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Improved Photodynamic Inactivation of Gram-Positive Bacteria Using Hematoporphyrin Encapsulated in Liposomes and Micelles

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Background and Objectives: Antimicrobial photodynamic inactivation (PDI) is a promising treatment modality for local infections. To increase the efficacy of photosensitizer, hematoporphyrin (Hp) was used as a model drug and encapsulated in liposomes and micelles. The bactericidal efficacy of the carrier-entrapped Hp was assessed against gram-positive bacteria.

Study Design/Materials and Methods: Hp was encapsulated in liposomes by a modified reversed-phase evaporation and extrusion method. Micelle-Hp was prepared by the reversed-phase evaporation method. Spectroscopic analysis was used to characterize the properties of Hp in PBS, liposome or micelle. The PDI efficacy was examined by using gram-positive pathogens including methicillin-susceptible, methicillin-resistant *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Streptococcus pyogenes*.

Results: The absorption and fluorescence emission spectra indicated that Hp encapsulated in liposomes and micelles is less likely to exist in aggregated form compared to that generally seen in an aqueous medium. Liposome- or micelle-Hp can induce complete eradication of the bacteria above a critical Hp dose, which is significantly lower than the dose required when using the non-encapsulated Hp. Furthermore, the PDI effect of the Hp encapsulated in micelles was superior to the Hp encapsulated in liposomes at lower Hp doses. Similar PDI results were also found in *S. epidermidis* and *S. pyogenes*.

Conclusions: Our results indicate that photosensitizer entrapped in micelle exert similar or better PDI efficacy than that of liposome, which indicates this formulation may be useful for the treatment of local infections in the future. Lasers Surg. Med. 41:316–322, 2009.

Key words: photodynamic inactivation; micelle; liposome; photosensitizer; bacteria

INTRODUCTION

Drug resistant pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) have become a serious threat due to its ability to develop high levels of resistance to several classes of antibiotics. The recent isolation of vancomycin-resistant [1] and mupirocin-resistant [2] strains of *aureus* highlights the urgent need for the new therapies of bacterial pathogens.

Photodynamic therapy (PDT) was originally developed as a therapeutic modality for cancer treatment [3]. The use of PDT has also been proposed as an alternative treatment to combat antibiotic resistance among pathogenic microbes and was specifically termed as photodynamic inactivation (PDI) [4]. The principle of PDT is based on the combined use of a photosensitizer and low-intensity visible light of an appropriate wavelength. After light irradiation, activated photosensitizers generate cytotoxic reactive oxygen species (ROS) to induce a bactericidal effect. Although the possibility to inactivate microbes by PDI has been known since the first days of PDT for more than 10 decades [5], it is only recently that this modality gained attention as a viable tool to eradicate infectious pathogens [6,7]. The main advantages of PDI are that bacteria can be eradicated almost instantly and the damage to adjacent host tissues can be avoided. PDI is effective against antibiotic-resistant and antibiotic-susceptible bacteria, and repeated photosensitization does not induce the resistance of the bacteria against the treatment [7]. Therefore, development of resistance to PDI in the target bacteria is not very likely to occur.

A number of photosensitizers such as rose bengal [8], methylene blue [9,10], toluidine blue [8,10], chlorine e6 [11], and hematoporphyrin [12] have been investigated for PDI against microbial pathogens. However, these

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photosensitizers have been found to form aggregates easily in aqueous medium, which may lead to a self-quenching effect on the excited state, thus reducing the yield of singlet oxygen ($^1O_2$) formation [13,14]. Specifically designed drug delivery systems can be designed to reduce the likelihood of aggregation of potential photosensitizing agents. Encapsulation techniques that have previously been applied to prevent the formation of aggregates include liposomes [15,16], polymeric micelles [17,18] and nanoparticles [19,20]. Due to the structure similarity with cell membrane, liposomes have been widely investigated as drug delivery systems after their initial discovery. By the end of the 20th century, researchers had largely overcome the instability and improved the targeting of liposomal drug delivery systems. Approaches include adding cholesterol for improved storage stability of the liposomes, covalently linking phospholipids with polyethylene glycol (PEG) for steric stabilization, and using antibodies or other ligands to improve the targeting.

