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Stem cells behavior on titanium surfaces modified by plasma

Análise do comportamento de células-tronco em superfícies de titânio modificadas por plasma

Juliana Carvalho SÁ
Adjunct Professor - Department of Dentistry – University of Rio Grande do Norte - Natal - RN - Brazil.

Naisandra Bezerra da SILVA
Hugo Alexandre de Oliveira ROCHA
Adjunct Professors - Department of Biochemistry - Federal University of Rio Grande do Norte - Natal - RN - Brazil.

Clodomiro ALVES JÚNIOR
Titular Professor - Department of Mechanical Engineering - Federal University of Rio Grande do Norte – Natal – RN - Brazil.

Abstract

Over the last decade, a considerable development of new technologies to the modification of dental implants has been observed contributing to reduce the healing process and their use in areas with low bone density. Among the new techniques, plasma nitriding has showed excellent results. In this study, a superficial modification of commercial pure titanium (Degree II), by using two different plasma treatments (planar and hollow cathode nitriding) was accomplished aiming at an optimization of the surface for biomedical applications. An evaluation of the chemical composition in all samples was carried out, in addition to a study of their roughness and texture. Then, stem cells were deposited onto these surfaces and a comparison among their properties and their biological behavior was accomplished. The results showed that the nitriding techniques produced significant changes in the superficial texture of the Ti samples. The roughness test presented better results in samples nitrided by hollow cathode technique. Statistically significant differences were found in the cell proliferation of samples nitrided by hollow cathode nitriding when compared to samples without treatment. The used techniques were, therefore, effective and directly influenced the characteristics of the titanium surface and consequently, the stem cell behavior.

Keywords

Titanium; plasma; dental implant.

Introduction

Osseointegrated implants are considered reliable tools for tooth replacement. For its success, it is necessary that there be a strong interaction between this biomaterial and its surrounding medium. Because of this reason, several studies appear aiming to find a biocompatible material without producing an adverse local or systemic biological response [1]. Among several materials, titanium is the most used in dental and orthopedic implants because of its higher properties such as biocompatibility, modulus of elasticity higher than bone, and high resistance to corrosion when compared to other metals and metallic alloys [2,3].

The surgical protocols for installing these implants proposed by Branemark have undergone alterations [4]. Currently, there are protocols in which an immediate load can be applied (installation and prosthesis activation in 48 hours). This change in the protocol was possible because of the greatest cautions taken during the surgical procedure, the development

of knowledge on the implant biomechanics, the modification in the implant design as well as the improvement of the techniques of modification of the implant surfaces [5]. The surface modification of this material results in a new Dentistry, where it is expected to decrease the non-functional time period of the implant, increase it applicability in alveolar bone with little quality, cause minimum discomfort to patient, and minimize the failure rates [1, 6-8].

According to Hunter et al.; Kunzler et al. and Brunette [9-11], the biological response may vary according to the cell type once it was found that the rough surfaces would favor the proliferation of osteoblasts while smooth surfaces would favor the proliferation of fibroblasts. It is believed that rough surfaces provoke an anchorage of proteins and consequently, provide a better adhesion of osteoblastic cells [12]. The explanation for this issue may be on the mechanism of bone formation proved by Kasemo [13]. Based on this mechanism, the first molecules to reach the biomaterial surface are the water molecules, which influence proteins, other molecules and cells further aggregating. Depending on the surface properties (smooth or rough; hydrophilic or hydrophobic), a different cellular behavior occurs.

In the search for surfaces providing this necessity to obtain a fast bone formation, several researches have been conducted. Among the different existing methods, those using plasma as energetic source are the ones that most advance in the last years, mainly because of its versatility. The plasma treatment provides an opportunity of largely varying the superficial properties of the materials by simply adjusting the experimental parameters, such as electronic density, energy and distribution of functions. This treatment is very used to increase the surface energy and clean the biomaterial surfaces. This constitutes a simple, dry technique, which does not harm the environment, of low cost and does not comprise the intrinsic properties of the biomaterial, affecting only its surface [14]. The aim of this study is to evaluate the superficial properties of titanium after two plasma treatment (planar and hollow cathode nitriding) and it effect on stem cell proliferation.

