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Anti-biofilm formation of a novel stainless steel against *Staphylococcus aureus*

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ABSTRACT

Staphylococcus aureus (*S. aureus*) is a bacterium frequently found proliferating on metal surfaces such as stainless steels used in healthcare and food processing facilities. Past research has shown that a novel Cu-bearing 304 type stainless steel (304CuSS) exhibits excellent antibacterial ability (i.e. against *S. aureus*) in a short time period (24 h.). This work was dedicated to investigate the 304CuSS's inhibition ability towards the *S. aureus* biofilm formation for an extended period of 7 days after incubation. It was found that the antibacterial rate of the 304CuSS against sessile bacterial cells reached over 99.9% in comparison with the 304SS. The thickness and sizes of the biofilms on the 304SS surfaces increased markedly with period of contact, and thus expected higher risk of bio-contamination, indicated by the changes of surface free energy between biofilm and the steel surfaces. The results demonstrated that the 304CuSS exhibited strong inhibition on the growth and adherence of the biofilms. The surface free energy of the 304CuSS after contact with sessile bacterial cells was much lower than that of the 304SS towards the same culture times. The continuously dissolved Cu^{2+} ions well demonstrated the dissolution ability of Cu-rich precipitates after exposure to *S. aureus* solution, from 3.1 ppm (2 days) to 4.5 ppm (7 days). For this to occur, a hypothesis mechanism might be established for 304CuSS in which the Cu^{2+} ions were released from Cu-rich phases that bond with extracellular polymeric substances (EPS) of the microorganisms. And these inhibited the activities of cell protein/enzymes and effectively prevented planktonic bacterial cells attaching to the 304CuSS metal surface.

1. Introduction

Stainless steel is widely used in a variety of manufacturing, engineering, and whole spectrum of industries, including energy, chemical engineering, medical devices, healthcare and environmental protection, marine, building construction, and agro-food systems [1,2]. However, within the healthcare and food-agro industry, the current publications or reports are more concentrating on the biofilm formation ability on materials such as plastics, fabrics, cloths and metals [2–8]. Many research papers have been published in order to demonstrate the antibacterial abilities of the Cu-bearing stainless steels (304CuSS) [9–11], which inhibit and weaken the biofilm formation particularly in the applications of healthcare and medical devices. It is a surprisingly scare that there is no research paper that could be found published across the entire agro-food and medical processing and packaging facilities.

Some bacteria can live and breed well on the surfaces of general materials such as plastics, ceramics and metals. Particularly, normal stainless steel products strongly support the bacterial growth in the biofilm formation for an extended period of time, which greatly increases the risk of bacterial cross-contamination, such as *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*)/*Methicillin-resistant Staphylococcus aureus* (*MRSA*), etc. Because of these, it is estimated that each year in the United States, approximately 9000 deaths are associated with healthcare, environmental related food contaminations [12]. Many studies have been focused on removing the pathogenic bacteria formed on stainless steels, as summarized in Table 1 [13–19]. However, these technologies cannot maintain their inhibition ability against sessile bacteria for a prolonged period, because a bacterial adhesion to a solid surface can lead to biofilm formation. And the ability of the biofilm formation is depending on not only the physiochemical properties of the bacterial cell surface, but also the substrate's chemical composition and physical properties underneath them as well [20], mainly including the substrate surface free energy, ionic charges, hydrophobicity, roughness, as well as the type of bacterial proteins presented [21]. Wherein, the surface energy is well governed by van der Waals and electrostatic forces [22].

The emphasis on eco-friendly environment and healthy food products is a pivotal movement to allow food manufacturers to consider this novel and antibacterial stainless steel owning to the limitations of the commercial SS on the uses of chemical disinfectants, chemical preservatives and other preservation methodologies (freezing and

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Table 1
Removing technologies of common pathogenic bacteria on the surfaces of stainless steels.

Methods	Common pathogenic bacteria	References
Chemical sanitizer	*Escherichia coli*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Cronobacter sakazakii*	[13,14]
Natural bactericidal agents	*Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli O 157:H7*	[17,19]
Surface treatment	*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Salmonella typhimurium*	[15,18]
Biological competition	*Listeria monocytogenes*	[16]

canning, etc.) [23]. Therefore the prevention of bio-contamination is crucial to various application environments.

