Efficacy of photodynamic inactivation against *Pseudomonas aeruginosa* with pulsed light and CW light excitation

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ABSTRACT

We compared methylene blue (MB)-mediated photobactericidal efficacies against *Pseudomonas aeruginosa* when using nanosecond pulsed light and CW light. In the intensity range of 10-200 mW/cm^2, there was no significant difference between two cases, while photobactericidal efficacy with nanosecond pulsed light was significantly lower than that with CW light at an intensity of 250 mW/cm^2. This is attributable to the saturated absorption of MB molecules due to high peak intensity of nanosecond pulsed light. On the basis of these results, we estimated the depth dependence of bacterial killing, showing that in the skin tissue region deeper than 1.5 mm, photobactericidal efficacy with nanosecond pulsed light was higher than that with CW light. This suggests that the advantage of using high-peak-intensity pulsed light for deep tissue treatment.

Keywords: PDT, pulsed laser, cw laser, saturated absorption, methylene blue, *Pseudomonas aeruginosa*

1. INTRODUCTION

Bacteria such as *S. aureus* and *P. aeruginosa* readily initiate infection in severe burn wounds and invade underlying tissue due to the destruction of cutaneous barrier and nutritious environment. For treatment of severe burns, therefore, control of infections is a critical issue. However, the problem associated with growing resistance against antibiotics in pathogenic bacteria needs alternative treatments; antimicrobial photodynamic therapy (APDT)[1,2] is one of the potential alternatives. PDT uses a combination of nontoxic dyes and visible light, producing reactive oxygen species to kill bacteria. By applying APDT, both antibiotics-sensitive and resistant bacteria can be photoinactivated[3] and multiple treatments might not induce a selection of resistant strains[4]. However, there is a common problem in PDT; treating deep tissue is difficult due to the limited optical penetration depth in tissue. In the case of PDT for tumor treatment, some researchers reported that high-peak-intensity pulsed laser light could penetrate to deeper tissue than continuous wave (CW) laser light, because of the saturated absorption of a photosensitizer[5-7]. However, there are only a limited number of reports on the use of pulsed light excitation for APDT[8, 9].

In this study, we investigated the efficacy of methylene blue (MB)-mediated photodynamic inactivation (PDI) against *Pseudomonas aeruginosa* using a nanosecond pulsed light excitation *in vitro*, and the results were compared with those obtained with a CW light excitation. In addition, we calculated the attenuation of light intensity in tissue to investigate the possibility of treating deep-located infections using nanosecond-pulsed light.

2. METHODS

We compared efficacies of MB-mediated PDI against *P. aeruginosa* using nanosecond pulsed light and that using CW light *in vitro*. In this study, MB with a constant concentration of 50 μM was used. To simulate the tissue depth-

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dependent photobactericidal effects with pulsed and CW light, we investigated bacterial survival fractions as a function of light intensity for both cases. We first performed experiments at a constant irradiation time of 120 sec. Secondly, to investigate whether we can obtain sufficient photobactericidal effects with low-intensity light irradiation for long period of time, we performed experiments at a constant total fluence of 30 J/cm^2.

As Pogue *et al*. pointed out[6], saturated absorption of photosensitizer molecules is considered to be an important factor to increase treatment depth in tissue. We calculated attenuation of light intensity in MB solution to estimate the saturated absorption. We measured the attenuation of light intensity in MB solution, and the results were compared with those of calculation to check the reliability of the simulation model used. Finally, we calculated the attenuation of light intensity in human skin using optical properties reported in the literature[16], and depth-dependent photobactericidal efficacies in skin were investigated.

2.1. *In vitro* experiments

P. aeruginosa (PSK strain) stored at -80℃ were thawed and suspended in saline. One hundred-μl MB solution was added to 100-μl aliquots of bacterial suspensions in a 24 well plate. Then the samples were incubated for 1 h in dark. The depth of samples in wells was approximately 1 mm. The concentration of bacteria was approximately 10^8 colony forming units per ml (CFU/ml) and the concentration of MB was 50 μM. A laser diode (HLD-2000MT, HILOGIC Inc.) was used as a CW light source and an optical parametric oscillator (OPO) pumped by the 3rd harmonics of a Q-switched Nd:YAG laser (Quantra-Ray MOPO-710 and Quantra-Ray GCR-290; Spectra Physics) was used as a nanosecond pulsed light source. The pulse duration was 5 ns and the repetition frequency was 30 Hz. The output wavelengths of LD and OPO were adjusted to 665 nm which corresponds to the absorption peak of MB molecules. Samples were irradiated in the average intensity range of 10-250 mW/cm^2. Experiments were performed under the two different irradiation conditions: (1) the constant irradiation time of 120 sec; (2) the constant total fluence of 30 J/cm^2. After irradiation, samples were serially diluted in saline and 100-μl aliquots were spread over the surface of nutrient agar. Plates were aerobically incubated for 18 h at 37ºC. Survival numbers of bacteria were evaluated by counting colonies on the plates.

