Promising *in vitro* performances of nickel-free nitrogen containing stainless steels for orthopaedic applications

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Abstract. The aim of the present work was to study the *in vitro* corrosion resistance in Hank's solution and biocompatibility of indigenized low-cost Ni-free nitrogen containing austenitic stainless steels (HNSs) and to compare with conventionally used 316L and 316LVM. The electrochemical behaviour was assessed using electrochemical impedance spectroscopy, potentiostatic polarization and scanning electron microscopy. The MTT assay [3-(4,5-dimethylthiazol 2-yl)-2,5-diphenyltetrazolium bromide] was performed using Dalton's lymphoma cell line for cytotoxicity evaluation and cell adhesion test. The resistance of surface film was raised by increasing nitrogen content in stainless steel (SS). The corrosion current density was decreased with increase in nitrogen content and corrosion potentials for HNS were observed to be more positive. Shallower and smaller pits were associated with HNS, indicating that nitrogen suppresses the pit formation. The HNS had higher cell proliferation and cell growth and it increases by increasing the nitrogen content. The surface wettability of the alloys was also investigated by water contact-angle measurements. The value of contact angles was found to decrease with increase in nitrogen content. This indicates that the hydrophilic character increases with increasing nitrogen content, which is further attributed to enhance the surface free energy that would be conducive to cell adhesion, which in turn increases the cell proliferation.

Keywords. HNS; EIS; 316LVM; implants.

1. Introduction

Austenitic stainless steels (e.g. AISI 316L), cobalt-based alloys, commercially pure titanium (CP-Ti) and Ti–6Al–4V alloy are most widely used metallic biomaterials. However, there is growing interest in Ti and its alloys, particularly as dental and orthopaedic implants because of their relatively lower modulus of elasticity, superior biocompatibility and higher corrosion resistance (Woodman *et al* 1984). The 316L grade of stainless steel (SS), however, is still the widely used material for internal fixation devices like fracture plates, screws, hip nail and artificial joints because of their good mechanical properties, corrosion resistance, easy processing, acceptable biocompatibility and very low cost as compared with those of other metallic implant materials (Disegi and Eschbach 2000; Schmidt *et al* 2001). The resistance of SS to corrosion is due to the presence of a thin oxide passive film on its surface (Milosev and Strehblow 2000; Olsson and Landolt 2003). But the passive film is highly susceptible to localized corrosion such as pitting, crevice corrosion

and stress corrosion cracking when used as implants (Thomann and Uggowitzer 2000; Kuroda *et al* 2002).

It has been reported that SS corrodes in body environment and releases iron, chromium and nickel (Sumita 1997). As we are aware that 316L contains 12–15% of nickel, which can be toxic to the human body if released. There are sufficient evidences that high levels of nickel ions in tissues can cause genotoxic and mutagenic activity or in contact with the skin can cause most widespread contact allergy (Vahter *et al* 2002; Takazawa *et al* 2003) and cancer (IARC 1999; Bal *et al* 2000). The problems caused by nickel-containing alloys have led to development of nickel-free austenitic SSs. To maintain the austenitic microstructure of the alloy, nickel reduction is balanced by the addition of austenite phase stabilizer, i.e. nitrogen and manganese. These nickel-free high nitrogen austenitic stainless steels (HNSs) are a potential alternative material owing to its better mechanical properties, very good corrosion resistance and excellent biocompatibility (Menzel *et al* 1996; Tschiptschin *et al* 1999; Thomann and Uggowitzer 2000). The addition of nitrogen has been reported to considerably increase the passive film stability and resistance towards pitting corrosion, crevice corrosion, intergranular corrosion and stress corrosion cracking in some media (Newman *et al* 1984; Clayton 1986; Newman and Shahrabi 1987; Mudali *et al* 1994, 1996; Baba *et al* 2002).

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Addition of nitrogen produces beneficial effects for the resistance to pitting corrosion by increasing the pitting potential, suppressing the metastable pits (Palit *et al* 1993; Ohno *et al* 1998; Pattersson 1999) and facilitating the re-passivation rate immediately after the breakdown of the passive film (Lee 2006). Nitrogen-containing steels also exhibit superior biocompatibility than nickel-containing alloys. Yamamoto *et al* (2004) and Ma *et al* (2012) have evaluated the cytocompatibility of nickel-free austenitic SS and observed that nickel-free SS had higher cell growth than nickel-containing steel. Wan *et al* (2010) and Ren *et al* (2011) have studied the effect of nitrogen content on blood compatibility of HNS including platelet adhesion and kinetic clotting time. They observed that as the nitrogen content in the alloy is increased, the amount of adherent platelets is further decreased and the initial clotting time becomes longer. *In vitro* studies on biocompatibility using primary osteoblasts and *in vivo* studies after bone implantation in the sheep tibia of steel, P558, indicate that P558 was much biocompatible with promising future as an implant material (Fini *et al* 2003, 2004).

