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Abstract. Photodynamic antimicrobial chemotherapy (PACT) is an effective method for killing bacterial cells in view of the increasing problem of multiantibiotic resistance. We herein reported the PACT effect on bacteria involved in skin infections using a zinc phthalocyanine derivative, pentalysine β-carbonylphthalocyanine zinc (ZnPc-$(Lys)_5$). Compared with its anionic ZnPc counterpart, ZnPc-$(Lys)_5$ showed an enhanced antibacterial efficacy *in vitro* and in an animal model of localized infection. Meanwhile, ZnPc-$(Lys)_5$ was observed to significantly reduce the wound skin blood flow during wound healing, indicating an anti-inflammation activity. This study provides new insight on the mechanisms of PACT in bacterial skin infection. [DOI: 10.1117/1.JBO.21.1.018001]

Keywords: photodynamic antimicrobial chemotherapy; zinc phthalocyanine; bacterial infection; skin; *Propionibacterium acnes*; *Staphylococcus aureus*.

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1 Introduction

The most common bacterial infections, such as cellulitis, abscesses, and postsurgical infections, are usually caused by pathogens like *Staphylococcus aureus*[1] and *Propionibacterium acnes*[2] that may lead to serious local and systemic complications.[3] For example, *S. aureus* can cause a range of illnesses, from minor skin infections to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, bacteremia, and sepsis.[4] In the past 60 years, antibiotics have been critical in the fight against infectious disease caused by these bacteria and other microbes. However, the broad application of antibiotics leads to bacterial resistance and becomes an increasing public health problem. Nowadays, about 70% of the bacteria that cause infections in hospitals are resistant to at least one of the drugs most commonly used for treatment. The threat of bacterium *S. aureus* is not only due to its distribution and pathogenicity but also to its ability to overcome antimicrobial agents.[5]

Antimicrobial peptides, also called host defense peptides, are an important component of the natural defenses of most living organisms against invading pathogens and have become an important research direction in the past two decades.[6,7] The discovery of natural antibacterial peptides, including histatins, defensins, cathelicidins, magainins, cecropins, and tachyplasins, has provided a new way to fight antibiotic-resistant microorganisms. The wide spectrum of antimicrobial activities reported for these molecules suggests that they could be used in the treatment of viral or parasitic infections. However, there are many general obstacles to move antimicrobial peptides to clinical applications,[8] including the toxicity against eukaryotic cells, the stability of the peptides *in vivo*, the potential for cross-resistance, and the high cost of production.

Photodynamic antimicrobial chemotherapy (PACT) is a new method for killing bacterial cells.[9] PACT treatment utilizes visible or near-infrared light at the appropriate wavelength to excite the nontoxic photosensitizer. The excited photosensitizer undergoes intersystem crossing to long-lived triplet states and, in the presence of oxygen, transfers its energy to molecular oxygen and generates reactive oxygen species such as singlet oxygen and hydroxyl radical, which are responsible for the killing of microbial cells nearby. One of the advantages of PACT in the inactivation of microorganisms is that both antibiotic-sensitive and -resistant strains can be successfully photoinactivated. The other advantage is that repeated photosensitization of bacterial cells does not induce a selection of resistant strains.[10] Although PACT is gaining increasing acceptance for the treatment of locally occurring infections such as psoriasis[11] and scleroderma[12] in dermatology, it is not, at present, a mainstream therapeutic option.

As a key component of PACT, an ideal photosensitizer should have high absorption coefficients in the near-infrared region, where light has deep penetration into tissues, and high photostability to minimize photobleaching. Phthalocyanines, a versatile class of macrocyclic compounds featured with a high fluorescence quantum yield, long triplet lifetimes, and high triplet quantum yields, are gathering growing interest as effective photosensitizers in targeted photodynamic therapy and

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imaging of tumors due to their longer wavelength band absorption (λ_{max} 600 to 700 nm) and higher extinction coefficients ($\varepsilon \sim 110{,}000$). Selectivity to target cells rather than host mammalian cells is another key property for the photosensitizer. In addition, photosensitizers with positive charges tend to bind to cells that carry negative charges on their surfaces.

