Biofilm formation by *Staphylococcus epidermidis* on nitrogen ion implanted CoCrMo alloy material

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Abstract: *Staphylococcus epidermidis* is the primary cause of medical device-related infections due to its adhesion and biofilm forming abilities on biomaterial surfaces. For this reason development of new materials and surfaces to prevent bacterial adhesion is inevitable. In this study, the adhesion of biofilm forming *S. epidermidis* strain YT-169a on nitrogen (N) ion implanted as well as on as-polished CoCrMo alloy materials were investigated. A medical grade CoCrMo alloy was ion implanted with 60 keV N ions to a high dose of 1.9×10^{18} ions/cm^2 at substrate temperatures of 200 and 400°C. The near-surface implanted layer crystal structures, implanted layer thicknesses, and roughnesses were characterized by XRD, SEM and AFM. The number of adherent bacteria on the surfaces of N implanted specimens was found to be 191×10^6 CFU/cm^2 for the 200°C and 70×10^6 CFU/cm^2 for the 400°C specimens compared to the as-polished specimen (3×10^6 CFU/cm^2). The adhesion test results showed that *S. epidermidis* strain YT-169a adhere much more efficiently to the N implanted surfaces than to the as-polished CoCrMo alloy surface. This was attributed mainly to the rougher surfaces associated with the N implanted specimens in comparison with the relatively smooth surface of the as-polished specimen. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 81A: 663–668, 2007

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INTRODUCTION

In recent years, the skin commensal *Staphylococcus epidermidis* have been recognized as an important medical pathogen in biomaterial-related infections.[1] The pathogenesis of *S. epidermidis* is associated with the biofilm forming abilities on biomaterial surfaces. Biofilm formation occurs in a two-step process. First, the bacteria rapidly adhere to the biomaterial surface reversibly by means of physicochemical interactions (van der Waals attraction forces, gravitational forces, the effect of electrostatic charge, hydrophobic interactions, ionic and dipole interactions).[2] Second, the bacteria proliferate and accumulate to form multilayered cell clusters on the surface by molecular and cellular interactions between cell surface structures (e.g. slime) and material.[2] Biofilm makes these embedded bacteria less accessible to the human defense system and decreases susceptibility against antibiotics.[3] In other words, multiple resistance to antibiotics was more frequent among slime producing *S. epidermidis* strains.[4] Metallic biomaterials especially placed in deep parts of the body with biofilm on their surface serve as an infection source and eventual removal of this material may be necessary.

Efforts to prevent bacterial adhesion and biofilm formation of biomaterials have mainly focused on the modification of their surfaces by coatings and surface modification techniques. Ion beam techniques can be used to reduce bacterial adhesion to the biomaterial surfaces. Previous studies report inhibition of bacterial adhesion and biofilm formation on metallic or polymer implants *in vitro* by coating and ion implanting surfaces of these materials with silver.[5,6]

Nitrogen (N) ion implantation can be an efficient surface treatment technique to form protective layers on the surface of CoCrMo alloy and to induce new surface properties without affecting bulk properties as only a small thickness of the material is involved. It is generally accepted that N ion implantation is an excellent method to enhance wear and corrosion resistances of a wide range of materials.[7] Several researchers have used ion beam applications in the

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medical area,[8,9] such as for orthopedic prostheses that require high wear resistance and excellent corrosion properties as well as biocompatibility.

In the present study, surface modification by N ion implantation is explored as a possible means of inhibiting bacterial adhesion to CoCrMo alloys. In particular, the adhesion of biofilm forming *S. epidermidis* strain YT-169a onto the nitrogen implanted surfaces will be investigated and compared with untreated (polished) CoCrMo material surface. Our study combines the use of X-ray diffraction techniques (θ/2θ XRD and GIXRD), cross-sectional scanning electron microscopy (SEM), and atomic force microscopy (AFM). Bacterial adhesion tests using *S. epidermidis* have been performed to evaluate the efficiency of N implanted surfaces to prevent and/or reduce bacterial adhesion and biofilm formation.

