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Citation: *Appl. Phys. Lett.* **98**, 021501 (2011); doi: 10.1063/1.3530434

View online: <http://dx.doi.org/10.1063/1.3530434>

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## Atmospheric pressure cold plasma as an antifungal therapy

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(Received 5 August 2010; accepted 22 November 2010; published online 11 January 2011)

A microhollow cathode based, direct-current, atmospheric pressure, He/O<sub>2</sub> (2%) cold plasma microjet was used to inactive antifungal resistants *Candida albicans*, *Candida krusei*, and *Candida glabrata* in air and in water. Effective inactivation (>90%) was achieved in 10 min in air and 1 min in water. Antifungal susceptibility tests showed drastic reduction of the minimum inhibitory concentration after plasma treatment. The inactivation was attributed to the reactive oxygen species generated in plasma or in water. Hydroxyl and singlet molecular oxygen radicals were detected in plasma-water system by electron spin resonance spectroscopy. This approach proposed a promising clinical dermatology therapy. © 2011 American Institute of Physics. [doi:10.1063/1.3530434]

*Candida* species (spp) are common yeasts found in human gastrointestinal, oral mucous, skin, and female genital trace.<sup>1</sup> It can cause mucocutaneous and cutaneous infections in both infants and adults. The high incidence of vaginal carriage of *Candida* spp in women's pregnancy will cause the infants' superficial colonization of *Candida*, which may develop mucocutaneous candidiasis such as thrush and diaper dermatitis.<sup>2</sup> Some high-risk infants will progress from colonization to systemic involvement. The increasing rate of *Candida* infection in adult is likely due to an increased prevalence of susceptible hosts, who receive intensive care, immunosuppressive therapy, and who have human immunodeficiency virus infections and receive broad-spectrum antibiotics.<sup>3</sup> Therapeutic strategies against *Candida* infection are very limited nowadays. They include systematic use of azole or topical use of imidazole.<sup>4</sup> On the other hand, the frequent prophylactic use of these drugs increases the infections caused by the drug-resistant *Candida* spp strains such as *C. krusei*, *C. glabrata*, and fluconazole-resistant *C. albicans* strains, which can usually result in the treatment failure of commonly used antifungal agents such as fluconazole or other azoles.<sup>5</sup>

In recent years, nonthermal plasmas have attracted much interest in biomedicine due to their potential applications in bacteria inactivation,<sup>6–9</sup> wound healing,<sup>10,11</sup> treatment of dental diseases,<sup>12,13</sup> and tooth whitening.<sup>14,15</sup> A few groups<sup>16,17</sup> have reported nonthermal plasma inactivation of *Candida albicans*. In this study, we present the fungicidal capability of a direct-current atmospheric pressure He/O<sub>2</sub> plasma microjet (PMJ) on *Candida* strains with fluconazole-resistance in air and in water. Antifungal susceptibility test and cell viability test 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl] (XTT assay) were performed after the plasma treatment. Electron spin resonance (ESR) spectroscopy was employed to evaluate the reactive species generated in the plasma-water environment.

The plasma device<sup>18</sup> comprises two copper tubes as electrodes separated by a ceramic tube and is driven by a dc negative-polarity high-voltage power supply through a 5 kΩ ballast resistor. Detailed schematic diagram of the device as well as the electrical circuit can be found in our earlier publications.<sup>8,9</sup> Premixed helium and oxygen gas mixture (volume ratio: 98% He and 2% O<sub>2</sub>, referred to as He/O<sub>2</sub> from hereon) was used as the working gas at a flow rate of 2.5 standard liters per minute (slm). When sustained in air, the visible length of the plasma microjet was approximately 25 mm (Ref. 18) at a sustaining voltage about 400 V and a discharge current of 35 mA. When immersed in water, the PMJ was sustained in a quasisteady gas cavity with approximately the same sustaining voltage (slightly fluctuating). Pictures of the He/O<sub>2</sub> PMJ sustained in air and in water can be found in Ref. 18 shows a picture of the He/O<sub>2</sub> PMJ sustained in water.