2.2. Calculation of light intensity attenuation

We calculated the attenuation of light intensity in a MB solution, based on the following equation (Beer's law),

$$I(z_{n+1}, t_n) = I(z_n, t_n) \exp(-\mu_a(z_n, t_n) \Delta z), \qquad (1)$$

where I is the light intensity, z is depth, t is time, and μ_a is absorption coefficient of the solution. The grid size Δz used to discretize this equation was 1.5 x 10^{-7} m and the time step Δt was 5.0 x 10^{-11} s. The initial value of μ_a was 5.1 cm^{-1}, corresponding to the absorption of the 50-μM MB solution at 665 nm. Figure 1 shows the energy diagram of photosensitizer and oxygen molecules reported by Sterenborg *et al*. Optical properties used for calculation is shown in Table 1.

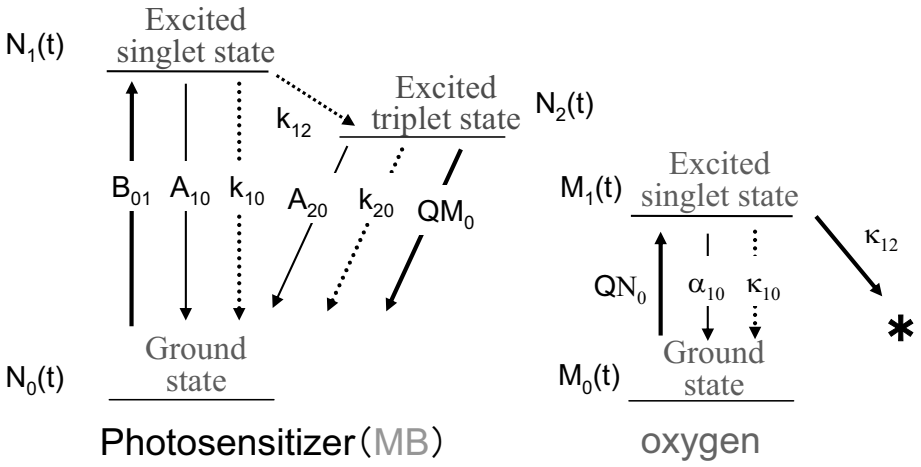


Figure 1. Energy diagram of the photosensitizer and oxygen molecules reported by Sterenborg *et al*. The asterisk represents the bound states of oxygen.

Table 1. Symbols used in the energy diagram (Fig 1) and constants used for simulation.

	Definition		Values
N_0	population of ground state MB		
N_1	population of excited singlet state MB		
N_2	population of triplet state MB		
M_0	population of ground state oxygen		
M_1	population of singet oxygen		
A_{ij}	Spontaneous transition rates		
k_{ij}	Radiationless transition rates		
α_{10}	Radiative transition rate		
κ_{ij}	Radiationless transition rates		
B_{01}	Stimulated transition rate	$B_{01} = \mu_a/N_a h\nu$	2.48×10^{-2} [$s^{-1}J^{-1}m^2$]
Q	quenching rate		1.7×10^9 [$s\ M^{-1}$] [11]
τ_f	the fluorescence decay time	$\tau_f = 1/(A_{10} + k_{10} + k_{12})$	35.8×10^{-9} [s] [12]
τ_t	the triplet decay time	$\tau_t = 1/(k_{20} + QM_0)$	30×10^{-6} [s] [13]
Φ_f	the fluorescence quantumn yield	$\Phi_f = A_{10}/(A_{10} + k_{10} + k_{12})$	0.02 [12]
Φ_t	the triplet quantumn yield	$\Phi_t = k_{12}/(A_{10} + k_{10} + k_{12})$	0.52 [14]
τ_Δ	the singlet oxygen life time	$\tau_\Delta = 1/(\alpha_{10} + \kappa_{10} + \kappa_{12})$	3.2×10^{-6} [s] [15]