Conjugation of antimicrobial peptides to phthalocynine photosensitizer is one strategy to develop antimicrobial therapy. ZnPc-$(Lys)_5$, a phthalocyanine derivative with five positive charges, prepared as a high purity single isomer, was previously reported[13] as an effective photosensitizer with high activity in both cultured tumor cells and experimental animal tumors.[14] To further investigate the photodynamic inactivation of ZnPc-$(Lys)_5$ on microorganisms involved in skin bacterial infections, we evaluated the antibacterial efficacy of ZnPc-$(Lys)_5$ *in vitro* and *in vivo* using the key pathogenic factors *P. acnes* and *S. aureus*. This study may provide a safe and effective approach for the treatment of bacterial skin infections.

2 Experimental Techniques

2.1 *Preparation of Photosensitizers*

According to our early published protocols,[14,15] the unsymmetrical ZnPc-$(Lys)_5$ was prepared via the activation of the carboxylic acid group on ZnPc-COOH by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide, and a coupling to pentalysine. The synthesized ZnPc-$(Lys)_5$ was characterized by ^{1}H NMR [AV-400, Bruker, 400 MHz, in dimethyl sulphoxide (DMSO)], ^{13}C NMR (75 MHz, in DMSO), FTIR (Magna-IR 750, Nicolett), and high-resolution mass spectra–electrospray ionization (DECAX-30000 LCQ Deca XP mass spectrometer). The UV–vis absorption spectrum of ZnPc-$(Lys)_5$ in dimethylfomamide was recorded from 300 to 800 nm using quartz cuvettes with 1-cm path length on a Lambda-35 UV/vis spectrometer (PerkinElmer, Massachusetts) in DMSO. Analytical high-performance liquid chromatography (HPLC) was carried out on a C-18 reversed-phase HPLC system (Dalian Elite Analytical Instruments Co., Ltd., Dalian, China; Column: SinoChrom ODS-BP 250×4.6 mm, 5 μm), using a linear gradient from 50% to 100% (v/v) of methanol:acetonitrile (1:1) at a flow rate of 1 ml/ min. The UV–vis absorption spectrum of photosensitizer ZnPc-$(Lys)_5$ in DMSO (Fig. 1) was typical of ZnPc with the strongest absorption at 678 nm (extinction coefficient of 118,380 $L \cdot mol^{-1} \cdot cm^{-1}$). Furthermore, singlet oxygen ($^1O_2$) is believed to be the major cytotoxic agent involved in photodynamic therapy. The quantum yield of singlet oxygen generation of ZnPc-$(Lys)_5$ was measured in reference to ZnPc, which has a quantum yield of 0.67 in DMSO.[16]

Zinc phthalocyanine tetrasulfonate (ZnPc-S_4) with an absorption peak at 680 nm was kindly provided by Professor Naisheng Chen of Fuzhou University, China.

2.2 *Microorganism and Culture Condition*

As the standard bacteria used in antibacterial activity tests according to the National Standard of the People's Republic of China in detection and control of pathogens (GB4789.28-2013), *P. acnes* (ATCC 6919) and *S. aureus* subsp. *aureus* (ATCC 6538) were purchased from Beijing Zhongyuan Ltd. *S. aureus* were cultured in nutrient agars, and *P. acnes* were cultured in thioglycollate medium in anaerobic bags.

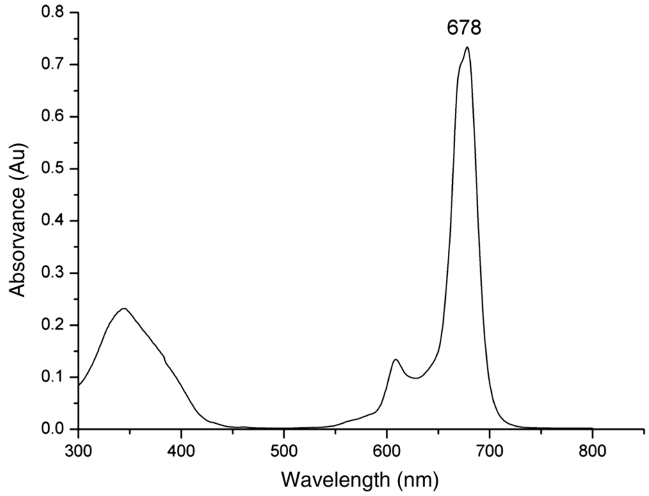

Fig. 1 UV–vis absorption spectrum of photosensitizer ZnPc-$(Lys)_5$ in DMSO.

