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ORIGINAL ARTICLE

A novel hydroxyapatite-binding antimicrobial peptide against oral biofilms

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Abstract

Objectives Novel synthetic antimicrobial peptides which consist of a new immunomodulatory peptide 1018 and two different modifications with hydroxyapatite-binding affinity were developed. We compared the effect(s) of these peptides against oral plaque biofilms and measured their effectiveness in killing biofilm microbes and in reducing biofilm volume.

Materials and methods The high affinity hydroxyapatite (HA)-binding peptide 1018 (SHABP), the mild affinity HA-binding peptide 1018 (MHABP), and peptide 1018 without additional amino acid sequence (peptide 1018) were synthesized. Oral multispecies biofilms were grown anaerobically for 3 days. The biofilms were exposed to three peptides at two different concentrations (0.65 and 3.25 μmol/L) for 24, 48, and 72 h. The biofilms were also treated for 3 or 9 min with the peptides (3.25 μmol/L). The percentage of killed biofilm bacteria and biofilm volume were determined by using LIVE/DEAD viability staining and confocal laser scanning microscopy.

Results SHABP was superior to MHABP and peptide 1018 in its killing efficacy of the pre-formed biofilms, especially at concentration of 3.25 μmol/L ($p < 0.05$). SHABP performed also better than MHABP and peptide 1018 in reducing the overall biofilm volume at both concentrations ($p < 0.05$). During the 3 days of long-term exposure, MHABP and peptide 1080 killed more bacteria in the top half of the biofilms, compared to bottom half. SHABP killed more bacteria in the bottom half (39%) of the biofilms than in the top half (29%) at day 1 ($p < 0.05$), whereas more bacteria were killed in the upper layers on days 2 and 3. SHABP killed a much higher percentage of plaque biofilm bacteria when used on 3-day-old biofilms for one or three times for 3 min than MHABP or peptide 1018 at high concentration ($p < 0.05$).

Conclusions The modified peptide 1018 with high HA-binding affinity had higher antimicrobial activity against biofilm microbes and reduced biofilm volume more than the other peptides tested.

Clinical relevance Modified peptide 1018 with high hydroxyapatite-binding affinity is a promising agent for use in oral antibiofilm strategies in the future.

Keywords Antimicrobial · Binding · Biofilm · Hydroxyapatite · Peptide 1018

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Introduction

Biofilms are three-dimensionally structured microbial communities encased in an extracellular matrix of microbial origin attached to surfaces. In the oral cavity, biofilms on tooth surfaces (hydroxyapatite) form the "bacterial plaque", which may lead, e.g., to caries [1], gingival infection, and periodontitis [2]. Recent molecular methods have revealed that almost all dental diseases are caused by dental biofilms that consist of multispecies microbial communities [3]. Consequently, eradication of the biofilms and microorganisms responsible for these infections is one of the primary goals of treatment. Microbial communities growing in biofilms are remarkably difficult to eradicate with antimicrobial agents [4, 5], and

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microorganisms in mature biofilms can be extremely resistant for reasons that have yet to be fully explained [6–10]. Additionally, the loading of antimicrobial agents onto materials has raised concerns about a possible link to increased bacterial resistance.

Cationic host defense peptides and their synthetic derivatives have been proposed to be alternative approaches in the treatment of infections [11]. There are more than 2100 host defense peptides (also termed antimicrobial peptides) in nature, and collectively, these have broad activities including partially independent immunomodulatory, direct antimicrobial, and anti-biofilm activities [12–14]. Since biofilms are difficult to eradicate once established, recent strategies have focused on peptides that are specifically designed to prevent biofilm formation or eliminate existing biofilms. Peptide IDR-1018 (VRLIVAVRIWRR-NH2), originally isolated as a novel immunomodulatory peptide, has broad antibacterial activity and inhibits the accumulation and accelerates the degradation of [(p)ppGpp], which is important for biofilm development [14]. Peptide 1018 has been shown to have moderate antibiofilm activity and inhibition of oral biofilms [15, 16]. One major obstacle to the success of the peptides as therapeutics in clinical trials is their inherent susceptibility to proteolytic degradation [17–19], especially in the oral environment, in which dosage retention is challenged by drug dilution with saliva flow [20] and drug inactivation by salivary enzymatic activity.

