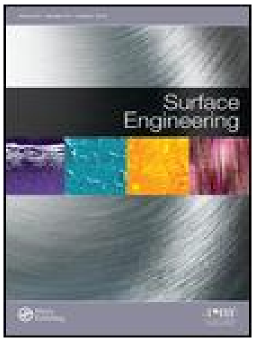

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In vitro study of cell behaviour on plasma surface modified titanium

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In recent years, several technologies that modify implant surfaces have been emerged. Among these techniques, the plasma nitriding process has been successfully applied in biomedical field. Nevertheless, its use in dental implants is quite limited owing to the high temperatures of the process (between 700 and 800°C), which causes distortion. In order to solve this problem, a new approach is proposed in the present paper, by which nitriding under a hollow cathode discharge is used to modify surfaces. Grade II Ti plates were submitted to nitriding under hollow cathode discharge conditions and treated at a temperature 450°C and pressure of 150 Pa for 1 h. These showed that plasma nitriding helped bring about a significant change in the surface texture of the treated plates. Furthermore, cell proliferation was 2·5 times as high as that of the untreated plates.

Keywords: Dental implants, Plasma nitriding, Osseointegration, Biomaterials, Cell behaviour

Introduction

The interaction between osteoblasts and a biomaterial depends on topography, chemical composition and surface structure of the materials.[1–3] These surface characteristics determine how the biological molecules will adsorb to the surface and, more particularly, determine the course of these adsorbed molecules. They also determine cell behaviour on contact.[1] When cells approach a surface they initially adsorb, they will adhere and spread. The first phase depends on the adhesion cells that influence their morphology and capacity to differentiate and proliferate.[3] Textured surfaces accelerate the initial healing phase because of greater protein adsorption, platelet accumulation and activation and fibre retention, thus promoting bone apposition in shorter periods of time.[4] A number of publications have reported significant failures of smooth surface implants when installed in maxilla with inadequate alveolar ridge height and low bone density (type IV bone); this has led to increased research and development of textured surfaces.[4]

Histologic and histomorphometric studies demonstrate that implants with rough surfaces obtain higher bone contact percentages in shorter time periods when compared to titanium implants with smooth or grooved surfaces.[5] It has been verified that roughness and porosity ~100 μm has promoted tissue growth within the pore. There are likely optimal surface roughness levels for different applications, but these remain as yet unknown.[6] Thus, surface topography cannot be quantified solely by roughness, as is often seen in the literature.[6,7] This may be explained by the osseointegration mechanism verified by Kasemo.[4] Based on this mechanism, the real precursor of osseointegration is the surface wettability of oxide caused by biological liquid and not roughness, as was previously imagined.[4] The water molecules are the first to reach the surface. Surface wettability influences the proteins and other molecules that appear a little later (in the order of micro or milliseconds). These biomolecules are soluble in water and have hydration (water) layers. The interaction between the surface water layer and the biomolecular water layer influences the fundamental kinetic processes and interface thermodynamics.[4,8] When the cells reach the surface they find themselves covered with proteins, whose layer has properties that are initially determined by preformed water layers.

Various studies have been developed in the search for surfaces that provide rapid osseointegration, modifying these surfaces using diverse processes[9] involving mechanical, chemical and physical methods for treating them, thus obtaining a wide diversity of textures.[10]

Among the techniques available for texturising surfaces, plasma nitriding has yielded encouraging results because of the excellent mechanical properties, chemical stability and biocompatibility achieved[11] when applied to titanium. Its use in hip, knee, shoulder and ankle implants has resulted in increased resistance to abrasion and reduced bacterial colonisation, when compared to other clinically used body implant surfaces.[12] A limitation, however, to the use of nitriding in dental implants is in the high temperature of the process, between 700 and 800°C, which may cause distortion of the implant due to its geometry and dimensional precision.[13]

An alternative solution to this problem is treatment using a hollow cathode design. This method consists in nitriding the material in a highly ionised atmosphere. High ion density causes higher energy surface bombardment.[14] This electrode configuration is used to increase

