One-Pot Synthesis of a Cyclic Antimicrobial Peptide-Conjugated Phthalocyanine for Synergistic Chemo-Photodynamic Killing of Multidrug-Resistant Bacteria

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Abstract: Multidrug-resistant (MDR) bacteria have posed a severe global health threat with high mortality. Among the emerging therapeutic methods, photodynamic therapy has shown to be a promising alternative with a negligible drug resistance reported so far. Herein, a robust one-pot approach was developed to synthesize a novel cyclic antimicrobial peptide-conjugated zinc(II) phthalocyanine-based photosensitizer. Our results showed that the novel conjugate composed of a cyclic bactenecin derivative and a photosensitizer could precisely target bacteria over mammalian cells. The conjugate also exhibited efficient antibacterial performance and a synergistic dual chemo- and photodynamic therapy against a broad spectrum of bacteria including ATCC-type strains and clinical isolates of MDR bacterial strains. These results suggest that our novel conjugate is a promising antibacterial agent to combat the menace of MDR bacteria. The abstract will be revised later

Multidrug-resistant (MDR) bacteria have become a serious threat to public health that is accounting for at least 700,000 deaths globally a year.[1] Apart from developing new antibiotics to combat these microorganisms, other therapeutic agents and alternative treatment modalities have also been explored, in particular those that involve different antimicrobial mechanisms.[2] Among the different strategies being studied, photodynamic therapy (PDT) has received considerable attention.[3] It relies on the uptake of a photosensitizer by target microbials. Upon light irradiation, the excited photosensitizer is able to transform molecular oxygen to highly cytotoxic reactive oxygen species (ROS), particularly singlet oxygen, for oxidizing cellular components, disrupting cell membrane, and damaging proteins and DNA. Compared to the conventional antibiotic-based therapy, antimicrobial PDT has the advantages of a broad spectrum of activity, an immediate onset of action, less adverse effects, and the unlikelihood of inducing resistance due to the multitargeted and nonspecific mode of action.

To enhance the binding with bacteria, cationic moieties are usually introduced to the photosensitizers. Through electrostatic interactions, they can bind strongly with the highly negatively charged outer membrane components, namely lipoteichoic acids and lipopolysaccharides of Gram-positive and Gram-negative bacteria, respectively.[4] This structural feature is particularly important for eradication of Gram-negative bacteria as they have an extra outer membrane composed of lipopolysaccharides that renders them generally less susceptible to the treatment than Gram-positive bacteria.[5] The cationic moieties can also

increase the solubility and reduce the aggregation of hydrophobic photosensitizers in aqueous media that would otherwise hinder the generation of ROS and eventually the therapeutic efficacy.[6] A number of cationic photosensitizers based on phthalocyanines,[7] porphyrins,[8] boron dipyrromethene derivatives,[9] chalcogenoviologens,[10] and aggregation-induced-emissive tetraphenylethenes[11] have been reported which generally show high antimicrobial activity against a spectrum of microbials, including the MDR ones.

As another approach to improve the properties and activities of photosensitizers, antimicrobial peptides (AMPs) have also been used as an active component. This special class of peptides generally exhibit an amphipathic structure consisting of both cationic and hydrophobic amino acid residues, which facilitates the binding and insertion to the membrane of bacteria, causing membrane penetration, disruption, and pore formation.[12] As a result, they have been widely recognized as promising antimicrobial agents. In fact, some of these peptides are FDA-approved and have been used clinically.[13] The conjugation of AMPs to photosensitizers not only can enhance their water solubility, but more importantly can impart a bacteria-targeting property, improve the potency, and reduce the nonspecific interactions with mammalian cells. Various AMPs such as apidaecin 1b,[14] $(KLAKLAK)_2$,[15] YI13WF,[16] and polymyxins[17] have been conjugated with photosensitizers to improve their bacteria targeting and bactericidal activities. However, most of these examples involve linear peptides which are subject to proteolytic degradation, resulting in low bioavailability.[18] The cyclic counterparts

are generally more resistant toward proteolysis and therefore are regarded as better candidates.[19] The cyclic lipopeptide polymyxins, for example, are very potent AMPs being regarded as the last therapeutic option for MDR bacterial infections. Unfortunately, the resistance to polymyxins has been increasing reported and these antibiotics are associated with nephrotoxicity.[20] Furthermore, the synthesis of polymyxins is challenging as it involves the use of unnatural amino acids and stringent cyclization conditions.[21] As a result, there has been a great demand of potent cyclic AMPs and efficient methodologies for their synthesis and conjugation with photosensitizers that can empower antimicrobial PDT for treating MDR bacterial infections.

