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Effects of NaOCl Aqueous Solutions and Ethyl Alcohol Solutions on Removing Protein Surface Contaminants and Re-establishing Antibacterial Activities of Copper-Alloyed Stainless Steel

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Effects of wiping copper-alloyed stainless steel surfaces with disinfectants to remove protein surface contaminants and re-establish their antibacterial activities were quantitatively studied. Disinfectants used were sodium hypochlorite aqueous solutions and ethyl alcohol aqueous solutions. Wiping with NaOCl aqueous solutions effectively removed protein surface contaminants. Ethyl alcohol aqueous solutions were also effective for cleaning, but their efficiency was less than that of NaOCl aqueous solutions. When the amount of residual surface contaminants was reduced to 0.4 ng/mm², the surfaces of the copper-alloyed stainless steel regained antibacterial activities to the same level as those in a clean surface condition.

Key words : Sodium hypochlorite / Ethyl alcohol / Antibacterial stainless steel / Protein surface contaminant / Environmental surfaces.

INTRODUCTION

Stainless steel is widely used for things or places that we touch with our hands, such as frames of chairs and beds, bedside lockers, door handles and various switches. General-purpose stainless steel is not antibacterial: pathogens remain viable on stainless steel surfaces and present a contamination hazard for considerable periods of time (Dietze et al., 2001; Higashino and Kamiya, 2011; Kramer et al., 2006; Kusumanigrum et al., 2003; Rose et al., 2003; Shams et al. 2007). Alloying with copper gives stainless steel some antibacterial activities, as has been well confirmed by several studies (Hong and Koo, 2005; Kawakami et al., 2010; Kielemoes and Verstraete, 2001; Noyce et al., 2006; Suzuki et al., 2001 and 2014). Introducing antibacterial stainless steel into frequently hand-touched

places has been considered to reduce risks of outbreaks of illnesses caused by pathogens via environmental surfaces. Surfaces of antibacterial stainless steel are contaminated with substances when they are touched and used, and the contamination inactivates the antibacterial activities. The surfaces of antibacterial stainless steel, hence, should be cleaned periodically so as to maintain their antibacterial activities. In a previous study (Kawakami et al., 2014), it was qualitatively shown that wiping with sodium hypochlorite aqueous solution was effective for re-establishing the antibacterial activities of copper-alloyed stainless steel, which had once been inactivated by surface contamination with protein and artificial oily substances.

In this study, NaOCl solution and ethyl alcohol aqueous solution (EtOH) were used as cleaning agents, and the effects of wiping with these agents to remove protein surface contaminants and enable protein-contaminated surfaces of a copper-alloyed stainless steel to recover their antibacterial activities

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were quantitatively studied.

MATERIALS AND METHODS

Sample materials and preparation of specimens

Samples subjected to experiments were copper-alloyed stainless steel (Nisshin Steel Co., Ltd., Tokyo, Japan) (Cu-SS) and a SUS 304 stainless steel (SUS304). The copper content of the Cu-SS was about 3.8 mass%. The SUS304 was selected as a reference as it has been widely used for places that are frequently touched by hands. The chemical compositions of the samples are summarized in Table 1.

Coupon specimens, 25 mm×25 mm×2 mm, were prepared from supplied plates. The test surfaces of specimens were polished by #1500 grit emery papers in order to obtain a uniform surface finish. The polished specimens were then subjected to ultrasonic cleaning in 70% EtOH followed by drying and disinfection under ultraviolet (UV) irradiation in a safety cabinet. In this study, sterilized Petri dishes served as the control.

Contamination and wiping

Bovine serum albumin (BSA) (Sigma-Aldrich Co., USA) was employed as the model protein contaminant. The BSA was dissolved in distilled water to a final concentration of 1.0 g/mL. The BSA solution of 0.1 mL was applied and spread evenly over the test surface of the specimen. The amount of applied BSA on the test surface was hence 160 ng/mm². The specimens were then dried in a thermostat kept at 24°C for 24 h.

