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Convenient Preparation of Bactericidal Hydrogels by Covalent Attachment of Stabilized Antimicrobial Peptides Using Thiol−ene Click Chemistry

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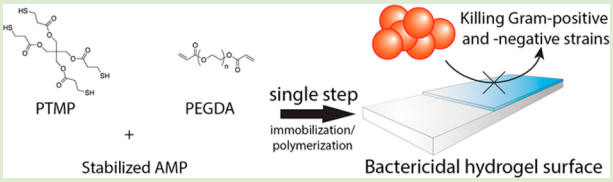
Supporting Information

ABSTRACT: This report describes the design and synthesis of a bactericidal poly(ethylene glycol)-based (PEG) hydrogel coating with covalently attached antimicrobial peptides (AMP) stabilized against proteolytic degradation. As such, mimics of the highly active AMP HHC10 (H-KRWWKWIRW-NH_2) were designed for optimal stability in human serum while retaining strong antimicrobial activity against *Staphylococcus aureus* and *Staphylococcus epidermidis*, the major causative agents of biomaterial associated infection. In order to investigate the selectivity of the AMPs, their hemolytic activity was determined. A N-terminal cysteine facilitated thiol−ene chemistry for a fast, single-step immobilization/photopolymerization strategy. The antimicrobial activity of the resulting thin layer hydrogel coating on a PET surface was established using the Japanese Industrial Standard (JIS) Z2801 assay, showing complete killing (>99.9%) of inocula of *S. aureus* ATCC 49230, *S. epidermidis* ATCC 35984, and *E. coli* ATCC 8739.



Colonization of biomedical implants by biofilm-forming bacteria often leads to biomaterial-associated infections. Both permanent, such as hip, knee, and dental implants, and temporary devices like urinary tract catheters and contact lenses are affected by a wide range of pathogens, despite the use of sterile devices and environments. The high costs of hospitalization, patient discomfort, and even mortality due to infections of especially the former biomaterials increase the need for antibacterially coated devices.[1,2]

Several device designs that meet this need involve the release of conventional antibiotics such as ciprofloxacin and gentamicin.[3,4] However, the rapid spreading of drug-resistant bacterial strains will make these devices less suitable for long-term usage.[5] Alternative antimicrobials, such as quaternary ammonium species, have interesting activity, yet these compounds are highly toxic.[6−8] Recently, silver nanoparticles have received much attention, but these are considered too toxic for clinical nontopical applications.[9−11] A combination of selectivity and broad spectrum antimicrobial activity to prevent the colonization of biomaterials can be achieved by the covalent attachment of antimicrobial peptides (AMPs) to a surface.[1]

These small, cationic peptides have gained increasing attention over the past two decades because of their ability to kill bacteria very rapidly and with high selectivity (e.g., low toxicity for mammalian cells).[12,13] As part of the host defense system they form the first line of defense against many pathogens. Several modes of action have been described, all starting with an interaction between the positively charged peptide and the negatively charged phospholipid part of the bacterial membrane. Subsequently, disruption of the membrane (e.g., by membrane depolarization, pore formation, etc.) can ultimately occur. Alternatively, peptides can be internalized and attack negatively charged targets such as RNA, leading to bacterial death. There are only very limited examples of inducing resistance.[14] However, one of the main drawbacks of this interesting class of potential antimicrobials is their poor stability in human serum. Approaches to decrease proteolytic degradation include the incorporation of D-amino acids,[15,16] β-amino acids,[17] or other unnatural amino acids,[18] as well as cyclization.[19]

HHC10 (H-KRWWKWIRW-NH_2), an antimicrobial peptide with high activity against multidrug resistant pathogens, was developed by the Hancock group and successfully tested both in vitro and in vivo.[20,21] Recent studies showed the potency of similar peptides as leachable antimicrobial agents[22−24] from titanium implants or as covalently attached peptides via polymer brushes using a multistep procedure.[25]

A convenient, single-step approach to immobilize AMPs onto cross-linked poly(ethylene glycol)diacrylate-based (PEGDA) hydrogels might be via thiol−ene photochemistry.[26]

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Table 1. LC99.9[a] Values for AMPs against *S. aureus*, *E. coli*, and *S. epidermidis* after 2 and 24 h Incubations

