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Enhanced killing of Escherichia coli using a combination of polyhexamethylene biguanide hydrochloride and 1-bromo-3-chloro-5,5-dimethylimidazolidine-2,4-dione

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One sentence summary: Bactericidal activity and mode of action of combined use of PHMB and BCDMH on Escherichia coli.

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ABSTRACT

The bactericidal activities of polyhexamethylene biguanide hydrochloride (PHMB), 1-bromo-3-chloro-5,5-dimethylimidazolidine-2,4-dione (BCDMH) and the combination of the two (designated as PB) were compared using Escherichia coli as the test organism. PB exhibited strong bactericidal activity: 10 mg/L PHMB combined with 8 mg/L BCDMH resulted in approximately 5.74 log₁₀ reduction (LR), whereas 320 mg/L PHMB or 20 mg/L BCDMH was about 5.53 and 6.56 LR, respectively. Analyses using scanning electron microscopy, flow cytometry and atomic absorption spectroscopy indicated that PB, PHMB and BCDMH disrupted cell membranes and changed membrane structure and permeability, resulting in the leakage of intracellular soluble proteins and ions. PB exerted stronger effects on potassium and magnesium leakage, membrane potential and permeability than BCDMH did. PB caused less protein leakage than PHMB did. These results suggest that at a relatively low concentration, PB exhibited good bactericidal activity and physiological effect on E. coli.

Keywords: combined disinfection; Escherichia coli; bactericidal activity; membrane integrity

INTRODUCTION

Polyhexamethylene biguanide hydrochloride (PHMB) is a broad-spectrum cationic amphipathic antibacterial agent. It has been broadly applied in water treatment, antifungal remedies, sanitization of beer glass, disinfection of solid-surfaces (Messick et al. 1999; Gilbert et al. 2001; Muller and Kramer 2008) and treating infective keratitis caused by Acanthamoeba (Elzezin, de Menezes and de Morais 2008). Biguanide disinfectants, including PHMB and chlorhexidine (Kuyyakanond and Quesnel 1992), have a damaging effect on membrane integrity (Gilbert and Moore 2005). Diluted PHMB (10 mg/L) can damage bacterial membranes, interact with bacterial DNA and impede bacterial proliferation (Gilbert et al. 2001; Chindera et al. 2016). Concentrated PHMB (>10 mg/L) can interact with acidic membrane lipids, resulting in membrane collapse and non-specific alteration of membrane integrity (Gilbert and Moore 2005; Allen, White and Morby 2006; O’Malley, Collins and White 2006). PHMB are effective against intracellular Staphylococcus aureus and has potent against intracellular methicillin-resistant Staphylococcus aureus (Kamaruzzaman, Firdessa and Good 2016). PHMB is non-
toxic and relatively safe to humans (Chindera et al. 2016). No resistant mutants have been reported after several decades of extensive use (Wessels and Ingmer 2013); however, high cost and large dose restrict its broader application. 1-bromo-3-chloro-5,5-dimethylimidazolidine-2,4-dione (BCDMH) is widely used for the treatment of swimming water and environmental disinfection. When BCDMH dissolves in water, HClO and HBrO are formed and can oxidise a variety of proteins. Ethylenediaminetetraacetic acid can significantly enhance the bacterial activity of BCDMH by increasing the permeability of bacterial cell membranes (Armon, Arbel and Green 1998). BCDMH is inexpensive and has strong bactericidal activity at low concentrations; however, it possesses a highly irritating odour and can cause contact dermatitis (Dalmau et al. 2012).

Phe et al. (2005) and Roth et al. (2010) explored the action mechanisms of PHMB and BCDMH at the cellular level. Ferreira et al. (2011) used scanning electron microscopy to examine cell surface changes in *Pseudomonas fluorescens* exposed to a quaternary ammonium compound. Berney et al. (2007), Bosshard et al. (2009) and Czechowska and van der Meer (2011) used flow cytometry to assess membrane permeability. Ferreira et al. (2011), and Hobbs et al. (2008) used atomic absorption spectroscopy to detect potassium and magnesium content.