METHODS

Materials

Medical grade CoCrMo alloy (ISO 5832-12) with a base chemical composition of 26% Cr, 6% Mo and balance Co was the material into which nitrogen was implanted. The specimens had a disc-like geometry with a diameter of 3 cm and a thickness of 0.3 cm. Before N implantation, all the specimens were polished to mirror-like quality with a mean surface roughness of about 3 nm based on AFM.

Nitrogen implantation

N ion implantation was carried out with a relatively simple, broad beam, ultrahigh current density implanter. The N ions generated by the implantation system were not mass analyzed, but primarily consist of N_2^+ (~70%) and N^+ (~30%). The polished CoCrMo alloy samples were ion implanted with 60 keV N ions to a high dose of 1.9×10^{18} N/cm^2 at substrate temperatures of 200 and 400°C. The N implantation conditions for the specimens of this study are summarized in Table I. The N implantation duration was 30 min. A constant processing temperature for a given specimen was assured mainly by control of the ion beam.[10]

TABLE I
Nitrogen (N) Ion Implantation Conditions[a]

Temperature (°C)	L_{SEM} (nm)	N Implanted Layer Phases	Ra (nm)
As-polished substrate		γ-(Co,CrMo), ε-(Co,Cr,Mo)	3.0
200	185	$(Co,Cr,Mo)_{2+x}N$ nitride	12.0
400	450	γ_N, $(Co,Cr,Mo)_{2+x}N$ nitride	8.0

[a]The polished CoCrMo alloy specimens were ion implanted with 60 keV N ions to a high dose of 1.9×10^{18} N/cm^2.

Mean roughness Ra was measured by AFM for both untreated (polished) and N implanted CoCrMo surfaces.

Characterization techniques

Near-surface N implanted layer phases, implanted layer thickness, and implanted layer surface roughness were analyzed by XRD, SEM, and AFM. XRD was done in both the symmetric θ/2θ and grazing-incidence (GIXRD) modes using a Philips X'pert XRD system in a step-scanning mode with Cu–Kα radiation. θ/2θ scan range for the samples investigated here was from 30° to 90°. GIXRD was carried out at the incident angle of $w = 1$ degree. The effective depth, which is ~sinω/μ (where μ is the linear mass absorption coefficient and is estimated to be 2540 cm^{-1} for CoCrMo alloy used in this research), probed by Cu–Kα X-ray beams at this incident angle is about 68 nm.[11] In the θ/2θ XRD geometry, the effective depths probed by Cu–Kα X-ray beams are estimated to be 0.51 and 1.50 μm for 2θ scan angles between 30° and 100°.[12] Cross-sectional SEM was done for measuring the N implanted layer thicknesses. Before the SEM analysis, specimens were polished, and etched electrolytically for 30 s. The type of chemical etchant was a mixture of HCl (5 mL) and H_2O_2 (100 mL). The surface roughness of the as-polished and N implanted CoCrMo specimens was measured by AFM (Nanoscope IV). At least three readings were taken for each surface tested. The AFM was also used to image the surface topography of the N implanted as well as as-polished specimens.

Bacterial adhesion test

In this study, *S. epidermidis* strain YT-169a, isolated from an endotracheal tube surface of a patient hospitalized in the intensive care unit of Department of Chest Diseases, Faculty of Medicine, Ege University, Turkey, was used as a biofilm forming bacteria. The bacteria were grown in 5 mL tryptic soy broth (TSB) at 37°C for 24 h. N ion implanted and as-polished specimens were sterilized by dry heat at 180°C for 2 h. The sterile specimens were transferred into 100 mL TSB supplemented with 1% sucrose and inoculated with 200 μL bacterial culture containing 1×10^9 colonies forming units (CFU). They were then incubated with bacteria at 37°C with shaking at 100 rpm for 14 h. At the end of incubation, the specimens were washed with sterile phosphate buffered saline (PBS) (8.5 g/L NaCl, 0.3 g/L KH_2PO_4, 0.6 g/L Na_2HPO_4 and 0.1 g/L peptone, (pH 7.0)) three times to remove non-adherent bacteria. They were then transferred into 25 mL PBS and the adherent bacteria were removed from the surfaces by scraping off by sterile cell scrapers. The removed bacteria were diluted to 10^{-3} and 10^{-4} with PBS and 100 μL of the diluted bacteria were inoculated to duplicate tryptic soy agar plates. The plates were incubated at 37°C for 24 h and the number of *S. epidermidis* colonies was counted, and quantified as CFU/cm^2. The test for each specimen was repeated three times.

