Effect of Heat Treatment on the Antibacterial Properties of 3Cr13MoCu Martensitic Stainless Steel (Postprint)

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Abstract

The influence of aging treatment on the antibacterial properties of 3Cr13MoCu martensitic stainless steel was investigated by means of antibacterial performance testing, hardness testing, transmission electron microscopy (TEM) observation, confocal laser scanning microscopy (CLSM) observation, and scanning electron microscopy (SEM) observation. The results indicate that with increasing aging temperature, the Cu-rich phase in 3Cr13MoCu martensitic stainless steel continuously grows, the bactericidal rate against *Staphylococcus aureus* continuously improves, but its hardness decreases rapidly. However, when aging treatment is performed at 500 °C, extending the aging time to 10–14 h leads to a continuous increase in the content of Cu-rich phase in the steel, and both its antibacterial properties and hardness continuously improve. Based on the antibacterial performance and hardness test results, the optimized heat treatment regime for 3Cr13MoCu stainless steel is determined as: solution treatment at 1080 °C for 30 min, water cooling + aging at 500 °C for 10–14 h, air cooling. Under this antibacterial heat treatment process, 3Cr13MoCu stainless steel exhibits excellent antibacterial performance, effectively killing planktonic bacteria while also inhibiting the formation of bacterial biofilms on the surface.

Full Text

Effect of Heat Treatment on Antibacterial Performance of 3Cr13MoCu Martensitic Stainless Steel

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Abstract

The effect of aging treatment on the antibacterial performance of 3Cr13MoCu martensitic stainless steel was investigated using antibacterial testing, Vickers hardness measurement, TEM observation, confocal laser scanning microscopy (CLSM), and SEM observations. The results showed that as aging temperature increased, Cu-rich phases in the 3Cr13MoCu martensitic stainless steel continued to grow, leading to progressively higher bactericidal rates against *Staphylococcus aureus*, but causing a rapid decline in hardness. However, when aging was performed at 500 °C, extending the aging time to 10–14 h increased the content of Cu-rich phases in the steel, resulting in simultaneous improvement of both antibacterial performance and hardness. Based on comprehensive evaluation of antibacterial and hardness test results, the optimal heat treatment regime for 3Cr13MoCu stainless steel was determined to be: solution treatment at 1080 °C for 30 min (water quenching) + aging at 500 °C for 10–14 h (air cooling). Under this optimized heat treatment, 3Cr13MoCu stainless steel exhibited excellent antibacterial performance, effectively killing planktonic bacteria while simultaneously inhibiting bacterial biofilm formation on its surface.

Keywords: Cu-bearing martensitic stainless steel, antibacterial performance, hardness, biofilm

Introduction

3Cr13Mo-type martensitic stainless steel exhibits high strength, hardness, and good corrosion resistance, making it particularly suitable for manufacturing medical instruments such as surgical scissors, bone rongeurs, scalpels, and dental curettes. However, in clinical practice, medical devices often become reservoirs for bacterial colonization and proliferation. Although all surgical instruments undergo rigorous sterilization before use, this cannot completely eliminate the possibility of patient infection, especially during prolonged surgical procedures where bacterial growth on instruments is inevitable. For military or civilian medical facilities such as field hospitals where effective disinfection measures cannot be guaranteed, the risk of bacterial infection is further amplified. According to data from the World Health Organization' s *Practical Handbook for Prevention of Hospital-Acquired Infections*, more than 14 million people worldwide suffer from nosocomial infections daily, with 60% of bacterial infections associated with medical devices. Moreover, material surfaces readily accumulate large numbers of bacteria that form biofilms, which exhibit extremely strong drug resistance and immune evasion capabilities, making them difficult to completely eradicate.

Copper ions possess potent bactericidal properties. Nan et al. found that when copper ions contact bacteria, they disrupt bacterial cell walls and membranes,