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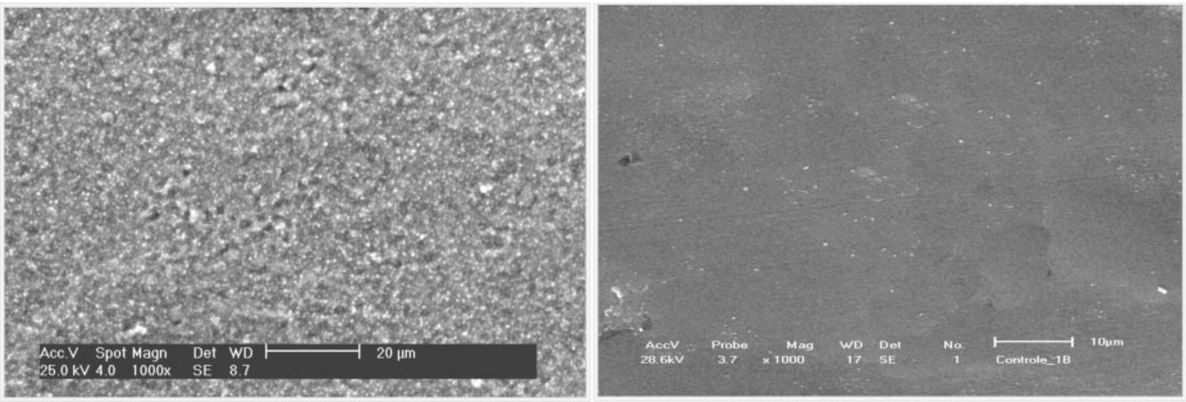


a b

1 Both plasma *a* nitrided titanium and *b* control surfaces observed under SEM

superficial damage and obtain a thermal gradient produced by the increased impact ionisation rate.[15]

In the present study, with dental implant purposes in mind, Ti plates were plasma nitrided using the hollow cathode discharge technique. Important characteristics such as wettability, roughness as well as cell behaviour have been analysed.

Materials and methods

In the present study 12 Ti cp (grade II) plates were used, measuring $15\times15\times1$ mm (height $\times$ width $\times$ thickness); these were divided into two groups: six plates using plasma nitriding under hollow cathode discharge and six untreated plates (control), all of which were used for *in vitro* observation of cell behaviour. The experimental apparatus used for plasma nitriding consists of a hermetically sealed chamber containing an anode in the upper flange and a cathode in a base.[16] The implant device to be nitrided was positioned on this base. The hollow cathode discharge configuration, consisting of a ring surrounding it with a lid on the upper part, was placed around the device. All the metallic parts were made of stainless steel, and the inner walls of the hollow discharge coated with Ti to avoid contamination. The distance between the surface of the sample and the upper lid, denominated here as distance between cathodes d_c, was fixed at 9 mm. The plates were treated at a pressure of 150 Pa, at 450°C, for 1 h. The temperature was measured by a chromel–alumel thermocouple in contact with the sample holder and controlled by the voltage and current between electrodes.

After completing the surface treatment, all the samples were submitted to a rigid cleaning protocol to remove the fats, proteins and carbohydrates using a solution of DEIV 3E under ultrasound conditions for 10 min, followed by washing in absolute alcohol and subjected to 10 min of ultrasound exposure. Another washing was performed in distilled water, followed by 10 min of ultrasound. Each step was repeated twice. The samples were then dried with a dryer for one minute and placed in packaging appropriate for hydrogen peroxide sterilisation. After sterilisation, the sessile drop technique for determining the static contact angle was performed for each of the samples. The samples were placed on a flat surface and using an adjustable volume digital micropipette with a 3·57% glucose solution (EURO-COLINS), 60% glycerine and dye, positioned perpendicularly and very near to the sample, 0·25 mL of solution was deposited onto its surface. In order to standardise the test and because the drops were very small, the change in angle was monitored at 1, 30 and 60 s.