Attenuation of light intensity in skin tissue was calculated, based on the following equations;

$$I(z_{n+1}, t_n) = I(z_n, t_m) \exp(-\mu_{effi(z_n, t_n)} \Delta z) \quad (2)$$

$$\mu_{eff}^2 = 3\mu_a (\mu_a + \mu_s'), \quad (3)$$

where μ_s' is the reduced scattering coefficient, which was fixed to 35.5 cm^{-1}. The initial value of μ_a was 5.1 cm^{-1}, which is the absorption coefficient of 50-μM MB solution at 665 nm.

2.3. Transmission measurement for MB solution

Quartz cuvettes filled with 50-μM MB solution were irradiated with nanosecond pulsed light and CW light. The optical path length of the cuvettes was 1 mm. We measured the light transmission in the average intensity range of 10-250 mW/cm^2. Transmittance was derived by deviding transmitted light intensity with incident light intensity.

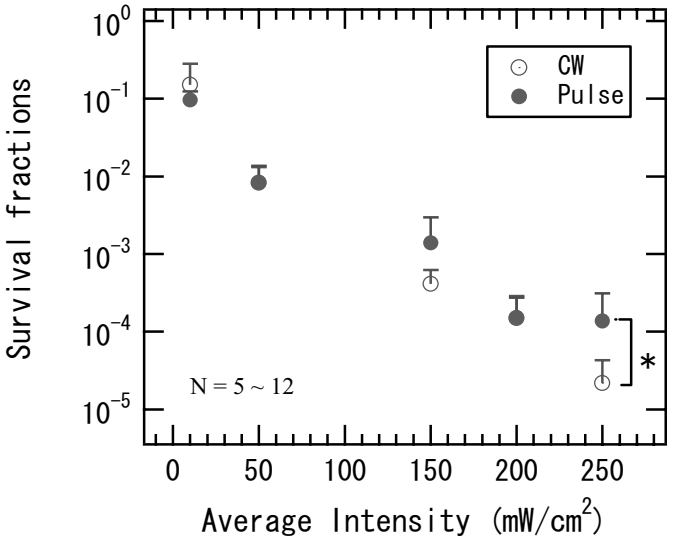
3. RESULTS AND DISCUSSIONS

3.1. *In vitro* experiment

Figure 2 shows the bacterial survival fractions as a function of average light intensity for a constant irradiation time of 120 sec. Both with nanosecond pulsed light and with CW light excitations, photobactericidal efficacy increased with increasing average intensity, indicating that PDT efficiency increases with increasing total fluence for a constant irradiation time. There was no significant difference between photobactericidal efficacy with nanosecond pulsed light and that with CW light in the intensity range of 10-200 mW/cm^2.

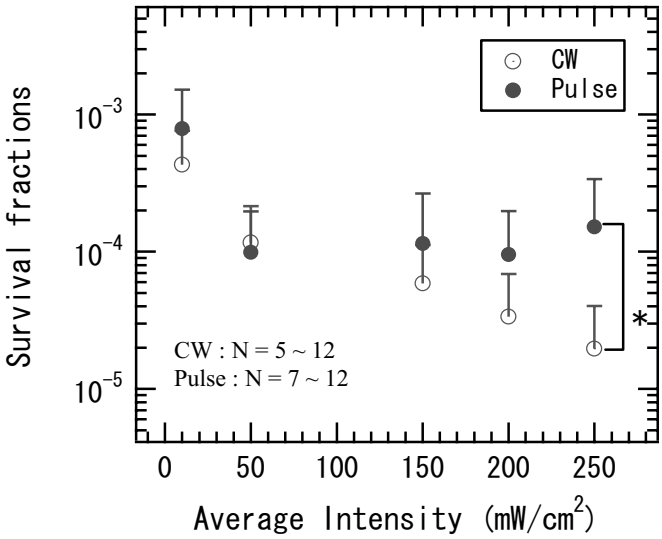
Figure 3 shows bacterial survival fractions as a function of average light intensity at a constant total fluence of 30 J/cm^2. Both with nanosecond pulsed light and CW light, photobactericidal efficacy at 10 mW/cm^2 was lower than those at 50-250 mW/cm^2 even for the same total fluence. This indicates that the PDT efficiency with high-intensity light irradiation for short period of time is higher than that with low-intensity light irradiation for long period of time for the same total fluence. With CW light, photobactericidal efficacy monotonically increased with increasing average light intensity.