For PDI, Ferro et al. [21] showed that disruption of the bacterial outer wall can be most efficiently achieved by using positively charged liposomes, analogous to the use of poly-lysine or poly-ethyleneimine [22,23]. A significant challenge is that encapsulating drugs in liposomes currently is not a one-formula-fits-all issue, and the composition of an optimal formula can only be obtained through tedious experimental trials. Other than cancer research, many studies do not favor the liposome delivery system because the phospholipids are quite expensive, inherently unstable, and the preparation is very difficult to scale up based on the bench experience. In fact, in some applications, less expensive starting materials, such as polymers, can be used instead of phospholipids to encapsulate the candidate compound [17,18]. The use of polymeric micelles is promising for the delivery of a photosensitizer because this system may improve drug solubility and prevent the formation of aggregates in the aqueous medium. Furthermore, preparation of polymeric micelles can be much less expensive and simpler compared to the use of liposomes, and scale-up is generally not a difficult issue.

In this investigation, we evaluated various encapsulation techniques for PDI using hematoporphyrin (Hp) as the model photosensitizer. The carrier systems included conventional liposomes, PEGylated liposomes, and the polymeric micelles. The present study showed that micelle-delivered photosensitizer exert similar or better PDI efficacy than the liposome-delivered photosensitizer against gram-positive bacteria.

MATERIALS AND METHODS

Materials

Hematoporphyrin dihydrochloride (Hp) was purchased from ChromaDex (Irvine, CA), and used as received. Phospholipids including 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000 (DSPE-PEG2000) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) were purchased from Avanti (Alabaster, AL). Sephadex cartridges pre-packed with G-50 silica gel were obtained from Pharmacia Biotech (Uppsala, Sweden). Pluronic F127 (PF127) was purchased from Wei Ming Pharmaceutical (Taipei, Taiwan). 4-nitroso-*N*,*N*-dimethylaniline (RNO), histidine, and all other chemicals were obtained from Sigma–Aldrich (St Louis, MO).

Microbial Strains and Growth Conditions

S. aureus (BCRC 10780; methicillin-susceptible), was purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). The other microbial strains used in this study were *Staphylococcus epidermidis* (ATCC 12228), *Streptococcus pyogenes* (ATCC 19615), and MRSA (ATCC 49476 and ATCC 33592). Tryptic soy broth (TSB) was used as the liquid medium for *S. aureus*, *S. epidermidis*, and MRSA. *S. pyogenes* was grown in Todd-Hewitt broth containing yeast extract.

Preparation and Characterization of Liposomes

Hp was encapsulated in liposomes by a modified reversed-phase evaporation and extrusion method [15]. Briefly, 0.3 mg of Hp solubilized in 50 μl methanol was added to the solution of 10 μmol DSPC with or without 0.2 μmol DSPE-PEG2000 dissolved in 240 μl chloroform in a round bound flask mounted to the evaporator. After removing the solvents, 1 ml 0.9% NaCl solution was added to the tube and the mixture was vortexed for 5 minutes, and passed through a 100 nm diameter polycarbonate membrane for 11 times. This converted the original vesicles ranging in 700–2000 nm diameter to small unilamellar vesicles (SUVs). The untrapped Hp and lipids were removed by size exclusion chromatography, and the liposome-Hp were stored at 4°C until use. The amount of Hp in the liposome was determined by UV–Visible spectrophotometry (Beckman COULTER DU800), and the lipid concentration was determined using the Bartlett assay [24]. Size distribution was measured with dynamic light scattering (Coulter N4 Plus Submicron, Beckman Coulter).