Thioglycollate medium is a semisolid nutrient medium containing a low concentration of agar to prevent convection of oxygen from the surface. The thioglycollate medium was boiled before use in order to drive off the oxygen and inoculated without excessive shaking of the medium after cooling.

A luminescent strain of *S. aureus* Xen29 (NCTC8532), a derivative of the biofilm forming *S. aureus* 12600 that possesses a stable copy of the modified *Photorhabdus luminescens* luxABCDE operon at a single integration site on the bacterial chromosome, was purchased from Caliper Life Sciences, Inc., and grown at 37°C using Luria–Bertani broth containing kanamycin (200 μg/ml to select for resistance encoded by the plasmid) to an absorbance of 0.5 at 600 nm corresponding to 1.44×10^8 organisms/ml.

2.3 *Cellular Uptake of Photosensitizer by Bacteria*

Aliquots of microorganism suspension [10^6 colony forming unit (CFU)/ml] were incubated in 96-multiwell plates (Falcon) with photosensitizer ZnPc-$(Lys)_5$ at different concentrations (10^{-7}, $10^{-6.5}$, and 10^{-6} M) for 1 h at 37°C. The exponentially growing cells were then washed with sterile phosphate buffer saline (PBS) before lysis with NaOH [0.1 N, 1.0 ml with 1% sodium dodecyl sulfate (SDS)] to give a homogeneous solution. The fluorescence of the cell extract was measured on a microplate reader (Synergy 4, BioTek Instruments). The concentration of cellular protein was determined using a bicinchoninic acid protein assay kit (Pierce, Thermo Fisher Scientific). Standard curves were made with cell lysates treated as above with known added amounts of bovine serum albumin. Results are expressed as nmol of phthalocyanine per mg cell protein.

2.4 *Antimicrobial Studies in Cultured Bacteria*

Bacteria suspensions ($\sim 10^6$ CFU/ml) were incubated in the dark at room temperature for 1 h with ZnPc-$(Lys)_5$ (at concentrations of 10^{-7}, $10^{-6.5}$, 10^{-6}, $10^{-5.5}$, and 10^{-5} M) followed by the light exposure using a light-emitting diode (LED) light source (SunDynamic, Inc., Qingdao) of 680 nm and with a power of 100 mW for 1 or 2 min (i.e., light dosages at 3 or 6 J/cm^2). After illumination of the appropriate wells, cell viability was determined by incubating agar plates overnight after applying 0.1 ml from one of the tubes in a bacterial dilution series. The percentage of cell survival was determined by comparing the colony counts from treated plates to the colony counts from control plates that were incubated without the conjugate and were kept in dark for periods equal to irradiation times.

2.5 *Antimicrobial Studies in Experimental Animals*

Sprague–Dawley rats (purchased from Shanghai SLAC Laboratory Animal Co. Ltd., Shanghai, China) were maintained and handled in accordance with the recommendations of an Institutional Animal Care and Use Committee (IACUC). The animals were allowed free access to water throughout the course of the experiments. The experimental procedures on rats were in accordance with PerkinElmer IACUC guidelines and approved veterinarian requirements for animal care and use. Rats ($n = 16$) weighing 150 ± 2 g were anesthetized with an intraperitoneal injection of pentobarbital (30 mg/kg, i.p.) before they were shaved on the back and treated with depilatory cream. Two excisional wounds were then made along the dorsal surface, using surgical scissors, by carefully opening the epidermal layer of the skin with one side connected. Each wound measured 8×8 mm with at least 5 mm of unbroken skin between wounds. The bottom of the wound was panniculus carnosus, with no visible bleeding. Localized infection on the wounds was induced according to our previously published protocol[13] with slight changes by inoculating a suspension (25 μl) of midlog phase bioluminescent Xen29 *S. aureus* ($\sim 10^8$ cells/ml in PBS) into each wound of the rats. The bacteria was allowed to attach to the tissue for 30 min, then 25 μl of ZnPc-$(Lys)_5$ (1 mM in PBS) or ZnPc-S_4 (1 mM in PBS) was added into the wounds. After a further 30 min were given to allow the conjugates to bind to and penetrate the bacteria under subdued room lighting, eight rats were illuminated using a 680-nm light source (SunDynamic, Inc., Qingdao) with an irradiance of 100 mW for 5 min/wound, giving a total light fluence of 15 J/cm^2 for each wound. The other eight rats were kept in their cages in the dark during the whole experimental period.