Material and Methods

Preparation of titanium substrate

Disks with pre-established dimensions (diameter: 15 mm; thickness: 1.5 mm) were cut from titanium Cp degree II cylinders. The samples were grounded using sandpapers from 80 to 2000 mesh. The chemical–mechanical polishing was carried out, using a mixture of colloidal silica (OP-S) and hydrogen peroxide (30%), during 30 min, until a roughness of 0.002 μm

Plasma treatment

After the polishing, the titanium disks were divided in three different groups (Table 1). On the first group (G1), the samples did not suffer any treatment. They were just grounded and polished. In the second group (G2) the samples were treated under the planar cathode configuration and in the third group (G3), the samples were treated using the hollow cathode configuration.

Table 1 – Conditions to the superficial treatment of titanium disks

	Without plasma treatment (G1)	Planar nitriding (G2)	Hollow cathode nitriding (G3)
Gases	-	20 % N_2 80% H_2	20 % N_2 80% H_2
Temperature	-	450°C	450°C
Time	-	1 h	1 h
Pressure	-	1.5 mbar	1.5 mbar

After polishing, the samples were placed into a stainless steel chamber (reactor), with 400 mm diameter and 400 mm length, hermetically sealed to receive the surface treatment. The system used for the plasma treatment consists of a high voltage DC source (potentiometer); a cylindrical vacuum chamber machined with stainless steel; a gas controlling system (flowmeter), a vacuum pump, a temperature meter (thermocouple) and pressure gauges (Figure 1). In this configuration, the plasma is generated between a negatively polarized bottom electrode (cathode) and an anode (top of the chamber), held at ground potential. The cathode works as the sample holder, and it consists in a circular electrode (A=254cm^2) coated with Ti sheet. The distance between the electrodes was 22 cm. Before each treatment, the system was pumped down by a two-stage rotatory pump, until it reached a residual pressure of 0.1 mbar. The gas flow (N2 and H2) was measured by a flowmeter (MKS instruments, Inc. Type 247). The applied DC voltage used was 550 V. The work pressure of 1.5 mbar, measured by

a Barocell Capacitance Manometer, was adjusted by manual valves. The temperature during the treatment was held constant at 450°C for 1h. It was measured by a chromel–alumel thermocouple, inserted in the sample holder, and was adjusted by varying the DC voltage applied between the electrodes. After the treatment, the plasma was turned off and the Ti samples were left inside the chamber to cool down for one and a half hour in vacuum. Then, the chamber was opened and the samples were taken out to the characterization.

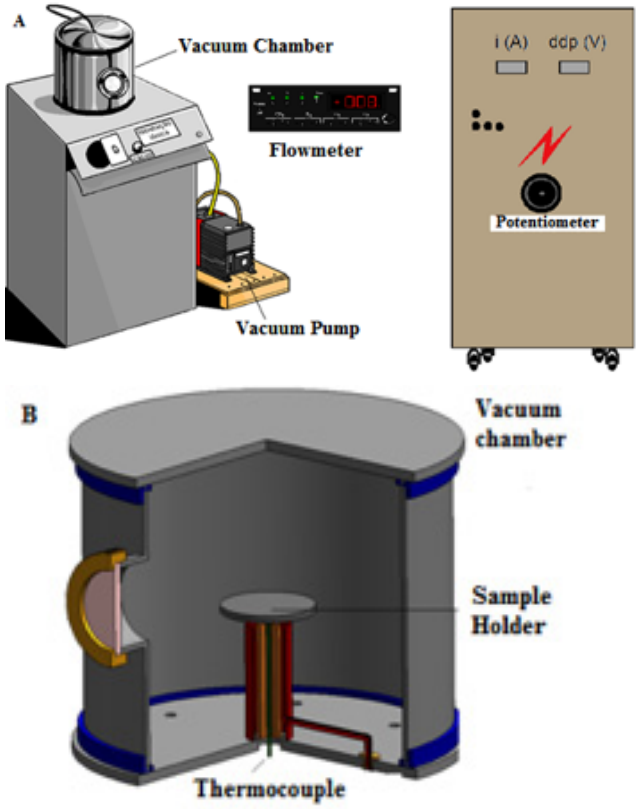

Figure 1 – (A) Schematic representation of the plasma reactor (B) Internal part of the steel vaccum chamber showing details of the sample holder and of the temperature meter (thermocouple).

