Improvement of titanium alloy for biomedical applications by nitriding and carbonitriding processes under glow discharge conditions

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Although titanium alloys are used in medicine, they present low wear resistance. In this paper we present the results of studies on surface layers produced by nitriding at three different temperatures, and by carbonitriding under glow discharge conditions in order to improve wear resistance, hardness, and to modulate microstructure and chemical composition of surface layers. A cell culture model using human fibroblasts was chosen to study the effect of such treatments on the cytocompatibility of these materials. The results showed that nitrided and carbonitrided surface layers were cytocompatible. Modulation of surface microstructure by temperature in the nitriding process and chemical composition of surface layers by carbonitriding led to differences in cellular behaviour. Cell proliferation appeared to be slightly reduced from the 6th day of culture on nitrided surfaces produced at 730 °C and 1000 °C, however after 12 days of culture, the best growth was on surface layers produced at 850 °C. The best viability was observed on the carbonitrided layer. The orientation and shape of the cells corresponded to surface topography. Nitriding and carbonitriding under glow discharge conditions may constitute interesting techniques allowing the formation of surface layers on parts with sophisticated shapes. They may also permit modulating surface topography in a way improving the features of titanium alloys for various applications in medicine.

1. Introduction

Successful use of titanium and its alloys in medicine stems from its promising effects in trauma treatment (endoprothesis, implants, stability plates) and use as parts in equipment and surgical tools. They offer good biocompatibility and high corrosion resistance. However, titanium and its alloys behave poorly in friction since titanium particles have often been detected in tissues and organs of organisms with titanium implants [1, 2]. As bone prostheses they are also bioactive by incorporating elements from the extracellular fluid (P, Ca, Si) into the oxide layer and, at least, by forming a Ca-P layer at the bone-metal interface [3], thanks to which titanium alloys are recognized as the most biocompatible metallic material.

Nevertheless, there is some controversy over their biocompatibility stemming from a few facts. Firstly, in the absence of wear, release of metal-derived elements that accumulate locally and/or systemically [4] is observed and in contrast with the other elements in the alloy, titanium is not excreted in the urine [5, 6]. Storage of elements in tissue surrounding a metallic prothesis can lead to inflammatory reactions and has been implicated in osteolysis [7, 8]. Secondly, elements in titanium alloys such as aluminium and vanadium are themselves inflammatory mediators [9]. This is why it is necessary to look for methods that can improve titanium and its alloys for medical applications.

Among engineering techniques, surface engineering by thermal spraying, ion implantation, PVD and discharge nitriding have marked significance [10, 11]. Nitriding under glow discharge in particular offers the most advantages since it allows uniform preparation of details having a sophisticated, shape-retaining surface layer with controlled thickness, structure, phase and chemical composition. Various phase and chemical

compositions can be obtained by modifications of the nitriding process, i.e. carbonitriding, oxinitriding and oxicarbonitriding under glow discharge conditions [11, 12]. Surface layers produced by glow discharge treatments are of a diffusion nature, which increases the adhesion between coating and substrate. The requirements for coatings in implants and prosthesis include good biocompatibility and regulated cell adhesion to the material surface. Biocompatibility for some nitrided layers obtained in different processes has already been shown [13–15].

In order to improve wear resistance and obtain good corrosion resistance and biocompatibility without toxic effects, surface treatments were performed on titanium alloys by nitriding and carbonitriding under glow discharge conditions. In the present work, cell cultures were used to evaluate the effects of microstructure, phase composition and topography of nitrided and carbonitrided layers produced under glow discharge conditions on biocompatibility by studying cell proliferation, viability, morphology and element storage.

2. Materials and methods

2.1. Materials

Specimens of the OT4-0 titanium alloy (0.4–1.4% Al, 0.3% Fe, 0.3% Cr, 0.12% Si, 0.5–1.3% Mn, Ti-balance) were subjected to a nitriding process under glow discharge conditions at temperatures: 730 °C, 850 °C and 1000 °C in a nitrogen atmosphere and 4 hPa pressure, and to a carbonitriding process at a temperature of 850 °C in a nitrogen + methane atmosphere. Untreated samples served as controls. All the materials studied were degreased and cleaned with ethanol and sterilized in an autoclave at 140 °C and 1500 hPa for 30 min.

2.2. Surface characterization

The materials were characterized according to the following criteria:

- Phase composition was determined by diffraction measurements using a Philips 1830 type X-ray diffractometer.
- Microhardness measurements were performed by means of a Vickers indenter under a 0.5 N load.
- Wear resistance was tested using the ''three roller-taper method'' [16]. In this test, friction is applied, under specified conditions, between three fixed cylindrical specimens (rollers) 8 mm in diameter and a rotating conical counter specimen (taper). Linear wear, expressed as wear depth, was determined by measuring the diameters of the ellipses formed on the surface of each roller. The results were then averaged. The standard deviation was about $\pm 0.5\,\mu m$. The counter specimen was made of AISI 45 steel, quench hardened and tempered to a hardness of 30 HRC. A unit load of 100 and 200 MPa were applied.
- The corrosion resistance test was conducted using the potentiodynamic method in 0.5 M NaCl solution. The polarization curves were determined at a temperature of 25 °C by polarization of the three samples with the investigated surface layers from a potential of -1000 mV towards anodic potentials at a potential varying rate of 50 mV/min using a Taccusel PRT-20 potentiostat. Prior to measurements the samples were kept in the test solution for 24 h in order to stabilize the corrosion potential. The potential was measured with respect to a saturated calomel reference electrode (SCE). For example, potentiodynamic polarization of nitrided layers produced at a temperature of 850 °C before and after the sterilization process were also performed in Dulbecco's medium (see culture technique). A temperature of 37 °C was maintained during the entire experiment.
- The morphology of the surface layers was observed using a Jeol JSM 35C scanning electron microscope. The microstructure was assessed by etching the cross sections of specimens using a mixture of HF, HNO_3 acids and H_2O, which were analysed using a Neophot 2 microscope.