leading to leakage of cellular contents and protein coagulation. Therefore, the core innovative concept of Cu-bearing metallic materials involves adding a certain amount of Cu to conventional medical stainless steels and subjecting them to appropriate heat treatment to impart excellent antibacterial functionality to the originally inert material, thereby inhibiting bacterial biofilm formation on stainless steel surfaces. In the late 1990s, Nisshin Steel of Japan first developed three series of Cu-bearing antibacterial stainless steels (NSSAM1, NSSAM, and NSS3) with good manufacturability and antibacterial performance, demonstrating strong bactericidal effects against common bacteria including *E. coli*, *S. aureus*, *Salmonella*, and *Pseudomonas aeruginosa*. Research in this field started later in China; Yang et al. conducted early studies on Cu-bearing antibacterial stainless steels and found that both ferritic and austenitic Cu-bearing stainless steels exhibited good antibacterial effects after aging treatment, achieving bactericidal rates exceeding 90% against common bacteria such as *E. coli* and *S. aureus*. This research team further developed novel biological functions for antibacterial metals, discovering that this type of material (Cu-bearing 316L stainless steel) could inhibit proliferation and migration of arterial smooth muscle cells, significantly reduce platelet adhesion, prolong coagulation time, and markedly promote proliferation and migration of vascular endothelial cells, thereby accelerating the reconstruction process of stent-implanted damaged blood vessels and reducing the tendency for in-stent restenosis. Ren Ling investigated the antibacterial performance of Cu-modified 317L stainless steel, demonstrating that with appropriate Cu addition and heat treatment, the material exhibited excellent antibacterial performance, capable of not only killing planktonic bacteria but also effectively inhibiting bacterial biofilm formation on the material surface, thereby alleviating bacterial infections caused by biofilms.

The heat treatment process for martensitic antibacterial stainless steel includes solution treatment and aging treatment. Solution treatment aims to fully dissolve Cu into -Fe, followed by rapid cooling to obtain an -Fe solid solution with supersaturated Cu atoms. During subsequent aging treatment, supersaturated Cu precipitates from the matrix to form -Cu phases, and the excellent antibacterial performance of Cu-bearing stainless steel is attributed to the extensive, uniformly dispersed precipitation of -Cu phases. Our previous research indicated that during aging of Cu-bearing martensitic stainless steel, lower aging temperatures resulted in insufficient precipitation of Cu-rich antibacterial phases that could not meet antibacterial performance requirements, whereas high-temperature aging, despite providing good antibacterial performance, caused rapid hardness decline due to high-temperature recovery effects, compromising the high strength and hardness that are practically valuable for martensitic stainless steel. Therefore, the key technical challenge of this research is how to maintain high hardness while achieving excellent antibacterial performance in martensitic antibacterial stainless steel. Selecting appropriate heat treatment processes to ensure this type of antibacterial stainless steel possesses both excellent antibacterial performance and good mechanical properties is of significant importance.

This work aims to explore the effects of different antibacterial heat treatment processes on the antibacterial and mechanical properties of 3Cr13MoCu-type Cu-bearing martensitic stainless steel, thereby obtaining an appropriate heat treatment regime that enables the steel to possess both excellent antibacterial performance and mechanical properties, providing theoretical guidance for applications of 3Cr13MoCu martensitic antibacterial stainless steel in relevant fields.

Experimental Methods

The experimental 3Cr13MoCu stainless steel was melted in a 25 kg vacuum induction furnace and cast into ingots. The ingots were forged at 1100 °C into 16 mm diameter bars for experimental use. The chemical composition of the experimental steel (mass fraction, %) was: Cr 13.76, Mo 0.79, Cu 3.66, C 0.32, Si 0.06, Mn <0.05, S 0.004, P 0.008, with Fe as the balance.

Antibacterial performance testing was conducted using the agar plate method (GB4789.2-94) with *Staphylococcus aureus* (ATCC25923) purchased from Guangzhou General Hospital. Test strains were taken from logarithmic-phase slant cultures. A standard bacterial suspension of 10 CFU/mL was co-cultured with 3Cr13MoCu stainless steel and control stainless steel (3Cr13Mo) at 37 °C for 24 h. The bacteria treated by these samples were then diluted to 10^3 CFU/mL and cultured on agar at the same temperature and humidity for 24 h, after which colony counts were determined from the petri dishes. The bactericidal rate calculation formula was as follows: where R is the bactericidal rate of the antibacterial stainless steel, C is the bacterial count on the control stainless steel, and A is the bacterial count on the 3Cr13MoCu stainless steel.

TEM samples were sectioned from specimens aged at 600 °C and 800 °C for 6 h. Sample preparation proceeded as follows: samples were ground with water-abrasive paper from 240 to 2000 grit to a thickness of 50 µm, then punched into 3 mm diameter discs and further ground until the disc surfaces were smooth. Finally, samples were dimpled to 10 µm thickness and ion-milled until micropores appeared in the central thin region. A JEM2010FX transmission electron microscope was used to observe the morphology and distribution of Cu-rich phases in the samples. Due to the small size and low contrast of Cu-rich phases, their crystal structure could not be readily analyzed by TEM diffraction techniques. Therefore, an energy-dispersive spectrometer (EDS) configured on the TEM was used for compositional analysis of Cu-rich phases at an operating voltage of 120 kV. Hardness testing was performed on a 401MVD Vickers hardness tester with a load of 4.903 N and loading time of 15 s.

