

Article



Construction and Characterizations of Antibacterial Surfaces Based on Self-Assembled Monolayer of Antimicrobial Peptides (Pac-525) Derivatives on Gold

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Abstract: Background: Infection that is related to implanted biomaterials is a serious issue in the clinic. Antimicrobial peptides (AMPs) have been considered as an ideal alternative to traditional antibiotic drugs, for the treatment of infections, while some problems, such as aggregation and protein hydrolysis, are still the dominant concerns that compromise their antimicrobial efficiency in vivo. Methods: In this study, antimicrobial peptides underwent self-assembly on gold substrates, forming good antibacterial surfaces, with stable antibacterial behavior. The antimicrobial ability of AMPs grafted on the surfaces, with or without glycine spaces or a primer layer, was evaluated. Results: Specifically, three Pac-525 derivatives, namely, Ac-CGn-KWRRWVRWI-NH₂ (*n* = 0, 2, or 6) were covalently grafted onto gold substrates via the self-assembling process for inhibiting the growth of Staphylococcus aureus (S. aureus) and Escherichia coli (E. coli). Furthermore, the alkanethiols HS(CH)₁₀SH were firstly self-assembled into monolayers, as a primer layer (SAM-SH) for the secondary self-assembly of Pac-525 derivatives, to effectively enhance the bactericidal performance of the grafted AMPs. The -(CH)₁₀-S-S-G₆Pac derivative was highly effective against S. aureus and E. coli, and reduced the viable amount of E. coli and S. aureus to 0.4% and 33.2%, respectively, after 24 h of contact. In addition, the immobilized AMPs showed good biocompatibility, promoting bone marrow stem cell proliferation. Conclusion: the self-assembled monolayers of the Pac-525 derivatives have great potential as a novel therapeutic method for the treatment of implanted biomaterial infections.

Keywords: antibacterial surface; self-assembled monolayers; antimicrobial peptides

1. Introduction

Bacterial infection is one of most serious issues that leads to the failure of implanted biomaterials (e.g., implants, artificial joints, contact fixators, and compression plates) in the clinic. Infections are frequently accompanied by the formation of bacterial bio-films on the surface of biomaterials, which protect the bacteria from antibiotics and host defense mechanisms, thereby leaving the patient more susceptible to infection [1]. Infections often lead to increased treatment costs, operation failures, and high mortality and morbidity [2]. Therefore, approaches that offer stable long-lasting antibacterial capacity, to prevent the formation of bacterial bio-films on the surface of implanted biomaterials, have a huge clinical impact [3,4].



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Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). Over the last few decades, many studies that focused on the inhibition of pathogenic bacterial infections have proposed several strategies to enhance the antibacterial performances of biomaterials, including external morphology modification, physical entrapment of antibacterial substances, and surface grafting of antibacterial substances via chemical treatments [5–7]. However, the current results are still not satisfactory because of the low antibacterial efficiency, low stability, short-lived antibacterial ability, or complicated manufacturing. Recently, antibacterial substances have been immobilized on gold or silicon substrates, in the form of self-assembled monolayers (SAMs), to impart antibacterial properties [8]. The self-assembly of antibacterial substances onto metal surfaces is a simple approach for achieving surface modification [9,10], where the antibacterial activity of the antimicrobial substance could not be affected by the coating process. Consequently, SAM has been widely proposed for various antibacterial applications [11,12].

The covalent immobilization method allows for stronger attachment of a molecule to the material surface, in comparison with non-covalent methods. Thus, covalent immobilization ensures that the conjugated molecules would not be easily released from the implanted biomaterials to the body, possessing long-lasting antibacterial activity [13]. Both non-metal compounds (e.g., antibiotics and selenium) and metals (e.g., zinc, silver, zirconium, and copper) have been applied as antibacterial coatings on biomaterial surfaces [14]. Silver has been highlighted as one of the most effective antibacterial elements, but its cytotoxicity has been reported as a dominant safety concern. In addition, the use of multiple antibiotics can reduce antimicrobial efficacy, by encouraging the emergence of multiple-drug-resistant pathogens [15]. Instead, antimicrobial peptides (AMPs) offer great advantages compared to traditional antibiotic drugs, such as minimal drug resistance, rapid sterilization, high efficiency, appropriate thermal stability, low molecular weight, low sensitivity to enzymatic hydrolysis, and no immunogenicity. Therefore, research on the application of AMPs as an alternative to conventional antibiotics have become popular in the recent years [16].