Enamel is the hardest substance in the human body and contains the highest percentage of minerals, 96%, with water and organic material composing the rest. The primary mineral in enamel is hydroxyapatite (HA) [21]. It has been established that the development and structural organization of a biofilm are influenced by the chemical nature of the substrate [17]. HA-binding heptapeptides, isolated by bio-panning phage display random heptapeptide library, have exhibited specific affinity to the tooth surface, making it a suitable molecule for tethering antimicrobial peptides onto tooth surface [22]. In the present study, novel synthetic antimicrobial peptides which consist of a new immunomodulatory peptide 1018 and two different modifications with HA-binding affinity were developed. We tested the effect(s) of these three peptides against oral plaque biofilms to assess if the ability to bind to hydroxyl apatite increases the antibiofilm properties of the peptide.

Materials and methods

Peptide synthesis

Peptide 1018 (peptide 478963: VRLIVAVRIWRR) and HA-binding peptides were synthesized (GL Biochem Ltd., Shanghai, China) using solid-phase 9-fluorenylmethoxy carbonyl (Fmoc) chemistry and purified to a purity of > 95% using reverse-phase high-performance liquid chromatography (HPLC) [15]. The two HA-binding modifications of peptide 1018 were synthesized by combining peptide 1018 sequence with a high affinity sequence CMLPHHGAC [22] and a medium affinity sequence CNPGFAQAC: peptide 478960: CAGHHPLMC-VRLIVAVRIWRR-NH2)(SHABP) and peptide 478961: CAQAFGPNC-VRLIVAVRIWRR-NH2) (MHABP). Peptide masses were confirmed by mass spectrometry. The peptides were resuspended in deionized water to make peptide stocks, from which peptide samples were taken and used in the experiments. All stocks remained sterile over time.

Minimal inhibitory concentration

The study was approved by the University of British Columbia Clinical Research Ethics Board (certificate H12-02430). All experiments were performed in accordance with relevant guidelines and regulations. Written informed consent was obtained from the participant for saliva and plaque bacteria collection in this study. Plaque samples were collected from one volunteer and grown overnight under anaerobic atmosphere (AnaeroGen; OXOID, Hampshire, UK) in brain heart infusion (BHI) broth [15, 16]. Bacteria were grown under anaerobic conditions at 37 °C for 24 h. The OD values of the plaque samples collected at the beginning of bacterial culture for minimal inhibitory concentration assays were 0.10 for BHI medium. Overnight cultures were diluted 1:100 in their growth medium and transferred to 96-well plates containing BHI broth and increasing concentrations (0, 1, 5, 10, 20, 40, and 80 μg/mL) of peptides in a biological safety cabinet (VWR General Purpose Digital Laboratory Incubators; VWR Inc., Radnor, Pennsylvania, USA) at room temperature. After 24 h of peptide treatment under anaerobic condition at 37 °C, bacterial growth was measured at an absorbance of 630 nm.

Biofilm model

Biofilms were grown anaerobically on sterile HA disks (0.38-in. diameter by 0.06-in. thickness; Clarkson Chromatography Products, Williamsport, PA, USA) as previously described [15, 16]. Saliva-coated HA disks were prepared by incubating each HA disk in the wells with saliva collected from the volunteer. Subgingival plaque was collected as well [15, 16]. The disks were incubated in BHI broth under anaerobic conditions at 37 °C for 3 days [15, 16] before being exposed to the peptides.

Long-term antibiofilm effect of peptides on preformed biofilms

Nine biofilm-covered disks (3-day-old biofilm) per group were exposed to the three peptides at two different concentrations (0.65 or 3.25 μmol/L) in BHI. Three disks for each

