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Characterization of Antibacterial Activity and Synergistic Effect of Cationic Antibacterial Peptide-resin Conjugates

Jeong Min Kim, Sujung Jang, Mi-Hwa Yang, Hyeongjin Cho, and Keun-Hyeung Lee*

Bioorganic Chemistry Lab., Department of Chemistry, Inha University, Inchon-City 402-751, Korea
**E-mail: leekh@inha.ac.kr*
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We synthesized peptide-resin conjugates (**1** and **2**) by immobilizing β-sheet antibacterial peptide and α helical antibacterial peptide on PEG-PS resin, respectively. Conjugate **1** showed considerable antibacterial activity in various conditions, whereas conjugate **2** did not exhibit antibacterial activity. The growths of various bacteria were inhibited by conjugate **1** even at lower concentrations than MIC. Conjugate **1** killed bacteria at MIC and had a potent synergistic effect with current antibacterial agents such as vancomycin and tetracycline, respectively. Overall results indicate that polymer surface modification using antibacterial β sheet peptide is a powerful way to prevent microbial contamination on polymer surfaces.

Key Words : Cationic antibacterial peptide, Antibacterial polymer, Surface modification, Synergistic effect, Antibacterial resin

Adsorption and proliferation of bacteria on polymer surfaces and possible host infection are major concerns in the field of medical devices.[1] There are many efforts to prevent bacteria adsorption and contamination on polymer surfaces.[2] Immobilization of antibacterial agents on polymer surface is a general method to prevent bacteria adsorption and contamination on polymer surfaces. Various types of antibacterial materials have been applied on polymer surfaces to prevent bacterial contamination of polymer surfaces. However, conjugation of most antibacterial materials on polymer surfaces considerably decreased antibacterial activities in comparison to those of unconjugated forms and sometimes the conjugated antibacterial materials did not show enough antibacterial activity to prevent growth of bacteria on polymer surfaces. As the targets of most antibacterial materials located inside of bacteria, immobilization on polymer surface prevents access of antibacterial materials from the targets of bacteria. Until now, more than 700 cationic antimicrobial peptides (CAPs) have been isolated from the host defense systems of invertebrate and vertebrate.[3-5] Most of these antimicrobial peptides are commonly positively charged at physiological pH and are able to adopt amphipathic α helical or β-sheet structures upon association with lipid bilayers. These antimicrobial peptides have received considerable attention because they show a different mode of antimicrobial mechanisms from those of current antibiotics. Even though their mode of action is not fully understood, the peptides increase the permeability of lipid bilayers of microorganisms, resulting in the death of microorganisms. They show several advantages over current antibiotics: fast killing, bactericidal activity, broad antimicrobial spectra, and synergistic effects with current antibiotics. Therefore, CAPs have been regarded as possible candidates for polymer surface modification to prevent microbial contamination on polymer surfaces. Since Doele *et al*. have reported the conjugation of α helical antibacterial peptides into a water insoluble resin.[6] Several independent research groups reported the modification of polymer surfaces using cationic antimicrobial peptides.[7-9] In the previous research, we synthesized peptide-resin conjugate by immobilizing an antibacterial peptide on PEG-PS resin.[10] The peptide-resin conjugate, like cationic antimicrobial peptides showed antibacterial activity without hemolytic activity and membrane perturbation activity.

In the present study, we chose an antibacterial β sheet peptide (Phe-Lys-Val-Lys-Phe-Lys-Val-Lys-Val-Lys-NH_2) and an antibacterial α helical peptide (Leu-Lys-Val-Val-Phe-Lys-Val-Leu-Phe-Lys-NH_2)[11] and synthesized peptide-resin conjugates (**1** and **2**) by immobilizing the β sheet peptide and the α helical peptide on PEG-PS resin, respectively. The antibacterial activity of both soluble and immobilized peptides toward bacteria was investigated in various conditions such as in the presence of albumin or serum proteins. The immobilized β sheet peptide (conjugate **1**) showed considerable antibacterial activity in various conditions, whereas the immobilized α helical peptide (conjugate **2**) did not exhibit antibacterial activity. Furthermore, we investigated the growths of various bacteria in the presence of conjugate **1** as a function of time and measured synergistic effects between conjugate **1** and current antibiotics such as vancomycin and tetracycline. Overall results indicate that polymer surface modification using antibacterial β sheet peptide is a powerful way to prevent microbial contamination on polymer surfaces.