AMPs are capable of inhibiting a large spectrum of microflora, including both Grampositive and Gram-negative bacteria, drug-resistant bacteria, and even fungi [17,18]. There are currently thousands of AMPs in the AMP database with known activity against microflora [19], but fewer than 100 have been evaluated in humans [17]. Most AMPs adhere to the membrane of bacterial cells for permeation, leading to either a small rupture or large-scale destruction to achieve cell death [20]. The highly effective membrane destruction mechanisms that are exhibited by AMPs do not occur independently, but are related to several properties of the peptide, including the amino acid sequence, size, structure and conformation, hydrophilicity, and hydrophobicity [21,22]. In addition to the direct antimicrobial ability, AMPs exhibit further promising characteristics, such as anti-absorptive and mineralization effects, which may impact the complex microbial environment during AMP treatment [23,24]. Pac-525 (Ac-KWRRWVRWI-NH₂) is a type of tryptophan (Try)-rich AMP that exhibits broad-spectrum antimicrobial activity against fungi, and Gram-positive and Gram-negative bacteria [5,25]. It is reported that Pac-525 could bind to negatively charged phospholipids strongly and destabilize the microbial membrane. Besides, the Try peptides have a strong ability to insert into membranes and affect the lipid polymorphism. More than that, they possess low cytotoxicity and high biosafety [26]. Therefore, Pac-525 has been widely used for its antibacterial properties in biomedical materials. For example, Li et al. prepared a Pac-525-loaded surface coating on titanium, via physical adsorption against *Porphyromonas gingivalis* biofilm formation [5]. He et al. developed a Pac-525-loaded PLGA microsphere that was encapsulated in a gelatin/chitosan nanofibrous guided bone regeneration membrane and mineralized collagen bone scaffold, presenting long-lasting high antibacterial activity for up to one month [25,27]. However, dissociative AMPs, applied in vivo, can undergo aggregation and peptide proteolysis, thereby compromising the antimicrobial efficiency [23]. Site-specific immobilization of AMPs, with the preservation of the integrity of the peptide backbone, should be more beneficial than random adsorption [28]. The immobilization of AMPs on metal and polymeric surfaces, including

titanium, stainless steel, silicon, and polyethylene terephthalate (PET), has been investigated, to achieve enhanced antibacterial characteristics [4]. Nevertheless, few studies have addressed the antibacterial properties of the conjugated Pac-525. Moreover, it is a vital important issue to develop a facile and moderate method to immobilize AMPs and preserve their antibacterial activities.

This study aimed to utilize the self-assembly characteristic of various Pac-525 (Ac-KWRRWVRWI-NH₂) derivatives, to form stable coatings on gold substrates via a covalent approach. Different numbers of glycine (G_n , n = 2 or 6) were used as a spacer for preserving the flexibility and stereochemical structure of Pac-525 after self-assembly. Besides, the SAM of alkanethiol HS(CH)₁₀SH (SAM-SH) was applied as a primer layer for the secondary self-assembly of Pac-525, to enhance the bactericidal performance of the grafted Pac-525 derivatives. The antimicrobial ability and biocompatibility of Pac-525, immobilized on gold substrates with and without the G_n spacers or primer layer (self-assembled monolayer of HS(CH)₁₀SH, SAM-SH), were compared.

2. Materials and Methods

2.1. Materials

AMPs (Pac-525, Ac-KWRRWVRWI-NH₂) with a purity of over 98% were customsynthesized by Qiangyao Bio-technology Co., Ltd. (Shanghai, China). An additional cysteine residue was attached to the AMP at the C-terminus of the bioactive sequence via a different number of glycine spacers (Table 1). The cysteine residue is referred to in this paper as -SH instead of its standard single-letter code C, to emphasize its role as a thiol donor. The alkanethiol HS(CH)₁₀SH (MW = 206. 41) was purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). Gold-coated silicon wafers ($0.5 \times 0.5 \text{ cm}^2$) that were used as gold substrates for AMP grafting were prepared via the sequential deposition of titanium (10 nm) and gold (40 nm) film utilizing electron beam evaporator (ANELVA L-400EK, Canon Anelva Corporation, Kanagawa, Japan). All other reagents were of analytical grade and were purchased from Chemical Reagent Co., Ltd. (Beijing, China).

Table 1. The sequences of the AMPs used in this study.

AMPs	Peptide Sequence
-Pac	Ac-C-KWRRWVRWI-NH ₂
-G ₂ Pac	Ac-CGG-KWRRWVRWI-NH ₂
-G ₆ Pac	Ac-CGGGGGG-KWRRWVRWI-NH ₂

The rBMSC model cells were purchased from Cyagen Bioscience Co., Ltd. (Guangzhou, China). Dulbecco's modified Eagle's medium (DMEM), antibiotics, trypsin-EDTA, and fetal bovine serum (FBS) were obtained from GIBCO Invitrogen Corporation/Life Technologies Life Sciences (Carlsbad, CA, USA).

2.2. Preparation of Self-Assembled Monolayers of Antimicrobial Peptides on Gold Substrates

The antimicrobial peptides undergo self-assembly on gold substrates via the stable covalent bonds between the sulfhydryl groups (-SH) of the cysteine on the AMPs and Au atoms of gold substrates. The gold substrates were ultrasonically cleaned in 75% ethanol solution for 30 min and then deionized water for another 30 min. The cleaned gold substrates were immersed in 0.6 mmol/L AMPs solution, and incubated at 37 °C overnight in a biochemical incubator (Table 1). The AMPs-immobilized gold substrates were gently rinsed with absolute ethanol in one step, then dried at room temperature, and used immediately.

2.3. AMPs Immobilization via Self-Assembled Alkanethiol Monolayers on Gold Substrates

The cleaned Au substrates were firstly immersed in alkanethiol $HS(CH)_{10}SH$ solution (40 mmol/mL) and incubated at 37 °C overnight. After rinsing with ethanol gently,

the Au substrates were then immersed in AMPs solutions (0.6 mmol/L) at 37 °C overnight. After incubation, the prepared gold substrates were gently cleaned with ethanol and then dried at room temperature.

