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Active Oxygen Detection for Sterilization Processing Using Silver Thin Film

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In this study, the quantification of active oxygen species (AOS) has been investigated using silver (Ag) thin films prepared on a glass substrate as an AOS-sensing detector. On the basis of the determined values, inactivation tests of microorganisms were performed under active oxygen atmosphere in an ultraviolet (UV) lamp system. From the results, it was confirmed that the formation of silver oxide (Ag_2O) derived from surface oxidation by the active oxygen influence was successfully quantified as an AOS level by inductively coupled plasma optical emission spectroscopy (ICP-OES). It was also verified that AOS exposure was an effective method for the inactivation of microorganisms.

1. Introduction

Active oxygen species (AOS) are used effectively in various industrial applications. The AOS ozone (O_3) has, for some time, been used for deodorizing, bleaching, water disinfection, and air purification. The hydroxyl radical (OH radical), another AOS, has been used for water purification and sterilization.[1,2] AOS refers to activated oxygen species that exhibit a higher reactivity than ground-state molecular oxygen, and includes, in a narrow sense, oxygen atoms (O), excited oxygen atoms (O^*), excited oxygen molecules (O_2^*), and O_3, and in a broad sense, the OH radical, superoxide (O_2^-),

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and hydrogen peroxide (H_2O_2).[3,4] The measurement of these AOS (except O_3, which has a relatively long life) has been considered difficult owing to their extremely high reactivity and short life span. Here, we propose the use of a silver-coated quartz crystal microbalance (Ag-QCM) to monitor AOS, particularly excited atomic oxygen ($O(^1D)$), in a typical production process. As a result of analyzing the surface oxidation condition of the Ag-QCM by scanning transmission electron microscopy (STEM), the formation of the oxidation layer was found to involve the following three steps: (a) the adsorption of AOS to the surface, (b) damage formation on the surface by the rapid volume expansion of the oxidation layer and the oxidation of unreacted Ag underlayers, and (c) the formation of a thick oxidation layer and the termination of QCM functionality by the eventual saturation of the oxidation reaction.[5] We also demonstrated a sterilizing effect of atomic oxygen O^*, generated by a low-pressure remote oxygen plasma, on spores of *Bacillus atrophaeus*, a spore-forming bacterium, to demonstrate the possibility of an industrial use for AOS.[6] As an AOS measurement method for this study, we developed an AOS sensing plate, and attempted to quantify AOS by measuring silver (Ag) oxidized by AOS, or oxidized silver (Ag_2O), using an inductively coupled plasma (ICP) emission spectrometer. On the basis of the AOS levels measured using the AOS-sensing plate, we then conducted an inactivation test on microorganisms using the AOS generated by ultraviolet (UV) lamps that emit vacuum-UV irradiation.

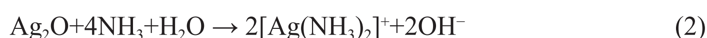
2. Test Methods and Devices

2.1 *AOS-sensing plate and measurement method*

2.1.1 *Principles of AOS measurement*

Metals are oxidized when they react with highly oxidizable molecules or atoms. Silver (Ag) is stable in air, but easily oxidized by the AOS. The AOS, *e.g.*, OH radicals, excited oxygen molecules (O_2^*), excited oxygen atoms (O^*), oxygen atoms (O), ozone (O_3), and superoxide anion (O_2^-), exhibit higher reactivity than ground-state molecular oxygen, and have potential abilities to oxidize Ag and proceed Ag_2O formation. As shown in eqs. (1) and (2), the Ag_2O generated by the reaction between Ag and AOS characteristically forms an amine complex and dissolves in ammoniacal solutions, while unoxidized Ag does not dissolve in the solutions and thus remains intact. These properties of Ag and Ag_2O are used to measure AOS level.

$$2Ag+O \rightarrow Ag_2O \tag{1}$$



$$Ag_2O+4NH_3+H_2O \rightarrow 2[Ag(NH_3)_2]^++2OH^- \tag{2}$$

2.1.2 *AOS-sensing plate*

In this study, we developed a sensing plate made of a thin glass plate coated with a Ag film, and used it as an indicator for detecting AOS. An alkali-free glass plate, 25 mm wide and 0.5 mm thick, was used as a base material. The plate was coated with a 100-nm-thick Ag film by an electrolytic plating method and used as an AOS-sensing plate.

