Article

Chitosan Inhibits the Rehabilitation of Damaged Microbes Induced by Photodynamic Inactivation

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Abstract: Photodynamic inactivation (PDI) combines the nontoxic photosensitizer (PS) and visible light to generate reactive oxygen species (ROS), which can cause oxidative damages in (on) microbial organisms. Previously, we have shown that chitosan can augment the bactericidal efficacy mediated by PDI against bacteria and Candida. In this study, we showed that the antimicrobial action of chitosan to augment PDI relates to the enlargement of cell surface destruction. The microbial cell surfaces exhibit severe irregular shapes after PDI in the presence of chitosan. Furthermore, increase in the concentrations or incubation time of chitosan significantly reduce the amounts of photosensitizer TBO required, indicating that chitosan could be a synergistic agent with PDI against human pathogens. A prolonged lag phase was found in PDI surviving microbial cells, in which chitosan can act to completely eradicate the cells. Once the impaired cells rebuild their cellular functions from PDI-induced damage, the increased cytotoxic effect of chitosan disappeared. Together, our results suggest that chitosan with an augmented bactericidal activity after PDI is to inhibit the rehabilitation of PDI surviving cells, leading to microbial death.

Keywords: Photodynamic inactivation, reactive oxygen species, chitosan, cell wall

1. Introduction

Infectious diseases caused by bacteria and fungi have become a serious problem in public health. Due to the emergence of resistance among human pathogens, conventional antimicrobial therapies have become less effective [1-3]. Indeed, many antibiotic and antifungal agent-resistant pathogens have be found and led to critical issue to public health, such as methicillin-resistant *Staphylococcus aureus* (MRSA) [4], multidrug-resistant *Pseudomonas aeruginosa* and pathogenic fungi [3, 5, 6]. Unlike the abundant choices of antibiotics, only four classes of drugs (flucytosine, azoles, echinocandins and polyenes) with relative toxicity to human cells are currently used to treat fungal infections [7]. Hence, development of a promising new drug or therapy against emerging infectious diseases or drug-resistant pathogens is urgent and required.

Photodynamic therapy (PDT) is an approved therapeutic modality for the treatment of cancers and noncancerous diseases[8, 9]. The mechanism of PDT action relies on oxygen, a nontoxic photosensitizer (PS) and a low intensity visible light[10]. Photosensitizers activated by specific wavelength of light undergo photochemical reactions with O_2 to generate reactive oxygen species (ROS)[9, 10]. The cytotoxic ROS could directly cause oxidative damage to cells, microbes or tissues [9,-11]. As summarized in numerous reviews, photodynamic inactivation (PDI) against microbial cells was regarded as a new antimicrobial modality and used for treating human infectious pathogens[9, 11, 12]. Specifically, several photosensitizers, such as acridine orange, chlorins,

phthalocyanines, rose bengal (RB), methylene blue (MB), and toluidine blue O (TBO), showed a great capability of controlling infectious diseases[13-16]. For instance, several clinical bacterial and fungal pathogens, including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Escherichia coli*, *Candida albicans* and many others can be efficiently controlled by PDI *in vitro*[11, 12, 17]. Furthermore, a photosensitizer PPA904-mediated PDI for treating chronic ulcers in a phase II trial revealed its great potential for clinical therapy [18]. Presently, neither cytotoxicity nor DNA damage to keratinocytes was found *in vitro* in MB-or TBO-mediated PDI, by which can cause significant killing of typical skin microbes[19-21]. Compared to bacteria, candida are eukaryotic cells and higher doses of photosensitizers or light irradiation are required for an effective killing, which might be harmful to human cells. Thus, combination of PDI and antimicrobial agent could be a promising treatment for infectious disease.

Chitosan [poly-(β-1/4)-2-amino-2-deoxy-D-glucopyranose] is a linear polysaccharide, which can be obtained by chitin deacetylation mediated by enzymatic hydrolysis of chitin deacetylase or NaOH alkaline conditions [22-24]. Due to its biodegradability, biocompatibility and nontoxicity properties, chitosan displays its various important pharmacological applications in biomedical uses, dietary supplement food, agriculture, cosmetics industries and drug delivery[24-27]. Furthermore, a broad antimicrobial spectrum of chitosan has been reported to be effective against Gram-positive, Gram-negative bacteria and fungi [28, 29]. In this regards, chitosan was used as an alternative antimicrobial treatment owing to its many exceptional advantages. Although chitosan against microorganisms shows a great commercial potential, the exact mechanisms of its antimicrobial activity are still not fully understood. Previously, we have shown that post-treatment of chitosan can significantly enhance the biocidal efficacy of PDI against different bacteria and *Candida* cells [30, 31]. However, the action mode of chitosan in augmenting the biocidal effect mediated by PDI is not clear. In this study, we demonstrated that the augmented cytotoxicity mediated by chitosan after PDI correlates to the increase in the chitosan concentrations and incubation time. Furthermore, PDI combined with chitosan could severely damage the microbial cell surfaces and induce a prolonged lag phase in PDI surviving cells. The augmented cytotoxicity of chitosan will not be found once the cell growth enters the exponential log phase. This study demonstrates the action mode of chitosan in increasing the antimicrobial efficacy and might provide a better strategy for antimicrobial treatment.

