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ORIGINAL ARTICLE

Enhanced germicidal effects of pulsed UV-LED irradiation on biofilms

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Abstract

Aims: The major objective of the study was to evaluate the enhanced germicidal effects of low-frequency pulsed ultraviolet A (UVA)-light-emitting diode (LED) on biofilms.

Methods and Results: The germicidal effects of UVA-LED irradiation (365 nm, 0·28 mW cm^{-2}, in pulsed or continuous mode) on *Candida albicans* or *Escherichia coli* biofilms were evaluated by determining colony-forming units. The morphological change of microbial cells in biofilms was observed using scanning electron microscopy. After 5-min irradiation, over 90% of viable micro-organisms in biofilms had been killed, and pulsed irradiation (1–1000 Hz) had significantly greater germicidal ability than continuous irradiation. Pulsed irradiation (100 Hz, 60 min) almost completely killed micro-organisms in biofilm (>99·9%), and 20-min irradiation greatly damaged both microbial species. Interestingly, few hyphae were found in irradiated *Candida* biofilms. Moreover, mannitol treatment, a scavenger of hydroxyl radicals ($OH^{\bullet}$), significantly protected viable micro-organisms in biofilms from UVA-LED irradiation.

Conclusions: The study demonstrated that pulsed UVA-LED irradiation has a strong germicidal effect (maximum at 100 Hz, over 5-min irradiation) and causes the disappearance of hyphal forms of *Candida*.

Significance and Impact of the Study: This study can assist in developing a low-frequency pulsed UVA-LED system to be applied to pathogenic biofilms for disinfection.

Introduction

Ultraviolet (UV) has long been applied as an effective treatment to disinfect wastewater and drinking water at clinical and industrial facilities (von Woedtke *et al.* 2003; Hijnen *et al.* 2006). A low-pressure mercury lamp, which emits germicidal UVC (shorter than 280 nm), is conventionally used as a light source. In recent years, UV-light-emitting diodes (LEDs) have been developed with the following many advantages: low cost, energy-efficient, long life, easy control of emission and no production of mercury waste (Hamamoto *et al.* 2007). The wave length of the commercial UV-LED is around 365 nm, UVA (315–400 nm). Some *in vitro* studies have clarified the germicidal effects of UVA against several bacteria or fungi (Berney *et al.* 2007; Azar Daryany *et al.* 2008, 2009). It has also been reported that UVA irradiation inactivates the bacteria by creating reactive oxygen species (e.g. H_2O_2, O^{2-} and OH^-) via the photooxidation of O_2, resulting in single-strand DNA breaks, whereas UVC irradiation directly causes DNA damage (World Health Organization, 1994).

Some bacteria and fungi develop biofilm formation on implanted biomaterials and medical devices in a highly clinically significant process, especially in oral and urinary regions (Douglas 2003; Hall-Stoodley and Stoodley 2009). Biofilm acts as a reservoir for persistent sources of infection. Lewis (2001) pointed out that such common

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infections as urinary tract infections (caused by *Escherichia coli* and other pathogens), common dental plaque formation and stomatitis (caused by *Candida albicans* and other pathogens), all of which are caused by biofilm, are hard to treat or frequently relapse. Less common but certainly more threatening biofilm infections cause serious morbidity and mortality. Although UV-LED is becoming more available for disinfection and sterilization in various fields and has planktonic germicidal effects on micro-organisms in medium (Hamamoto *et al.* 2007; Mori *et al.* 2007), the germicidal effects of continuous- or pulsed-wave UV irradiation on biofilm remain unclear. Recently, UV light has been emerging as a preventative strategy offering new hope in the management of biofilm-related infections and achieving success in the disinfection of water (Lakretz *et al.* 2010) and optical instruments (Patil *et al.* 2007).