In the present study, *in vitro* corrosion behaviour and biocompatibility of in-house prepared nickel-free austenitic SS for application as biomaterials was evaluated. Usually, steels which are used for biomedical applications contain low carbon ($\leq 0.03\%$). But, some newly developed high nitrogen medical grade SSs also contain carbon. For example, P558 alloy contains about 0.20 wt% carbon and F2581 contains 0.15–0.25 wt% carbon (ASTM F 2581-07; Thomann and Uggowitz 2000). To analyse the effect of carbon with nitrogen, one alloy which contains high nitrogen (0.34 wt%) as well as carbon (0.48 wt%) and two more alloys having low carbon (≤ 0.08 wt%), but high nitrogen (0.43 wt% N and 0.52 wt% N) were selected for the present study. The results were compared with conventional high-quality nickel grades (316L and 316LVM) being used in these applications. The techniques used for the characterization of the corrosion resistance were electrochemical impedance spectroscopy (EIS) and potentiostatic polarization. Finally, SEM was used to understand the surface morphology of the alloys after corrosion studies. The MTT assay was performed using DL cell line for cytotoxicity evaluation and cell adhesion test. The surface wettability was also investigated by water contact-angle measurement.

2. Experimental

2.1 Materials

Three austenitic SSs (316 MnN₁, 316 MnN₂ and 316 MnN₃) with different nitrogen contents (0.34 wt% N, 0.43 wt% N and 0.52 wt% N) were prepared in the form of ingots, respectively, by melting iron and ferro-alloys (Cr, Mo, Mn, high nitrogen ferro-chrome, etc.) in appropriate proportions to meet the aimed composition of alloy in an induction furnace. These ingots were solutionized at 1050–1070 °C to avoid segregation. Then, specimens of 80 × 15 × 6 mm were cut from ingot, hot-rolled at 1050 °C to reduce the thickness by 50–80%, solution treated at 1050–1070 °C for 30 min, and, then, water quenched. The 316L and 316LVM SSs were obtained from Mishra Dhatu Nigam Ltd. (MIDHANI), Hyderabad, India. These steel were received in hot-rolled and annealed condition and used for the study as such. The chemical compositions of all five austenitic SSs are shown in table 1. Coupons of 10 × 10 mm size were cut from them and polished successively with emery papers of 600–800–1000–1200 grades. Specimens were washed with deionized water, followed by acetone, and dried in air.

2.2 Electrochemical corrosion

2.2a Electrodes and electrolyte: Each coupon was soldered onto one surface with an insulated copper wire and carefully coated with epoxy resin, leaving the other surface (which had a surface area of 1 cm²) exposed for corrosion studies. The exposed metal surface was then mirror polished on revolving selvyt cloth using alumina of 0.3 μ , washed with deionized water followed by acetone and dried just before test. These specimens were used as working electrodes for the electrochemical corrosion studies. The electrochemical experiments were carried out in Hank's solution of pH value of 7.4 ± 0.2 , prepared in deionized water (Gurappa 2002). The chemicals used to prepare the solution were of analytical reagent grade and were used without further purification. The solution of about 200 mL was used for electrochemical tests and the temperature was maintained at 37 °C (98.4 F), which is equivalent to human body temperature (Gurappa and Kumar 2000).

Table 1. Composition of stainless steels (wt%).

Sample	C	Cr	Mn	Ni	Si	Mo	S	P	N	Fe
316L	0.021	17.19	1.56	14.0	0.71	2.77	0.004	0.015	0.060	Bal.
316LVM	0.021	17.24	1.68	14.42	0.24	2.83	0.004	0.007	0.07	Bal.
316MnN ₁	0.48	19.32	12.77	0.05	0.26	3.32	0.006	0.009	0.34	Bal.
316MnN ₂	0.08	19.11	11.85	0.08	0.25	3.02	0.004	0.010	0.43	Bal.
316MnN ₃	0.017	18.28	11.92	0.04	0.07	3.24	0.003	0.008	0.52	Bal.