Biofilm formation by bacteria on the surfaces of the N implanted and as-polished materials was also visualized by SEM. For this purpose, both N ion implanted and as-polished specimens were incubated with bacteria in 100 mL TSB supplemented with 1% sucrose at 37°C with shaking at 100 rpm for 24 h. They were then washed with PBS and dried on silica gel at 37°C for 24 h.

RESULTS

XRD analysis

Figure 1 shows the XRD (θ/2θ) and GIXRD results for the N implanted specimens at substrate temperatures of 200 and 400°C. Included in the same figure are the results for the as-polished, untreated CoCrMo alloy sample. In Figure 1, the substrate peaks are labeled as "γ-(hkl)" for the fcc γ-(Co,Cr,Mo) phase and "ε-(hkl)" for the hcp ε-(Co,Cr,Mo). The XRD results for the specimen implanted at 400°C show additional peaks labeled as γ_N. These new peaks are due to the formation of an interstitial phase (nitrogen atoms occupying octahedral sites in fcc lattice). The γ_N phase formation is similar to that observed for N implantation of 304 stainless steel at 400°C where γ_N forms from the γ-(Fe,Cr,Ni) parent structure.[13] Note that the parent structure of the substrate material in this study is γ-(Co,Cr,Mo). Based on Ref. 13, the γ_N phase was observed when the substrate temperature was held near 400°C. At lower and higher implantation temperatures the γ_N is not produced. Higher implantation temperatures (≥450°C) lead to CrN, while lower implantation temperatures (~200–350°C) result in a hexagonal nitride phase, ε-$(Fe,Cr,Ni)_{2+x}N$.[13]

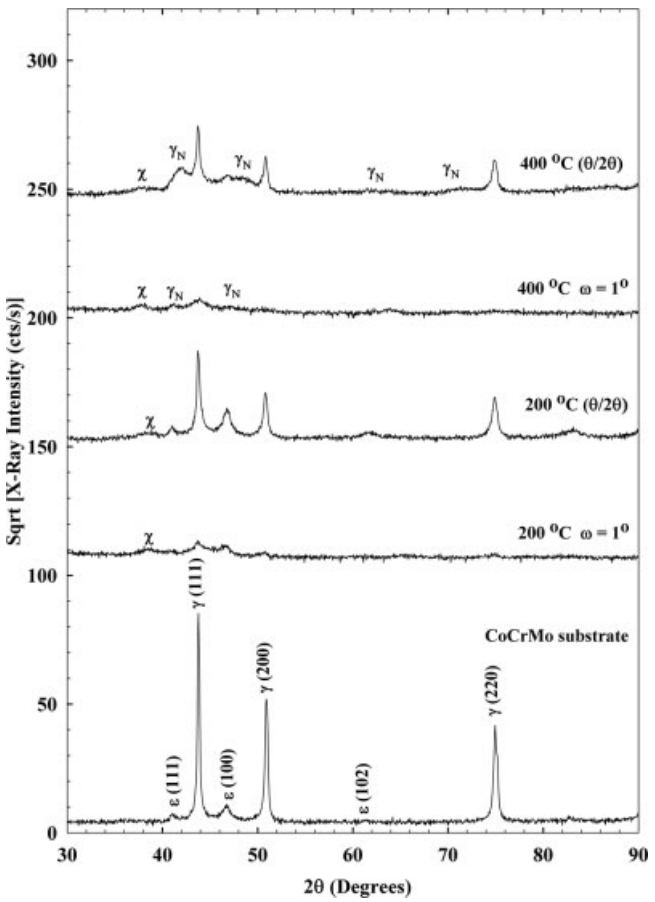

Figure 1. XRD and GIXRD data for the as-polished and nitrogen implanted specimens. Note that the square-root of the intensity is plotted to reveal the weaker peaks more clearly.