		S. aureus ATCC 49230 UAMS-1		*E. coli* ATCC 8739		*S. epidermidis* ATCC 35984	
entry	peptide	2 h	24 h	2 h	24 h	2 h	24 h
1	HHC10	4	4	4	1	4	2
2	Ac-HHC10	2	2	1	1	2	1
3	retro-HHC10	2−15	4	1	1	8	1
4	Ac-retro-HHC10	4	4	1	1	4	2
5	inverso-HHC10	4	2−8	2	1	4	2
6	D-allo-inverso-HHC10	4	4	4	4	4	2
7	Ac-inverso-HHC10	2	2	4	2	2	2
8	retro-inverso-HHC10	4	4	2	1	15	2
9	CysHHC10[b]	8	4	8	4	4	2
10	inverso-CysHHC10[b]	4−8	4−8	4−8	2−8	4−8	2−8
	controls[c]						
	magainin II	15	15	2	2	15	15
	BP2-M1	2−4	4	1	1	4	2
	ciprofloxacin	>60	2	2	0.1	4	0.2

[a]Defined as the lowest concentration of AMP (in μM) that killed 99.9% of an inoculum of 1×10^6 CFU/mL within 2 or 24 h. [b]Disulfide formation was observed by MALDI. [c]BP2M1[32] and magainin II[33] were selected as control AMPs for their known activities. Ciprofloxacin was chosen to compare AMP activities with a conventional antibiotic. All incubations were performed in duplicate.

The step-growth mechanism and mild reaction conditions, low temperature, fast polymerization, and tolerance for oxygen are indicative of the suitability of this polymerization reaction for many biomolecular applications. RGD-peptides attached to this hydrogel already showed promising integrin binding results.[26] Additionally, the hydrophilic character of poly(ethylene glycol) (PEG) hydrogels comprise an alternative approach for antifouling purposes.[27] The biocompatibility of such systems has been described before.[28]

In this study, we describe the application of thiol−ene chemistry for the development of one of the first hydrogel networks containing highly active antimicrobial peptides with increased proteolytic resistance.

First, we synthesized a series of modified HHC10 peptides and investigated their antimicrobial properties, selectivity, and stability. Subsequently, the most promising peptide was selected for use in a hydrogel network. As such, a cysteine residue on either side of the peptide is a valid anchoring point.[26,29] Thus, the thiol−ene click reaction was carried out between the PEGDA and cross-linker in the presence of cysteine-containing HHC10. Conveniently, the PEG-spacer may ensure that the antimicrobial peptides have sufficient freedom to orientate toward the bacterial membrane.[30] Antibacterial activity of the obtained coatings was studied by a surface antimicrobial activity assay (JIS Z2801).[31]

All synthesized mimics of antimicrobial peptide HHC10 were subjected to an antibacterial activity assay in vitro against *Escherichia coli* and biofilm-forming *Staphylococcus aureus* and *Staphylococcus epidermidis*, species commonly related to biomaterial-associated infections. The concentration of AMP killing 99.9% of the inocula (99.9% lethal concentration; LC99.9) after 2 and 24 h incubation closely resembled the minimum inhibitory concentrations (MIC) reported earlier,[20,32,33] although there are small discrepancies that can be attributed to differences in assay conditions and bacterial strains.

All HHC10 mimics showed microbicidal activities at low μM concentrations (1−8 μM) against *E. coli*, *S. aureus*, and *S. epidermidis* (Table 1). The small difference between the activity after 2 and 24 h incubation indicates a fast mode of action (e.g., disruption of the transmembrane electropotential by the positive charge of the AMP, as was discussed before[34]) against both Gram-positive and Gram-negative strains. Moreover, the inverso-HHC10, containing nonproteinogenic D-amino acids, showed similar activities as HHC10, thereby demonstrating the lack of a specific target sensitivity toward different stereo isomers.[34] Similarly, retro-inverso HHC10 showed comparable bactericidal activities, indicating the reversed orientation of the backbone amide bond was not affecting the activity.[35] Furthermore, based on the retained bactericidal activity, the reversed amino acid sequence of retro-HHC10 excludes the possibility that a sequence-specific mechanism is needed for antimicrobial activity of this peptide. In addition, the importance of β-branched D-isoleucine was investigated by replacing it with the more affordable D-allo-isoleucine. The antimicrobial activity of the corresponding AMP, D-allo-inverso-HHC10 was retained when compared to inverso-HHC10. Elongating the peptide sequence with a *N*-terminal cysteine to be used for immobilization purposes (CysHHC10 and inverso-CysHHC10) showed a slight decrease in activity, possibly partly due to disulfide formation, as was evidenced by MALDI (see Supporting Information, SI, Figure 1).