2.2b Apparatus and procedures: The electrochemical experiments were carried out using a standard three-electrode cell assembly, in which platinum wire and dip-type saturated calomel electrodes (luggin capillary) were used as counter and reference electrodes, respectively. The studies were performed using CHI760C electrochemical workstation with DC software for corrosion. The working electrode was held in the test solution at 37 °C for 5 h, prior to each experiment, to attain stabilized open-circuit potential (E_{OC}). The temperature of the cell was controlled thermostatically using a thermostat (JULABO Labortechnik GmbH, West Germany) at the experimental temperature. The potential of working electrode was measured against saturated calomel electrode (SCE). EIS measurements were carried out in a frequency range from 10^5 to 1 Hz under potentiostatic conditions, with an amplitude of 5 mV peak to peak, using a.c. signal at E_{OC} . ZSimpwin software was used for simulating the experimental EIS data.

Potentiostatic polarization curves were obtained by changing the electrode potential automatically from -1.2 to 2 V vs E_{OC} at a scan rate of 0.01 V s $^{-1}$. A constant distance of approximately 1–2 mm between the tip of luggin capillary and working electrode surface was maintained throughout the experiment. The current was passed through the cell at different pre-determined values through the counter electrode and the equilibrium potential between the working and reference electrodes was measured. Potential values at a given current were found to be generally constant within 1 min. The corrosion potential (E_{corr}), corrosion current density (I_{corr}) as well as other parameters were automatically extracted from the polarization curves by the tafel extrapolation. All the measurements were repeated at least three times and a fresh solution was used for the new test. Finally, scanning electron microscopy (SEM), FEI Quanta 200FEG, was used to understand the surface morphology of SSs, after corrosion studies.

2.3 Contact angle measurement

Contact angles were measured with Kruss F-100 tensiometer for thin strip ($20 \times 10 \times 1$ mm ^{3}) of SS samples at room temperature in water medium. Three different experiments were done for each kind of sample and the measured results were averaged.

2.4 Cytocompatibility evaluation

Fetal bovine serum (FBS) was obtained from Invitrogen, Grand Island, NY, USA. Con-A, PMA and MTT were purchased from Sigma Chemical Company, Bangalore, India. RPMI-1640 culture medium was obtained from HiMedia, Mumbai, India. All other chemicals stated otherwise were obtained either from Super Religare Laboratory (SRL), Mumbai, India, or HiMedia, Mumbai, India. A spontaneously occurring T cell lymphoma

commonly called as Dalton's lymphoma (DL) were harvested from DL-bearing mice after 18 days of post-DL cells transplantation, when yield is maximum, and subjected for experimental setting.

In vitro biocompatibility of austenitic SS samples was evaluated using DL cell line. The cells were maintained and cultured in RPMI-1640 medium supplemented with 10% FBS, antibiotic and antifungal (100 units per mL penicillin, 100 μ g per mL streptomycin and 0.025 μ g per mL amphotericin), at 37 °C in a humidified chamber in 5% CO $_2$ atmosphere. For experimental setting and/or for subculture, cells were harvested, washed and seeded at a density of 0.5×10^6 cells per well in a standard proliferation assay. The coupons of SSs were sterilized by autoclaving at 121 °C for 20 min and immersed in RPMI-1640 medium containing 10% FBS at 37 °C in 5% CO $_2$ in separately located bottom of 24-well plate for 48 and 72 h. Thereafter, the extract was used to carry out the cytotoxicity/viability assays. Proliferation/cytotoxicity assay was performed in a 96-well microtiter plate seeded with 0.5×10^6 cells per wells and 10 μ L of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added to freshly prepared PBS (5 mg/mL) and incubated for 6–12 h, thereafter cells were centrifuged for 5 min at 100g at 4 °C. Supernatant was then carefully discarded without disturbing the dark-blue formazan crystals and 200 μ L of DMSO was added to each well to dissolve the formazan crystals and absorbance was taken at 595 nm. All experiments were carried out at least three times and three samples of each type were used for each time point. Mean and standard deviation of the optical density (OD) values obtained for three replicates of each sample were calculated. The viability of the cells can be calculated by the following equation

$$\text{Cell viability (\%)} = \frac{\text{OD}_{595}(\text{sample})}{\text{OD}_{595}(\text{control})} \times 100. \quad (1)$$

For cell attachment test, the SS coupons were separately located into the bottom of 24-well microplate/35 mm petri dish. DL cells at the density of 1×10^6 cells per mL were seeded onto the coupons and cultured in 5% CO $_2$ at 37 °C in a humidified chamber for 1, 3 and 5 days, respectively, and thereafter cell suspension was discarded and the samples were washed three times in cold PBS to remove any unattached cell. Adherent cell populations were then fixed in 2.5% of glutaraldehyde solution for 45 min and dehydrated using a graded ethanol series (50, 60, 70, 80, 90 and 100% of ethanol) for 10 min each. Samples were dried in air and observed under SEM.