Three types of *Candida* strains, namely, *C. albicans* BMU 02971, *C. krusei* ATCC6258, and *C. glabrata* BMU 00271 being either resistant or dose-dependent susceptible to fluconazole, were used in the experiments. Furthermore *C. albicans* SC5314 (susceptible to fluconazole) was used as the control. Each *Candida* strain was grown for 2 days on sabouraud dextrose agar (SDA) at 35 °C to ensure the viability and purity. When treated in air, 100 μl diluted suspension (1 × 10<sup>4</sup> CFU/ml) was spread evenly onto SDA via a sterile plastic transferring loop. This initial concentration was chosen on purpose so that all results from various treatment times can be fitted into the colony-forming unit (CFU) count detection limit in a later stage. The agar plate was maintained at 1 cm from the exit nozzle of the device, where the temperature was below 40 °C. This is not a critical temperature for the survival of the fungi. The plasma treatment was limited to a 2 × 2 cm<sup>2</sup> square area ("treated area") at the center of a 9 cm diameter Petri dish, with a treatment time ranging from 0 to 10 min. No dehydration was observed. For the treatment in water, conidial suspension was diluted to a concentration of (1–3) × 10<sup>6</sup> CFU/ml. 20 ml suspension was treated with He/O<sub>2</sub> PMJ from 0 to 4 min. Treatment

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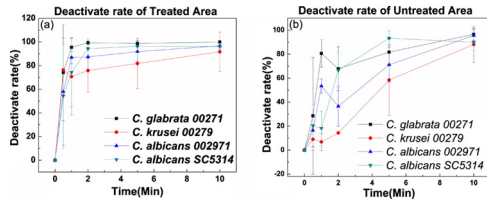


FIG. 1. (Color online) Inactivation rates of four *Candida* strains treated with He/O<sub>2</sub> (2%) PMJ in air in (a) treated area and (b) untreated area.

details for both cases can be found in our previous publications.<sup>8,9</sup> After the plasma treatment, 200  $\mu$ l suspensions were aspirated out and further diluted 1000- and 100-fold to perform antifungal susceptibility test and colony counts, respectively. All experiments were repeated at  $\geq 3$  times for statistical analysis.

Inactivation rate is defined as  $100\% \times (1 - \text{CFU}_{\text{treated}} / \text{CFU}_{\text{control}})$ . When treated in air, the inactivation rates in treated areas and untreated areas are plotted in Figs. 1(a) and 1(b), respectively. In treated area, an initial fast increase of the inactivation rate within the first minute of was observed for all four *Candida* strains, followed by a much slower change of inactivation rates. It appears that different strains respond to plasma treatment slightly differently, with *C. glabrata* reaching 100% inactivation in 2 min, while *C. krusei* only reaching 91% after 10 min plasma treatment. Interestingly, in the untreated area, although the initial inactivation was not as prominent as it was in the treated area, significant inactivation rates ( $\sim 90\%$ ) were observed after a 10 min plasma treatment. We suspect that in the treated area, both charged particles and reactive species (short lived and long lived) participate in the inactivation. In the untreated area, however, only longer lived reactive species (such as O<sub>3</sub>) can contribute to the inactivation.<sup>8</sup> Helium metastability ( $\sim 20$  eV) has a longer lifetime, can essentially transport laterally to the untreated area, and can interact with oxygen in air via stepwise excitation to create antifungal species on-site. Nevertheless, it is possible to deactivate the strains in the untreated area, but a bigger dosage seems to be necessary to achieve an equivalent inactivation rate as in the treated area.

The inactivation rates of the *Candida* strains treated in water are plotted in Fig. 2(a). A fast inactivation was observed for all strains in the first 30 s. 100% inactivation was achieved between 1 and 2 min treatment.

To verify the death of the microbes, XTT colorimetric assay (Sigma, St. Louis, MO) was used to evaluate the cellular viability of the *Candida* strains.<sup>19</sup> Figure 2(b) shows the results of XTT assay of the four fungi strains at different plasma treatment times. The results correspond to CFU counts except for high statistical errors observed between 30 s and 2 min plasma treatment. Nevertheless, zero metabolic

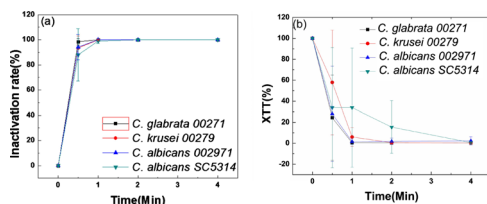


FIG. 2. (Color online) Inactivation rate (a) and XTT assay (b) of four *Candida* strains treated with He/O<sub>2</sub> (2%) PMJ in water.