However, it must be noted that after immobilization via thiol−ene photopolymerization, this oxidation can no longer take place. Preservation of the activities of *N*-terminally acetylated peptides indicated that the positive charge of the amine was not necessary for antimicrobial activity and, thus, can be utilized for immobilization purposes.

The cytotoxicity of the AMPs was determined by adding HHC10 or its mimic peptides to sheep red blood cells followed by measuring the release of hemoglobin. In general, all AMPs tested at 2.5 to 10 times the LC99.9 concentration showed very low hemolysis of sheep blood erythrocytes (Figure 1). All tested peptides showed less than 2% lysis as compared to the control (1% Triton X-100). These results indicate that reversing the amino acid sequence as in the retro-HHC10 and/or side chain orientation for the (retro-)-inverso-HHC10 peptides did not affect the selectivity of the tested AMPs. Reduction of the net positive charge from +5 to +4 in the *N*-acetylated peptides also did not influence toxicity, thereby

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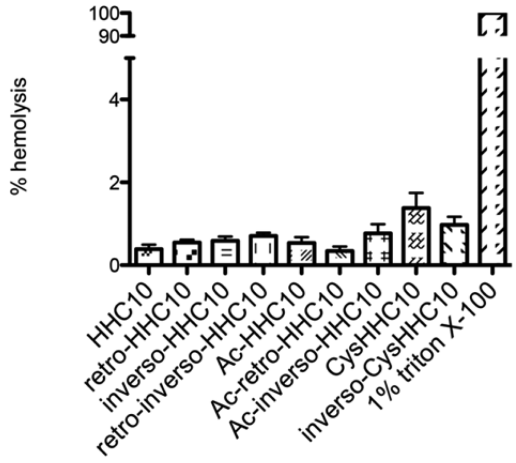


Figure 1. Hemolytic activity of a range of HHC10-derived AMPs tested at 15.6 μg/mL (2.5−10 × LC99.9) after 1 h.

making these HHC10 mimics potentially suitable candidates for immobilization.

In view of its high bactericidal and low hemolytic activity, next, the sensitivity to proteolytic degradation was determined of inverso-HHC10 by measuring the percentage of remaining intact peptide by HPLC after incubation in 25% (vol/vol) aqueous pooled human serum at 37 °C.[18] Incubation was carried out for 24 h to monitor the time-course of degradation and compare to the proteolytic stability of HHC10 (Figure 2). HHC10 was fully degraded within 4 h. In contrast, inverso-HHC10 was not significantly degraded after 4 h. Moreover, after 24 h, the intact peptide was largely present.

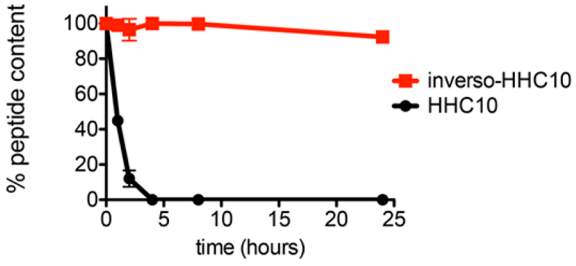


Figure 2. Stability of 150 μg/mL HHC10 and inverso-HHC10 in 25% pooled human serum over time.

In order to covalently attach the stabilized AMP to a hydrogel network, the sequence was elongated at the N-terminus with a cysteine residue to afford inverso-CysHHC10, which was added to a mixture of PEGDA/pentaerythritol tetrakis(3-mercaptopropionate) (PTMP) and photoinitiator in methanol and subsequently added to the surface of a poly(ethylene terephthalate) (PET) sheet. Next, photopolymerization was carried out for simultaneous hydrogel formation and peptide immobilization (Scheme 1). Three different amounts of inverso-CysHHC10 (leading to 0.2, 1, and 10 wt % hydrogels, respectively) were used to assess the optimal peptide concentration for antimicrobial activity in vitro using the JIS Z 2801 assay.[31] Prior to testing, hydrogel samples were washed for 24 h in water to remove any remaining unbound peptide. HPLC analysis showed no detectable amount of peptide after the first washing (see SI, Figure 2). Subsequent incubation with *S. aureus* demonstrated a 6-log reduction of bacteria of the 10 wt % AMP containing coating as compared to the blank hydrogel without AMP (Figure 3). Similar