The XRD results for the specimen implanted at 200°C show a peak labeled "χ" for the $(Co,Cr,Mo)_{2+x}N$ phase. The XRD peaks associated with this phase are very weak (low intensity) and broad suggesting a distribution of nitrogen in a very thin implanted layer (about 100 nm or less based on the SEM results to be discussed later). The $(Co,Cr,Mo)_{2+x}N$ phase is referred to as the hexagonal nitride phase and is quite similar to the epsilon nitride phase $(Fe,Cr,Ni)_{2+x}N$, which forms in the surface layers of nitrogen implanted 304 SS.[13]

To reveal the N implanted layer phases at the near surface, GIXRD of the N implanted specimens was carried out. The GIXRD results at the incident angle of $\omega = 1$ degree for the specimens N ion implanted at 200 and 400°C are also shown in Figure 1. The GIXRD scans indicate that the top N implanted layer (~70 nm) for the specimen implanted at 200°C substrate temperature is mainly composed of the $(Co,Cr,Mo)_{2+x}N$ nitride phase. The GIXRD results also show some contribution coming from the substrate phase due to the increased penetration depth at this angle. The XRD (θ/2θ) results in Figure 1 for the specimen implanted at 400°C also show a small peak (χ) believed to be mainly due to the $(Co,Cr,Mo)_{2+x}N$ nitride phase. As can be seen from Figure 1, this peak is revealed much more clearly at grazing incident angle geometry.

SEM analysis

Figure 2 shows the cross-sectional SEM results for the N implanted specimens at the implantation temperatures of 200 and 400°C. These pictures quite clearly reveal N implanted layers with a relatively uniform thickness. The N implanted layer thickness obtained from the picture is about 150 nm for the sample implanted at 200°C. The SEM photos were taken sequentially over a span of several grains to look for N implanted layer thickness variations. So, based on several SEM photos, the average N implanted layer thickness for the 200°C sample is 185 nm. The SEM picture for the specimen implanted at 400°C quite clearly reveal the extremely uniform nature of the N implanted layer, which is mainly composed of the γ_N phase based on the XRD results. The γ_N layer thickness found from the SEM picture in Figure 2 is about 400 nm, while the average γ_N layer thickness is estimated to be 450 nm. The average layer thicknesses for the N implanted specimens determined by the cross-sectional SEM analysis are given in Table I.

AFM analysis

In Figures 3 and 4, 3D AFM images of specimens' surfaces before and after N ion implantation are com-

