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Original Article

Effect of Gold Nanoparticles and Hydroxypropylmethylcellulose on the Activity of Two Novel Designed Antimicrobial Peptides

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Abstract

Infectious wounds are one of the issues that significantly burden population lives and finances each year. Even though there have been significant breakthroughs in wound healing dressing, wound infections continue to be an issue. One of the latest techniques is using contemporary dressings containing antimicrobial agents to expedite wound healing and prevent infection. In the present study, the effect of gold nanoparticles (AuNPs) and hydroxypropylmethylcellulose (HPMC) as the main expedient of a hydrogel formulation on the bioactivity of two newly designed antimicrobial peptide (AMPs) was investigated. AuNPs were produced using two methods of chemical and biological synthesis. AuNPs were characterized individually and in the presence of AMPs in a stepwise manner. The antibacterial activity of these combinations against *Staphylococcus aureus* and *Acinetobacter baumanii* was evaluated. The results demonstrated that the stability of green-synthesized AuNPs was significantly superior to that of chemically synthesized AuNPs in presence of AMPs. In addition, the antibacterial activity of AMPs changed when combined with AuNPs and HPMC compared to its free state. This alteration was different based on the AMP identity and the combination composition. In the case of AMP1, designed based on regenerating islet-derived protein 3-alpha (REG3A), addition of AuNPs could enhance the antimicrobial activity. However, in the presence of another AMP (designed based on Cathelicidin-2), activity variations did not adopt with a distinct pattern. In general, the best antimicrobial activity was observed on the *A. baumanii* when a combination of green synthesized AuNPs, AMP derived from Cathelicidin-2 and HPMC was applied. In conclusion, since the inclusion of hydrogel and nanoparticles in the most combination conditions resulted in the efficacy reduction of AMPs, further efforts in selecting a suitable polymeric component should be made to develop an effective and inexpensive wound dressing formulation for this designed AMP.

Keywords: Peptide; Hydrogel; Nanoparticle; Formulation; *Staphylococcus aureus*; *Acinetobacter baumanii.*

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1. Introduction

Practical and affordable wound therapy is crucial, since the prevalence of chronic wounds and the resulting financial burden have been raised up. Wound healing includes an initial assessment, cleaning, debridement, administration

of therapeutic agents, and therapeutic dressing. The wound healing process depends on choosing the proper dressing and therapeutic agent to absorb the exudate and keep the wound moist and free of infection [1].

The commercially available wound dressings are frequently available as films, foams, hydrogels, and hydrofibers. They can seal the wound and surrounding skin moisture, which benefits angiogenesis, epidermal cell migration, and proliferation. Additionally, these advanced dressings can be activated with various therapeutic agents to improve wound healing more efficiently. Pharmaceutical formulations for wound dressing regularly include antimicrobial substances, such as antibiotics, naturally occurring molecules like honey, and metal nanoparticles (NPs), to treat infections and promote wound healing [2-4]. Although there are many different wound dressing treatments, few are particularly effective for treating chronic wounds. Consequently, applying a new approach to treat chronic ulcers and properly stop persistent infections is imperative [5].

In this regard, antimicrobial peptides (AMPs) are unique therapeutic alternatives. They are endogenous peptides produced by biological systems to defend the host against harmful microorganisms. AMPs are also characterized as host defense peptides because they are critical in the development of the innate defenses of the immune system. In fact, the primary mode of action against pathogenic microorganisms is the disruption of the cell membranes. Along with their antimicrobial function, AMPs may also play a role in reducing inflammation, immune system activation, and wound healing. Due to their unique mode of action and different qualities compared to common antibiotics, AMPs can be considered a new and promising generation. Although they have several advantageous characteristics, AMPs have limited medicinal development due to their poor catalytic stability and limited penetration across biological barriers. Chemical modifications such as peptide cyclization, non-protein amino acids, peptidomimetics, lipidation, etc., are frequently used to address these disadvantages, especially in improving enzymatic stability [6].

Nanoparticles offer an additional possible treatment for multidrug-resistant bacteria. Nanoparticles on their own (e.g., silver and gold) are known to exhibit antibacterial properties and operate via various mechanisms. Thus, anchoring AMPs on metallic nanoparticles may constitute an alternative strategy for combating antibiotic-resistant bacteria and enhancing the antimicrobial activity of both constituents [7-9].