2.3. Cell culture technique

Human skin fibroblasts were grown from explants of skin biopsies taken from subjects who had given informed consent. Cells were cultured in Dulbecco's medium containing 15% fetal bovine serum in an atmosphere of 95% air and 5% CO_2. At confluence, the cells were harvested, counted and reseeded at a density of 1×10^6 cells/cm^{-2} in 5 ml medium on the upper surface of control samples of OT4-0 alloy and samples with nitriding layers produced at: (a) 730 °C, (b) 850 °C, (c) 1000 °C and carbonitriding surface layers produced at (d) 850 °C. The cells were cultured 2, 6, 12 days before the discs were removed. The cells growing on discs were fixed for scanning electron microscopy or removed for light microscopy. They were investigated for cell proliferation, viability, morphology and presence of titanium. There were seven samples for each investigation.

2.4. Cell culture characterization

- Cell proliferation and morphology. Cells seeded on the samples were fixed in 1.5% glutaraldehyde in cacodylate buffer (pH 7.3) for 30 min at 4%, postfixed in tannin and osmium tetroxide, dehydrated in ethanol series, air dried and coated with gold before examination by scanning electron microscopy (SEM) (Jeol JSM 35C). The cell number count was expressed per square millimeter. The data are presented as means.
- Cell viability. Cells were removed from samples with 0.05% trypsin in 0.02% EDTA solution, rinsed with culture medium, stained with methylene blue and counted using a Burker's camera. Methylene blue stain is a stain, that penetrates into dead cells. The results were expressed as the number of living and dead cells per milliliter. The data were than calculated as % of living cells. Statistical comparisons between groups were carried out by Student's paired *t*-test. For all comparisons an alpha-level of 0.05 was considered indicative of statistically significant difference.
- Sample toxicity. Cells seeded on a cell culture flask wall were fixed in absolute ethanol for 10 min, rinsed in

running water, stained with eosin—hematoxylin, dehydrated in ethanol, air dried and DPX closed. Analysis of cell density seeded around the samples was done under a light microscope connected to a PC computer. The number of cells was quantitated on three 1 mm^2 areas each on the border with the sample and 5 mm from the sample by computer image analysis using Multiscan software.

- Cell X-ray microanalysis. After 12 days of culture cells were detached using a trypsin—EDTA solution and centrifuged. The cell pellet was fixed for SEM, dehydrated and air dried. The culture medium was lyophilized. Fibroblast clumps or culture medium were placed on a carbon specimen using carbon paste. Microanalysis were performed at 20 kV by 100 sec analysis using AN 10000 Oxford microanalyser working with a Jeol JSM35C scanning microscope. Spectrums were obtained from a point analysis of three regions in samples.

3. Results

The microstructures of the surface layers produced on the OT4-0 titanium alloy by nitriding and carbonitriding processes assessed by etching the cross sections of specimens are shown in Fig. 1. The nitriding processes gave layers of the $TiN + Ti_2N + \alpha Ti(N)$ type with a TiN (external zone) thickness of about 2–5 μm and hardness from 1700 to 1900 HV0.05, whereas carbonitriding produced $Ti(CN) + Ti_2N + \alpha Ti(N)$ layers with a Ti(CN) thickness about 5 μm and hardness of 2300 HV0.05.

The layer topography analyses under scanning electron microscopy are shown in Fig. 2. These varied depending on the process conditions. Process parameters were found, therefore, to influence surface topography (Fig. 2) and the carbon, nitrogen and titanium contents of the layers (Fig. 3). The microstructure of carbonitrided layers showed the better regularity and homogeneity.

The results of corrosion resistance of the nitrided and carbonitrided layers measured in a 0.5 M NaCl solution are shown in Fig. 4, and in Dulbecco's solution before and after sterilization in Fig. 5. As can be seen, carbonitrided and nitrided layers presented good corrosion resistance, which was not effected by sterilization.

The corrosion resistance of surface layers depends on their morphology, i.e. the microstructure, surface topography and chemical composition (Figs. 1–3), and these can fully be controlled by modifying the parameters of the glow discharge process. We can see from Fig. 4 that, during the glow discharge assisted treatments, the corrosion resistance of nitrided and carbonitrided layers produced on the titanium alloy OT4-0 has not been deteriorated or even be increased. The high corrosion resistance of titanium alloys is very beneficial especially in medical applications. This advantage results from the ability of titanium alloys to be passivated. It should however be noted that the oxide layers formed due to passivation have thickness up to 150 nm, whereas nitrided and carbonitrided layers can be up to 100 μm thick and in addition show a high resistance to frictional wear (Fig. 6). The corrosion resistance of nitrided and carbonitrided layers depends on their structural defects (microcracks, pores present in the TiN and Ti(CN) zones of the layer), which can entirely be eliminated by glow discharge assisted treatments. In all the samples examined the values of stationary potentials and corrosion currents under free-corrosion conditions were similar and ranged from -250 mV to -150 mV, and from 0.4 $\mu A/cm^2$ to 0.7 $\mu A/cm^2$ respectively.