S. aureus, a typical Gram-positive *coccus*, is one of the most common pathogens associated with serious foodborne diseases and has long been considered as a major issue in public health risks [24,25]. It is often found on the surfaces of stainless steels used in the food processing industry worldwide [26,27]. More importantly the antibiotic-resistant or disinfectant resistant bacteria can easily form new pathogenic *S. aureus* (e.g. MRSA), which is a devastating concern causing serious problem in the clinical medicine, hospitals and agro-food industries [28–30].

Staphylococcal biofilm formation is one of the critical processes in the control of bacterial inhibition by the disinfectant and antibacterial materials. This biofilm formation comprises two steps, beginning with the initial attachment to a contact surface, followed by the accumulation of multi-layered cell clusters — namely intercellular adhesion [31]. Once the formation of biofilm occurs, the resistance of bacteria towards antibacterial material or agent is enhanced. According to the work of Pastoriza et al. [32], the establishment of the poison food caused by *S. aureus* depends on the ability of the strain to survive on a colonized substrate, multiply under a variety of conditions and produce organic EPS. This is a complex mixture of biomolecules, such as proteins, humic-like substances, polysaccharides, uronic acid, lipids and glycoproteins, which surround the bacterial cell [33]. Therefore, fewer biofilms on the stainless steel substrate may reduce the risk of bio-contamination towards healthcare and food processing facilities.

Previous papers on the 304CuSS showed it a strong antibacterial ability against *S. aureus* with the antibacterial rate over 99.9% within 24 h [34–36]. However, there has been no work on whether or not the 304CuSS can mitigate the formation of the biofilm after an extended period of exposure to *S. aureus*. Therefore, the objectives of this work are to investigate the adhesion behavior of *S. aureus* on the surface of the 304CuSS by using a standard direct plate counting (JIS Z 2801-2000 or GB/T 21510-2008). The Cu^{2+} release from the 304CuSS was examined by inductively coupled plasma-mass spectrometer (ICP-MS), contact angle measurement for surface free energy (SFE), and fourier transformed infrared spectroscopy (FTIR) for identifying the variations of functional groups after sessile bacteria were contacted with steels, and morphologies of the bacteria were observed by a scanning electron microscope (SEM).

2. Material and methods

2.1. Sample preparation

The samples used in this study were the 304CuSS (0Cr18Ni9Cu3.8) and the 304SS (0Cr18Ni9) and they were solution treated at 1040 °C for 30 min followed by a water quenching process. And then the samples were aged at 700 °C for 6 h followed by air-cooling. These heat treatments allowed the 304CuSS to have a strong bactericidal ability resulting from a certain amount of saturated Cu-rich precipitates [34] that have been formed in the 304CuSS, as shown in Fig. 1 [37]. The size of samples was 10.0 mm in diameter and 5.0 mm in thickness,

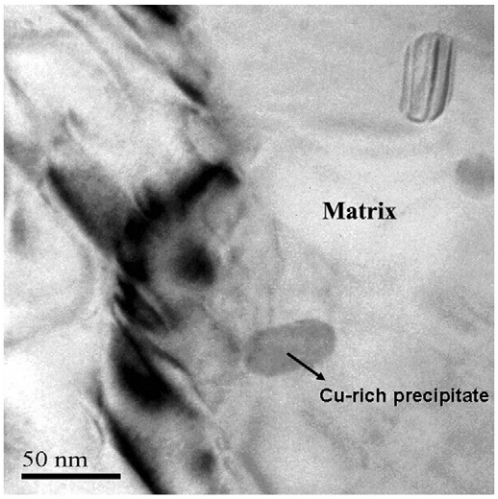

Fig. 1. Cu-rich precipitates from a Cu-bearing Stainless steel matrix after the thermal/heat treatment [37].

and they were grounded by a series of grit SiC papers (400, 600, 800 and 1000). The samples were then sterilized by an autoclave at 120 °C for 20 min before tests.

