the implant and surrounding tissue were obtained as described previously\(^23\). In short, the implants were separated from the tissue and both the implant and tissue were used for quantitative culture of bacteria. The implants were vortexed briefly in 0.5 ml of PBS to remove non-adherent bacteria, and then sonicated in fresh PBS for 5 min in a water bath sonicator (Elma Transsonic T460, 35 kHz; Elma) to dislodge all bacteria. The tissue samples were homogenized in 0.5 ml of PBS using 5 zirconia beads (Ø 2mm, BioSpec Products) and the MagnaLyser System (Roche),
with 3 cycles of 30 s at 7000 rpm, with 30 s cooling on ice between cycles. The sonicates and homogenates were 10-fold serially diluted and 50 µl was plated on agar. In addition, the sonicated implants and 50 µl of each homogenate were cultured in 5 ml of TSB containing 0.5% (v/v) Tween 80 for 48 h at 37 °C. The numbers of cultured bacteria were expressed as log CFU per sample. In case the highest dilution (i.e. 10⁵-fold) yielded non-countable numbers of colonies, the log number of CFU was displayed as >6. For statistical analysis a log CFU value of 6.5 was given to these samples. The lower limit of detection was 10 CFU. This value was assigned to samples positive in broth and negative in blood agar plate culture. For statistical analysis and to visualize the data on a logarithmic scale, a value of 1 CFU per sample was assigned when no growth had occurred in either culture system.

STATISTICAL ANALYSIS
All statistical analyses were performed with Graphpad Prism. Two-sample comparisons were made using a Kruskal-Wallis test followed by a Mann Witney rank sum test. The significance of differences between the frequencies of categorical variables was determined using Fisher’s exact test, and between percentages with ANOVA. For release kinetics, curves were fitted using linear regression analysis. For all tests, p-values of ≤0.05 were considered significant.

RESULTS
SAAP-145 AND SAAP-276 KILL MDR S. AUREUS AND PREVENT BIOFILM FORMATION
SAAP-145 and SAAP-276 were highly effective against a panel of clinical strains of S. aureus, including MDR strains, killing ≥99.9% of the bacteria at 0.8 – 1.6 µM concentrations in PBS, and at 3.2 – 25.6 µM in PBS with 50% plasma (Table 3).

Anti-biofilm effects were assessed in biofilm-culture medium, specifically designed to investigate anti-biofilm effects independent of bacterial killing. In this medium bacteria were not killed up to the highest tested concentration (12.8 µM), but a clear reduction of biofilm formation by S. aureus JAR060131 was observed at ≥1.6 µM of SAAP-145 and ≥3.2 µM of SAAP-276 as compared to incubation without peptides (Figure 1).

SAAP-145 AND SAAP-276 DO NOT INDUCE RESISTANCE IN S. AUREUS
As resistance development is a major concern, we tested the potential of S. aureus
JAR060131 to develop resistance to our new peptides and – for comparison – to rifampicin and doxycycline, two antibiotics often used in the prevention and treatment of BAI. The MIC_{RPMI} of all 4 independent replicates of *S. aureus* for SAAP-145 and SAAP-276 was identical at passage 1 and passage 22, i.e. 1.875 µM with a maximal 4- and 2-fold increase, respectively, in certain passages during the experiment (Figure 2). Similar results were found for doxycycline: the MIC_{RPMI} of all replicates of *S. aureus* for doxycycline remained 0.125 µg/ml with an 8-fold increased MIC_{RPMI} for one isolate at certain passages during the experiment. In contrast, the MIC_{RPMI} of all *S. aureus* replicate cultures for rifampicin increased from 0.125 µg/ml to ≥512 µg/ml within 6 passages and remained at this level for the following passages. These results demonstrate that *S. aureus* JAR060131 can quickly develop resistance to rifampicin, but not to SAAP-145, SAAP-276 and doxycycline.