Cyclic peptides are usually prepared by intramolecular cyclization through amide bond formation and metal-catalyzed coupling.[22] These synthetic approaches, however, involve the use of unnatural amino acids and relatively stringent and harsh reaction conditions, usually resulting in low efficiency. Recently, an efficient peptide cyclization strategy has been reported which involves site-selective alkylation of the sulfhydryl side chains of two cysteine residues with a bis(bromomethyl)benzene unit to form a monocyclic structure.[23] Based on this cyclization strategy, we report herein a facile one-pot approach to synthesize a bactenecin-conjugated phthalocyanine-based photosensitizer. Bactenecin (RLCRIVVIRVCR) is a β-hairpin cyclic AMP composed of a central hydrophobic loop and positive charges located at both ends that are brought in proximity by a disulfide bridge.[24] The cationic character strongly

hitches the peptide to negatively charged bacterial surface, while the hydrophobic loop interacts with cytoplasmic membrane, inducing perturbation of the inner membrane of bacteria.[25] This peptide exhibits a broad spectrum of action and the cyclic structure is important for the antibacterial activity against Gram-negative bacteria.[26] It was reported that its analogue RRLCRIVWVIRVCRR (**Bac**) with additional two arginine and one tryptophan residues showed enhanced antibacterial activity,[26a] and hence this sequence was employed. By using a bifunctional linker with a bis(bromomethyl)benzene unit and a cyclopentadiene moiety, the cyclization of this linear peptide sequence through the aforementioned site-selective alkylation of the two cysteine residues and further conjugation with a maleimide-substituted phthalocyanine via the maleimide-cyclopentadiene Diels-Alder reaction could be achieved in situ effectively (Figure 1). Owing to various desirable characteristics, zinc(II) phthalocyanines have been well documented as superior photosensitizers.[27] It was found that the two therapeutic components could induce synergistic chemo- and photodynamic cytotoxic effects against a broad spectrum of bacterial strains, including clinical isolates of MDR bacterial strains. To our knowledge, only a few examples of cyclic AMP-photosensitizer conjugates have been reported[17,28] and the synergistic actions of the two components have rarely been investigated.[29] Please check if these are the only examples

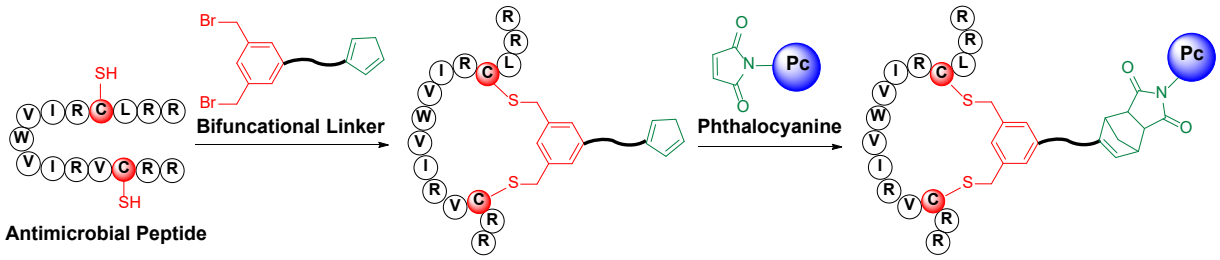


Figure 1. Schematic representation of the one-pot AMP cyclization and phthalocyanine conjugation.

This in situ cyclization-coupling process involves three components. The antimicrobial peptide RRLCRIVWVIRVCRR (**Bac**) was prepared manually by using a modified 9-fluorenylmethoxycarbonyl solid-phase peptide synthesis protocol. The bifunctional linker cyclopentadiene-substituted bis(bromomethyl)benzene **Cp-BB** was prepared by nucleophilic mono-substitution of 1,3,5-tris(bromomethyl)benzene with sodium cyclopentadienylide. The maleimide-substituted phthalocyanine **Mal-Pc** was synthesized according to Scheme S1 in Supporting Information, which involved mixed cyclization of two phthalonitriles, deprotection of the amino group, followed by condensation with 6-maleimidohexanoic acid. The course of the preparation of the target conjugate **cBac-Pc** as monitored by HPLC is shown in Figure 2. The linear peptide **Bac** (1 mM) was first treated with **Cp-BB** (1 mM) in a mixture of *N*,*N*-dimethylformamide (DMF) and borate buffer (pH 10.0) (1:1, v/v). After 1 h, a new peak corresponding to the cyclopentadiene-substituted cyclic peptide **Cp-cBac** was observed in the HPLC chromatogram with a high percentage of conversion. Without purification, the

maleimide-substituted phthalocyanine **Mal-Pc** was added to afford the target conjugate **cBac-Pc** via a Diels-Alder reaction in 79% HPLC yield after stirring at ambient temperature overnight. The product was isolated using HPLC in 27% yield and characterized with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. For comparison, the cyclic Bac (**cBac**) without the phthalocyanine moiety was also prepared similarly by treating the linear peptide **Bac** with 1,3-bis(bromomethyl)benzene. The experimental details are given in Supporting Information. Any comparison that you can make with other cyclic peptide syntheses?

