High Nitrogen Stainless Steel as a Preferred Substratum for Bacteria and Other Microfouling Organisms

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High Nitrogen steels (HNS) have gained importance in recent years as construction materials. Their resistance to pitting corrosion makes them better candidates for industrial use. In the medical field, these materials are welcomed as their use can minimize the risk of nickel allergy. Nitrifying bacteria are a group of bacteria widely distributed in soils, freshwater, seawater *etc.* and since they derive their energy by oxidation of nitrogen compounds, they pose a threat to these materials. Experiments on the adhesion of nitrifying bacteria on HNS were carried out following coupon exposure method in the laboratory. *Nitrobacter winogradskyi* (nitrite oxidizing bacteria) and *Nitrosomonas europaea* (ammonia oxidizing bacteria) were used for the adhesion studies. In addition, adhesion of a heterotrophic strain, *Pseudomonas* sp. also was studied for comparison. Materials used for the experiment were AISI type 304 L, 304 LN, and high nitrogen stainless steels. The results showed that *Nitrobacter winogradskyi*, *Nitrosomonas europaea* and *Pseudomonas* sp., invariably, adhered more on the high nitrogen stainless steel coupons compared to the other two types of coupons tested. During the initial stages, *i.e.* on the first day, the trend was not so clear, but as the exposure period increased the pattern has clearly evolved. The area of adhesion showed a gradual decrease going from HNS, to 304 L through 304 LN coupons. The acidic metabolic products of nitrifying bacteria might interact with the surface of HNS, disturbing the equilibrium of ammonium ion formation, thereby leading to localized corrosion. Exposure to a natural freshwater pond showed a heavy microfouling load on HNS coupons. The consequences of preferential adhesion of bacteria and other microfouling organisms on the corrosion resistance of HNS are discussed. As a possible means to mitigate bacterial adhesion on HNS, silver containing high nitrogen stainless steel was tested. The results suggested a significant decrease in bacterial adhesion on silver containing HNS compared to 304 L and HNS.

KEY WORDS: high nitrogen stainless steels; bacterial adhesion; microbiologically influenced corrosion; nitrifying bacteria; microfouling; silver containing high nitrogen stainless steel.

1. Introduction

Nitrogen is a universal alloying element in steels. It has both detrimental (various embrittlement phenomena) and beneficial (creep resistance, solid solution hardening, grain growth *etc.*) effects.¹⁾ Steels containing more than 0.08 mass% nitrogen in ferritic matrix or 0.4 mass% nitrogen in an austenitic matrix are considered as high nitrogen stainless steels (HNS).²⁾ Recently these types of stainless steels are being advised for many commercial purposes, as they possess improved structural stability and corrosion resistance than other classes of steels.²⁾ Also, in some cases, human body in contact with nickel containing stainless steels develops a kind of hypersensitivity with symptoms of inflammation, eczema, irritation *etc.* This is called “nickel allergy” and it occurs in contact with/while wearing wrist-watches, jewelry, implant materials *etc.*³⁾ Nickel free stainless steel in which the element Nickel is replaced by nitrogen is a possible answer to this phenomenon. Mechanisms by which nitrogen as an alloying element increases the pitting corrosion resistance are reported such as consumption

of proton by nitrogen to form ammonium ion helping in passivating the pit nucleus^{4,5)}; nitrogen going into solution as nitrate ion thereby inhibiting pitting or segregation of nitrogen in the passive film playing a role in suppressing the dissolution of it.⁴⁾ In general, nitrogen compounds formed as a result of nitrogen in HNS are responsible for the corrosion resistance. As any other material, HNS also are prone to bacterial adhesion and colonization when they are introduced into a non-sterile environment. A group of bacteria of particular interest is nitrifying bacteria. They are present universally, in different environments such as soil, freshwater, seawater, mud layers, inside corroded bricks, and on concrete surfaces such as cooling towers *etc.* They are unicellular in nature with an ability to form micro colonies embedded in polysaccharide matrix or slime layer. Two physiological groups exist; the first one oxidizes ammonia while the second oxidizes nitrite.