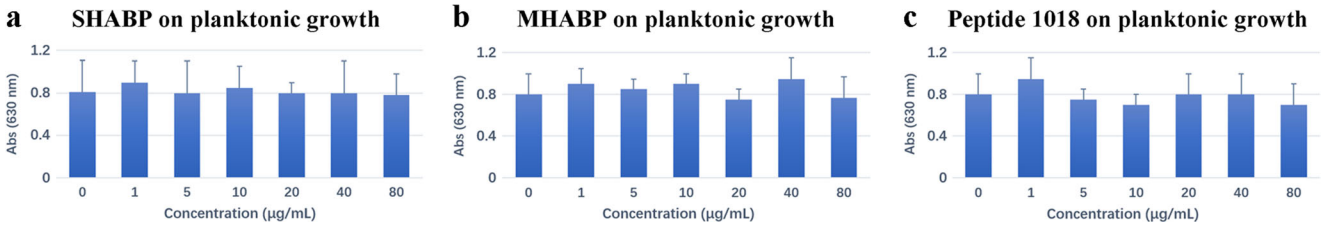

Fig. 1 Effect of increasing peptide concentrations on the planktonic growth of plaque bacteria in BHI broth after 24 h

peptide concentration were incubated with the peptide for 24, 48, and 72 h in BHI under anaerobic conditions at 37 °C [15, 16]. Control specimens with no peptide (only BHI + sterile water) were included for the same time periods.

Short-term antibiofilm effect of peptides on preformed biofilms

Short-term exposure to the peptides was performed as previously described [16]. Briefly, twelve 3-day-old plaque biofilm disks were rinsed in phosphate-buffered saline (PBS) at pH 7.0 for 1 min. Disks were then immersed in 1 mL of 3.25 μmol/L (= 5 μg/mL) of peptides for 3 min for either one or three subsequent treatments, with a 1-min wash in PBS between each exposure to the peptides. Six disks treated with sterile water were used as the negative control group.

Confocal laser scanning microscopy examination of biofilms

Following the exposure to the above solutions, all specimens were rinsed gently in 0.85% physiological saline and then stained with a 1:1 mixture of SYTO 9 and propidium iodide (BacLight LIVE/DEAD Bacterial Viability kit, Molecular Probes, Eugene, OR, USA) following the manufacturer's instructions. Images of the stained samples were taken by a CSLM (FV10i-LIV, Olympus, Canada) at 480/500 nm for SYTO 9 and 490/635 nm for propidium iodide, respectively. Five random areas were chosen from each specimen and scanned at a resolution of 512 × 512 pixels with 5-μm step size from the top to the bottom of the biofilm for each chosen area. Three samples were observed for each group and five randomly selected areas of each sample were scanned. Three-dimensional volume stacks were reconstructed with the Imaris