2.2. Bacterial culture and direct plate counting

A Gram-positive *S. aureus* ATCC25923 was selected and its solution was incubated overnight at 37 °C, and then diluted into the Luria-Bertani (LB) medium as a standard approach [38] with a concentration of 3.0×10^5 CFU/ml. The number of adhered bacteria on samples was determined by a direct plate-counting method (JIS Z 2801-2000). To fit into this standard test, the steel samples were placed first into a 24-well plate, and then 1 ml of the LB medium that incubated bacterial solutions was dropped into each well of the plate for co-culture. These plates were incubated at 37 °C for 2, 4, and 7 days separately to facilitate the biofilm formation.

After removing the bacterial cells with the sterile distilled water for three times, adhered bacteria were then swabbed and serially diluted onto LB plates. Bacterial colonies were counted and results were expressed in log (CFU/ml). Three parallel samples were used for different time points.

2.3. Contact angles and surface free energy analysis

Contact angle was measured by using the Sessile-Drop methodology by a contact angle analyzer (OCA-20, Dataphysics, Germany). The SFE of a solid can be calculated by the following expression [39]:

$$\gamma_L(1+\cos\theta) = 2\sqrt{\gamma_S^d\gamma_L^d} + 2\sqrt{\gamma_S^p\gamma_L^p} \quad (1)$$

where the $\boldsymbol{\gamma}_L$ is an experimentally determined surface free energy of the liquid, θ is the contact angle, $\boldsymbol{\gamma}_S^d$ is the dispersion component of the surface free energy of the solid, γ_L^d is the dispersion component of the surface free energy of the liquid, $\boldsymbol{\gamma}_S^p$ is the polar component of the surface free energy of the solid, and $\boldsymbol{\gamma}_L^p$ is the polar component of the surface free energy of the liquid.

The shapes of the droplets on the stainless steels were calculated both the left and right contact angles from the shapes of the droplets with an accuracy of $\pm 0.1°$. The planktonic bacteria liquids, LB medium and other substances were removed through washing with sterile distilled water for 15 s three times, and the average value of the contact angle at four points on the surface was regarded as the mean value of the contact angle for each treated sample at 25 °C [40,41]. Contact

angles were measured at time points of beginning 2, 4 and 7 days, respectively.

2.4. Surface analysis

The variations of the functional groups from the bacterial cells after *S. aureus* was acted with the steel samples were analyzed by using FTIR. The spectra data were collected from FTIR data system (TENSOR 27, Bruker, Germany with diffusion reflectance accessory within the range of 400–4000 cm^{-1}).

Before the test, the bacteria co-cultured with the steel samples were harvested after 7 days of incubation by centrifuging in 5000 G force. After 7 days of incubation, each sample was withdrawn and immersed in sterile distilled water for 15 s three times to release planktonic bacteria, culture medium and other impurities covered on the surfaces. The adhered bacteria were collected by thoroughly rubbing their surfaces with two moistened swabs, which were resuspended in sterile distilled water by vigorously vortexing for 30 s followed by oven drying [40,41]. The samples were then grounded in an agate pestle and mortared with KBr. The transmittance spectra of the samples were recorded as a function of wave number given a resolution of 2 cm^{-1} at 25 °C.

In order to determine the amount of Cu^{2+} ions released from the 304CuSS after exposure to *S. aureus* solution for 2, 4 and 7 days separately, an inductively coupled plasma mass spectrometer (ICP-MS, OPTIMA3000, USA) was used sensitively enough to detect 1 ppb (1×10^{-6} g/l) of Cu^{2+} ions [35]. The surface area of 3 cm^2 of the samples and 1 ml of the bacterial solution were prepared for this test. After action for different times, 100 μl of the bacterial solution was taken out and fully mixed for 30 s by an oscillator, and then diluted 50 times before the ICP test. Three parallel samples for both steels were examined and the data obtained presented the mean ± SD. Meanwhile, biofilms on the steel surfaces were analyzed by a field emission scanning electron microscope (SEM, JSM-6301F, Oxford, England).

2.5. Statistical data analysis

All data in this study presented the mean ± SD of three experimental replicates.