In a research, an AMP (surfactin; SFT) and 1-dodecanethiol (DT) coated gold nanodots (AuNDs) showed stronger antibacterial activity than the AMP alone. SFT/DT-AuNDs were efficiently active against microorganisms with and without antibiotic resistance. Furthermore, *in vivo* healing investigations of methicillin-resistant *S. aureus*–infected wounds indicated that SFT/DT–AuNDs promoted quicker healing, more efficient formation of collagen fibers, and better epithelialization [10]. Furthermore, de Alteriis *et al.* investigated the efficacy of AuNPs coated with indolicidin (AuNPs–indolicidin) against infectious *Candida albicans* biofilms and found that the

exposure to the nanocomplex strongly reduced *C. albicans* ability to build biofilms and degraded already-developed mature biofilms [11]. Moreover, Otari *et al.* used a green chemistry method to synthesize AMP-coated AuNPs-alginate biohydrogel. This compound showed significant bactericidal activity against pathogenic bacteria [12].

Casciaro *et al.* discovered that covalently coupling Esc (1-21), a product of the frog skin AMP esculentin-1a, to soluble AuNPs [AuNPs@Esc(1-21)] through a poly(ethylene glycol) linker increased the free peptide efficacy against *Pseudomonas aeruginosa* by 15-fold magnitude, while causing no toxicity to human keratinocytes. In addition, AuNPs@Esc(1-21) exhibited dramatically increased tolerance to enzymatic degradation and the capacity to destroy the bacterial membrane at extremely low concentrations. This study revealed for the first time that peptide-coated AuNPs could exhibit wound-healing capabilities on a monolayer of keratinocytes [13].

AMPs in water-based gel formulations can be beneficial for wound healing because it has been established that a moist environment is desirable for wound rehabilitation. The presence of minimum components in gel formulation, which lowers the possibility of harmful interactions in the formulation, is another advantage [14]. On the other hand, a viable strategy to deal with antibiotic resistance is a combination of several therapeutic substances. Therefore, in this study, we investigated the antibacterial activity of the combination of peptide, gold nanoparticles (AuNPs), and HPMC. For this purpose, we synthesized AuNPs using two different approaches and their combination with two antimicrobial peptides designed in our previous studies was evaluated. The AMP1 (LVSARIRCPK) and mCHTL(131-140) (RKWLRKIRRWRK) were previously *in silico* designed using Regenerating islet-derived protein 3-alpha (REG3A; UniProtID: KBQ0641) [15] and Cathelicidin-2 (UniprotID: Q2IAL7) as protein templates, respectively.

2. Materials and Methods

2.1. Reagents and microorganism strains

Chloroauric acid ($HAuCL_4$) was purchased from Sigma (USA) and Hydroxypropyl methylcellulose (HPMC), trisodium citrate, the bacterial growth media of LB (Luria-Bertani) liquid, and agar media were supplied from Merck Chemical (Germany). The AMP1 (LVSARIRCPK) and mCHTL(131-140) (RKWLRKIRRWRK) were chemically synthesized. GenScript (China) was commissioned to carry out the chemical synthesis.

Staphylococcus aureus (ATCC25923), *Pseudomonas aeruginosa* (ATCC27853), and *Escherichia coli* (ATCC25922) were purchased from culture collection of Pasteur institute of Iran, and *Acinetobacter baumanii* bacteria, which had been already isolated from hospital samples and amplified in the microbiology lab of pharmacy school, Shahid Beheshti University of Medical Sciences. This bacterium has been previously identified and confirmed by differential culture media included Sheep blood agar and MacConkey agar [16-18].

2.2. AuNPs synthesis using Turkevich method ($AuNPs_1$)

The AuNPs were prepared based on the Turkevich method [19]. Briefly, 20 µL of

$HAuCL_4$ was dissolved in 50 mL of distilled water under magnetic stirring (1000 rpm) until the reaction temperature reached the boiling point of water. Then, 2 mL of warmed 1% sodium citrate aquatic solution was added, and the solution was heated for 20 minutes under magnetic stirring (1000 rpm) till it turned wine-red.

2.3. AuNPs synthesis using a macromolecular fraction of Spirulina culture medium ($AuNPs_2$)

The process introduced by Dananjaya *et al.* [20] was slightly modified to synthesize the AuNPs. Using distilled water, solutions containing 5 mg/mL of *Spirulina* polysaccharide (SP) and 4 mM of $HAuCl_4.3H_2O$ were produced. The mixture of 10 mL of SP solution and 2 mL of $HAuCl_4.3H_2O$ solution was heated in a water bath at 90 °C for approximately 5 minutes until the color changed from pale yellow to purple-red, indicating the generation of AuNPs.