<table>
<thead>
<tr>
<th>Strain</th>
<th>PBS</th>
<th>Plasma</th>
<th>PBS</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SAAP-145</td>
<td>SAAP-276</td>
<td>SAAP-145</td>
<td>SAAP-276</td>
</tr>
<tr>
<td>JAR060131</td>
<td>1.6</td>
<td>0.8</td>
<td>12.8</td>
<td>(12.8-25.6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6.4</td>
<td>(6.4-12.8)</td>
</tr>
<tr>
<td>LUH14616 (MRSA)</td>
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<td>0.8</td>
<td>6.4</td>
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<td>(12.8-25.6)</td>
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<tr>
<td>LUH15101 (MRSA)</td>
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<td>1.6 (0.8-1.6)</td>
<td>6.4 (3.2-25.6)</td>
<td>3.2 (3.2-12.8)</td>
</tr>
</tbody>
</table>

Table 3. Antimicrobial activity of SAAP-145 and SAAP-276 in PBS or in PBS with 50% human plasma against different (MDR) *S. aureus* clinical strains. Results are expressed as the lethal concentration (LC) 99.9, i.e. the lowest peptide concentration that killed ≥99.9% of the bacteria within 2 h. LC99.9 values are medians (and ranges) of at least three independent experiments. If no range is indicated, the LC99.9 was identical in all experiments.

![Figure 1. Prevention of *S. aureus* JAR060131 biofilm formation by SAAP-145 (black) and SAAP-276 (grey). Results are expressed as percentage of biofilm quantity, as measured using crystal violet staining, relative to the biofilm quantity in untreated control incubations (OD_{595nm} of 0.95 ±0.33), which was set at 100%. Values are means and standard errors of the mean of three independent experiments, each performed in 6-fold. Biofilm quantity was significantly (p ≤ 0.0001) reduced by ≥1.6 µM and ≥3.2 µM of SAAP-145 and SAAP-276, respectively.](image)
SAAP-145 AND SAAP-276 PERMEABILIZE THE BACTERIAL CYTOPLASMIC MEMBRANE

Binding of LL-37 to the bacterial cytoplasmic membrane leads to permeabilization and ultimately killing of the bacterium\textsuperscript{36,37}. To assess whether SAAP-145 and SAAP-276 interact with the membrane in a similar fashion, we used differential scanning calorimetry (DSC) on dipalmitoyl-phosphatidylglycerol (DPPG) liposomes, which mimic the cytoplasmic membrane of bacteria\textsuperscript{38}. The thermotropic phase behavior of DPPG is altered when a membrane-active peptide interacts with the phospholipid bilayer\textsuperscript{39}. Indeed, exposure to SAAP-145 and SAAP-276 resulted in loss of the pre-transition of DPPG, which is very sensitive to the incorporation of foreign molecules into the lipid matrix (Figure 3a). SAAP-145 induced similar thermotropic behavior as was previously described for OP-145, i.e. upon heating a minor increase of the main transition temperature accompanied with an increase of the

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**Figure 2.** Development of resistance in *S. aureus* JAR060131 to SAAP-145 and SAAP-276 and the antibiotics rifampicin and doxycycline. Results are expressed as fold-change in minimal inhibitory concentration (MICRPMI) compared to the MICRPMI at the start of the experiment of 4 independent incubations (indicated by the different symbols) for 22 serial passages in modified RPMI. The MICRPMI at the start of the experiment was 1.875 µM for the peptides and 0.125 µg/ml for the antibiotics.
main transition enthalpy, and upon cooling two phase transitions (Supplementary Table 1), which suggests the formation of a quasi-interdigitated structure\(^{38}\). A different behavior was detected for SAAP-276, which caused the main transition temperature as well as enthalpy to be markedly decreased upon heating (Supplementary Table 1; Figure 3a). This observation is typical for bilayers, where incorporation of the peptide induces a strong perturbation of the hydrocarbon chain region. In summary, these findings suggest that SAAP-P145 resides rather in the membrane interface inducing chain-interdigitation in the gel phase and membrane thinning in the fluid phase, whereas SAAP-276 penetrates deeper into the bilayer core perturbing the hydrocarbon chain packing. This is in line with SAAP-276 having a larger hydrophobic region than SAAP-145, facilitating penetration of the former into the bilayer (Figure 3b).

