Accumulation of copper and silver onto cell body and its effect on the inactivation of *Pseudomonas aeruginosa*

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Abstract *Pseudomonas aeruginosa*, a gram-negative rod bacterium, is a causative agent of waterborne pneumonia and presents high tolerance against conventional disinfectants. The inorganic biocidal reagents, copper and silver, were applied to inactivate *P. aeruginosa* inoculated in a synthetic drinking water (SDW). Additionally, the relationship of the specific amount of accumulated copper and silver reagents (Cs) on *P. aeruginosa* with inactivation profile was elucidated in this study. Flow cytometry (FCM) following staining with SYTO 9 and PI was used for detection of bacterial viability and density. Individual copper and silver reagents, and their combination, exhibited excellent biocidal abilities even at the concentration of 0.05 mgCu/L and 0.005 mgAg/L. The critical amounts of accumulated disinfectant (Cs) were calculated at 2.82 × 10⁻⁷ mgCu/cells and 5.13 × 10⁻⁸ mgAg/cells, at an incubation of 70 h. Consequently, the role of disinfectant on the inactivation of *P. aeruginosa* and the assessment of biocidal ability of copper, silver, and their combination were successfully explained by evaluating the terms Cs and Cc.

Keywords Copper; flow cytometry; inactivation; *Pseudomonas aeruginosa*; silver

Introduction

The Gram-negative rod bacterium *Pseudomonas aeruginosa*, belonging to the Pseudomonadaceae, is normally detected in natural water and leads cause for pneumonia infection by the route of human inhalation of water mist contaminated with it. Additionally, *P. aeruginosa* is focalised as the important microbe because of the organism’s inherent resistance to various disinfectants (Kievit et al., 2001; Teitzel and Parsek, 2003; Head and Yu, 2004). In order to prevent human contacts with pathogenic bacteria, great amounts of drinking water are chlorinated daily to inactivate pathogens in water treatment systems. However, numerous researches have reported on the disadvantages of chlorination, because of recolonization and health hazards, especially the damage of disinfection byproducts including trihalomethanes, haloacetic acids, bromate and chlorite formed during the reaction of chlorine with natural organic matter in the source water (Kajino et al., 1999; Singer, 1999; Zebger et al., 2005). Among advanced inorganic biocidal agents, copper and/or silver are currently studied as one of the substitutable methods for chlorination in water disinfection. Many laboratory works report the superior efficiencies for the inactivation of pathogenic bacteria at much lower concentrations than the guideline of copper and silver for drinking water recommended by WHO (Kim et al., 2004). The mechanisms of biocidal reaction of copper and silver are hypothetically explained as: (i) depression of nutrient uptake; (ii) inhibition of cell division; (iii) interference of proton transfer; and (iv) bonding to DNA increasing the stability of the double helix (Rusin et al., 2003).

Bacterial viability is normally classified depending on the integrity of cell membrane, and simultaneous double staining of nucleic acid using SYTO 9 (green, permeable) and PI (red, impermeable) have been widely studied for differentiation of microorganic viability
based on the permeability of two fluorescent molecular probes into cell membrane (Teitzel and Parsek, 2003). The combination of those fluorescent properties with the flow cytometric (FCM) technique has been introduced recently for extensive and rapid assessment of the microbial viability, as well as the density, from various environmental samples (Amor et al., 2002).

Unfortunately, most of the laboratory works regarding biocidal copper and silver reagents were focused on their efficiencies or other environmental effects, such as pH, temperature, and microbial properties, while the accumulation profiles of copper or silver onto bacterial cells and their relation to inactivation behaviour were not yet clearly elucidated. Therefore, we tried to estimate the applicability of copper and silver for disinfection of drinking water in this study, especially the accumulation profiles of biocidal copper and silver reagents onto P. aeruginosa. Their effects on the inactivation were investigated with installation of FCM.

Materials and methods

Bacterial strains and inoculation

Pseudomonas aeruginosa (ATCC 10145) was carefully cultured in Luria-Bertani (LB) broth medium for 1 d at 37.5 ± 1°C and adjusted to the log phase (Hoefel et al., 2003). Exponentially grown model microbe was inoculated onto synthetic drinking water (SDW) at a density of 1.0 × 10⁵ CFU/ml and incubated at 25 ± 1°C for 24 h. This step was repeated three times using SDW to remove the residual effects of LB broth medium, and finally P. aeruginosa was adjusted at the density of 2.0 × 10⁷ CFU/mL in test tubes (glass material tightened with cap) with 30 ml of SDW, respectively. The microbial density during these steps multiplied to 1.0 × 10⁹ CFU/ml from initial bacterial density (1.0 × 10⁵ CFU/mL). In order to reject unexpected or unexplainable effects for the inspection of properties of model microbe and transmission profile of copper and silver, SDW with copper (0–1.0 mgCu/L as copper sulfate) or silver (0–0.1 mgAg/L as silver nitrate) compositions were used. The SDW contained organic compounds with a concentration of 1 mgTOC/L as glucose and inorganic compounds (995.07 μg/L KNO₃, 129.92 μg/L KH₂PO₄, 44.29 μg/L Na₂SO₄, 18.34 μg/L CaCl₂·2H₂O, 41.81 μg/L MgCl₂·6H₂O, 9.68 μg/L FeCl₃·6H₂O, 19.07 μg/L KCl, 0.4 μg/L CoCl₂·6H₂O, 0.54 μg/L CuCl₂·2H₂O, 21.94 μg/L MnSO₄·5H₂O, 0.21 μg/L ZnCl₂, and 0.13 μg/L (NH₄)₆Mo₇O₂₄·4H₂O) (Sathasivan et al., 1997).