On the other hand, it has been discussed that extremely low-frequency (<300 Hz) waves and vibrations including an alternative magnetic field could cause biological effects (Markkanen *et al.* 2001; Bohrerova *et al.* 2008; Sharifian *et al.* 2009). Recent studies on UV disinfection have focused on comparing the germicidal efficiency of continuous and pulsed UV exposure against diverse pathogens, e.g., *E. coli*. It is considered that pulsed UV application is a more efficient and effective method than continuous-wave UV (Bialka and Demirci 2008). Several studies have also suggested that pulsed-wave UV may more effectively inactivate pathogens than continuous-wave UV under the same exposure conditions (Fine and Gervais 2004; Bohrerova *et al.* 2008); however, some opposing results showed no difference in microbial inactivation between continuous- and pulsed-wave UV light irradiations (Otaki *et al.* 2003; Wang *et al.* 2005). This divergence between the reports regarding microbial inactivation using UV light irradiation may reflect different light sources; therefore, it remains a controversial issue.

In this study, we focused on low-frequency pulsed UV-LED as a light source and clarified the germicidal effects of low-frequency pulsed UV-LED irradiation on *in vitro C. albicans* and *E. coli* biofilm using a novel UV-LED disinfection system.

Materials and methods

Micro-organism strains and biofilm formation

Candida albicans CAD1, a clinical isolate, and *E. coli* K12, a laboratory strain, were used for biofilm development in this study. *Candida albicans* CAD1 suspension ($2{\cdot}0 \times 10^7$ colony-forming units; CFU ml^{-1}) was prepared as described previously (Hirota *et al.* 2005). To develop biofilms, the suspension was diluted in yeast nitrogen base (YNB)/100 mmol l^{-1} glucose medium supplemented with 2·5 mmol l^{-1} *N*-acetylglucosamine (Glc*N*Ac) (Hogan *et al.* 2004; Yoshijima *et al.* 2010) at a final concentration of $5{\cdot}0 \times 10^3$ CFU ml^{-1}. One millilitre of *C. albicans* suspension was added to each well of a 24-well culture plate with a type I collagen coating coverslip (Celldesk LF1; Sumitomo Bakelite Co., Tokyo, Japan) and was aerobically incubated for 3 days at 37°C. For *E. coli* biofilm development, 1 ml of *E. coli* K12 suspension ($1{\cdot}0 \times 10^7$ CFU ml^{-1}) in Luria–Bertani broth was added to each well and aerobically incubated for 2 days as described previously. The numbers of *C. albicans* and *E. coli* in biofilms developed on Celldesk were $2{\cdot}5$–$3{\cdot}5 \times 10^6$ CFU per Celldesk and $2{\cdot}5$–$3{\cdot}0 \times 10^7$ CFU per Celldesk, respectively.

UV-aid LED disinfection system and UV irradiation

The UV-LED disinfection system developed in this study is composed of a current regulator (Model F30PV; FLC ElectronicsAB, Partille, Sweden), function regulator (WF1973; nF, Yokohama, Japan) and UV-LED device (NCSU033A(T), wave length: 365 nm; Nichia Chemical, Tokushima, Japan) (Fig. 1a). In this study, the current value was set to 700 mA, and the UV beam from the

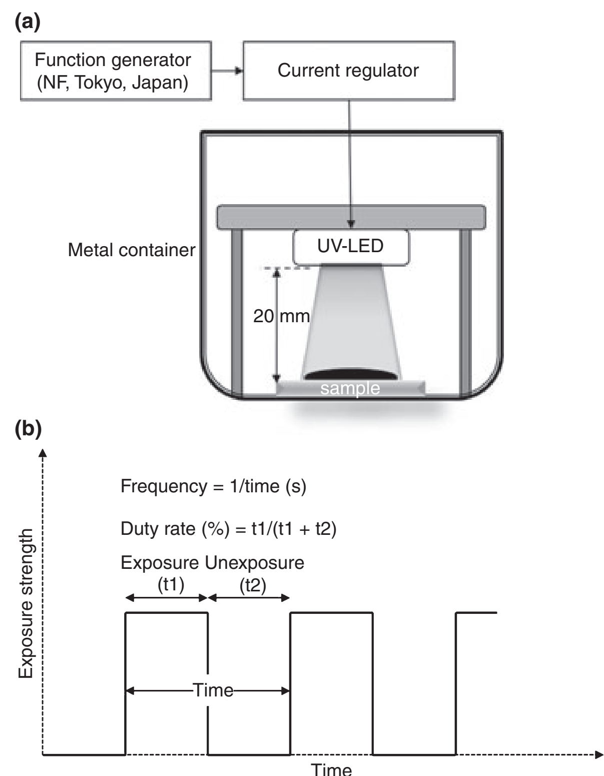

Figure 1 (a) UV-light-emitting diode (LED) disinfection system; (b) description of pulsed UV-LED.