2.1.3 *AOS analysis method*

An exposed AOS-sensing plate, which generates Ag_2O on the Ag surface, was dipped in dissolved oxygen-free aqueous ammonium, and then the solution was heated to dissolve the plate for the elution of Ag_2O. The amount of Ag left in the ammonium solution was then quantified using an inductively coupled plasma optical emission spectrometer (ICP-OES, type: SPS7800, Seiko Instruments Inc.), and the AOS level was calculated from the obtained Ag level. A Ag standard solution of known concentration was serially diluted to prepare Ag solutions of different concentrations. The emission intensity of each Ag solution was then measured using the ICP-OES at 328.1 nm wavelength, and the relationship between the obtained emission intensity and the Ag concentration was evaluated and used as a standard curve. The AOS level per unit area is calculated using

$$M = (m_1 - m_0)/(2 \cdot A \cdot S), \tag{3}$$

where M indicates the AOS level per unit area (mol/cm^2); m_1, the detected Ag level in the sample solution (g); m_0, the detected Ag level in the blank test solution (g); A, the atomic mass of Ag (107.9 g/mol); and S, the area of the sensing plate (cm^2).

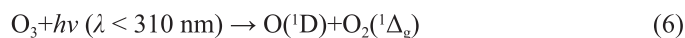
2.2 *AOS generator*

2.2.1 *AOS generator and generation principles*

Figure 1 shows the AOS generator (hereafter 'test box') used in this study. Inside the test box, one 4 W AOS-generating UV lamp (UV lamp-A) and two 6 W UV lamps (UV lamp-B) are arranged, and the top is equipped with a fan for stirring the air inside. UV lamp-A is a low-pressure mercury lamp used for AOS generation, which emits UV at 184.9 nm wavelength (vacuum-UV irradiation), and at 253.7 nm wavelength, which is known to be effective for sterilization. In contrast, UV lamp-B is also a low-pressure mercury lamp, but the UV at 184.9 nm wavelength is blocked by an outer bulb, and the lamp only emits UV at 253.7 nm wavelength. The mechanism of AOS generation induced by UV is generally explained as follows.[7,8] The ground-state oxygen molecule ($O_2(^3\Sigma_g)$) is dissociated, by UV at 240 nm wavelength or lower, from the oxygen atom $O(^3P)$, which then reacts immediately with molecular oxygen to generate O_3 (eqs. (4) and (5)). O_3 is dissociated by UV at 310 nm wavelength or lower, and generates an excited oxygen molecule ($O_2(^1\Delta_g$; $^1O_2$) and excited oxygen atom ($O(^1D)$) (eq. (6)). Therefore, a UV lamp that emits UV at both 184.9 and 253.7 nm wavelengths enables a continuous generation of AOS. Since both the $^1O_2$ and $O(^1D)$ are AOS, a string oxidizability is predictably generated by the exertion of the AOS on a target.

$$O_2(^3\Sigma_g) + h\nu\ (\lambda < 240\ \text{nm}) \rightarrow O(^3P) + O(^3P) \tag{4}$$

$$O_2(^3\Sigma_g) + O(^3P) \rightarrow O_3 \tag{5}$$



$$O_3 + h\nu\ (\lambda < 310\ \text{nm}) \rightarrow O(^1D) + O_2(^1\Delta_g) \tag{6}$$

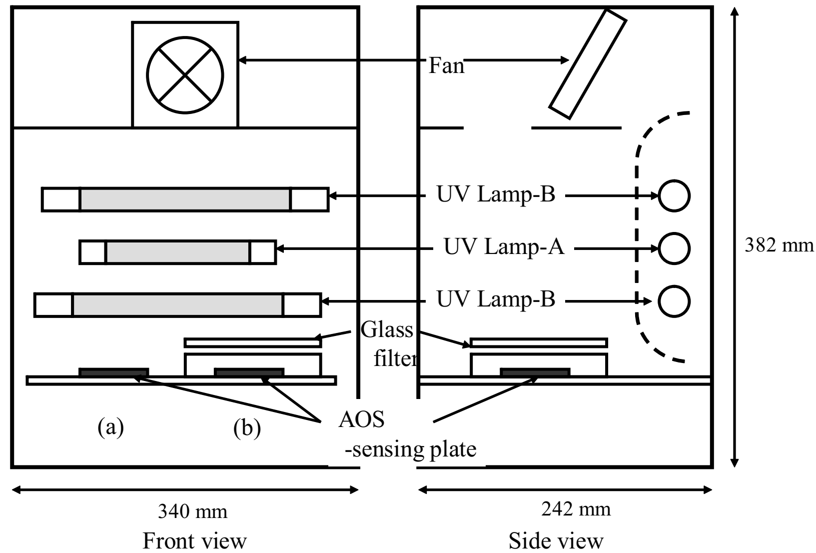


Fig. 1. Experimental setup of UV lamp apparatus (test box).