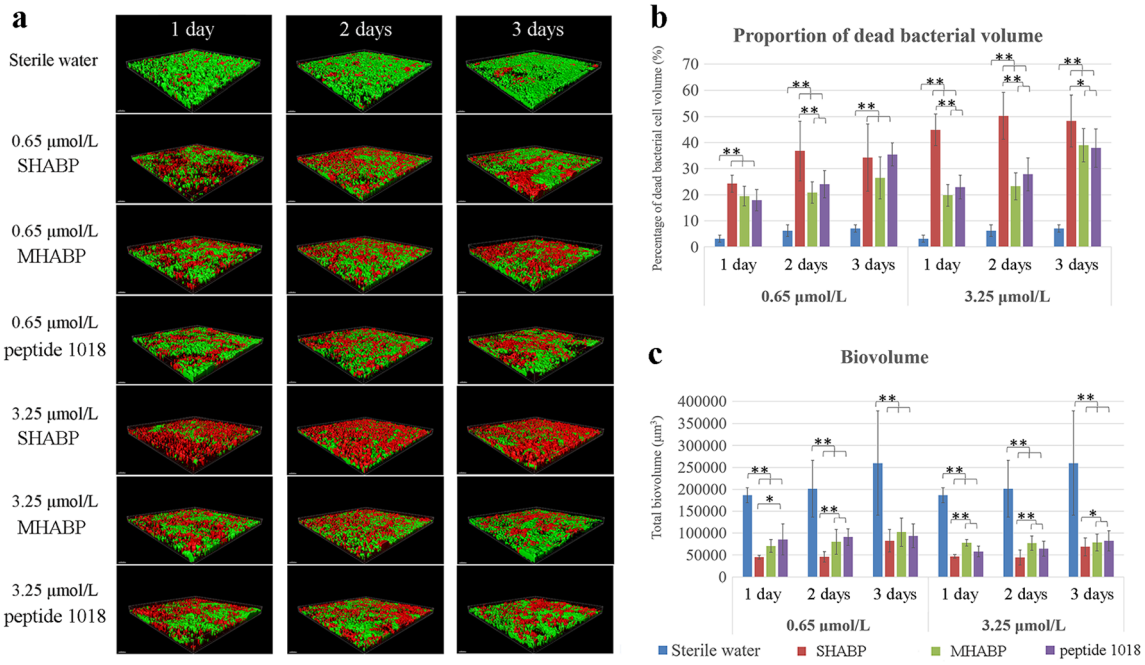

Fig. 2 Long-term antibiofilm effect of peptides on biofilms grown for 3 days before the exposure. **a** Confocal microscopy images of biofilm development on disks over 3 days (biofilm age 4, 5, and 6 days) in the presence of peptides or water. **b** The proportion of dead biofilm bacterial cell volume during the 3-day exposure. **c** Total biofilm volume 1, 2, and 3 days after exposure of the 3-day-old biofilm to the three peptides or water (biofilm age 4, 5, and 6 days)