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LED device was emitted at a distance of 20 mm from the target biofilm for exposure. Either a continuous mode or a pulsed mode was applied to irradiate the biofilm. The intensity of UV-LED light at 365 nm in continuous or pulsed mode was measured and set to 0·28 mW cm^{-2}. Therefore, the total dosages of pulsed and continuous mode were same. Exposure pulses were employed with frequencies of 0·1, 1, 10, 100 and 1000 Hz (in multiples of 10) and duty rate of 25, 50 and 75% (Fig. 1b). Duty rate means the percentage of the exposure time in total operating time and was defined by the equation shown in Fig. 1b. For example, the oscillation time in each pulsed mode (duty rate 50%) was twice as long as the irradiation time in continuous mode. The total actual exposure dosage over a fixed period of time was the same in all frequency pulsed and continuous modes.

After incubation, each biofilm formed on Celldesk was exposed to all exposure conditions: frequencies, duration and duty rate of UV-LED irradiation at room temperature in an enclosed sterile metal container. As a control, unexposed biofilms were placed in the container during the same period.

Cell growth and viability of micro-organisms

To evaluate the viability of the tested micro-organism cells in exposed biofilm, biofilms were treated with phosphate-buffered saline (PBS) supplemented with 2·5% trypsin to obtain single-cell suspensions. One hundred microlitres of appropriately diluted suspension was spread on Sabouraud glucose agar (for *C. albicans*) or trypticase soy agar (for *E. coli*) and aerobically incubated for 48 h at 37°C. Under each experimental condition, these experiments were performed six times in duplicate.

Treatment of exposed biofilms with mannitol

It has been reported that UVA-LED (wavelength: 365 nm) induces cellular membrane damage and growth delay indirectly by increasing the levels of reactive oxygen species, such as hydroxyl radicals ($OH^{\bullet}$) (Hamamoto *et al.* 2007). To evaluate the role of hydroxyl radicals in the inactivation of biofilms by UV-LED irradiation, biofilms were treated with 100 μl of 10 mmol l^{-1} mannitol, a scavenger of $OH^{\bullet}$, or PBS in each well for 5 min before UV irradiation by pulsed UV at selected frequencies or by continuous UV, and the survival rate was calculated.

Scanning electron microscopy (SEM)

The biofilms of *C. albicans* or *E. coli* formed as described earlier were irradiated at 1 or 100 Hz for 5 or 20 min. Unexposed and exposed biofilms were aerobically incubated overnight at 37°C. Then, the biofilm was rinsed to remove loosely adherent cells and fixed in 2·5% glutaraldehyde solution overnight at room temperature. Subsequently, the biofilms were dehydrated through a graded series of ethanol solution to 100%, air-dried and coated with Au ion for SEM analysis. SEM was carried out with a Miniscope TM-1000 electron microscope (Hitachi, Tokyo, Japan).

Statistical analysis

All data were analysed using one-way ANOVA and Turkey's multiple comparison test with SPSS software (ver. 12.0; SPSS Japan Inc., Tokyo, Japan). Significant difference was accepted at $P < 0·05$.

Results

Inactivation effect of UV-LED exposure frequency on biofilm

We investigated the inactivation effect of UV-LED exposure frequency on microbial biofilms. Figure 2 shows the survival rates of *C. albicans* and *E. coli* cells in biofilm irradiated in continuous mode (for 2·5 min) or pulsed mode (for 5 min, 0·1–1000 Hz and in multiples of 10) using the UV-LED system.