2.2.2 *Structural analysis and measurement method*

We used an X-ray diffract-meter (model: MiniFlex, Rigaku Corporation) to analyze the crystal structure of the substance produced on the AOS-sensing plate (silver thin film). In addition, a UV illuminometer (model: UVR-2, TOPCON) was used to measure the UV illumination on the AOS-sensing plate.

2.3 *Microorganism test method*

2.3.1 *Preparation of microorganism samples and test indicators*

Four strains of microorganisms with different properties were used to examine the inactivation effect. We used a Gram-negative bacterium (*Escherichia coli*, NBRC 3972), a Gram-positive bacterium (*Staphylococcus aureus*, NBRC 13276), a Gram-positive spore-forming bacterium (*Bacillus subtilis*, NBRC 3134), and a black mold (*Aspergillus niger*, NBRC 9455). All the strains were purchased from the Biological Resource Center (NBRC), the National Institute of Technology and Evaluation (NITE). *E. coli* was cultured in soybean-casein digest (SCD) agar media under prescribed conditions, at 36°C for one day, and *S. aureus* was cultured at 30°C for two days. These bacteria were then harvested and dissolved in a phosphate buffer (pH 7.0) for subsequent use as test samples. *B. subtilis* was also cultured under prescribed conditions, at 30°C for seven days, and then heat-inactivated at 80°C for 15 min to inactivate the trophozoites, after which the spores alone were obtained and used as a test solution. *A. niger* spores were cultured under

prescribed conditions, at 25°C for 7–14 days, in potato dextrose agar (PDA) media, and the cultured spores were then scraped out into sterilized water containing a detergent (0.05% Tween80), and filtered through a sterile gauze for subsequent use as a test solution. Next, 10 mL of each of the bacterial and spore solutions, at prescribed concentrations, was filtered through 37 mm membrane filters (0.45 μm pore diameter) to prepare test indicators. The number of bacteria was adjusted to include approximately 10^5 colony-forming units (cfu) in the test indicators.

2.3.2 *Inactivation test method using the test box*

Test indicators were placed inside the test box to initiate the experiment. We conducted a direct UV- and AOS-exposure test (Fig. 1(a)) and an indirect exposure test (Fig. 1(b)) in which UV is blocked by a borosilicate glass located ~5 mm above the test sample. The former test (Fig. 1(a)) demonstrates the effect of AOS including the impact of UV, and the latter (Fig. 1(b)) shows the effect of AOS only.

2.3.3 *Assessment method of the inactivation effect*

Following the tests, the test indicators (membrane filters) were transferred to centrifuge tubes containing 20 mL of sterilized water, stirred well, and sonicated for 10 min to elute the microorganism samples from the indicators into the water. The samples were then serially diluted to appropriate concentrations, smeared on PDA or SCD agar media, and cultured under prescribed conditions. Subsequently, we counted the number of colonies formed on the media and evaluated the rate of inactivation.

3. Results and Discussion

3.1 *Measurements using the AOS-sensing plate*

3.1.1 *Exposure of the AOS-sensing plate in the test box*

Figure 2 shows the appearance of the AOS-sensing plate exposed to UV inside the test box and the measured Ag_2O levels (AOS levels). It was observed that the brightness of the Ag plate decreased in response to the increase in exposure time, and the color of

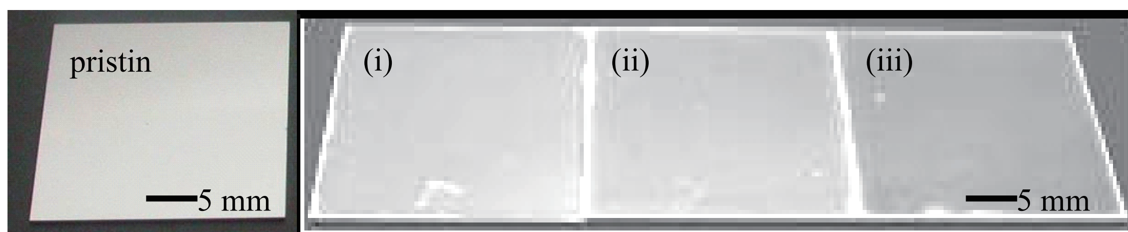


Fig. 2. AOS-sensing plate samples before and after exposure to the UV lamp: (i) 6.8×10^{-8}, (ii) 2.3×10^{-7}, and (iii) 2.8×10^{-7} mol/cm^2.