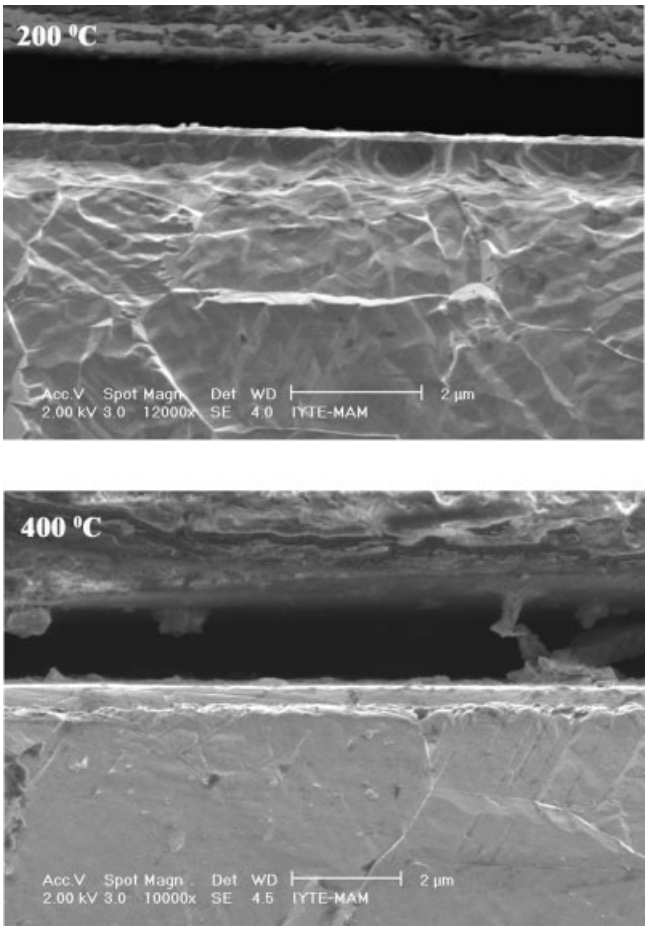

Figure 2. Cross-sectional SEM images for the specimens nitrogen implanted at substrate temperatures of 200 and 400°C.

pared. These figures clearly demonstrate different surface topography of samples in 3D view. The AFM image of the as-polished surface shows that it is

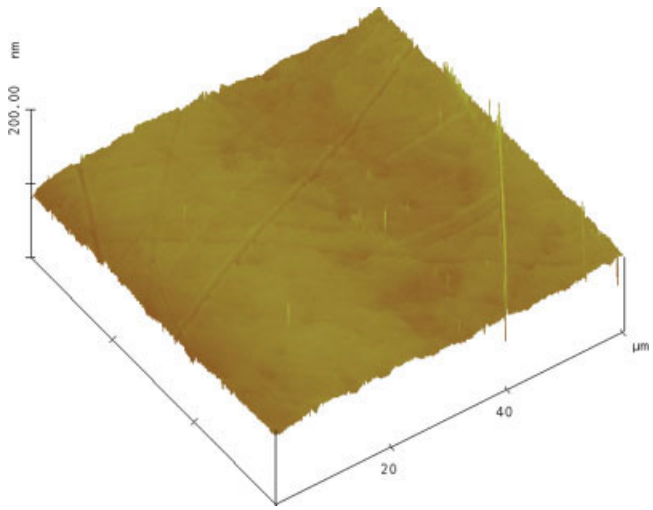

Figure 3. Three-dimensional AFM image of the as-polished (unimplanted) CoCrMo substrate alloy surface. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