3. Results and discussion

3.1. Adhesion of bacteria

The number of adhered bacteria on stainless steel was found significantly changed after the steel samples were co-cultured with the *S. aureus* solution during a different time period, as shown in Fig. 2. The number of adhered bacteria was almost invisible at the beginning of contact with both steels. While the number of sessile bacteria sharply increased on the 304SS after it was placed into the bacterial solution for 7 days, the bacterial concentration was increased from 6.93 ± 0.28 log (CFU/ml) (2-days exposure) to 7.91 ± 0.16 log (CFU/ml) (7-days exposure), demonstrating that the 304SS did not show any inhibition or antibacterial performance against *S. aureus*. In contrast, the number of adhered bacteria on the 304CuSS showed much less increase from 3.28 ± 0.13 log (CFU/ml) (2-days exposure) to 4.39 ± 0.12 log (CFU/ml) (7-days exposure), showing that the 304CuSS exhibited much better antibacterial ability compared to the 304SS when exposed to the bacterial solution under the same time period. Further observation also clearly demonstrated that the 304CuSS effectively inhibited the biofilm formation on its surface, hence lowered the risk of bacterial contamination. The ability of this antibacterial stainless steel to kill both *E. coli* and *S. aureus* in the aqueous environment in a shorter period of time was shown by a previous paper [34], which confirmed that the 304CuSS can also kill planktonic bacterial cells and inhibit the biofilm formation or succession on its surface.

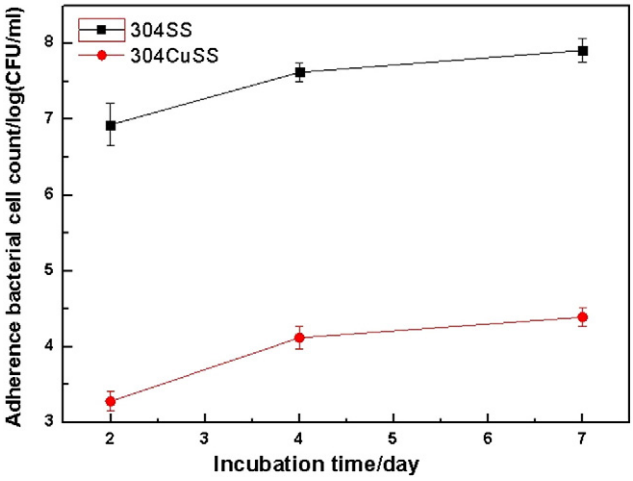

Fig. 2. Variations in the number of adhered bacteria (log_{10} CFU/ml ± SD) after contacting with stainless steel samples for different time points (days).

3.2. Biofilm formation

The attachment of sessile bacterial cells from the solution to the metal surface is a significant step in the biofilm formation process. Fig. 3 shows a series of SEM images of samples after exposure to the LB medium with *S. aureus* from 2 days to 7 days, indicating a dynamic process of the biofilm formation and succession on the stainless steel surfaces.

2 days exposure: Sessile bacteria on the 304SS surface were as shown in Fig. 3a, after 2 days exposure, a large quantity of sessile bacterial

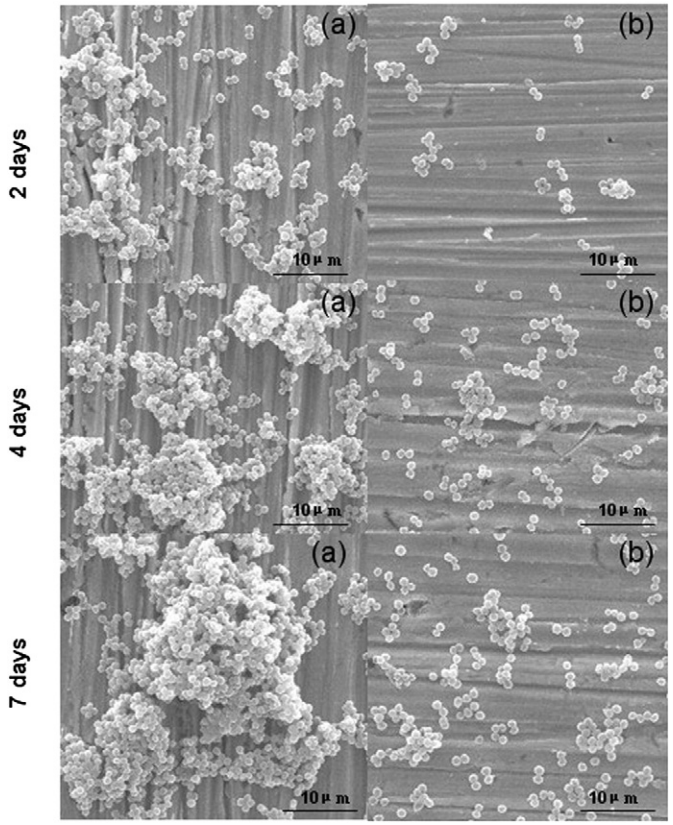

Fig. 3. Variations of bacterial morphologies after samples of 304SS (a) and 304CuSS (b) were exposed to *S. aureus, and the images were taken* for different time points from day 2 to day 7.