To observe bacterial biofilm attachment on sample surfaces, DAPI (4' ,6-diamidino-2-phenylindole) staining was performed on antibacterial stainless steel and control stainless steel samples after co-culture with bacteria, followed by CLSM observation. DAPI rapidly penetrates bacterial cell membranes and binds to double-stranded DNA molecules, with a minimum absorption wavelength of 358 nm and maximum emission wavelength of 461 nm, producing

blue fluorescence. Sample preparation was as follows: antibacterial stainless steel and control stainless steel samples were placed in 24-well plates, and 1 mL of *S. aureus* suspension at 10 CFU/mL was added to each well and cultured at 37 °C for 24 h. In a darkroom, 1 µL of dissolved DAPI dye was added to achieve a concentration of 1 µg/mL. After staining for 15 min, samples were removed from the 24-well plates and rinsed three times with PBS buffer (NaCl 8 g/L, KCl 0.2 g/L, Na HPO 1.56 g/L, KH PO 0.2 g/L), then dried in the dark at room temperature before CLSM observation.

An S-3400N scanning electron microscope (SEM) was used to observe bacterial morphology and characteristics on sample surfaces after culture. Sample preparation was as follows: antibacterial stainless steel samples co-cultured with bacteria for 24 h were fixed with 4% glutaraldehyde at room temperature for 4 h, then sequentially dehydrated with 25%, 50%, 75%, and 100% ethanol aqueous solutions and dried at room temperature for 24 h. All experimental samples were gold-sputtered before observation.

2.1 Effect of Aging Temperature on 3Cr13MoCu Stainless Steel Properties

The bactericidal effects of 3Cr13MoCu stainless steel against *S. aureus* after solution treatment at 1080 °C for 30 min (water cooling) followed by aging at 500, 600, 700, and 800 °C for 6 h (air cooling) are shown in [Figure 1: see original paper]. The figure reveals that after 24 h of co-culture with ordinary 3Cr13Mo stainless steel, the culture medium exhibited extensive bacterial colony growth ([Figure 1a: see original paper]). Due to the bactericidal action of 3Cr13MoCu stainless steel, bacterial colony numbers decreased significantly after 24 h of co-culture ([Figure 1b: see original paper]). As aging temperature increased, the number of bacterial colonies growing on the culture medium after co-culture with 3Cr13MoCu stainless steel continued to decline ([FIGURE:1c–f]). Statistical results of bactericidal rates are presented in [Figure 2: see original paper]. The data show that 3Cr13MoCu stainless steel subjected only to solution treatment exhibited a low bactericidal rate of only 59%, indicating insufficient antibacterial activity. After aging treatment, the antibacterial performance improved significantly, reaching 74% after aging at 500 °C. When aged above 600 °C, the bactericidal rate exceeded 90%, demonstrating strong antibacterial performance.

Hardness test results are shown in [Figure 3: see original paper]. The data indicate that 3Cr13MoCu stainless steel after solution treatment alone had relatively high hardness, with a Vickers hardness of 542 HV. After aging at 500 °C for 6 h, hardness reached its peak value of 557 HV. However, with further increases in aging temperature, hardness began to decline rapidly. After aging at 600 °C, hardness decreased to 325 HV. When aging temperature increased above 700 °C, hardness dropped below 300 HV.

Ren Ling' s research on Cu-bearing antibacterial stainless steel demonstrated

that the Fe/Cu microcell effect formed between precipitated Cu-rich phases and the matrix during aging promotes copper ion dissolution. Copper ions dissolved from the material surface destroy bacterial cell membranes and walls upon contact, ultimately causing coagulation of cellular proteins and bacterial death. Therefore, the presence and content of Cu-rich precipitates determine the antibacterial performance of Cu-bearing stainless steel.