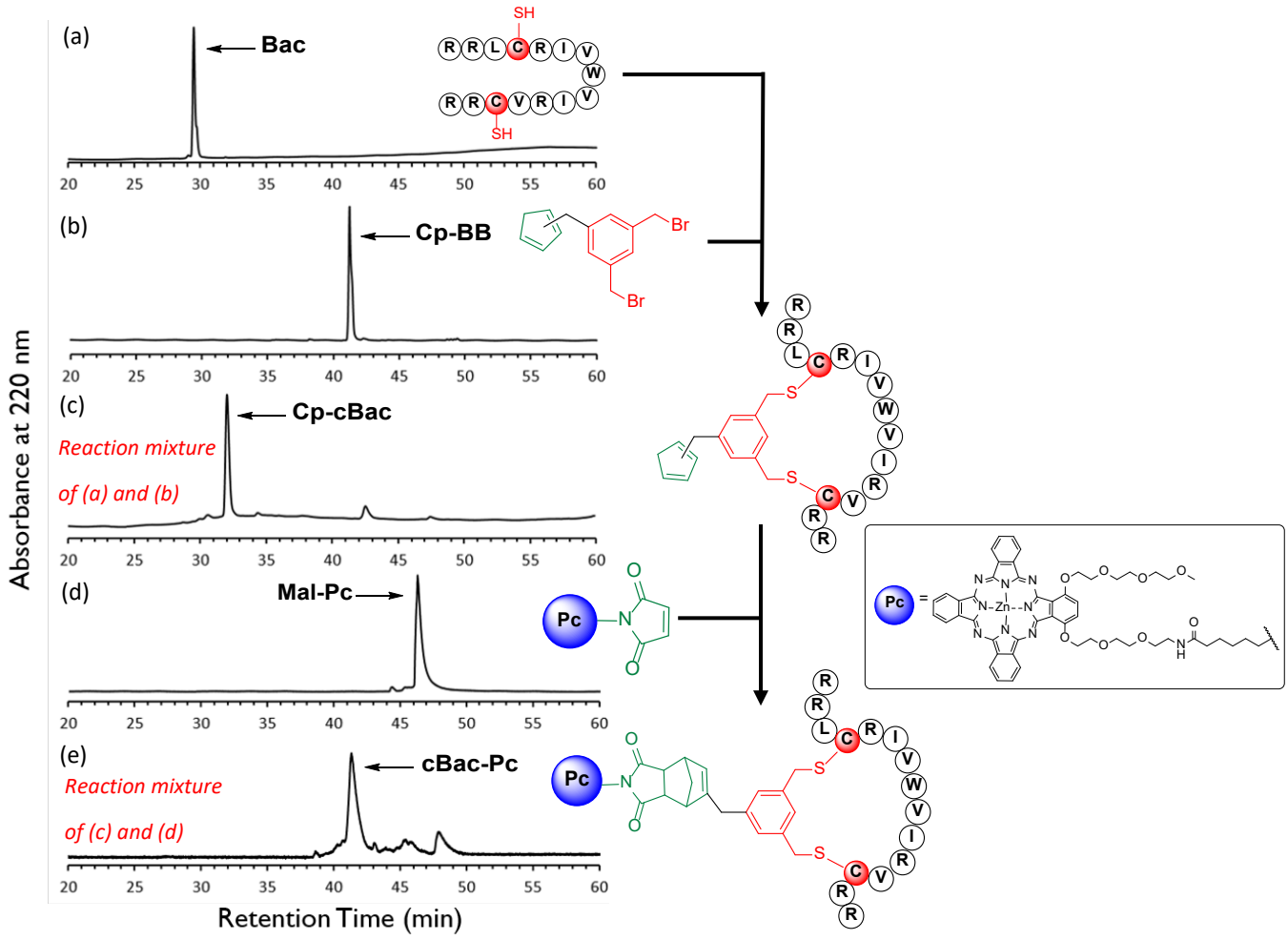


Figure 2. Monitoring the course of the formation of **cBac-Pc** using HPLC.

The electronic absorption and fluorescence spectra of **cBac-Pc** were measured in DMF and phosphate buffered saline (PBS) at pH 7.4 with Tween 80 (0.1%, v/v) added to increase the water solubility of the conjugate, using **Mal-Pc** as a reference compound for comparison (Figure S1). Both compounds gave typical absorption spectra of non-aggregated phthalocyanines in DMF, showing an intense and sharp Q-band at 688 nm, which strictly followed the Lambert-Beer's law (Figure S2). The Q-band was slightly diminshed in PBS for both compounds. Upon excitation at 610 nm, both compounds showed a fluorescence emission at ca. 700 nm. The fluorescence quantum yield (Φ_F) in DMF was determined to be 0.20 (for **cBac-Pc**) and 0.21 (for **Mal-Pc**), relative to the unsubstituted zinc(II) phthalocyanine (ZnPc) (Φ_F = 0.28 in DMF).[7a] To evaluate the efficiency of these compounds in generating singlet oxygen, 1,3-diphenylisobenzofuran (DPBF) was used as the singlet oxygen scavenger and the absorbance of DPBF at 415 nm was monitored spectroscopically along with time. It was found that both compounds could induce photodegradation of DPBF in both media (Figure S3). The singlet oxygen quantum yields (Φ_Δ) of **cBac-Pc** and **Mal-Pc** were found to be 0.58 and 0.60, respectively, relative to ZnPc (Φ_Δ = 0.56 in DMF).[7a] All these data are summarized in Table 1, which shows that the additional cyclic peptide of **cBac-Pc** did not cause significant effects on the spectroscopic and photosensitizing properties of the phthalocyanine core. The high

singlet oxygen generation efficiency, particularly in aqueous media, is important for the photobactericidal activities. We can comment on these spectral properties which are typical for 1,4-disubstituted ZnPc

Table 1. Electronic absorption and photophysical data for **cBac-Pc** and **Mal-Pc** in DMF.