2. Results

2.1. PDI in combination with chitosan treatment dramatically enhanced the cidal ability

To optimize the synergistic killing ability of PDI and chitosan to *S. aureus*, *P. aeruginosa* and *C. albicans*, different concentrations of TBO were incubated with microbial cells for 30 min, washed with PBS and then irradiated with red LED light (630 ± 5 nm). Microbial cells were further treated with or without chitosan following light irradiation. The surviving CFUs were shown in Figure 1. As expected, *C. albicans* with a larger cell surface require a 10-fold concentration of TBO to reduce 2~3 logs of viable cells compared to those of *S. aureus* and *P. aeruginosa*. Furthermore, microbial cells under the combined treatment of PDI and chitosan caused a complete eradication compared to those treated with PDI or chitosan alone. The chitosan concentration required for the complete killing of *S. aureus*, *P. aeruginosa* and *C. albicans* is 0.025%, 0.25% and 0.25%, respectively. These results further support our previous findings that addition of exogenous chitosan can synergistically increase the PDI biocidal efficacy against Gram (-), Gram (+) bacteria and fungi [30, 31].
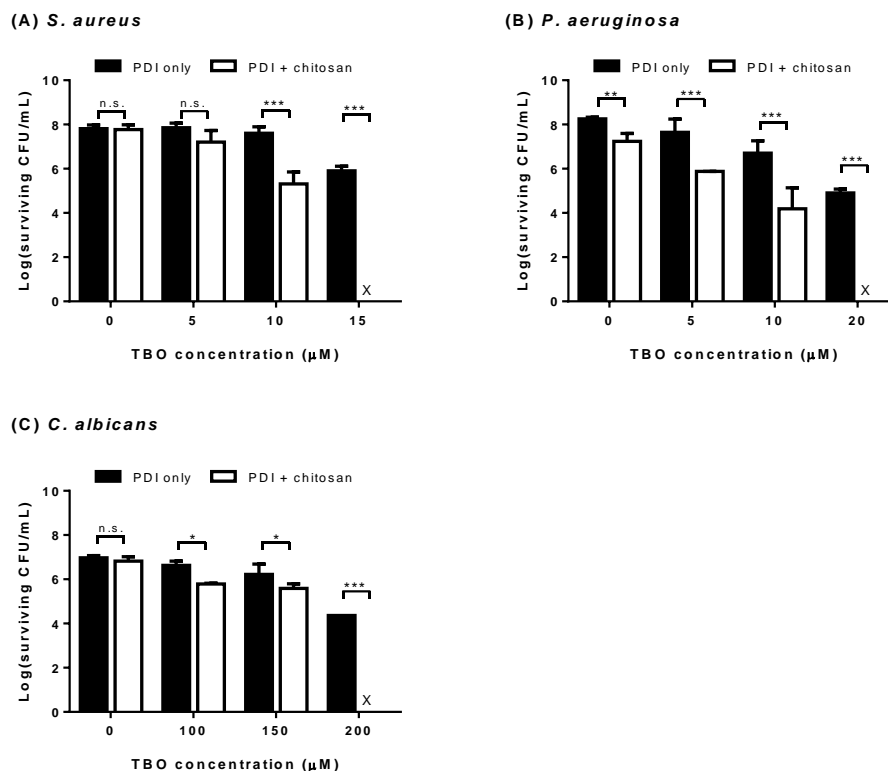
(A) *S. aureus*

(B) *P. aeruginosa*

(C) *C. albicans*

Figure 1. Chitosan augments the killing efficacy of PDI. Planktonic cells of *S. aureus* **(A)**, *P. aeruginosa* **(B)**, and *C. albicans* **(C)** subjected with TBO-mediated PDI under the light dose of 50 J/cm^2. Following PDI, microbial were further treated with chitosan for 30 minutes. The concentration of chitosan used for *S. aureus* and *P. aeruginosa* is 0.025% and 0.25%, respectively. For *C. albicans*, 0.25% chitosan was used for the post incubation after PDI. Each sample was then measured its surviving rate by plate count. At least 3 repeated experiments were performed to determine the surviving CFU/mL. Each value is the mean from three independent experiments ± standard deviation, X indicates the complete killing of cells. n.s., no significance; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