Figure 3. SAAP-145 and SAAP-276 cause rapid membrane permeabilization and killing of _S. aureus_. a, Effect of SAAP-145 and SAAP-276 on the phase transition of DPPG liposomes upon cooling. Results of one representative experiment out of three experiments. b, Helical wheel projections indicating the hydrophilic and hydrophobic region along the helical axes. Charged amino acids are shown in purple and apolar amino acids in green-yellow, P and Q in red. Hydrophobicity expressed as transfer free energy of peptides from water to n-octanol (\(\Delta G_{\text{WOCT}}\) in kcal/mol) calculated from the whole residue hydrophobicity scale taking into account the contribution of the C-terminal amidation and N-terminal acetylation are shown in the wheel\(^{60}\). c, Leakage of ANTS/DPX from POPG liposomes in the presence of SAAP-145 (squares) and SAAP-276 (triangles). Results are expressed as percentage leakage of the fluorescent dye relative to the total amount of the fluorescent dye. Values are means and standard deviations of three independent experiments. d, Membrane permeabilization in _S. aureus_ JAR060131 after addition of 1.6 \(\mu\text{M}\) SAAP-145 (squares) and SAAP-276 (triangles) as measured by propidium iodide (PI) influx during 5 min. Results are means and standard deviations of three independent experiments. e, Killing of _S. aureus_ JAR060131 after 0.5 – 5 min exposure to 1.6 \(\mu\text{M}\) of SAAP-145 (squares) or SAAP-276 (triangles), or no peptide (circles) as negative control. Results are expressed as numbers of viable bacteria in log CFU/ml, and are means and standard deviations of three independent experiments. The dashed line indicates the level of the 1000-fold (i.e. 99.9%) reduction in numbers of CFU of the LC99.9.
It is generally accepted that electrostatic interactions play an important role in the interaction of antimicrobial peptides and components of the bacterial cell membrane. Therefore, we tested the degree of peptide-induced permeabilization of bacterial membrane mimics using large unilamellar vesicles composed of the major lipid components of the staphylococcal membrane, palmitoyl-oleoyl-phosphatidylglycerol (POPG), loaded with the fluorescent marker ANTS/DPX. SAAP-145 and SAAP-276 caused similar leakage profiles of ANTS/DPX as a measure of membrane permeabilization from POPG vesicles, with complete permeabilization of the vesicles at 2 µM, with SAAP-276 being slightly more effective than SAAP-145 (Figure 3c).

We verified the proposed permeabilization of the membrane by the SAAPs in live *S. aureus* bacteria using flow cytometric analysis of propidium iodine (PI) influx, an indication of membrane permeabilization. Both SAAP-145 and SAAP-276 permeabilized the membranes in a time-dependent manner (Figure 3d). Within 60 s after exposure to 1.6 µM of SAAP-276 all *S. aureus* cells were permeabilized. SAAP-145 permeabilized the bacteria at a slightly slower rate: 1.6 µM induced permeabilization in approximately 60% of cells after 60 s. This fast permeabilization of the membrane was associated with rapid killing of *S. aureus* after exposure to the peptides: 1.6 µM of SAAP-145 or SAAP-276 killed >99.9% of the bacteria within 30 or 120 s, respectively (Figure 3e). These data indicate that binding of SAAP-145 and SAAP-276 to the bacterial membrane leads to permeabilization of the membrane, which results in death of the bacteria within minutes.

**INJECTED SAAP-145 REDUCES IMPLANT BUT NOT PERI-IMPLANT TISSUE COLONIZATION BY *S. AUREUS* JAR060131 IN MURINE BAI MODEL**

We determined the ability of SAAP-145 and SAAP-276 to reduce colonization by *S. aureus* of titanium implants and the surrounding tissue in a mouse subcutaneous implant infection model.