Preparation for LIVE and DEAD bacteria

Two sterilised test tubes (15 mL) containing P. aeruginosa (3 × 10⁶ CFU/mL) were used to yield artificial live and dead conditions. Isopropyl alcohol (70%, mixing every 15 min for 1 h at room temperature then washing with distilled water by using centrifuge) was used as the completely dead condition. In the case of live condition, distilled water without isopropyl alcohol was used.

Detection of bacterial properties

Nucleic acid of P. aeruginosa was stained with two kinds of fluorescent molecular probes, SYTO 9 and propidium iodide (PI) (LIVE/DEAD BacLight Bacterial Viability Kits, L-7012, Molecular Probes, Inc., USA) for detection of bacterial properties and biocidal ability of disinfectants by using a flow cytometric installation. PI and SYTO 9 were simultaneously added to the samples at a concentration of 1.5 μg per 1 mL sample, and incubated at room temperature for 15 min in the dark. Prepared samples were then analysed with flow cytometry (FCM, EPICS ALTRA flow cytometer, Beckman Coulter Inc., USA) equipped with a 488 nm argon laser for cell sorting. The emitted fluorescence
was split into three different channels: PMT2 (green, SYTO9) at 510–540 nm, PMT4 (orange, PI) at 615–640 nm, and PMT5 (red, PI) at 660–690 nm. Flow count™ (assayed concentration was 1,052 particles/µl, Beckman Coulter) was applied for cell counting. Plate culture colony counting tests with LB broth (agar added) were also carried out to compare with the density of P. aeruginosa detected by FCM.

**Determination of specific amounts of accumulated disinfectant (C<sub>s</sub>)**

The functions of initially injected disinfectant were determined as three categories in this study; residual disinfectant (C<sub>1</sub>), accumulated disinfectant onto P. aeruginosa cell body (C<sub>2</sub>); and adsorbed on the surface of test tube (C<sub>3</sub>). The hypothetical assumptions were that the formation of biofilm on the surface of test tube and loss of disinfectants to the outside of the experimental boundary did not occur during the tests. The membrane filter of pore size 0.2 µm (Whatman) was used to experimentally differentiate the accumulated disinfectant. The value of disinfectant analysed without filtration (C<sub>f</sub>) was regarded as the sum of C<sub>1</sub> and C<sub>2</sub>, and, measured after filtration (C<sub>f</sub>), was regarded as C<sub>1</sub>. Thereby, the disinfectant accumulated onto P. aeruginosa can be determined as the subtraction of C<sub>f</sub> from C<sub>t</sub>. This calculated value was then divided by the initial density of P. aeruginosa (2.0 × 10<sup>7</sup> cells/mL), and finally defined as the specific amount of accumulated disinfectant (C<sub>s</sub>). Therefore, the term (C<sub>s</sub>) could be theoretically regarded as the accumulated disinfectant per one flow cytometric signal unit (or colony) of P. aeruginosa. From the control test, complete rejection of P. aeruginosa (over 99.9999%) was detected when the membrane filter was used, while the concentration of disinfectants was not affected by a filtration. The values of copper and silver were analysed by inductively coupled plasma mass spectroscopy (ICP-M/S 4400, Hewlett Packard Inc., USA).

**Results and discussion**

**Application of FCM on the detection of bacterial properties**

The applicability of FCM for simultaneous detection of bacterial viability and density at artificial yield conditions by quantificational mixture between live and dead samples was estimated as illustrated in Figure 1(a). The ratios of live and dead detected by FCM were well familiarised, though 25% of dead composition was included in 100% of live sample. Additionally, the density of P. aeruginosa detected by FCM combined with SYTO 9
and PI when copper and silver was applied was also well represented ($R^2 = 0.9803$) of the value detected by traditional plate culture colony counting in the range of $0–4.64 \times 10^7$ cells(or CFU)/mL (Figure 1(b)).

**Inactivation profile of copper and silver**

The bacterial cultivability of *P. aeruginosa* without disinfectants multiplied from $1.85 \times 10^7$ to $4.64 \times 10^7$ cells/mL for 70 h in SDW, while it decreased at least $-4$ orders of log$_{10}(Nt/No)$ within 3 h after injection of disinfectants (Figure 2). The minimum concentration of copper for complete inactivation of *P. aeruginosa* (at the detection limit, $-7.0$ as Log$_{10}Nt/No$) after 3 h was observed at concentrations of 0.4 mgCu/L and it decreased to 0.15 after 8 h, while the inactivation ability of silver after incubation of 3 h ceased near $-4$ even at the highest concentration. The complete inactivation of *P. aeruginosa* by silver reagents was observed after 8 h at the concentration of 0.01 mgAg/L. Interestingly, the minimum concentration for complete inactivation of *P. aeruginosa* when the combination of silver (or copper) in copper (or silver) significantly decreased from 0.4 to 0.14 mgCu/L and 0.013 mgAg/L after 3 h; therefore it was considered that the combination of copper and silver accelerated the inactivation ability (Figure 2(c)).