One of the best ways to promote bactericidal activity for existing bactericides is combined use. For example, the combined effects of glutaraldehyde with three surfactants were tested on *Daphnia magna*, and an additive effect was observed (Boillot and Perronin 2008). When two or more bactericides act together, it is possible to maximise the advantages or overcome certain disadvantages. So far, the combined use of PHMB and BCDMH has not been explored. In this study, we tried to combine PHMB with BCDMH at various ratios and compared the combined bactericidal activity with that of PHMB or BCDMH alone.

**MATERIALS AND METHODS**

*Escherichia coli* inoculums

*Escherichia coli* 8099 was purchased from the Entry-Exit Inspection and Quarantine Bureau (Shanghai, China). Frozen bacterial samples were reconstituted in 50 mL of sterile tryptone soya broth and incubated at 37°C for 18 h. Single colonies were obtained using the streak plate method on tryptone soya agar (TSA), (SinoPharm chemical reagent co., Ltd, China) and one was used for enlarged culture with nutrient broth at 37°C for 18 h in a shaking incubator.

**Quantitative suspension test**

The *E. coli* cell suspensions were diluted with tryptone saline solution (TPS), (SinoPharm chemical reagent co., Ltd, China), centrifuged at 1077 × g for 15 min and washed twice with TPS. The pellet was resuspended in TPS to obtain a suspension with an optical density of ~0.6 at 600 nm. The bacterial suspension was mixed with 3% bovine serum albumin (BSA), (SinoPharm chemical reagent co., Ltd, China) at the ratio of 1:1. Then, 1 mL of the resulting cell suspension was mixed with 4 mL of a solution containing PHMB (Taizhou Sunny Chemical Co., Ltd, China), BCDMH (Taixing Jiansheng Fine Biological Technology Co., Ltd, China) or both (designated as PB) and incubated for 3 min. Then, 0.5 mL of the above mixture was mixed with 4.5 mL of Dey/Engley Neutralizing Broth, containing 0.5% Tween 80, and incubated for 10 min at room temperature. The neutralised solution was serially diluted (1:10) with TPS, and then 1 mL of each dilution was mixed with 15 mL of molten (40°C-45°C) TSA and poured into sterile Petri dishes and incubated at 37°C for 48 h and counted colonies.

**Scanning electron microscopy**

The cell suspension was mixed with PB or sterile hard water, neutralised and incubated as described in the quantitative suspension test section. Cells treated with sterile hard water are designated as CK. The solutions were centrifuged at 1077 × g for 15 min, and the pellet was washed twice with PBS. The pellets were suspended in 100 μL of PBS. Field emission scanning electron microscopy, using field emission scanning electron microscope (SU 8010; Hitachi, Tokyo, Japan) was conducted using a previously described protocol (Ferreira et al. 2011). Magnification was set at 20 000 and 50 000×.

**Flow-cytometric measurements**

Syto9 and propidium iodide (PI) were purchased with the Live/Dead Baclight Bacterial Viability Kit (Invitrogen, California, USA). Bis-(1,3-dibutylbarbituric acid) trimethine oxonol [DiBAC4(3)] was purchased from Sigma (USA). The Moflo XDP High Performance Flow Cytometer was from Beckman Coulter (USA). The samples were prepared, neutralised and centrifuged at 13 000 × g for 2 min (Wang et al. 2010). The pellet was washed and suspended in 0.85% NaCl. Syto9 and PI were used to assess membrane integrity (Simoes, Pereira and Vieira 2005; Czechowska and van der Meer 2011). Staining was conducted according to previously described procedures (Berney, Weilbmann and Egli 2006; Berney et al. 2008). In the procedure using Syto9/PI, untreated samples (CK) were used as a positive control for viable cells, and samples, treated with 70% isopropanol, were used as a negative control for dead cells. In the procedure using DiBAC4(3), heat-killed cells (90°C for 3 min) were used as the inactivated control, and untreated samples (CK) were used as positive control. Cell numbers were maintained between 10 000 and 15 000 using Summit 5.2.