For *C. albicans* biofilm, the survival rates of all exposed groups were <10%. In particular, at 10 and 100 Hz, the rates were below 1%, and there was no significant difference between 10 and 100 Hz ($P = 0·731$). These experimental results show that UV-LED exposure from 1 to 1000 Hz in pulsed mode had more inactivation efficiency

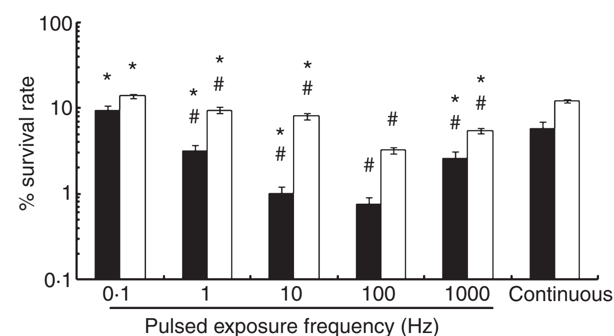

Figure 2 Inactivation effect of UV-light-emitting diode (LED) exposure frequency on *Candida albicans* (■) and *Escherichia coli* (□) biofilms. Biofilms were irradiated in continuous mode (for 2·5 min) or pulsed mode (for 5 min, 0·1–1000 Hz, in multiples of 10) using the UV-LED system. Error bars indicate SDs. Asterisks indicate significant differences *vs* the 100 Hz group (*, $P < 0·01$). # indicates significant differences *vs* the continuous group (#, $P < 0·01$).

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against *C. albicans* biofilm than continuous mode for the same effective exposure time. Interestingly, the inactivation rate increased from 0·1 to 100 Hz in a frequency-dependent manner, peaked at 100 Hz (99·25%) and then declined at 1000 Hz.

For *E. coli* biofilm, the survival rates at 1–1000 Hz were <10%, and UV-LED exposure at 100 Hz showed the strongest inactivation effect on *E. coli* biofilm. A similar tendency was observed between the values of all exposed *E. coli* group and those of the *C. albicans* group (Fig. 2).

Inactivation effect of UV-LED exposure duration on biofilm

We determined the inactivation effect of UV-LED exposure duration on microbial biofilm. Figure 3 shows the survival rates of *C. albicans* and *E. coli* cells in biofilm irradiated in continuous or pulsed mode (at 100 Hz and 50% duty rate) using UV-LED system for the indicated periods. The inactivation efficiency in continuous and pulsed mode increased in an exposure duration-dependent manner.

For *C. albicans* biofilm, the survival rate in continuous mode ranged from 1 to 10% for 2·5-to 30-min irradiation, which is equivalent to 5- to 60-min pulsed exposure at 50% duty rate, while the pulsed mode at 100 Hz covered the range from 0·1 to 1%. We also observed that the mean inactivation rate of irradiation at 100 Hz for 60 min was high (99·93%; data not shown). These results indicate that the inactivation efficiency in pulsed mode at 100 Hz exceeded that in continuous mode, and the difference between modes seemed to slightly increase with the exposure duration.

For *E. coli* biofilm, the survival rate in continuous mode also ranged from 1 to 10% for 2·5- to 30-min irradiation, while the survival rate in pulsed mode further decreased as the duration increased, especially from 20 to 60 min.

In particular, the germicidal efficiency of 5-min exposure in pulsed mode on *C. albicans* and *E. coli* biofilms was 6·34- and 2·53-fold higher than in continuous mode, respectively. For both biofilms, 60-min exposure to pulsed UV-LED at 100 Hz was required to achieve an inactivation rate of 99·9%.

Inactivation effect of UV-LED duty rate on biofilm

To further investigate the effect of the duty rate on the inactivation of microbial cells in biofilm, germicidal efficiency after 5-min irradiation in pulsed mode at 100 Hz was evaluated at duty rates of 25, 50 and 75% and compared to continuous mode. As shown in Fig. 4, the mean values of the survival rates among all exposed groups of *C. albicans* biofilm were significantly different ($P < 0{\cdot}05$). While the mean value of the inactivation rate in continuous mode was 91·53%, that of all pulsed groups exceeded 99·0% and were increased in a duty rate-dependent manner.