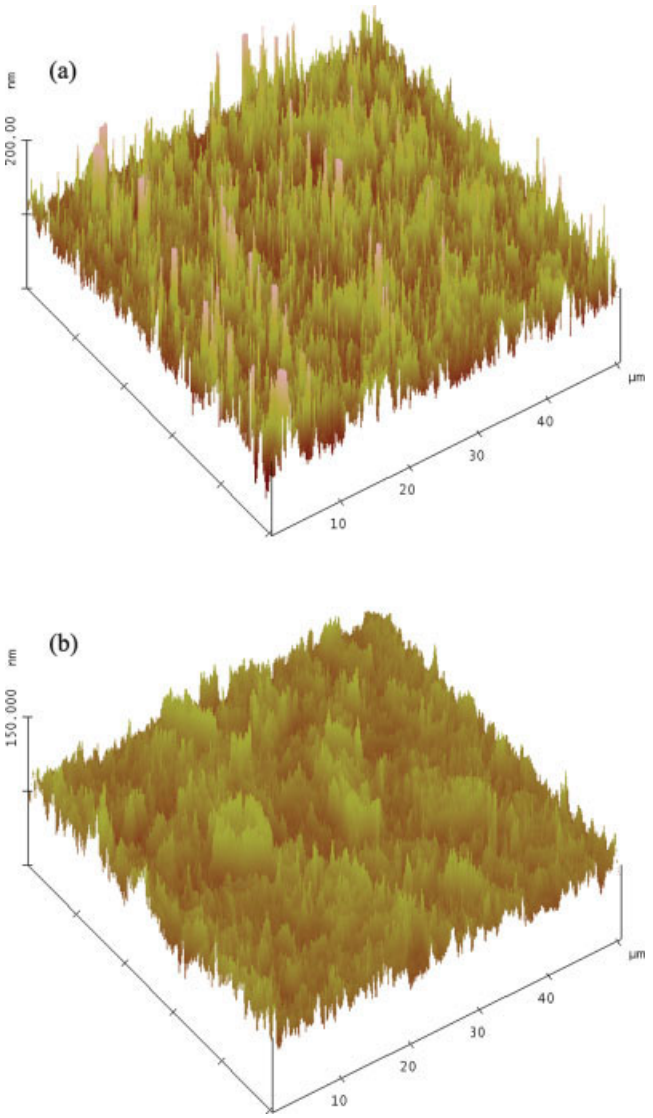

Figure 4. Three-dimensional AFM images of the N implanted specimens at substrate temperatures of (a) 200°C and (b) 400°C. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

rather smooth and featureless. The average roughness (Ra) value for the as-polished surface is found to be 3 nm. The 3D AFM images in Figure 4 clearly indicate that surface roughness is strongly increased by N ion implantation. The Ra values calculated from these images are about 12 and 8 nm for the specimens implanted at 200 and 400°C, respectively. The roughness values for the as-polished as well as N implanted specimens can also be found in Table I.

Bacterial adhesion test

The number of attached bacteria to the as-polished (unimplanted) and N implanted surfaces were quantified by bacterial adhesion test. It is found that the mean number of bacteria on the surfaces of N

implanted specimens at 200 and 400°C specimens were 191×10^6 CFU/cm^2 and 70×10^6 CFU/cm^2, respectively. The number of bacteria on the as-polished CoCrMo alloy surface is found to be 3×10^6 CFU/cm^2.

DISCUSSION

The bacterial adhesion test results presented above suggest that the N implanted layers/surfaces may not be beneficial for preventing or reducing bacterial adhesion on CoCrMo alloy surfaces or both. The results clearly indicate that *S. epidermidis* strain YT-169a adhere much more efficiently to the surfaces of N implanted specimens. This is mainly attributed to the much rougher surfaces of the N implanted specimens compared to the rather smooth surface of the as-polished specimen. The test results further indicate that the bacteria adhere strongly to the surface of the specimen implanted at 200°C compared to that implanted at 400°C. This is also believed to be due to the roughness differences between these two samples; as can be seen from Table I, the specimen implanted at 200°C has a larger surface roughness (12 nm) compared to the specimen implanted at 400°C (8 nm).

The SEM images in Figure 5 show biofilm formation by *S. epidermidis* strain YT-169a on the surfaces of the N implanted specimens. These images clearly indicate the cell-to-cell interactions by slime formation by the bacteria and the physicochemical interactions between the bacteria and the surface of the N implanted material. It is known that these cellular and physicochemical interactions play a significant role in biofilm formation.[2]

There are varying opinions as to the effect of surface characteristics on bacterial adhesion. While a number of researchers report a positive correlation between adhesion and increased surface roughness, others report no correlation between surface roughness and the ability of bacteria to attach.[14,15] A recent research study investigating adhesion of thermo-resistant streptococci on 316L and 304L stainless steels found that adhesion could not be related to increases in surface roughness but surface topography around a critical size close to the diameter of the bacterial cells may entrap bacteria.[16] Another research study mentions that electropolished surfaces are more resistant to bacterial adhesion than are those with a higher surface roughness.[17]

