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Design and synthesis of novel antibacterial peptide-resin conjugates

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Abstract—We synthesized a novel peptide-resin conjugate by immobilizing β-sheet antibacterial peptide on PEG-PS resin. The peptide-resin conjugate, similar to cationic antimicrobial peptides, demonstrated unique properties such as potent antibacterial activity, no hemolytic activity, lipid membrane perturbation activity, and potent synergism with vancomycin. Specially, the peptide-resin conjugate showed a more increased lipid membrane perturbation activity in comparison to unbound β-sheet antibacterial peptide.

Over the past two decades, more than 700 cationic antimicrobial peptides (CAPs) have been isolated from the host defense peptides of invertebrate and vertebrate sources.[1,2] CAPs are positively charged at physiological pH and able to adopt an amphipathic α helical or β-sheet structure upon association with lipid bilayers.[2] Even though the mode of the action of CAPs was not fully elucidated, these peptides are supposed to act on the cytoplasmic membranes of microorganisms and thereby increase the permeability of lipid bilayers.[3] As they feature several improvements to current antibiotics, such as fast killing, bactericidal activity, broad antimicrobial spectra, and a great synergism with currently available antibiotics, CAPs have attracted considerable research attention as potential and novel antimicrobial agents.

Microbial adsorption and proliferation on polymer surfaces and possible host infection are additional major concerns in the field of medical devices.[4] Many efforts have been made to lower microbial adsorption and to prevent microbial contamination on polymer surfaces.[5] Immobilization of antimicrobial agents by covalent bonding into polymer surface was one of general methods to prevent microbial contamination on polymer surfaces. As CAPs have several improved properties over current antibiotics, CAPs were immobilized into resins to investigate surface activity[6–8] and various polymers that mimicked the structure of CAPs were synthesized.[9] Doele et al. have reported the conjugation of α helical antibacterial peptides into a water insoluble resin.[6] Even though the peptide-resin conjugate showed antibacterial activities, the activities of the conjugates were decreased at least 50-fold in comparison to the activity of the unconjugated peptides and the minimum inhibition concentrations (MICs) for various bacteria were greater than 1000 μg/mL. In addition, the cytotoxicity of the conjugates was not reported. Antimicrobial oligomers were synthesized by coupling reaction of polymaleic anhydride chain with antimicrobial tetrapeptides.[8] The oligomers exhibited a marginal antibacterial activity with a weak hemolytic activity but the MICs for bacteria were not reported. Most importantly, the resin peptide conjugates and the oligomers did not show the unique features of CAPs such as the increased permeability of lipid membranes and potent synergism with current antibiotics.

In the present study, we chose amphipathic β-sheet peptides (**4**, Phe-Lys-Val-Lys-Phe-Lys-Val-Lys-Val-Lys) as the immobilization candidate instead of amphipathic α helical peptides and conjugated the peptide into water swelling PEG-PS resins and investigated the properties of the peptide-resin conjugate. The β-sheet peptide is an analog of antibacterial decapeptide identified by using synthetic combinatorial library technology.[10] This peptide exhibits a potent antibacterial activity without hemolytic activity and has a weak, nonspecific binding for serum proteins in comparison to α helical peptides.[11] To improve the access of immobilized peptides for target microorganisms and to prevent nonspecific peptide

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binding, the peptide is immobilized on PEG-PS resin.[12] Figure 1 shows the structures of the peptide-resin conjugates (**1**–**3**) and peptides (**4**, **5**).

We synthesized peptide-resin conjugates **1** and **2** by immobilizing **4** and **5** on PEG-PS resin (90 μm), respectively. A scramble peptide **5** has the same amino acid composition as the β-sheet peptide but peptide **5** exhibited neither antimicrobial activity nor secondary structure.[11] To investigate the effect of the resin on the activity, the β-sheet peptide was conjugated into MBHA resin (conjugate **3**). Peptide-resin conjugates were synthesized by using Fmoc-chemistry. When we synthesized the peptide-resin conjugate, 1% (mole ratio) of Rink amide MBHA resin was mixed with PEG-PS resin or MBHA resin, respectively, to characterize its synthesis (Scheme 1). The coupling reaction of the amino acids to the resin was repeated until no color change in ninhydrin test was observed. The deprotection 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 resin was cleavaged at this step and among the PEG-PS or MBHA resins the peptides separated from the Rink amide MBHA resin were 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.[13] HPLC and mass spectra results revealed the successful synthesis and purity (>95%) of the conjugates **1** and **2**. HPLC indicated the successful synthesis and purity (>95%) of the conjugate **3**, however, mass spectrum revealed that conjugate **3** included the desired peptide and the small amount of the shorter, deleted peptide. Thus, the activities measured by the conjugate **3** reflected the combined activities of the desired peptide and the shorter deleted peptide.