The hollow cathode nitriding (G3) was carried out by using the following procedure: a circular titanium plate containing four legs, similar to a table (Figure 2), was placed on the sample-holder. It was established a distance between the sample surface and the top flange. In this study, it is called cathode/cathode distance (Dcc), and it was fixed in 9 mm. By using this technique, the electrons are successively repulsed (zigzag movement) between the sample holder and the cathode resulting in a high ionization rate.

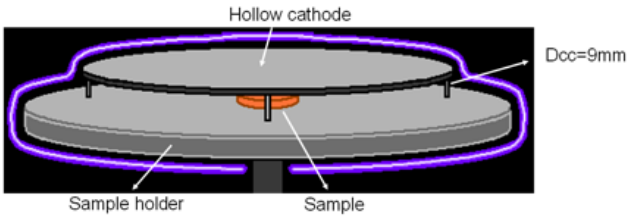

Figure 2 – Schematic representation of the hollow cathode configuration.

In planar nitriding (G2) the sample is placed directly onto the sample holder without other auxiliary device.

Chemical Composition

The chemical analysis was carried out by EDS (Energy Dispersive Spectrometer) using a SEM (Scanning Electron Microscope) Philips, model XL-30 ESEM. In this test type, an electron beam focuses on the sample and the most external electrons of the atoms and constituting ions are excited, changing the energetic levels. By returning to its initial position, they release the energy acquired, which is emitted in wavelength in the x-ray spectrum. A detector installed in the SEM vacuum chamber measures the energy associated to this electron. Because the electrons of a given atom have distinct energies, it is possible, in the beam incidence point of view, to determine which chemical elements are present in that place. Therefore, 3 different areas of each sample were assessed.

Topography and roughness measurement

Topography and roughness analysis were accomplished using an Atomic Force Microscope (AFM), model SPM 9600, Shimadzu. The AFM provided the surface topography (2D and 3D). Photomicrographs of three different areas of each sample with 5 µm scanning were obtained. In each 2D photograph, 2 lines were drawn to determine the rough profiles. The texture was also evaluated through a scanning electron microscope (SEM) (Philips, model XL-30 ESEM), at x 2000 magnification.

Obtainment of the stem cells

Stem-cells were extracted from the umbilical cords of the Januario Cicco Maternity of the Federal University of Rio Grande do Norte. The cords were transported into collection caps composed of 2% of glucose, 20% of human albumin (Sigma), 10% of penicillin-streptomycin-Fungizone (Sigma) under refrigeration (4°C) until the culture cell laboratory. After washing, in a

new sterile collection cap, the vein of the umbilical cord was cauterized and washed twice with PBS and finally, clamped at its distal extremity. The vein was filled with 1% of collagenase solution (Sigma) in PBS and its proximal extremity was closed. After incubation at 37°C for 20 minutes, the collagenase solution was drained and the endothelial and sub-endothelial cells were collected and washed with PBS. The cell suspension was centrifuged at 1500 rpm for 10 minutes at 25°C and the cell pellet was resuspended in α-MEM (Gibco) supplemented with 20% of fetal bovine serum (HyClone), 2 mM L-glutamin (Gibco) and 100 U penicillin/ streptomycin (Sigma). Following, the cells were counted and plated in culture flasks of 75 cm2. After 4 days of culture. The medium was changed and the non-adhered cells were removed. After 21 days with medium changes weekly performed, the cells were trypsinized (5 mg trypsin/ ml of PBS), washed in PBS, resuspended in medium and plated again in new flasks of 75cm2 for expansions. After 4 days of expansion, the cells were plated onto titanium discs.

Cellular proliferation test through AlamarBlue Assay

The AlamarBlue Assay was employed to measure the cellular proliferation. The AlamarBlue Assay was designed to measure quantitatively the proliferation of various human and animal cell lines, bacteria and fungi. It incorporates a fluorimetric/calorimetric growth indicator based on detection of metabolic activity. Specifically, the system incorporates an oxidation/reduction indicator that both fluoresces and changes color in response to chemical reduction of growth medium resulting from cell growth [15].