**Accumulation of copper and silver onto *P. aeruginosa***

The accumulation profiles of disinfectant corresponding to the changes of the initial concentration of disinfectants were assessed. The rapid accumulation was observed during 8 h after starting the tests, and the specific amount of accumulated disinfectants ($C_s$) increased proportional to the increase of the initial concentration of injected disinfectants. Furthermore, the values of $C_s$ diminished significantly when two disinfectants were combined, even though the inactivation abilities showed higher than individual uses (data not shown).

The relationship between the specific amount of accumulated disinfectant and inactivation ability is shown in Figure 3. The biocidal abilities of copper and silver at the same $C_s$ value increased significantly corresponding to the time courses. The inactivation abilities at $C_s$ of $8.4 \times 10^{-8}$ and $2.3 \times 10^{-8}$ µgCu/cells, were $-1.8$ and $-2.3$ order of log$_{10}(Nt/No)$ after 3 h, respectively, but they approximately increased to the detection limit ($-7.0$) after 8 h. Moreover, the inactivation abilities of $C_s$ of silver

![Figure 2](https://iwaponline.com/wst/article-pdf/54/3/29/431815/29.pdf)
(<3.7 × 10^{-8} \mu gAg/cells) were accomplished to the detection limit, even though they did not reach the detection limit at any Cs values after 3 h incubation. It was considerable that those phenomena were derived from the requirement of time for the biocidal reaction between disinfectants and microbe after the rapid accumulation of disinfectants.

Based on the calculated Cs values, the critical amount of accumulated disinfectant (Cc) was determined as described in Table 1. The critical amount of accumulated disinfectant, Cc, was the minimum value of Cs for satisfying the complete inactivation (reaching the detection limit, ~7.0) of initial density of \textit{P. aeruginosa} (cells_i). After 3 h, Cc obtained from copper tests showed at 1.07 × 10^{-6}; it decreased to 2.82 × 10^{-7} \mu gCu/cells, after 70 h incubation. In the case of silver, the value of Cc when 8 h had passed was indicated as 2.89 × 10^{-7} and finally reduced to 5.13 × 10^{-8} \mu gAg/cells_i. The value of Cc of silver after 3 h was similar to that of copper obtained after 70 h. Consequently, it was considerable that the biocidal ability of silver for inactivation of \textit{P. aeruginosa} during the unit time was much higher than that of copper. The acceleration of inactivation ability derived from the combination of copper and silver could also be explained clearly by using the term of Cc. The overall values of Cc when copper and silver were combined were significantly smaller than those when copper or silver was used, and combined copper and silver acted as a biocidal reagent simultaneously after accumulated on \textit{P. aeruginosa} cell body (Table 1).

**Table 1** Comparison of critical amount of accumulated disinfectants between individual application of copper and silver and their combination

<table>
<thead>
<tr>
<th></th>
<th>Copper only (\mu g/cells_i)</th>
<th>Silver combined (\mu g/cells_i)</th>
<th>Silver only (\mu g/cells_i)</th>
<th>Copper combined (\mu g/cells_i)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 h</td>
<td>1.07 × 10^{-6}</td>
<td>3.09 × 10^{-7}</td>
<td>N.D*</td>
<td>4.01 × 10^{-7}</td>
</tr>
<tr>
<td>8 h</td>
<td>5.32 × 10^{-7}</td>
<td>3.31 × 10^{-7}</td>
<td>2.89 × 10^{-7}</td>
<td>1.77 × 10^{-7}</td>
</tr>
<tr>
<td>24 h</td>
<td>3.08 × 10^{-7}</td>
<td>1.50 × 10^{-7}</td>
<td>1.12 × 10^{-7}</td>
<td>5.39 × 10^{-8}</td>
</tr>
<tr>
<td>70 h</td>
<td>2.82 × 10^{-7}</td>
<td>1.33 × 10^{-7}</td>
<td>5.13 × 10^{-8}</td>
<td>3.29 × 10^{-8}</td>
</tr>
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\*Initial density of \textit{P. aeruginosa} detected by FCM technique

\*N.D.: not defined

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**Figure 3** Inactivation profiles of \textit{P. aeruginosa} corresponding to the variation of the specific amount of accumulated (a) copper and (b) silver reagents (Cs)
Conclusions
The application of FCM with SYTO 9 and PI for the simultaneous detection of bacterial viability and density was well associated with the traditional plate culture colony counting method. Both copper and silver seemed to be good biocides at combating *P. aeruginosa*, and efficiencies were significantly improved when copper and silver were combined. By introducing the terms of $C_s$ and $C_c$, it was possible to clearly understand the relationship between quantificational variations of disinfectants and inactivating profiles against *P. aeruginosa*.

References