Preparation and Characterization of Micelles

Reversed-phase evaporation method was used to encapsulate Hp into micelles [25]. Briefly, 0.5 mg of Hp and 100 mg of PF127 were dissolved in 2.5 ml co-solvent system (chloroform:methanol = 4:1, v/v) in a round bottom flask. The solvent was removed by rotary evaporation to form a thin film. The film was hydrated using 1 ml of distilled water at room temperature to give a final 10% (w/v) micelle solution, and this process was carried out in a sonication bath for 20 minutes. The hydrated preparation was kept overnight at room temperature and then passed through a 0.2 μm PVDF filter to remove the free Hp since the Hp that is not entrapped within the micelles undergoes aggregation and does not pass through these filters [25]. Both UV–Visible absorption and fluorescence spectra of the filtrate were used to verify that Hp in the filtrate is in monomeric form. Hp concentration in the filtrates was calculated by measuring its absorption at 397 nm after extracting into methanol. The size distribution of the Hp-containing micelles was measured by dynamic light scattering.

Spectroscopic Analysis

Studies on the absorption and fluorescence spectra of Hp were carried out in PBS, methanol, and all the encapsulated samples. Absorption spectra were measured with the UV–Visible spectrophotometer (Beckman Coulter, Fullerton, CA). Fluorescence spectra were obtained on a Fluorolog®-3 luminescence spectrometer (Horiba Jobin Yvon, Edison, NJ).

PDI of Bacterial Cells

For PDI, the bacterial cells in the stationary phase were harvested by centrifugation of broth culture, washed thrice with PBS and suspended in PBS to produce a cell suspension containing 10^8 CFU/ml. In a typical experiment, 0.1 ml of a cell suspension of the bacteria containing approximately 10^8 CFU per ml was transferred into a well. Then, 0.1 ml of the PBS solution (pH 7.4) containing Hp was added to the well. The samples were incubated in the dark for 30 minutes unless otherwise specified, and then irradiated at room temperature. The light source used for Hp irradiation consisted of high power LED array with the wavelength centered at 635 ± 5 nm, which delivered at an irradiance of 60 mW/cm^2 [26]. For chlorine e6 PDI, the light source consisted of high power LED with the wavelength centered at 653 ± 5 nm, which delivered at an irradiance of 15 mW/cm^2. Irradiated as well as non-irradiated bacterial cells were serially diluted 10-fold with PBS and the colonies formed after 18 hours of incubation at 37°C were counted.

Bacterial Cell Survival Assay

CFU of a bacterial suspension was counted using the following standard protocol: aliquots (10 μl) of appropriate dilutions (from 10^{-1} to 10^{-5}) were plated on TSB agar plates and incubated at 37°C in darkness for 18 hours. The survival fraction was calculated as N_{PDI}/N_0, where N_{PDI} is the number of CFU per ml after photodynamic inactivation and N_0 is the number of CFU per ml in the initial sample. The dark toxicity of the substrates, defined as the intrinsic toxicity of the compounds in the absence of light, was monitored by evaluating the survival fraction of incubated but non-illuminated bacterial samples, and calculated as N_{DARK}/N_0, where N_{DARK} is the number of CFU per ml of the non-illuminated samples. All results are expressed as the mean ± standard deviation. Differences between two means were assessed for significance by the two-tailed Student's t-test and a value of $P<0.05$ was considered significant.

TABLE 1. Characteristics of Nanocarriers Encapsulated With Hp

Formulation	Entrapment efficiency (%)	Particle size[a] (nm)
Liposome	80.2 ± 4.7	123.9 ± 34.8
PEGylated liposome	79.8 ± 4.4	123.7 ± 10.5
Micelle	71.2 ± 7.5	57.5 ± 7.4

[a]Diameter (mean ± SD).

RESULTS

Characteristics and Spectral Properties of Hp in the Nanocarriers

Hp was encapsulated into three nanocarrier systems: conventional liposomes composed of DSPC (abbreviated as liposome-Hp), stealth liposomes using PEGylated phospholipids in addition to DSPC (abbreviated as PEGylated liposome-Hp), and polymeric micelles using Pluronic F127 (abbreviated as micelle-Hp). Liposome-Hp and PEGylated liposome-Hp each yielded a fairly homogenous population of small unilamellar vesicles with an average diameter of approximately 120 nm and an entrapment efficiency of ~80% (Table 1). The size of the micelle-Hp (~58 nm) was smaller than the liposomes and the entrapment efficiency was greater than 70%. The absorption and fluorescence spectral properties of Hp in PBS, methanol and various nanocarrier systems are shown in Table 2. It has been demonstrated that the aggregated and monomeric Hp can be differentiated by the differences in absorption and emission spectra. The aggregation of Hp in the aqueous medium such as PBS is characterized by the absorption peak at 374 nm. However, the maximum absorption peak is red shifted to 395 nm in methanol indicating a lower extent