Materials and Methods

Synthesis of Peptide-resin Conjugates. Peptide-conjugates were synthesized by solid phase synthesis using Fmoc chemistry. The peptide chain was assembled on PEG-PS

resin (90 um). When we synthesized the peptide resin conjugate, 1% (mole ratio) of Rink amide MBHA resin was mixed with PEG-PS resin to characterize its synthesis. All Fmoc-amino acid derivatives were purchased from Novabiochem (San Diego, CA). The side chain protection was tert-butyloxycarbonyl (Boc) for Lys. The coupling reaction of the amino acids to the resin was repeated until no color change in ninhydrin test was observed. The deprotection and cleavage of the peptide resin conjugates was achieved by treatment with a mixture of TFA:H_2O (95:5, v/v). The peptide on the Rink amide MBHA resin was cleaved at this step and then the peptide was analyzed by analytical HPLC and MALDI TOF mass spectrometer to investigate the conjugate purity and to confirm the successful synthesis of the peptide resin conjugate. HPLC and mass spectra confirmed the successful synthesis of the conjugates with high purity (> 95%). Conjugate **1** (MALDI TOF-MS: calcd 1249.83, obsd 1250.30 $[M+H]^+$), Conjugate **2** (MALDI TOF-MS: calcd 1219.81, obsd 1220.21 $[M+H]^+$).

Antibacterial Assay. Briefly, *in vitro* antibacterial assay was done by a modified microdilution technique with using 96-well microplate (Nunc, Denmark). Antibiotic medium 3 (M3; pH 7.0 at 25 ºC, Difco) was used as antibacterial assay media. Freshly grown cells on antibiotic medium 3 agar plate were suspended in physiological saline to 10^4 cells per 1 mL of 2 × concentrated medium and used as the inoculum. Resin conjugates swelled and added to the wells (100 μL per well) and the wells were serially diluted twofold. After inoculation (100 μL per well, 1×10^4 cells per mL), plates were incubated at 37 ºC for 24 h, and the absorbance at 620 nm was measured by ELISA reader (Spectra, Austria) to assess cell growth. The minimal inhibition concentration (MIC) was determined as the lowest concentration of the peptide (resin conjugates) that completely inhibited the growth of the test organism. Magainin II was used as a reference compound in this assay. All MICs were determined from two independent experiments performed in duplicate. MICs for the conjugates report only the total amount of peptide bound on the resin based on Fmoc titration result. These values are not measurements of for contact and inhibition of the microorganisms.

Hemolytic Activity. Packed human erythrocytes were washed three times with buffer (150 mM KCl, 5 mM Tris-HCl, pH 7.4) and then packed erythrocyte was suspended in 10 volume of the same buffer (stock cell suspension). For antibiotic treatment, the cell stock suspension was 25-fold diluted with the same buffer and preincubated at 37 ºC in water bath for 15 min. The final concentration of erythrocyte was approximately 0.4% (v/v). Then increasing amounts of the test samples were added. After incubation for 1 hour at 37 ºC, samples were centrifuged at 4,000 × g for 5 min and the absorbance of supernatant was determined at 540 nm. Hemolysis effected by 0.1% Triton X-100, was considered as 100% and well-known hemolytic peptide. Melittin was used as a reference compound for 100% lysis of erythrocyte in this assay. Heat-inactivated serum (serum proteins) was prepared as follows: blood was collected from five mice and allowed to clot at room temperature for 4 h and then centrifuged for 5 min at 1500 × *g*. The supernatant was collected and incubated at 50 °C for 30 min.

Result and Discussion

Synthesis of Peptide-resin Conjugates. Previously, we synthesized an amphipathic β sheet peptide (Phe-Lys-Val-Lys-Phe-Lys-Val-Lys-Val-Lys-NH_2) and an amphipathic α helical peptide (Leu-Lys-Val-Val-Phe-Lys-Val-Leu-Phe-Lys-NH_2).[11] Both peptides consisting of ten amino acids showed potent antibacterial activity without hemolytic activity. We synthesized peptide-resin conjugate **1** and **2** by immobilizing the amphipathic β sheet peptide and the amphipathic α helical peptide on PEG-PS resin bead (90 μm), respectively (Figure 1). As described in experimental section, the successful synthesis and high purity of the peptide-resin conjugate were confirmed by analyzing HPLC and mass spectra of the cleaved peptides from the resins. We calculated the peptide loading level of the resins (nmol/g) using Fmoc titration method.[12] The concentration of the conjugate in the assay was calculated using the assay medium volume and the calculated amount of the peptides on the bead on the basis of the peptide loading level of the resins (nmol/g).