Injection of SAAP-276 along the infected implants resulted in a 0.5-log, but not significant, lower median number of CFU on the implants, whereas in mice receiving SAAP-145 the median numbers of viable bacteria on the implants were significantly lowered, by approximately 98% (*p* = 0.0028; Figure 4). Colonization of the peri-implant tissue by the bacteria was not altered by the peptides.

**SAAP-145 AND SAAP-276 ARE RELEASED FROM PLEX COATINGS IN A CONTROLLED FASHION**

Next, the peptides were formulated in the previously described PLEX coating23,25,28 adapted
Figure 4. Effects of SAAP-145 and SAAP-276 on colonization of implant and peri-implant tissue by *S. aureus* JAR060131 in mice. Immediately following subcutaneous implantation of the titanium implant, $1 \times 10^6$ CFU *S. aureus* were injected along the implants. One hour thereafter, 50 µl of 4.4 mM SAAP-145 or SAAP-276, or PBS as control, were injected along the implants. After 24 h, the animals were sacrificed, biopsies containing the implants with the surrounding tissue were taken, the implants were removed and sonicates of the implants and homogenates of the peri-implant tissue were quantitatively cultured. Results are expressed as the frequency of culture-positive samples and as the number of viable bacteria retrieved from the implants (left) and peri-implant tissues (right). The horizontal lines represent the median values. **, significantly ($p \leq 0.01$) different from the PBS-injected control group as calculated by the Mann-Whitney rank sum test for the log CFU values. No significant differences were detected by the Fisher’s exact test for the frequencies of culture-positive samples.

Figure 5. Cumulative release of NBD-labelled peptides from PLEX-coated TCP granules. TCP granules with a PLEX coating containing fluorescently labelled SAAP-145 (black) or SAAP-276 (grey) were incubated in distilled water for 1 h. Thereafter, the water was collected and refreshed daily for subsequent 30 days and the amount of fluorescently-labelled peptide in the water samples was measured spectrophotometrically. Results are expressed as the percentage cumulative release during 30 days of SAAP-145 and SAAP-276, relative to the total amount of peptide incorporated in the coating. Values are means of two replicates. The linear fit of the percentage cumulative release from day 4 – 30 ($R^2$ of 0.92 for SAAP-145 and of 0.99 for SAAP-276) are shown as black lines.
to the specific peptides (Table 2). In vitro release experiments using TCP granules coated with the SAAP-145- and SAAP-276-containing PLEX coatings revealed that approximately 52% of SAAP-145 and 69% of SAAP-276 were released from the PLEX coatings during the first 3 days, followed by a constant rate ($R^2$ 0.92 and 0.99, respectively) of approximately 0.6% release daily during the subsequent 27 days (Figure 5). Together, approximately 73% and 86% of the loaded SAAP-145 and SAAP-276, respectively, were released from the PLEX coatings over 30 days.

**SAAP-145- AND SAAP-276-PLEX COATINGS REDUCE IMPLANT AND TISSUE COLONIZATION BY S. AUREUS JAR060131 IN MURINE BAI MODEL**

We assessed the *in vivo* efficacy of the SAAP-145-PLEX and SAAP-276-PLEX coatings on colonization of titanium implants and the peri-implant tissue by *S. aureus* JAR060131 in the murine subcutaneous implant infection model 1 day after implantation and inoculation. The number of culture positive implants were significantly lower for mice receiving implants coated with SAAP-145-PLEX ($p = 0.0045$) and SAAP-276-PLEX ($p = 0.0016$) than for mice receiving implants with the placebo-PLEX coating (Figure 6). Mice with the SAAP-145-PLEX- and SAAP-276-PLEX-coated implants also had significantly lower levels of bacterial colonization of the implants (SAAP-145: $p < 0.0001$, SAAP-276: $p = 0.0002$) and of the surrounding tissues (SAAP-145: $p = 0.0015$, SAAP-276: $p = 0.0061$) than mice receiving the placebo-PLEX-coated implants. Moreover, the bacterial colonization of the SAAP-145-PLEX-coated implants was significantly lower ($p = 0.0423$) than of the non-coated implants. No significant differences were observed in the bacterial counts from either the implants nor from the tissues of mice carrying placebo-PLEX-coated implants as compared to the respective counts from mice with non-coated implants.