Compound	λ_{abs} (nm) (log ε)	λ_{em} (nm)[a]	Φ_F[b]	Φ_Δ[c]
cBac-Pc	339 (4.72), 621 (4.54), 688 (5.30)	700	0.20	0.58
Mal-Pc	340 (4.73), 621 (4.54), 688 (5.30)	700	0.21	0.60

[a] Excited at 610 nm. [b] Using ZnPc as the reference (Φ_F = 0.28 in DMF). [c] Using ZnPc as the reference (Φ_Δ = 0.56 in DMF).

To investigate the bacteria-binding behavior of the conjugate **cBac-Pc**, ATCC-type Gram-negative *Escherichia coli* (*E. coli*) (ATCC 25922) and Gram-positive *Staphylococcus aureus* (*S. aureus*) (ATCC 25923) were used for the study. The bacteria were incubated with **cBac-Pc** or **Mal-Pc** used as the negative control for 30 min, and then their fluorescence images were examined using confocal laser scanning microscopy. As shown in Figure 3a, strong fluorescence of **cBac-Pc** was observed for both the bacterial strains, while the bacteria incubated with **Mal-Pc** showed negligible fluorescence. The results clearly indicated that the cyclic peptide in the conjugate could function as an effective bacteria-targeting ligand, promoting the binding toward these bacteria. The week fluorescence of **Mal-Pc** in bacteria could be attributed to the absence of cationic moieties, leading to weak binding interactions with the bacterial membrane.

A good antimicrobial agent should have high selectivity toward bacteria but low binding affinity toward the host. Hence, this ability of **cBac-Pc** was evaluated using a coculture method with RAW 264.7 murine macrophage cells as the model mammalian cells. As shown in Figure 3b, **cBac-Pc** could only adhere to the *E. coli* and *S. aureus*, displaying strong fluorescence in these bacteria, while fluorescence could hardly be observed around and inside the RAW 264.7 cells. These results further verified that the conjugate could selectively recognize and adhere to the bacteria, leaving the surrounding mammalian cells untouched, which is favorable for selective bacteria inhibition to minimize potential side effects. Make comparison with other AMP-photosensitizer conjugates

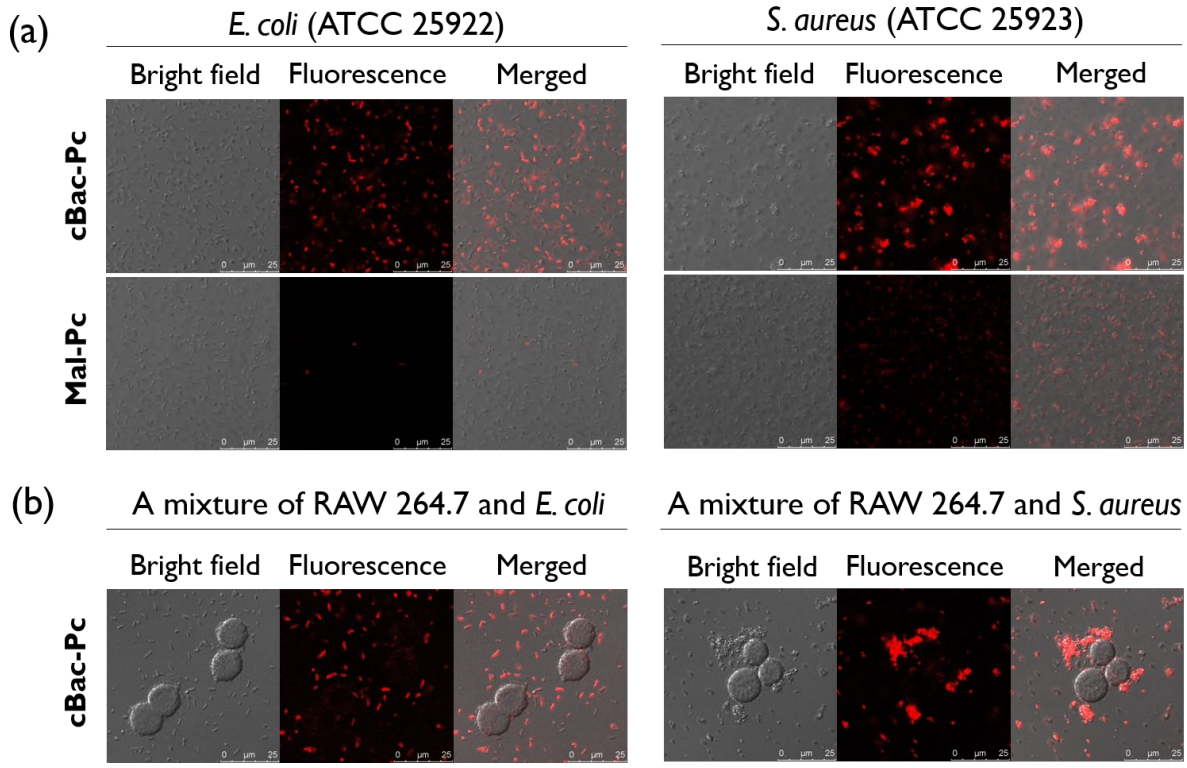


Figure 3. (a) Bright field, intracellular fluorescence, and the merged images of *E. coli* and *S. aureus* after incubation with **cBac-Pc** or **Mal-Pc** (0.5 μM) for 30 min. (b) Bright field,

intracellular fluorescence, and the merged images of a mixture of RAW 264.7 cells and *E. coli* or *S. aureus* after incubation with **cBac-Pc** (0.5 μM) for 30 min.