The results obtained from examinations of the resistance to frictional wear of the $TiN + Ti_2N + \alpha Ti(N)$ and $Ti(CN) + Ti_2N + \alpha Ti(N)$ layers on the

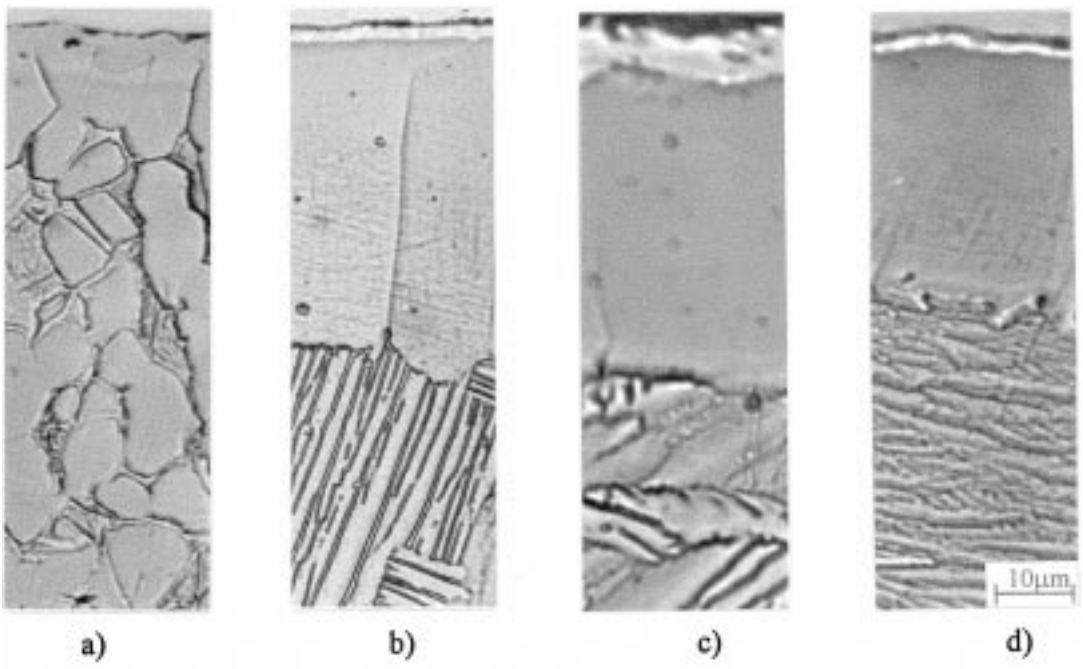

Figure 1 Microstructure of the surface layers produced by nitriding at 730 °C (a), 850 °C (b), 1000 °C (c) and carbonitriding at 850 °C (d) under glow discharge conditions.

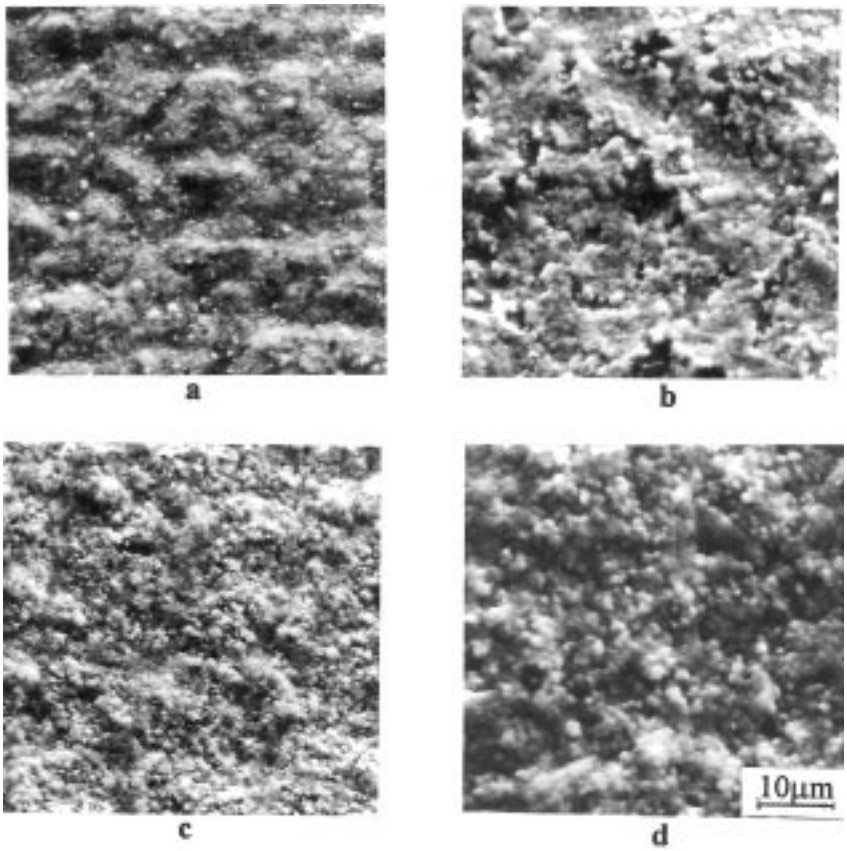

Figure 2 SEM photographs of the surface layers nitrided at 730 °C (a), 850 °C (b), 1000 °C (c), carbonitrided at 850 °C (d).