We measured antibacterial activities of the peptide-resin conjugates and peptide using a modified microdilution method.[14] We calculated the quantity of peptides bound on the resin based on the Fmoc titration method.[15] When we measured antibacterial activity, we added the amount of the conjugate into the medium volume on the basis of calculated values of the total peptide quantity on the resin. Table 1 shows the antibacterial activities of the conjugates and peptides. The MICs of the β-sheet peptide are similar to the previous reported values.[11] Conjugate **1** showed a considerable antibacterial activity. The MICs of conjugate **1** ranged from 25 to 200 μg/mL. The antibacterial activities of conjugate **1** were decreased by 8- or 64-fold in comparison to those of unbound peptide **4**. The activity of conjugate **1** was comparable to that of magainin II, a well-known antibacterial peptide. The lack of activity of conjugate **2** (scramble peptide immobilized to PEG-PS resin) up to 400 μg/mL indicated that the amphipathic β-sheet structure is a critical factor for the activity. Conjugate **3** (β-sheet peptide immobilized to MBHA resin) showed no activity, indicating that water swelling property of PEG-PS resin is a critical factor for maintaining the activity of immobilized peptides. To check the cytotoxicity against mammalian cells, conjugate **1** was added to erythrocytes and the level of hemolysis was measured (Fig. 2). Nonhemolytic peptide **4** showed no hemolytic activity in this condition, whereas the cytotoxic peptide, melittin,[16] caused 100% lysis at concentrations exceeding 25 μg/mL. The conjugate **1** showed no hemolytic activity up to 200 μg/mL.

To investigate whether or not the conjugates act on lipid membranes, we investigated interactions between the conjugate with large unilamellar vesicles (Fig. 3). As bacterial lipid membranes were negatively charged, we prepared large unilamellar vesicles (LUVs) consisting of phosphatidylglycerol (PG) as previously described in the literature.[17] After the addition of the conjugates or peptides, the release of fluorescence dye, calcein encapsulated in vesicle, was determined by measuring

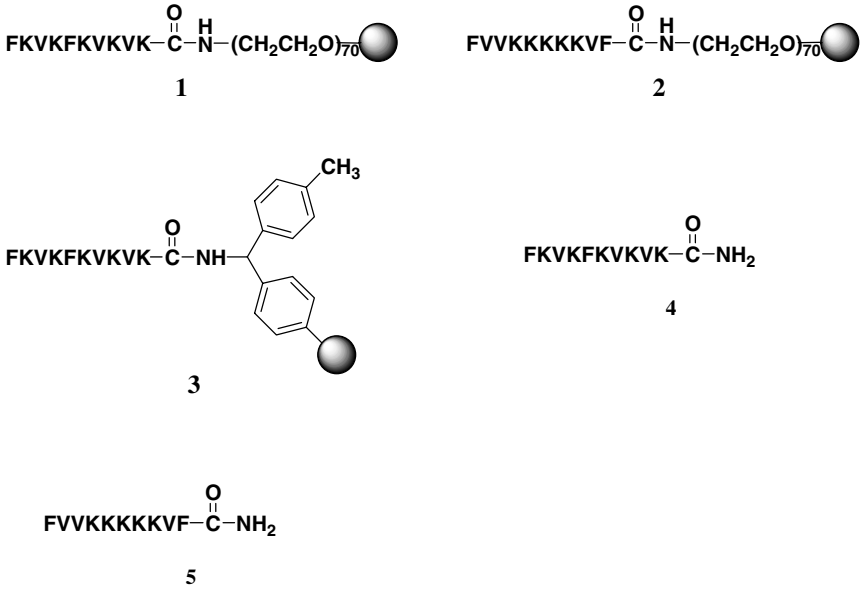
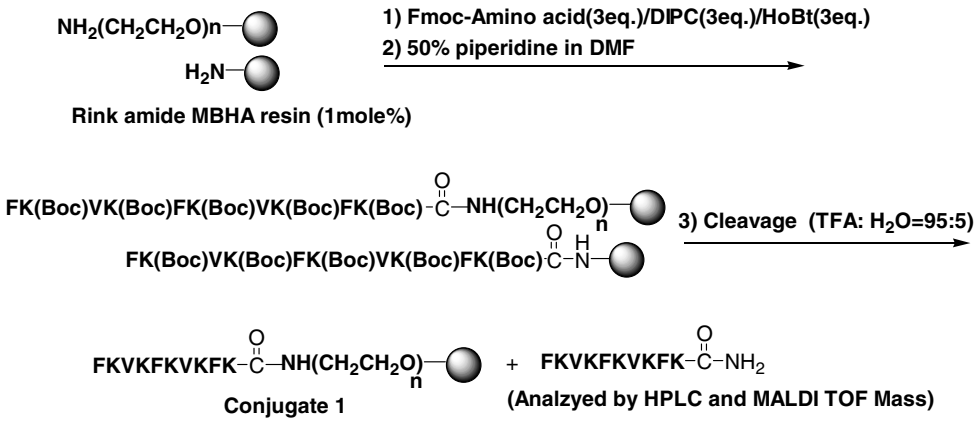