For *E. coli* biofilm, the inactivation characteristics of all exposed groups were similar to those for *C. albicans*. All mean values of the inactivation rate in pulsed mode exceeded 90%, while continuous mode was lower.

Effect of mannitol treatment on biofilm irradiated by UV-LED

To evaluate the role of hydroxyl radicals, which indirectly lead to microbial cellular membrane damage and microbial growth delay, in the inactivation of microbial biofilms by UV-LED irradiation, the biofilms were treated

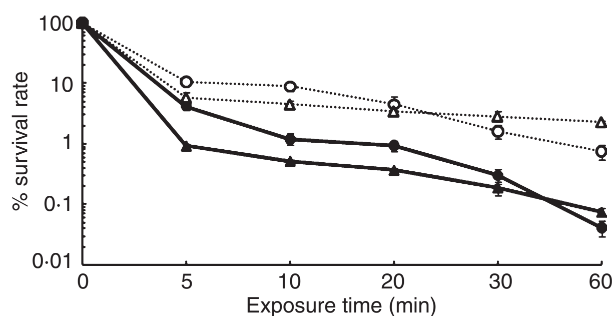

Figure 3 Inactivation effect of UV-light-emitting diode exposure duration on *Candida albicans* and *Escherichia coli* biofilms. (△) *C. albicans* and (○) *E. coli* biofilms irradiated in continuous mode. (▲) *C. albicans* and (●) *E. coli* biofilms irradiated in pulsed mode at 100 Hz. Error bars indicate SDs.

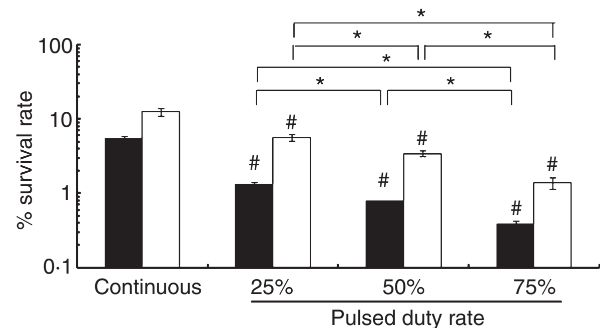

Figure 4 Inactivation effect of UV-light-emitting diode (LED) exposure duty rate on *Candida albicans* (■) and *Escherichia coli* (□) biofilms. Biofilms were irradiated in continuous mode (for 2·5 min) or pulsed mode (for 5 min, at 100 Hz) using the UV-LED system. Error bars indicate SDs. Asterisks indicate significant differences (*, $P < 0{\cdot}05$) between the indicated groups. # indicates significant differences *vs* the continuous group (#, $P < 0{\cdot}05$).

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with 10 mmol l^{-1} mannitol for 5 min before UV-LED irradiation at 100 Hz. As shown in Fig. 5, both microbial survival rates in mannitol treatment groups were significantly higher than the untreated control (PBS) groups ($P < 0{\cdot}01$). For example, the mean survival rates of *C. albicans* biofilm in mannitol and PBS treatment groups were 7·23 and 0·88%, respectively, indicating that mannitol treatment, which scavenges hydroxyl radicals, protected *C. albicans* cells in biofilm against injury by 5-min UV-LED irradiation at 100 Hz and that hydroxyl radicals increased by UV-LED irradiation largely underlie this fungal inactivation mechanism. Moreover, the inactivation characteristics of mannitol treatment groups of *C. albicans* and *E. coli* biofilm irradiated by pulsed UV-LED at the tested frequencies or with continuous UV-LED were similar to those of PBS treatment groups.