Titanium disks were cleaned using distilled water and sterilized in an autoclave during 30 min. After this, they were placed in plates of 24 wells to the proliferation tests. Onto these disks and onto the control well (plastic), it was added 104 cells/cm2. After three and seven days it was introduced the AlamarBlue solution to determinate the reductant potential. Furthermore, the data were analyzed using spectrophotometer FEMTO 600s and through equations to determinate the reductant potential.

$$PR = \frac{(\varepsilon ox)_2\lambda . A_1\lambda - (\varepsilon ox)_1\lambda . A_2\lambda}{(\varepsilon red)_1\lambda . A'_2\lambda - (\varepsilon red)_2\lambda . A'_1\lambda} . 100$$

PR = Percent Reduced
(εox) = Molar extinction coefficient of AlamarBlue Oxidized form
(εred) = Molar extinction coefficient of AlamarBlue Reduced form
λ = wavelength
A = Absorbance

Statistical Analysis

The statistical analysis was performed through Student-Newman-Keuls test, with level of significance of 5%.

Results

In this study, the chemical composition of titanium disks, carried out by EDS, indicated that titanium was the element predominant in all the samples. Relative percentage of some other elements was also found (Table 2). Nitrogen was found because N2 was used to treat the sample and oxygen was also found because the planar nitriding treatment caused reactivity in the sample surface by forming an oxide layer. Small amount of silicon was found because of the polishing was executed through silicon carbide sandpapers.

Table 2 – Relative percentage (in weight) of the elements present in titanium samples

	N (%)	O (%)	Si (%)	Ti (%)
Without plasma treatment (G1)	0	0	0.24	99.76
Planar nitriding (G2)	3.09	2.84	0.59	93.42
Hollow cathode nitriding (G3)	3.10	6.66	0.64	89.61

The SEM microscopy (carried out with the magnitude of 2000x) was accomplished in order to observe the texture of the surfaces before and after the treatments. Samples present in groups G1 and G2 presented a smooth and uniform texture (Figure 3A). Samples from G3 group presented a rougher texture, containing precipitates (white spots) finely dispersed (Figure 3B), indicating a higher bombarding on the surface due to the hollow cathode configuration.

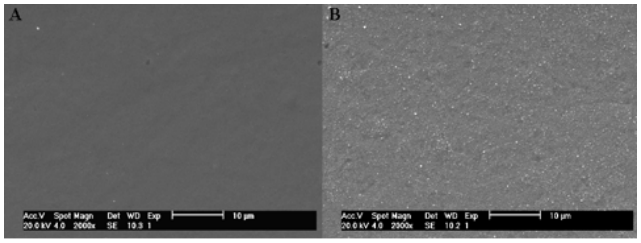

Figure 3 – Surface topography of the samples analyzed by SEM. (A) Textures of G1 and G2. (B) Texture of G3.

AFM was carried out in order to obtain more information on texture and roughness in the new surface of the material (Figure 4, Figure 5 e Figure 6).

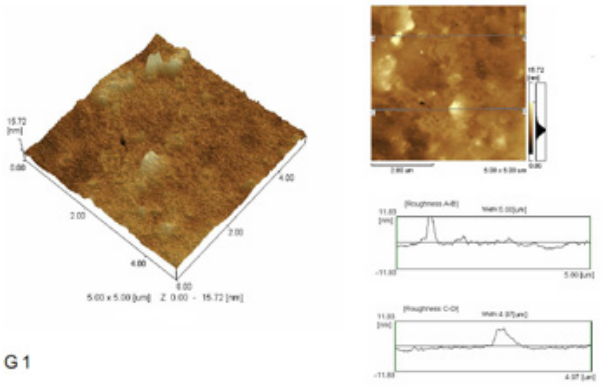

Figure 4 – Texture and roughness profile to the G1 samples analyzed by AFM.

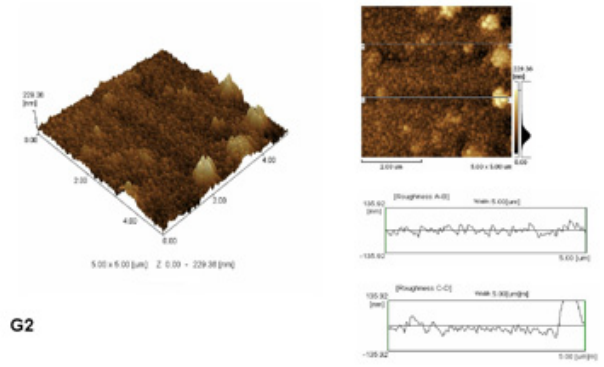

Figure 5 – Texture and roughness profile to the G2 samples analyzed by AFM.