TABLE 2. Spectral Properties of Hp in Various Solvents/Nanocarriers

	UV–Vis spectra		Fluorescence spectra[a]
Solvent/carriers	Soret band (nm)	$\lambda_{397}/\lambda_{374}$[b]	Major position (nm)
PBS[c]	374	0.85	614
Methanol[c]	395	1.27	627
Liposome[d]	397	1.23	623
PEGylated liposome[d]	397	1.25	623
Micelle[d]	397	1.25	624

[a]Excitation: 397 nm, slits: 2 nm, PMT: 850 V.
[b]Ratio of monomer to dimer (absorbance at 397 nm/374 nm).
[c]Solvent.
[d]Nanocarriers suspended in 0.9% NaCl.

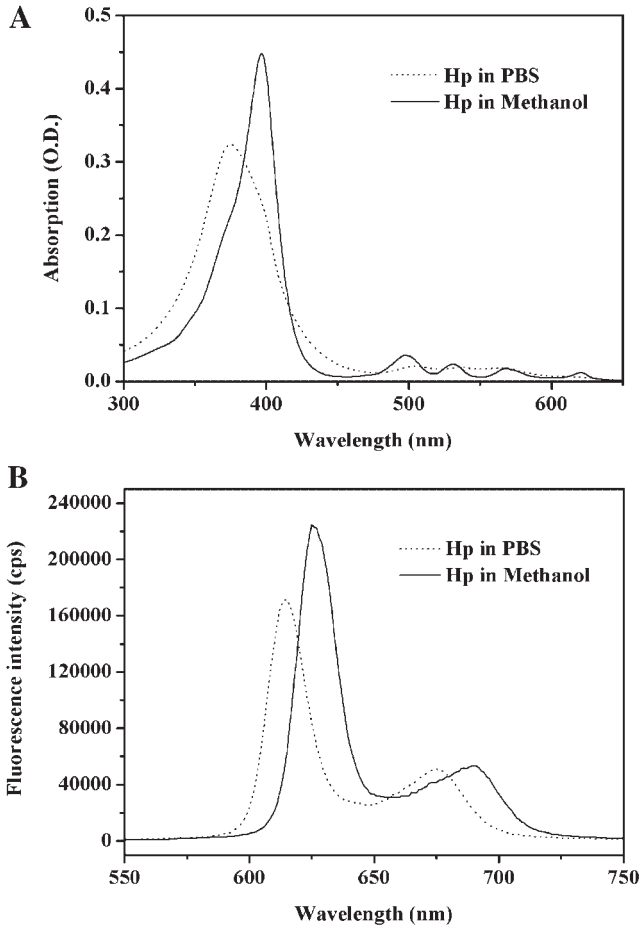

Fig. 1. Absorption (**A**) and fluorescence emission (**B**) spectra of Hp in PBS and methanol. Absorbance spectra were collected using different concentrations of Hp in PBS (12 μM) and methanol (3 μM) because the spectral intensity of the aggregated form was much lower than that of the monomer form. The fluorescence spectra were obtained from 1 μM Hp in PBS and methanol.

of aggregation [27]. As reported, the major fluorescence spectra of Hp in PBS is at 614 nm [28]. Our studies showed that the maximum Hp Soret band in PBS and methanol is at 374 and 395 nm, respectively (Fig. 1A). Meanwhile, the main fluorescence emission of Hp is shifted from 614 nm in PBS to 627 nm in methanol (Fig. 1B). In various nanocarrier systems, the maximum Hp Soret band is at 397 nm, closer to that in methanol and more than 20 nm different from that in PBS (Table 2). The degree of monomeric to aggregated Hp was further evaluated by taking the absorbance ratio at 397 and 374 nm. The ratio of Hp in PBS was 0.85, and the value for Hp in methanol and the nanocarriers were around 1.23 – 1.27. Higher value of the absorbance ratio indicated that more Hp is in the monomeric status. Furthermore, compared to Hp in PBS, there is a significant fluorescence band shifting to a longer wavelength range (~623 nm) when Hp was encapsulated in the nanocarrier systems. The absorption and fluorescence emission spectra all indicated that when Hp was encapsulated into liposomes or micelles, it is less likely to exist in aggregated form compared to that generally seen in an aqueous medium.