2.4. Samples

The SAMs samples were divided into 8 groups for all examinations, except the inhibition zone and inhibition ratio assay tests. Specifically, the groups included an untreated gold substrate (Au) and a gold substrate immobilized with SAM of HS(CH)₁₀SH (SAM-SH) as negative controls, and the substrates immobilized with -Pac, $-G_2Pac$, $-G_6Pac$, $-(CH)_{10}$ -S-S-G₂Pac, or $-(CH)_{10}$ -S-S-G₆Pac as experimental samples.

2.5. Characterization of AMP-Functionalized Gold Substrates

The hydrophilicity of the SAMs samples was evaluated based on water contact angle (WCA) (VCA Optima surface analysis system, AST products Inc., Billerica, MA, USA) [29], where the average value from six measurements was used. The nanostructure of the SAMs was analyzed using atomic force microscopy (AFM) with triangular silicon nitride cantilevers in tapping mode (FastScan, Bruker, Karlsruhe, Germany). The scanning line number and rate were fixed at 256 and 1 Hz, respectively. The morphology images were flattened and studied using the NanoScope Analysis 1.8 software package (Bruker, Karlsruhe, Germany). The surface chemical composition of the SAMs was evaluated using X-ray photo-electron spectroscopy (XPS, K-Alpha, Thermo Fisher Scientific, Waltham, MA, USA). The results of XPS were also calculated by quantification tests. The formula was as follows:

$$Grafting Density_{Au-S} = Area_{peak1} / Area_{sum} \times Atomic_{S/Au}$$
(1)

The two-step self-assembling established stable immobilization through disulfide bonds between SAM-SH and AMPs (-(CH)₁₀-S-S-GnPac-NH₂, n = 0, 2, 6). Moreover, the grafting density of S–S bond could be evaluated simply. The formula was as follows:

Grafting Density_{S-S} =
$$\frac{\left(\operatorname{Area}_{sum} - \operatorname{Area}_{peak2}\right)/3}{\operatorname{Area}_{peak2}} \times \operatorname{Grafting Density}_{Au-S}$$
 (2)

The explanation of Formulas (1) and (2) is given in the corresponding section of results.

2.6. Antibacterial Properties

2.6.1. Bacteria

S. aureus (ATCC 6538) and *E. coli* (ATCC 44102) were used as representative Grampositive and Gram-negative bacteria, respectively. These bacteria were selected based on their ability to adhere, proliferate, and establish a biofilm, and because these strains commonly cause implant-associated infections [30]. *S. aureus* and *E. coli* were cultured in a Luria-Bertani (LB) medium containing yeast extract (5 g/L), tryptone (10 g/L), and NaCl (10 g/L) at 37 °C 200 rpm overnight. The bacterial inoculum was adjusted to OD₆₀₀ of 0.1, which corresponds to ~1 × 10⁸ CFUs/mL in LB.

2.6.2. Inhibition Zone Assay

The cultured *S. aureus* and *E. coli* were evenly dispersed on the surface of agar plates (LB with 2% agar) uniformly. Oxford cups (inner diameter = 6 mm) were placed in the center of the agar plates, and 0.2 mL AMPs solution (Pac, $-G_2Pac$ or $-G_6Pac$; 0.6 mmol/L) was poured into the Oxford cup. Ultrapure water was used as the negative control. The plates were incubated for 24 h, and the inhibition zones around the cylinders were measured in triplicate.

2.6.3. Inhibition Ratio Assay

The antibacterial activity of the AMP-functionalized gold substrates against *S. aureus* and *E. coli* was evaluated, where SAMs samples included an untreated gold substrate (Au) as a negative control, and the -Pac, -G₂Pac, -G₆Pac, -(CH)₁₀-S-S-Pac, -(CH)₁₀-S-S-G₂Pac, -(CH)₁₀-S-S-G₆Pac immobilized substrates as the experimental samples. The inhibition ratio was calculated by immersing each sample in 200 μ L bacterial suspension (10⁴ CFU/mL) and incubating in a 48-well plate at 37 °C for 24 h. After incubation, the samples were moved to new 48-well plates and washed lightly two times by LB. Then, the samples were rinsed and ultrasonically cleaned in 1 mL of LB for 5 min in 1.5 mL tubes, respectively. The suspension was attenuated 1000-fold, and 200 μ L bacterial diluent was evenly dispersed on the surface of an agar plate. The plate was incubated for 12 h, and the number of bacteria was calculated manually via antibacterial quantification tests. The inhibition ratio (R) was calculated as follows:

$$\% \mathbf{R} = \frac{(A-B)}{A} \times 100 \tag{3}$$

where *A* is the average number of bacteria on the Au control, and *B* is the average number of bacteria on the experimental samples.