For the Cu-SS, BSA contaminated specimens with EtOH treatment were also prepared: the entire test surface of the BSA contaminated Cu-SS specimen was covered with 0.1 mL of 99.5% EtOH, and then the EtOH was evaporated in the thermostat.

Cleaning agents used for wipe cleaning were NaOCl solution at several chlorine concentrations and pH, EtOH at several concentrations, and 0.1 M sodium hydroxide aqueous solution (pH12).

A sterile wipe (ITW Texwipe, Kennesville, NC USA) was folded once to produce a rectangle of 80- by 145-mm. The sterile wipe loaded with 0.5 mL of the cleaning agent and was fixed to a crockmeter (Atlas Electric Device Co., Chicago, IL USA). The specimen was set on the crockmeter so that the test surface of the specimen was pressed against the sterile wipe. The specimen was then reciprocally moved on the sterile wipe surface for a specific number of cycles. The distance of the reciprocal movement was 75 mm.

Antibacterial tests

Antibacterial tests were carried out on the basis of ISO 22196. The strains used were *Escherichia coli*

TABLE 1. Chemical compositions of samples [mass%].

Sample	C	Si	Mn	Cr	Ni	N	Cu
Cu-SS	0.011	0.52	1.78	18.14	9.37	0.011	3.78
SUS304	0.04	0.05	0.91	18.08	8.09	—	—

(NBRC 3972) and *Staphylococcus aureus subsp. aureus* (NBRC 12732). A precultured bacterial suspension of 0.05 mL, with about 1.0×10^6 colony forming units per mL (CFU/mL) in bacterial concentration, was inoculated on the test surface of a coupon specimen. The inoculum was then covered by a sterilized polyethylene film, 20- by 20-mm, in order to make the inoculum form a thin layer. The inoculated bacteria were incubated for 24 h at a temperature of $35 \pm 1^\circ\text{C}$. After the incubation, the test surface and the polyethylene film were rinsed with Soybean Casein Digest broth with Lecithin and Polysorbate 80 medium (Nihon Pharmaceutical Co., Ltd., Japan.) at 3.0 mL and 2.0 mL, respectively. The rinse solutions were combined and then subjected to plate counting technique with standard agar medium in order to count the number of viable bacterial cells. The number of viable bacteria after exposure to the test surface was expressed as total colony forming units per specimen.

Measuring the amount of BSA remaining on a test surface after wipe-cleaning

The swab method (Urano et al., 2010) was employed to retrieve BSA remaining on test surfaces after the wiping treatment. An entire test surface was wiped with a polyethylene micro-porous swab (As One Co. Ltd., Osaka, Japan) loaded with 0.055 mL of 0.1 M NaOH solution. The BSA retrieved on the swab was washed off by 30 min ultrasonification in 1 mL of 0.1 M NaOH solution containing 1.0 mg of sodium dodecyl sulfate. Modified Lowry Protein Assay Kit (Thermo Fisher Scientific Inc., Illinois, USA) was used to measure the amount of BSA in the NaOH solution. The amount of residual BSA was expressed as the mass of BSA per unit test surface area.

All chemicals were reagent grade and used as obtained without further purification. They were obtained from Wako Pure Chemical Industries, Ltd., Japan, unless otherwise stated.

Friction force measurement

Coupon specimens, 50 mm×50 mm×2 mm, were prepared from the Cu-SS plates and subjected to dynamic friction force measurements. The specimen pressed on an EtOH-loaded sterile wipe was horizontally moved at a constant rate of 1.0 mm/sec by an Instron type materials testing machine. The value of the

dynamic friction force was recorded by the materials testing machine at a sampling rate of 10 Hz, and then averaged over the sampling period.

Statistical Analysis

All data reported were the averages of at least three replicate data sets. Error bars represent the standard deviation. The Scheffé test was employed for multiple comparison test and a p -value of less than 0.05 was considered statistically significant. Prior to application of the Scheffé test, analysis of variance was carried out to confirm the significance of the data.

RESULTS AND DISCUSSION

Recovery of antibacterial activities

Experimental results obtained from antibacterial tests are shown in Fig. 1.