2.2. Combination treatment of PDI and chitosan caused a more severe damage on cell surface

It has been known that generation of ROS after photodynamic reaction causes oxidative damages to lipids, protein and DNA of microbial cells [32]. However, the biocidal action of PDI combined with chitosan against microorganisms remains unclear. Therefore, we performed transmitted electron microscopy (TEM) to examine the cellular morphologies of microbial cells treated with PDI or chitosan alone or under the combined treatment. As shown in Figure 2, there is no or mild damage on the cell surfaces of *S. aureus*, *P. aeruginosa* and *C. albicans* treated with PDI or chitosan alone, whereas post-incubation with chitosan after PDI caused a more severe corruption on cell surfaces, suggesting that chitosan might augment the damage on the cell surface induced by PDI.
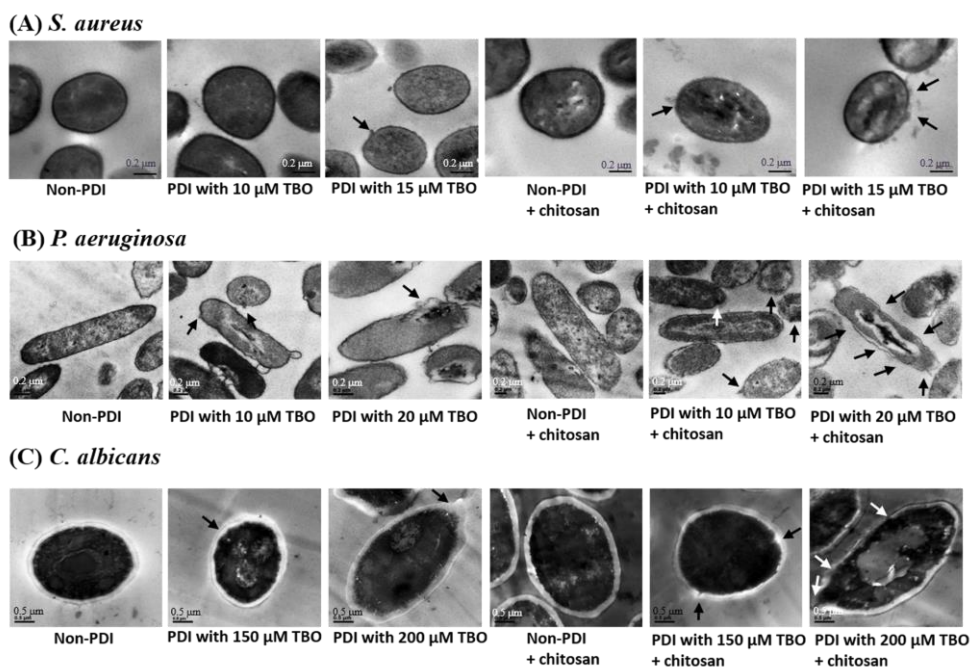
Figure 2. TEM showed that PDI combined with chitosan caused a severe damage on cell surface. Images of *S. aureus* **(A)**, *P. aeruginosa* **(B)**, and *C. albicans* **(C)** were taken after TBO-mediated PDI, chitosan or combined treatment of PDI and chitosan. Samples with PDI, chitosan or combined treatment were pre-fixed with glutaraldehyde and osmium tetroxide, followed by dehydration for in a graded acetone series. Samples were embedded, trimming with Ultracut E and mounted on copper grids and were observed using a transmission electron microscopy. Control experiment was conducted in absence of any treatment. Arrows indicate irregular cell surfaces.

2.3. Increase in the incubation time or concentration of chitosan enhances PDI-induced cytotoxicity

As shown above, PDI combined with chitosan exhibits an augmented killing effect against microbial cells. We then further examined whether the increase biocidal activity correlates with the concentration or incubation time of chitosan. To this end, we performed a low-dose PDI against bacteria and *C. albicans* by incubating with 10 μM and 150 μM of TBO, respectively. As shown in Figure 3A, 10 μM TBO-mediated PDI resulted in 1-log reduction in *S. aureus*. In the presence of 0.025% chitosan, PDI-induced cytotoxicity increases in an incubation time-dependent manner, in which a complete cell killing was found after incubated for 90 min. Similar results were also found in *P. aeruginosa* and *C. albicans* under the combination of PDI and 0.25% chitosan.
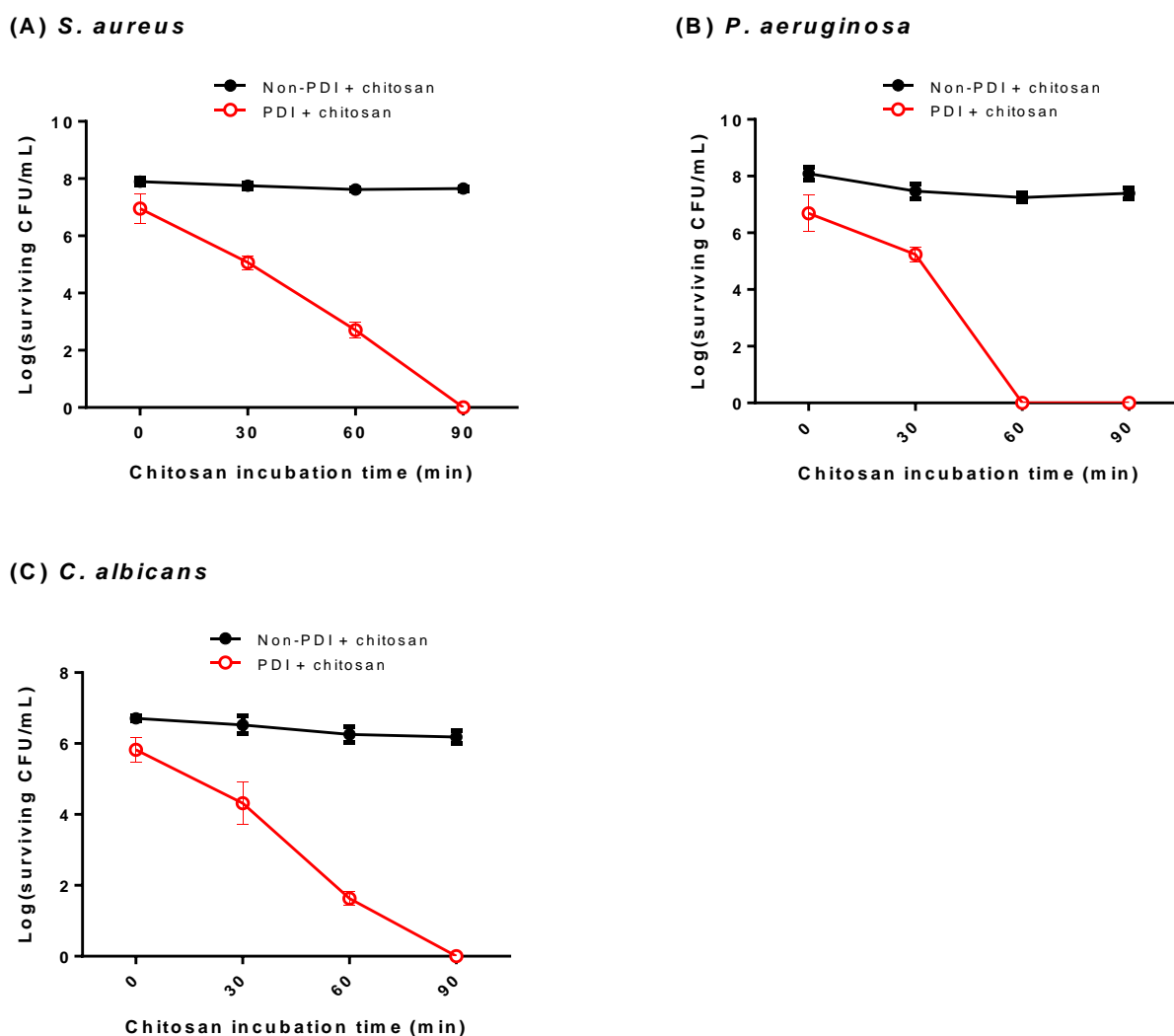
(A) *S. aureus*