Being encouraged by the high selectivity toward bacteria of **cBac-Pc**, we further examined its antimicrobial activities. This study was performed against a panel of bacterial strains, including the aforementioned ATCC-type *E. coli* and *S. aureus*, as well as clinical isolates of MDR *E. coli* (19-117441) and methicillin-resistant *S. aureus* (MRSA) (19-364714), with or without light irradiation. The non-peptide-conjugated phthalocyanine **Mal-Pc** and the non-phthalocyanine-conjugated cyclic peptide **cBac** were used as the controls in order to reveal the dual therapeutic effects. A fix amount of bacteria suspension, i.e. 10^5 colony forming unit per mL (CFU mL^{-1}), was incubated with different concentrations of these compounds at 37 ^oC for 30 min, followed by light irradiation ($\lambda > 610$ nm, fluence rate = 21 mW cm^{-2}) for 5 min (for the two Gram-positive bacterial strains) or 30 min (for the two Gram-negative bacterial strains) or just leaving in the dark at room temperature. After these treatments, a standard plate colony-counting method was used to determine the percentage of live bacteria (Figure S4). Figure 4 shows the dose-dependent survival curves against the four bacterial strains expressed in the percentage of CFU mL^{-1}. For the Gram-negative *E. coli*, the dark antibacterial activity of **cBac-Pc** was similar to that of **cBac**, showing that the phthalocyanine core in **cBac-Pc** did not significantly affect the antimicrobial activity of the peptide after conjugation. Upon light

irradiation to activate the photodynamic process, the killing efficiency of **cBac-Pc** was significantly enhanced with a minimum inhibitory concentration (MIC), defined as the lowest concentration of a compound that could prevent visible growth of a microorganism at defined conditions, of 4 μM. This value is similar to those for the protophophyrin IX conjugated with two Y113WF peptides.[16] just give this as an example. Please find out more references to make the comparison. The inhibition ability of **Mal-Pc** was limited even upon light irradiation, which is consistent with the results of confocal microscopic study (Figure 3a), showing that **Mal-Pc** exhibited a low binding affinity to the bacteria. The ROS generated from **Mal-Pc** could not attack the bacterial membrane due to their short lifetime and action range.[30] For the Gram-positive *S.* aureus, the antibacterial activity of **cBac-Pc** in dark was slightly higher than that of **cBac**, showing an enhanced activity after conjugation with the phthalocyanine. Upon light irradiation, this conjugate exhibited a 10^5-fold reduction in CFU mL^{-1} and was able to kill all bacteria at 500 nM. The significantly higher potency of **cBac-Pc** toward the Gram-positive bacteria could be attributed to their lack of an extra outer membrane as the Gram-negative counterpart, making them more susceptible to the treatment.

To increase the clinical relevance, the antibacterial activity of **cBac-Pc** was also examined against two clinically isolated MDR bacterial strains. The results for the MDR *E. coli* are similar to those for the ATCC-type *E. coli* with a MIC value of 4 μM. It is worth noting that this MDR strain did not show resistant to both **cBac** and **cBac-Pc**, although it is resistant to

some commonly used antibiotics such as cefotaxime, cefuroxime, ciprofloxacin, and levofloxacin.[31] On the other hand, the potency of **cBac-Pc** against the clinically isolated MRSA was slightly lower than that of the ATCC-type *S. aureus*, showing a MIC value of 1 μM. However, this conjugate remained highly photocytotoxic against MRSA and the bacteria did not exhibit resistance toward this compound.