creatures were observed from planktonic bacterial cells growing into clusters. Contrary to this, on the 304CuSS surface, only a small quantity of planktonic bacteria appeared as shown in Fig. 3b (top).

7 days exposure: Island-like biofilms gradually adhered to the 304SS and there was a remarkable increase in both of the thickness and sizes as shown in Fig. 3a (lower), however, in contrast, the morphology and structure of the biofilms on the 304CuSS were not much changed upon such prolonged exposure (Fig. 3b, lower).

Previous work showed that the volume of bacterial biofilms contains a higher composition of glycocalyx matrix (75–95%) than those of bacterial cells (5–25%), which may be concentrated in either of the lower or the upper section of the biofilm [42]. Moreover, Marshall et al. [43] reported that biofilm development and succession on solid surfaces are ultimately dependent upon the population growth of the initial surface-colonization and the production of extracellular polymers by the colonized organisms. This might explain why the adhesion capability of *S. aureus* to the 304SS was stronger compared to that of the 304CuSS, i.e., the sessile bacterial cells were easier attached to the 304SS than those attached to the 304CuSS in which the released Cu^{2+} ions inhibited the biofilm formation [36].

3.3. FTIR analysis

FTIR spectra of bacterial cells on the surface of stainless steels can be used to analyze the chemical interactions of molecules on the cell wall, the membrane and the cytoplasm of the bacteria. Structural changes in the biofilms after 7 days of contact in the present study were assessed by FTIR, as shown in Fig. 4. Notably, bacterial cell walls contain similar types of functional groups as those in EPS [33], which formed the first barrier that comes into direct contact or interact with metals in the aqueous environment, in order to protect the interior microbial cells [44]. The adhesion of EPS films to metal surfaces is partially facilitated and affected by the interactions of metal ions with the functional groups of the bacterial polymers [45].

Based on these antibacterial mechanisms and the FTIR spectrum, the bands appearing at 3430, 2922, 1654 and 1077 cm^{-1} for 304CuSS were assigned to be the N–H of amide in proteins, C–H of CH_2 in fatty acids, Amide I in proteins and CH–OH bending, respectively. The key indication is that all these peaks were decreased sharply when they were in comparison to those of the 304SS [46]. These are obvious indications of the dynamic process of the bacterial apoptosis. The absorption peaks at 1412 and 1254 cm^{-1} for both samples are characteristics of C–O–C stretching of the glucosamine residue and N–H deformation [47,48], which were not changed with an increase of the time point. The outer membrane of *S. aureus* is composed of lipoteichoic acids, phospholipids and proteins [49,50], and its FTIR spectrum was quite complex and consisted of some of the broad bands that arise from the superimposing the absorptions from various macromolecules [46]. After contacting 7 days with the 304CuSS, the bacterial biological system was severely damaged according to the rapid variations of N–H of the amide in proteins, C–H of CH_2 in fatty acids, Amide I in proteins and CH–OH bending peaks. A strong decrease in the number of metabolic active cells after incubation with the 304CuSS also indicated a possible mechanism against *S. aureus*, which is related to the alteration of metabolic activity leading to protein damage. This showed a reduction in positive cells, and thus the biofilms were clearly destroyed at the same time. This phenomenon corresponds to the change in bacterial adhesion with increase of the time (Fig. 2).