**Potassium and magnesium analysis**

The potassium and magnesium standards were purchased from America o2si Smart Solution (AA grade). The Zeenit 700P atomic absorption instrument was obtained from Analytik (Jena, Germany). The instrument was calibrated using potassium standards of 0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/L (final concentration), and magnesium standards of 0, 0.05, 0.10, 0.15, 0.20 and 0.25 mg/L (final concentration), prepared with sterile 0.05 M N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) buffer (Sangon Biotech (Shanghai) Co., Ltd, China), pH 7.4. Linear relationships were observed between the emission, potassium concentration and magnesium concentration (y = 0.5904x + 0.014, R² = 0.9946 for K+ and y = 1.8228x + 0.0187, R² = 0.9939 for Mg²+). Disinfection solutions were prepared using HEPES buffer. Each bacterial suspension was prepared in HEPES buffer at a final concentration of 8 × 10⁸ CFU/mL. The following were performed as Ioannou described procedures (Ioannou, Hanlon and Denyer 2007). The reaction times were 0, 1, 3, 5, 10 and 20 min. After filtration, K+ and Mg²+ in the supernatant were analysed using a Zeenit 700P atomic absorption instrument in flame emission mode (parameters for the potassium ion: wavelength, 766.5 nm; slit, 0.7 nm high; air-acetylene flame; parameters for the magnesium ion: wavelength, 285.2 nm; slit, 1.5 nm; air-acetylene flame).

The optical density of the bacterial suspension was measured using a UV spectrophotometer (Jasco, Tokyo, Japan) in the range of 200–800 nm. The pH of the bacterial suspension was measured using a pH meter (model 545, Mettler Toledo, Akron, Ohio, USA).

**Flow-cytometric measurements**

Syto9 and propidium iodide (PI) were purchased with the Live/Dead BacLight Bacterial Viability Kit (Invitrogen, Califor-
Table 1. PB preparation and bactericidal activity analysis.

<table>
<thead>
<tr>
<th>PHMB (mg/L)</th>
<th>LR</th>
<th>BCDMH (mg/L)</th>
<th>LR</th>
<th>PB (mg/L)</th>
<th>BCDMH</th>
<th>LR</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>2.66 ± 0.28</td>
<td>5</td>
<td>0.00 ± 0.12</td>
<td>8</td>
<td>10</td>
<td>6.39 ± 0.26</td>
</tr>
<tr>
<td>20</td>
<td>3.87 ± 0.38</td>
<td>10</td>
<td>0.98 ± 0.34</td>
<td>10</td>
<td>10</td>
<td>5.84 ± 0.92</td>
</tr>
<tr>
<td>80</td>
<td>4.12 ± 0.27</td>
<td>15</td>
<td>4.20 ± 0.81</td>
<td>15</td>
<td>10</td>
<td>6.16 ± 0.46</td>
</tr>
<tr>
<td>160</td>
<td>4.92 ± 0.55</td>
<td>20</td>
<td>6.56 ± 0.51</td>
<td>10</td>
<td>8</td>
<td>5.74 ± 0.31</td>
</tr>
<tr>
<td>320</td>
<td>5.53 ± 0.86</td>
<td>25</td>
<td>6.99 ± 0.10</td>
<td>10</td>
<td>12</td>
<td>5.93 ± 0.98</td>
</tr>
</tbody>
</table>

Values are the log_{10} reduction (i.e., LR) in viable counts due to different disinfectants treatment and are calculated from the average log_{10} for untreated control—average log_{10} of disinfectant-treated. PHMB and BCDMH were used separately and bactericidal activities were compared with combined use of them. In PB group different PHMB and BCDMH were used to combine in order to obtain equivalent bactericidal effect with PHMB and BCDMH when used alone and try to use as little as BCDMH, as BCDMH gives strong irritant smell.