According to solid-state transformation theory, precipitation in alloys is a diffusion-nucleation-growth type transformation with compositional changes. Viswanathan et al. studied the precipitation process of Cu-rich phases in iron alloys, showing that Cu-rich phase precipitation has no incubation period, meaning nucleation occurs without a free energy barrier and the activation energy for precipitation is very low. The growth of Cu-rich phases in Cu-bearing antibacterial stainless steel primarily depends on diffusion of Cu atoms in steel. Since Cu atoms exist as substitutional atoms in steel, their diffusion occurs near vacancies. According to diffusion theory, the diffusion coefficient of Cu atoms largely depends on vacancy concentration at different temperatures. After high-temperature solution treatment at 1080 °C and rapid cooling, 3Cr13MoCu stainless steel obtains a supersaturated solid solution while simultaneously generating a substantial number of supersaturated vacancies. These supersaturated vacancies greatly accelerate Cu atom diffusion during aging treatment, providing favorable nucleation sites for Cu-rich precipitate formation. However, after solution treatment, most Cu remains dissolved in the stainless steel matrix and cannot contact bacteria, resulting in low bactericidal rates. After aging treatment, antibacterial performance improves rapidly due to precipitation of Cu-rich phases.

Hornbogen et al. proposed that during thermal aging of Cu-bearing steel, initially formed Cu-rich segregation zones are metastable bcc-structured phases maintaining coherency with the ferrite matrix. These Cu-rich zones grow with increasing aging temperature, and when reaching a critical size, transform into fcc-structured -Cu precipitate particles. Once fcc precipitate particles form, they grow into spherical particles approximately 30 nm in diameter, then develop into rod-like morphologies. According to general precipitation strengthening principles, the transitional structure of Cu-rich segregation zones maintaining coherency with the matrix is characteristic of peak-aged structures. This suggests that at 500 °C, Cu-rich precipitates remain relatively small and coherent with the matrix, elevating stainless steel hardness to its peak. With increasing aging temperature, Cu-rich phases gradually grow while tempering softening begins. Consequently, as aging temperature increases, dislocation density and other defects gradually decrease, reducing strengthening and hardness effects. This manifests as gradually improving antibacterial performance but rapidly declining hardness in 3Cr13MoCu stainless steel.

To further investigate precipitation behavior of Cu-rich phases in 3Cr13MoCu stainless steel, TEM observations were conducted on precipitate morphology. As shown in [Figure 4a: see original paper] and [Figure 4b: see original paper], Cu-

rich phases readily precipitate at grain boundaries and other defect sites such as quenched vacancies and dislocations. At 600 °C aging, Cu-rich precipitates had grown into spherical particles approximately 30 nm in diameter ([Figure 4a: see original paper]). When aging temperature increased to 800 °C, spherical Cu-rich phases evolved into rod-like morphologies with sizes increasing to approximately 300 nm ([Figure 4b: see original paper]), consistent with the previous hypothesis that Cu-rich segregation zones transform into precipitate particles above 600 °C, weakening their pinning effect on dislocations. Therefore, as aging temperature increases, antibacterial performance of martensitic antibacterial stainless steel continuously improves while hardness declines rapidly.

EDS analysis results for Cu-rich phases and the stainless steel matrix are shown in [FIGURE:4c–e]. At 600 °C aging, Cu content in Cu-rich phases was 10.5%, increasing to 40.5% at 800 °C aging, both significantly higher than the 2.0% Cu content in the 3Cr13MoCu stainless steel matrix. However, this does not necessarily indicate that Cu content in Cu-rich phases increases with aging temperature, because during EDS analysis, the converged beam spot area is larger than the Cu-rich phase. When precipitates are smaller, TEM electrons more easily penetrate the Cu-rich phase and strike the matrix, meaning the actual Cu content in Cu-rich phases should be greater than measured values when precipitates are small.

2.2 Effect of Aging Time on 3Cr13MoCu Stainless Steel Properties

Based on antibacterial performance and hardness test results for 3Cr13MoCu stainless steel aged at different temperatures, the steel exhibited good antibacterial performance when aged above 600 °C but suffered rapid hardness decline, losing its practical value of high strength and hardness. To enable the experimental steel to maintain high mechanical performance while possessing excellent antibacterial properties, 3Cr13MoCu stainless steel was aged at 500 °C for various durations. The bactericidal effects against *S. aureus* are shown in [Figure 5: see original paper]. The results demonstrate that as aging time extended, bacterial colony numbers on culture media after co-culture with 3Cr13MoCu stainless steel continuously decreased. Statistical analysis of bactericidal rates revealed that after 10 h aging, the steel achieved a 94% bactericidal rate against *S. aureus*, exceeding 99% after 14 h aging, demonstrating strong antibacterial performance. Hardness test results showed that hardness continuously increased with prolonged aging time, reaching 600 HV after 10 h aging and 620 HV after 14 h aging.