the plate turned yellow ochre. The reproducibility of the AOS levels detected by the sensing plate was satisfactory when measured under identical conditions. However, the resulting color tones of the Ag plate were not necessarily the same in appearance; therefore, AOS levels cannot be predicted simply by the change in the color tone of the sensing plate.

3.1.2 *Results of X-ray diffraction analysis of the AOS sensing plate*

Figure 3 shows the X-ray diffraction patterns of the Ag plate surface before and after its exposure to UV lamp-A in air, and after in nitrogen atmosphere. Before the exposure to UV in air, five peaks representing Ag were detected (Fig. 3(a)). After the exposure to UV in air, several peaks representing Ag_2O were detected in addition to the peaks representing Ag (Fig. 3(b)). However, no peak representing Ag_2O was detected after the exposure to UV in the nitrogen atmosphere (Fig. 3(c)), indicating that Ag on the Ag plate is stable for the exposure to UV without air.

3.1.3 *Preparation of the AOS standard curve using the AOS-sensing plate*

Figure 4 shows the relationship between the exposure time and the detected AOS level when the AOS-sensing plate was exposed to AOS in the test box. A direct positive correlation was observed between the exposure time and the detected AOS level. Nevertheless, when the detected Ag_2O level exceeds 3.0×10^{-7} mol/cm^2, the slope does not conform as closely to the linear curve.

3.2 *Results of AOS exposure of microorganisms*

3.2.1 *Survival curves of microorganisms exposed to AOS*

Figure 5 shows the survival curves of each microorganism exposed to AOS under the direct UV- and AOS-exposure conditions (Fig. 5(a)), and the indirect UV-exposure condition wherein a borosilicate glass was located on the test indicator (Fig. 5(b)). The illumination level of UV detected by a UV illuminometer when directly exposed was 0.01 mW/cm^2. In both cases in Figs. 5(a) and 5(b), an increase in AOS level decreased the number of bacteria in an exponential manner. The order of microorganisms that were inactivated earlier, or that required less AOS to be inactivated, was *E. coli* < *S. aureus* < *A. niger* < *B. subtilis* spores. This order was consistent in cases of both direct and indirect exposures. According to the tests of O_3 sterilization, which is known as an oxidation process, it has been reported that the susceptibilities to the O_3 in microorganisms are Gram-negative, Gram-positive, and spore-forming bacteria in descending order.[9] Compared with these results, the AOS inactivation shows a similar tendency to the O_3 sterilization in microorganisms.

3.2.2 *SEM images of microorganisms under AOS exposure*

Figure 6 shows scanning electron microscopy (SEM) images of the respective microorganisms before and after their exposure to AOS. The samples were directly exposed to all the UV lamps for 2 h, which is sufficient for inactivating all the microorganisms. As a result, many *E. coli* cells showed morphological changes, such as fissures on the cells, after exposure to AOS. This is probably due to the fact that