(B) *P. aeruginosa*

(C) *C. albicans*

Figure 3. Increase in the chitosan incubation time can dramatically enhanced the killing effect. The concentration of photosensitizer TBO used in PDI was 10 μM in *S. aureus* **(A)** *and P. aeruginosa* **(B)**. For *C. albicans* **(C)**, 150 μM of TBO was used for PDI. After light irradiation (50 J/cm²), Chitosan were added and further incubated for different time. The concentration of chitosan used for *S. aureus* and *P. aeruginosa* is 0.025% and 0.25%, respectively. For *C. albicans*, 0.25% chitosan was used for the post incubation after PDI. As the time indicated, microbial cells were removed for plate count. Each value is the mean obtained from three independent experiments ± SD.

Furthermore, we assessed the increased level of cytotoxicity against microbial cells pre-treated with PDI under different concentrations of chitosan. As shown in Figure 4, less than 1-log reduction of viable cells was observed against bacteria (*S. aureus* and *P. aeruginosa*) and *C. albicans* in the presence of 10 μM and 100 μM TBO, respectively. However, in the presence of chitosan, increased cytotoxicity was found in PDI-treated cells, which showed in a concentration-dependent manner. Complete eradication of *S. aureus* and *C. albicans* could be found by increasing the concentration of chitosan to 0.25% and 0.75%, respectively (Figure 4A and 4C). Although, 0.75% chitosan did not completely eradicate the *P. aeruginosa* under the PDI condition of 10 μM TBO plus 50 J/cm² light dose (Figure 4B). 20 μM TBO mediated PDI combined with 0.25% chitosan can completely eradicate the *P. aeruginosa* (Figure 1B), suggesting the augmented bactericidal effect of chitosan require certain level of damage induced by PDI.
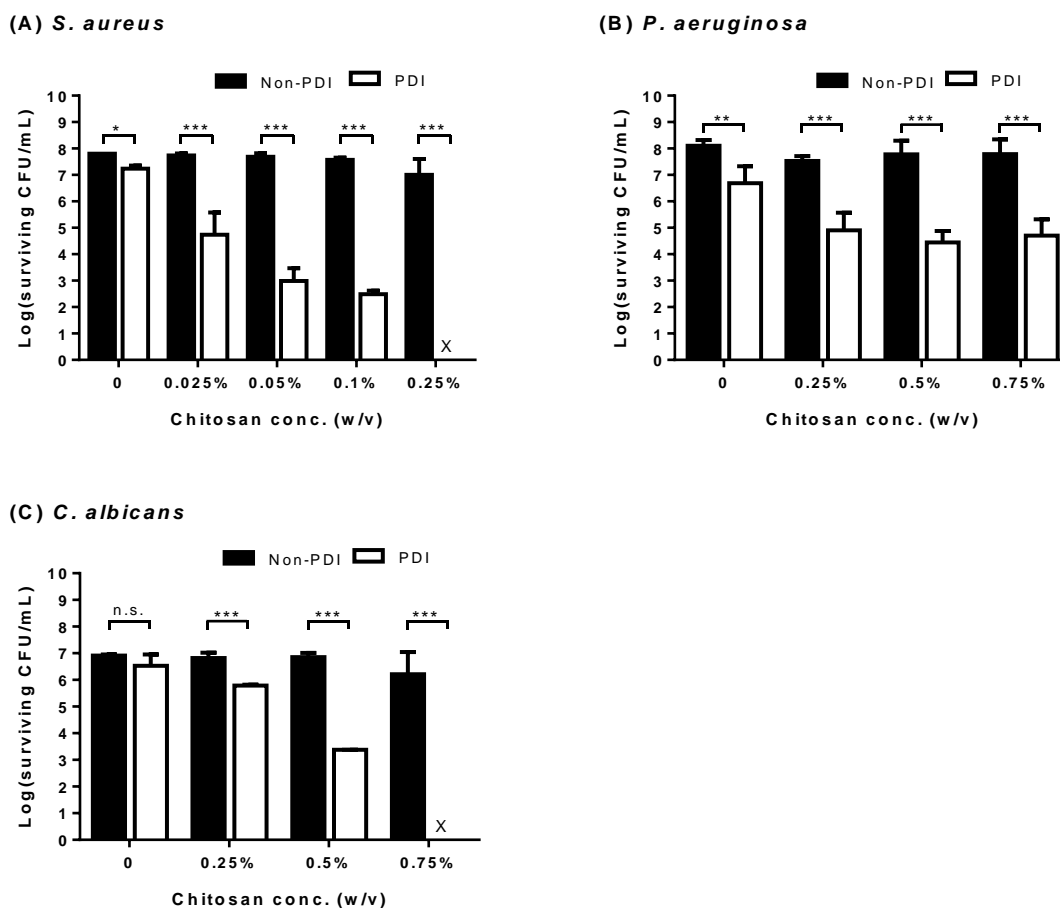
(A) *S. aureus*