2.6.4. Live/Dead Bacterial Cell Stain

The SAMs samples were immersed in 200 μ L bacterial suspension (10⁴ CFU/mL) and incubated at 37 °C for 24 h. The liquid supernatant was discarded, and the incubated bacterial surface was rinsed with PBS and stained with SYTO 9 and PI solutions according to the manufacturer's protocols. The live and dead bacterial cells were visualized with confocal laser scanning microscopy (CLSM, ZEISS, Gottingen, Germany) at 200× magnification (objective magnification (20×), ocular magnification (10×)).

2.6.5. Field-Emission Scanning Electron Microscopy

The surface morphology of the bacteria was visualized using field-emission scanning electron microscopy (FE-SEM, Carl Zeiss, Oberkochen, Germany). The SAMs samples were immersed in 200 μ L bacterial suspension (10⁴ CFU/mL) and incubated at 37 °C for 24 h. The incubated bacterial surface was rinsed with PBS and the samples were fixed in 4% glutaraldehyde overnight. The samples were rinsed with PBS, dehydrated in different concentrations of ethanol (30%, 50%, 70%, 80%, 90%, 95%, and 100%) for 20 min each, placed in tertiary butyl solutions (25%, 50%, 75%, and 100% tertiary butyl dissolved in absolute ethyl alcohol), and freeze-dried. The bacteria were sputter-coated with gold (JEOL JFC-1200 fine coater, Tokyo, Japan) and the morphological structure of the bacteria was observed using FE-SEM at an accelerating voltage of 10 KV.

2.6.6. Reactive Oxygen Species

The SAMs samples were immersed in 200 μ L bacterial suspension (10⁴ CFU/mL) and incubated at 37 °C for 24 h. The liquid supernatant was discarded, and the incubated bacterial surface was rinsed with PBS and stained with 2,7-Dichlorodi-hydrofluorescein diacetate (DCFH-DA) solution according to the manufacturer's protocol. The bacteria were visualized using CLSM at 200× magnification (objective magnification (20×), ocular magnification (10×)) (IX-71, Olympus Co., Ltd., Shinjuku, Tokyo, Japan). The reactive oxygen species (ROS) areal ratios (R2) were calculated as follows:

$$R2 = B2/A2 \times 1000\%$$
 (4)

where *A*2 is the average area of the image and *B*2 is the average area of the fluorescence image of the samples.

2.7. Biocompatibility

2.7.1. Preparation of Samples

The SAMs samples were sterilized in 75% ethyl alcohol under ultraviolet (UV) irradiation for 24 h. The rat bone marrow mesenchymal stem cells (rBMSC) model cells were cultured in DMEM supplemented with 10% (v/v) FBS, 100 IU/mL penicillin, and 100 IU/mL streptomycin in a humidified incubator at 37 °C and 5% CO₂.

2.7.2. Cell Morphology

Morphological examination of the cultured rBMSCs on the SAMs samples was performed using FE-SEM and CLSM after 48 h cell culturing. The cytoskeleton stained samples were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 5 min at room temperature, and stained with SYTOX Green (Sigma-Aldrich Co., LLC., Rockville, MD, USA) and Alexa Fluor 546 phalloidin (Invitrogen, Waltham, MA, USA), to visualize the nucleus and F-actin, respectively. The stained cytoskeleton was observed using CLSM.

2.7.3. Live/Dead Cell Stain

The rBMSCs were seeded on sterilized samples in a 48-well cell culture plate at a density of 1.2×10^4 cells/well, then cultured for 24 h. The liquid supernatant was discarded, and the sample was rinsed with PBS and stained with Calcein-AM and PI solution according to the manufacturer's protocol. The samples were incubated at 37 °C for 15 min and observed using CLSM at 400× magnification (objective magnification (40×), ocular magnification (10×)).

2.7.4. Cell Proliferation Assay

The rBMSCs were seeded on the sterilized samples in a 48-well cell culture plate at a density of 1.2×10^4 cells/well for up to 5 days. The cell numbers were measured on day 1, 3, and 5 (cell counting kit-8 (CCK-8); Beyotime Institute of Biotechnology, Haimen, China). The samples were removed from the culture medium, rinsed with PBS, and immersed in 250 µL CCK-8 solution and incubated for 3 h at 37 °C. The incubated solution was transferred to a 96-well cell culture plate and analyzed using a microplate reader at 450 nm.

2.8. Statistical Analysis

Some data were calculated as mean \pm standard deviation based on a minimum of three samples per sample group. Statistical analysis was performed using analysis of variance (ANOVA), where Tukey or Dunnett's T3(3) tests were calculated using SPSS software for windows (IBM7 SPSS version 17).

3. Results

3.1. Characterization of the AMP-Functionalized Gold Substrates

The AMP-functionalized surfaces were prepared via a self-assembly technique (Figure 1). The WCA measurements showed that the hydrophilicity of the AMP-functionalized surfaces increased in comparison with the Au substrate or primer layer (SAM-SH), demonstrating that the peptides were successfully self-assembled on the substrates. Besides, it is noted that the surface hydrophilicity increased slightly in the SAMs of the AMP with a longer spacer length, which implied that the spacers might be beneficial for promoting the self-assembly of the Pac-525 peptides. Moreover, the SAMs of Pac-525, formed on the primer layers, were more hydrophilic than those formed directly on the Au substrates, indicating that the primer layer probably improved the self-assembly of the Pac-525 derivatives (Figure 2A).

