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Cytotoxicity study of plasma-sprayed hydroxyapatite coating on high nitrogen austenitic stainless steels

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Abstract Stainless steel has been frequently used for temporary implants but its use as permanent implants is restricted due to its low pitting corrosion resistance. Nitrogen additions to these steels improve both mechanical properties and corrosion resistance, particularly the pitting and crevice corrosion resistance. Many reports concerning allergic reactions caused by nickel led to the development of nickel free stainless steel; it has excellent mechanical properties and very high corrosion resistance. On the other hand, stainless steels are biologically tolerated and no chemical bonds are formed between the steel and the bone tissue. Hydroxyapatite coatings deposited on stainless steels improve osseointegration, due their capacity to form chemical bonds (bioactive fixation) with the bone tissue. In this work hydroxyapatite coatings were plasma-sprayed on three austenitic stainless steels: ASTM-F138, ASTM-F1586 and the nickel-free Böhler-P558. The coatings were analyzed by SEM and XDR. The cytotoxicity of the coatings/steels was studied using the neutral red uptake method by quantitative evaluation of cell viability. The three uncoated stainless steels and the hydroxyapatite coated Böhler-P558 did not have any toxic effect on the cell culture. The hydroxyapatite coated ASTM-F138 and ASTM-F1586 stainless steels presented cytotoxicity indexes ($IC_{50\%}$) lower than 50% and high nickel contents in the extracts.

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Introduction

Metals are found in abundance in nature and are usually present in the human tissue. The human body can exert control over the metal level and allergenic reactions are activated at the moment when the body considers that the maximum, tolerable level, of a certain metal has been exceeded. Many metals found in the human body as traces, e.g. iron, zinc, copper, manganese, nickel, cobalt, molybdenum, titanium and chromium [1] are essential for the human life. However, when present in larger amounts they can induce toxic reactions.

The incidence of clinical problems induced by metal sensitization is not totally known. However, the occurrence of allergic reactions, caused by metal is relatively high; 15% of the population suffer from nickel allergy and 8% from chromium allergy [2]. Some reports indicate that patients with metal sensitizition can use metallic prostheses without presenting problems [3].

The occurrence of allergic reactions depends on the way the metallic elements are found inside the body. When in contact with the skin and other tissues, metals and metallic salts can bond to proteins of the host tissue and produce large molecules capable of activating allergies [4]. Although nickel ions can be eliminated quickly from the body in urine, allergies and inflammations in the skin and in the mucous membranes, caused by nickel, are frequently reported [5]. On the other hand, chromium ions are eliminated less rapidly remaining in the tissue. Hexavalent chromium is the most biologically active and toxic form of chromium in the human body [2]. It can react with the cells, reducing itself to trivalent chromium, accumulating in the body and inducing high levels of toxicity.

Stainless steels used in implants, normally have nickel in concentrations between 8–14 wt.%, chromium up to 20 wt.%, small amounts of manganese and molybdenum, and maximum of 0.03 wt.% carbon. A passive layer of chromium oxide is formed at the surface of stainless steels, inhibiting cation dissolution; however, when the layer is dissolved by the action of aggressive environments, only the ions present in the film (Cr and Fe) are released. Accordingly [6], as long as nickel is not contained in the steel's passive layer nickel ions present in the bulk material are not released in saliva.

High nitrogen stainless steels are known for their excellent mechanical properties and corrosion, pitting corrosion and corrosion-fatigue resistance [7]. Many reports concerning allergic reactions caused by nickel led to the development of nickel free austenitic stainless steels [8, 9].

When stainless steels are used in orthopedic implants osseointegration does not occur. In order to increase osseointegration of stainless steels, implants have been coated with hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ [10]. Many different processes have been used to deposit hydroxyapatite, plasma spray being the most used industrially among them. Hydroxyapatite plasma coatings present excellent mechanical and physical properties.

The aim of this work was to evaluate the cytotoxicity of plasma-sprayed hydroxyapatite coating on high nitrogen austenitic stainless steel, using the neutral red uptake method.

Experimental

Sample preparation

ASTM F138, ASTM F1586 and Böhler P558 stainless steels, with compositions shown in Table 1, were used as subtracts. Uncoated samples were 6 mm in diameter and 9 mm in length. Hydroxyapatite coated samples were 9 mm in diameter and 60 mm in length.