3. Results and discussion

3.1 Electrochemical corrosion

3.1a Electrochemical impedance spectroscopy (EIS): To investigate the relative stability of the passive films

Figure 1. Bode plots of different SSs in Hank's solution at 37 °C: (a) Bode phase plots and (b) Bode modulus plots.

Figure 2. Equivalent electric circuits for quantitative evaluation of EIS spectra.

formed on the surface of different SSs, impedance spectra were recorded at the passive potential after passive film generation. Figure 1(a and b) show Bode (phase and modulus) diagrams of the recorded EIS spectra of SS samples at 37 °C in Hank's solution. All spectra were analysed in terms of their equivalent electric circuits (EECs) presented in figure 2. In fitting procedure, when only one time constant was used in modelling of the EIS spectrum, a large discrepancy between the experimental and modelled data was observed. However, when the EEC presented in figure 2 was used, the agreement between the experimental (shown by point symbols) and modelled (shown by solid line) data was excellent. Because the impedance data of a solid/electrolyte interface often reveal a frequency dispersion that is attributed to a 'capacitive dispersion', the capacitance is expressed in terms of the constant-phase element (CPE) (Jorcin *et al* 2006). The impedance of the CPE is given by

$$Z_{\text{CPE}} = \frac{1}{Q}(j\omega)^{-n}, \quad (2)$$

where Q is the magnitude of the CPE, j the imaginary unit, ω the angular frequency and n the CPE exponent.

Table 2 presents the values of electrochemical impedance parameters obtained from the fitting procedure. The EEC used describes the behaviour of a process characterized by two time constants, namely, the high-frequency

(HF) time constant ($\text{CPE}_1 - R_1$) and low-frequency (LF) time constant ($\text{CPE}_2 - R_2$). Based on the EEC parameter values obtained by fitting the experimental data and also based on the available literature (Omanovic and Roscoe 1999, 2000; Abreu *et al* 2004), the physical meaning of the HF time constant can be associated with faster charging/discharging and charge-transfer processes, respectively, represented by the double layer/space charge capacitance (CPE_1) and charge transfer resistance (R_1) parallel combination. On the contrary, the LF time constant ($\text{CPE}_2 - R_2$) can be related to slower mass-transport processes in the oxide phase, namely, diffusion of electroactive species (metal ions or oxygen-containing species, i.e. oxygen and metal vacancies, respectively) through the formed passive film. Therefore, the HF time constant can be associated with the properties of the electrode/electrolyte interface, while the LF time constant reflects the mass-transport processes through the formed passive oxide film. The CPE is a combination of properties related to both the surface and the electroactive species, and is independent of frequency. The results of the fitting procedure indicate that impedance measurements can detect the duplex nature of the oxide layer formed on these types of steels, composed of an inner chromium-rich film which behaves as a p -type semiconductor and an outer iron-rich film which behaves as an n -type semiconductor (Azumi *et al* 1986; Ge *et al* 2001; Sudesh *et al* 2008; Ha *et al* 2009). The values of impedance for HNS were higher than that of 316L, and increased with nitrogen content; meanwhile, the phase angle shifted towards higher values. R_s is the resistance of the electrolyte between the working and the reference electrodes, which was obtained almost same for all the tested samples. Taking into account the physical meaning of the EEC, the sum of the two resistances (R_1 and R_2) is related to the total resistance of surface to general corrosion, which is higher for HNS, indicating the formation of a more

Table 2. Results of the parameters obtained from the fitting of the equivalent electric circuit to the experimental impedance data obtained for different stainless steels in Hank's solution at 37 °C.

Stainless steels	R_s ($\Omega \text{ cm}^2$)	CPE_1 (F/cm^2)	n_1	R_1 ($\Omega \text{ cm}^2$)	CPE_2 (F/cm^2)	n_2	CPE_2 R_2 ($\Omega \text{ cm}^2$)	R_{ct} ($10^5 \Omega \text{ cm}^2$)
316L	3.21	2.91×10^{-6}	0.86	105.3	2.64×10^{-5}	0.80	3.34×10^4	0.15
316LVM	3.74	3.60×10^{-6}	0.98	108.2	3.25×10^{-5}	0.74	3.63×10^4	0.30
316MnN ₁	2.29	1.46×10^{-5}	0.67	372.2	3.43×10^{-4}	0.80	4.12×10^5	0.65
316MnN ₂	2.79	4.5×10^{-5}	0.83	533.3	4.38×10^{-4}	0.81	5.20×10^5	1.73
316MnN ₃	3.36	7.71×10^{-5}	0.98	685.5	5.27×10^{-4}	0.94	8.42×10^5	2.20