Dark Toxicity of Hp in the Nanocarriers

A suitable photodynamic inactivation should induce the antimicrobial effect only upon light illumination. Therefore, the photosensitizer intended to be placed in contact with the target microbe should present minimal toxicity when not exposed to light. In this study, the bacteria were incubated in the dark with all the Hp preparations under the studied concentration for 30 minutes and no detectable dark toxicity was found in any studied condition (Fig. 2).

Photodynamic Inactivation Against Gram-Positive Bacteria

To examine the PDI effects of PEGylated liposome-Hp, liposome-Hp and micelle-Hp, we used three gram-positive species, *S. aureus*, *S. epidermidis*, and *S. pyogenes*, which are all important pathogens. Illumination of *S. aureus* following incubation with different concentrations of various Hp formulations caused a significant decrease in viability ($P<0.05$) under the light dose of 50 J/cm^2, compared to the group of Hp in PBS (Fig. 3). The most important difference in the PDI against *S. aureus* is that micelle-Hp has better bactericidal effects than the two liposomes or the PBS group at the lower Hp concentrations. At a concentration of 0.25 μM, the micelle-Hp was able to completely eradicate the microbe, while both the PEGylated liposome-Hp and the liposome-Hp required a higher concentration (0.5 μM) to achieve the same result. Hp in PBS group showed the least potency in bacterial killing among all of the test samples. Similar enhancements in bacterial killing with the micelle were found in

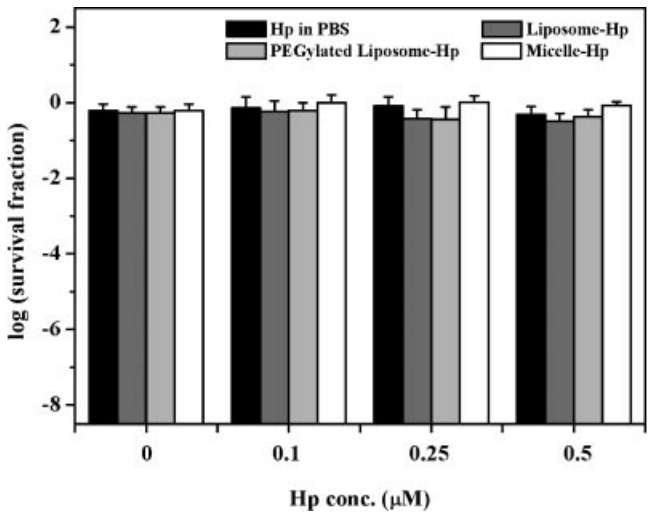

Fig. 2. Dark toxicity of *S. aureus* after incubated with various formulations of 0.5 μM Hp for 30 minutes ($n=3$).

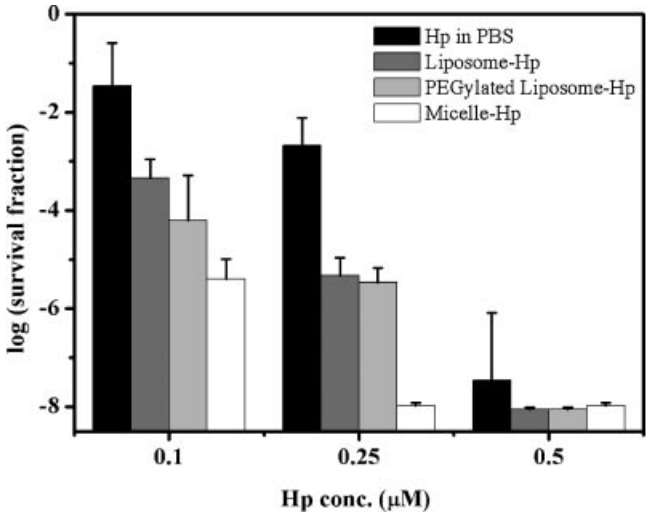

Fig. 3. Cell survival fraction of *S. aureus* after incubated with various formulations of Hp (0.1, 0.25, and 0.5 μM Hp equivalents) for 30 minutes and subjected to 50 J/cm^2 of the red light illumination. Values are means of three independent experiments and bars are SEM.