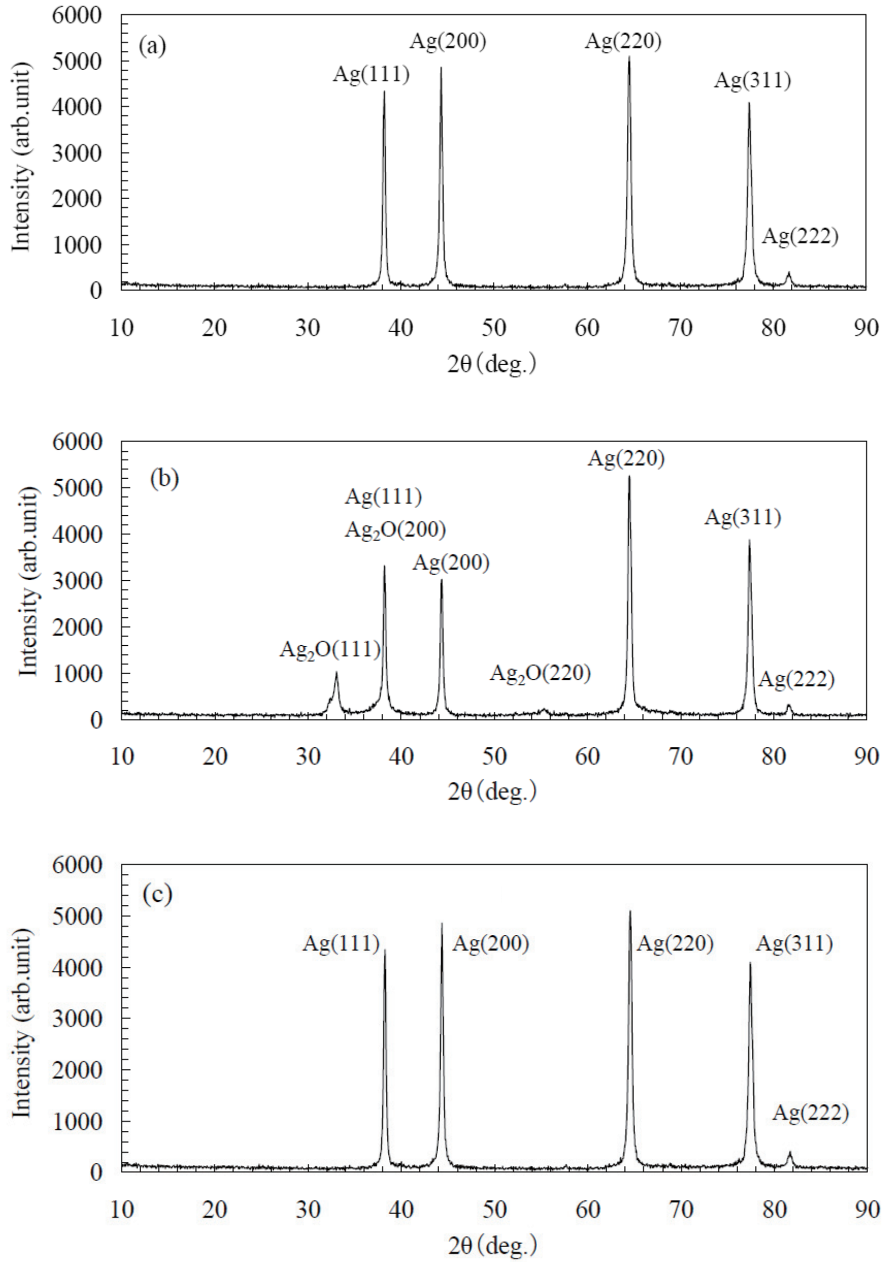


Fig. 3. X-ray diffraction analysis of the Ag film plate before and after UV lamp-A exposure in air and in nitrogen atmosphere: (a) before and (b) after exposure in air and (c) after exposure in nitrogen atmosphere.

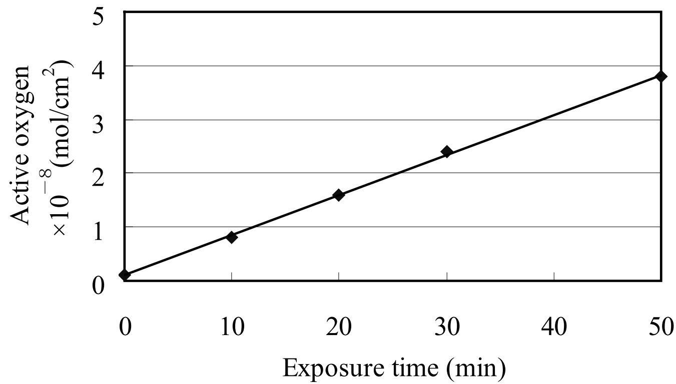


Fig. 4. Detection of active oxygen using the silver plate.