Figure 3. Values of R_{ct} of different SSs in Hank's solution. **Figure 4.** Polarization curves for different SSs in Hank's solution at 37 °C.

protective oxide layer on the surface. The charge transfer resistance (R_{ct}) is related to the rate of corrosion reactions at the passive potential. As illustrated in figure 3, the R_{ct} increases with increase of nitrogen content in the SS. It was observed that the corrosion resistance of HNS is

high, even in the presence of carbon indicating that carbon might not have an effect on the protective layers on the surface of HNS. Meanwhile, some authors observed that the exchange of electrons from blood proteins to the electrode surface of a medical device could induce blood coagulation (Shih *et al* 2003). The addition of nitrogen improved the R_{ct} of SS, which would restrain the occurrence of electrons exchange and inhibit the possibility of thrombosis formation.

3.1b Potentiostatic polarization study: Figure 4 shows the polarization curves of different SSs at 37 °C in Hank's solution. The figure shows that the polarization current is significantly low before the pitting occurs and then the current increases to a high level. Relevant polarization parameters were determined from the tafel extrapolation of polarization curve for the different steels and their values are recorded in table 3. Maximum corrosion rate was observed for the 316L and it decreases with increase in nitrogen content. In essence, the SS with highest nitrogen exhibits the minimum corrosion rate. It has been observed that the values of I_{corr} for SS samples decreased with increase in nitrogen content and is highest for 316L. The E_{corr} for HNSs is more positive and it increases with increase in nitrogen content, indicating that HNS are more corrosion resistant than 316L and 316LVM in Hank's solution. These results are in close agreement to the results obtained by EIS studies.

Another important requirement for selecting any material for biomedical application is to determine the breakdown potential (E_{bd}), i.e. potential at which the passive film breaks and anodic current strongly increases. In fact, in the presence of chloride ions, the passive layer becomes unstable at this potential and it produces a localized rapid attack of the metal with the formation of pitting. In this study, the maximum breakdown potential is observed for 316MnN₃, and decreases with decrease in nitrogen content. But the minimum breakdown potential was observed for 316MnN₁ and it is lower than that of 316L; however, its I_{corr} value is low and E_{corr} value is more positive. This indicates that the passive film on the surface of carbon-containing SS is unstable at the particular applied potential. This behaviour is different from the obtained results of EIS.

Table 3. Polarization parameters for different stainless steels in Hank's solution at 37 °C.

Stainless steels	E_{corr} (V vs SCE)	I_{corr} ($\mu\text{A cm}^{-2}$)	β_c (V dec $^{-1}$)	β_a (V dec $^{-1}$)	Corrosion are (mils y $^{-1}$)	E_{bd} (V vs SCE)
316L	-0.655	5.556	5.546	3.202	2.359	0.403
316LVM	-0.585	5.169	5.655	3.461	2.247	0.489
316MnN ₁	-0.369	3.668	4.979	4.045	1.667	0.194
316MnN ₂	-0.309	2.906	5.198	4.288	1.321	0.437
316MnN ₃	-0.236	2.663	5.597	4.559	1.210	0.523

In comparison with 316L, the HNS showed evidently much lower passive current density, suggesting that the passive film formed on the HNS is more protective. In general, during the pit propagation, the local environment is acidified by the hydrolysis of the dissolving metal, and the metal inside the pit is maintained in an actively dissolving state according to

It is believed that nitrogen in the steel dissolves; it consumes protons in the pit to form ammonia, thus, preventing a lowering of the pH in the pit, which contributes to the suppression of acidification inside the pit (Osozawa *et al* 1975; Osozawa and Okato 1976; Palit *et al* 1993). It is also speculated that surface films are stabilized through passivation or nitrogen enrichment of the film/metal interface to prevent the attack of chloride ions (Lu *et al* 1983; Vanini *et al* 1994; Olsson 1995). The increase of nitrogen content enhanced the corrosion resistance of SS, thus, improving the biocompatibility. It has also been observed from the results that the 316LVM is more corrosion resistant than 316L. Similar results were obtained in our previous study (Talha *et al* 2012). There are many other factors that affect the corrosion behaviour of steels such as impurities, microstructure, electrolytes, pH values and so on. It is well known that some inclusions on the surface of stainless steels accelerate pitting. 316LVM is a vacuum-melted alloy (VM = vacuum melted), so it has less inclusions compared to 316L. Hence, the impurity elements such as S, P and oxides in 316LVM steels is lower, and then the nucleation sites for pitting, such as MnS inclusions, were probably reduced (Yu 1987; Shahryari *et al* 2008), which decreased the susceptibility to pitting attacks.