3.4. Cu^{2+} ions dissolution

The antibacterial ability of the 304CuSS is reflected by the Cu ion dissolution from the steel matrix [36,51]. Fig. 5 shows the variation of Cu^{2+} ions dissolved from the 304CuSS during the exposure period. The dissolution of Cu ions from the 304CuSS varied from 3.1 to 4.5 ppm in the ICP tests which was far more than 1.5 ppm that was enough to kill bacteria within 7 h [35]. It can be concluded that the concentration of Cu^{2+} ions gradually increased with exposure time in the bacterial solution because the Cu-rich phases were uniformly and diffusively precipitated in the steel matrix [37]. Once the Cu-rich phases on the steel surface became depleted, the requisite ones continue to be supplied from the inside steel, which continuously inhibits the formation and succession of the biofilms. In addition, the amount of Cu^{2+} ion dissolution in later day points becomes lower and the overall amount of Cu ions as well as bacterial holding of the Cu ion concentration was well below the required minimum limitation standard issued by WHO [52], and thus guarantees safe usage or biologically safe applications of the 304CuSS.

In light of the finding that metal adsorption behavior is heavily dependent on the properties of bacterial cell functional groups, the metal-EPS binding mechanisms may be well extended to explain metal and the bacterial cell interactions. The Cu^{2+} ion demonstrated a high adsorption potential on microbial EPS, which might be associated with its size and charge density [53]. In general, the Cu^{2+} ion with a strong binding affinity is preferentially adsorbed over other species in either single or multi-metallic system, while its adsorption performance is less affected by the presence of other metals or metal elements [54]. The FTIR results suggested that Cu^{2+} ion can form complexes with carboxyl functional group of bacterial EPS, shown clearly through the changes in peak intensity of FTIR spectral band at both 1412 cm^{-1} (associated with the stretching vibration of C═O bond from a carboxylic

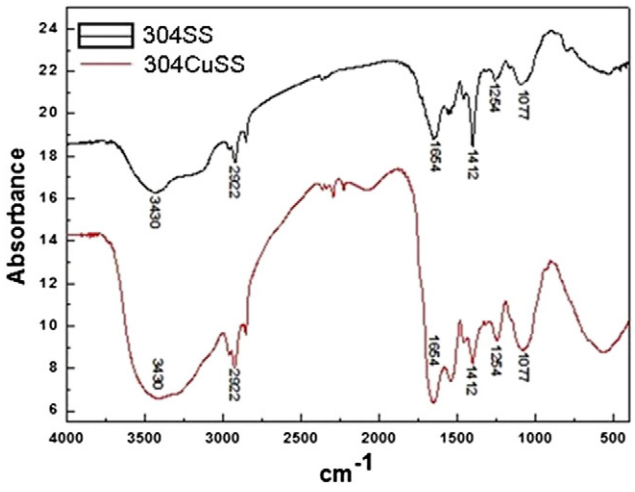

Fig. 4. FTIR spectra of bacterial biofilms on sample surfaces after 7 days exposure on steel surfaces.

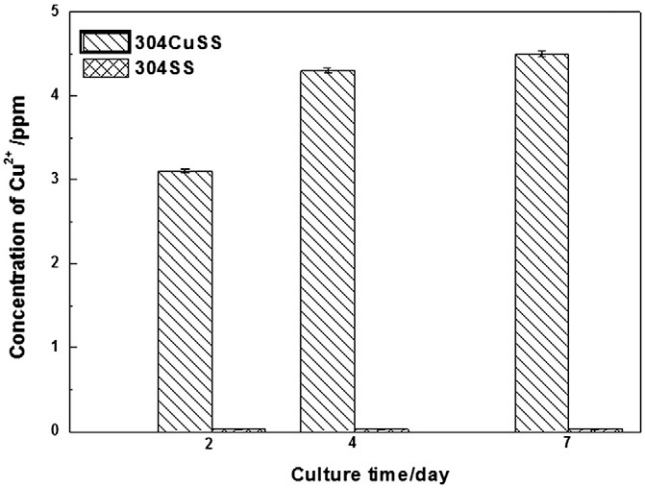

Fig. 5. Dissolution of Cu^{2+} ions from 304CuSS exposure to *S. aureus* for different time points.

