Enhanced inactivation of bacterial spores by atmospheric pressure plasma with catalyst TiO$_2$

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1. Introduction

Over the years, a considerable number of studies have been performed on the inactivation of microorganisms [1–8]. One of the conventional inactivation methods is thermal treatment using dry heat or an autoclave, but this may cause thermal damage to some treated objects and usually takes a long time [1,2]. Ultra-violet (UV) photons can kill microorganisms without direct contact to materials, but bacterial spores usually have a high degree of UV resistance. Since there are also screening problems, some new approaches have been attempted [1,3].

One of the alternative methods is atmospheric pressure plasma. Plasma is a capable sterilizing method to inactivate a wide range of microorganisms [4–7]. Among many physical inactivation sources such as heat, UV photons, charged particles, and reactive species, the reactive oxidation species have been known as the most effective sterilization source [4–6]. Therefore, oxygen gas is often additionally added to the supply gas to enhance the sterilization effect. In the presence of too much oxygen, however, the plasma characteristics tend to become altered to show higher breakdown voltage and gas temperature causing the plasma to be less stable [9]. Hence, development of a new means for enhancing the inactivation efficiency without affecting the plasma characteristics is imperative. On the other hand, as a non-plasma method, photo-catalytic oxidation inactivation using metal oxides such as titanium dioxide (TiO$_2$) has offered a number of positive features. The photo-catalytic inactivation of Gram-negative $Escherichia$ $coli$ and Gram-positive $Lactobacillus$ $helveticus$ by TiO$_2$ with 365 nm UV photons is one of many examples [8]. However, this method takes a long time to inactivate microbes and contaminated materials.

Recently, TiO$_2$ has shown synergetic effects with the plasma, i.e., enhanced destruction of benzene and toluene was achieved by combining the plasma and the photo-catalytic metal oxides, compared to the use of plasma alone [10]. Although prior studies have been made on the destruction of chemical and pharmaceutical materials by simultaneous use of plasma and catalysis [10], little is known about the inactivation of microbes such as bacterial spores. In this work, experimental results are presented showing the enhanced inactivation of $Bacillus$ $subtilis$ spores by the catalyst TiO$_2$ utilized together with atmospheric pressure plasma; the results are compared with the plasma only treatment case. This result may be easily applicable to inactivating various microorganisms including $Bacillus$ $anthracis$ spores, which have the potential to be used in bioterrorism. The TiO$_2$ concentration effects are also shown along with brief discussions of the mechanism of the TiO$_2$ plasma-catalysis.

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2. Experimental

2.1. Bacterial spore suspension and catalyst preparation

Based on the method of Nicholson and Setlow [11], *B. subtilis* spore samples were prepared and cultured in a medium containing 1 g Bacto nutrient broth (Difco), 10 ml 10% (w/v) KCl, 10 ml 1.2% (w/v) MgSO4·7H2O, 1 ml 1 M Ca(NO3)2·4H2O, 1 ml 0.01 M MnCl2·4H2O, and 1 ml 1 mM FeSO4·7H2O in 1 l of double distilled water (ddH2O). The number density of the spore suspension was approximately 2 × 10⁹ spores/ml.

The catalyst TiO2 powder (JUNSEI, extra pure anatase form, 150 nm typical size) of a certain mass concentration was added to the *B. subtilis* spore suspensions, keeping the same spore number density. To prepare a uniform suspension, the same ratios of spores and TiO2 particles were mixed using a vortex mixer (SI-0236, Scientific Industries).

The treatment sample was prepared by applying 10 μl (5 droplet × 2 μl, N₀ = 2 × 10⁶ spores/ml) of the spore suspension to a slide glass and letting it dry for about 1 h. After the treatments by plasma, UV, or heat, the spores were taken off the slide glass and submerged in 50 ml ddH2O. Then, the treated spores were cultured on a nutrient agar at 37 °C for 24 h for quantification of the treatment results. The nutrient agar was prepared by mixing 8 g Bacto nutrient broth (Difco) and 15 g Agar powder (JUNSEI) in 1 l of ddH2O.

2.2. Experimental set-up for plasma treatment

The schematic of the atmospheric pressure plasma source and three types of *B. subtilis* spore samples are illustrated in Fig. 1. Each sample has the same initial spore density (2 × 10⁶ spores/ml). A detailed description of the plasma characteristics is found in our previous report [4]. The atmospheric pressure plasma was generated at 13.56 MHz radio-frequency (rf) in ambient air with either helium or argon supply of 6 lpm. The plasma has a relatively large area (110 mm × 15 mm) and low discharge current and gas temperature, applicable to thermally sensitive materials. The gap distance between the powered electrode and the spore sample was fixed at 2 mm to satisfy the sufficient uniform discharge generation condition.