Commercial hydroxyapatite powder (Steach chemical), with average particle size of 8.5 μm and surface area of 65.42 g/m^2 was used. The hydroxyapatite powders were kept in an oven at 40°C for one hour before coating. The powder was plasma sprayed using a Metco 4MP system; ventilation with air was used all the time to keep the environment clean and to prevent sample heating. Before spraying, the substrates were grit blasted using alumina (Al_2O_3) and ultrasonically cleaned in acetone. The deposition was performed manually with horizontal movements. Five passes were used. The parameters used for coating deposition are shown in Table 2.

Table 2 Plasma spray parameters

Deposition parameter	Value
Voltage	60 V
Current	400 A
Spray distance	100 mm
Primary gas	Hydrogen

Transverse sections were mounted in bakelite, metallographicaly prepared and polished up to 1 μm diamond paste. The coatings were protected with electrolytic nickel plate before mounting. Scanning electron microscopy (SEM) was carried out in a Philips XL30TMP to examine the morphology of the coating and energy dispersive spectroscopy (EDS) analysis was performed to measure changes in the chemical composition of the coatings. X-ray diffractometry (XRD) was carried out in a Philips MPD 1880 equipment with a CuKα tube, to identify the phases present in the coating. The coating crystallinity was calculated, evaluating the area bellow the most intense picks of XRD patterns (2θ within 25–37°). After deposition, the coatings were thermal treated at 600°C for one hour, to improve the hydroxyapatite crystallinity.

Cytotoxicity test

The biocompatibility study was based on International Standardization Organization, ISO 10993 [11, 12]. The cytotoxicity test was carried out on coated and uncoated stainless steels extracts using the neutral red uptake method. The samples were cleaned and submitted to sterilization by γ radiation. The extracts were prepared by immersion of these samples with 0.5 cm^2 of exposed area for each ml of cell culture medium (MEM) supplemented with 5% fetal calf serum (SBF) and kept at 37 ± 1°C for 10 days, in a humidified Binder CB150 incubator with 5% CO_2. Positive and negative controls were used to confirm the good performance of the assay. A 0.02% phenol solution was used as positive control and presented toxicity and polished titanium sheets, used as negative control, presented no toxic effect. The cell

Table 1 Chemical composition of the stainless steels

Element (wt%)	Cr	Mn	Mo	N	C	Si	Ni	Nb
ASTM-F138	17.50	1.69	2.70	0.068	0.01	0.16	14.50	—
ASTM-F1586	21.06	3.62	2.44	0.37	0.017	0.16	10.60	0.40
Böhler P558	16.70	9.90	3.30	0.48	0.16	0.40	0.014	—

line used was NCTC clone 929 from American Type Culture Collection bank. The serially diluted samples extract (100; 50; 25; 12.5 e 6.25%) were put in contact with cell culture in a 96 wells microplate and it was incubated for 24 h at 37°C in a humidified atmosphere with 5% CO_2. The samples and the controls were tested in triplicate. After 24 h, the extract was replaced by 0.2 ml MEM with 50 μg neutral red/ml. After 3 h of incubation, period for incorporation of neutral red by no injured cells, the medium of the microplate was discarded and the plate was washed twice with phosphate-saline buffer (PBS) and after with a solution of 1% $CaCl_2$ in 0.5% formaldehyde. In each well was added 0.2 ml of extraction solution containing 1% acetic acid in 50% ethanol. This solution extracts incorporated neutral red from the cells. The optical density (OD) of the microplate was measured in a Spectrophotometer Sunrise, Tecan, in 540 nm.

The extracts samples were also analyzed by atomic emission spectroscopy on Spetroflame da Spectro, to determine the amount of metal dissolved and compared with the cytotoxicity results.

Results and discussion

Samples characterization

The morphology of the cross-section of the as-sprayed hydroxyapatite coatings, deposited on the three stainless steels samples are shown in Fig. 1. One can observe three regions, the substrate (S), the coating (HA) and the electrolitic nickel plate (PN). For the three steels, the coating thickness was 120 μm approximately. The EDS chemical analysis (not shown) indicated calcium, phosphorus and oxygen, the basic components of hydroxyapatite. The coatings in all three specimens showed partially melted particles, flattened particles, spherical particles and well distributed pores through all the layer. In Fig. 1, the interface between the high nitrogen austenitic stainless steel and the hydroxyapatite layer can be observed. The black regions around the interface are constituted by Al_2O_3 particles that remained after grit blasting the surface, before coating.