(B) *P. aeruginosa*

(C) *C. albicans*

Figure 4. Increases in chitosan concentration could enhance the killing ability against *S. aureus* and *C. albicans*. Planktonic cells of *S. aureus* **(A)**, *P. aeruginosa* **(B)**, and *C. albicans* **(C)** subjected with TBO-mediated PDI were then incubated with different concentrations of chitosan for 30-min. For PDI, 10 µM TBO was used in *S. aureus* and *P. aeruginosa*. For *C. albicans*, 100 µM TBO was used. After light irradiation (50 J/cm^2), cells were further incubated with different concentration of chitosan as indicated for 30 min. Each value is the mean obtained from three independent experiments ± SD, X indicates the complete killing of cells. n.s., no significance; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

2.4. PDI resulted in a prolong lag phase in the PDI surviving cells

As noted, the augmented cytotoxicity of chitosan in PDI-treated cells relates to the damaged level induced by PDI. It is not clear whether the PDI-induced damage will affect the reproductive abilities of cells survived from PDI. We therefore further examined the growth curves of PDI surviving cells. After PDI, microbial cells were introduced into fresh culture medium, then performed plate count to determine the viable cells at different incubation time points. As shown in Figure 5, a prolonged lag phase was found in the microbial cells survived from PDI, compared to that of cells without PDI treatment. The most predominant effect was in *C. albicans* treated with PDI, having 10-h delay to enter the exponential (log) phase, whereas 2- and 6-h delay were found in PDI-treated *S. aureus* and *P. aeruginosa*, respectively. Although PDI-treated cells have a prolonged lag phase, the pattern of exponential growth was similar after leaving the lag phase, suggesting the PDI-induced damage is temporarily. These results indicate that PDI might initiate some pro-survival strategies in microbial cells to recover from the damage thereby prolonging the lag phase.
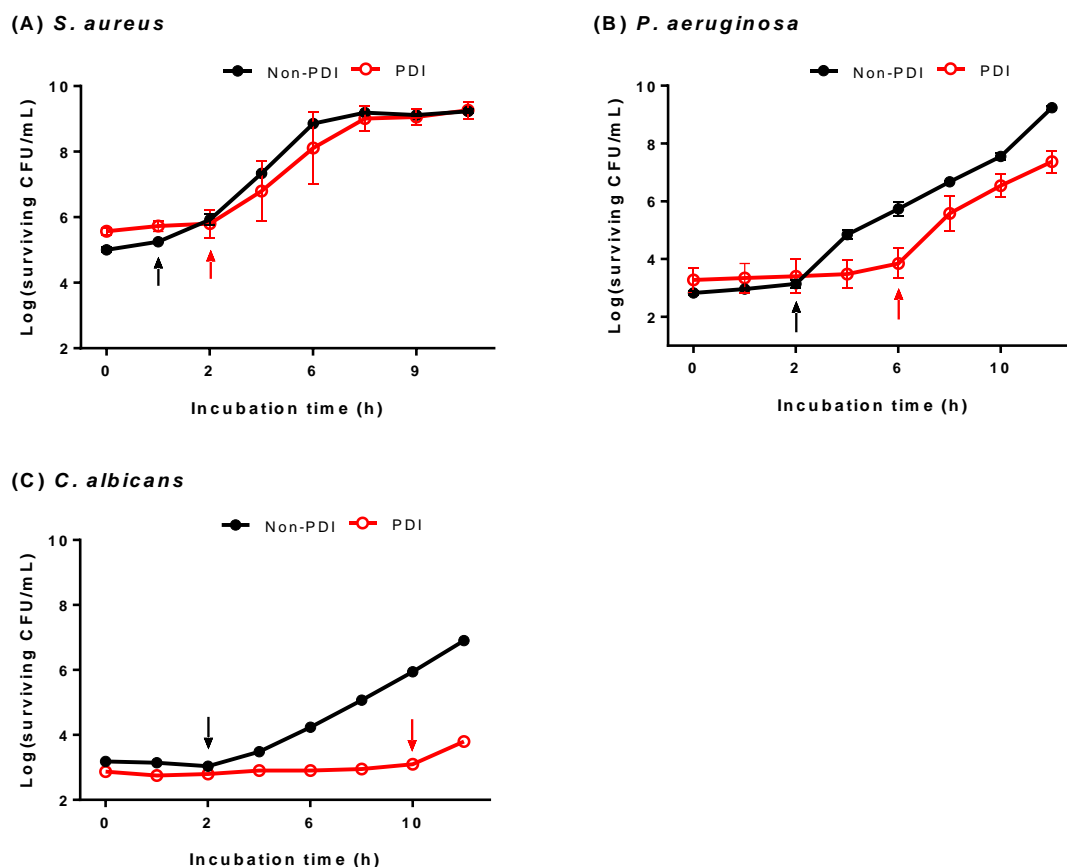
(A) *S. aureus*