Soluble protein content

Protein concentration was measured using the Bradford assay (Bradford 2015). A linear relationship was observed between absorbance and the concentration of BSA (y = 1.8554x + 0.024, R^2 = 0.9979). Absorbances at 595 nm were measured with a UV/vis spectrophotometer (VARIAN 100UV-VIS, Australia).

Data analysis

The software SPSS 20 was used for the statistical analysis. All data were expressed as mean ± standard deviation. Least significance difference and Duncan’s tests were performed to assess the homogeneity of variance, and the confidence interval was set to 95%. Differences, with P values less than 0.05, were considered statistically significant.

RESULTS AND DISCUSSION

PB preparation and analysis of bactericidal activity

PHMB and BCDMH were mixed at various ratios. Quantitative bactericidal analysis and culture counts on nutrient agar were used to assess bactericidal activity on Escherichia coli. PHMB (320 mg/L) or BCDMH (20 mg/L) was used for comparison; each bactericide can result in approximately ≥5.0 log_{10} reduction (LR; ~5.53 and 6.56, respectively) and meets the required standards for bactericides. The bactericidal activity of PB, containing 8 mg/L BCDMH and 10 mg/L PHMB, was nearly identical to that of 320 mg/L PHMB or that of 20 mg/L BCDMH (Table 1). Thus, 8 mg/L BCDMH and 10 mg/L PHMB were combined for subsequent analyses. For ≥5.0 LR, the effective dose of BCDMH or PHMB in PB was much lower than that of BCDMH or PHMB alone, suggesting that an enhanced bactericidal activity resulted from combining PHMB and BCDMH.

Organic materials or organic pollutants usually exist in the environment to weaken disinfection effect. To simulate the disinfection environment in the field, 0.5 mL of 3% (w/v) BSA was added to the disinfection system. PHMB, a type of bis-biguanide, is ineffective against some Gram-negative bacteria, such as Pseudomonadaceae and Providencia spp. (Thomas and Stickler 1978; Mitchell, Brown and Skurray 1998). Chlorination from BCDMH will potentially cause formation of carcinogens and trihalomethanes (Gopal et al. 2007). If two or more bactericides act together, the environmental toxicity of BCDMH residuals is reduced (Boillot and Perrodin 2008; Wang et al. 2010). Compared with BCDMH or PHMB alone, PB can achieve a similar LR at a much lower concentration. The effective doses of PHMB or BCDMH, in PB, were 1/32 of PHMB and 2/5 of BCDMH, respectively, indicating that PB can be used as an effective bactericide.

Scanning electron microscopy

Scanning electron micrographs of E. coli, treated with bactericides, are shown in Fig. 1. The membranes of cells, exposed to the bactericides, changed significantly. Compared with those of untreated cells, the membranes of PB-treated cells appeared rougher, more wrinkled, partially collapsed and deformed. Additionally, PB caused parts of the membrane to protrude. Similar phenomenon was observed in PHMB-treated cells, while the membranes of BCDMH-treated cells remained relatively...
smooth. These results suggest that PHMB alone, or PHMB in PB, was the main contributor to the destruction of the membrane surface. The differences between the treatments appeared more obvious at 50,000× magnification. Our results show that some E. coli cells, treated with PB, PHMB or BCDMH, were longer than the untreated cells, suggesting that PB, PHMB or BCDMH induced bacterial SOS response (Chindera et al. 2016).

Membrane potential

Membrane potential was analysed by flow cytometry using DiBAC₄(3) staining (Fig. 2). Depolarised E. coli cells, with DiBAC₄(3) signal concentrated within the area indicated by the arrow, are shown in Fig. 2A. DiBAC₄(3) is a membrane-potential-sensitive lipophilic and anionic bis-oxonol dye and can only be taken up by depolarised cells or cells with disrupted cytoplasmic membranes (Berney et al. 2008). Compared with untreated cells, the depolarised proportion of cells, treated with PB or PHMB, increased sharply at 1 min, and then remained relatively stable. The proportions of depolarised cells were significantly lower in both the control and BCDMH-treated groups.