3.1c Scanning electron microscopy (SEM) analysis: At the end of the polarization tests, surfaces of all samples were observed by SEM in order to analyse the pitting corrosion on surface of SSs. Figure 5(a–e) shows the SEM micrograph of the different SS surfaces after the polarization test in Hank's solution. SEM micrographs confirm the pitting corrosion on the surface of SSs. Pits were in fact found on all the steel sample surfaces, but few pits were observed for the steel having highest nitrogen. However, the surface of the 316L and 316LVM corroded much more in Hank's solution, which reveals the

presence of many open perforated pits and pits were deeper and bigger than those on nitrogen-bearing steels. It is very interesting to note that the samples which have higher carbon content also have very less number of pits indicating that nitrogen suppresses the pit formation, even in presence of carbon. This indicates that the nitrogen-bearing alloy is suitable for biomedical applications.

3.2 Contact angle

The values of contact angle obtained with distilled water are shown in table 4. It can be seen that all the differences are statistically significant for the experimental materials, among which the HNS is more hydrophilic than 316L and 316LVM. Increasing nitrogen content enhances the hydrophilic character, i.e. lower contact angle (CA) values.

3.3 Cytocompatibility evaluation

Figure 6 represents the results of cell proliferation on various surfaces of SS samples after 48 and 72 h, determined by the MTT test. Results indicate that the cell proliferation increases significantly ($P \leq 0.05$ – 0.001) in a time-dependent manner for all the prepared samples. All the samples permitted cell proliferation and no sample exerts any cytotoxic effect on cell proliferation/viability. The significant difference in cell proliferation between 316L and HNS is observed and it is higher for HNS than that of 316L and 316LVM, and increases with increasing nitrogen content. The results demonstrate that all samples do not cause any reduction or inhibition in cell viability or proliferation. Interestingly, cell proliferation/viability was detected higher in carbon-containing sample than in 316L, 316LVM and even than in 316MnN₂, indicating that the carbon does not exert any negative effect on cell proliferation and enhances the proliferation/viability of cells. The difference between the numbers of proliferating DL cells on the surface of different SS coupons for 1, 3 and 5 days was examined, which indicate that adhesion of cells increases in a time-dependent manner and has been observed significantly higher on fifth day of cells incubation as compared to cells incubated for one and three days as shown in figure 7. Significant increase in the number of cells with time on all the sample

Figure 5. SEM micrographs of (a) 316L, (b) 316LVM, (c) 316LMnN₁, (d) 316MnN₂ and (e) 316MnN₃ after polarization test in Hank's solution.

Table 4. Contact angle values (degree) obtained with distilled water on the surface of different SSs.

316L	316LVM	316MnN ₁	316MnN ₂	316MnN ₃
79.68	77.41	67.14	69.18	64.19

surfaces suggest that all samples do not exert any growth inhibitory effect on DL cells proliferation. The number of adherent cells population was observed higher on HNS than on 316L and 316LVM. This was confirmed by count-

ing the number of cells in six random areas of 0.03 cm² in the SEM images of the different samples and determining the average (figure 8). Therefore, results obtained from the present studies suggest that the number of adherent cells population at different coupons of SS-containing nitrogen was higher due to the high rate of cells proliferation, which was confirmed by SEM results. There was no evidence of changes in cell morphology or damage. The corrosion resistance of SSs is enhanced with nitrogen content, which represented a reduction of corrosion

products available to interact with the biological environment. Therefore, HNS could exhibit better cytocompatibility with increasing nitrogen content, in comparison to 316L and 316LVM.

It has been observed that HNS with different nitrogen content has an influence on the surface energy value, which in turn influenced the cytocompatibility. With increasing nitrogen content, the surface wettability and total surface free energy (SFE) increase (Ma *et al* 2012). The increase in SFE is due to increase in polar component (γ^p), which increases with nitrogen content in the alloy. High polar components improve the wettability of material (Wan *et al* 2010). Oxide layer with high surface energy on the surface of biomaterials is hydrophilic because of binding structural water and formation $-OH$ and $-O^{2-}$ groups in its outermost layer (Zhao *et al* 2007). In general, the biomaterial with high surface energy is conducive to cell adhesion and spreading. Surface energy modulates protein adsorption, which further regulates cell adhesion, cell spreading and proliferation (Kennedy *et al* 2006). This can have consequences for the *in vivo* response to a material. Higher surface energy and increased wettability have been shown to enhance interaction between an implant surface and its biological environment (Baier *et al* 1984). It is clear from contact angle measurements that nitrogen in steel influences the hydrophilic character of steel and it increases with

nitrogen content, which in turn influences the cytocompatibility. Thus, it is deduced that HNS exhibits better cell adhesion and cytocompatibility with increasing nitrogen content. Further, the samples containing carbon and nitrogen show more cell proliferation and cell adhesion, indicating that carbon has no adverse effect and somewhat also enhances the cell proliferation. These results

Figure 6. OD values of DL cells on different SSs determined by MTT assay after 48- and 72-h incubation.