For SUS304 specimens, the counts of bacterial cells recovered from polished surfaces (B) and BSA-contaminated surfaces (C) were not significantly different from that value from the control (A). None of the cleaning treatments resulted in a significant change in the bacterial count (D - I).

For the Cu-SS specimens, the bacterial count recovered from as polished surfaces (B) was significantly lower than that value from the control (A). The bacterial count from the BSA-contaminated Cu-SS surfaces (C) was not significantly different from the values from the control. Wiping with neither dry sterile wipes (D) nor sterile wipes loaded with distilled water (E) changed the bacterial count. Wiping with NaOCl solution (500 ppm in available chlorine concentration and pH12) significantly reduced the bacterial count, and the bacterial count from the Cu-SS surfaces wiped four cycles with sterile wipes loaded with NaOCl solution (G) was not significantly different from the value from the newly polished surfaces (B). Such results indicate that wiping with NaOCl solution was effective in re-establishing antibacterial activities which had been deactivated by BSA surface contamination. Contrary to the efficiency of NaOCl solution, EtOH at both concentrations tested did not significantly change the bacterial count (H and I).

As disinfectants used in the wipe cleaning affected the recovery of antibacterial activities that had been deactivated by BSA surface contamination, the effects of disinfectants on removing BSA surface contaminants from Cu-SS surfaces were investigated.

Effects of cleaning agents on removing BSA surface contaminants from the Cu-SS surfaces

Figure 2 shows the amounts of BSA remaining on test surfaces wiped with dry sterile wipes.

FIG. 1. Experimental results obtained from antibacterial tests. (a) *E. coli* and (b) *S. aureus*. (A: control, B: as polished, C: BSA-contaminated, D: BSA + dry wiping $\times$ 20 cycles, E: BSA + wiping with distilled water $\times$ 4 cycles, F: BSA + wiping with NaOCl (500 ppm in available chlorine concentration) $\times$ 1 cycle, G: BSA + wiping with NaOCl (500 ppm in available chlorine concentration) $\times$ 4 cycles, H: BSA + wiping with 10 % EtOH $\times$ 4 cycles, I: BSA + wiping with 70 % EtOH $\times$ 4 cycles)

FIG. 2. The amount of residual BSA after wiping the surfaces with dry sterile wipes.

The dry wipes removed a small amount of applied BSA; about 140 ng/mm² (90% of the applied BSA) remained on the test surfaces independently of the

FIG. 3. The amount of residual BSA on the surfaces wiped with sterile wipes loaded with distilled water (○) and on surfaces firstly treated with 99.5% EtOH and then wiped with sterile wipes loaded with distilled water (●).

FIG. 4. The amount of residual BSA after wiping with sterile wipes loaded with 0.1 M NaOH solution (pH12).

number of wipe cycles.

Figure 3 shows the amount residual BSA on test surfaces wiped with sterile wipes loaded with distilled water (Figure 3 also shows the amount of residual BSA on test surfaces that were firstly treated with 99.5% EtOH and then subjected to wiping with distilled water. This will be discussed later).

The amounts of residual BSA on the surfaces wiped more than two cycles were about 10 ng/mm² almost independently of the number of wipe cycles. The surfaces were not clean enough to re-establish the antibacterial activities as shown in Fig. 1.

The amounts of residual BSA after cleaning with sterile wipes loaded with 0.1 M NaOH solution (pH12) are shown in Fig. 4.

The amount of residual BSA after four wipe cycles was about 2 ng/mm². The value was less than the value for the specimens wiped with sterile wipes loaded with distilled water (Fig. 3).

Figure 5 shows the amounts of residual BSA on test surfaces wiped with sterile wipes loaded with NaOCl.

FIG. 5. The amount of residual BSA after wiping with sterile wipes loaded with NaOCl solution. Symbols: □, 50 ppm (pH12); △, 500 ppm (pH12); ○, 5000 ppm (pH12); ▲, 500 ppm (pH2.6); ●, 500 ppm (pH7.7).