The present study finds no correlation (positive or negative) between bacterial adhesion and the N implanted layer phases. A detailed analysis of the nature of the N implanted layer phases as well as their surface characteristics such as hydrophobicity, electrical charge, and passive film seems to be necessary. For the as-polished CoCrMo alloy, its native oxide layer

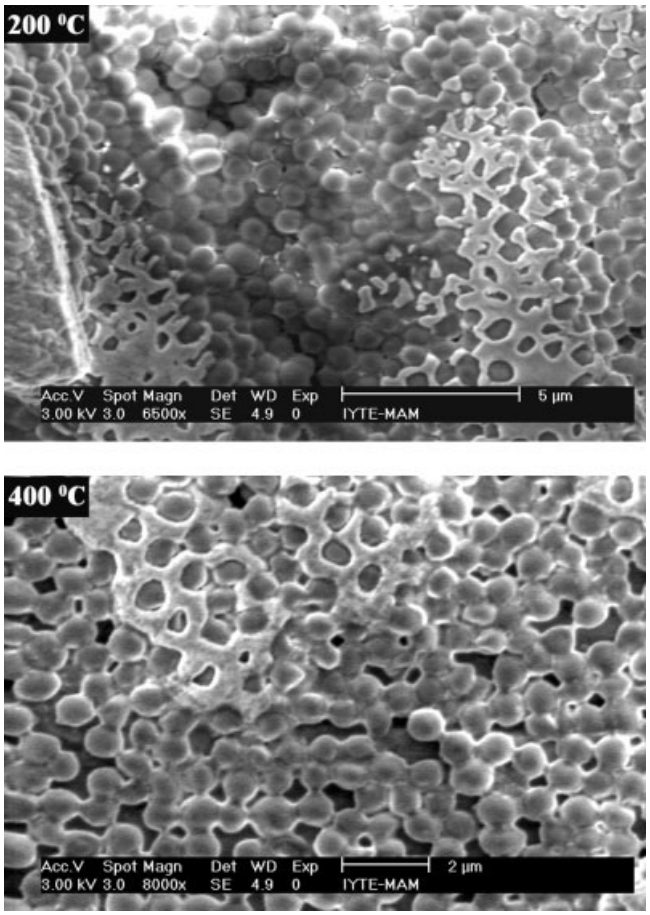

Figure 5. SEM images showing bacterial adhesion and biofilm formation on the nitrogen implanted specimens at 200 and 400°C.

(predominantly composed of Cr_2O_3 with some minor contributions from Co and Mo oxides) might also play a role in attracting less bacteria compared to the N implanted surfaces. However, the study in Ref. 16 found that the native oxide layer on stainless steel enhances bacterial adhesion compared to the surface whose oxide layer was damaged or removed by an acid treatment. There is no conclusive evidence on the formation of oxide layer(s) on the N implanted surfaces investigated in this research study. A thin oxide layer would probably form on the surfaces of the N implanted specimens in air after the implantation. It would be important to investigate the physiochemical characteristics of the N implanted as well as as-polished surfaces since it is believed to be an important factor governing bacterial adhesion.[2,16]

CONCLUSIONS

In this study, the adhesion of biofilm forming ability of *S. epidermidis* strain YT-169a on N implanted

CoCrMo surfaces was investigated. The bacterial adhesion test showed that *S. epidermidis* strain YT-169a adhere preferentially to the N implanted surfaces compared to the as-polished substrate alloy material and more bacteria adhered to the specimen implanted at 200°C than to the specimen implanted at 400°C. The SEM images also indicated thick biofilm formation on the surfaces of the N implanted CoCrMo specimens. Stronger bacterial adhesion behavior of the N implanted surfaces compared to the as-polished surface was attributed mainly to the rougher surfaces associated with the N implanted specimens compared to the relatively smooth surface of the untreated CoCrMo alloy material. The present study finds no correlation between bacterial adhesion and the N implanted near-surface crystal structures. In order to establish a correlation, a much more detailed analysis of the N implanted surfaces is required.

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