**SAAP-145- AND SAAP-276-RELEASING PLEX COATINGS REDUCE MDR S. AUREUS LUH15101 COLONIZATION IN MURINE BAI MODEL**

As the PLEX coating improved antibacterial effects of the peptides against *S. aureus* JAR060131 in mice carrying titanium implants, we subsequently assessed the effects of these coatings on colonization of the implants and the peri-implant tissue by the MDR *S. aureus* LUH15101 an example of a ‘worst case’ pathogen. This strain is resistant to a wide range of antibiotics (Table 1), including doxycycline. Since in the previous experiment no effect was noted of the placebo-PLEX coating, we eliminated this additional control from the present experiment. We included doxycycline-releasing PLEX-coated implants for
Figure 6. Effects of SAAP-145- and SAAP-276-PLEX coatings on colonization of titanium implants and peri-implant tissues by \textit{S. aureus} JAR060131 in mice. Immediately following subcutaneous implantation, $1 \times 10^6$ CFU of \textit{S. aureus} were injected along the non-coated, placebo-PLEX-, SAAP-145-PLEX- or SAAP-276-PLEX-coated implants. The PLEX-coatings contained $589 \pm 118$ µg of SAAP-145 and $666 \pm 90$ µg of SAAP-276. After 24 h, the animals were sacrificed, biopsies containing the implants with surrounding tissue were taken, the implants were removed and sonicates of the implants and homogenates of the peri-implant tissue quantitatively cultured. Results are expressed as the frequency of culture-positive samples, and as the numbers of viable bacteria retrieved from the implants (a) and the peri-implant tissues (b). The horizontal lines represent the median values. *, significantly (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$) different from the non-coated or placebo-PLEX-coated control groups as calculated by the Mann-Whitney rank sum test for the log CFU values and by the Fisher’s exact test for the frequencies of culture-positive samples.
Figure 7. Effects of SAAP-145-, SAAP-276- and doxycycline-PLEX coatings on colonization of titanium implant and peri-implant tissues by MDR *S. aureus* LUH15101 in mice. Immediately following subcutaneous implantation of the non-coated, doxycycline-PLEX- (doxy), SAAP-145-PLEX- or SAAP-276-PLEX-coated implants, $1 \times 10^6$ CFU of MDR *S. aureus* were injected along the implants. The PLEX-coatings contained 348 ± 81 µg of doxycycline, 554 ± 125 of SAAP-145 and 493 ± 175 µg of SAAP-276. After 24 h, the animals were sacrificed, biopsies containing the implants with surrounding tissue were taken, the implants were removed and sonicates of the implants and homogenates of the peri-implant tissue were quantitatively cultured. Results are expressed as the frequency of culture-positive samples, and as the numbers of viable bacteria retrieved from the implants (a) and the peri-implant tissues (b). The horizontal lines represent the median values. *, significantly ($p \leq 0.05$); **, $p \leq 0.01$; ***, $p \leq 0.001$) different from the non-coated or doxycycline-PLEX-coated control groups as calculated by the Mann-Whitney rank sum test for the log CFU values. No significant differences were detected by the Fisher’s exact test for the frequencies of culture-positive samples.
comparison. Mice carrying doxycycline-PLEX-, SAAP-145-PLEX- or SAAP-276-PLEX-coated implants had approximately 2- ($p = 0.0153$), 3.5- ($p = 0.0005$) and 2.7-log ($p = 0.0051$) lower median numbers of viable bacteria on the implants than animals carrying non-coated implants (Figure 7a). The bacterial counts on the SAAP-145-PLEX-coated implants were significantly lower ($p=0.0111$) than those on the doxycycline-coated implants.