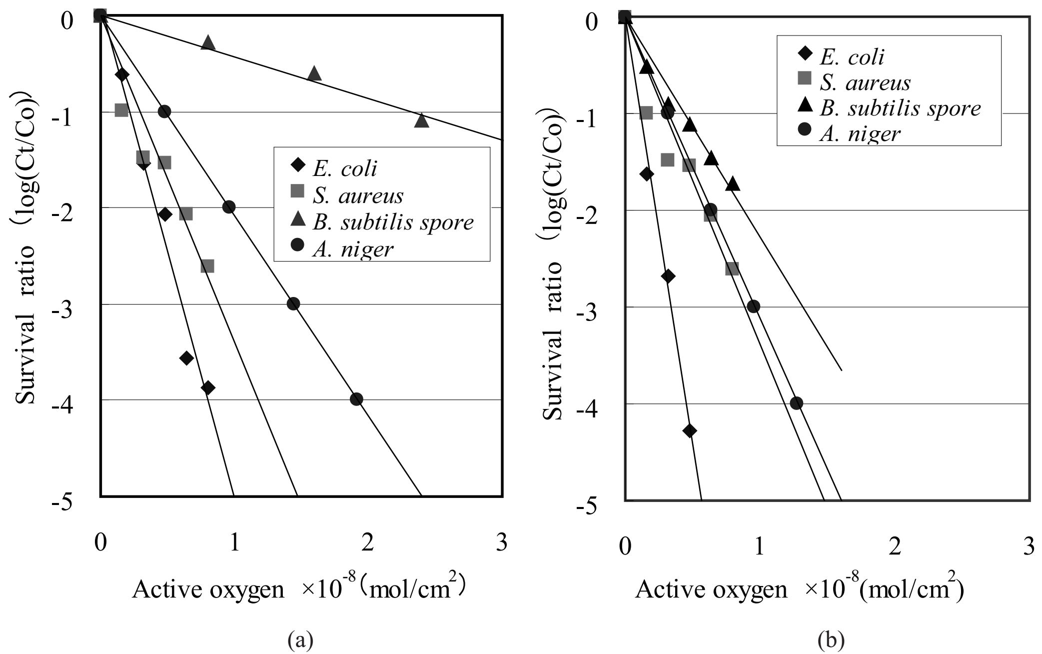


Fig. 5. Survival curves of microorganisms when exposed to AOS: (a) placed directly and (b) placed under the glass.

E. coli is a Gram-negative bacterium covered with a thinner cell membrane than the other microorganisms, and this is one of the reasons why *E.coli* is more susceptible to AOS. Many *S. aureus* cells shrank after exposure. In contrast, no significant morphological change was observed in *A. niger* and *B. subtilis* after their exposure to