To measure the bacteria load after PACT treatment, we cut out the epidermis layer of the skin from rats under anesthesia. After being shredded into small pieces, the tissues were mixed with PBS, and the bioluminescence signals were measured on a Synergy™ 4 multimode microplate reader (BioTek Instruments, Winooski, Vermont). The viability of bacteria after PACT treatment was measured by comparing the luminescence signal to that of a control group treated with saline. All rats were kept in cages after the treatment. Their wound areas were measured in two dimensions each day for 12 days. To precisely observe the early wound healing after different treatments, the laser speckle contrast imaging (LSCI) system (MoorFLPI-2, Moor Instruments Ltd., United Kingdom) was used to monitor cutaneous blood flow in the wound area after PACT treatment (~2 weeks later). The spatiotemporal characteristics of blood flow under the wound area were addressed.

3 Results and Discussion

3.1 *Preparation of Photosensitizer ZnPc-$(Lys)_5$*

The structure of ZnPc-$(Lys)_5$ was confirmed by NMR spectroscopy and mass spectrometry as described previously.[13–15] Furthermore, the quantum yield for singlet oxygen photogeneration for ZnPc-$(Lys)_5$ in DMSO was determined to be 0.64 ± 0.02, which is similar to that for ZnPc (0.67),[16] showing that the coupling of a positively charged pentalysine group does not significantly alter the generation of singlet oxygen.

3.2 *Cellular Uptake of Photosensitizer by Bacteria*

The uptake profile of a photosensitizer is important in the clinical setting in order to adjust the dose for effective treatment.[17] It is believed that bacteria *P. acnes* and *S. aureus* are the main pathogenic factors involved in some skin infections, like cellulitis, abscesses, and postsurgical infections. In the current study, we measured the uptake of photosensitizer ZnPc-$(Lys)_5$ in the above-mentioned bacteria. Meanwhile, the negatively charged ZnPc compound, ZnPc-S_4, was used as a control. The uptake of ZnPc-$(Lys)_5$ was shown in a dose-dependent manner with higher concentration of ZnPc leading to higher cellular uptake on both bacteria we measured (Fig. 2). Compared with the anionic photosensitizer ZnPc-S_4, ZnPc-$(Lys)_5$ exhibited much higher cellular uptake amounts in both bacteria (*S. aureus* and *P. acnes*). This enhanced uptake amount of ZnPc-$(Lys)_5$ is likely due to the binding of the positively charged ZnPc-$(Lys)_5$ to the outer membrane of bacteria that bears a strong negative charge.[18] Furthermore, we noticed that the uptake amount of ZnPc-$(Lys)_5$ (at 1 μM) in *S. aureus*

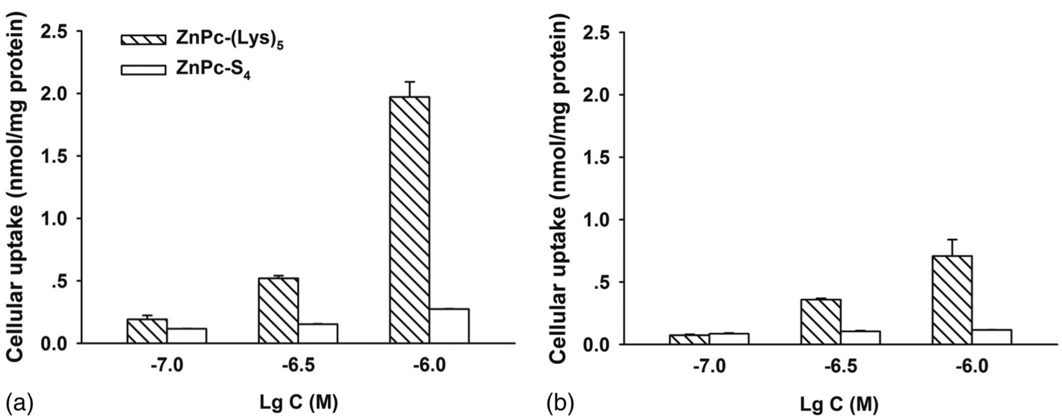

Fig. 2 Dose-dependent cellular uptakes of ZnPc-$(Lys)_5$ by bacteria (a) *S. aureus* and (b) *P. acnes*. Bacteria were incubated for 1 h with various concentrations of ZnPc-$(Lys)_5$ and were washed and dissolved in 0.1-N NaOH—1% SDS for quantization of ZnPc-$(Lys)_5$ using fluorescence signals.