Antibacterial Activities of Conjugates and Peptides in Various Conditions. As peptide-resin conjugates were precipitated in buffer solution, we measured antibacterial activity of peptide-resin conjugates and the peptides with and without shaking, as shown in Table 1. Magainin II was used as a positive control, whereas PEG-PS resin bead was used as a negative control in this assay.

The immobilized β sheet peptide on resin (conjugate **1**) showed potent antibacterial activity against tested bacteria with shaking (94-100 RPM) and without shaking. The activity of the soluble β sheet peptide was slightly changed by shaking. This result revealed that shaking had no considerable effect on the activity of conjugate **1**. Unexpectedly, the immobilized α helical peptide on resin (conjugate **2**) did not exhibit antibacterial activity up to 400 μg/mL, whereas the α helical peptide showed potent antibacterial activity. PEG-PS resin bead did not show activity regardless of shaking, indicating that antibacterial activity of conjugate **1** was not caused by PEG-PS resin containing amino group but conjugated the β sheet antibacterial peptide. Considering the second structures of most cationic antibacterial peptides, no activity of conjugate **2** was an unexpected result because cationic antibacterial peptides mostly adopted amphipathic α helical structures. The difference of activity between conjugate **1** and **2** may be due the difference of antibacterial

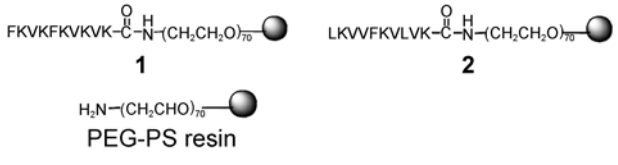

Figure 1. Structure of peptide-resin conjugates and resin.

Table 1. Antibacterial activities of conjugates and peptides[a]

Name		Minimum inhibitory concentration (μg/mL)			
		Micrococcus luteus (ATCC 9341)	*Staphylococcus aureus* (ATCC 6538)	*Escherichia coli* (ATCC 25922)	*Pseudomonas aeruginosa* (ATCC 9027)
Conjugate **1**	-	100	25	200	100
	Shaking	100	25	200	100
Conjugate **2**	-	> 400	> 400	> 400	> 400
	Shaking	> 400	> 400	> 400	> 400
β sheet peptide	-	1.56	3.12	6.25	3.12
	Shaking	3.12	1.56	6.25	3.12
α helical peptide	-	1.56	1.56	25	50
	Shaking	1.56	3.12	25	50
Magainin II	-	50	50	25	50

[a]The size of PEG-PS resin is 90 μm and the peptide loading level of conjugate **1** is 0.14 mmol/g. The shaking speed is 94-100 RPM.

Table 2. Antibacterial activities of conjugates and peptides in the presence of plasma protein (12.5%, w/w)

Name	Minimum inhibitory concentration (μg/mL)			
	M. luteus (ATCC 9341)	*S. aureus* (ATCC 6538)	*E. coli* (ATCC 25922)	*P. aeruginosa* (ATCC 9027)
Conjugate **1**	100	100	200	200
Conjugate **2**	> 400	> 400	> 400	> 400
β sheet peptide	6.25	6.25	12.5	12.5
α helical peptide	12.5	6.25	100	> 200
Magainin II	100	100	200	200

mechanism between α helical antibacterial peptides and β sheet antibacterial peptides. α helical antibacterial peptides inhibited the growth of bacteria by ion channel formation in lipid membranes of bacteria, whereas β sheet antibacterial peptides did their antibacterial action by shallow penetration into lipid membranes of bacteria.[3] The conjugation of α helical antibacterial peptides may prevent the ion channel formation of the peptide in lipid membranes of bacteria. Conjugate **1** and **2** did not show hemolytic activity up to 400 μg/mL, whereas mellitin (25 μM) caused 100% lysis of erythrocytes.

The antibacterial activities of most antibacterial agents have been decreased in the presence of serum proteins.[13] Thus, we investigated the antibacterial activity of conjugate **1** in the presence of serum proteins. As shown in Table 2, the activities of the antibacterial β sheet peptide toward bacteria were 2- to 8-fold reduced in the presence of serum proteins (12.5%, w/w). The binding of cationic antibacterial peptides to serum proteins might result in the decrease of the effective concentration of the peptides for bactericidal activity.[13] However, the antibacterial activities of the immobilized β sheet peptide (conjugate **1**) toward bacteria were not considerably decreased in the presence of serum proteins compared to those of the unconjugated β sheet peptide and well-known antibacterial peptide, magainin II measured in the presence of serum proteins. Albumin among plasma proteins played an important role in the binding and transporting of fatty acid, peptides, and proteins.[14] Albumin induced a considerable increase of MIC of a small cationic antibacterial peptide for *S. aureus*.[15] Albumin was known to have no effect on the growth of bacteria and the decrease of antibacterial activity of the peptide was mainly due to competing binding of the peptide to albumin.[16] Thus, we measured antibacterial activity of conjugate **1** in the presence of human serum albumin.