(B) *P. aeruginosa*

(C) *C. albicans*

Figure 5. Prolonged recovery time in the PDI surviving cells. Cells of *S. aureus* **(A)**, *P. aeruginosa* **(B)**, and *C. albicans* **(C)** treated with or without PDI. For PDI, 15 μM and 20 μM TBO was incubated with *S. aureus* and *P. aeruginosa*, respectively. For *C. albicans,* 400 μM TBO was used for PDI. After light irradiation (50 J/cm^2), cells were sub-cultured in a fresh culture medium. During the incubation, cells were collected every 2 h to perform plate count. Black and red arrows indicate the time that PDI and non-PDI treated cells enter the exponential growth phase, respectively.

2.5. Chitosan inhibits the recovery of the damaged cells

As shown above, cell wall damage induced by PDI was augmented by chitosan, which prolongs the lag phase of surviving microbial cells. In this regards, we speculate that the augmented cytotoxicity mediated by chitosan might relate to the damage in the PDI surviving cells. To examine the susceptibility to chitosan in surviving cells, chitosan was added at different time point after PDI. Notably, 2-, 6- and 10-h was required for PDI-treated *S. aureus, P. aeruginosa* and *C. albicans* to enter the exponential (log) phase, respectively (Figure 5). As shown in Figure 6, chitosan could completely eradicate the surviving cells of *S. aureus* in the first 2 h after PDI. After that, the augmented cytotoxicity induced by chitosan gradually decrease and the cell rehabilitation recovered. Compared to *S. aureus*, at least 6 and 10-h was required for the rehabilitation of damaged *P. aeruginosa* or *C. albicans*, respectively.
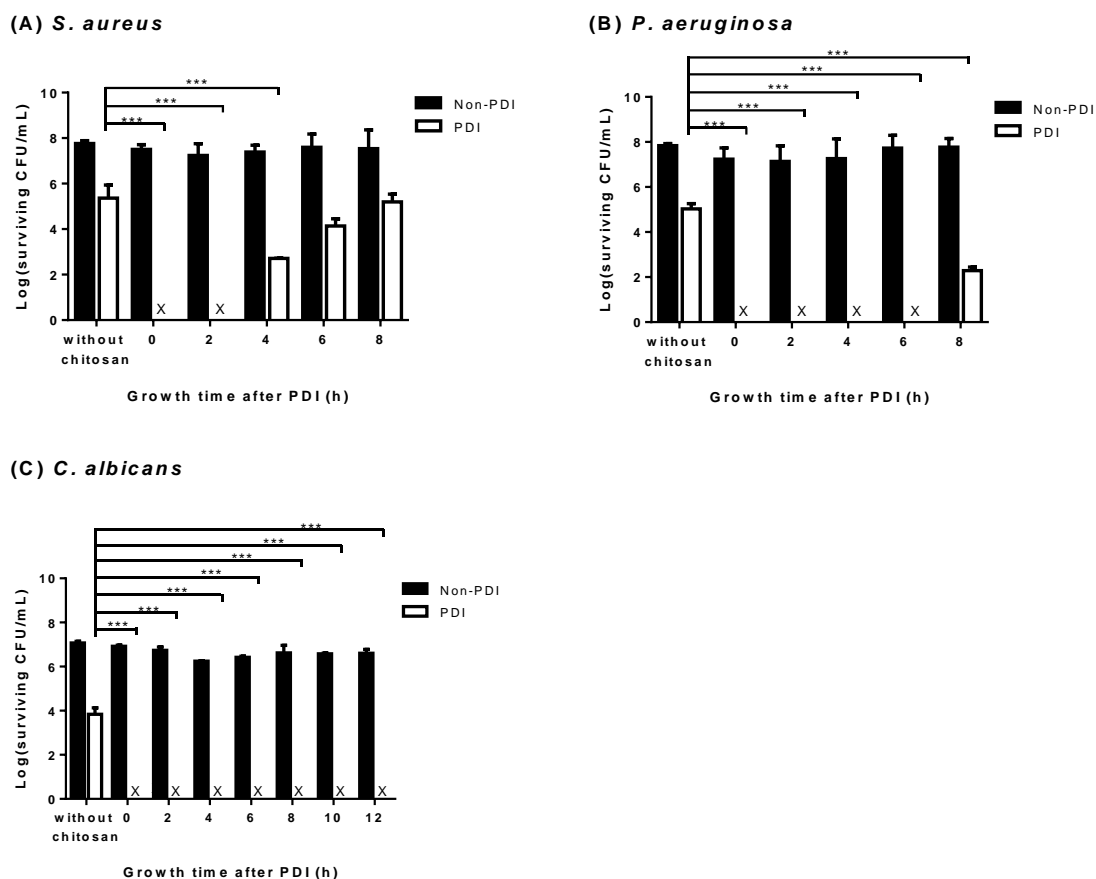
(A) *S. aureus*