2.4. AuNPs characterization

The hydrodynamic diameter and the zeta potential of nanoparticles were determined using the zeta sizer nano-ZS (Malvern instrument, UK) at scattering angle of 90° and 1 cm pathlength. A one to 10 diluted suspension of AuNPs was analyzed. The data was analyzed automatically, and the average value of the observed sizes across 30 runs was displayed [21]. Both bare AuNPs and AuNPs conjugated with mCHTL(131-140) and AMP1 peptide were analyzed.

2.5. HPMC gel formulation of peptide and AuNPs

Hydroxypropyl methylcellulose (HPMC) gel was prepared using a modified version of the casting procedure described by Fonseca *et al.* [22, 23]. First, an HPMC dispersion was produced by mixing 4 g with 100 mL of distilled water and stirring the mixture on a magnetic stirrer at 1000 rpm for 30 minutes at 25 °C. Glycerol was added after the HPMC dispersion previously heated at 70 °C for 5 minutes. The temperature of the reaction was then gradually raised to 85 °C and lowered to 55 °C. Finally, different ratios of peptide, HPMC, and AuNPs were combined to produce various series of samples. The final composition of the samples is listed in **Table 1**.

Table 1. Different samples used in this study.

Sample	Content
1	peptide
2	$AuNPs_1$ (Synthesized by citrate method)
3	$AuNP_2s$ (synthesized by SP)
4	Peptide + $AuNP_{1s}$
5	Peptide + $AuNP_2s$
6	Peptide + $AuNP_{1s}$ + HPMC
7	Peptide + $AuNP_2s$+ HPMC
8	$AuNP_{1s}$ + HPMC
9	$AuNP_2s$ + HPMC
10	Peptide + HPMC
11	HPMC

2.6. Evaluation of the stability of AuNPs in the presence of the peptide

One µL of the peptide solution with a concentration of 0.1 mg/mL was gradually added to 1 mL of AuNPs dispersion, until the nanoparticles aggregated and the mixture turned purple. After peptide addition in each step, the mixture sonicated for 5 minutes.

2.7. Evaluation of antibacterial activity

Antibacterial activity was evaluated using the microdilution method. An initial 100 µL of Muller-Hinton broth medium (MHB) was added into each well of a 96-well microplate. Serial dilutions of the different samples including peptide, peptide + AuNPs, peptide + AuNPs + HPMC, HPMC, and AuNPs were prepared in different concentrations (500 µg/mL to 0.97 µg/mL) which were loaded in separated wells in triplicate. 10 µL of a 1:20 dilution of 0.5 McFarland suspension (1×10^8 CFU/mL), containing 5×10^6 CFU/mL, were added to each well. The plates were incubated for 24 hours at 35±2 °C before the bacterial growth was measured by reading the absorbance at 630 nm. *Staphylococcus aureus* (ATCC25923) (as a gram-positive bacterium) and *Acinetobacter baumanii* (a gram-negative Bacterium) were used in this study.

2.8. Statistical analysis

All experiments were conducted in triplicate and, where applicable, and numerical data were presented as the mean ± SD. Using GraphPad Prism 9.4.1 (GraphPad Software, San Diego, CA, USA), statistical analysis was conducted. Multiple comparisons were made with a two-way analysis of variance (ANOVA) using Tukey post Hoc test. The *p-value* <0.05 was considered as significant.

3. Results and Discussion

3.1. Characterization of AuNPs and peptide interaction with AuNPs

The average size and charge of the naked $AuNPs_1$ produced by sodium citrate solution were 41.49 nm and -34 mV, respectively. When combined with the peptide, the average size increased to 96.93 nm, and its zeta potential showed a decrease to -27.5 mV. Then again, *Spirulina* polysaccharide-produced naked $AuNPs_2$ had an average size of 137 nm and zeta potential of -15.8 mV, and the size and charge did not show remarkable changes after peptide addition (161 nm and -15.8 mV). However, the NPs dispersity increased as peptide coating on $AuNPs_2$ would not be the same on all AuNPs and occur differently based on the first coating developed during green synthesis of AuNPs. The permanent macromolecular fluid layer affixed to the particles was thought to be the cause of greater diameter and different charge of $AuNPs_2$.