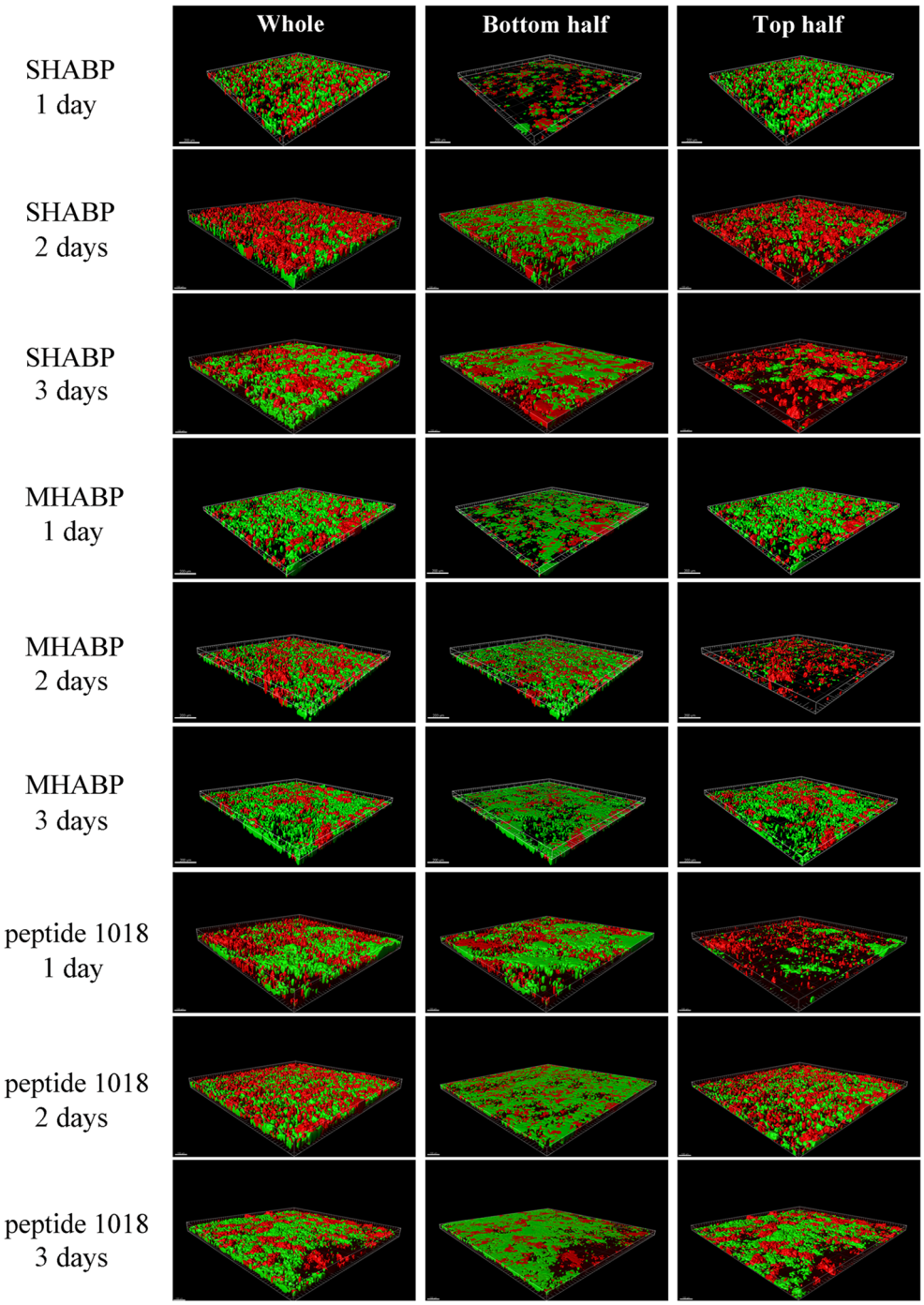

Fig. 3 Confocal microscopy images of bottom and top halves of biofilms after the 3-day-old biofilm was challenged by 3-day continuous exposure to 3.25 μmol/L of the three peptides. In the images below, the biofilms from left to right are 4, 5, and 6 days

7.2 software (Bitplane Inc., St Paul, MN, USA), and the total volume of the biofilm (red and green fluorescence) was measured. The proportion of dead bacteria was indicated by the proportion of red fluorescence of the total of green and red fluorescence [5, 9, 10, 15, 16]. As for the long-term high-concentration treatment test, the whole stack from the top to the bottom of the biofilm for each area was divided into two equal portions: the upper and lower half. The corresponding proportion of dead bacteria and biovolume of both halves were calculated.

Statistical analysis

Statistical analysis was performed with SPSS 16.0 software for Windows (SPSS, Chicago, IL, USA). Homogeneity of variance was determined using Levene's test. One-way ANOVA was applied, and post hoc multiple comparisons were used to isolate and compare the results (mean ± standard deviation) at the $P < 0.05$ significance level.