As shown in Table 3, the presence of serum albumin (800

Table 3. Antibacterial activities of conjugates and peptides in the presence of serum albumin (800 μg/mL)

Name	Minimum inhibitory concentration (μg/mL)			
	M. luteus (ATCC 9341)	*S. aureus* (ATCC 6538)	*E. coli* (ATCC 25922)	*P. aeruginosa* (ATCC 9027)
Conjugate **1**	100	25	50	100
Conjugate **2**	> 400	> 400	> 400	> 400
β sheet peptide	1.56	3.12	12.5	6.25
α helical peptide	3.12	6.25	50	100
Magainin II	100	100	100	100

μg/mL) induced the increase of MIC values of the β sheet antibacterial peptide by 2 or 4 times, whereas the conjugate **1** retained antibacterial activity in the presence of albumin. The result indicated that non-specific binding of the β sheet antibacterial peptide to albumin or serum proteins was prevented by conjugation to PEG-PS resin.

Bacteria Growth in the Presence of Conjugate 1 as a Function of Time. We investigated inhibition activity of conjugate **1** on the growth of bacterial cells. Figure 2 displayed the bacterial growth in the presence of conjugate **1** as a function of time.

The growth of *S. aureus* was fully suppressed for 30 hrs by conjugate **1** at low concentration (6.25-12.5 μg/mL). *S. aureus* has not grown for 48 hrs in the presence of conjugate **1** (25-50 μg/mL), which indicated that conjugate **1** irreversible inhibited the growth of *S. aureus* at the same concentration as the MIC. The inhibition of the growth by conjugate **1** was rapid and concentration dependent. The growth inhibition of *M. luteus*, *E. coli*, and *P. areuginosa* by conjugate **1** was also observed at lower concentration than MICs. The maximum number of bacterial cells grown in the presence of conjugate **1** at the lower concentration than MIC was much lower than that of bacterial cells grown in the absence of conjugate **1**. No growth of bacteria such as *M. luteus*, *E. coli*, and *P. areuginosa* was monitored in the presence of conjugate **1** at MICs, which supports that conjugate **1** has the same minimum bactericidal concentration and minimum inhibition concentration.

Synergistic Effects of Conjugate 1 with Current Antibacterial Agents. As CAPs might act on lipid membranes of microorganism, CAPs exhibited the potent synergistic effect with current antibacterial agents. We investigated the synergistic effect of conjugate **1** with vancomycin and tetracycline (Table 4). The MIC of vancomycin for *M. luteus* was 3 μg/mL, whereas 0.125 μg/mL vancomycin completely inhibited the growth of *M. luteus* in the presence of 0.39 μg/