Bacterial adhesion and consequent formation of biofilms on material surfaces can result in microbiologically influenced corrosion.^{6,7)} It is also known that elemental composition on the surfaces act as chemical cues by which bacter-

ial adhesion is facilitated or mitigated.⁸⁾ As the pitting corrosion resistance of HNS depends on the ammonia or nitrite formed, bacterial adhesion especially that of nitrifying bacteria poses threat to these advanced materials. Bacterial adhesion could be triggered by the presence of nitrogen compounds and utilization of ammonia/nitrite, might leave the surface prone to localized corrosion. Moreover, adhesion of bacteria onto HNS surfaces is detrimental from the point of view of bacterial infection as they are used as implant materials and also as a material to prevent nickel allergy. Therefore, adhesion of nitrifying bacteria over the HNS surface was studied keeping AISI type 304 L and 304 LN stainless steels as control. Adhesion of heterotrophic bacteria, *Pseudomonas* sp. was also studied for comparison. An attempt was also made to find out the mitigating effect if any on bacterial adhesion by silver containing HNS.

2. Materials and Methods

2.1. Materials Used and Experimental Coupons

AISI type 304 L, 304 LN, 0.4% HNS and silver containing HNS were the materials used for the present study. Composition of the High Nitrogen Steels used in the study is given in **Table 1**. Experimental coupons were prepared by cutting the above materials into 30×10 mm size. They were polished to 1000 grit finish by emery paper, degreased by ultrasonic cleaning in acetone, washed, dried and kept in a desiccator until the experiment. Just before the start of the experiment, coupons were sterilized with 70% ethanol and were dried under UV in a laminar flow sterile air chamber. Duplicate sets of the above materials were used for the experiment.

2.2. Bacterial Strains and Experimental Medium

Nitrobacter winogradskyi (IFO 14297), *Nitrosomonas europaea* (IFO 14298) and *Pseudomonas* sp. (isolated from an MIC failure case and maintained in JWRI, Osaka University) were used for the experiment. Details of the composition of the respective media used are given in **Table 2**. In the case of *Pseudomonas* sp. the medium was diluted to get a concentration of 0.1% (v/v). This was done to reduce the pace of growth of *Pseudomonas* sp. so that it is in par with the other two strains tested.

2.3. Experimental Methods

Coupon exposure studies were carried out to estimate the bacterial adhesion over the coupon surfaces as well as the changes in surface characteristics such as pitting. Two separate experiments were carried out in the laboratory *i.e.* to estimate the area of bacterial adhesion and pitting and the free corrosion potential fluctuation over a prolonged period of exposure to the respective bacteria in the respective media. In addition, field studies have also been carried out by exposing coupons in a freshwater pond located within the Osaka University campus.

2.3.1. Bacterial Adhesion Studies

Bacterial adhesion studies were done for a period of 16 d. Replacement of half the volume of the nutrient media was done after a week. Coupons polished and sterilized as described above were introduced aseptically into the medium inoculated with the respective bacteria. Different types of

Table 1. Chemical composition of HNS used in the study (mass %).

Alloying elements	HNS 0.4%	Silver free HNS	Silver containing HNS
C	0.02	0.054	0.055
Si	0.15	0.35	0.34
Mn	6.0	1.05	1.05
Ni	10.0	8.60	8.60
Cr	23.0	18.30	18.30
Mo	2.0	----	----
N	0.48	0.2016	0.2108
V	0.14	----	----
P	----	0.028	0.028
S		0.005	0.005
Al		0.001	0.001
O		0.0060	0.0058
Ag			0.099

Table 2. Chemical composition of different media used.

<i>Nitrosomonas europaea</i>	<i>Nitrobacter winogradskyi</i>	<i>Pseudomonas</i> sp.
Medium No.240	Medium No. 239	Nutrient broth (Difco)
(NH ₄) ₂ SO ₄0.5g	NaNO ₂1g	Peptone.....5g
NaCl.....0.3g	NaCl.....0.3g	Beef Extract...3g
K ₂ HPO ₄1g	K ₂ HPO ₄0.5g	Distilled Water...1L
MgSO ₄ ·7H ₂ O.....0.3g	MgSO ₄ ·7H ₂ O.....0.5g	pH7.0
FeSO ₄ ·7H ₂ O.....0.03g	MnSO ₄ ·nH ₂ O.....0.002g	
CaCO ₃7.5g	Fe ₂ (SO ₄) ₃0.005g	
Distilled Water....1L	Distilled Water1L	
pH8.0	pH7.5	

coupons were introduced in the same flask to keep the experimental conditions same for all of them. Temperature of incubation was 28°C and the experimental flasks were wobbled gently (85 rpm). Intermittent samplings were done after 1, 4, 8 and 16 d and the coupons were stained with 0.01% acridine orange, a fluorescent dye, and observed under an epifluorescence microscope. Images were taken randomly choosing 20 different fields using a CCD camera. Analysis was done on duplicate sets of coupons. Percentage area of bacterial adhesion was computed using image-processing software.