Figure 1. Structure of peptide-resin conjugates and peptides.



Scheme 1. Synthesis scheme of conjugate 1.

Table 1. Antibacterial activities of peptide-resin conjugates and peptides

Name	Minimum inhibitory concentration (μg/mL)			
	Staphylococcus aureus (ATCC 6538)	*Micrococcus luteus* (ATCC 9341)	*Pseudomonas aeruginosa* (ATCC 9027)	*Escherichia coli* (ATCC 25922)
1	100	25	200	100
2	>400	>400	>400	>400
3	>400	>400	>400	>400
4	1.56	3.12	6.25	3.12
5	>400	>400	>400	>400
Magainin II	50	50	25	50

The size of PEG-PS resin bead is 90 μm and the peptide loading levels of conjugates **1** and **2** are 0.14 mmol/g. The peptide loading level of conjugate **3** is 0.50 mmol/g.

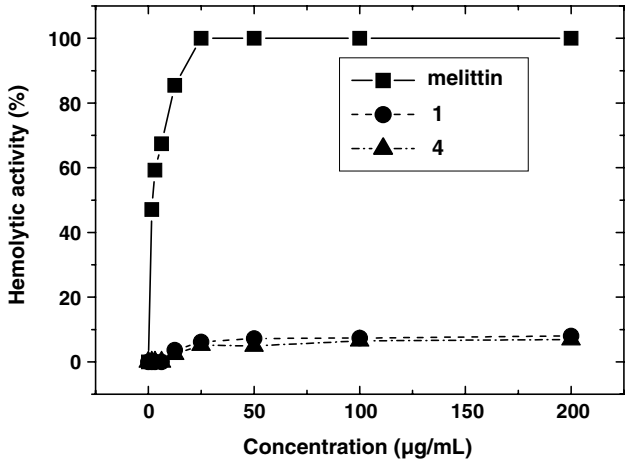


Figure 2. Hemolytic activity.

the decrease of self-quenching on Perkin-Elmer LS55 Luminescence spectrometer equipped with stirrer (Ex: 490 nm, Em: 520 nm). As shown in Figure 3, peptide **4** induced a leakage in a concentration dependent manner. Interestingly, conjugate **1** induced a leakage, whereas conjugates **2** and **3**, with no antibacterial activity, did not in the same condition (data not shown). This result suggests that conjugate **1** acts on the lipid membrane of microorganisms. Even though conjugate **1** induced dye release from the vesicles, the leakage potency and dye release kinetic were somewhat different from those induced by the soluble peptide **4**. It takes a relatively long time for conjugate **1** to induce 100% release of entrapped dye in comparison to the time for the peptide to induce 100% release. The initial leakage rate was dependent on the quantity of the bound peptide on the resin; however, the leakage rate in the middle was similar regardless of the quantity. Interestingly, conjugate **1** induced 100% release of entrapped dye, regardless of the resin-bound peptide quantity. The conjugated peptide on the resin, unlike a soluble peptide, demonstrated sustained leakage power. It seemed to be necessary to elucidate the detailed mode of the action of the β-sheet peptide and conjugate **1**. The mode of the action of the peptide and conjugate **1** is under current investigation.