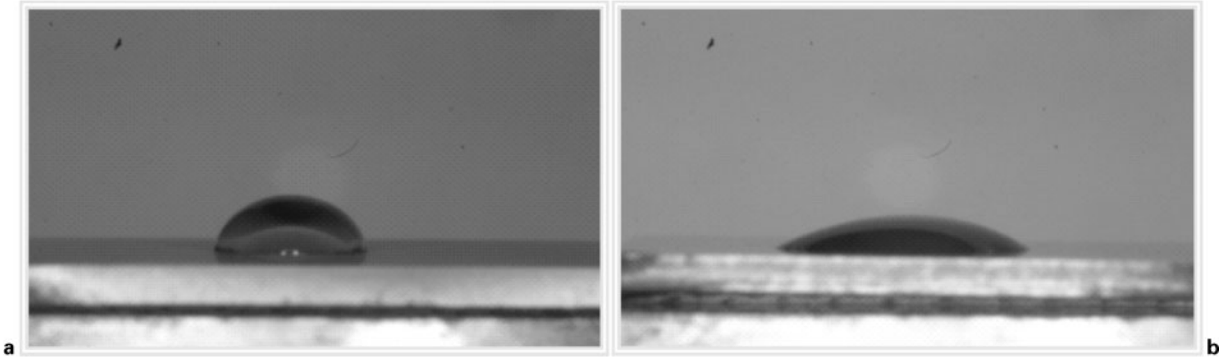
To analyse the roughness, the R_a parameter was measured using a Taylor Hobson SURTRONIC 3+ surface profilometer with a cutoff value of 0·25. The measurements were taken in three different directions, at angles of $\sim120°$ with dispersion in these three different directions.

The present study used an Osteo-1 cell line, provided by the department of oral pathology of the faculty of dentistry at the Universidade de São Paulo (USP). The cells were cultivated in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum and 1% antibiotic–antimycotic solution. In order to stimulate their growth the cells were incubated in a humid environment (stove) at 37°C, in an atmosphere containing 95% oxygen and 5% carbon dioxide. The culture medium was changed daily since the nutrients were consumed as soon as they grew. The culture medium was placed into a Neubauer chamber for cell counting using trypan blue dye exclusion method,[17] the nitrided samples and control samples were placed in 12 wells. All the samples were sterilised by gamma radiation. In this experiment, for observing the cell behaviour, 1×10^4 Osteo-1 cells per well were plated on all the samples in wells of 25 mm diameter. All 12 wells were counted 24, 48 and 72 h after plating. Using an Olympus BX 60M (Japan) optical microscope coupled to a computer running Image-Pro analysis software, version 4.51.22 for Windows. Cell proliferation was analysed by counting the number of sample adhering cells at different experimental times (one, two and three days) in the two groups, in duplicate. These data were used to study the cell behaviour of both experimental groups.