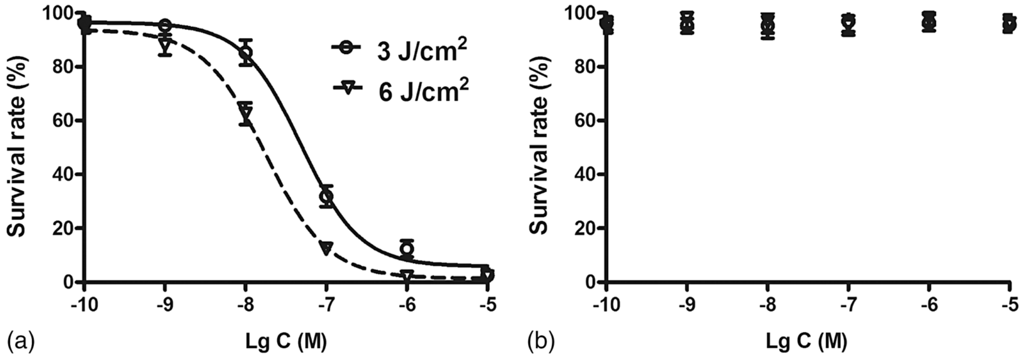

Fig. 3 (a) Dose-dependent antimicrobial effects of ZnPc-$(Lys)_5$ and (b) ZnPc-S_4 on *S. aureus* under two different light doses (3 and 6 J/cm^2, respectively).

(1.97 nmol/mg protein) was about 2.7 times more than that in *P. acnes* (0.73 nmol/mg protein).

3.3 *Antimicrobial Studies in Cultured Bacteria*

With increasing numbers of small, naturally occurring antibacterial peptides being discovered, the mechanism of action of these peptides is being intensively investigated.[19–21] A common factor in all the structures is the polycationic charge due to lysine, histidine, and arginine residues in the amino acid sequence, and the polycationic charge is probably responsible for their initial binding to bacteria. In this study, we conjugated a pentalysine moiety to ZnPc to selectively target bacteria for photodestruction and tested the antibacterial activities of ZnPc-$(Lys)_5$ under two different light dosages (3 and 6 J/cm^2) against *S. aureus*.

Figure 3 shows the antibacterial activities of ZnPc-$(Lys)_5$ [Fig. 3(a)] and ZnPc-S_4 [Fig. 3(b)] against *S. aureus* at different light dosages (3 and 6 J/cm^2, respectively). It is obvious that ZnPc-$(Lys)_5$ inhibited the bacterial growth in light dosage-dependent and photosensitizer dose-dependent manners [Fig. 3(a)]. In contrast, ZnPc-S_4 did not show significant toxicity to *S. aureus* under the same conditions with the same light and photosensitizer dosages [Fig. 3(b)]. The pronounced photodynamic effect of the cationic ZnPc-$(Lys)_5$ on *S. aureus* may be due to the electrostatic attraction between the photosensitizer and the negatively charged membrane of the bacterium.

Moreover, ZnPc-$(Lys)_5$ did not show significant toxicity to the bacterium in the absence of light illumination (data not shown), suggesting a broad safety margin of ZnPc-$(Lys)_5$ with 10-μM concentration followed by a light dosage at 6 J/cm^2.

We further investigated the antimicrobial activities of ZnPc-$(Lys)_5$ against *P. acnes*, the primary pathogen responsible for cystic acne, under a light dose at 6 J/cm^2 using a colony-counting method. It showed that irradiation of *P. acnes* cells treated with ZnPc-$(Lys)_5$ (at a concentration of 1 μM) caused a 5 to 6 log reduction of the CFU (Fig. 4). In comparison, the survival fraction of *P. acne* was about only 4 log reduction of the CFU, which was much smaller than that of *S. aureus*. This result was in line with the different amount of cellular uptakes we observed (Fig. 2).