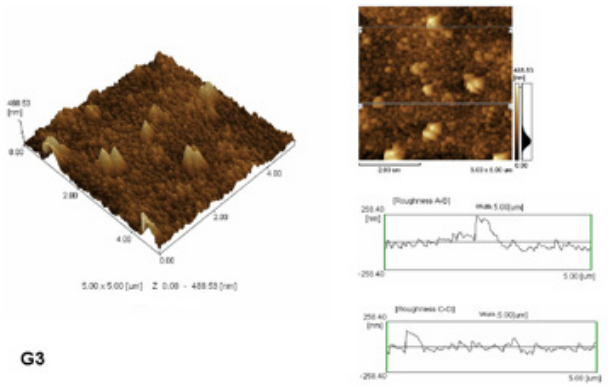

Figure 6 – Texture and roughness profile to the G3 samples analyzed by AFM.

G1 group presented samples with smooth surface, with maximum peak of 15.72 nm, without evidence of directional grooves and crystals due to the sanding and polishing. Through the roughness profile it can be observed peaks and valleys containing low intensity and regularly distributed onto the surface, revealing a smooth and uniform aspect. In G2 group, intense peaks and valleys were produced (maximum peak of 229.36 nm). In G3 group it was observed the most intense roughness profile compared to the other techniques. Maximum peaks of 488.53 nm were found and the roughness parameters were the highest values, compared to the other techniques. It can be observed, therefore, that hollow cathode nitriding (G3) produced more defects in the surface than the other techniques of nitriding.

The cellular proliferation through AlamarBlue Assay was performed at three and seven days (Table 3). The number shown represents the reduction potential of alamar, in percentage. The greater this number, the greater the cellular proliferation.

Table 3 - Cell proliferation through AlamarBlue Assay

	G1	G2	G3
Three days	7.6 ± 1.02	7.9 ± 1.65	10.5 ± 2.11
Seven days	18.5 ± 3.5	14.1 ± 2.24	26.1 ± 2.06

It can be observed that both at three and seven days, there was a greater proliferation in G3 group samples. Student-Newman-Keuls test found statistically significant differences among G3 when compared with G1 and G2 ($p<0.05$). On the other hand, there were no statistically significant differences when G1 was compared with G2 ($p>0.05$).

Discussion

In this study it can be observed that the plasma treatment through hallow cathode nitriding produced a surface with higher superficial texture alteration. This happens because of the high rate of ionizing, since the electrons contained in plasma are successively reflected between the two cathodic surfaces [16]. Electrons are repulsed from the central cathode (that can be the sample in treatment) to the external cathodes. When the electrons are close to these external cathodes, they are constantly repealed, promoting a zigzag movement that will increase the ionization rate in this area. A high density of ions means, in this case, a higher bombarding in the surface, producing a higher amount of defects when compared to planar nitriding (G2). These results are according to previous studies accomplished by Guerra Neto [6].

It is also observed, in this research, that the

roughness influenced on the cellular response. These data are in agreement with those of the studies of Korovessis and Deligianni [17]. It is known that the roughness affects the first stages of vascularization of tissues around the implant immediately after the surgery, determining the migration, alignment, orientation, adhesion and finally, the rate of proteins production and subsequent cellular function [7].

Silva [18] conducted a study through a mechanical profilometer and atomic force microscopy by comparing the roughness values before and after the titanium plasma treatment (planar cathode and cathodic cage nitriding). The data obtained were significantly different among the samples with and without treatment and the samples exposed to plasma.

Guerra Neto [19] performed a study comparing the roughness of nitrated titanium surfaces by hallow cathode and planar nitriding with titanium samples without treatment. Firstly, the samples showed a roughness of about 0.2 μm. These data were obtained through profilometer. By performing the planar nitriding, the roughness was almost the same. However, by executing the hallow cathode nitriding through a ring-shape cathode, the roughness was almost twice the initial value, from 0.2 μm to 0.4 μm.