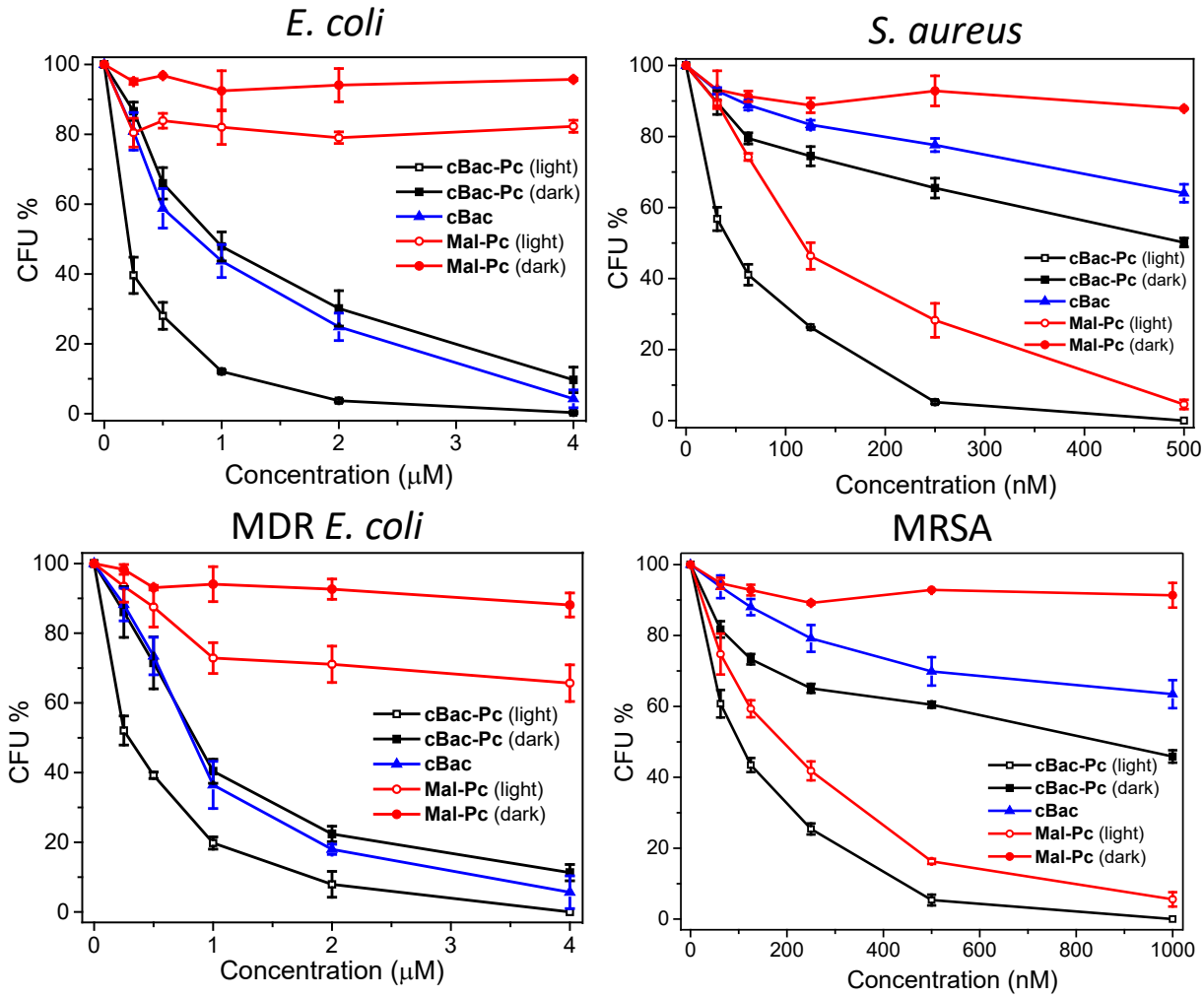
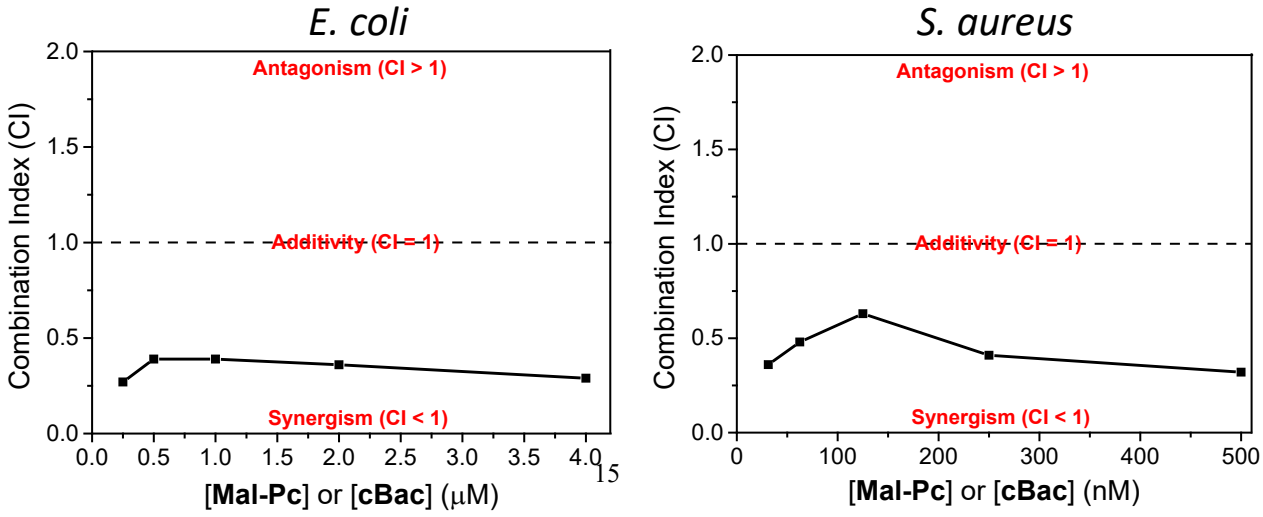


Figure 4. CFU inhibition of **cBac-Pc**, **cBac**, and **Mal-Pc** at different concentrations against *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923), clinically isolated MDR *E. coli* (19-117441),

and clinically isolated methicillin-resistant *S. aureus* (MRSA) (19-364714) with or without light irradiation. Data are expressed as the mean value ± standard error of the mean of three independent experiments, each performed in triplicate.

To reveal whether the two antibacterial components of **cBac-Pc** exhibited synergistic effects, the combination indices (CI) were calculated for all the four bacterial strains using the corresponding dose-dependent survival curves shown in Figure 4. A value of CI greater than, equal to, or less than unity indicates an antagonistic, additive, or synergistic effect, respectively.[32] It can be seen in Figure 5 that the CI values were less than unity for all the four bacterial strains, suggesting that the cytotoxic effect of the cyclic peptide and the photodynamic action of the phthalocyanine in **cBac-Pc** clearly work in a synergistic manner. Although many AMD-photosensitizer conjugates have been reported, the synergistic action of the two antimicrobial components has rarely been demonstrated.[29] This property is highly desirable for antimicrobial agents.