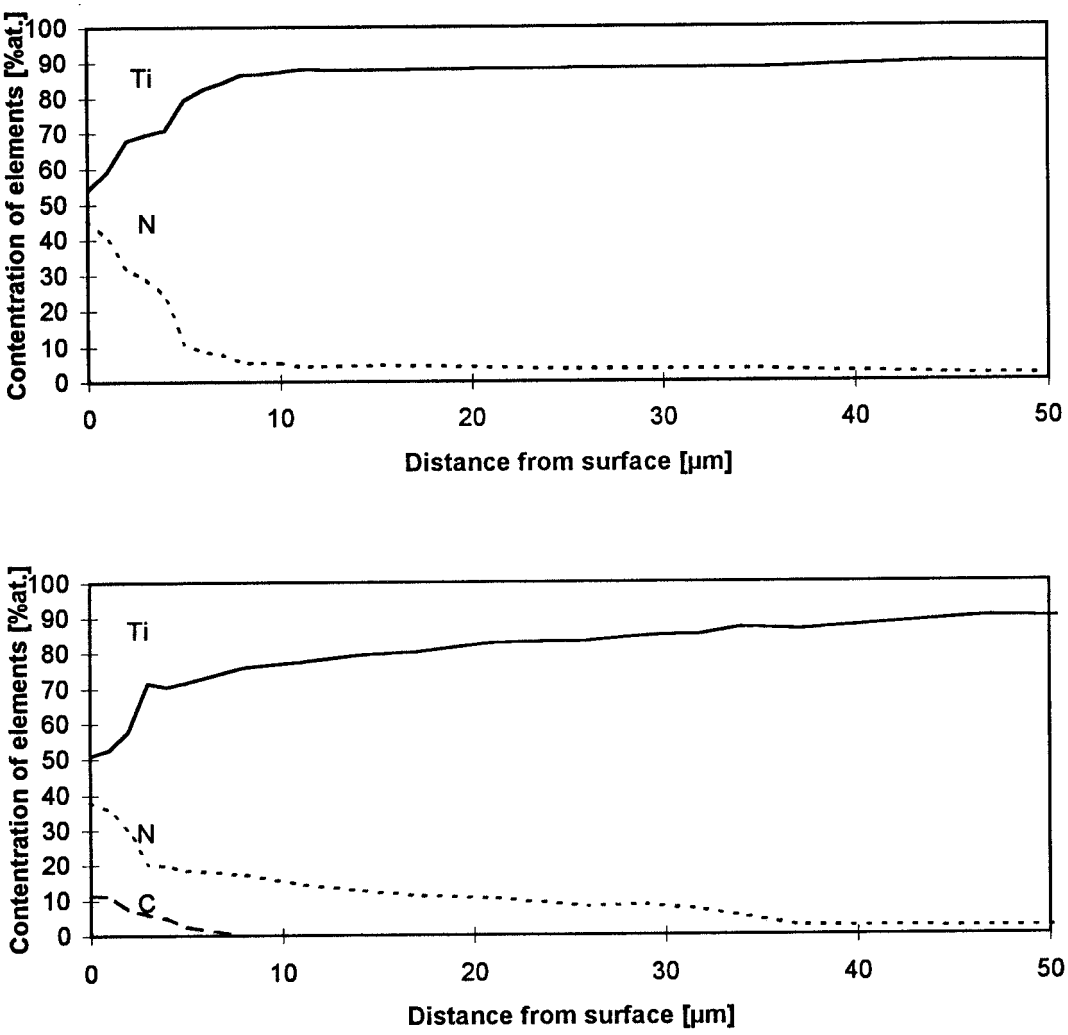

Figure 3 Chemical compositions of the nitrided (a) and carbonitrided (b) layers produced at 850 °C on OT4-0 titanium alloy.

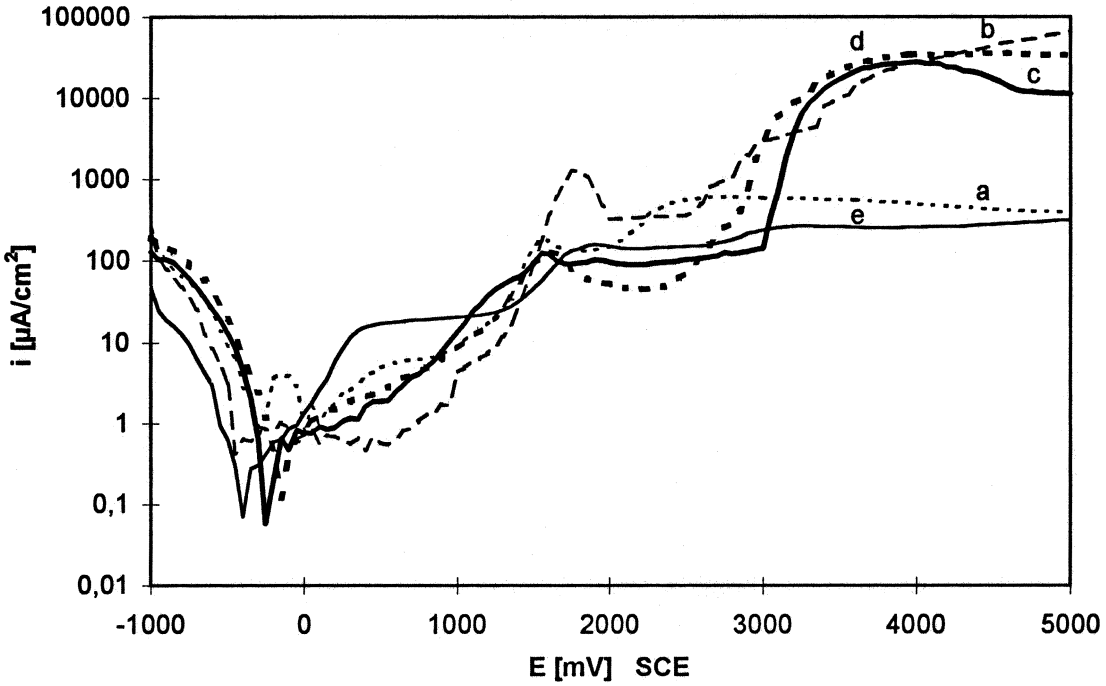

Figure 4 Polarization curves of nitrided layers produced at 730 °C (a), 850 °C (b), 1000 °C (c) and carbonitrided layers produced at 850 °C (d) in comparison with OT4-0 titanium alloy (e) measured in 0.5 M. NaCl.

OT4-0 alloy and the titanium alloy alone are shown in Fig. 6. A significant increase of wear resistance offered by the plasma carbonitriding and nitriding processes was observed. Moreover, it did not change after sterilization. At a load of 200 MPa the specimens of the OT4-0 titanium alloy underwent seizure after 10 min.