The depolarised proportion of cells is presented in Fig. 2B. PB affected the membrane potential. At 1 min, the proportions of depolarised cells in the PB group were 81.67%, distributed between that of PHMB-treated group (96.79%) and that of BCDMH-treated group (28.84%). This result indicates that PHMB was the main contributor to the change in membrane potential. Similar phenomenon has been demonstrated in cells treated with PHMB (Gilbert, Pemberton and Wilkinson 1990).

Membrane integrity

Flow cytometry, using Syto9 and PI, was employed to analyse membrane integrity. The data from three independent experiments are shown in Fig. 3; the proportion of permeabilised cells is shown in Fig. 3B. At 5 min, the proportion of permeabilised cells in the PB group was 85.45% that of the PHMB group, and much higher than that of the BCDMH group.

Membrane disruption may lead to inevitable changes in membrane integrity and electric potential. Cell membrane damage by free chlorine was illustrated to be dose-dependent (Wang et al. 2010); damage by chlorine solution and the proportion of damaged cells have been investigated (Phe et al. 2005). The results suggested that BCDMH does not have notable effect on membrane integrity and on cell depolarization, and the effects of PB and PHMB were significant (Figs 2 and 3). Although PHMB in PB was only 3.13% (10/320), but permeabilised cell and depolarised cell ratios were 85.45% and 84.38% those of PHMB group, indicating that PHMB in PB plays main role in changing membrane integrity and depolarizing cell with the help of BCDMH. Perhaps, BCDMH helps PHMB with changing membrane integrity and depolarizing cell by oxidizing membrane protein and lipid.
Leakage of ions

The results of potassium and magnesium leakage, obtained using the atomic absorption instrument in flame emission mode, are shown in Fig. 4. Potassium and magnesium leakage, induced by PB and PHMB, reached its maximum at 1 min, and was significantly higher than that induced by BCDMH (Fig. 4A and B). The leakage induced by PB was similar to that caused by PHMB, suggesting that PHMB in PB was largely responsible for inducing leakage in cells treated with PB.

Potassium and magnesium leakage is used as an indicator of the damage to the cytoplasmic membrane (Ioannou, Hanlon and Denyer 2007; Hobbs et al. 2008; Ferreira et al. 2011). Bisbiguanides can cause potassium ion and proton leakage (Hugo and Longworth 1964; Hugo and Longworth 1965; Hugo and Longworth 1966). The results of the ion leakage assessment agree with those of the scanning electron microscope analysis, and the assessment of membrane potential and permeability, suggesting that greater leakage was caused by PHMB or PB;
PHMB is associated with strong destructive effect on the cell membranes.

Soluble protein content

Soluble protein content in the supernatant, after centrifugation, is shown in Fig. 4C. Compared with soluble protein leakage in the PHMB group, protein leakage in the PB group was only 18.26% of that in the PHMB group, but greater than that in the BCDMH group. BCDMH is an oxidizing bactericide that functions largely via free chlorine and has less destructive effects on the membrane; thus, it is likely that BCDMH caused only slight leakage of ions and soluble proteins.

The effect of PB on protein leakage was weaker than that of PHMB. This may be because the amount of PHMB in the PB group was only 1/3 of that in the PHMB group, and the membrane destruction in the PB group was so slight that only small molecules or ions could leak rapidly with ease.

In summary, PB can quickly and efficiently kill E. coli at low concentrations. The PHMB, contained in PB, functions in altering the bacterial membrane integrity and membrane potential, leading to the leakage of proteins and ions. Aided by PHMB, the BCDMH, contained in PB, can enter E. coli cells easily and act on the various types of intracellular macromolecules more directly. Mutual promotion of PHMB and BCDMH highlights the combined effect of BCDMH with PHMB and accelerates the process of cell death.

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Conflict of interest. None declared.

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