Bactericidal Mechanism of Ag/Al₂O₃ against *Escherichia coli*

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The bactericidal process of Ag/Al₂O₃ to *Escherichia coli* has been investigated to clarify the bactericidal mechanism. In SEM images, the configuration of *E. coli* cells contacting with the catalyst surface was quite different from that contacting with AgNO₃ solution, which indicated that the Ag⁺ eluted from the catalyst did not play an important role in the bactericidal process. The bactericidal experiments strongly confirmed the contribution of multiform reactive oxygen species (ROS) (super oxide dismutase (SOD) and catalase as the scavengers for O₂^{•−} and H₂O₂, respectively) to bactericidal effect on the catalyst surface. Furthermore, the surface modification of Ag/Al₂O₃ by ultraviolet and formaldehyde influenced the bactericidal effect obviously, which not only confirmed the bactericidal mechanism of catalytic oxidation but also provided evidence for the synergistic effect between Ag and Al₂O₃ on the catalyst surface.

Introduction

Ag/Al₂O₃ is an effective inorganic bactericide.¹ Our previous works have shown that it could not only inactivate the most bacteria making contact with its surface, but also decompose their organic remains into CO₂ in air, at room temperature. Through the primary research for the mechanism of the bactericidal process of Ag/Al₂O₃, we realized that it might be the reactive oxygen species (ROS) on the catalyst surface, as also found in the photocatalysis of TiO₂,^{2,3} that damaged the membranes of the cells, made bacteria inactive, and then decomposed the remains of bacteria into CO₂ as the final product.^{4,5}

Although we insisted that the bactericidal mechanism referring to catalytic oxidation was more remarkable in air, the toxicity of Ag⁺ eluted from the catalyst to cells was difficult to be excluded. Therefore, in order to differentiate the two bactericidal processes, catalytic oxidation and the toxicity of Ag⁺, SEM was used to detect the configuration of the *Escherichia coli* cells damaged by either catalyst or Ag⁺ solution. At the same time, bactericidal experiments with ROS scavengers were undertaken to investigate the bactericidal effect of ROS formed on the catalyst surface. The catalyst consisted of Ag and Al₂O₃: the valency of Ag can be changed by UV irradiation, and Al₂O₃ can chemically absorb formaldehyde. Making use of these characteristics of the catalyst, we investigated their influence on the bactericidal effect of Ag/Al₂O₃ and explored the bactericidal mechanism ultimately.

Experimental Section

Catalyst Preparation. Ag/Al₂O₃ (Ag 5 wt %) were prepared by an impregnation method. The wet sample was dried at 120 °C for 12 h and then was calcined in a muffle furnace at 600 °C for 3 h. Before using, 200 mg of Ag/Al₂O₃ powder was pressed into wafers of ca. 20 mg/cm². XRD was used to investigate the silver phase on γ-Al₂O₃. As reported in our previous work,^{4,6} only the γ-Al₂O₃

phase was detected when the silver loading was 5 wt %, but the Ag and Ag₂O phases were clearly observed at 2θ of 33.768°, 38.128°, 44.368°, and 64.468° with 8 wt % Ag loading. The absence of diffraction lines of silver phase on 5 wt % Ag/Al₂O₃ catalyst indicates that silver is at a very high degree of dispersion. The silver particles (ranging from 15 to 20 nm) distributed evenly on the surface of Al₂O₃ were also observed by TEM measurement.⁶ The Al₂O₃ without Ag was also prepared by the aforementioned method, and the later experiments were all applied to both Ag/Al₂O₃ (loading Ag) and Al₂O₃ (unloading Ag) for contrast.

SEM Observation. The bactericidal processes of catalysts and Ag⁺ were compared using the scanning electron microscope (SEM). A 20 μL portion of the *E. coli* suspension prepared as outlined previously was respectively applied on the surface of Ag/Al₂O₃, Al₂O₃, and a piece of glass with 20 μL of AgNO₃ solution (5 mg/L). It had been reported that the concentration of Ag⁺ eluted from the Ag/Al₂O₃ wafer was less than 5 mg/L.⁴

Before SEM measurement, the SEM specimen was prepared by following the standard procedures described elsewhere.^{7,8} The applied wafers and glass were fixed with glutaraldehyde and osmium tetroxide, drained with ethanol/water with the concentrations of ethanol increasing. The absolute ethanol was replaced by dimethoxy-methane, and the samples were dried to critical point with CO₂. The wafers were glued onto stages with conductive silver and metallized with gold. The samples were microscoped and photographed with a scanning electron microscope (Hitachi S-3000N).