Figure 2 shows the XRD patterns of hydroxyapatite coatings before and after thermal treatment at 600°C for one hour, together with the pattern of the hydroxyapatite powder. The XRD patterns of the as-coated samples show peaks of crystalline hydroxyapatite, calcium oxide and beta and alpha tricalcium phosphate as well as a hump corresponding to amorphous hydroxyapatite [13]. After thermal treatment, the results indicate the crystallization of the amorphous hydroxyapatite, corresponding to an increase in the diffraction peak intensities of crystalline hydroxyapatite. The biggest intensity calcium oxide CaO peak at $2\theta = 37.5°$ [14, 15] and a trace of beta tricalcium phosphate and alpha tricalcium phosphate, still remained.

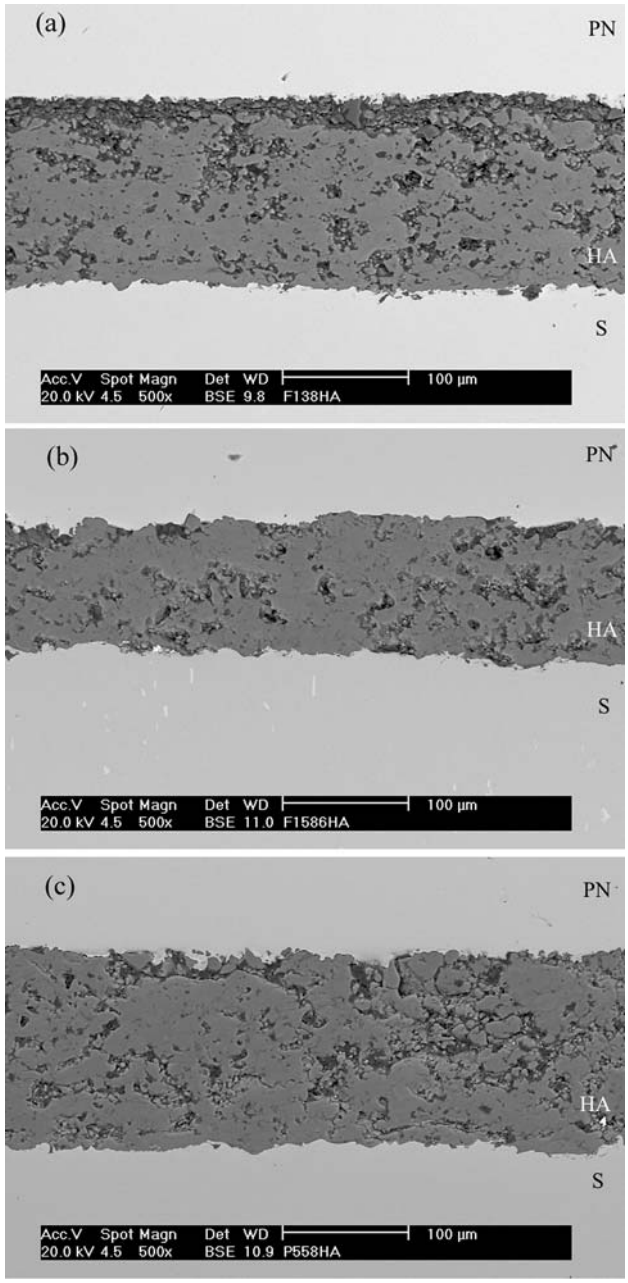

Fig. 1 SEM cross-sectional microestruture of the as-sprayed hydroxyapatite coatings deposited on high nitrogen austenitic stainless steels (a) F138, (b) F1586, (c) P558. Substrate (S), hydroxyapatite coating (HA) and plate electrolitic nickel (PN).

If the coating is absorbed, the implant can move inside the bone releasing coating particles in the surrounding body tissues [15]. The other calcium phosphate phases present greater dissolution rates [16] than crystalline hydroxyapatite. Thus, the hydroxyapatite coating must have the biggest reasonable degree of crystallinity. A thermal treatment at 600°C can be used to increase crystallinity.