Nevertheless, the increase in size recorded by DLS was not substantial after peptide addition to $AuNPs_1$. However, the findings of stability assay demonstrated that $AuNPs_2$ are more stable than $AuNPs_1$. In the presence of more than 100 pg of the peptide, $AuNPs_1$ color quickly changed to purple, which indicate the aggregates formation, developed to complete NPs precipitation over the time. In comparison, $AuNPs_2$ remained stable with no color change, even after adding 500 µg of the peptide. It seems that despite their larger size, they could tolerate more concentration of peptide without aggregation. This may be the consequence of the shape of AuNPs, which resulted in different stability. It was confirmed that different morphologies of AuNPs have a distinct metastable area in that condition, they show the best colloidal stability [24].

In our current work, the surface of AuNPs was coated with two different AMPs, AMP1

and AMP2 that named mCHTL (131-140). The chemosynthesis ($AuNPs_1$) and green-synthesis ($AuNPs_2$) methods were utilized for the production of AuNPs. In the presence of high concentrations of the AMP, $AuNPs_1$ formed a precipitate quickly. Only less than 100 pg of both peptides could be loaded on 1 mL of $AuNPs_1$, while the NPs stability did not change. Higher concentrations caused the aggregation of AuNPs. However, $AuNPs_2$ were stable at high peptide concentrations. The presence of lysine and arginine in the peptide sequence may be responsible for the precipitation of $AuNPs_1$. It has been demonstrated that amino groups have well interacted with free gold surfaces. In addition, studies showed that cationic peptides could cause the precipitation of nanoparticles [25]. The effect of this interaction on the antimicrobial activity of AMP was more significant in mCHTL(131-140) compared to AMP1, which had only three lysine (K) and arginine (R) residues, compared to eight number of R and K residues in mCHTL(131-140).

Moreover, in this study, the interaction of AMP with differently synthesized AuNPs was just investigated for the second AMP, mCHTL (131-140). Results showed that green synthesized nanoparticles with mCHTL (131-140) were much more stable than AuNPs produced by chemical reduction with sodium citrate. It was previously reported that through the green synthesis of AuNPs, *Spirulina* polysaccharide, with hydroxyl and carbonyl functional groups, can convert Au^{3+} ions into Au^0 neutral atoms. These polysaccharides can be then immobilized on Au^0 caps [26]. In addition, these functional groups can compete with the gold atoms for interaction with the lysine and arginine of the peptide. Therefore, the probability of interaction of gold atoms with peptide amino acids decreases, increasing the probability of AuNPs instability.

3.2. Antimicrobial activity of AuNPs

The effect of AMP1 combination with the $AuNP_{1s}$ on the antimicrobial activity against *S. aureus*, *P. aeruginosa*, and *E. coli* showed that the addition of $AuNPs_1$ could significantly (*p*-value < 0.001) improve the antimicrobial activity of peptides on all three bacteria (**Figure 1**). Although the addition of AuNPs significantly improved the antibacterial activity (*p*-value <0.001). In the case of AMP1, the addition of $AuNPs_1$ improved the antimicrobial activity, against both Gram-positive and Gram-negative bacteria. Likewise, Shamaila *et al.* used sodium borohydride as a reducing agent to synthesize AuNPs and clarified how Gram-negative and Gram-positive bacteria would be lysed by AuNPs [27].

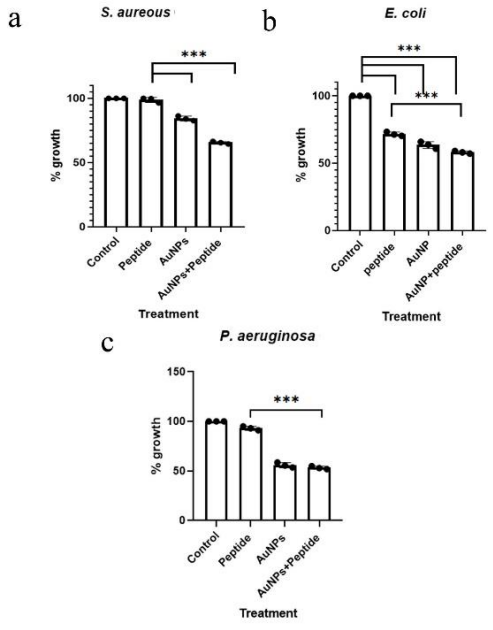

Figure 1: The inhibitory effect of $AuNP_{1s}$, AMP1, and their combination on the growth of S. *aureus*, *P. aeruginosa*, and *E. coli*. *** *p*-value <0.0001.