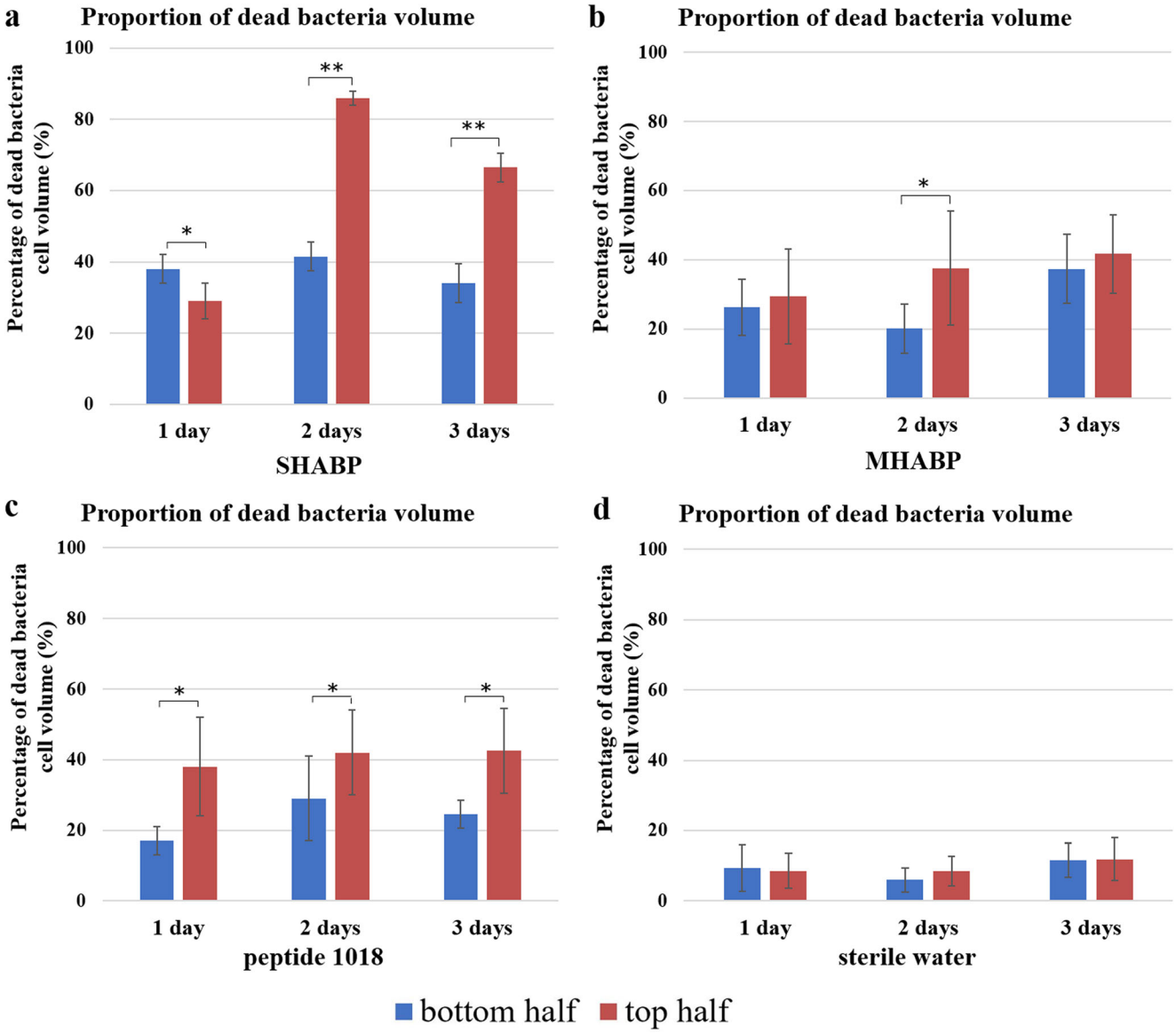

Fig. 4 The proportion of biofilm dead cell volume. **a** SHABP; **b** MHABP; **c** peptide 1018; **d** sterile water

Results

None of the three peptides, even at a concentration as high as 80 μg/mL, inhibited planktonic growth of dental plaque samples in BHI broth (Fig. 1). There was no significant difference to negative control where no peptides were added.

In the long-term, continuous exposure test, SHABP was superior to MHABP and peptide 1018 in killing biofilm bacteria, especially at high concentration in all experiments (Fig. 2a, b; $p < 0.05$). The killing by SHABP occurred already during the first 24 h of exposure, and little changes in dead bacterial population within the biofilm were observed after the first day (Fig. 2). With MHABP and peptide 1018, killing on day 3 was markedly greater than after 24- and 48-h exposure, yet significantly less than with SHABP (Fig. 2).

Exposure of the 3-day-old biofilm to the peptides reduced the volume of the biofilms considerably with both peptide concentrations, and after 1-day exposure to the peptides, the biofilm volume was in all cases far below 50% of the control group biofilm, which was exposed to water only (Fig. 2c). Slight but insignificant increase in biofilm volume was detected after 2 and 3 days of exposure to the peptides (Fig. 2c). At day 3 of peptide treatment with SHABP, the biofilm volume was only ca. 25% of the control biofilm (Fig. 2c). All three peptides caused reduction in biofilm volume, and the differences between the peptides were smaller than the differences in their ability to kill biofilm bacteria (Fig. 2b).

Data from the stacks of CLSM scans through the biofilms were divided into bottom and top halfs and analyzed separately to understand whether there were differences in the activity