3.4 *Antimicrobial Studies in Experimental Animals*

The method using genetically engineered bacteria that emit luminescence to monitor bacterial numbers and viability in real time in living animals so far has been demonstrated in several models.[22,23] In the current studies, we evaluated the *in vivo* efficacy of ZnPc-$(Lys)_5$ on skin disorder-related bacteria using a localized infection animal model by inoculating the luminescent strain of *S. aureus* (Xen29) into excisional wounds on the back of rats. We estimated that the luminescence signal was linearly proportional to live bacterial CFU in the range of 10^3 to 10^8 CFU. In our experiments, about 2 million CFU of the luminescent *S. aureus* from a midlog culture in 25 μl were applied onto each wound (8×8 mm) made on the back

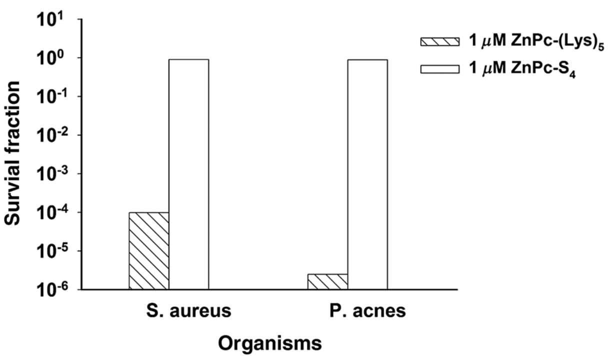

Fig. 4 Effects of ZnPc-$(Lys)_5$ (1 μM) on organisms related to bacterial skin disorder under a light dose at 6 J/cm^2. Survival fractions are expressed as ratios of CFU from bacteria treated with ZnPc-$(Lys)_5$ or ZnPc-S_4 and light over CFU of bacteria treated with neither.

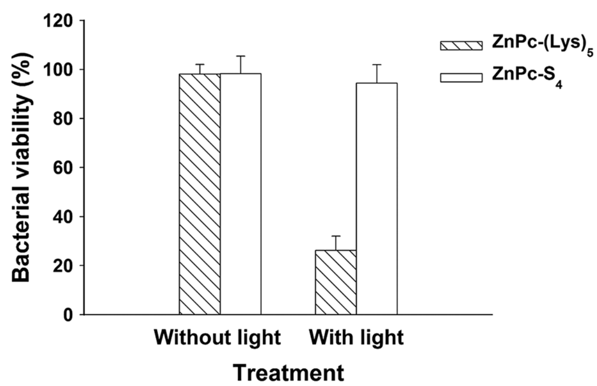

Fig. 5 Reduction of viable luminescent *S. aureus* strain (Xen29) in infected incision wounds on rats upon incubation with ZnPc-$(Lys)_5$ (1 μM) and light exposure (680 nm, 15 J/cm^2). Data points are means of values from wounds on rats, and bars are standard error of the mean (SEM).

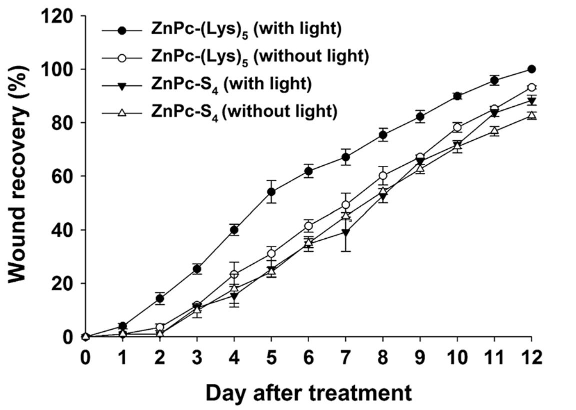

Fig. 6 Effects of ZnPc-$(Lys)_5$ (1 μM) on wound recovery of *S. aureus* infected excisional wounds with rats in the presence or absence of light irradiation (680 nm, 15 J/cm^2). Each wound was infected with 2×10^6 CFU *S. aureus* 30 min before the photodynamic treatment with photosensitizers. Wounds were measured in two dimensions every day after the infection, and the areas were calculated. Data points are means of values from the corresponding wound on rats and bars are SEM.