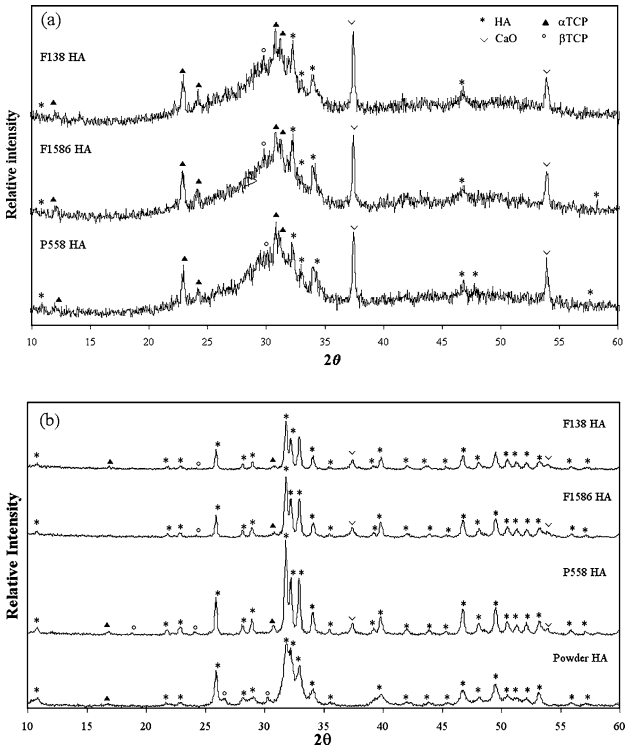

Fig. 2 XDR patterns of hydroxyapatite coatings (a) before and (b) after thermal treatment at 600°C for one hour and for the hydroxyapatite powder.

The degree of crystallinity of all coatings increased up to 97% and the presence of 3% of other phases is not critical as the ASTM F1185-88 standard allows up to 5% of other phases in the coating. The thermal treatment did not change the structure of the metallic substrate, as carbide precipitation was not detected and did not occur.

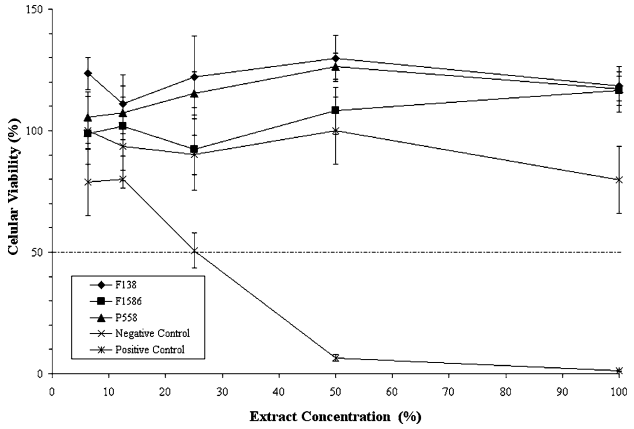

Fig. 3 Cellular viability curves of high nitrogen austenitic stainless steels using neutral red uptake technique in cytotocixity assay.

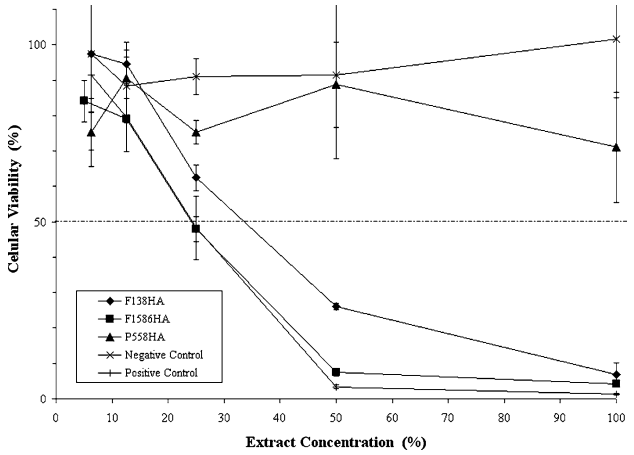

Fig. 4 Cellular viability curves of plasma-sprayed hydroxyapatite coating on high nitrogen austenitic stainless steels using neutral red uptake technique in cytotocixity assay.

Cytotoxicity test

The determination of the cell viability and/or cell growth is used as a tool for the measurement of the cells behavior when in contact with implanted devices. In the cytotoxicity test, by neutral red uptake technique, quantitative measurement of the cell viability can be made based on statistical data, increasing the reliability of the test [11].

The cytotoxic effect was evaluated by cell viability percentage, calculating the average of the optical density of each extract concentration in relation to a group of control cells. A plot of the variation of the % of cell viability with the concentration of the extract was made allowing to determination of the cytotoxicity index ($IC_{50\%}$). $IC_{50\%}$ is the extract concentration that produces 50% of death or lyses in the cell population after the test.

Figure 3 shows the cellular viability curves of the uncoated stainless steels. The results showed that none of the uncoated stainless steels presented any toxic effect. In the cytotoxicity assay, they showed the same behavior of the negative control.