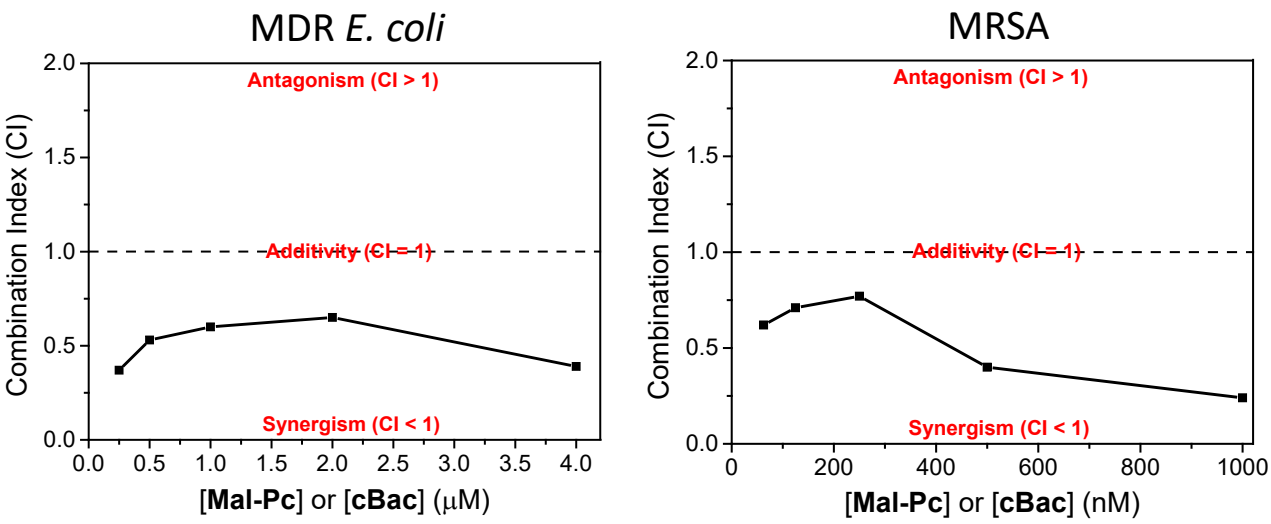
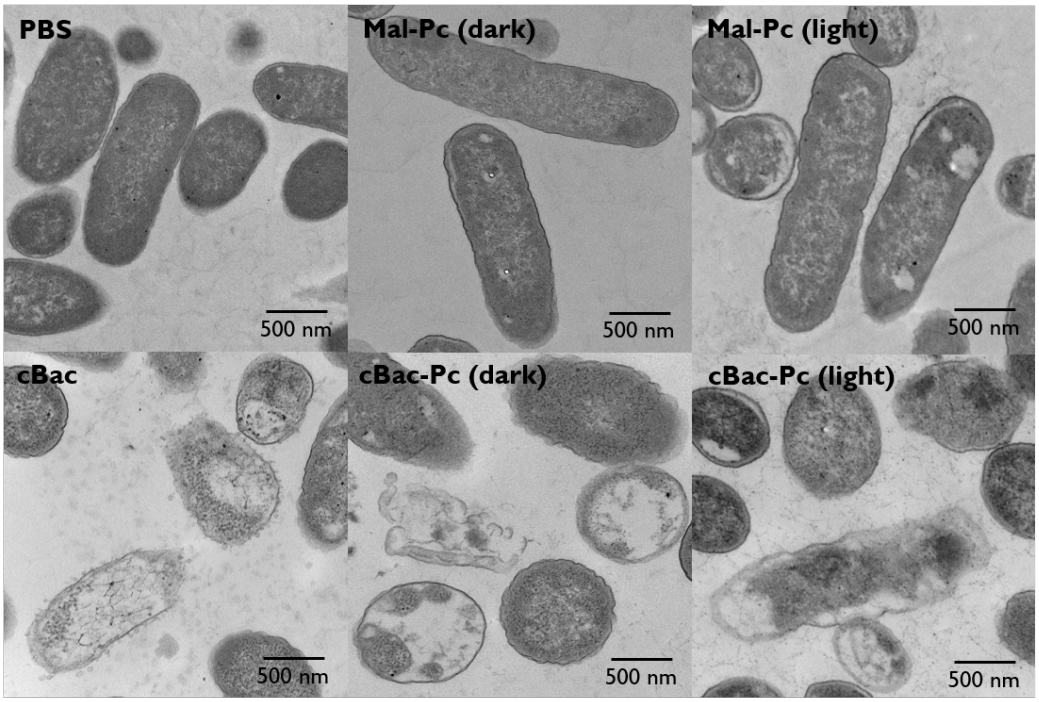


Figure 5. Combination indices calculated according to the dose-dependent survival curves for *E. coli*, *S. aureus*, MDR *E. coli*, and MRSA at different concentrations of **Mal-Pc** and **cBac** with a fixed ratio (1:1) in the conjugate **cBac-Pc**.