Biological investigation of human fibroblasts cultured on nitrided and carbonitrided and untreated titanium alloy samples (OT4-0) revealed differences in their behaviour between treated and control materials. Treated samples appear to be more cytocompatible than untreated titanium alloy. Final cell densities on samples counted on days 2, 6, 12 of culture were higher on coated samples than on titanium alloy (Fig. 7). There were 39, 23, 61 and 32% fewer fibroblasts on titanium alloy than on TiN + TiαN + αTi(N) layers produced at 730, 850, 1000 °C and Ti(CN) + Ti_2N + αTi(N), respectively. For samples covered with nitrided layers obtained at 730 °C and 1000 °C and carbonitrided layer obtained at 850 °C, a decrease in fibroblast growth from the sixth day of

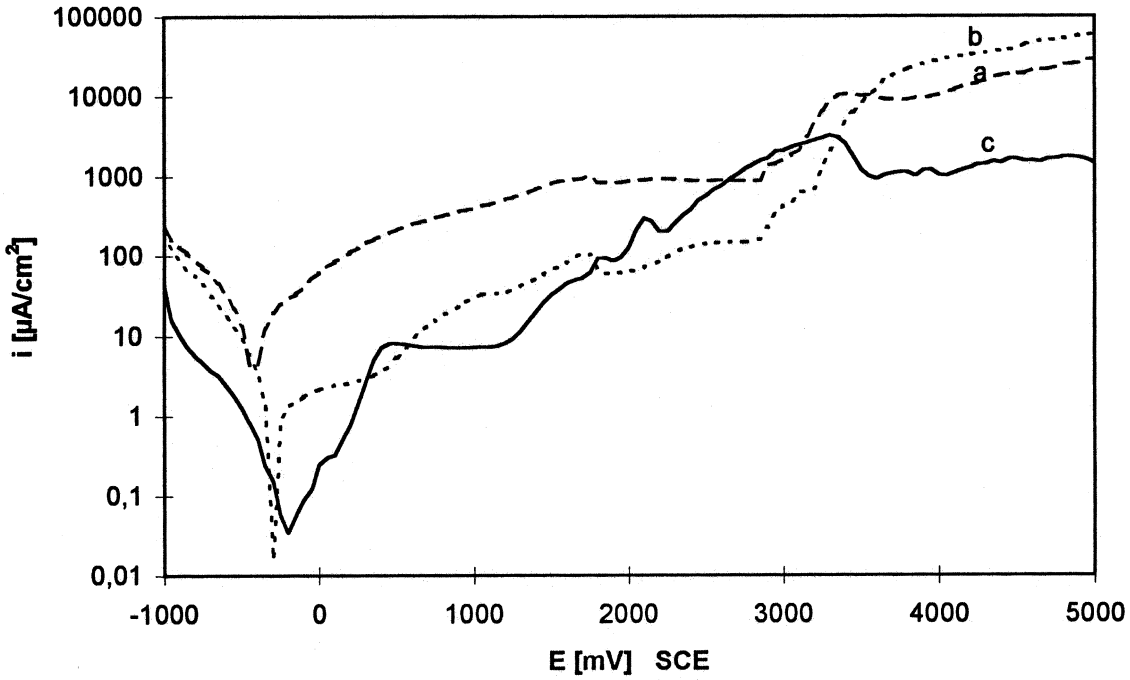

Figure 5 Potentiodynamic polarization curves of nitrided layers produced at 850 °C (a), carbonitrided layers (b) and OT4-0 titanium alloy (c) measured in Dulbecco's culture medium.