Results and discussion

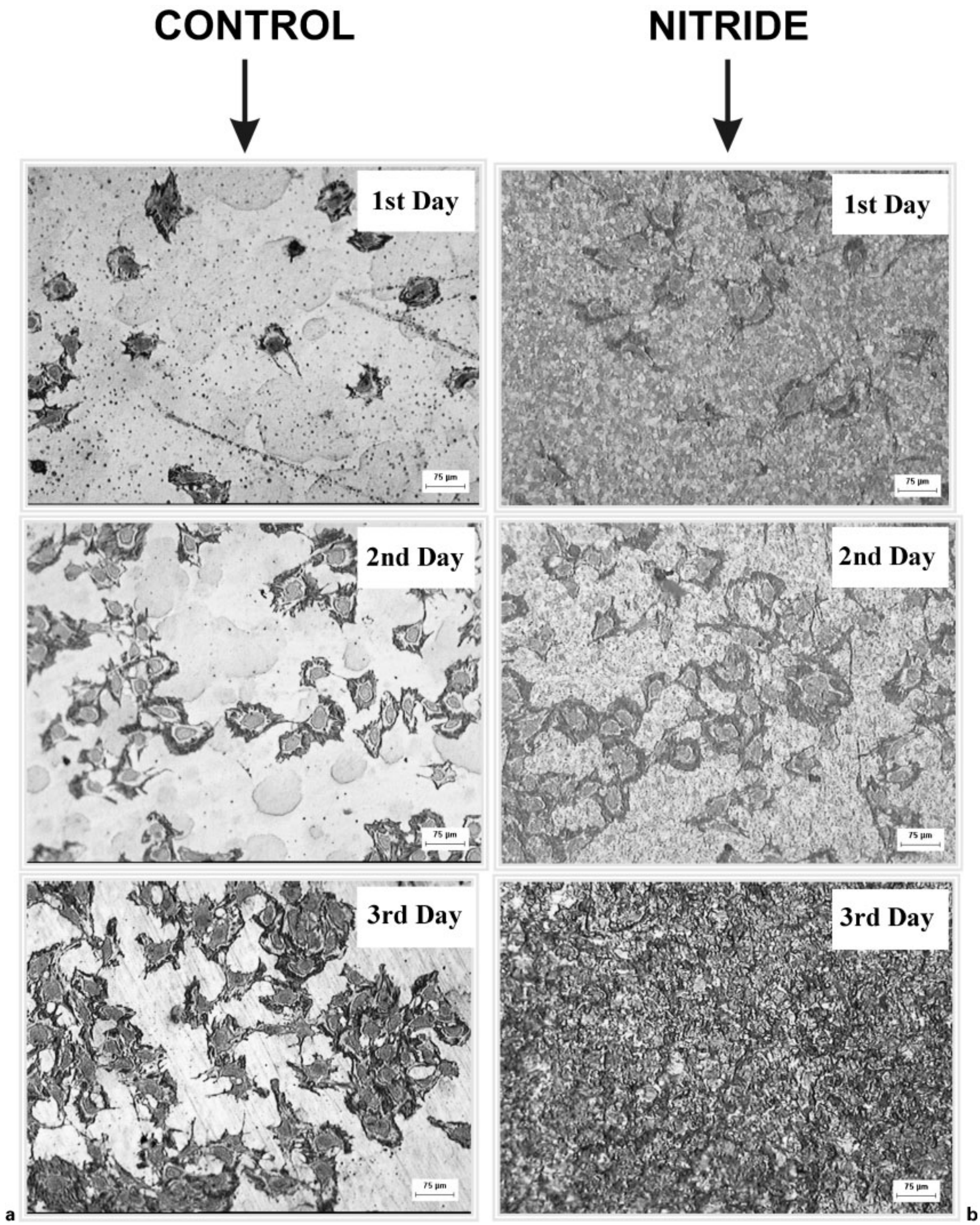
Plasma nitrided titanium surfaces observed under SEM exhibit a rough texture, with precipitates (white spots) finely dispersed and with a mean free space between them of 5 µm (Fig. 1). The presence of these precipitates, as well as their distribution, may be an important factor in assessing wettability and its relation to roughness.

It is known that surface roughness has an influence on the osseointegration of texturised surfaces.[18] Although roughness influences surface topography, good surface