To better understand the antibacterial actions of **cBac-Pc**, the bacterial morphology of *E. coli* and *S. aureus* before and after the various treatments was also examined using transmission electron microscopy (TEM). As shown in Figure 6, both bacterial strains showed a smooth surface and an intact membrane for the PBS control group. Upon treatment with the non-peptide-conjugated **Mal-Pc**, the morphology of *E. coli* was not significantly changed both in the absence and presence of light irradiation. For *S. aureus*, while the bacterial cells remained intact in the dark, they were slightly fragmented after light irradiation. Upon treatment with **cBac** or **cBac-Pc** in the dark, a wrinkled membrane surface and bacteria fragmentation were observed. Upon further light irradiation after incubation with **cBac-Pc**, the damage was

exacerbated and debris was generated from the leakage of intracellular milieu. These results suggested that the high antibacterial property of **cBac-Pc** could be attributed to the physical damage on the bacterial cell wall induced by the cyclic peptide followed by *in situ* photodynamic action caused by the phthalocyanine core.

(a)



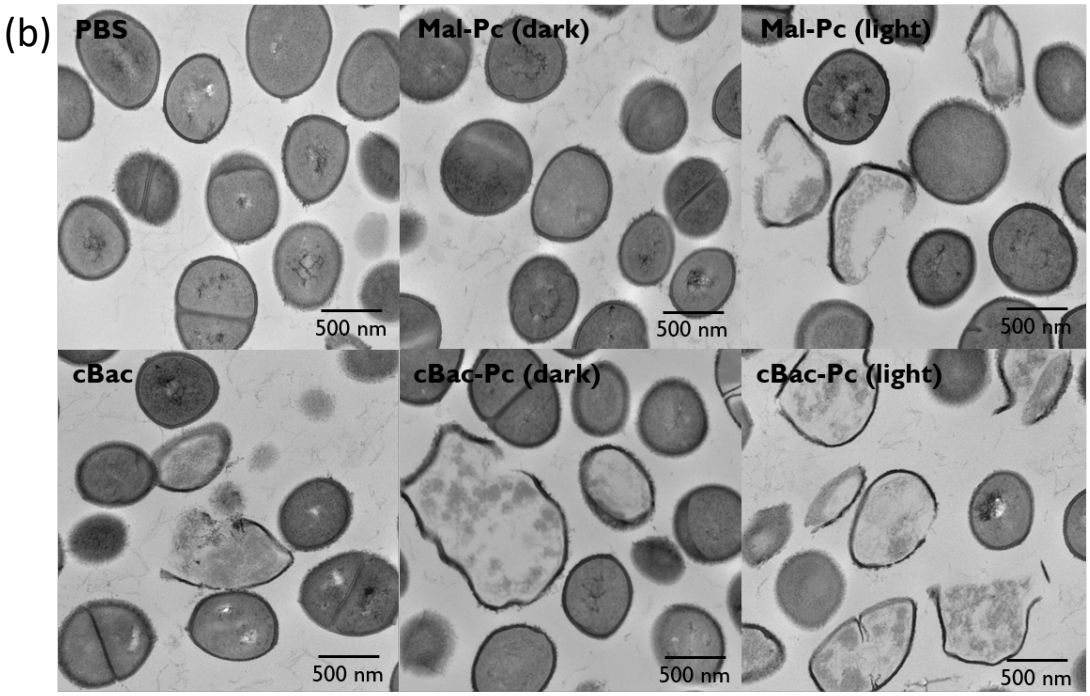


Figure 6. TEM images of (a) *E. coli* and (b) *S. aureus* after incubation with **cBac-Pc**, **cBac**, or **Mal-Pc** (at 2 μM for *E. coli* or 250 nM for *S. aureus*) for 30 min with or without light irradiation ($\lambda > 610$ nm, fluence rate = 21 mW cm^{-2}) for 30 min (for *E. coli*) or 5 min (for *S. aureus*).

Finally, the specificity of **cBac-Pc** between bacterial and mammalian cells was further examined by assessing its hemolytic property toward mouse red blood cells and its cytotoxicity against RAW 264.7 cells. Figure S5a shows the results for the former study, which also includes the results for the two control compounds **cBac** and **Mal-Pc** for comparison. It can be seen that **cBac-Pc** showed negligible hemolysis at 4 μM, which is about the MIC of this conjugate for *E. coli*, and the hemolysis was still less than 20% at 50 μM in the dark, though the conjugate exhibited slightly more hemolysis than **cBac** and **Mal-Pc**. For the cytotoxicity study (Figure S5b), the cell viability was only decreased by about 20% for all the three compounds at 50 μM

in the absence of light irradation. All the results suggest that **cBac-Pc** was not significantly toxic toward these mammalian cells at its effective dose, demonstrating its high biocompatibility.

In summary, we have synthesized a novel cyclic antimicrobial peptide-conjugated phthalocyanine through an in situ cyclization of a facile one-pot procedure. The conjugate (**cBac-Pc**) exhibited a high selective binding affinity toward bacterial over mammalian cells and demonstrated a high bactericidal activity against the Gram-negative *E. coli* and the Gram-positive *S. aureus*, together with two clinically isolated MDR bacteria. The inhibition mechanism was based on the synergy of physical destruction and photodynamic therapy. The results showed that this novel conjugate could serve as a promising therapeutic agent to combat bacterial infections with good biocompatibility toward mammalian cells. Will revise further later

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Conflict of interest

The authors declare no conflict of interest.

Keywords: antibacterial activity · cyclization · peptides · photodynamic therapy · phthalocyanines

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Table of Contents