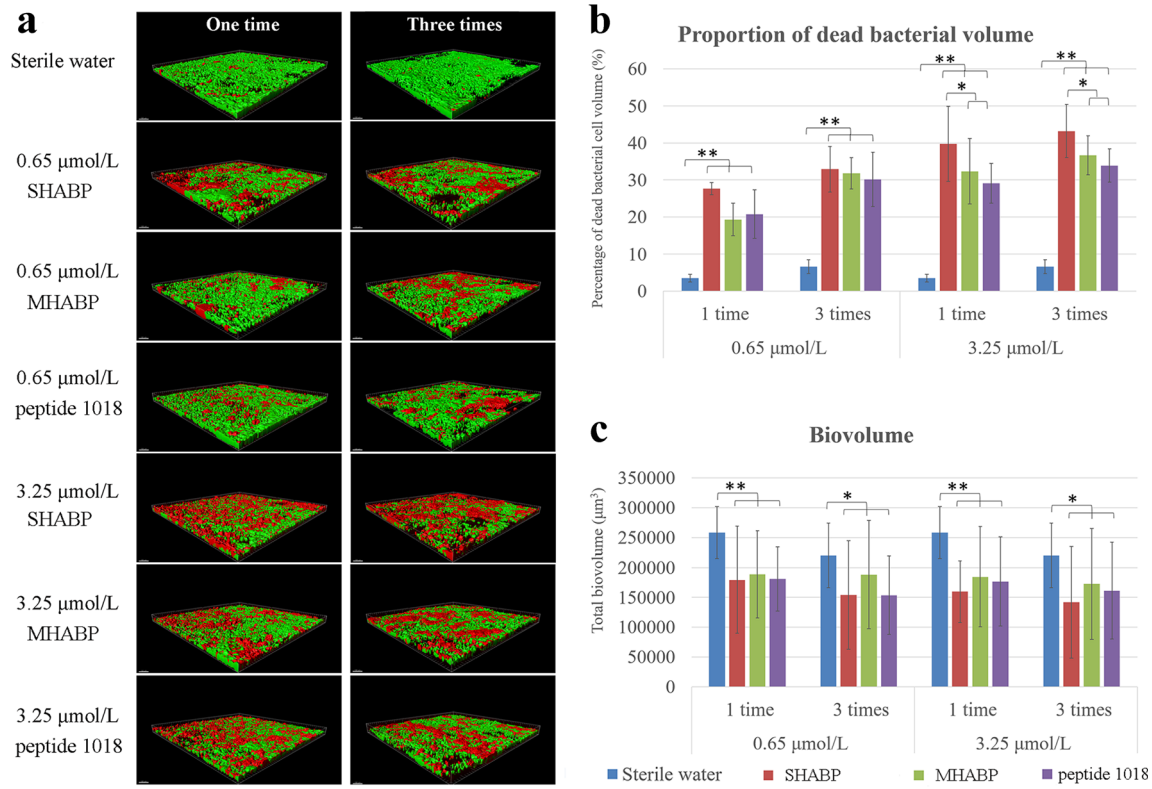

Fig. 5 Short-term antibiofilm effect of peptides on 3-day-old biofilms. **a** CLSM images of biofilms after one or three times exposures to the peptides. **b** The proportion of dead bacterial cells after different peptide treatments. **c** Total biovolume of biofilm formed after one time or three times treatment

of the peptides in different layers of the biofilm. All peptides on day 3 killed more bacteria in the top half than in the bottom half, but the difference was stronger with SHABP and peptide 1018 than with MHABP (Figs. 3 and 4; $p < 0.05$).

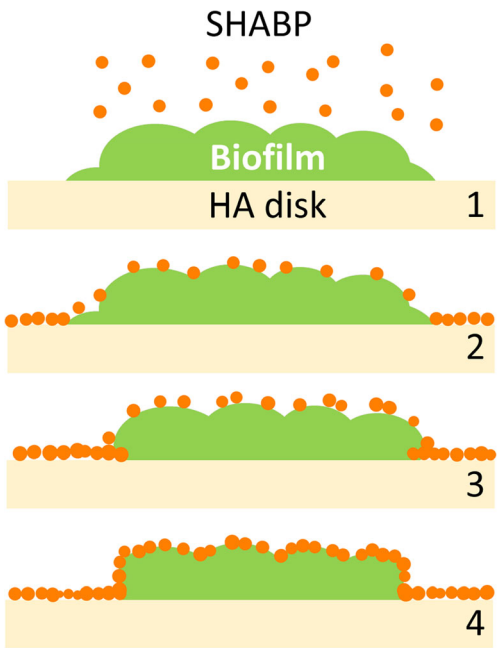

Fig. 6 Schematic diagram showing the different stages of SHABP binding to the biofilm and HA surfaces

The effects of short-term exposures of 3 and 9 min on the 3-day-old biolfim are shown in Fig. 5. The proportion of dead bacterial cells was dependent on the agent of exposure (Fig. 5a, b) and peptide concentration; SHABP killed a higher percentage of plaque biofilm bacteria than MHABP or peptide 1018 at the higher peptide concentration (Fig. 5b; $p < 0.05$). The difference in killing with the peptide (3.25 μmol/L) between 3- and 9-min exposure time was insignificant within each peptide. The biofilm volume was reduced equally much after 3- and 9-min exposure; the reduction was less than after long-term exposure of 1–3 days (Figs. 2c and 5c).