Bacterial Culture and Bactericidal Experiment. *E. coli* (ATCC8099) was used as the bacterial strain and supplied by the Chinese Center for Disease Control and Prevention. *E. coli* was inoculated on the incline of LB agar (Fluka Co. 61746) and incubated for 18 h at 37 °C. The bacterial lawn on culture medium incline was washed off with 5 mL of sterile water. The *E. coli* suspension prepared by this method was about 10⁸ colony forming units per milliliter (CFU/mL).

A 20 μL portion of the *E. coli* suspension was applied onto the catalyst wafer surface, and the contacting times were 0.5, 2, 5, 10, and 20 min. The survival cells were shaken off by WH-2 Genie Vortex Shaker (2000 r/min amplitude: 6 mm) from the catalyst wafers into 5 mL of 0.9% NaCl aqueous solution to eliminate the effect of Ag⁺ and then were plated on LB agar plates. The plates were at 37 °C for 24 h before counting. All experiments were repeated three times.

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Figure 1. SEM images of *E. coli* cells after contacting with (a) Ag/Al₂O₃, (b) Al₂O₃, and (c) AgNO₃ solution for 30 s, and after contacting with (d) Ag/Al₂O₃, (e) Al₂O₃, and (f) AgNO₃ solution for 96 h.

Results and Discussion

SEM Observation. It was observed that the configuration of the cells on the catalysts was quite different from the configuration of cells contacting Ag⁺ solution at room temperature for 30 s and 96 h (Figure 1). Exposure to the Ag/Al₂O₃ surface for only 30 s resulted in cells badly collapsed and led to the release of intracellular constituents. Figure 1a shows the typical appearance of cells damaged by strong oxidants such as •OH and O₂^{•−}.^{7,9} Although the damage of cells on Al₂O₃ was not as serious as that on Ag/Al₂O₃, the cells shrank after 96 h (Figure 1e). However, the cells in contact with Ag⁺ solution for 96 h were still glossy and plump (Figure 1f). Because the configuration of cells on Ag/Al₂O₃ was absolutely different from that in contact with Ag⁺ (5 mg/L), it could be deduced that the toxicity of Ag⁺ eluted from the catalyst to the *E. coli* cells was not the dominant factor in the bactericidal process of Ag/Al₂O₃.

Effect of ROS Scavengers. The SEM images had supplied sufficient evidence to confirm that the toxicity of the Ag⁺ (5 mg/L) eluted from Ag/Al₂O₃ made little contribution to the bactericidal effect against *E. coli*. Considering that Ag/Al₂O₃, as an oxidative catalyst, should have the capability to transform O₂ into ROS which might contain superoxide anions (O₂^{•−}), hydrogen peroxide (H₂O₂), and hydroxyl radicals (•OH),^{1,8} we supposed that ROS involved on the catalyst surface must contribute to the bactericidal effect of Ag/Al₂O₃ and play an important role in the bactericidal mechanism.

In this experiment, 200 units/mL super oxide dismutase (SOD) and 175 units/mL catalase were used as the scavengers for superoxide anions (O₂^{•−}) and H₂O₂ respectively. The effect of the addition of ROS scavengers on the bactericidal activity after 30 min is shown in Figure 2. In the case of Ag/Al₂O₃, the bactericidal effect was weakened after the introduction of SOD,

Figure 2. Effect of ROS scavengers on the bactericidal activity of Al₂O₃ and Ag/Al₂O₃ after 30 min.

compared with the control experiment without any ROS scavengers at the corresponding time intervals. When the two scavengers (SOD and catalase) were both introduced, the bactericidal effect became further weakened. This result indicates the formation of O₂^{•−} and H₂O₂ on the catalyst surface and their contribution to the bactericidal effect. It was reported that silver ions could catalyze the decomposition of H₂O₂, thus resulting in the formation of •OH and/or O₂^{•−}, when Ag⁺ and H₂O₂ were used simultaneously.^{10,11} Since both Ag⁺ and H₂O₂ were detected in the present system, •OH should also be involved in the bactericidal process, and devoted to the inactivation of *E. coli*. That is the reason why the bactericidal effect was not completely inhibited by the two scavengers mentioned above. In the case of Al₂O₃, little effect of the addition of scavengers on the bactericidal activity was also observed, indicating a small amount of ROS existence, which is in accordance with the reports on the Al₂O₃ surface.^{12,13} However, the absence of silver in this system resulted in a lower bactericidal activity.

Effect of Ultraviolet Irradiation. Freshly prepared Ag/Al₂O₃ looks white, but Ag/Al₂O₃ will become black after irradiation by ultraviolet (UV, 280–300 mW/cm², λ = 365 nm) for 24 h. The reason is that Ag on the surface of Al₂O₃, most of which exists as oxide,⁶ was highly dispersed at the nanometer degree. This kind of silver oxide is very sensitive to light and can be easily reduced into silver by light. Thus, it is the silver nanoparticles that make the catalyst appear black in macroscopy.