2.3.2. Corrosion Studies

Corrosion studies were carried out for 8 weeks. Coupons for corrosion potential measurement were also introduced in the same manner as for the bacterial adhesion studies and the electrical contact was established using metallic wires attached to them and sealed with silicon gum. While doing so, precautions were taken to avoid galvanic corrosion effects. Intermittent potential measurement (Ag/KCl elec-

trode; 3.33 mol/L KCl) was carried out using a potentiostat.

2.3.3. Surface Analysis

Surface of the coupons after exposure to the respective bacteria for 30 d was observed under a Scanning electron microscope for the presence of pits due to corrosion. Surface of the coupons exposed to sterile medium for the same number of days (medium control) were also observed for formation of pits in order to test the corrosion effect of the media used.

2.3.4. Field Study

Coupon exposure studies were carried out for a period of one month, in a fresh water pond of approximately 500 m² area located within the campus of Osaka University, Osaka, Japan. This is a perennial pond with an average depth of 3 m. The pond is fed by intermittent rainfall. Experimental coupons (in duplicates) were fixed in a coupon fixing assembly and were suspended from a platform at ~1 m depth. The coupons after retrieval at fixed intervals were brought to the laboratory in an immersed condition in the pond water and observed immediately under an environmental scanning electron microscope (NIKON ESEM-2700; serial number 210205). A point method,⁹⁾ wherein, dots at 1 mm² interval were put on a transparent sheet of the size of the images was laid over the images to calculate the area cover of the microfouling organisms.

2.3.5. Bacterial Adhesion Studies on Silver Containing HNS

Bacterial adhesion studies on silver containing stainless

steel coupons were carried out in a separate experiment following the coupon exposure method. HNS and silver containing HNS coupons were exposed to the bacteria (*Nitrobacter winogradskyi*, *Nitrosomonas europaea* and *Pseudomonas* sp.) for 16 d with intermittent sampling. The composition of the materials used is given in Table 1. Silver containing coupons were introduced in different flasks to avoid the effect of leached out silver ions on bacterial adhesion on other coupons. Coupon retrieval, staining and microscopic observation were the same as described earlier.

3. Results

3.1. Bacterial Adhesion Studies

The results showed that *Nitrobacter winogradskyi*, *Nitrosomonas europaea* and *Pseudomonas* sp., invariably, adhered more on the high nitrogen stainless steel coupons compared to the other two types of coupons tested. During the initial stages, i.e. on the first day, the trend was not so clear, but as the exposure period increased a clear pattern evolved. The area of adhesion (average) showed a gradual decrease going from HNS, to 304 L with 304 LN coupons showing a medium level of adhesion. The preference of HNS coupons for adhesion was more pronounced in the case of *Nitrobacter winogradskyi* than the other two types of bacteria, at an exposure time of 16 d. On HNS coupons, *Nitrobacter winogradskyi* showed an area cover of 69.88%, with 8.48% and 8.03% in 304 LN and 304 L, respectively. *Nitrosomonas europaea* and *Pseudomonas* sp. adhered to

Fig. 1. Bacteria seen adhered to HNS and 304 L coupons after 16 d of exposure to (a) *Nitrosomonas* sp., (b) *Nitrobacter* sp. and (c) *Pseudomonas* sp.

Fig. 2. Percentage area of adhesion of bacteria on different coupons as a function of exposure time.

42.08% and 43.43% of HNS; 22.48% and 22.16% of 304LN and 12.67% and 11.64% of 304L coupons, respectively (Figs. 1 and 2). The variation in the bacterial attachment by the end of the study *i.e.* after 16 d was statistically significant with HNS showing very high bacterial settlement compared to the rest of the materials (One-way ANOVA, $F=9.84$, $p<0.05$; $F=11.16$, $p<0.05$ and $F=3.01$, $p=0.1$, respectively for *Nitrobacter* sp., *Nitrosomonas* sp. and *Pseudomonas* sp.).