2.3. Photo-catalyst analyses

The characteristic changes in the plasma-treated TiO2 particles were analyzed by X-ray diffraction (XRD, RIGAKU, D/MAX-RB 12 kW), UV–vis absorbance spectrum (JASCO, V-570), high resolution dispersive Raman microscope (Horiba Jobin Yvon, LabRAM HR UV/vis/NIR), and photoluminescence (PL) spectrum. The XRD diffraction pattern was measured at an angle of 2θ, of which the angle was scanned from 20° to 60° with a speed of 1°/min. The Raman spectrum was obtained using an Ar ion laser of 514.5 nm wavelength with an output power of 10 mW as an exciting source. The UV–vis absorbance spectra of the original and the treated TiO2 samples were measured for the wavelength range of 300–700 nm. The PL spectrum was analyzed using a He–Cd laser of 325 nm wavelength with 17 mW output power. The TiO2 mass concentration was fixed at 1 mg/ml.

3. Results and discussion

3.1. Survival curves in various plasma conditions with and without TiO2

Fig. 2(a) depicts survival curves of the *B. subtilis* spores treated by helium and argon plasmas with and without TiO2 (1 mg/ml) addition [as depicted in samples (a) and (b) of Fig. 1]. The decimal reduction time (D-value) for inactivating 90% of the microbial population in the sample is plotted in Fig. 2(b). As shown in the figure, the helium plasma (gray) is much less efficient than the argon plasma (blue and red), which agrees with the results of other works [5]; the inactivation speed is enhanced by the increase of the input power, as expected. At 100 W, the D-value is decreased from 435 to 333 s by the helium plasma and decreased from 26 to 15 s by the argon plasma, each with TiO2 added, which demonstrates more than 40% inactivation enhancement compared with the use of plasma alone. One interesting thing to note is that in contrast to the conventional notion, in which the treatment sample should be in contact with the catalyst to expect inactivation, it is found that there are still catalyst effects even when the spore suspensions and the catalyst TiO2 are not mixed but spatially separated on the slide glass [as depicted in Fig. 1 sample (c)]. When this sample was...
treated at about 5 mm distance between the spore suspensions and TiO2, the D-value was still decreased from 26 s to about 19 s for an argon plasma (100 W, 6 lpm). Such results are very promising from an application point of view and need to be studied further.

3.2. Field emission scanning electron microscope (FE-SEM) images

Fig. 3 illustrates the morphological change of the B. subtilis spores pictured using a field emission scanning electron microscope (FE-SEM, HITACHI, S-4800). Fig. 3(a) and (b) represent the controlled spore samples and those spore samples treated by He/TiO2 plasma at an input power of 100 W for the exposure time of 120 s, respectively. The gas temperature was less than 75 °C [4]. The FE-SEM image of the treated sample clearly demonstrates a number of spores that were physically damaged (indicated by arrows) by the plasma and TiO2 particles.

3.3. TiO2 concentration effect

Several different concentrations of TiO2 were attempted to find the catalyst density at which the largest inactivation enhancement is achieved. The optimal TiO2 concentration for the most effective inactivation of the B. subtilis spore was found to be 1 mg/ml, as shown in Fig. 4(a). It was found that both a too low concentration (0.5 mg/ml) and a too high concentration (10 mg/ml) are less effective due to the insufficient production of oxygen radicals and also due to the low exposure of the spores being covered by the TiO2 particles [as depicted in the circle of Fig. 3(b)].

3.4. Plasma photo-catalytic effect (plasma activation of TiO2)

To account for the catalytic activation of TiO2 by the plasma, the plasma photo-catalytic effect was investigated. In general, TiO2 is known to be activated by UV photons. In order to assess the role of UV photons emitted from the plasma, a fused silica plate was placed between the plasma and a detector to allow only the UV photons to pass through. Comparison of the measured UV intensity between the cases with and without the fused silica plate showed almost identical intensity levels [4]. Fig. 4(b) clearly presents the negligible TiO2 activation by the plasma-emitted UV reaching the treatment sample through the fused silica plate, compared with the plasma treatment results. We also investigated the thermal–catalytic effect by putting the B. subtilis spore sample into a thermal furnace set at 180 °C, which is the same as the gas temperature of the argon plasma at 100 W. The negligible TiO2 activation by heat is also seen in Fig. 4(b). On the other hand, the combination of the plasma and TiO2 reduced the D-value by 40%, as depicted in Figs. 2(a) and 4(b). This means that TiO2 is activated by the plasma, not by heat or by the UV photons emitted from the plasma.

Yet, the oxygen related emission line intensities from the plasma were increased with the TiO2 particles present. To understand the outcome of the TiO2 activation and thus the enhanced B. subtilis spore inactivation, spectral intensities of the optical plasma emission were obtained using a spectrometer (Oriel MS125™) with a charge-coupled device detector (CCD). The measured spectral lines were hydroxyl radical (OH, 306 nm), excited atomic argon (Ar I, 696 nm), and excited atomic oxygen (O I, 777 nm) lines emitted from the Ar and the Ar/TiO2 plasmas (100 W, 6 lpm) during the exposure time of 2 s. As depicted in Fig. 5, the intensities of the OH and O I lines, normalized by the Ar I line, are significantly increased in the presence of TiO2 at 120 s. This result indicates that the TiO2 particles are activated to produce reactive oxygen radicals by the plasma, but not by the plasma-emitted UV or heat.
3.5. TiO₂ characterization change by atmospheric pressure plasma

One of the possible explanations for the plasma activation of TiO₂ is the lowering of its activation energy by the plasma. In general, the anatase form catalyst TiO₂, having a band gap energy of 3.2 eV, creates electron–hole pair energy states with a given external energy source such as UV photons [8,10,12–16]. However, it is reported that its activation band gap energy can be lowered by plasma (13.56 MHz, 500 W, 2 Torr, 673 K, hydrogen plasma) to (2.02–2.45) eV, which corresponds to the wavelength range of (506–614) nm [14]. This resulted from the formation of oxygen vacancy states that are located between the valence and the conduction bands. This means that the broader shifted energy band in the visible spectral range is more effective at creating electron–hole pair energy states.