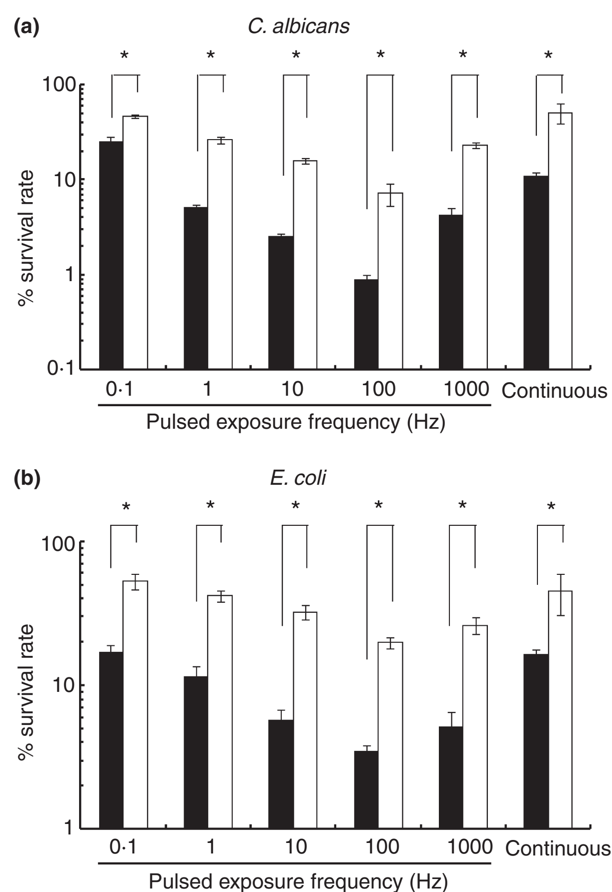

Figure 5 Effect of mannitol treatment on (a) *Candida albicans* and (b) *Escherichia coli* biofilms irradiated by UV-light-emitting diode (LED). Biofilms were irradiated in continuous mode (for 2·5 min) or pulsed mode (for 5 min, 0·1–1000 Hz, in multiples of 10) using the UV-LED system. Error bars indicate SDs. Asterisks indicate significant differences ($P < 0{\cdot}01$) between the mannitol treatment group (□) and untreated control group (■).

SEM observation

To confirm microbial cell morphology in biofilm after 5- and 20-min UV-LED irradiation at 1 and 100 Hz, SEM observation was performed. As shown in Fig. 6, SEM observation showed that the visible amounts of *C. albicans* and *E. coli* on the Celldesk after UV-LED irradiation at 1 and 100 Hz for 5 min were slightly decreased when compared with those of the unexposed control, indicating that UV-LED irradiation inhibited quantitative growth in *C. albicans* and *E. coli* and consequently delayed the formation of biofilm. This decrease was in an exposure duration-dependent manner. In particular, under high magnification (×2000), we observed that most *C. albicans* and *E. coli* cells irradiated at 100 Hz for 20 min were disrupted and formed small clumps of denatured micro-organism cells, and some of their surfaces appeared perforated (Fig. 6a). In contrast to these irradiated *Candida* cells, the control samples showed biofilm composed of intact *Candida* cells with daughter cells and hyphal formation; however, few hyphae were found in exposed *C. albicans* samples. These observations may imply that UV-LED irradiation inhibited hyphal formation in *Candida* biofilm and mature biofilm formation by both micro-organisms.

Discussion

There are two modes of application of UV light: continuous UV mode, which is the conventional one and delivers the UV light in a continuous mode, or pulsed UV mode, in which the UV light is energized by electrical charge stored in a capacitor and released as intermittent short pulses, thus increasing the instantaneous energy intensity (Krishnamurthy *et al.* 2007). Thus, pulsed UV light has more instantaneous energy than continuous UV light for the same total energy supplied. Therefore, pulsed UV light treatment is more effective and rapid way of inactivating micro-organisms than conventional or continuous UV light sources because the energy is multiplied manyfold. Although UV irradiation has been applied as an effective method of disinfection and sterilization for decades, no reports have clarified the inactivation effect of low-frequency pulsed UV irradiation on biofilm. In this study, we first established a simple and standard *in vitro* biofilm model for developing *C. albicans* and *E. coli* biofilm and investigated the enhanced germicidal effects of low-frequency pulsed UV-LED, our novel disinfection system, against biofilm. We sought to determine the susceptibility of biofilms to this system and the inactivation efficiency by exposure frequency, duration and duty rate in pulsed mode compared to continuous mode in this system.