More than $1 \times 10^6$ CFU were cultured from the tissues surrounding non-coated implants. The numbers of bacteria in the tissues surrounding the doxycycline- and SAAP-145-PLEX-coated implants were not significantly lower than those in tissues surrounding non-coated implants (Figure 7b), but from the peri-implant tissue of SAAP-276-PLEX-coated implants at least 1.3-log lower median numbers of CFU ($p = 0.0021$) were retrieved.

**DISCUSSION**

Strategies to prevent biomaterial-associated infections (BAI) include the development of antibiotic-releasing implant coatings\(^{11,40}\). However, such coatings may promote the emergence of antibiotic resistance\(^{41}\). This clearly shows the need for coatings releasing novel antimicrobial agents less likely to induce resistance, such as antimicrobial peptides (AMPs)\(^{13}\).

In the present study we selected two of the recently developed Synthetic Antimicrobial and Anti-biofilm Peptides, SAAP-145 and SAAP-276\(^{24}\), to prevent *S. aureus* BAI. These peptides were shown to be highly effective against (MDR) *S. aureus* clinical strains and to prevent biofilm formation by *S. aureus in vitro*.

SAAP-145 and SAAP-276 rapidly permeabilized the membrane of *S. aureus* bacteria, resulting in killing within minutes. This permeabilization, likely leading to disruption of the membrane, implies that the peptides are highly effective against dividing as well as non-dividing metabolically-inactive bacteria residing in a biofilm. It has long been thought that because of this mechanism of action not involving specific target molecules in the bacterial cell, resistance development to antimicrobial peptides is very unlikely. However, resistance to AMPs in bacteria can occur, for example as a result of proteolytic degradation, export by efflux pumps, surface modifications increasing positive charge\(^{42,43}\) or synthesis of biofilm matrix molecules impeding peptide access to the bacteria\(^{44}\). This commonly confers moderate levels of non-specific resistance\(^{44}\). More specifically, bacterial resistance against the human cathelicidin LL-37 has been reported, including bacteria-induced down regulation of LL-37 expression in host cells, up-regulation of efflux pumps as well as degradation of LL-37 by proteolytic enzymes produced by *S. aureus*\(^{45}\). In our study we therefore investigated
possible resistance development of *S. aureus* to our new peptides, using the antibiotics rifampicin and doxycycline as comparators. No resistance development against doxycycline was noted, but rifampicin readily induced resistance, as expected for this antibiotic. No resistance development was seen for SAAP-145 and SAAP-276 against *S. aureus*.

A number of AMPs has already reached clinical phase 2 or 3 testing, and application of AMPs in antimicrobial coatings for biomaterial is a subject of increasing interest. However, incorporation of AMPs in controlled release coatings for orthopedic and trauma devices has not yet been extensively developed. *In vitro* studies with micro-porous calcium phosphate coatings loaded with the broad spectrum AMP Tet213 on titanium implants had antimicrobial activity against *S. aureus* and *Pseudomonas aeruginosa*, and controlled release of this peptide, linked to collagen, from a multilayer coating on titanium inhibited early *S. aureus* biofilm formation. To the best of our knowledge, *in vivo* studies with this type of release coatings have not yet been reported.

We previously reported that a PLEX coating with a controlled release of OP-145, a peptide related to SAAP-145 and SAAP-276, reduced mouse implant colonization and protected rabbits from *S. aureus* osteomyelitis. As OP-145 has reduced antimicrobial activity in presence of biological fluids, we developed a set of novel synthetic peptides with good antimicrobial and anti-biofilm activities under physiological conditions. From this set, SAAP-145, SAAP-148 and SAAP-276 were the most promising candidates. We used SAAP-145 and SAAP-276 for incorporation in a PLEX coating applied to titanium implants in order to prevent BAI. These PLEX coatings were modelled according to the OP-145-PLEX coating featuring an initial burst release followed by a constant low level of OP-145 release and showed a similar release profile *in vitro*. The SAAP-145- and SAAP-276-PLEX-coated implants indeed reduced numbers of bacteria retrieved from the implants (both peptides) and the tissue (SAAP-276) at 1 day post infection, confirming efficacy of the peptides released in the initial burst. Efficacy at later time points remains to be established in such models as the rabbit intramedullary nail infection model, in which the PLEX coating with the related peptide OP-145 showed efficacy after 28 days. The SAAP-145-PLEX and SAAP-276-PLEX coatings were effective against *S. aureus* JAR060131 as well as the MDR *S. aureus* LUH15101 on the implants. Unexpectedly, the doxycycline-PLEX-coated implants reduced the numbers of the doxycycline-resistant MDR *S. aureus* LUH15101 strain on the implants. This can be explained by the high local concentrations of doxycycline released from the doxycycline-PLEX coating. Similar results were observed in earlier studies in rabbits.