TABLE I. The MIC (g/ml) of fluconazole for *Candida* spp at various plasma treatment times.

<i>Candida</i> strains	0 s	30 s	1 min	2 min	4 min
<i>C. glabrata</i> 00271	$\geq 64$	4	$\leq 0.125$	$\leq 0.125$	$\leq 0.125$
<i>C. krusei</i> 00279	32	16	16	$\leq 0.125$	$\leq 0.125$
<i>C. albicans</i> 002971	$\geq 64$	1	$\leq 0.125$	$\leq 0.125$	$\leq 0.125$
<i>C. albicans</i> SC5314	0.5	0.5	$\leq 0.125$	$\leq 0.125$	$\leq 0.125$

activity was achieved at 4 min, indicating the complete inactivation of the fungi.

Antifungal susceptibility test was performed following Clinical and Laboratory Standards Institute recommended reference standard M27-A3 with minor modification.<sup>20</sup> The motivations are twofold: (1) it is possible that some fungi survive after a certain dosage of plasma treatment. They are possibly, however, modified by the plasma and are more susceptible to traditional antifungal treatment. (2) Clinical trials combine traditional treatment methods and the developing technique. As the toxicity and safety study of the plasma treatment is still lacking, one might want to reduce the exposure to plasma to a minimum dosage while achieving a considerable reduction of the dosage of traditional antifungal therapy. Fluconazole powder (Fuyang Genebest Chemical Industry Co. Ltd., China) was prepared to desired concentrations (64–0.125 mg/l) during each experiment. The viability of fungi was again evaluated with XTT assay. *C. krusei* 00279 was used as quality control for antifungal susceptibility test. Table I shows MIC (the minimum inhibitory concentration required to inhibit 50% of fungal growth after 2 day incubation). A drastic reduction of MIC is observed for all fungi strains after a 1–2 min plasma treatment.

The plasma-liquid system is a highly complex environment. Some attempts have been made to try to understand the plasma-water interaction as well as the associated physical and chemical processes.<sup>21</sup> In the current system, the overall water temperature was measured to increase with the plasma treatment time and reach equilibrium (approximately 30 °C) in 6 min. This temperature, however, is not sufficient for the inactivation of fungi in water via pure thermal effects. The pH value of the water (monitored with a microprocessor pH meter) decreased slightly from 7.4 to 6.2 in 10 min. Hydrogen peroxide in water was evaluated via a H<sub>2</sub>O<sub>2</sub> test kit (model HYP-1; HACH Co., Loveland, CO) to be around 2 mg/l, which is not sufficient to influence the fungi growth in water either.<sup>22</sup>

Reactive oxygen species (ROS) are well accepted in the research community as the key killing agent for microbes.<sup>23,24</sup> Both hydroxyl radical ( $\bullet\text{OH}$ )[E(OH) = 2.8 eV] and singlet oxygen  $\{\text{O}_2(^1\Delta_g), \text{E}[\text{O}_2(^1\Delta_g)] = 0.98 \text{ eV}\}$  tend to attack unsaturated fatty acids on cell membrane. Their presence can compromise the function of the membrane lipids and cause the transportation of ions and polar compounds into the cell.<sup>25</sup> In our recent work,<sup>26</sup> it was found that the oxidative stress pathway is directly involved in eukaryotic yeast resistance to plasma processing. ESR spectroscopy was used to evaluate ROS generated by PMJ in the aqueous environment. 5,5-dimethyl-1-pyrroline-N-oxide (DMPO, Sigma Aldrich Co., Ltd.) was used as the  $\bullet\text{OH}$  spin-trap reagent and added into the system prior to the plasma treatment. The spin-trapped adduct DMPO-OH typically