The stress field caused by mismatch between Cu-rich precipitates and the matrix is the primary strengthening source for 3Cr13MoCu stainless steel during 500 °C aging. The effect of Cu-rich precipitates on hardness during aging can be explained by Mott-Nabarro theory, where μ is the shear modulus of precipitate particles, r is precipitate particle radius, f is precipitate volume fraction, is the mismatch function, and b is the magnitude of the Burgers vector for dislocations. Liu Yongqian' s research on 3Cr13-type Cu-bearing stainless steel indicated that

during low-to-medium temperature aging, the formation and growth rate of Cu atom enrichment zones increased rapidly with aging time, causing rapid hardness increase in Cu-bearing stainless steel. When the precipitation amount of Cu-rich phases reached a certain level, the volume fraction of Cu-rich segregation zones ceased to change while the growth rate of Cu-rich phases gradually slowed, leading to gradual stabilization of hardness changes. This suggests that during 500 °C aging of 3Cr13MoCu stainless steel, both the size and volume fraction of Cu-rich precipitates increased with aging time, and the interaction between Cu-rich phases and dislocations significantly increased hardness. After 10 h aging, the steel not only achieved over 94% bactericidal rate but also maintained high hardness. Therefore, the optimal heat treatment regime for 3Cr13MoCu stainless steel was determined to be: solution treatment at 1080 °C for 30 min (water cooling) + aging at 500 °C for 10–14 h (air cooling).

2.3 Biofilm Observation

To analyze bacterial attachment on 3Cr13MoCu stainless steel surfaces after optimal heat treatment, DAPI staining was performed on experimental steel samples (aged at 500 °C for 10 h) after 24 h co-culture with *S. aureus*, followed by CLSM observation. As shown in [Figure 6: see original paper], control stainless steel without antibacterial action exhibited massive bacterial attachment after 24 h co-culture, while samples co-cultured with 3Cr13MoCu stainless steel showed very sparse bacterial attachment. This result demonstrates that 3Cr13MoCu stainless steel can inhibit bacterial attachment on its surface, thereby preventing bacterial biofilm formation.

To further observe bacterial biofilm morphology on 3Cr13MoCu stainless steel surfaces, SEM observations were performed on samples after bacterial co-culture. As shown in [Figure 7: see original paper], control stainless steel surfaces were covered with numerous spherical *S. aureus* bacteria, with biofilm formation at some locations, while 3Cr13MoCu stainless steel surfaces showed very few bacteria, confirming that 3Cr13MoCu stainless steel can inhibit bacterial biofilm formation on its surface.

Bacterial biofilms are microbial community aggregates formed by single or multiple bacterial species adapting to natural environments, primarily composed of polysaccharide-protein complexes that form membrane-like structures irreversibly attached to lesion or device surfaces. Biofilm formation involves three main processes: cell adhesion, microbial colony generation, and extracellular polymeric matrix encapsulation. Bacteria adhered to material surfaces in biofilms exhibit extremely strong drug resistance and immune evasion, providing protection for embedded bacteria and creating a natural barrier against penetration of antibacterial drugs into bacterial cells. Antibacterial drugs cannot fundamentally resolve infections caused by bacterial biofilms, and treatment becomes increasingly difficult as bacterial drug resistance strengthens. Cu-bearing antibacterial stainless steel can dissolve a certain amount of copper ions in physiological environments. While killing planktonic bacteria

around the stainless steel, copper ions effectively reduce bacterial numbers on the surface and can even overflow from the interface between biofilm and material to directly kill bacteria within the biofilm, thereby inhibiting bacterial biofilm formation on the surface and effectively preventing bacterial infections. Since 3Cr13MoCu stainless steel after optimal antibacterial heat treatment exhibits not only excellent antibacterial performance and high hardness but also inhibits bacterial biofilm formation on its surface, this steel has broad application prospects in medical instruments, dining utensils, kitchenware, and other fields.

Conclusions

1. As aging temperature increases, Cu-rich phases in 3Cr13MoCu stainless steel continue to grow, leading to continuously improving antibacterial performance but progressively increasing softening and corresponding hardness decline.
2. During aging at 500 °C, as aging time extends, the precipitation of Cu-rich phases in 3Cr13MoCu stainless steel increases, resulting in simultaneous improvement of both antibacterial performance and hardness. Therefore, the optimal heat treatment regime for 3Cr13MoCu stainless steel is determined to be: solution treatment at 1080 °C for 30 min (water cooling) + aging at 500 °C for 10–14 h (air cooling).
3. Under the optimized antibacterial heat treatment condition, 3Cr13MoCu stainless steel exhibits excellent antibacterial performance, capable of killing planktonic bacteria while effectively inhibiting bacterial biofilm formation on its surface.

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Note: Figure translations are in progress. See original paper for figures.