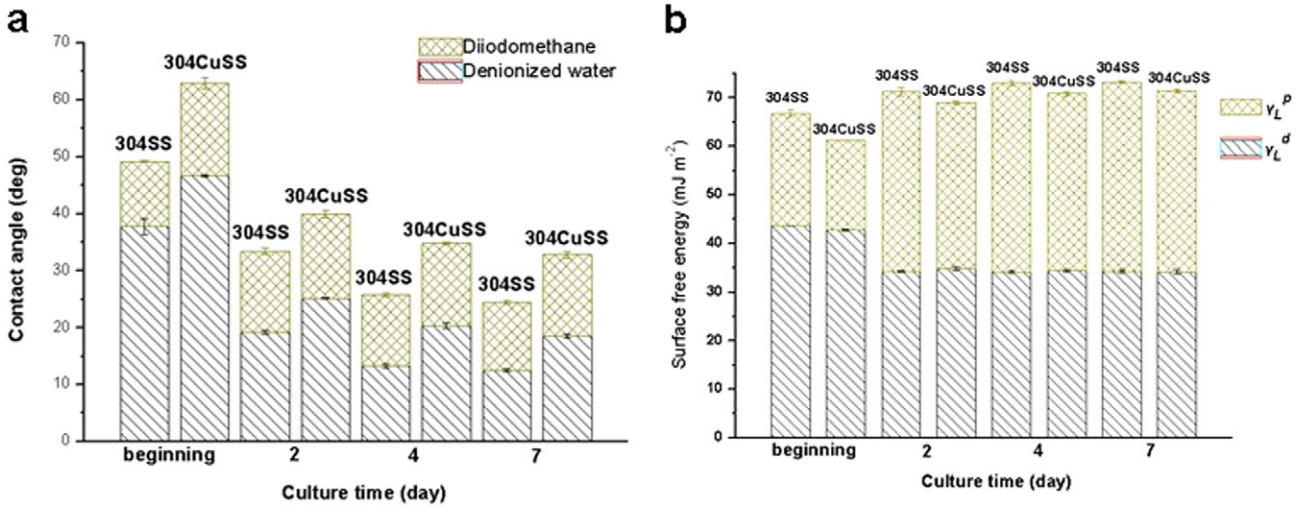

Fig. 6. Surface analysis of the stainless steel samples after exposure to *S. aureus* for different time points, (a) showed the level of contact angles, and (b) the surface free energy (SFE).

group and deformation vibration of –OH from alcohol and phenol groups), as well as the 1077 cm^{-1} (attributed to the stretching vibration of –OH group) [55].

The hypothesis for the Cu^{2+} ion to produce a functional binding in the killing process of bacteria might be to inhibit the bacterial EPS growth at the first stage, and then was followed by penetrating into the EPS and the cell membrane wall to lead to the death of bacteria by altering its protein properties.

3.5. Surface properties

Surface contact angle is an important factor for the attachment and growth of bacteria on material surfaces [39]. Based on Eq. (1), the γ_S can be obtained by determining both unknown γ_S^d and γ_S^p from the results of the contact angles, and using the γ_L^d and γ_L^p for the two probing liquids as 21.8 and 51.0 mJ m^{-2} for the deionized water, and 48.5 and 2.3 mJ m^{-2} for the diiodomethane, respectively [56]. The original roughness of the surfaces on the 304CuSS was 42 ± 2 μm and the 304SS was 46 ± 1 μm provided by the Institute of Metal Research (IMR), which was incapable of affecting the testing results of the contact angle and surface free energy. As shown in Fig. 6 the contact angles were measured in order to determine the physicochemical properties of the stainless steels affected by Cu^{2+} ion after exposures to *S. aureus* for different time points.

As shown in Fig. 6, the contact angles of the probe liquids on the surface of stainless steels decreased while the surface free energy (SFE) increased after contacting with bacteria for different time periods. After stainless steel interaction with the bacterial solution for 2 days, there was only a small difference of SFE between the two steels, and the mean value of SFE (71.3 mJ m^{-2}) for the 304SS was slightly higher than that for the 304CuSS (69.0 mJ m^{-2}). With the increase of the time points, the mean values of SFE for both steels increased, demonstrating that the SFE on the 304SS was always slightly higher than that of the 304CuSS, even for a relatively longer period of exposure to *S. aureus*. This factor was considered as an attribution to the subtle change in the physical and chemical properties on the steel surfaces after contacting with bacteria. In the biofilm formation process, it was observed that some trace organics were firstly adsorbed onto the surface to form a conditioning layer, which directly led to the SFE alternation. And the results also showed that the bacteria was more easily adsorbed onto the surface of the 304SS than that of the 304CuSS, which was in coherence with reports by Hamaza et al. [57] and Milne et al. [58], which demonstrated that when the surface energy becomes lower, bacterial adhesion turns to be weaker. Tang et al. [59] also verified that the changes of contact angle and SFE were attributed to the changes in surface functionalities of stainless steel after thermal treatment. Once the biofilms are formed on sample surfaces, they do not easily detach. Other works found that the amount and the structure of adsorbed proteins produced impact on the adhesion nature [60] and