However, as AMP1 did not affect the most important organisms involved in wounds, i.e. *S. aureus*, we changed the target peptide to mCHTL (131-140), which inherently exhibited more potent antibacterial activity compared to AMP1. Then, antibacterial activity against *S. aureus* and *A. baumanii* was evaluated for different samples (**Table 1**). The growth inhibitory effects of both AuNPs (chemically synthesized $AuNPs_1$ and green synthesized $AuNPs_2$) on bacterial targets were not significant. Although $AuNPs_2$ at the highest concentration, 500 µg/mL, inhibited the growth of *A. baumanii* by about 42%, it had no inhibitory effect on *S. aureus*. No inhibitory effect was observed for $AuNPs_1$ on both bacteria.

Nevertheless, it has been previously shown that AuNPs synthesized by both chemical and biological methods possess antibacterial properties [28, 29], however, none of the AuNPs we synthesized exhibited significant antibacterial properties and our results were not consistent with previous studies. For example, using the leaf extract of *Viola betonicifolia*, Wang *et al.* produced AuNPs (VB-AuNPs). Their findings confirmed high antioxidant, antibacterial, antifungal, and biofilm inhibitory activities of VB-AuNPs, which synthesized by plant leaf extract, compared to the commercially available chemically produced AuNPs [30]. In addition, previous study in contrast showed that AuNPs synthesized using the polysaccharide extract of *Spirulina maxima* as the reducing agent could also exhibit fungicidal activity against *Candida albicans* [20].

3.3. Effect of the interaction between peptide and AuNPs with HPMC on antimicrobial activity

In current study, HPMC was selected as a moisture-retaining component for wound dressings due to its promising potentials reported so far [31-33]. The results indicated that the peptide antibacterial efficacy generally decreased in the presence of HPMC. It is likely that the gel released the peptide in insufficient quantities to eradicate the bacteria. This challenge needs more investigation to be removed. The MIC values of the mCHTL (131-140) peptide against *A. baumannii* and *S. aureus* were 7.8 µg/mL and 250 µg/mL, respectively. However, HPMC by itself did not exhibit any antibacterial activities on *A. baumannii* and *S. aureus*. Moreover, the mCHTL (131-140) peptide inhibitory activity slightly diminished, and its MIC against *A. baumannii* increased to 15.62 µg/mL, when loaded on HPMC. Additionally, the calculated MIC was remained unchanged at 15.62 µg/mL after adding $AuNPs_2$ to mCHTL (131-140) and HPMC (sample 7 contained peptide, $AuNPs_2$, and HPMC). In contrast, samples 4 and 6, composed of peptide + $AuNPs_1$ and peptide+ $AuNPs_1$+ HPMC, respectively, showed lack of antibacterial effects.

In different manner, after loading the mCHTL (131-140) on HPMC, its ability to inhibit *S. aureus* remained unchanged, however adding $AuNPs_2$ to the peptide and HPMC composition (sample 7) decreased the MIC to 125 g/mL. In contrast, the combination of peptide, $AuNPs_1$, and HPMC (sample 6) did not show any antibacterial effect. The results are shown in **Figure 2**.