(B) *P. aeruginosa*

(C) *C. albicans*

Figure 6. Chitosan inhibits the recovery of the PDI surviving cells. For PDI, 15 μM and 20 μM TBO was incubated with *S. aureus* (A) and *P. aeruginosa* (B), respectively. For *C. albicans* (C), 400 μM TBO was used for PDI. After light irradiation (50 J/cm^2), cells were sub-cultured in a fresh liquid medium. Chitosan was then added at the time indicated and incubated for 30 min. After incubation, cells were washed with PBS and performed plate count. Each value is the mean obtained from three independent experiments ± SD, X indicates the complete killing of cells. ***, P < 0.001.

3. Discussion

Management of infectious diseases caused by bacteria and fungi has confronted a serious problem due to the emergence of new strains and drug-resistant isolates which hamper the clinical treatment. Hence, development and searching of new drugs or alternative therapeutic methods to control the microbial infection have become a demanding work. Antimicrobial photodynamic inactivation against a broad range of human pathogens has been reported and recognized as a promising therapeutic modality[11, 12, 17, 31, 33, 34]. It is clear that production of ROS during the PDI is the major cytotoxic agent that leads to microbe destruction[11, 12]. Besides, ROS production from PDI has been shown to reduce the microbial virulence[34-36] and germ tube formation in *C. albicans* [34, 37]. These reports implicate that ROS not only is a toxic compound for microbes but also involve in modulating gene expression.

Although the antimicrobial properties of chitosan have been documented in several reviews[38, 39], the mechanism of chitosan in antimicrobial activity is still not fully elucidated. It has been shown that degrees of deacetylation of chitosan correlate with its antimicrobial activity [26]. Furthermore, within the lower pH solution, chitosan forms stronger cationic polymer and is able to interact with negative-charged microbial cell surface, thereby inhibiting microbial growth [40, 41]. By analyzing the genetic profiling of *S. simulans* and *S. aureus* challenged with chitosan, Raafat *et. al.* [42] demonstrated that the antibacterial activity of chitosan relates to its binding to the bacterial cell wall and membrane that further interferes with bacterial energy metabolism and electron transfer chain.

Furthermore, transcriptional responses of chitosan-treated *Saccharomyces (S.) cerevisiae* revealed that some cell wall- and stress-relative genes, such as Cin5p/Yap4p, Crz1, and Rlm1p transcription factors are involved in chitosan response [43]. Interestingly, chitosan-treated *S. cerevisiae* also exhibited less susceptibility to β-1,3-glucanase cell wall degrading enzyme, suggesting that cell membrane is more likely the binding target for chitosan [43]. Previously, we have shown that post-treated with chitosan can dramatically increase the antimicrobial effect of PDI against bacteria and *C. albicans* [30, 31]. In this study, we further demonstrated that chitosan enhances the damage on bacterial and fungal surfaces after PDI (Figure 2). These results indicate that chitosan might exert its antimicrobial effect by interfering the cell wall function and PDI-induced damage at cell wall further augment its biocidal effect.

The cell wall in a bacterium or a fungus not only provides a tensile strength for maintaining a definite shape but also protects microbes from environmental stress and immune evasion [44, 45]. Thus, differences of microbial cell walls and the permeability barriers are responsible for the susceptibility to the antimicrobial PDI. Compared to Gram-positive bacteria, most photosensitizers are less effective against Gram-negative bacteria [46-49]. The cell wall of Gram-negative bacteria consists of many-layered structures, including peptidoglycan, LPS, and lipoproteins, which might prevent the action of photosensitizer as well as chitosan [11, 47]. This might explain why the increased concentration of chitosan in *P. aeruginosa* did not cause a strong bactericidal effect (Figure 4), though a longer incubation time could result in complete cell killing (Figure 3).

Here we demonstrated the action mode of chitosan in augmenting the lethal effect of PDI to infectious organisms. We found that a longer recovery time in the lag phase is required for the PDI surviving microbes to step into the exponential growth stage, suggesting that some pro-survival or pro-death factors are initiated in response to PDI. (Figure 5). Addition of the exogenous chitosan in the lag phase results in a significantly augmented cytotoxicity (Figure 6). In this scenario, we argue that some pro-survival or pro-death factors might be suppressed or enhanced by chitosan, subsequently abolishing the damage repair and leading to cell deaths. However, once the exponential log stage began and cells have regained its physiological function, inhibitory effects of chitosan was unavailing. These results therefore suggest a more interesting issue: the potential mechanisms by which chitosan inhibits these pro-survival factors. In the future, exploration of how chitosan exhibits a great antimicrobial activity and how chitosan enhances the effectiveness of PDI will provide useful guidelines for us to develop a better way to manage infectious diseases.