S. epidermidi and *S. pyogenes* (Fig. 4). In a pattern similar to *S. aureus,* Hp in PBS and the two liposome groups are less effective compared to the micelle-Hp.

Photodynamic Inactivation on MRSA

The response to PDI using Hp in various formulations against two methicillin-resistant strains of *S. aureus* (MRSA ATCC 49476 and MRSA ATCC 33592) is shown in Figure 5. The PDI effect using the nanocarrier-Hp showed an enhanced antimicrobial effect on the two MRSA bacteria, consistent with the results found for *S. aureus*. If we consider the formulations in which the Hp concentration was 0.5 μM, there was an approximately 2-log reduction in viable counts with the Hp in PBS group, while both liposome formulations and the micelle formulation, exhibited a 4–5 log reduction (Fig. 5). When the Hp dose is increased to 1 μM, large reductions in viability of the two MRSA strains are observed for all formulations with no significant difference between them.

Photodynamic Inactivation Against *S. aureus* Using Encapsulated Chlorine e6

As shown above, encapsulation of Hp in PEGylated liposomes and PF127 micelles enhanced the PDI efficacy of Hp. To further verify the feasibility of using these nanocarriers in PDI, we then encapsulated another type of photosensitizer chlorine e6 (Ce6) into PEGylated liposomes and PF127 micelles. As shown in Figure 6, compared to the group of Ce6 in PBS, the micelle-Ce6 was able to completely eradicate *S. aureus* at the concentration of 0.5 μM, indicating the feasibility of using the micelle-forming agent to avoid the likelihood of aggregation of the photosensitizer.