In the study of Lê Guehennec et al. [20] smooth titanium discs (Ra= 160 nm) were compared with sandblasted discs (Ra=2060nm). It was demonstrated that the adhesion, scattering and cellular proliferation occurred more rapidly on the roughness surfaces

Khang et al. [21] executed a careful study on the influence of roughness on the adhesion of bone cells. Pure titanium was deposited onto glass generating surfaces with different roughness (smooth discs, discs with roughness at nanometric and micrometric level). It was found that the bone cells adhered better to the rougher surfaces.

Manso-Silvan et al. [22] conducted a study by covering TiAlV alloys with TiN and analyzed the behavior of the stem-cells onto the surfaces covered by TiN. It was found a highter adhesion on the surfaces covered by TiN.

Currently, studies have fairly researched on the physical, chemical and mechanical alterations coming from the superficial modifications of the tooth implants and their effects on the cellular behavior. By performing an analysis of the topography, wettability, and roughness in relation to the cellular proliferation, it is verified that the literature exhibited controversial results. Some studies showed that the smooth surfaces favor the cellular proliferation [23,24]. However, De Santis et al. [25] and Hatano et al. [26] observed higher proliferation in cells plated onto roughness surfaces and Rosa and Beloti [27] did not verify the difference in this proliferation under roughness conditions.

Although the cellular response in different surfaces is largely researched, the studies are limited by the use of the terms “smooth or rough”, that is, surfaces with roughness values that little varies among them. Additionally, the substrates are produced by different techniques, creating different types of surfaces [28].

In this study, a direct relationship between roughness and cellular proliferation was found. In these samples with higher roughness, there is a higher proliferation rate of stem cells. It is important to emphasize that this roughness difference is at nanometric level; however, this was relevant for the occurrence of a preference of stem cells by this type of surface. According to Dalby et al. [29] and Schwartz [30], the microtopography has high power of altering the adhesion, movement, morphology, apoptosis, macrophage activation and genic activation, therefore influencing on the cellular behavior.

Conclusion

The results obtained by plasma treatment confirmed the viability of this technique and its positive effect on initial cellular events present in the osseointegration process. This method was able to produce modifications on the surface of titanium samples and still maintained the characteristics of biocompatibility on the new surface, as demonstrated by the cell proliferation tests carried out in conditions established for this study. The hollow cathode nitriding produced higher superficial modifications on the titanium surfaces, influencing significantly on the cell proliferation. This technique of surface modification can be used, therefore, at industrial scale, aiming to improve the quality of the healing process surrounding the implant and consequently its clinical success.

Acknowledgements

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Resumo

Nos últimos anos tem-se observado um grande crescimento em tecnologias para modificação de superfícies de implantes dentais visando reduzir o tempo de espera pela cicatrização, assim como possibilitar seu uso com sucesso em áreas de baixa densidade óssea. Dentre esses métodos de modificação, a nitretação por plasma tem apresentado ótimos resultados. No presente trabalho, estudou-se superfícies de titânio comercialmente puro (Grau II) modificadas através de dois tratamentos por plasma diferentes (nitretação planar e nitretação em cátodo oco), com o objetivo de obter uma otimização da superfície para aplicações biomédicas. Uma avaliação da composição química e um estudo da rugosidade e textura destas amostras foram realizados. Em seguida, depositou-se células-tronco sobre essas superfícies e uma comparação entre as novas propriedades obtidas e a proliferação celular foi feita. Os resultados mostraram que a nitretação por plasma produziu mudanças significativas na textura superficial das amostras de titânio. A rugosidade foi superior nas amostras nitretadas em cátodo oco. Encontrou-se diferença estatisticamente significante na proliferação celular das amostras nitretadas em cátodo oco quando comparadas com as amostras sem tratamento. Essas técnicas de modificação são, portanto, efetivas e possuem influência direta nas características da superfície e no comportamento de células-tronco.

Unitermos:

Titânio. Plasma. Implantes dentários.

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Corresponding Author
Juliana Carvalho Sá
Adjunct Professor - Department of Dentistry – University of Rio Grande do Norte State - Natal - RN - Brazil.
e-mail: julianasa.uern@hotmail.com
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