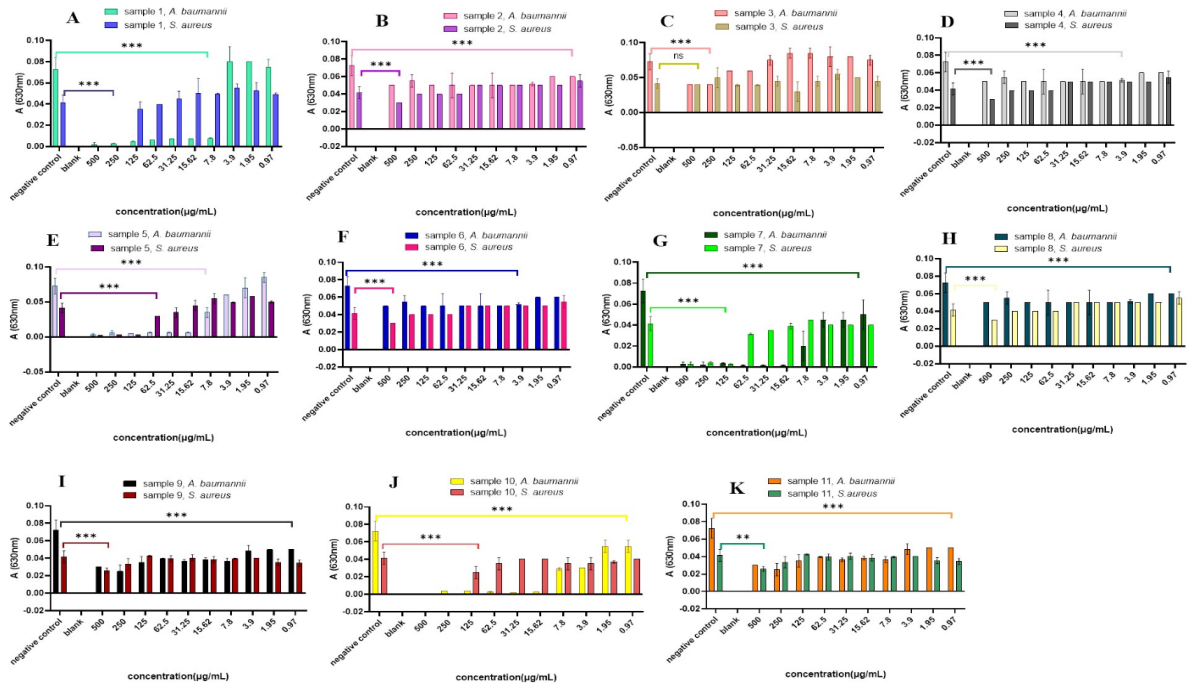

Figure 2. The inhibitory effect of samples 1-11 on the growth of *A. baumannii* and *S. aureus.* A to K: The antibacterial activity of samples 1 to 11, against *A. baumannii* and *S. aureus*. Different colors were used to show the effect on each bacterial target. (ns) *p*-value >0.05; ** *p*-value <.001; *** *p*-value <0.0001.

It was also unexpectedly observed that the antimicrobial activity decreased when mCHTL (131-140) combined with both $AuNPs_1$ and $AuNPs_2$. It may be the result of the interaction of lysine and arginine, which are essential for AMPs antimicrobial properties, with the surface of AuNPs. In addition, the most antimicrobial peptides are typically unstructured in aquatic solutions. As a result, the peptide is randomly bound to the surface of AuNPs that might decline the peptide antibacterial activity [34]. Besides, regarding the low stability of $AuNPs_1$ in the presence of peptide, a much lower concentration of peptide was used compared to free peptide. Therefore, it seemed reasonable that the samples of $AuNPs_2$ exhibited a higher bactericidal effect than the $AuNPs_1$.

The mCHTL (131-140) and its combination with AuNPs in an HPMC gel-based formulation showed the best activity against *A. baumannii* which is an aerobic, Gram-negative bacillus, opportunistic pathogen. This pathogen has a high incidence among immunocompromised individuals, and patients with a prolonged (> 90 days) hospital stand and is usually resistant to most first-line antibiotics [35]. Although this formulation cannot be recommended for wound healing, which usually contain *S. aureus* as the main infectious pathogen, however, it could be developed to achieve efficient treatment for hospitalized *A. baumannii* infections.

4. Conclusion

In fact, antimicrobial peptides have garnered considerable interest as one of the most promising options to replace conventional antibiotics. Using these peptides in dressings can aid in hastening wound healing. Nevertheless, some drawbacks of antimicrobial

peptides have restricted their medical applications. Peptides can be coupled with other substances, such as metal nanoparticles, to eliminate some defects. In addition, two distinct procedures were used to produce AuNPs, and the stability of these two types of AuNPs in the presence of the peptide was examined. To improve the performance of AMPs and enhance their resistance to enzymatic digestion, synthetic peptides were coated on AuNPs. Green synthesized AuNPs were considerably more stable than chemically synthesized AuNPs in the presence of the peptide, and the peptide function was sustained more effectively in the presence of bio-synthesized AuNPs. Furthermore, the green production of AuNPs using *Spirulina* polysaccharides is a straightforward, inexpensive, eco-friendly, and one-step process. Therefore, green NPs manufacturing is superior and more cost-effective than the citrate reduction method. The Results of this study did not confirmed the optimal performance of the formulation of HPMC, AuNPs, and AMP, therefore, more studies regarding the development of novel efficient wound dressing are essential for novel designed AMP. Interaction of AMPs with AuNPs may result in the hindrance of important amino acids involved in the antibacterial activity or induce changes in the AMP conformation that resulted in the reduction of their bioactivities. Removal of these undesirable interactions should be considered in the future studies. Moreover, the network structure of HPMC gel and its related pH might influence the stability and antimicrobial activity of AMP. This challenges should be more studied in the following investigations.

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Conflict of interest

The authors declare to have no conflict of interest.

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