Discussion

In the present study, peptide 1018 with established antibiofilm activity was modified by adding two peptide sequences to the 1018 core, one with high affinity and one with medium affinity to HA (22), in order to examine if the modifications would alter the activity of peptide 1018 against biofilms grown on HA surfaces.

The three peptides did not inhibit the planktonic growth of plaque bacteria suspended in BHI broth as there was no difference in the growth of the bacteria with or without the three peptides up the high concentration of 80 μmol/L (Fig. 1). The concentration required to prevent biofilm formation by

peptide 1018 was far below the MIC reported for planktonic cells using microplate assays [23]. Another previous study [15] showed that at 5 μg/mL, peptide 1018 reduced the oral biofilm biovolume to less than 50% of the untreated controls, and even the lowest 1018 concentration of 1 μg/mL resulted in almost 50% reduction in biofilm volume. In the present study, two concentrations 0.65 μmol/L and 3.25 μmol/L were used, which correspond to 1 μg/mL and 5 μg/mL of peptide 1018, respectively.

SHABP triggered a high percentage of cell death on 3-day-old biofilm as measured after 1 day of exposure was started. SHABP killed twice as many bacteria as the other two peptides at 24 and 48 h. After 3 days of exposure, SHABP still had killed significantly more bacteria than the other peptides, but the difference was smaller than at days 1 and 2. SHABP also displayed strong anti-biofilm activity when used only for 3 and 9 min. At the higher concentration, SHABP killed ca. 40% of the biofilm bacteria, which was significantly more than the other two peptides (Fig. 5).

Biofilm formation is greatly affected by the environment. In the present study, SHABP showed strong performance in reducing the volume of the preformed biofilm and in preventing further biofilm growth during the 3 days of exposure. SHABP was considerably more potent than MHABP and peptide 1018, which did not differ much from each other.

During the exposure to the peptide(s), the reaction-diffusion-limited penetration of agents into a biofilm may result in only low levels of exposure to the antimicrobial agent in the deeper regions of the biofilm. A previous study [22] found that the high affinity HA-binding heptapeptide (with the amino acid sequence of CMLPHHGAC) had a high affinity to HA and a more significant control over crystalline formation and morphology of the peptide. The peptide also possesses some degree of disordered or randomly coiled structure and exhibits a greater degree of conformational instability [22]. These molecular features may have an impact on the HA-binding affinities. However, the reason or mechanisms for the better performance of SHABP is not clear. It may be speculated that SHABP binds quickly on HA surface and penetrates into the bottom of biofilm at the first day exposure (Fig. 6). The bottom half of biofilms had a slightly higher proportion of killed bacteria compared to the top half after 1 day of SHABP exposure. However, after the first day, more killing occurred in the top half of the biofilm. The mechanisms by which high-affinity binding may affect killing in various parts of the bioflm is presently not understood.

One limitation of this study is that the plaque came from one donor. However, our previous studies have shown that the source and possible differences in the species composition of these multispecies biofilms had little impact on the susceptibility of the biofilm bacteria to antimicrobial agents [9, 10, 16].

Conclusion

A new molecular bioconjugate consisting of a novel immunomodulatory peptide 1018 and its HA-binding modification was studied. The modified peptide was found to improve antimicrobial activity and reduce biofilm formation on HA surfaces. The results indicate that the antibiofilm peptide 1018 with the contact-active modification may be a promising agent for use in oral antibiofilm strategies in the future.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Ethics permission was obtained from the University of British Columbia Office of Research Services, Clinical Research Ethics Board (certificate number H15-02793).

Informed consent Informed consent was obtained from the volunteer providing oral plaque in this study.

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