The results for hydroxyapatite coated high nitrogen stainless steels are shown in Fig. 4. The P558 steel coated with hydroxyapatite also did not present cytotoxicity. In the other hand ASTM F138 and ASTM F1586 stainless steels coated with hydroxyapatite presented toxic effects with cytotoxicity index $IC_{50\%}$ of 34 and 25, respectively.

The results obtained by atomic emission spectroscopy, for a 100% extract concentration are shown in Table 3. The extract of uncoated steels and coated P558, showed that the metal levels were similar to those observed in the cell culture medium MEM. The extract of hydroxyapatite coated stainless steels ASTM F138 and ASTM F1586 presented high levels of chromium and nickel, elements considered by a number of authors [6, 17] as toxic.

The results shown in Table 3, higher levels of Cr and Ni in the extract of the toxic samples (HA coated F138 and

Table 3 Chemical analysis of extract of samples

	Metal (ppm)				
Sample	Fe	Cr	Ni	Mo	Mn
F138 HA	1.054 (±0.000)	1.681 (±0.004)	0.314 (±0.003)	0.032 (±0.010)	0.041 (±0.001)
F1586HA	1.864 (±0.015)	2.750 (±0.005)	0.478 (±0.003)	0.091 (±0.001)	0.173 (±0.001)
P558HA	3.400 (±0.014)	0.127 (±0.008)	0.007 (±0.001)	0.260 (±0.001)	1.400 (±0.000)
F138	0.076 (±0.004)	<0.008 (±0.002)	0.016 (±0.004)	<0.023 (±0.006)	0.010 (±0.003)
F1586	0.070 (±0.004)	<0.008 (±0.003)	0.010 (±0.002)	<0.023 (±0.009)	0.010 (±0.003)
P558	0.028 (±0.001)	<0.008 (±0.001)	0.008 (±0.001)	<0.023 (±0.010)	0.030 (±0.001)
MEM	<0.008 (±0.002)	<0.008 (±0.007)	<0.006 (±0.004)	<0.023 (±0.019)	<0.002 (±0.001)

F1586), are in accordance with the results presented above, concerning % of cell viability.

The Cr content in the extract of the coated P558 steel is greater than MEM, but not sufficient to cause toxicity. The Ni content in the extract of coated and uncoated P558 steel is similar to MEM. This is due to a very low nickel content in the P558 stainless steel.

The average chromium content in human tissues is approximately 0.2 ppm [1]. The amount of chromium in the extract of the hydroxyapatite coated F138 and F1586 was greater than 0.2 ppm and can cause injury to the cell culture during the test. The extracts of hydroxyapatite coated F138 and F1586 presented also nickel levels greater than the normal level in the human tissue, 0.1 ppm [1]. The iron levels found in all extracts were much greater than those found in MEM, although they do not cause cell damage. The iron is an essential metal and is found in tissues in levels up to 70 ppm [1].

Figure 2b. shows the presence of CaO in the XRD patterns for thermal treated coatings. Calcium oxide has no biocompatibility, consequently it should be avoided [16]. This phase appears in the hydroxyapatite coatings deposited on the three stainless steels, but only the F138 and F1586 presented toxicity. Therefore it could be deduce that toxic response is due to release of Cr and Ni more than the remained calcium oxide.

The coated steels ASTM F138 and ASTM F1586 presented cytotoxicity. Probably when stainless steel was submitted to plasma spray deposition some changes in the substrate structure and composition did happen, releasing some metal ions. For the P558 stainless steel, this kind of event did not happen, probably due to the absence of nickel and lower chromium content.

The high levels of metallic ions measured in the extract can also be explained, by diffusion through a network of interconnected pores in the hydroxyapatite. A pH reduction can take place, inside the pores, due to an increase in solution concentration (e.g. chloride ions belonging to MEM), breaking the steel passive film and affecting the corrosion resistance [18, 20]; this process can increase the concentration of ions released by corrosion.

Before deposition all three steels did not present toxicity. When the temperature is increased, some metallic ions of the F138 and F1586 stainless steels, may have been released reamining in the hydroxyapatite coating. After MEM imersion for 10 days, these ions released in solution by lixiviation damaged the cells in the microplate during the test.

Conclusions

Based on the results it can be concluded:

- ASTM F138, ASTM F1586 and P558 stainless steels did not present toxicity in the cytotoxicity test.
- After plasma-spray hydroxyapatite coating the ASTM F138 and ASTM F1586 stainless steels presented toxic effects.
- The hydroxyapatite plasma-sprayed coated P558 high nitrogen stainless steel showed the best results in the cytotoxicity test.

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