Staphylococci persisting in peri-implant tissue are an important source for recurrence
of infection\textsuperscript{40,54,55}. These bacteria can be present in biofilms or even within host cells, like macrophages, surrounding the implants\textsuperscript{56,29}. Therefore, we also investigated whether the novel peptides were effective in tissue. SAAP-145 and SAAP-276 reduced the numbers of viable \textit{S. aureus} JAR060131 in peri-implant tissue when these peptides were released from PLEX coatings, but not when they were injected along the implants. This was also observed for the number of viable MDR \textit{S. aureus} LUH15101 in the tissue around the SAAP-276-PLEX coated implants. This clearly illustrates the benefit of the PLEX coating allowing controlled and prolonged release of the peptides. In contrast to the SAAP-276-PLEX-coated implants, the doxycycline-PLEX-coated implants did not reduce the numbers of the doxycycline-resistant MDR \textit{S. aureus} in the peri-implant tissue. This underlines the potency of the SAAP-276-PLEX coating in the combat of BAI caused by MDR staphylococci. The SAAP-145-PLEX coating was more potent than the doxycycline-PLEX coating in reducing the numbers of CFU on the implant, and showed similar results in clearing bacteria from the peri-implant tissue. Therefore, we consider the SAAP-145- and SAAP-276-PLEX coatings very promising for further development as an antimicrobial system preventing BAI even by MDR \textit{S. aureus}. The coatings might also provide additional protection from infection of implants used in revision surgery.

The novel SAAPs have potential as alternatives to conventional antibiotics also in other types of infection. Systemic application of AMPs by intravenous injection is considered difficult in view of their short half-life due to degradation and rapid removal from the circulation by the liver and kidneys and by potential cytotoxicity following high dose delivery. Consequently, local administration of the SAAPs is the most common route of administration, including delivery on mucosal surfaces, such as the airways and bladder, and in dermal ointments on different types of skin wounds, such as infected burns, surgical wound infections and diabetic foot ulcers. Indeed, topical application of SAAP-148 ointments proved to be safe and highly efficacious in eradicating MRSA and \textit{Acinetobacter baumannii} in a murine abraded skin infection model, as well as in acute and biofilm-associated \textit{ex vivo} human skin infection models, which mimic the infected skin of the patient as closely as possible\textsuperscript{24}. The stability, safety and efficacy of SAAPs may be improved through innovative formulation strategies and delivery systems. Several types of nano-carriers have been found successful in the delivery of AMPs including LL-37 and peptides derived thereof\textsuperscript{57–59}. Together, our SAAPs with mechanisms of action with low risk of resistance development offer promising alternatives to standard anti-infective therapies and preventive strategies for implants.
CONCLUSION

The present PLEX coatings releasing the novel LL-37-derived peptides SAAP-145 and SAAP-276 are promising alternatives for coatings releasing conventional antibiotics to prevent biomaterial-associated infections by both antibiotic-sensitive and MDR *S. aureus* without selecting for resistant bacteria.

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SUPPLEMENTARY DATA

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*Supplementary Table 1.* Differential scanning calorimetry (DSC). Tm, main phase transition temperature; ΔHm, main phase transition enthalpy. Data are the representative results of three independent experiments with standard deviations for Tm within 0.1 °C and for ΔH less than 10%. <sup>a</sup>) peptide-enriched domains; <sup>b</sup>) enthalpy of individual transitions could not be resolved.
REFERENCES


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