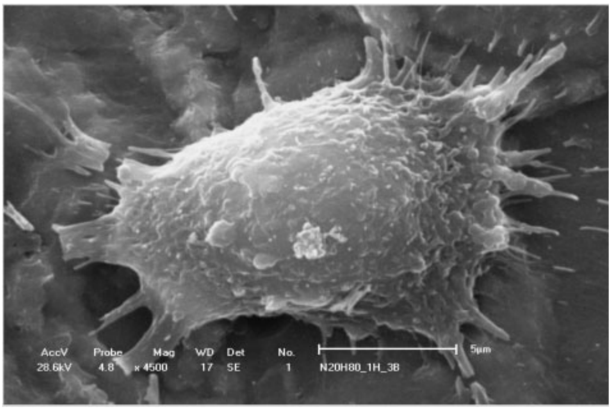
a control; *b* nitride

2 Sessil drop when spread onto nitrided and non-nitrided surface after 60 s

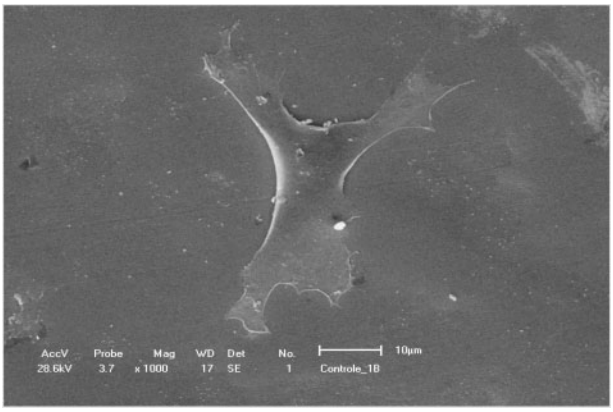


a control; *b* nitride

3 Evolutive stage of cell proliferation



4 Adhered cells for nitrided test samples



5 Adhered cells for control test samples

wettability is not only related to surface roughness. Studies on Ti surfaces with high roughness have shown diminished fibronectin absorption, a well known cell adhesion protein.[19] Another study investigated four different implant surfaces and concluded that acid attacked surfaces (R_a=0·62 μm) produced a better performance than those that had roughnesses with higher R_a values.[5] It can be concluded that increased roughness does not necessarily mean improved osseointegration.

In the present study, the roughness R_a on plates nitrided under hollow cathode discharge found was 0·45 μm, while the roughness found on the untreated plates was 0·22 μm. Although this value is small when compared to other values in the literature, the surfaces treated by this technique yielded better wettabilities than those treated by conventional techniques.[20]

Figure 2 illustrated the sessil drop when spread onto the nitrided and non-nitrided surface, after 60 s. For this time, a contact angle of 45° was verified for the non-nitrided surface, whereas for the nitrided surface this value was only 19°. The difference between contact angles may be attributed to the fact that the nitriding under hollow cathode discharge process promotes large topographic modifications. Since wettability is directly associated to the contact angle, this result is important for successful osseointegration, given that the mechanism initiates with the contact of water molecules with the biomaterial surface.[4]

Figure 3 presents the evolutive stage of cell proliferation for the first three days of culture on nitrided and untreated surfaces. The first piece of evidence is that the nitrided surface is biocompatible, since adhesion and proliferation occurred. The first day of culture revealed a slight increase in the number of cells on the nitrided plate when compared to control. On the second day, evidence of greater cell proliferation on the nitrided plate was observed, in addition to slight cell stretching for better adaptation to the plate surface. On the third day, proliferation was considerably greater on the nitrided plate than on the control, forming a carpetlike layer over the entire plate. The control plate contained a number of unfilled spaces.

In regard to the morphologic aspect of the adhered cells, numerous stress fibres were observed on the cell periphery that ensured their focal adhesion. Surface irregularities seem to guide cell adhesion in a differentiated manner. Voluminous, round and/or elongated cells were observed interacting with and spreading themselves over surface irregularities (Fig. 4).

Finally, it was observed that on the untreated test sample, the cell (Fig. 5) spreads itself over the surface with an elongated shape; no stressed fibre emission is seen, only stretching in order to occupy a greater surface area.

Conclusions

Based on the present study, the following can be concluded about Ti plate surfaces during plasma nitriding under hollow cathode discharge.

1. It is biocompatible and suitable for biomaterial use because of its cell adhesion and proliferation.
2. It was verified that both cell adhesion and proliferation in nitrided plates was greater than that of untreated plates.
3. The nitrided samples produced wettability results greater than those of the non-nitrided samples.
4. The rapid spreading of the sessil drop after 60 s in the nitrided samples did not occur in the untreated samples.
5. Plasma treated Ti samples exhibited increased surface roughness, when compared to the untreated surface.

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