Figure 7. SEM images of DL cells cultured on different SS surfaces after 1, 3 and 5 days incubation.

Figure 8. Average number of cells counted in six random areas of 0.03 cm² of each SS sample.

are in close agreement with those obtained by Buhagiar *et al* (2011). They studied S-phase surface layers formed on 316LVM and HNS by plasma surface alloying with nitrogen, carbon and both carbon and nitrogen and reported that osteoblast cells prefer surfaces that are rich in both nitrogen and carbon.

4. Conclusions

The results indicated the formation of a more protective oxide layer on the surface of HNS which was demonstrated by the higher values of R_1 and R_2 . HNS exhibited higher value of R_{ct} . The EIS spectra are best fitted using an electrical equivalent circuit (EEC) characterized by two branches (CPE , R in parallel). This EEC is consistent with the model of a two-layer structure for the passive film. The values of the I_{corr} for SS samples decreased with increase in nitrogen content. The E_{corr} for nitrogen bearing SS are more positive. The maximum corrosion rate was observed for the 316L and it decreases with increase in nitrogen content. The maximum breakdown potential was observed for 316MnN₃, and the minimum breakdown potential was observed for 316MnN₁. It has also been observed from the results that the 316LVM is more corrosion resistant than 316L. As to the pits, morphology, shallower and smaller pits were associated with HNS compared with 316L and 316LVM. The steel having more carbon content show less number of pits, indicating that nitrogen suppresses the pit formation even in the presence of carbon. The HNS had higher cell proliferation and cell growth and it increases by increasing nitrogen content. The value of contact angles was found to decrease with increase in nitrogen content. This indicates that the hydrophilic character increases with increase in nitrogen content, which is further attributed to enhance

the surface free energy, which would be conducive to cell adhesion, which in turn increases the cell proliferation. Therefore, HNS could show better corrosion resistance and biocompatibility due to the reduction of metallic ions and corrosion products available to interact with the biological environment. However, further studies are essential for these materials before using it for biomedical applications.

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References

- ASTM F 2581-07, Standard specification for wrought nitrogen strengthened 11 manganese-17 chromium-3 molybdenum low-nickel stainless steel alloy bar and wire for surgical implants (UNS S29225)
- Azumi K, Ohtsuka T and Sato N 1986 *Trans. Jpn. Inst. Met.* **27** 382
- Abreu C M, Cristobal M J, Losada R, Novoa X R, Pena G and Perez M C 2004 *J. Electroanal. Chem.* **572** 335
- Baier R E, Meyer A E, Natiella J R, Natiella R R and Carter J M 1984 *J. Biomed. Mater. Res.* **18** 327
- Bal W, Kozowski H and Kasprzak K 2000 *J. Inorg. Biochem.* **79** 213
- Baba H, Kodama T and Katada Y 2002 *Corros. Sci.* **44** 2393
- Buhagiar J, Bell T, Sammons R and Dong H 2011 *J. Mater. Sci: Mater. Med.* **22** 1269
- Clayton C R 1986 *Passivity mechanisms in stainless steels: Mo-N synergism*, Report No. N00014-85-K-0437. New York
- Disegi J A and Eschbach L 2000 *Injury* **31** 4
- Fini M, Aldini N, Torricelli P, Glavaresi G, Borsari V, Lenger H, Bernauer J, Glardino R, Chiesa R and Cigada A 2003 *Biomaterials* **24** 4929
- Fini M *et al* 2004 *J. Biomed. Mater. Res. B Appl. Biomater.* **B71** 30
- Gurappa I and Kumar M K 2000 *Evaluation of corrosion resistance of plasma sprayed alumina, magnesia-stabilized zirconia and yttrium-stabilized zirconia coatings on 316 stainless steel for biomedical applications. Proceedings of Global 2000 Corrosion Meeting* (Mumbai, India: Quest Publications), vol 1, p 561
- Ge H, Zhou G and Wu W 2001 *Appl. Surf. Sci.* **211** 321
- Gurappa I 2002 *Mater. Charac.* **49** 73
- Ha H Y, Jang H, Kwon H S and Kim S J 2009 *Corros. Sci.* **51** 48
- IARC 1999 *Monographs on the evaluation of carcinogenic risks to humans: surgical implants and other foreign bodies* (Lyon) vol. 74, p 65