4. Materials and Methods

4.1. Strains and reagents

The SC5314 *C. albicans* strain was grown in 50 mL yeast peptone dextrose (YPD) at 37℃. *Staphylococcus aureus* (BCRC 10780) and *Pseudomonas aeruginosa* (ATCC 27853) were grown in tryptic soy broth (TSB) at 37℃. Chitosan used in this study was purchased from Shin Era Technology (Taipei, Taiwan). As described previously, its molecular weight is ~20 kDa and the degree of deacetylation of chitosan is ~90% [30, 31]. The photosensitizer of toluidine blue O (TBO) used in antimicrobial photodynamic inactivation and all other chemicals were obtained from Sigma-Aldrich Chemical C. (MO, USA), unless otherwise stated.

4.2. PDI in Planktonic Cells of microbial cells

Overnight cultures of *C. albicans* harvested by centrifugation at 6000 × g for 10 min were washed with phosphate-buffered saline (PBS; pH7.4) and resuspended in PBS. 0.1 mL of *C. albicans* cells (10^7 CFU/mL) were transferred into a 96-well plate [31]. Preparation s of *S. aureus* and *P. aeruginosa* for PDI followed the same procedure, but the final cell numbers were 10^8 CFU/mL [30]. 0.1 mL of photosensitizers TBO dissolved in PBS with different concentrations were added into each sample. Samples were incubated in the dark environment for 30 min at 25℃ with rotation speeds of 100 rpm. The samples were then centrifuged with 12,000 × g for 1 min, washed with PBS and resuspended in 200 μL PBS. Samples were subjected to a high-power LED light irradiation with a wavelength at 630

± 5 with a final irradiance of 50 J/cm². PDI and non-PDI samples were diluted and plated on YPD and TSB for *C. albicans* and bacteria, respectively, to determine the antimicrobial ability. Experiments were performed at least 3 times and all results were expressed as the mean ± SD. Survival Assays were subjected to statistical analysis using student's t test.

4.3. Effect of Chitosan on TBO-mediated PDI

To test the antimicrobial effect of chitosan on bacteria and *C. albicans*, chitosan (1% *w/v*) dissolved in 1% acetic acid were prepared [30, 31]. *S. aureus*, *P. aeruginosa* and *C. albicans* treated with or without TBO-mediated PDI were further incubated with chitosan with different concentrations or incubation time to test the survival rates of each microbe. Experiments were performed at least 3 times and all results were expressed as the mean ± SD. Survival Assays were subjected to statistical analysis using student's t test.

4.4. Survival Assay

Colony-forming units (CFU) of *S aureus*, *P. aeruginosa* and *C. albicans* suspensions after PDI and non-PDI with or without chitosan treatment were presented as the following description. 10 µL of each sample with appropriate dilutions plated on YPD or TSB plates for *C. albicans* and bacteria, respectively, were incubated at 37℃ in the dark environment for 18 h. The surviving rate was expressed as follows: (cell number after PDI or PDI-chitosan treatment) / (initial sample cell number) [30, 31]. Also, to evaluate the toxicity of TBO in the dark and minimize the intrinsic experimental error, each sample was monitored and determined its surviving numbers of the non-illuminated samples. Experiments were performed at least 3 times and all results were expressed as the mean ± SD. Survival Assays were subjected to statistical analysis using student's t test.

4.5. Transmitted Electron Microscopy (TEM)

Overnight cultures of microbial samples harvested by centrifugation at 6000 × g for 10 min were washed with phosphate-buffered saline (PBS; pH7.4). Microbial cells treated with chitosan, PDI or in combination treatment were pre-fixed with 3% glutaraldehyde for 2 h. Samples were washed with PBS for 3 times and then fixed with 1% osmium tetroxide for 1.5 h, followed by dehydration for 5 min in a graded acetone series (30% and 50%) and 15 min in a graded acetone series (70%, 90% and 100%) and incubated for 15 min each in acetone. A 3:1 (overnight), 1:1 (4 h) and 1:3 (4 h) mixture of acetone and Spurr were added in the dehydrated sample during the infiltration. Samples were embedded, trimming with Ultracut E and mounted on copper grids. Samples were observed using a transmission electron microscopy (HITACHI H-7650, Japan). Control experiment was conducted in absence of any treatment.

4.6. Effects of Chitosan on the Recovery of TBO-Mediated PDI Microbial cells

The remaining surviving cells of PDI-treated and non-PDI-treated *S. aureus*, *P. aeruginosa* and *C. albicans* were re-inoculated in TSB or YPD medium, respectively. At the time indicated, chitosan was added into bacteria or *C. albicans*, incubated for 30 min. Bacterial and Candida cells were then plated on the TSB and YPD plates, respectively, to determine their survival rates and chitosan inhibitory efficacy. Experiments were performed at least 3 times and all results were expressed as the mean ± SD. Survival Assays were subjected to statistical analysis using student's t test.

Author Contributions: CHL and HFC conceived and designed the experiments. MHL carried out the experimental works. CPC and MS performed the TEM experiments. CHL and HFC participated in preparing

the manuscript. All the authors read and approved the final manuscript. CTC conceived the study, participated in its design and coordination and finalized the draft of the manuscript.

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