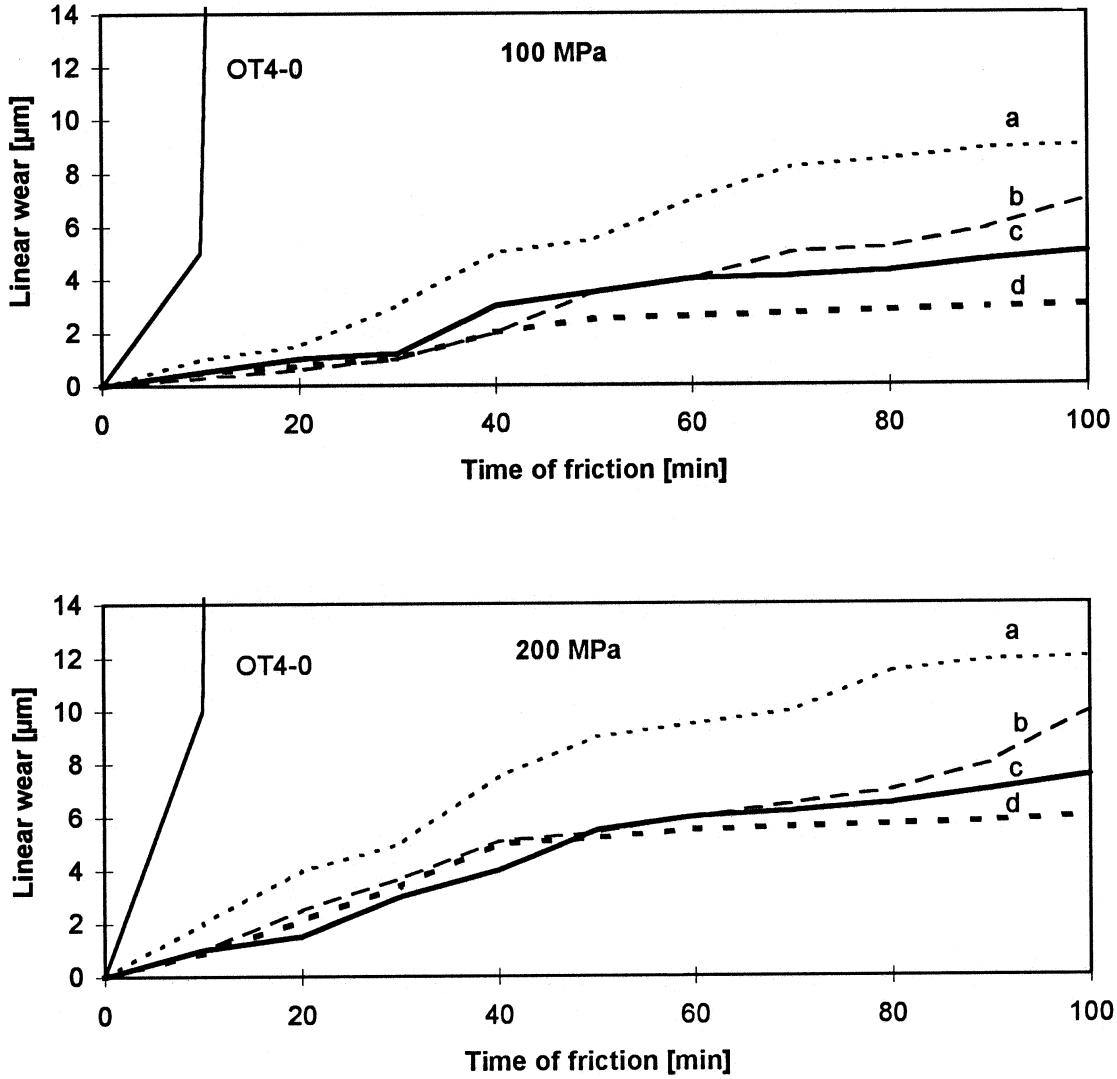

Figure 6 Wear resistance of the nitrided layers produced at 730 °C (a), 850 °C (b), 1000 °C (c) and carbonitrided layers (d) in comparison with OT4-0 titanium alloy, unit loads: 100 and 200 MPa.

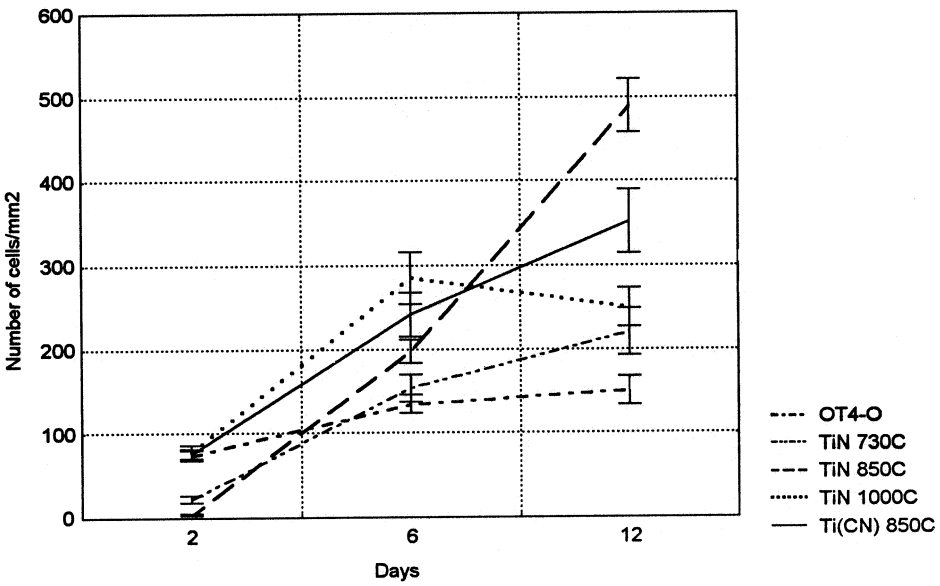

Figure 7 Proliferation of fibroblasts on nitrided at 730 °C, 850 °C, 1000 °C and carbonitrided at 850 °C surface layers and untreated samples of OT4-0 titanium alloy.

TABLE I Viability of fibroblasts (%) growing on samples covered by surface layers produced in the process of nitriding and carbonitriding and untreated samples of OT4-0 titanium alloy. • $p < 0.05$ v tissue culture plate, ° $p < 0.05$ v OT4-0 titanium alloy, ▲ $p < 0.05$ v carbonitrided surface layer, ◆ $p < 0.05$ v nitrided at 730 °C surface layer

Substratum			Cells viability (%)	
			6 days	12 days
$TiN + Ti_2N + \alpha Ti(N)$	730°C	$n = 7$	100	92.3 (0.3)•°▲◆
$TiN + Ti_2N + \alpha Ti(N)$	850°C	$n = 7$	100	87.5 (0.5)•▲◆
$TiN + Ti_2N + \alpha Ti(N)$	1000°C	$n = 7$	100	85.3 (0.4)•▲◆
$Ti(CN) + Ti_2N + \alpha Ti(N)$	850°C	$n = 7$	100	98.0 (0.2)°▲
OT4-0		$n = 7$	98.3 (0.1)	87.8 (0.5)•°
Tissue culture plate		$n = 7$	100	98.1 (0.1)•

culture was observed. This seems to indicate that the topographic features of the surface layers produced at these temperatures have some inhibitory effects.

Viability was analysed during the entire experiment and presented in Table I. It was the same for nitrided and carbonitrided samples till day 6 of culture. The percentage of living cells decreased on day 12 of culture for nitrided samples by 8% to 15.1% and for carbonitrided ca. 2%.

SEM study allowed visualizing fibroblast morphology, attachment and spreading on investigated samples. After 12 days of culture, cells formed a monolayer covering the surface with a different orientation and polarity depending on surface topography (Fig. 8a, b, c). Fibroblasts growing on coatings with irregular surfaces were elongated—spindle shaped with no protrusions.