of anesthetized rats. The antibacterial efficacy of ZnPc-$(Lys)_5$ on skin disorder-related bacteria was evaluated by measuring the bacterial load right after the PACT treatment and monitoring the wound size for 12 days after the PACT treatment in order to detect the wound healing rate. We observed that ZnPc-$(Lys)_5$ dramatically reduced the bacterial load in the wounds upon light irradiation (680 nm, 15 J/cm^2), whereas ZnPc-$(Lys)_5$ without light irradiation or with the anionic photosensitizer ZnPc-S_4 (in the presence or absence of light irradiation) did not show an antibacterial effect upon the excisional wounds on the back of rats (Fig. 5). These results show that the cationic ZnPc-$(Lys)_5$ has a strong antimicrobial effect in animal models and is more effective than anionic photosensitizer ZnPc-S_4.

In the presence of light, ZnPc-$(Lys)_5$ dramatically promoted wound healing in rats from the second day after the treatment compared with ZnPc-S_4 (Fig. 6). This accelerating effect on wound healing is much stronger in the presence of light (680 nm, 15 J/cm^2) compared to the case without the irradiation. Such wound-healing promotional activities were previously reported with two different photosensitizers (5-aminolevulinic acid at 5 mg/kg and hematoporphyrin derivative at 5 mg/kg) in an open excision wound model with rats.[24] Interestingly, two other reports[25,26] showed that PACT treatment did not have any effect on wound healing. The reason for these conflicting results is not yet clear; it could probably be explained by the use of different photosensitizers and requires further study.

We noticed that the wound recovery rate was significantly enhanced from the second day to the fifth day after PACT with ZnPc-$(Lys)_5$ (Fig. 6) and slowed down afterward. This observation is possibly due to the metabolism of ZnPc-$(Lys)_5$ and suggests that PACT with ZnPc-$(Lys)_5$ applied repeatedly for a certain interval of time (∼five days) would promote the recovery of infected incision wounds on rats.

To further evaluate the wound healing process in bacterial skin infections with PACT treatment, we imaged the blood flow under the wound area upon all wounds healing (2 weeks later) using an LSCI system, which has been used widely to image cutaneous blood flow.[27,28] We observed that PACT with ZnPc-$(Lys)_5$ in the presence of light irradiation (680 nm, 15 J/cm^2) significantly reduced the blood flow under the wound area (∼1.4-fold blood flow as normal skin, Fig. 7(B-c),

