Materials Science and Engineering C 35 (2014) 77–84

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Effect of cold working on biocompatibility of Ni-free high nitrogen austenitic stainless steels using Dalton's Lymphoma cell line

Mohd Talha [a], Sanjay Kumar [b], C.K. Behera [a], O.P. Sinha [a,*]

[a] Centre of Advanced Study, Department of Metallurgical Engineering, Indian Institute of Technology (Banaras Hindu University) Varanasi 221005, Uttar Pradesh, India
[b] Centre of Advanced Study, Department of Zoology, Banaras Hindu University, Varanasi 221005, Uttar Pradesh, India

ARTICLE INFO

Article history:
Received 22 June 2013
Received in revised form 5 October 2013
Accepted 19 October 2013
Available online 1 November 2013

Keywords:
HNS
316LVM
Cytotoxicity evaluation

ABSTRACT

The aims of the present work are to explore the effect of cold working on in-vitro biocompatibility of indigenized low cost Ni-free nitrogen containing austenitic stainless steels (HNSs) and to compare it with conventionally used biomedical grade, i.e. AISI 316L and 316LVM, using Dalton's Lymphoma (DL) cell line. The MTT assay [3-(4,5-dimethythiazol 2-yl)-2,5-diphenyltetrazolium bromide] was performed on DL cell line for cytotoxicity evaluation and cell adhesion test. As a result, it was observed that the HNS had higher cell proliferation and cell growth and it increases by increasing nitrogen content and degree of cold working. 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 and degree of cold working. This indicates that the hydrophilic character increases with increasing nitrogen content and degree of cold working which further attributed to enhance the surface free energy (SFE) which would be conducive to cell adhesion which in turn increases the cell proliferation.

1. Introduction

Austenitic stainless steel (e.g. 316L) is one of the representatives of metallic biomaterials and is widely used as dental and orthopedic devices as well as cardiovascular stents because of its good mechanical properties, corrosion resistance, easy processing, and acceptable biocompatibility and very low cost as compared with other metallic materials. These conventional stainless steels (SSs) contain nickel (12.0–15.0%) which causes negative reactions in the body at normal physiological conditions [1–3]. Developments of new metallic materials with high mechanical properties but without nickel are attracting attentions of both material scientists and clinical doctors, and among them nickel-free high nitrogen austenitic stainless steels (HNSs) are apparent alternative materials. To maintain an austenitic microstructure in the alloy nickel reduction is balanced by an addition of nitrogen and manganese. Nitrogen containing steels are one of the potential materials for biomedical applications which exhibit attractive mechanical properties, good corrosion resistance and adequate biocompatibility [4–11]. Ren [12] and Peng Wan [13] studied the effect of nitrogen content on blood compatibility of SSs including platelet adhesion and kinetic clotting time and suggested that HNS exhibits better blood compatibility than 316L and 317L. They demonstrated that as the nitrogen content increases, the number of adherent platelets decreased correspondingly and the initial clotting time becomes longer. Further, in-vitro studies in cytogenetic effects, the Ames test and in-vivo studies after bone implantation in the sheep tibia indicate that a new nickel-free nitrogen containing stainless steel, P558, is devoid of mutagenicity and genotoxicity [9,10,14,15] indicating that it was very biocompatible with a promising future as an implant material. Also comparing with the cytocompatibility of Fe–Cr–Mo–N, Fe–Cr–Mo and 316L, SS indicate that Fe–Cr–Mo–N had higher cell growth than 316L in static and dynamic conditions [16]. Nickel was detected in the extracting media of 316L and Fe–Cr–Mo while there was no nickel detected in that of Fe–Cr–Mo–N alloys.

Hydrophilicity is an important factor for a biomaterial, since it promotes cell growth and proliferation, hence it is directly related to biocompatibility. In a scanty literature, very few reports are available regarding SSs which indicate that improvement of surface wettability will influence the albumin adhesion and enhance the cell compatibility [17–19]. The cytocompatibility of materials is closely related to the surface properties, particularly the surface energy as demonstrated by Tianchi Ma et al. in their studies [20]. They obtained values of contact angle with distilled water of HNS and 317L and detected in the results that HNS is more hydrophilic than 317L. Increasing the nitrogen content in the alloy enhances the hydrophilic character of HNS surface i.e. increases surface free energy (SFE).