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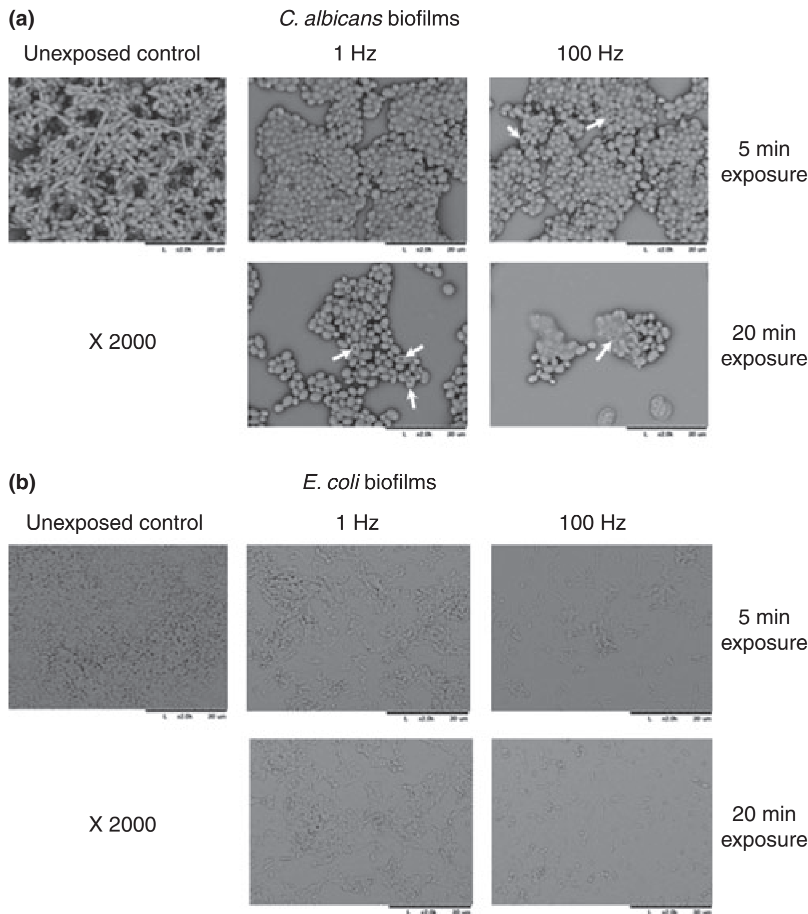

Figure 6 Scanning electron microscopy micrographs of (a) *Candida albicans* and (b) *Escherichia coli* biofilms on Celldesk (Magnification, ×2000). Biofilms were irradiated in pulsed mode (for 5 or 20 min) using the UV-light-emitting diode system. Some of *C. albicans* surfaces appeared perforated (arrows).

Here, we demonstrated that the UVA-LED system is an efficient method for the inactivation of both *C. albicans* and *E. coli* biofilm. Both pulsed and continuous UV-LED irradiation for 5 min had strong inactivation effects on micro-organism biofilm. By comparison with the continuous mode, significantly greater germicidal efficiency in pulsed mode was revealed in the frequency spectrum from 1 to 1000 Hz (Fig. 2). In particular, the experimental results of the inactivation rates indicated that 5-min pulsed UV-LED irradiation at 100 Hz could be applied in a clinical setting as an effective and rapid disinfection method against microbial biofilm. In agreement with this result, the rapid germicidal effects of pulsed UV irradiation have also been shown, with the maximum reduction of *E. coli* in fruit samples of 3·9 $\log_{10}$ CFU g^{-1} at 72 J cm^{-2} after 60-s irradiation using Xenon pulsed UV light (Bialka and Demirci 2008). It was also suggested that the differences in germicidal efficiency between previous reports and our results may be because of the different UV light source. To date, the inactivation mechanism of pulsed UV light is not well understood. It has been considered that the predominant inactivation mechanism of conventional UV light is through the thymine dimer formation within the cells DNA (photochemical), which leads to bacterial death (Rowan *et al.* 1999). However, additional inactivation mechanisms of pulsed UV light have been proposed through the localized heating (photothermal) and the constant disturbance of high intensity pulses on bacterial structure (photophysical) (Krishnamurthy *et al.* 2007). Because of these additional inactivation mechanisms, pulsed UV light is 4–6 times effective than continuous UV light in terms of microbial inactivation (Fine and Gervais 2004). Our present findings and previous reports suggest that pulsed UV light could be an alternative to conventional methods with a potential to inactivate pathogens in biofilm. There is no report showing the most effective frequency of pulsed UV-LED on the inactivation of micro-organisms in biofilm. Present result shows that 5-min pulsed UV-LED irradiation at 100 Hz had the most effect on the inactivation of both *C. albicans* and *E. coli* cells in biofilm (Fig. 2). We presume that the susceptibility of micro-organism in biofilm to UV-LED irradiation may depend on the frequency of UV-LED. Further studies regarding this question need to addressed and conducted.