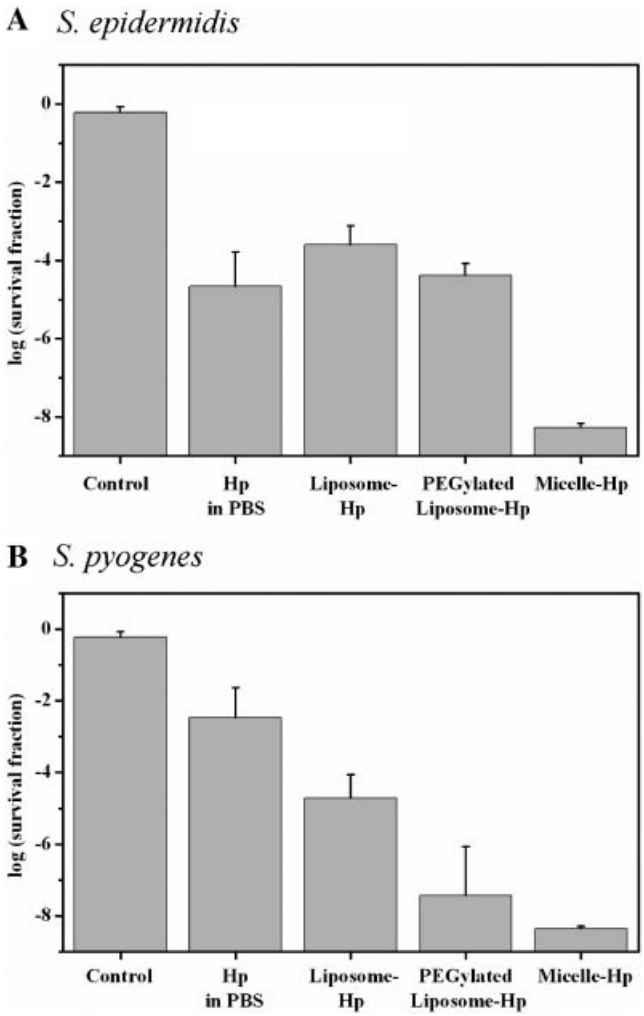

Fig. 4. PDI of *S. epidermidis* (**A**) and *S. pyogenes* (**B**). After incubation with various formulations of Hp for 30 minutes, bacteria were subjected to 50 J/cm^2 of the red light illumination. Note that, different concentration of Hp was used for *S. epidermidis* (0.25 μM Hp equivalents) and *S. pyogenes* (0.1 μM Hp equivalents). Values are means of three independent experiments and bars are SEM.

DISCUSSION

Most photosensitizers are observed to aggregate in aqueous medium. This may result in self-quenching of the excited state molecules and reduction in subsequent ROS production. To increase the efficacy of PDI, it is preferable to prepare the photosensitizer in its monomeric form by formulating in suitable carriers. In this study, we encapsulated Hp into liposomes and micelles. The spectral analysis of the encapsulated Hp indicated that the encapsulation process was able to retain Hp molecules in their monomeric form in these carriers. Furthermore, the observed ability of PDI to markedly reduce cell growth clearly indicate that encapsulation of Hp may exert a significant bactericidal effect.

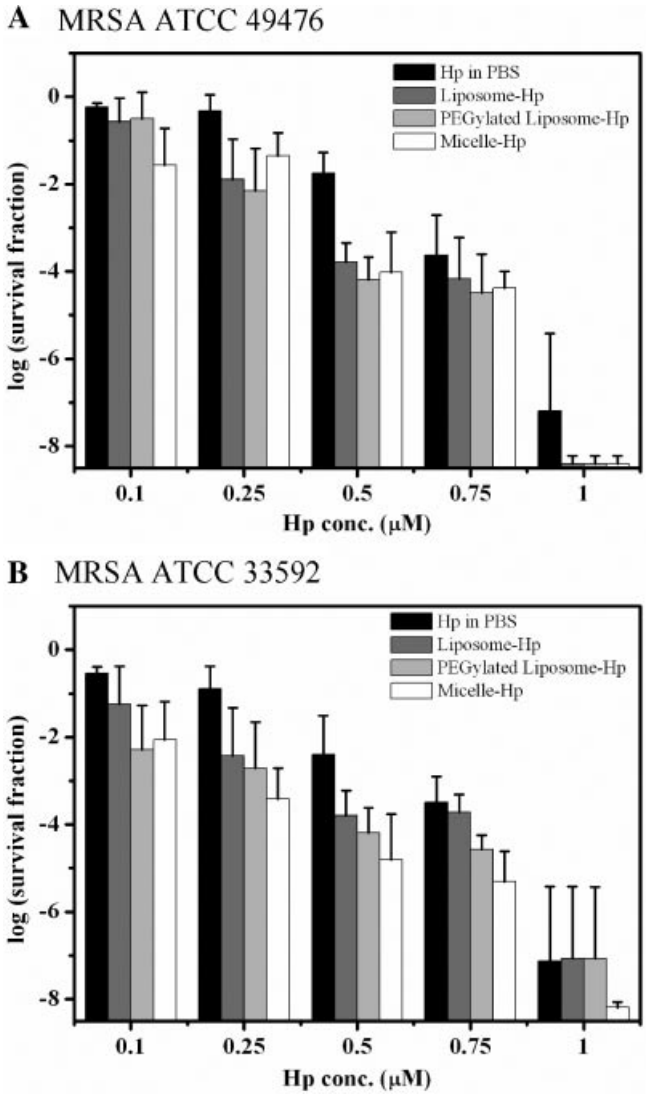

Fig. 5. Cell survival fraction of MRSA ATCC 49476 (**A**) and MRSA ATCC 33592 (**B**) after incubated with various formulations and concentrations of Hp for 30 minutes and subjected to 50 J/cm^2 of the red light illumination. Values are means of three independent experiments and bars are SEM.