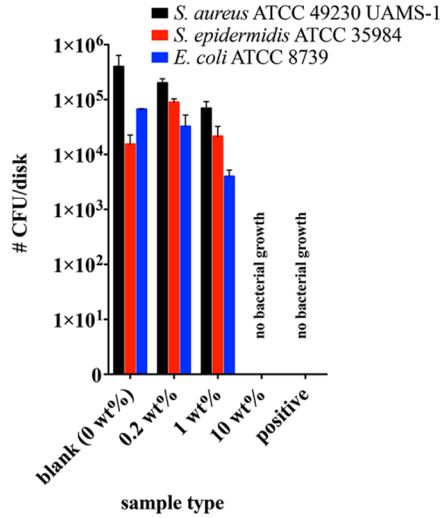
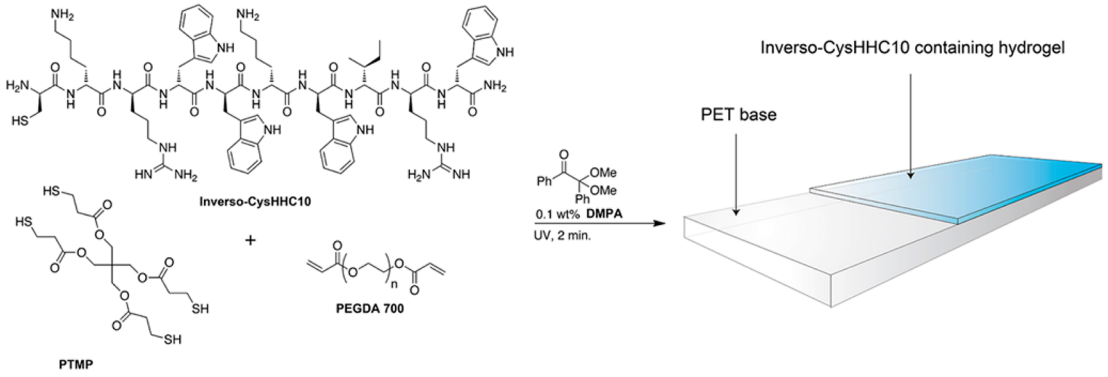


Figure 3. Antibacterial activity of inverso-CysHHC10 containing hydrogels.

bactericidal activity was found against *S. epidermidis* and Gram-negative *E. coli*. A solution of Ac-HHC10 AMP exceeding the LC99.9 concentration by >500-fold was added to a blank hydrogel as a positive control.

Scheme 1. One-Step Crosslinking and Immobilization of Inverso-CysHHC10[a]



[a]Reaction takes place between thiol and alkene.

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In conclusion, this study describes the synthesis and in vitro biological evaluation of a range of HHC10-derived antimicrobial peptides showing a wide tolerance in side chain and backbone orientations with respect to their antibacterial properties and selectivity. Inverso-HHC10 was shown to be stable in serum, whereas HHC10 itself was rapidly degraded. The bactericidal activity of the peptides against *S. aureus*, *S. epidermidis*, and *E. coli* demonstrated the potential for applications against these biofilm-forming bacteria often encountered in biomaterial-associated infection. Moreover, inverso-CysHHC10 was incorporated into a PEG-hydrogel using thiol−ene photoclick chemistry in a single-step procedure. The resulting AMP-hydrogels showed potent bactericidal activity against Gram-positive *S. aureus* and *S. epidermidis* and Gram-negative *E. coli* in vitro. It was thus also demonstrated that this class of antimicrobial peptides retain biological activity when immobilized in a hydrogel network.

Moreover, this research has shown the potency of incorporating stabilized antimicrobial peptides in a hydrogel with a single-step immobilization/polymerization strategy for the development of soft antimicrobial coatings.

■ ASSOCIATED CONTENT

Supporting Information

Further experimental details and HPLC and MALDI-TOF data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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