- Jorcin J B, Orazem M E, Pebere N and Tribollet B 2006 *Electrochim. Acta* **51** 1473
- Kuroda D, Hiromoto S, Hanawa T and Katada Y 2002 *Mater. Trans.* **43** 3100
- Kennedy S B, Washburn N R, Simon Jr C G and Amis E J 2006 *Biomaterials* **27** 3817
- Lu Y C, Bandy R, Clayton C R and Newman R C 1983 *J. Electrochem. Soc.* **130** 1774
- Lee J B 2006 *Mater. Chem. Phys.* **99** 224
- Mudali U K, Reynders B and Stratmann M 1994 *Mat. Sci. Forum.* **723** 185
- Menzel J, Kirschner W and Stein G 1996 *ISIJ Int.* **36** 893
- Mudali U K, Dayal R K, Gnanamoorthy J B and Rodriguez P 1996 *Mat. Trans. JIM* **37** 1568
- Milosev I and Strehblow H H 2000 *J. Biomed. Mater. Res.* **52** 404
- Ma T, Wan P, Cui Y, Zhang G, Li J, Liu J, Ren Y, Yang K and Lu L 2012 *J. Mater. Sci. Technol.* **28** 647
- Newman R C, Lu Y C, Bandy R and Clayton C R 1984 *Proceedings of the ninth International congress on metallic corrosion* (Ottawa, Canada: National Research Council), vol 4, p 394
- Newman R C and Shahrabi T 1987 *Corros. Sci.* **27** 827
- Osozawa K, Okato N, Fukase Y, Yokota K and Boshoku-Gijyutsu 1975 *Corros. Eng.* **24** 1
- Osozawa K, Okato N, Stahele R W and Okada H (eds) 1976 in *Passivity and its breakdown on iron and iron base alloys* (NACE), p 135
- Olsson C O A 1995 *Corros. Sci.* **37** 467
- Ohno H, Tanabe H, Sakai A, Misawa T and Zairyo-to-Kankyo 1998 *Corros. Eng.* **47** 584
- Omanovic S and Roscoe S G 1999 *Langmuir* **15** 8315
- Omanovic S and Roscoe S G 2000 *J. Coll. Interf. Sci.* **227** 452
- Olsson C O A and Landolt D 2003 *Electrochimica Acta* **48** 1093
- Palit G C, Kain V and Gadiyar H S 1993 *Corrosion* **49** 977
- Patterson R F A J 1999 *Corros. Sci.* **41** 1639
- Ren Y, Wan P, Liu F, Zhang B and Yang K 2011 *J. Mater. Sci. Technol.* **27** 325
- Sumita M 1997 *Orthop. Surg.* **48** 927
- Schmidt C, Ignatius A and Claes L E 2001 *J. Biomed. Mater. Res.* **54** 209
- Shih C C, Shih C M, Su Y Y and Lin S J 2003 *J. Biomed. Mater. Res.: Part A* **67** 1320
- Shahryari A, Omanovic S and Szpunar J A 2008 *Mater. Sci. Eng.* **C28** 94
- Sudesh T L, Wijesinghe L and Blackwood D J 2008 *Corros. Sci.* **50** 23
- Tschiptschin A, Aidar C, Neto F and Alonso-Falleiros N 1999 *Mater. Sci. Forum* **569** 318
- Thomann U I and Uggowitzer P J 2000 *Wear* **239** 48
- Takazawa K, Miyagawa H and Hariya A 2003 *Jpn. Soc. Artif. Organs.* **6** 71
- Talha M, Behera C K and Sinha O P 2012 *J. Chem. Pharmaceut. Res.* **4** 203
- Vanini A S, Audouard J P and Marcus P 1994 *Corros. Sci.* **36** 1825
- Vahter M, Berglund M, Akesson A and Liden C 2002 *Environ. Res.* **88** 145
- Woodman J L, Jacobs J J, Galante J O and Urban R M 1984 *J. Orthop. Res.* **1** 421
- Wan P, Ren Y, Zhang B and Yang K 2010 *Mater. Sci. Eng.* **C30** 1183
- Yu Z S 1987 *Applications of rare earths in iron and steel* (in Chinese), (Beijing: Metallurgical Industry Press) p 282
- Yamamoto A, Kohyama Y, Kuroda D and Hanawa T 2004 *Mater. Sci. Eng.* **C24** 737
- Zhao G, Raines A L, Wieland M, Schwartz Z and Boyan B D 2007 *Biomaterials* **28** 2821