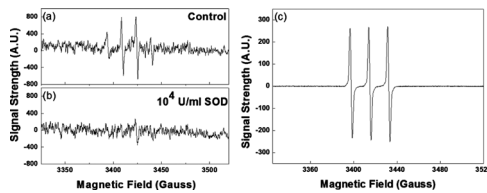


FIG. 3. Electron spin resonance spectra: (a) DMPO-OH signal showing the existence of  $\bullet\text{OH}$ ; (b) with the addition of  $10^4$  U/ml SOD, DMPO-OH signal diminishes; and (c) TEMPO signal showing the existence of singlet oxygen.

shows a quartet pattern with a line intensity ratio of 1:2:2:1 and hyperfine coupling constants of  $a_N = a_H = 1.49$  mT in the ESR spectrum,<sup>27</sup> as shown in Fig. 3(a). Superoxide anion radical ( $\bullet\text{O}_2^-$ ) was reported to exist in a helium plasma-water system and participate in the inactivation of *E. Coli* via its protonized conjugate HOO at an onset pH of 4.7[9, 24]. Unfortunately, no obvious  $\bullet\text{O}_2^-$  was detected in our study. However, when  $10^4$  U/ml superoxide dismutase (SOD, S4636, Sigma Aldrich Co. Ltd.) was added into the system prior to the plasma treatment, considerable reduction of the DMPO-OH signal was observed [Fig. 3(b)], indicating that  $\bullet\text{O}_2^-$  exists in the system but converts quickly into  $\bullet\text{OH}$ . 2,2,6,6-tetramethylpiperidine (TEMP, Sigma Aldrich Co., Ltd.) was used to spin-trap singlet oxygen. The resulting adduct TEMPO usually shows a triplet pattern with a line intensity ratio of 1:1:1 and hyperfine coupling constants of  $a_N = 1.72$  mT in the ESR spectrum,<sup>28</sup> as shown in Fig. 3(c). The intensities of DMPO-OH and TEMPO signals at various PMJ treatment times are presented in Figs. 4(a) and 4(b), respectively. The signal intensity of DMPO-OH increases from 0 to 50 s, while the signal intensity of TEMPO increases in the initial 20 s and decreases from 20 s onward. The change of liquid condition during the plasma treatment and the exhaust of TEMP due to excessive oxidative species may have led to this result. Nevertheless, we believe that the creation of strong oxidants such as  $\bullet\text{OH}$  and  $\text{O}_2(^1\Delta_g)$  has led to a faster inactivation of the fungi in water.

In summary, this study showed that a microhollow cathode discharge based direct-current helium/oxygen (2%) plasma microjet can be generated and sustained in air and in water. In both cases, inactivation of *Candida* spp strains with fluconazole-resistance was observed. The treatment of fungi in water, however, showed more effective results. This may lead to a promising antifungal therapy in fungal infection, especially in those caused by the drug-resistant strains.

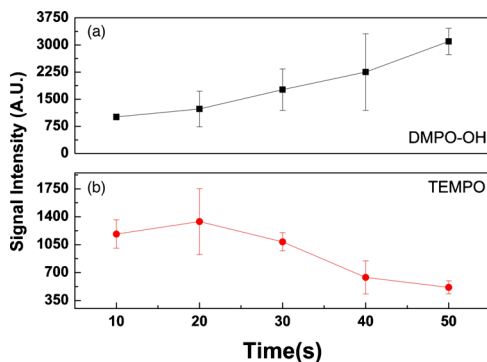


FIG. 4. (Color online) The signal intensities of (a) DMPO-OH and TEMPO at various PMJ treatment times.

P.S. and Y.S. contributed equally to this work. This research was sponsored by the Bioelectrics Inc. (U.S.A.), National Basic Research Program (Grant No. 2007CB935602, and the China International Science and Technology Cooperation (Grant No. 2008KR1330). The development of the PMJ device was funded in part by the Electro-Energetic Physics Program of the U.S. Air Force Office of Scientific Research (AFOSR) under Grant No. FA9550-08-1-0332. The acknowledgment is also made to the donors of the American Chemical Society Petroleum Research Fund for the partial support of this research.

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