The three-dimensional (3D) nanostructure of the AMP-functionalized surfaces was visualized using AFM in tapping mode (Figure 2B). The AMP-functionalized surfaces were not as smooth as the Au control, and the nanostructure of the AMP-functionalized surfaces

with the spacers or primer layer was visibly denser, representing the higher grafting density of the AMPs, which was consistent with the results of WCA.

The bonding of the AMP onto the Au substrate, via the interaction between gold and sulfur, was confirmed by XPS analysis (Figure 2C). The spectral region of S2p showed two peaks (S1 and S2) included in the S2p3/2 and S2p1/2 spin-orbit split levels [31,32]. The two peaks separation of 1.2 eV, and the deconvolution of the S2p3/2 and S2p1/2, showed a typical intensity ratio of 2:1 [33,34]. Each peptide contains only one S atom, while the HS(CH)₁₀SH contains two S atoms, so the densities of the assembled peptides could be quantified by XPS analysis, which could be estimated according to the area ratio. In Figure 2C, the S1 and S2 peaks showed two different chemical stations of S atoms in the assembled peptides. The S1 peak at 161.84 \pm 0.35 eV corresponded to an Au-S bond between the thiol group and the gold surface, which indicated the existence of chemical combination. Meanwhile, the S2p3/2 peak at 163.64 \pm 0.11 eV (S2) indicated the presence of the S-H bond from the peptides physisorption, and the unformed S-S bond of HS(CH)₁₀SH on the gold surface. Most of the photoelectron signals of XPS come from the surface region, and all the samples were tested in the same penetration depth of X-ray. The $Atomic_{S/Au}$ means the atomic ratio of S and Au in each sample. Formulas (1) and (2) were designed as follows: For Formula (1), it is easily understood that $((Au - S)/S) \times (S/Au) = (Au - S)/Au$. The grafting density of Au-S (Table 2) was calculated according to Formula (1). The two-step self-assembling established stable immobilization, through the disulfide bonds between SAM-SH and the AMPs (-(CH)₁₀-S-S-GnPac-NH₂, n = 0, 2, 6). Moreover, each two-step self-assembling unit has three S atoms (two in the linker and one in the AMP). The grafting density of the S-S bond (Formula 2) could be evaluated simply, in that $((S-S)/(Au-S)) \times ((Au-S)/Au) = (S-S)/Au$. The grafting density of S-S (Table 2), in the per unit area of Au, was calculated according to Formula (2). After the analysis of the densities, we found that all the peptides showed nice self-assembling properties on the gold substrates. Compared with one-step self-assembling, the two-step self-assembling indicated a significantly increased density of the Au-S bond.



Figure 1. Schematic diagram of the structure of the AMPs and self-assembling processes on gold Scheme 6538. and *E. coli* (ATCC 44102) were used as a representative of Gram-positive and Gram-negative bacteria, to evaluate antimicrobial ability of AMP-functionalized surfaces.



Figure 2. Surface characterization of the AMP-functionalized substrates. (**A**) The water contact angles measurement; (**B**) AFM images; (**C**) XPS spectra analysis.

3.2. Antibacterial Activity of the AMP-Functionalized Surfaces

Typical inhibition zones were observed around the Oxford cups containing the AMP (-Pac, -G₂Pac, -G₆Pac) solutions, while no inhibition was observed for the ultrapure water control (Figure 3). The obvious inhibition of *E. coli* (Figure 3A) and *S. aureus* (Figure 3B) was evaluated, based on the diameter of the antibacterial ring, which was measured in triplicate. Specifically, the diameter of the inhibition zone for the control group, against both *E. coli* and *S. aureus*, was 0.78 ± 0.01 and 0.78 ± 0.02 cm, respectively, while the -Pac solution was 1.31 ± 0.01 and 1.37 ± 0.06 cm, -G₂Pac was 1.34 ± 0.05 and 1.38 ± 0.02 cm, and -G₆Pac

Α

was 1.35 ± 0.06 and 1.31 ± 0.03 cm, respectively. The differences in the diameters of the antibacterial rings were not obvious, which indicated that the antibacterial activities of the Pac-525 derivatives, against both *E. coli* and *S. aureus*, were not affected by the involvement of glycine spacers.

Table 2.	The analy	ysis of XPS	peak of	different SAMs.
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SAMs	-Pac	-G ₂ Pac	-G ₆ Pac	-(CH) ₁₀ - -S-S-Pac	-(CH) ₁₀ - -S-S-G ₂ Pac	-(CH) ₁₀ - -S-S-G ₆ Pac	-(CH) ₁₀ SH
Atomic S/Au	0.24	0.34	0.45	0.34	0.52	0.51	0.62
Area of peak 1	6765.78	4267.14	7369.58	6964.18	6378.79	9172.12	3321.83
Area of peak 2	7346.28	7111.32	17485.07	10822.13	15535.65	18727.04	9494.15
Area of sum	21569.10	18498.09	35511.22	24316.05	30810.29	34699.79	21790.82
Grafting density Au-S	0.08	0.08	0.09	0.10	0.11	0.13	0.09
Grafting density S-S	-	-	-	0.06	0.09	0.08	-

В



Figure 3. The antibacterial ability of the Pac-525 derivatives by the inhibition zones assay against E. coli (A) and S. aureus (B).