With nanosecond pulsed light excitation, on the other hand, photobactericidal efficacy increased with increasing average light intensity at up to 50 mW/cm^2, but further increase in photobactericidal efficacy was not observed at higher intensities; there was a significant difference in photobactericidal efficacy between pulsed and CW light irradiations at 250 mW/cm^2. The decreased PDT efficiency with pulsed light is attributable to the saturated absorption of MB molecules.



* : $p < 0.05$
Mann-Whitney U-test

Fig. 2. Bacterial survival fractions as a function of average light intensity. Irradiation time was 120 sec. The concentration of MB was 50 μM. Data shown as mean ± SD.



* : $p < 0.05$
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Fig. 3. Bacterial survival fractions as a function of average light intensity. Irradiated total fluence was 30 J/cm^2. The concentration of MB was 50 μM. Data shown as mean ± SD.

3.2. Attenuation of light intensity

Figure 4 shows the calculated light intensity attenuation in a 50 μM MB solution at 250 mW/cm^2. In the all depth region, intensity of nanosecond pulsed light was higher than that of CW light, indicating that high-peak-intensity pulsed light causes the transient decrease in absorption due to the saturated absorption.

Figure 5 shows the light transmittance through a 1-mm thick MB solution at a concentration of 50 μM. Both with pulsed and CW light, measured and calculated transmittances show good agreements. With CW light, transmittance was almost constant at ~60 %. With nanosecond pulsed light, on the other hand, transmittance increased with increasing average light intensity, reaching nearly 80% transmittance at 250 mW/cm^2. Increased transmittance with nanosecond pulsed light is attributable to the saturated absorption of MB molecules.

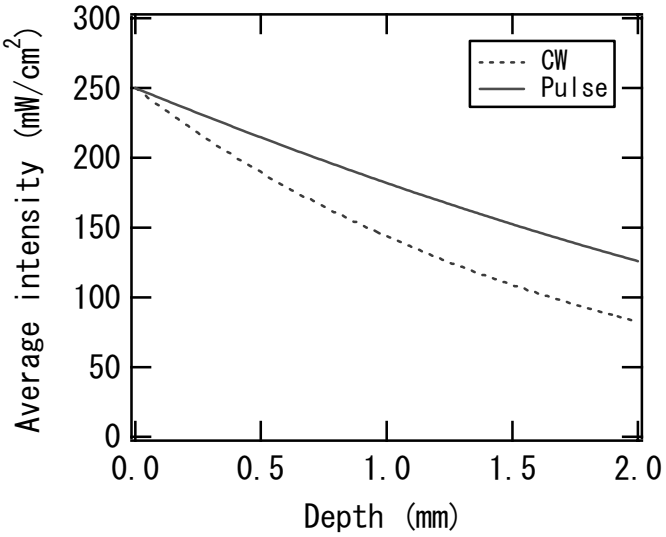


Fig. 4. Calculated light-intensity-attenuation in MB solution. Incident light intensity was 250 mW/cm^2. The concentration of MB was 50 μM.

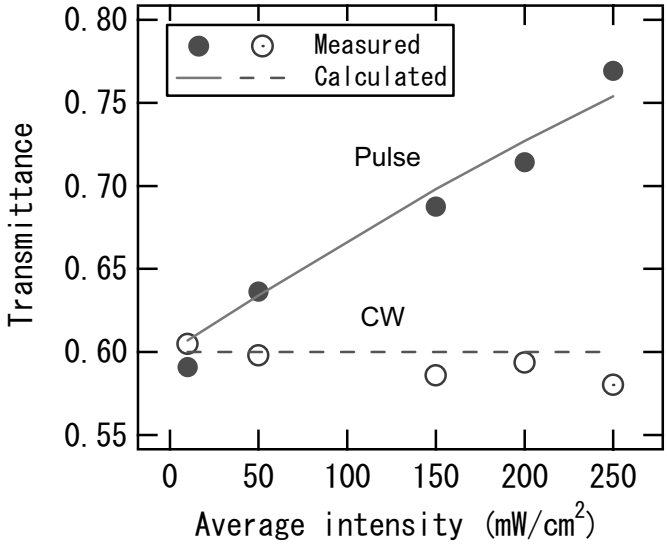


Fig. 5. Transmittance through a 1-mm thick MB solution. The concentration of MB was 50 μM. Open and closed circles indicate measured values with CW light and nanosecond pulsed light, respectively.
Solid line and dashed line indicate calculated values with CW light and nanosecond pulsed light, respectively.