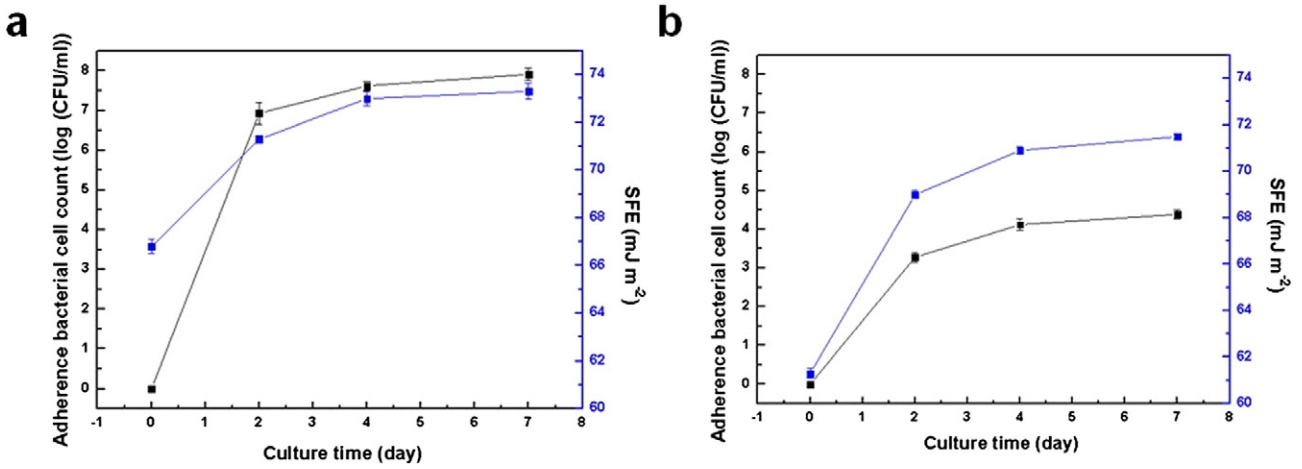

Fig. 7. Variations of SFE and the number of colonies adhesive to 304SS (a) and 304CuSS (b) for different time points.

the surface energy of the substrate [61], corresponding to the variations of FTIR spectra on the 304CuSS after 7 days of exposure to *S. aureus*. The excellent antibacterial capabilities of the 304CuSS allowed the material to inhibit the formation and propagation of biofilm on its surface, which is in an agreement with the variations of morphologies observed by SEM (Fig. 2).

Furthermore, a marked increase between the adhesive bacterial colonies and SFE towards different time points was also observed, as shown in Fig. 7. With increase of time points, both SFE and bacterial colonies increased i.e., the more bacteria are absorbed to the steels, the more increase in SFE appeared on the substrate. These results indicate that after interaction with *S. aureus* over a prolonged period of time, the risk of bacterial contamination on the surface of the 304SS is obviously higher than that of the 304CuSS.

4. Conclusions

The antibacterial/anti-biofilm ability of the 304CuSS exposed to the culture with *S. aureus* was investigated in this research in a comparison of the 304SS. It was found that when the exposure time points were increased, the antibacterial rate of the 304CuSS against adherence bacterial cells could reach above 99.9% in comparison with the 304SS. Furthermore, the thickness and size of the biofilms on the 304SS were markedly increased, which obviously showed an accelerated biocontamination in contrast with the 304CuSS. The bacteria adhesion ability on the 304SS was greater compared to that of the 304CuSS. Furthermore, these may further promote bacteria cell death, followed by significant inhibition of the EPS formation and succession of the biofilms on the 304CuSS surface. Especially, the concentration or amount of Cu^{2+} ion dissolution will not put human health at risk. This may support a new development trend by providing better or more efficient sanitary materials and facilities for innovative practices in the healthcare and food processing industries.

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