In order to evaluate the antibacterial effects of the SAMs of AMPs, the bacteria, after 24 h of culturing on different substrates, were counted and compared (Figure 4). Additionally, the inhibitory ratios were also measured, as listed in Table 3. The inhibitory ratios of the -Pac, $-G_2Pac$, $-G_6Pac$, $-(CH)_{10}$ -S-S-Pac, $-(CH)_{10}$ -S-S- G_2Pac , and $-(CH)_{10}$ -S-S- G_6Pac against *E. coli*, were 24% ± 33.8%, 24.9% ± 4.6%, 50.2% ± 2.0%, 58.0% ± 4.8%, 59.5% ± 4.8%, and 99.6% ± 0.3%, respectively. As for *S. aureus*, they were 20.8% ± 5.3%, 21.4% ± 5.1%, 39.8% ± 1.6%, 55.3% ± 3.8%, 59.1% ± 1.1%, and 66.8% ± 0.4%, respectively. In comparison with the Au substrate, all the AMP-functionalized substrates showed obvious antibacterial activity. The longer the length of the glycine spacers, the better the antibacterial activity. Besides, the existence of a primer layer dramatically improved the antibacterial activities of the self-assembled AMPs. Therefore, the -(CH)₁₀-S-S-G₆Pac group had the highest antibacterial ratio, both against *E. coli* and *S. aureus*, among all the groups.

Bacteria

-Pac

-G₂Pac

-(CH)₁₀-

-S-S-G₆Pac

Coli (%) S. aureus (%)	$\begin{array}{c} 24.\ 3\pm 3.8\\ 20.\ 8\pm 5.3\end{array}$	$\begin{array}{c} 24. \ 9 \pm 4.6 \\ 21. \ 4 \pm 5.1 \end{array}$	$50.\ 2 \pm 2.0 \\ 39.\ 8 \pm 1.6$	$58.\ 0 \pm 4.8 \\ 55.\ 3 \pm 3.8$	$59.5 \pm 4.8 \\ 59.1 \pm 1.1$	99. 6 ± 0.3 66. 8 ± 0.4
A 1200	· · · ·	***	B	, ,	· · · · · ·	**
of Escherichia - 000 Co - 008 - 009 - 009 - 009 - 009 - 009 - 000 - 009 - 000 - 00		***	- R 1400 - - SS - - SJ1200 - - OJ1000 - - Q1000 - - Q100	Ē,	*	
400 - 400 - 940 - 200 - 1 - - - 0 -			- S - - 5 600 - - 194 400 - - 194 400 - - 194 200 - - 194 200 - - 194 200 - - 194 200 -			
	Pac -G ₂ Pac -G ₆ Pac -(CH	H) ₁₀ -S(CH) ₁₀ -S(CH) ₁₀ -S-	Au	-Pac -G ₂ Pac -G ₆	Pac -(CH) ₁₀ -S(CH) ₁₀ -S-	-(CH) ₁₀ -S- Au

Table 3. The inhibitory ratios of different substrates.

-G₆Pac

-(CH)₁₀-

-S-S-Pac

-(CH)₁₀-

-S-S-G₂Pac

Figure 4. The antibacterial ability of the AMP-functionalized surfaces against *E. coli* (**A**) and *S. aureus* (**B**) by bacterial counting (* p < 0.05, ** p < 0.01 and *** p < 0.001).

The typical morphologies, especially the membrane integrity of the bacteria after 24 h of culturing on different substrates, were examined by FE-SEM. As shown in Figure 5A,C, the cells remained intact, with smooth and clear membranes, in the control group (Au and SAM-SH), indicating that the Au and SAM-SH surfaces had no antibacterial activity, although the density of the bacteria on the -Au surface was much higher than that on the -SH surface. In contrast, the numbers of bacteria on the AMP-functionalized surfaces decreased obviously in comparison with the Au surface, especially in the -(CH)₁₀-S-S-G₆Pac group. Moreover, most of the attached bacteria showed damaged appearances with membrane variations or disruptions. The viability of the bacteria had obvious damages, most of them were still alive on the -Pac and -G₂Pac surfaces, while the number of dead bacteria increased on the surfaces with longer glycine spacers or a SAM-SH primer layer. The -(CH)₁₀-S-S-G₆Pac group presented the best antibacterial efficiency, with most the bacteria dead. The results were obtained for both *S. aureus* and *E. coli*.

With the influences of the immobilized AMPs, the intracellular oxidative stress of the bacteria may have had changes, which were related to the physiological change and viability of the bacteria. After culturing for 24 h, the fluorescence images of the ROS were examined, and are shown in Figure 6A (*E. coli*) and Figure 6B (*S. aureus*), and the quantitative analysis of the fluorescence images of the ROS area ratios was also measured, and is listed in Table 5. The qualitative and quantitative intracellular oxidative stress analyses confirmed the positive ROS production in the AMP-functionalized surfaces groups, indicating that the immobilized AMPs could trigger intracellular ROS burst. Consistent with the live/dead staining results, the -(CH)₁₀-S-S-G₆Pac group had the highest ROS production, for both *E. coli* and *S. aureus*.