3.2. Corrosion Studies

Free corrosion potential fluctuated on prolonged exposure to different bacteria (Fig. 3). In all the cases, compared to sterile controls, the corrosion potential of coupons exposed to bacteria showed a shift towards more positive values. Among the 304L and HNS coupons exposed to the bacteria, HNS coupons showed more positive potential. Especially, in the case of exposure to *Nitrosomonas* sp., the ennoblement was significant as compared to the control material. However, none of the coupons attained a free corrosion potential, which could initiate pitting in them.

3.3. Surface Analysis

Results on the surface observation revealed that pitting corrosion was not significant on any of the experimental coupons. HNS coupons exposed to both sterile and non sterile media were devoid of severe pitting after 30 d exposure (Fig. 4).

Fig. 3. Variation in free corrosion potential as a function of exposure time (Con-Control; Expt-Experimental; A-*Nitrosomonas* sp., B-*Nitrobacter* sp., C-*Pseudomonas* sp.).

3.4. Field Study

Figure 5 shows the surfaces of coupons exposed to the freshwater pond for 30 d observed through ESEM. Compared to 304L stainless steel surfaces, microfouling load was more on HNS surfaces. The approximate percentage cover of 304L and HNS coupons estimated from ESEM images were 73.81% and 96.4% respectively. The microfouling community included microalgae commonly found in freshwater habitats such as *Navicula* sp., *Nitzschia* sp., *Acanthos* sp., *Acanthos* sp., *Pinnularia* sp. etc. A selection of species was noticed over the HNS coupons whereas on 304L SS various common microfouling algae were seen. Both HNS and 304L SS showed pitted surfaces after a period of one-month exposure.

3.5. Bacterial Adhesion Studies on Silver Containing HNS

Figure 6 shows the results of bacterial adhesion studies on silver containing HNS. Compared to HNS coupons, silver containing HNS coupons showed lesser area of bacterial adhesion. Unlike the bacterial adhesion studies carried out earlier to find out the difference in % area, this experiment showed a continuous increase in % area of adhesion as a function of exposure time in all the coupons tested.

Fig. 4. Environmental Scanning Electron microscope (ESEM) images of HNS coupon surfaces after an exposure period of 30 d to sterile media (A, B) and inoculated with *Nitrosomonas* sp. (C) and *Nitrobacter* sp. (D).

Fig. 5. Environmental Scanning Electron microscope (ESEM) images showing freshwater microorganisms attached to different coupons exposed to a freshwater pond for a period of one month.

Fig. 6. Percentage area of adhesion of bacteria on silver free (SFHNS) and silver containing (SCHNS) HNS coupons as a function of exposure time (A - *Nitrosomonas* sp., B - *Nitrobacter* sp., C - *Pseudomonas* sp.).

4. Discussion

Bacterial adhesion studies revealed that there is a preference of attachment by all three kinds of bacteria used in this study for HNS surfaces. Nitrifying bacteria derive their energy by oxidizing nitrogen compounds.¹⁰⁾ HNS surfaces immersed in aqueous medium provides a rich substratum for these type of microbes as there is production of ammonia and nitrites which are the essential compounds for the bacteria used in this study. In the case of *Pseudomonas* too, the percentage area of adhesion was more on HNS coupons compared to 304 L or 304 LN SS. According to Sreekumari *et al.*,¹¹⁾ elemental segregation could trigger bacterial adhesion. The grain boundary preference observed for bacterial attachment in this report might have been due to the elemental segregation on grain boundaries. Studies on the bacterial adhesion on sulfur enriched welds showed that it is a preferred site for bacterial adhesion¹²⁾ and is more susceptible to MIC.¹³⁾ Sulfur is an essential element for bacterial metabolism and would be acting as a chemical cue to attract bacteria. On the contrary, addition of toxic alloying elements such as copper or silver makes stainless steel antibacterial.^{14,15)} These reports and others¹⁶⁾ give evidence on the effect of elemental composition on bacterial adhesion to metal surfaces and MIC. In the present study the element nitrogen or its compounds might be playing the main role in bacterial adhesion *i.e.* on to the surfaces of HNS coupons. Other elements differing in composition among the experimental materials are not reported to have any affinity towards the experimental strains.