The synergistic effect of conjugate **1** with vancomycin was investigated against Gram-positive bacteria strain, *Staphylococcus aureus* (*S. aureus*) because vancomycin exhibits activity only against Gram-positive bacteria. Vancomycin is the antibiotic of last choice for the treatment of life-threatening infections with methicillin-resistant *S. aureus*.[18] The MIC of vancomycin for *S. aureus* was 3 μg/mL, whereas 0.0156 μg/mL vancomycin completely inhibited the growth of *S. aureus* in the presence of 3.12 μg/mL conjugate **1**, as shown in Table 2. Interestingly, this result revealed that in the presence of 3.12 μg/mL conjugate **1**, the activity of vancomycin for *S. aureus* was increased by 190-fold. The fractional inhibitory concentration (FIC) index[19] of conjugate **1**

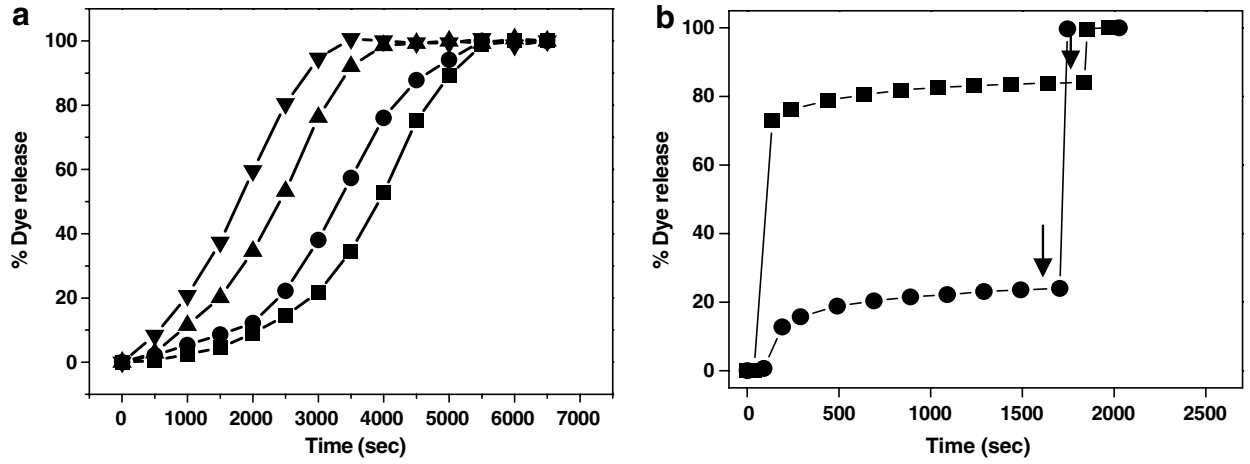


Figure 3. Dye release percentage from PG LUVs induced by (a) conjugate **1** (■, 5.0 nmol; ●, 6.0 nmol; ▲, 39.0 nmol; ▼, 160 nmol) and (b) peptide **4** (●, 0.8 μM; ■, 8.0 μM). The samples were added into 40 μM LUVs in Tris buffer solution (pH 7.4) at 22 °C. The arrow indicates the addition of TX-100.

Table 2. Synergistic activity of conjugate **1** and vancomycin against *S. aureus*

Conjugate **1** (μg/mL)	*Vancomycin* (μg/mL)					
	0.25	0.125	0.0625	0.0313	0.0156	0.0078
6.25	−	−	−	−	−	−
3.12	−	−	−	−	−	+
1.56	+	+	+	+	+	+
0.78	+	+	+	+	+	+
0.39	+	+	+	+	+	+
0.195	+	+	+	+	+	+

+, − indicate growth or no growth, respectively.

for vancomycin, against *S. aureus* was calculated as 0.036. Generally, an FIC value below 0.5 indicates that the two compounds have a synergistic activity. For example, FIC values of various antibacterial agents for several bacteria ranged from 0.03 to 2.[20] The pretty low FIC index (0.036) indicated that the conjugate **1** had the potent synergism with an important antibiotic, vancomycin against *S. aureus*. As the conjugate **1** may act on the lipid membranes of bacteria, the conjugate, similar to CAPs, is expected to show the potent synergism with other current antibacterial agent.

In the present study, we synthesized a new peptide-resin conjugate and characterized its properties. To the best of our knowledge, this is the first report of the successful synthesis of a peptide-resin conjugate able to maintain the unique properties of CAPs such as antibacterial activity, no hemolytic activity, increased permeability of lipid membranes, and potent synergism with vancomycin. Our results indicate that β-sheet antibacterial peptide is a possible candidate for polymer surface modification to prevent microbial contamination.

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