Table 4. Quantitative analysis of fluorescence images of ROS fluorescent area ratios.

Bacteria	-Pac	-G ₂ Pac	-G ₆ Pac	-(CH) ₁₀ - -S-S-Pac	-(CH) ₁₀ - -S-S-G ₂ Pac	-(CH) ₁₀ - -S-S-G ₆ Pac	-(CH) ₁₀ SH	Au
E. coli (‰) S. aureus (‰)	$\begin{array}{c} 15.99 \pm 2.45 \\ 2.71 \pm 1.23 \end{array}$	$\begin{array}{c} 14.67 \pm 2.36 \\ 2.89 \pm 1.83 \end{array}$	$\begin{array}{c} 28.89 \pm 3.81 \\ 17.26 \pm 2.35 \end{array}$	$\begin{array}{c} 20.23 \pm 4.60 \\ 6.50 \pm 1.81 \end{array}$	$\begin{array}{c} 22.21 \pm 5.83 \\ 6.51 \pm 2.83 \end{array}$	$\begin{array}{c} 57.44 \pm 3.65 \\ 43.97 \pm 6.35 \end{array}$	$\begin{array}{c} 0.13 \pm 0.11 \\ 0.26 \pm 0.13 \end{array}$	$\begin{array}{c} 0.13\pm0.12\\ 0.24\pm0.11\end{array}$



Figure 5. The typical SEM images and live/dead fluorescence images of *E. coli* (**A**,**B**) and *S. aureus* (**C**,**D**) on different SAM substrates after 24 h of culturing.



Figure 6. The intracellular ROS levels of E. coli (A) and S. aureus (B) on different SAM substrates after 24 h of culturing.

E. coli (%) 15.99 ± 2.45 14.67 ± 2.36 28.89 ± 3.81 20.23 ± 4.60 22.21 ± 5.83 57.44 ± 3.65 0.13 ± 0.11 0.13 ± 0.13 S. aureus (%) 2.71 ± 1.23 2.89 ± 1.83 17.26 ± 2.35 6.50 ± 1.81 6.51 ± 2.83 43.97 ± 6.35 0.26 ± 0.13 0.24 ± 0.13	Bacteria	-Pac	-G ₂ Pac	-G ₆ Pac	-(CH) ₁₀ - -S-S-Pac	-(CH) ₁₀ - -S-S-G ₂ Pac	-(CH) ₁₀ - -S-S-G ₆ Pac	-(CH) ₁₀ SH	Au
	E. coli (‰) S. aureus (‰)	$\begin{array}{c} 15.99 \pm 2.45 \\ 2.71 \pm 1.23 \end{array}$	$\begin{array}{c} 14.67 \pm 2.36 \\ 2.89 \pm 1.83 \end{array}$	$\begin{array}{c} 28.89 \pm 3.81 \\ 17.26 \pm 2.35 \end{array}$	$\begin{array}{c} 20.23 \pm 4.60 \\ 6.50 \pm 1.81 \end{array}$	$\begin{array}{c} 22.21 \pm 5.83 \\ 6.51 \pm 2.83 \end{array}$	$\begin{array}{c} 57.44 \pm 3.65 \\ 43.97 \pm 6.35 \end{array}$	$\begin{array}{c} 0.13 \pm 0.11 \\ 0.26 \pm 0.13 \end{array}$	$\begin{array}{c} 0.13\pm0.12\\ 0.24\pm0.11\end{array}$

Table 5. Quantitative analysis of fluorescence images of ROS fluorescent area ratios.

3.3. In Vitro Biocompatibility

To confirm the biosafety of these antibacterial substrates in biomedical applications, the biocompatibility of the AMP-functionalized substrates was then evaluated by the in vitro cell culture of rBMSCs. The typical cell morphologies that are cultured on samples are shown in Figure 7A (FE-SEM images), Figure 7B (CLSM images), and Figure 7C (live/dead fluorescence images). It is revealed that the rBMSCs had a typical stem cell morphology, and good attachment and survival on all the AMP-functionalized surfaces, indicating that the immobilized AMPs had no cytotoxicity. Additionally, as shown in Figure 7D, the rBMSCs had good proliferation behaviors within 5 days of cell culturing. More than that, the cell number on the AMP-functionalized surfaces was significantly higher than that on the Au or SAM-SH surfaces, which may be due to the -NH₂ groups of the AMPs on the surfaces for negative-charged cell attachment and spreading.



Figure 7. The biocompatibility of the SAM substrates evaluated by in vitro cell culture of rBMSCs. (**A**) Typical SEM images of rBMSCs on different substrates; (**B**) CLSM images of rBMSCs stained with Rhodamine Phalloidin for F-actin (red) and SYTOX Green for nuclei (green); (**C**) live/dead fluorescence images of rBMSCs after 48 h of cell culturing, green for live cells and red for dead cells; (**D**) cell proliferation of rBMSCs within one week by CCK-8 assay.