There were two significant findings in this study. First, at lower Hp concentrations, the micelle group could still exert a complete bactericidal PDI effect compared to the liposome groups. In gram-positive bacteria, it has been found that macromolecules with a molecular weight in the 30–60 kDa range can readily diffuse near the plasma membrane [29]. In this regards, the smaller size of micelle may have the advantage to reside more closely to the plasma membrane of microbes. Thus, after light irradiation, eradication of the microbes could be achieved at a lower micelle-Hp concentration. Therefore, the smaller size micelle-Hp may be advantageous for bactericidal efficacy. The other finding is that the PDI susceptibility of MRSA strains was lower than the other antibiotic-susceptible strains, though PDI is accepted as non-specific and effective against both antibiotic-susceptible and resistant bacteria. In the future, it is worthwhile to extensively examine the PDI susceptibility among different MRSA strains.

For photodynamic treatment of neoplastic lesions, photosensitizers encapsulated in liposomes have been developed and proven to yield a more pronounced and selective targeting to tumor tissues [30]. However, the cost of lipids and the preparation processes might pose as barriers to the marketing of such products for antimicrobial clinical applications. Polymeric micelles have emerged as a carrier system to deliver photosensitizers for anti-tumor treatment [31]. The most popular polymer that has been used is the amphiphilic block copolymer series such as poloxamers. Poloxamers has been known to self-assemble into polymeric micelles in an aqueous environment, [32]. Meanwhile, poloxamers are non-ionic, water-soluble and inert surfactants, and are listed as inactive ingredients by the U.S. Food and Drug Administration (FDA) [33]. In this study, we used commercially available poloxamer PF127 to solubilize and encapsulate Hp and found this encapsulation can enhance the PDI effect of the model photosensitizer Hp. The increased PDI efficacy was also found by using PF127 to encapsulate chlorine e6 (Fig. 6). These results indicate that PF127 as the micelle-forming agent could be used to avoid the likelihood of aggregation of potential photosensitizer. PF127 is less expensive compared to the phospholipids required for liposome preparation. In addition, preparation of polymeric micelles is much cheaper and simpler compared to liposome preparation, which is generally much more costly and time-consuming. Since the potential of PDI in bactericidal treatments may be closely related to the cost and efficacy of the treatment, our present

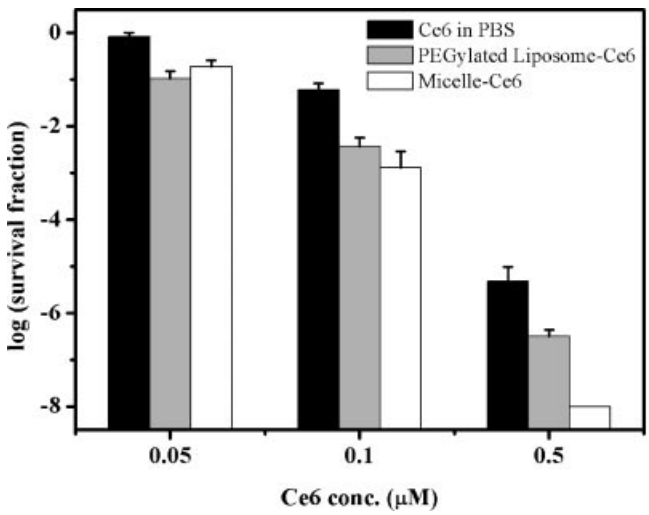

Fig. 6. Cell survival fraction of *S. aureus* after incubated with various formulations of Ce6 (0.05, 0.1, and 0.5 μM Ce6 equivalents) for 30 minutes and subjected to 10 J/cm^2 of light illumination. Values are means of three independent experiments and bars are SEM.

studies indicate that micelle delivery systems may be useful for clinical applications.

Multi-antibiotic resistance of pathogens is a rapidly growing and alarming phenomenon, and PDI may be a useful modality for treating localized infections when antibiotic treatments are limited or ineffective. Because the manufacture of polymeric micelles is simple and relatively inexpensive, micelle delivery systems are a promising carrier system suitable for potential clinical PDI application. In the future, using photosensitizers encapsulated in micelles together with a convenient and easy-to-use LED light source is an attractive development for the treatment of localized microbial infection.

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