Wiping with NaOCl solution effectively reduced the amount of residual BSA on test surfaces: under all conditions tested, the amounts of residual BSA were less than 6 ng/mm². Increase in the available chlorine concentration and pH reduced the amount of residual BSA. When the test surfaces were wiped four cycles with sterile wipes loaded with NaOCl solution, i.e. under conditions of 500 ppm in available chlorine concentration and pH12, the amount of residual BSA was about 0.4 ng/mm². In consideration of the results obtained from the antibacterial tests, 0.4 ng/mm² of residual BSA is clean enough for Cu-SS to regain its antibacterial activities to the same level as those in a clean surface condition.

The cleaning efficiency of NaOCl solution depends on the concentrations of dissociated OCl⁻ ions and hydroxide ions OH⁻ (Fukuzaki et al., 2007; Urano and Fukuzaki, 2005). Sodium hypochlorite, in a higher pH range, dissociates and forms hypochlorite ion OCl⁻. As it is a strong oxidizer, the dissociated OCl⁻ ion decomposes organic molecules into low-molecular-weight fragments by oxidative action, making organic molecules more easily detach from solid surfaces. Hydroxide ions give both contaminant substances and solid surfaces negative charges, increase the repulsion force acting between them, and as a result accelerate the detachment of contaminant substances from solid surfaces. Dissociated OCl⁻ ions and OH⁻ ions, in addition, act synergistically to accelerate the detachment of organic molecules from solid surfaces (Fukuzaki, 2014). Such theories can explain our experimental results: the NaOCl solutions with higher available chlorine concentration and pH showed greater cleaning efficiency, and more effectiveness effective than NaOH solutions with the same pH.

The amounts of residual BSA after wiping with EtOH-loaded sterile wipes are shown in Fig. 6.

FIG. 6. The amount of residual BSA after wiping with sterile wipes loaded with EtOH. (a) 99.5%, (b) Symbols: ○, 10%; ●, 25%; ◇, 50%; △, 70%.

The amount of residual BSA was about 56 ng/mm^2 for the surfaces wiped four cycles with 99.5 % EtOH-loaded sterile wipes (Fig. 6 (a)), and the value decreased with the decrease in EtOH concentration to about 6.8 ng/mm^2 for the surfaces wiped four cycles with 10 % EtOH (Fig. 6 (b)), which was only slightly greater than the value for the test surfaces wiped four cycles with distilled water. Such results indicate that EtOH is effective in removing BSA, but its efficiency is lower than that of NaOCl solution and distilled water.

The amounts of residual BSA on the test surfaces firstly treated with 99.5% EtOH and then subjected to wiping with distilled water are shown in Fig. 3. The amount of residual BSA was much less than the values for the specimens wiped with 99.5% EtOH and the same as the values for the specimens wiped with distilled water. Such results indicate that EtOH presumably has less effect on the adherence of BSA on stainless steel surfaces, even though EtOH in higher concentrations might denature the BSA molecules.

In order to study the adverse effects of EtOH concentration on cleaning efficiency, the dynamic friction force during wiping was measured and the results are summarized in Fig. 7.

FIG. 7. Dynamic friction force. The abscissa is the mass of loaded liquid divided by the mass of a sterile wipe into which the liquid was loaded. Symbols: ●, distilled water; ○, 10% EtOH; △, 25% EtOH; ▲, 50% EtOH; ▽, 75% EtOH; □, 99.5% EtOH.

The abscissa in the figure is the liquid content of sterile wipes, i.e. the mass of loaded liquid divided by the mass of a sterile wipe to which the liquid was loaded. The liquid content for preparing specimens for antibacterial tests and for residual BSA measurements was about 150%. The value of friction force decreased with the increase in EtOH concentration. Such results presumably indicate that abrasion, by which surface contaminant substances are mechanically removed from solid surfaces, decreased if sterile wipes were loaded with EtOH in higher concentrations.

We conclude that NaOCl solution is effective in removing BSA surface contaminants and re-establishing the antibacterial activity of Cu-SS surfaces. Ethyl alcohol aqueous solution is also a good cleaning agent, but its efficiency is lower than that of NaOCl solution.

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