4. Discussion

Infection is one of the most common causes of implanted biomaterial failure, which will aggravate the deficiency of the bone mass surrounding the implants. Traditional antibiotics are often used to combat infection, but the rise in antibiotic resistance has reduced the effectiveness of this solution. Alternatively, AMPs that have little drug resistance are regarded as effective candidates for treating infections, when applied as a coating on the implant surface [3,13]. However, AMPs tend to diffuse quickly when they are used in vivo, and have been associated with aggregation and protein hydrolysis issues that compromise the antimicrobial efficiency. Therefore, conjugating AMPs on surfaces is preferred, to prevent AMPs diffusion and denaturation, while inhibiting infections on implanted medical materials.

In this study, cysteine-terminated AMPs underwent good self-assembly on the Au substrate, via Au-S chemical bonds, directly forming uniform peptide monolayers. In order to maintain AMP activity, glycines were inserted between the cysteine and the AMP sequence, as spacers, to guarantee the structure and flexibility of AMPs on the substrates $(-Pac, -G_2Pac, and -G_6Pac)$, which were beneficial for the self-assembly of AMPs as well. In addition, to further improve the flexibility of AMPs and the quality of the SAM-AMPs, self-assembling monolayers of SH-terminated alkanethiols (SAM-SH) were firstly made, to provide a primer layer for the secondary grafting, via S-S chemical bonds between the alkanethiols and AMPs, forming the SAM-AMPs on the SAM-SH (-(CH)₁₀-S-S-Pac, -(CH)₁₀-S-S-G₂Pac, and -(CH)₁₀-S-S-G₆Pac). The AFM and XPS examinations indicated that the involvement of glycine spacers did not disturb the self-assembly of the AMPs, the three substrates of -Pac, $-G_2$ Pac, and $-G_6$ Pac had similar peptide densities. While the primer layer of SAM-SH provided a more compact layer for the secondary self-assembly of AMPs, therefore, both the peptide density and flexibility were higher than those on primary SAM-AMPs substrates, which may have had positive effects on the antibacterial activities of the AMP-functionalized substrates. The antibacterial assays confirmed that the antibacterial activities of the surfaces increased with the spacer length and with the involvement of the primer layer. The $-(CH)_{10}$ -S-S-G₆Pac substrate showed the best antibacterial efficiency against both E. coli and S. aureus. Although the AMP-functionalized surfaces had obvious and effective antibacterial activity, they exhibited excellent biocompatibility at the same time. The bone marrow stem cells had very good attachment, viability, and proliferation on these surfaces, indicating that the AMPs did not attack mammalian cell membranes.

The antibacterial mechanism of the AMP-functionalized substrates was also evaluated preliminarily. The SEM images showed that the membranes of the bacteria could be the target of the AMPs. The rugged or broken bacterial membranes and the debris of bacterial membrane can be clearly observed as well. The ROS staining results were consistent with the antibacterial assays, implying that AMPs finally triggered the production of intracellular ROS and cell death.

Many previous studies have reported the antibacterial activity of the immobilized AMPs [35]. In this study, we developed a facile, efficient, and energy-saving approach, to modify biomaterial surfaces via self-assembling AMPs on Au substrates. Au substrates have been considered as ideal substrates for constructing SAMs with high uniformity and quality. Therefore, here, we prepared SAMs of AMPs on Au substrates that could guarantee the quality of the SAMs with similar grafting densities, for comparisons. Actually, gold, as a type of very good biomedical metal with unique biocompatibility, has been widely used in many kinds of biomedical applications, including gold nanoparticle probes, gold electrodes, and gold-coated medical devices. It is very easy to modify the surfaces of these devices, via the self-assembly of AMPs, to endow them with antibacterial activities. Beyond that, this method could also be applied to other metals. We confirmed that the antibacterial activity of antimicrobial peptides still remained after self-assembly, while the efficiency was tightly related to the flexibility and the stereochemical structure of the AMPs. Therefore, the longer spacer may contribute to the higher antibacterial efficiency, on the premise of good self-assembly on the surfaces. Besides, it is the first time that SAM-SH has been used

as a primer layer for the secondary self-assembly of AMPs. It is exciting that the SAM of the antimicrobial peptides on the SAM-SH showed outstanding antibacterial performance, especially for *E. coli*, with over a 99.6% inhibitory ratio. Nevertheless, it should be noted that the stability of the SAMs of the AMPs on the surfaces of devices may be affected during the implantation processes, especially by press-fitting into a defect, which will probably compromise the long-term antibacterial properties. The antibacterial application in vivo and their long-term stability need further evaluation, which is on the way in our laboratory.

5. Conclusions

In this study, we constructed a simple and energy-saving method to successfully fabricate antibacterial surfaces on gold substrates, via the one-step self-assembly of cysteineterminated AMPs (Ac-CG_nKWRRWVRWI-NH₂, n = 0, 2 or 6) or two-step surface modification using the SAMs of alkanethiols HS(CH)₁₀SH and cysteine-terminated AMPs. The samples with the self-assembled Pac-525 derivatives had antibacterial ability, and the -(CH)₁₀S-S-G₆Pac was highly effective against *S. aureus* and *E. coli*, reducing them to 33.2% and 0.4%, by contact in a short time span, respectively. In addition, the AMP-functionalized surfaces had an ideal biocompatibility with good cell adhesion, spreading, and proliferation of rBMSCs.

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