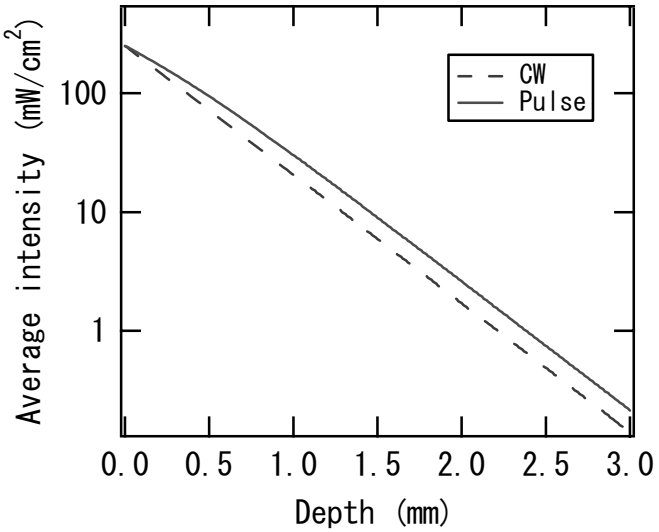
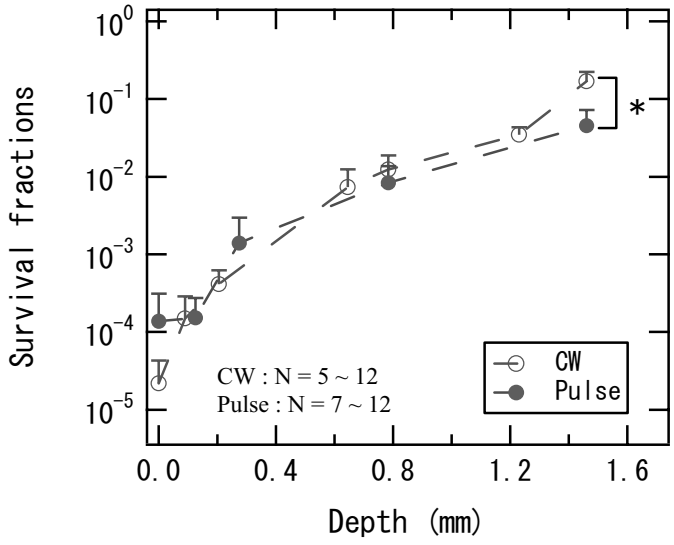


Fig. 6. Calculated light-intensity-attenuation in skin. Incident light intensity was 250 mW/cm^2.
The concentration of MB was 50 μM.

Figure 6 shows the simulated light-intensity-attenuation in human skin. Attenuation in the skin is larger than that in the MB solution (Fig. 4) due to scattering effect in the skin. In the region deeper than 1 mm, average intensity of nanosecond pulsed light is approximately 1.5 times higher than that of CW light.

3.3. Depth dependence of photobactericidal efficacy

Figure 7 shows the depth dependence of photobactericidal efficacy, which was derived from the results shown in Fig. 2 and 6. Photobactericidal efficacy with nanosecond pulsed light was slightly higher than that with CW light in the region deeper than 0.8 mm; there was a significant difference between pulsed and CW light excitatons at a depth of 1.5 mm. Since the reduced scattering coefficient of skin tissue (μ_s' = 35.5 cm^{-1}) is much higher than the absorption coefficient of MB (μ_a = 5.1 cm^{-1}, 50 μM), the effect of saturated absorption is lowered in skin.



* : $p < 0.05$
Mann-Whitney U-test

Figure 7. Depth dependence of photobactericidal efficacy.
The irradiation time was 120 sec and the concentration of MB was 50 μM. Data shown as mean ± SD.

4. CONCLUSION

We compared photobactericidal efficacies with nanosecond pulsed light and CW light. Photobactericidal efficacy with nanosecond pulsed light excitation was slightly higher than that with CW light excitation in the region deeper than 0.8 mm; there was a significant difference at a depth of 1.5 mm. By optimizing the irradiation conditions, advantage of using pulsed light for deep tissue treatment can be improved.

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