The color change, visible to the naked eye, of the plasma-treated TiO₂ powder from white to light gray in our experiment is consistent with other reports [15,16]. The color change is related to Ti³⁺ formation, which is an indication of oxygen vacancy generation [13,15].

The XRD patterns depicted in Fig. 6(a) show that the dominant phase of the TiO₂ particle is the anatase form. The phase transformation from anatase to rutile took place by the atmospheric pressure plasma during an exposure time of 120 s at 100 W rf power. The weight fraction of the rutile phase in the TiO₂ particle was estimated by the formula 1/[1 + 0.884(𝐴_ἄντας/𝐴_ᵣᵣᵪ)], where 𝐴_ἄντας and 𝐴_ᵣᵪᵪ represent the integrated intensities of anatase (1 0 1) and rutile (1 1 0) peaks, respectively [17,18]. Although the TiO₂ particles were in the extra pure anatase phase, the weight fraction of the rutile phase was increased by about 34% due to the argon plasma.

The Raman spectra depicted in Fig. 6(b) clearly show the phase transformation from anatase to rutile by the plasma. Five strong peaks were observed at 143, 195, 395, 515, and 638 cm⁻¹, which explicitly indicates the existence of the anatase particles in the sample. However, a small peak at 448 cm⁻¹ was observed in the
argen plasma-treated (100 W, 120 s) TiO$_2$ sample. This peak indicates that the rutile particles are produced from the anatase particles by phase transformation. Since the defect sites of TiO$_2$ play a main role in the phase transformation [18], the increase in the rutile phase peak indicates existence of defect sites. In general, the phase transformation from anatase to rutile usually occurs in high temperature (>550 °C) calcinations by thermal fluctuations of Ti and O atoms in the anatase TiO$_2$ particles [18,19]. Yet, although the atmospheric pressure plasma has a low gas temperature (Ar 100 W, <180 °C), both XRD and Raman measurements in this study show that the atmospheric pressure plasma somehow provides a sufficient energy to create the defect levels.

The UV–vis absorbance spectra show an increase in the visible range absorbance for the plasma-treated TiO$_2$ sample (100 W, 10 min) when compared with the raw TiO$_2$ sample, as presented in Fig. 7(a). This result was also seen in the previous report using a low-pressure plasma (13.56 MHz, 400 W, 0.2 Torr, nitrogen plasma) for different treatment times (10, 30, 60, 120 min) at 400 °C [15]. The increase in the visible range absorbance suggests that the defect levels are created in the TiO$_2$ particle by the argon plasma.

To verify the defect level formation in TiO$_2$, photoluminescence spectra were obtained as presented in Fig. 7(b). The PL spectra of the non-treated and the plasma-treated (Ar 100 W, 1, 2 and 10 min) TiO$_2$ samples show two broad peaks emitted from the anatase phase: one in the visible range (450–650 nm) centered at 525 nm (or 2.36 eV) and the other near the absorption edge at 405 nm (or 3.06 eV) of TiO$_2$. These PL spectra are consistent with other investigations [20]. As depicted in the figure, the intensity of the smaller energy peak (or the visible band) is significantly increased while the higher energy peak is changed little. The intensity increase in the visible band can show the oxygen vacancy related defects [21]. Therefore, we suggest that the increase of the number of TiO$_2$ particles with the defect levels may be brought about by the atmospheric pressure plasma treatment. As a result, the defect energy levels help to generate more free electrons and active oxygen radicals by activating the TiO$_2$ particles at lower energy, resulting in a better bacterial spore inactivation.

4. Conclusion

Enhanced inactivation of B. subtilis spores was achieved by atmospheric pressure plasma aided by the catalyst TiO$_2$, resulting in a 40% decrease in the D-value compared to the use of plasma alone. Activation of the TiO$_2$ catalyst by UV or heat induced by the atmospheric pressure plasma is found to be negligible. Measurements of the significantly enhanced OH and O I spectra emitted from the plasma with TiO$_2$ addition suggest abundant production of the reactive oxygen radicals, which are known to be the dominant inactivation means of microorganisms. The XRD patterns, Raman spectra, UV–vis absorbance spectra, and PL spectra demonstrate that the oxygen vacancy related defects are formed inside the TiO$_2$ particle by the atmospheric pressure plasma. Since the defect levels have a lower energy, TiO$_2$ particles are activated more easily, and therefore, the reactive oxygen radicals are generated more in the plasma/TiO$_2$ system.

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