In accordance with this characteristic, the bactericidal experiment with white and black catalyst wafers was undertaken to detect the relationship between bactericidal effect and the silver species formed on the Al₂O₃ surface. After pretreatment by UV irradiation, the bactericidal effect of Ag/Al₂O₃ declined obviously (Figure 3), while for that of Al₂O₃ no influence was observed (Figure 4). This indicates that Ag at the oxidized state was beneficial for the bactericidal activity. This is in accordance with our former assumption that oxidized Ag species with high oxidizing potential could activate the adsorbed oxygen more efficiently.^{1,4}

Effect of Formaldehyde Adsorption. In this experiment, the catalyst wafers (Ag/Al₂O₃ and Al₂O₃) were pretreated in a flow of 100 ppm of HCHO (He balance, 40 mL/min) at 38 °C (the purpose of increasing the temperature is to enhance the concentration of adsorbed HCHO) for 12 h. The bactericidal experiment was carried out immediately after pretreatment.

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Figure 3. Bactericidal effect of Ag/Al₂O₃ (a) irradiated and (b) not irradiated by UV.

Figure 4. Bactericidal effect of Al₂O₃ (a) irradiated and (b) not irradiated by UV.

Figure 5. Bactericidal effect of Ag/Al₂O₃ (a) treated and (b) not treated by HCHO.

As shown in Figure 5, a crossing was clearly observed at about 7 min on the bacterial survival curves of treated and untreated Ag/Al₂O₃ wafers. Before 7 min, the treated wafer exhibited a worse bactericidal effect than the untreated one, with a slow decrease in surviving bacteria. After 7 min, the surviving bacteria on the treated wafer decreased more dramatically than that on the untreated one. Inactivation of 100% of *E. coli* was achieved on the treated wafer in 20 min. However, no crossing was observed in the curves of Al₂O₃ (Figure 6).

It had been reported in our previous research about elimination of HCHO on Cu/Al₂O₃ catalyst that HCHO could adsorb on the surface of Al₂O₃ and easily turn into formate (HCOO⁻) in the presence of Cu at room temperature.¹⁴ Accordingly, in the case of Ag/Al₂O₃, it could be deduced that HCOOH was formed on

Figure 6. Bactericidal effect of Al₂O₃ (a) treated and (b) not treated by HCHO.

the catalyst surface with the consumption of ROS, and even took up the bactericidal reactive site on the surface of Ag/Al₂O₃, making the catalyst lose its bactericidal ability before the crossing. Although the bactericidal activity of formate cannot be ignored, it would be delayed for a certain time (7 min as seen in Figure 5) to display its bactericidal ability after the crossing, resulting in a sharp decrease of surviving bacteria. In the case of Al₂O₃, HCHO as a commonly used chemical disinfectant demonstrated obvious bactericidal effect after the adsorption on the Al₂O₃ surface reached saturation (about 3 min as shown in Figure 6a).

It can be confirmed from the above results that Al₂O₃ joined in the bactericidal process in addition to Ag, which suggests two possibilities: (1) a synergistic effect of Ag and Al₂O₃; (2) separate contributions to the bactericidal effect. However, the second possibility can be negated since the crossing point did not appear in control experiment (Figure 6), indicating that HCHO did not influence the bactericidal effect of the Al₂O₃ without Ag before 3 min. Furthermore, the complete inactivation was not achieved in 20 min on the Al₂O₃ surface compared to that on the Ag/Al₂O₃ surface (Figure 5a). That is to say, the synergistic effect existed between Ag and Al₂O₃, and the bactericidal activity was obviously enhanced by the presence of Ag species on the Al₂O₃ surface, which is in good accordance with the above results.

Conclusions

The difference between the configuration of *E. coli* cells making contact with the catalyst surface and the AgNO₃ solution indicates that the toxicity of Ag⁺ eluted from the catalyst could be ignored in the bactericidal process in air. We supposed that the ROS on the surface of the catalyst would be concerned in our research for bactericidal mechanism. The contribution of multiform ROS (O₂^{•-} and H₂O₂) on the catalyst surface was confirmed from the result of the bactericidal experiment introduced ROS scavengers. The worsened bactericidal effect of the Ag/Al₂O₃ surface modified by ultraviolet light and formaldehyde proved the beneficial effect of Ag in oxidation state and the synergistic effect between Ag and Al₂O₃ on the catalyst surface, which gave more evidence to the catalytic oxidation bactericidal mechanism.

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