Analysis of cell culture morphology and proliferation around the discs showed that fibroblasts were uniformly spread around all samples.

Titanium was absent from the spectra of elemental microanalysis of clumps of fibroblasts or lyophilised culture medium from experiments with nitrided and carbonitrided layers (Fig. 9a, b), which suggests that the layers are resistant to biological conditions. Titanium released from untreated samples of OT4-0 alloy was found to accumulate in cells and culture medium (Fig. 9c).

4. Discussion

Recently it has been suggested that future designs of biomaterials should be focused on the use of new wear-resistant material combinations. In these experiments, nitrided or carbonitrided layers produced under glow discharge conditions on OT4-0 alloy samples with possible applications in medicine were tested. The produced layers presented improved wear and corrosion resistance. In our experiments, fibroblasts were directly

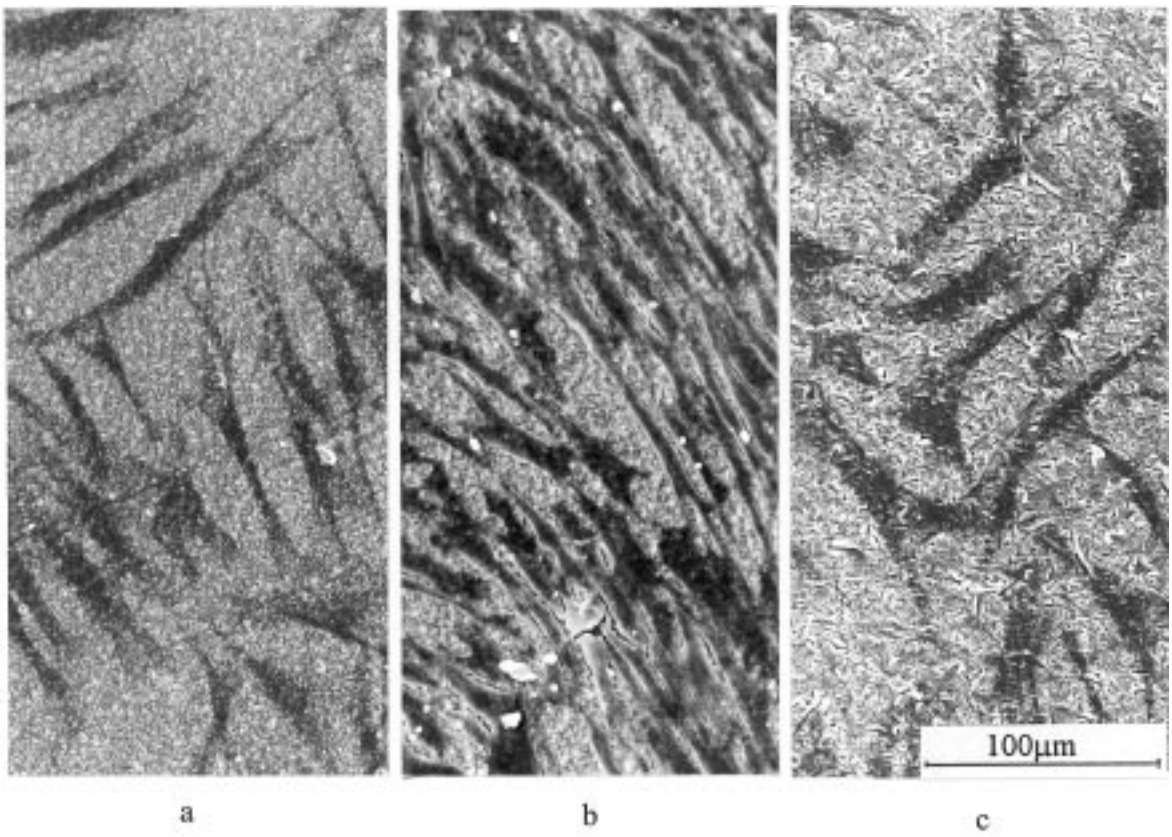

Figure 8 Spreading of fibroblasts on surface nitrided layers produced at 850 °C (a) and 1000 °C (b), and titanium alloy (c) on day 6 of culture.