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Regarding the inactivation effect of UV-LED irradiation duration, both *C. albicans* and *E. coli* cells in biofilm were significantly reduced in a duration-dependent manner by irradiation in both pulsed and continuous mode (Fig. 3). In particular, our results showed that 5-min UV-LED irradiation killed over 95% of both micro-organisms, and the germicidal efficiency of 5-min exposure in pulsed mode on *C. albicans* and *E. coli* biofilm was 6·34- and 2·53-fold higher than in continuous mode, respectively.

Previous studies showing the mechanisms of UVA disinfection have implied that UVA indirectly damages DNA by creating reactive oxygen compounds (e.g. H_2O_2, O_2^- and $OH^\bullet$) via photooxidation of O_2, resulting in single-strand breaks in DNA (Douki *et al.* 2003; Courdavault *et al.* 2004; Mouret *et al.* 2006). We hypothesized that these mechanisms are also applicable to biofilm disinfection and tried to treat biofilm with mannitol, a scavenger of $OH^\bullet$, before UV irradiation to confirm this mechanism. Our results showed that mannitol treatment before pulsed UVA-LED irradiation at different exposure frequencies as well as continuous mode irradiation protected micro-organisms in biofilm against damage by irradiation, indicating that $OH^\bullet$ created by UV-LED irradiation is one mechanism of UVA disinfection against micro-organism biofilm (Fig. 5).

Another important feature of the pathogenesis of *C. albicans* infection is its ability to switch between yeast and hyphal forms (Berman 2006). The development of hyphal forms was proved to affect *C. albicans* biofilm integrity (Blankenship and Mitchell 2006; Paramonova *et al.* 2009) and allowed direct invasion of tissue or medical devices and is associated with enhanced pathogenicity (Romani *et al.* 2003). This may explain why it is difficult to establish conventional methods to inactivate pathogenic *C. albicans* biofilm and to effectively remove hyphal forms of this fungus. Therefore, we established a hypha-rich *Candida* biofilm model by supplementing with Glc*N*Ac, which is a known *in vitro* inducer of yeast-to-hypha transition (Kumar *et al.* 2000; Biswas *et al.* 2007). SEM observations showed that UV-LED irradiation had effective antifungal properties and inhibited hyphal formation in *Candida* biofilm (Fig. 6). In particular, irradiation with pulsed UV-LED at 100 Hz damaged the fungal cells to a great extent.

Taken together, the present study established a *C. albicans* and *E. coli* biofilm model and showed that our UV-LED disinfection system was significantly effective to inactivate biofilm formed *in vitro* and proposed that pulsed UV-LED, especially at around 100 Hz, may be an effective alternative to UV irradiation as a disinfection method for inactivating pathogenic biofilm. Other studies are also planned to be performed under more closely controlled clinical conditions, such as co-cultured biofilm, as reported by Thein *et al.* (2006), and biofilms formed on medical or dental materials with saliva treatment or grown *in situ*. This UV-LED system is expected to be applied to the disinfection of pathogenic biofilm in various clinical situations.

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