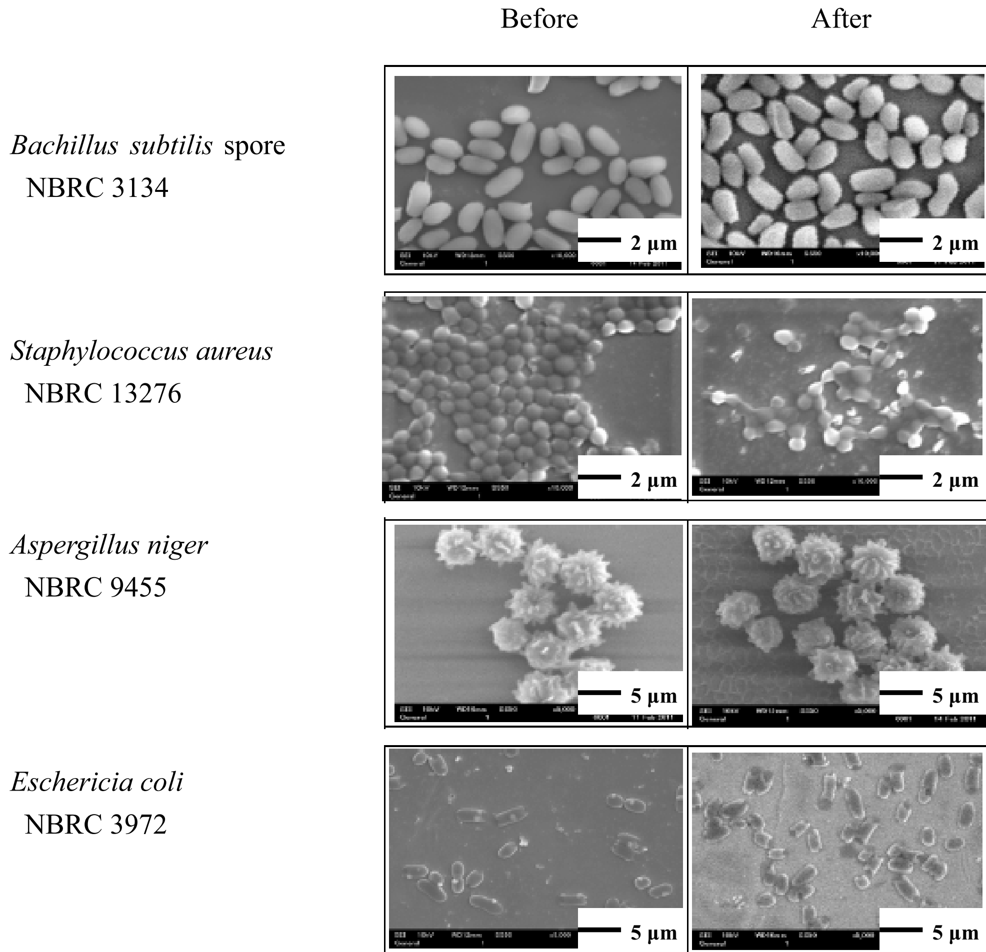


Fig. 6. SEM images of microorganisms before and after exposure to AOS.

AOS. Microorganisms that form spores, such as *B. subtilis*, are known to have extremely rigid cell membranes, and thus, even when all the *B. subtilis* cells were inactivated, no significant morphological change was observed on the SEM images. UV exposure is known to inactivate microorganisms without changing their morphological structure. The exposed UV is absorbed by nucleic acids, such as deoxyribonucleic acid (DNA), which encode the genes of microorganisms; this induces the formation of pyrimidine dimers and thus damages the base pairs. As a result, the functions required for biological maintenance and cell division are inhibited and microorganisms finally become cell-lethal.[10,11] The effect of AOS on microorganisms is that the AOS physically destruct the cell membranes of the microorganisms through oxidation reactions. In addition, AOS

also serve as a factor that inhibits functions indispensable for biological maintenance, such as metabolic networks and pathways, and transcriptional controls.[12] These results thus indicate that the AOS generated by UV lamps effectively inactivate various microorganisms, such as Gram-positive and -negative bacteria, spore-forming bacteria, and molds. We consider that it is important to clarify the mechanisms of the AOS inactivation of microorganisms.

4. Conclusion

In this study, it was found that the AOS generated by UV lamps can be measured using an AOS-sensing plate coated with a Ag film, and effectively inactivates microorganisms including bacteria and true fungi, with the following results.

(1) The AOS generated by UV lamps could be quantified using a Ag-coated AOS-sensing plate and described using the number of moles per unit area (mol/cm^2).

(2) Exposure to AOS alone (not necessarily with UV) could sufficiently inactivate the microorganisms.

(3) Microorganisms, such as bacteria and molds, were inactivated more effectively by a higher level of AOS, and a positive correlation was observed between the AOS level and the inactivation effect.

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References

1 I. Soumiya: Ozone Handbook (Sanyu-shobou, Tokyo, 2004) Chap. 14, p. 221.
2 S. Nakayama: Production and Application of OH Radical Species (NTS, Tokyo, 2008) Chap. 3, p. 231.
3 H. Sugimistu: Application and Foundation of Ozone (Korin, Tokyo, 1996) p. 20.
4 K. Asada, M. Nakano and K. Kakinuma: The Measurement Manual of Reactive Oxygen Species (Kodansha, Tokyo, 1992) p. 5.
5 H. Matsumoto, Y. Shibata, F. Suzuki, K. Yoshino, M. Matsuoka, T. Iwasaki, S. Kinoshita, K. Noda and S. Iwamori: J. Vac. Soc. Jpn. **53** (2010) 206.
6 H. Matsumoto, K. Yoshino, N. Sakai, T. Iwasaki, S. Kinoshita, K. Noda and S. Iwamori: J. Mater. Sci. Soc. Jpn. **47** (2010) 42.
7 H. Okabe: Photochemistry of Small Molecules (Wiley Interscience, New York, 1978) p. 177.
8 T. Matsunaga, K. Hieda and S. Nikaido: Photochem. Photobiol. **54** (1991) 403.
9 T. Sasaki, A. Nakamura and K. Mise: Sterilization and Killing of Microorganisms (JSA, Tokyo, 1998) p. 279.
10 N. Egami: Living Things and Radiation (University of Tokyo Press, Tokyo, 1975) Chap. 6, p. 104.
11 S. Uchida: Radiat. Biol. Res. Commun. **32** (1997) 183.
12 V. K. Matus, M. A. Martynova, A. M. Mel'nikova and S. V. Konew: Dokl. Akad. Navuk. **33** (1989) 277.