Metallurgical parameters like cold working (c.w.), inclusions and alloy composition might affect the biocompatibility of austenitic SSs. SSs undergo different levels of cold working during the final manufacturing stage of components. During cold working, strain-induced martensite and residual stresses are significantly introduced on the surface of the alloy [21] which may affect the cytocompatibility of the alloy. Cold working in general introduces deformation sub-structures like dislocation networks, twins, deformation bands, cell structures and emerging slip steps at the surfaces [22,23]. The effect of cold working on

* Corresponding author. Tel.: +91 9452825977; fax: +91 542 2369478.
E-mail address: opsinha.met@itbhu.ac.in (O.P. Sinha).

0928-4931/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.msec.2013.10.017

cytocompatibility of austenitic SSs has still remained an unexplored area of research. The purposes of present studies were to compare in-vitro cytocompatibility of HNS with conventional AISI 316L and 316LVM, used for biomedical applications and to evaluate the effect of cold working on cytocompatibility and cell adhesion of these alloys using Dalton's Lymphoma (DL) cell line. The surface wettability was also investigated by water contact angle measurement. Usually, steels which are used for biomedical applications contain low carbon (≤0.03%) but some newly developed high nitrogen SSs contain carbon. For example, P558 alloy contains about 0.20 wt.% carbon and F2581 contains 0.15–0.25 wt.% carbon [5,24]. That is why we have also studied the synergetic effect of carbon and nitrogen on cytocompatibility and cell adhesion. Therefore, one alloy which contains high nitrogen (0.34 wt.%) as well as carbon (0.48 wt.%) and other two alloys having low carbon (≤0.08 wt.%) but high nitrogen (0.43 wt.% and 0.52 wt.%) were selected for the present study along with conventional 316L and 316LVM.

2. Experimental

2.1. Reagents and culture media

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 obtain from HiMedia, Mumbai, India. All other chemicals stated otherwise were obtain either from Super Religare Laboratory (SRL), Mumbai, India or HiMedia, Mumbai, India.

2.2. Cell lines and animal used

A spontaneous occurring T cell lymphoma commonly called as Dalton's Lymphoma (DL) was harvested from DL bearing mice after 18 days of post DL cell transplantation, when yield is at maximum and subjected to experimental setting.

2.3. Materials

Three austenitic stainless steels ($316MnN_1$, $316MnN_2$ and $316MnN_3$) 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 ferrochrome, etc.) in appropriate proportions to meet aimed composition of alloy in an induction furnace. These ingots were solutionized at 1050–1070 °C to avoid segregation. Then specimens of 80 × 12 × 2 mm slices 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 respectively. 316L and 316LVM stainless steels were obtained from Mishra Dhatu Nigam Ltd. (MIDHANI), Hyderabad, India. These steels were received in hot rolled and annealed conditions and used for study in the same conditions. The chemical compositions of all five austenitic SSs are shown in Table 1. All the alloy specimens were cold rolled to 10% and 20% reductions in thickness values. Coupons of 10 × 10 mm size were cut from them, polished successively with emery papers 600–800–1000–1200 grades. Specimens were washed with de-ionized water followed by acetone and dried in air.

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.

2.4. Contact angle measurement

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

2.5. Biocompatibility evaluation

2.5.1. Cell proliferation assays (MTT test)

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% fetal bovine serum (FBS), anti-biotic and antifungal (100 U per mL penicillin, 100 μg per mL streptomycin and 0.025 μg 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 microtitter plate seeded with 0.5×10^6 cells per wells and 10 μL of MTT [3-(4,5-dimethythiazol 2-yl)-2,5-diphenyltetrazolium bromide] was added in freshly prepared PBS (5.0 mg/mL) and incubated for 6–12 h, thereafter cells were centrifuged at 100 ×*g* for 5 min at 4 °C. Supernatant was then carefully discarded without disturbing the dark-blue formazan crystals and 200 μL of DMSO was added to each well to dissolve the formazan crystals. 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:

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

2.5.2. Cell attachment

The stainless steel coupons were separately located in the bottom of 24-well micro plate/35 mm Petri dish. DL cells at the density of 1×10^6 cells per mL were seeded onto the coupons and culture 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 cells. Adherent cell populations were then fixed in 2.5% of glutaraldehyde solution for 45 min and dehydrate 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 scanning electron microscope (FEI Quanta 200 FEG).

3. Results and discussion

3.1. Contact angle

The values of contact angle obtained with distilled water are shown in Table 2. It can be seen that all differences are statistically significant for the experimental samples, among which the HNS is more hydrophilic than 316L and 316LVM. Increasing nitrogen content enhances the hydrophilic character, i.e. lower contact angle values. Moreover, the values of contact angles slightly decrease with increase in the degree of cold working indicating that the hydrophilic character of the surface increases to some extent.