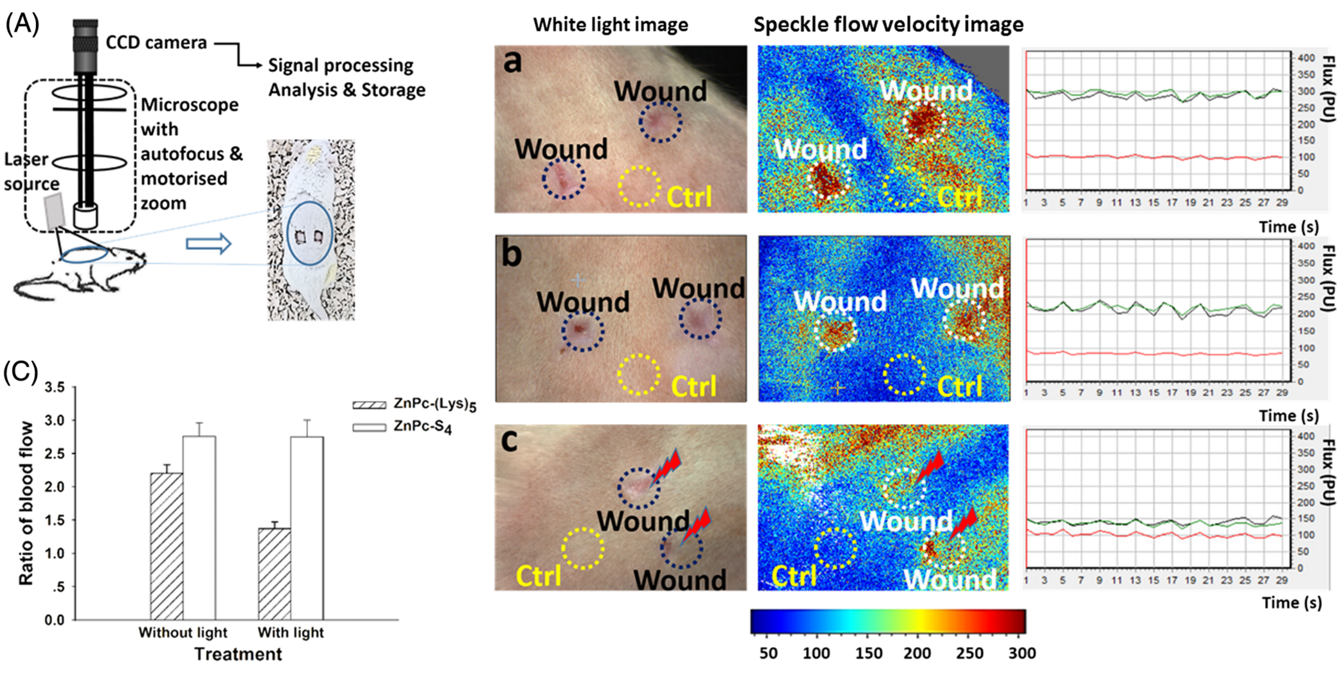

Fig. 7 *In vivo* speckle imaging of *S. aureus* infected excisional wounds with rats treated with ZnPc-$(Lys)_5$ or ZnPc-S_4. (A) Schematic diagram of the laser speckle imaging system. (B) Three representative speckle images of the wound-skin rats treated with ZnPc-S4 (a) or ZnPc-$(Lys)_5$ with (c) or without (b) irradiation (680 nm, 15 J/cm^2). (C) Analysis of the blood flow ratio under wound-skin of rats 2 weeks after PACT with ZnPc-$(Lys)_5$ or ZnPc-S_4.

whereas PACT with $ZnPc-S_4$ showed ∼2.8-fold blood flow as normal skin in the presence or absence of light irradiation [Fig. 7(B-a)]. These results demonstrated that PACT with $ZnPc-(Lys)_5$ significantly relieved the symptoms of inflammation during the wound healing process [Fig. 7(C)]. Furthermore, we observed that PACT with $ZnPc-(Lys)_5$ in the absence of light irradiation partially reduced the blood flow under the wound area [∼2.2-fold blood flow under normal skin, Fig. 7(B-b)], indicating that ambient light illumination alone was helpful in promoting the wound healing process under PACT with $ZnPc-(Lys)_5$.

The LSCI system used in the current study allows for real-time monitoring of the skin microvascular structural and functional information. It shows great potential for monitoring blood flow, but the spatial resolution suffers from the scattering of tissue.[28,29] A combination method of LSCI and skin optical clearing is now attracting increasing attention.[28,30,31] The physiological effect of the optical-clearing agents on skin, such as inflammation, seems limited but requires further evaluation.[32]

Typically, PACT treatment uses laser light sources. For example, a recent report showed the effectiveness of laser therapy with a wavelength of 850 nm in the prevention of caries and gingivitis in adolescents.[33] We used LED light sources with a power of 100 mW in this study and found it strong enough to produce significant antimicrobial photodynamic effects, in combination with our cationic photosensitizer $ZnPc-(Lys)_5$. The use of battery-powered, inexpensive, and portable LED light sources may make possible a low-cost and efficient deployment of systems to be used for wound decontamination.

4 Conclusions

With the widespread occurrence of antibiotic resistance among pathogenic bacteria, additional methods of killing bacteria in wounds are being sought vigorously. We herein report the use of a zinc phthalocyanine derivative, pentalysine β-carbonylphthalocyanine zinc $ZnPc-(Lys)_5$), in PACT, the promising antimicrobial effects on bacterial infected skin, and the increased rate of healing after PACT treatment in an animal model. In conclusion, our study demonstrated that phthalocyanine-based photosensitizer shows a specific affinity for bacterial cells. Compared with $ZnPc-S_4$, $ZnPc-(Lys)_5$ showed more efficient uptake by bacterial cells and enhanced antimicrobial effects *in vitro*, as well as a significantly increased healing rate of *S. aureus* infected excisional wounds on rats. Overall, $ZnPc-(Lys)_5$ is a promising antimicrobial photosensitizer for the safe and effective treatment of bacterial skin infection.

Acknowledgments

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