High nitrogen steels are manufactured with a view to improve pitting resistance. The mechanism by which the pitting resistance is attained is by preventing acidification and active dissolution of metals by ammonia formation.⁴⁾ This is established by the raising of pH due to NH_4^+ ions. Another mechanism proposed is that the corrosion resistance is established by the nitrite formed.⁴⁾ Yet another mechanism is that the presence of nitrogen in the passive film makes it less sensitive to pitting.⁴⁾ Whichever the said mechanism is acting, the colonization of bacteria, especially nitrifying bacteria leaves the surface devoid of the formed compounds, and thus, would be detrimental to the surfaces as far as corrosion resistance is concerned. Nitrifying bacteria form nitric acid as their metabolic products and once it is formed it would be breaking the balance in neutralization of acidic environment by ammonia. The free corrosion potential measurement results showed that bacterial adhesion had a direct impact on shifting the potential to more positive values. Compared to sterile controls, the coupons exposed to bacteria showed higher potential values. Also, HNS coupons showed a significant positive shift in their corrosion potential compared to the control material exposed to the same bacteria. This observation is important as far as the influence of bacterial adhesion on potential ennoblement is concerned. On HNS coupons, bacterial adhesion was more compared to 304 L SS. However, the corrosion potential measurement did not reveal a rise in potential, to a range, which would lead to pitting within the period of observation. SEM observations revealed occasional pit initiation on HNS coupons but were not severe.

In the case of field study, the surfaces of HNS were seen

pitted by 30 d. In the field the attachment was so severe compared to laboratory studies, which might have led to passive film dissolution, and pitting within 30 d. Bacterial adhesion or microfouling acts as the preliminary step in the initiation of biocorrosion or microbially influenced corrosion. Therefore, to have a heavy load of micro fouling is a detrimental factor. In the laboratory study, though pitting corrosion was not spotted by 30 d, in longer duration the heavy bacterial biofilm load would be triggering MIC. As nitrifying bacteria and the *Pseudomonas* sp. tested in the present study are exopolymer producers, the chances are more for the formation of oxygen concentration cells and consequent pitting.

High nitrogen stainless steels are also targeted as advanced materials, which could be used to combat nickel allergy. It is also advised as a good candidate as biomaterials. Considering the application of HNS as implants on human body, the bacterial adhesion leading to infection is a matter of concern. Corrosion resistance and replacement of nickel by nitrogen are two important aspects of preference of this material. Nitrogen acting as a positive factor for bacterial adhesion is of significance as per the results of this study. Bacterial adhesion, microfouling and the consequent corrosion might act as a hindrance to the material performance. Bacterial adhesion in general, and nitrifying bacteria in particular, makes the material susceptible to pitting corrosion by removal of nitrogen compounds on which the corrosion resistance property of HNS is based on. Bacterial colonization on HNS adversely affects its performance as biomaterials or in case of nickel allergy as it might lead to infections.

Antibacterial stainless steels are getting popular and are being used in hospital and domestic appliances, old age homes *etc.* Copper or silver is the main toxic elements used in manufacturing antibacterial stainless steels.^{14,15,17)} High nitrogen stainless steels containing silver as an alloying element, was thought of as a preventive measure to the problem of heavy bacterial adhesion to HNS surfaces. Bacterial adhesion was found to be significantly lower in the case of silver containing HNS compared to HNS suggesting a toxic effect on bacteria preventing them from fouling the surface. However, changes in material properties as well as corrosion resistance due to the addition of silver were not studied during the present study. Therefore, further study is essential in order to ascertain the material properties of the silver containing HNS before it can be used as HNS capable of abating bacterial colonization.

5. Conclusions

(1) Bacterial adhesion and microfouling studies on high nitrogen steels showed that they were the preferred substratum for adhesion compared to 304 L SS by both nitrifying bacteria and *Pseudomonas* sp.

(2) Free corrosion potential ennoblement was more in the case of coupons exposed to bacteria compared to bacteria free controls. Increase in potential was more pronounced in HNS coupons compared to 304 L SS suggesting an influence of bacterial adhesion on corrosion properties.

(3) In the field study, HNS coupons showed more microfouling growth with a selection of species, compared to the control 304 L coupons. Severe pitting was also noticed.

(4) High nitrogen stainless steels containing silver as an alloying element, showed lesser bacterial adhesion than its silver free counterpart.

(5) Further investigations on the material properties of silver containing HNS is necessary to ascertain its use to mitigate bacterial adhesion on HNS surfaces.

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