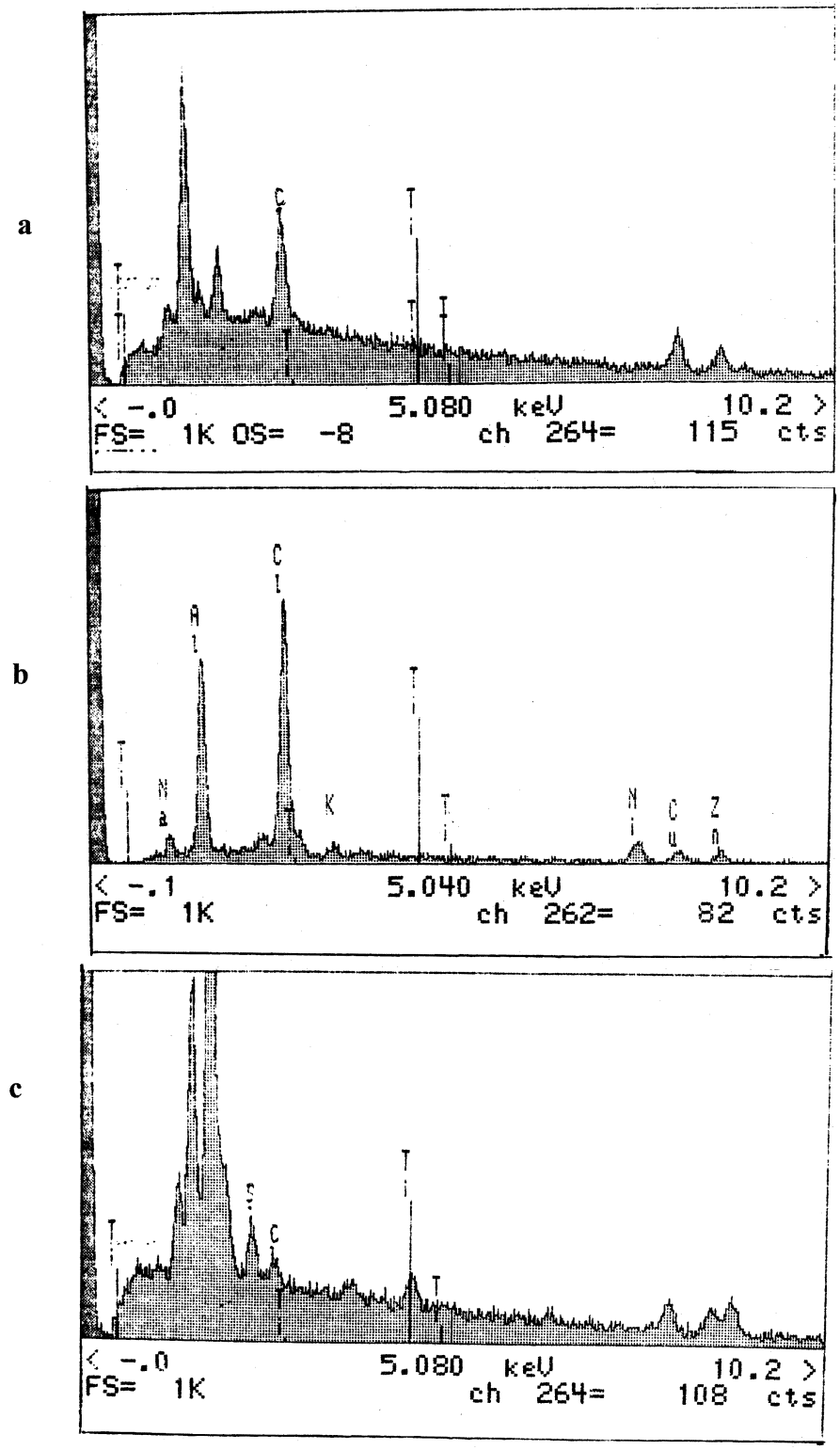

Figure 9 Elemental microanalysis of fibroblasts detached from nitrided layer produced at 850 °C (a), carbonitrided layer produced at 850 °C (b) and titanium alloy OT4-0 (c).

exposed to the materials. They showed good proliferation and better viability when they were exposed to samples covered with nitrided and carbonitrided layers than when exposed to untreated samples of titanium alloy.

Since biomaterials have different applications in medicine, features such as microstructure, roughness and chemical composition of the surface layer can be crucial. In the presented investigations, these features were tested *in vitro* for three $TiN + Ti_2N + \alpha Ti(N)$ type surface layers produced at different process temperatures (730, 850, 1000 °C). Fibroblast growth on these layers suggests that the differences relate to surface, microstructure, topography and roughness. The final cell densities were highest on $TiN + Ti_2N + \alpha Ti(N)$ produced at 850 °C with a semismooth surface, rather than on surfaces with very fine or large grains produced at 730 and 1000 °C. Also, the decrease of cell proliferation on

nitrided layers produced at 730 and 1000 °C from day 6 of culture seems to show that the morphology of these surfaces has some inhibitory effects. It is known that surface topography of titanium nitride layers can be contact guidance [17–19] and that changes in surface roughness have a strong influence on cellular behaviour [19]. Einsenbarh's *et al*. [19] experiments demonstrated that even very small changes in the topography of a surface in the range of 1 μm were implicated in different cellular reactions to the substratum. In our investigations we observed different cellular viability on surfaces with different topography and elemental composition. Carbonitrided layers presented a greater homogeneity and density of the upper layer than nitrided layers, which was followed by higher cell proliferation and viability.

Decreased viability of fibroblasts growing on untreated samples of titanium alloy after 6 and 12 days indicated cellular toxic effect of this material, which is consistent with titanium ion release to the medium and its presence in the cells, evidenced by microanalysis. Comparable viability of fibroblasts growing on control tissue culture plate to layers carbonitrided and nitrided at 730 °C after 12 days, indicated that microstructure and topography of these layers do not interact with cell surface glycoconjugates to induce pathological growth as apoptotic processes. However, decreased viability of fibroblasts growing on surfaces produced by nitriding at 850 and 1000 °C after 12 days, suggests that interaction of layer topography with cell surface needs more experiments on apoptotic processes in the cells.

The morphology of fibroblasts growing directly on surface layers and untreated samples was normal. However the contact surface between cells and substrate was reduced—cells appeared spindle-shaped on the more irregular surface obtained by nitriding at 1000 °C and these needs further analysis, e.g. by a morphometric program. Similar observations have been made by other investigators [14, 19, 21–23].

Analysis of fibroblast proliferation on the border with samples showed no toxic effect of any of the investigated materials. This study has also demonstrated good corrosion resistance in a biological medium of both nitrided and carbonitrided coatings. However, good corrosion resistance does not exclude metal ion release, which was observed in untreated samples of OT4-0 alloy. The produced layers protected samples from release of titanium in the cell culture environment.

Features of improved wear and corrosion resistance together with biocompatibility with human fibroblasts in culture can be an indication to test these materials in *in vivo* experiments for medical applications. In particular, the method of nitriding and carbonitriding under glow discharge conditions allows the production of surface layers on parts with sophisticated shapes and modulation of surface topography for various applications in medicine. Since ion release, cytotoxic, allergic activities are what finally determine the biological safety of all materials that can be used in the human body, the presented coatings after preliminary *in vitro* study could be considered for further *in vivo* experiments on animals as an improved material for various possible clinical applications.

Acknowledgments

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