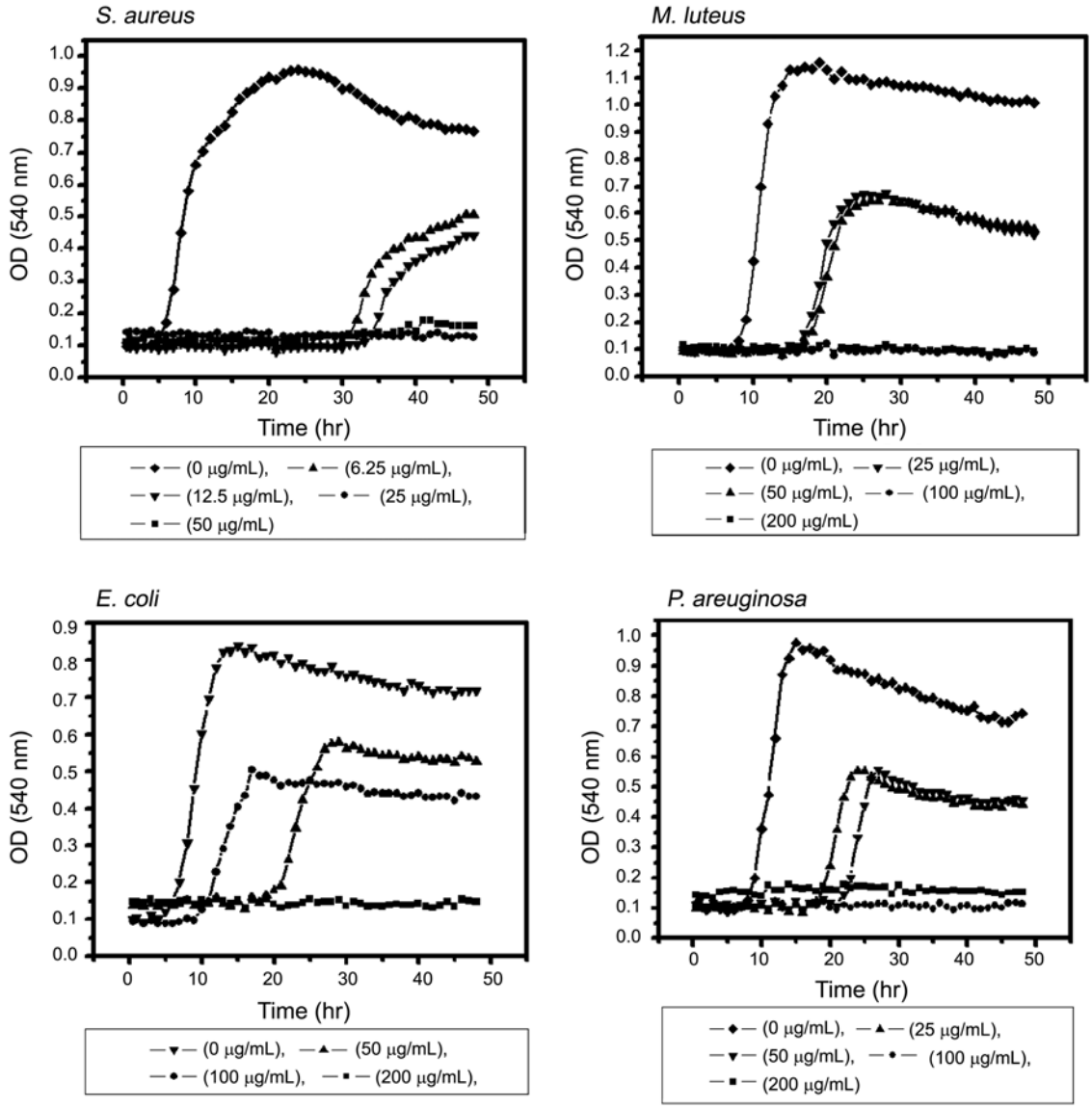

Figure 2. The growth of bacteria in the presence of conjugate **1**.

Table 4. FIC indexes for conjugate **1** in combination with current antibiotics

Organism	Current antibiotics	FIC index
M. luteus (ATCC9341)	Tetracycline	0.050
	Vancomycin	0.050
S. aureus (ATCC6538)	Tetracycline	0.078
	Vancomycin	0.13

mL conjugate **1**. This result revealed that in the presence of 0.39 μg/mL conjugate **1**, the activity of vancomycin for *M. luteus* was increased by 24 fold. The fractional inhibitory concentration (FIC) index of conjugate **1** for vancomycin against *M. luteus* was calculated as 0.05.[17]

The MICs of tetracycline for *S. aureus* and *M. luteus* were 0.5 μg/mL, respectively. Conjugate **1** showed a potent synergistic effect with tetracycline; 0.0078 μg/mL tetracycline completely inhibited the growth of *M. luteus* in the presence of 3.12 μg/mL conjugate **1**. The fractional inhibitory concentration (FIC) index of conjugate **1** for tetracycline against *S. aureus* and *M. luteus* was calculated as 0.078 and 0.05, respectively. Generally, an FIC value below 0.5 indicates that both antibacterial agents have a synergistic effect. For example, FIC values of various antibacterial agents for several bacteria ranged from 0.03 to 2.[18] The pretty low FIC index (0.05) indicated that the conjugate **1** had potent synergistic effects with current antibiotics such as vancomycin and tetracycline .

Conclusions

We synthesized peptide-resin conjugates by immobilizing cationic antibacterial peptides on resin and characterized their antibacterial activities in various conditions and synergistic effects with current antibiotics. Conjugate **1** showed antibacterial activity without hemolytic activity and potent synergistic effects with vancomycin and tetracycline. Conjugate **1** maintained antibacterial activity in the presence of serum proteins and albumin. Furthermore, conjugate **1** inhibited the growth of various bacteria at the lower concentration that MICs. Our results indicate that polymer surface modification using antibacterial β sheet peptide is a powerful way to prevent microbial contamination on polymer surfaces.

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