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# SYNTHESIS AND BIOCOMPATIBILITY PROPERTIES OF BISMUTH-TITANIUM THIN FILMS OBTAINED BY SOL-GEL PROCESS

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## ABSTRACT

In this article, the biocompatibility Bismuth-titanium coatings is evaluated. The thin films were obtained by the sol-gel method in different concentrations of the precursors, and deposited by spin-coating onto substrates AISI 316 L. The biocompatibility testing as cell adhesion and cytotoxicity as MTT(3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) were performed by fibroblasts cells and the osteoblast cell line for establishing biocompatibility levels in both cell types and therefore, establish the possible applications in the field of medicine.

**Key words**: Bismuth, Titanium, Sol-gel, Cytotoxicity, Fibroblasts, Osteoblast.

## INTRODUCTION

Biomedical prosthetic devices are used in human body to carry out the functions that are no longer performed by the original human parts[1]. Metals are used in the human body mainly for orthopedic purposes and their degradation by wear and corrosion must be negligible so that they can be used for various practical applications[1]. Among various metallic materials used for orthopedic devices, 316L stainless steel (SS) is one of the most commonly used[1], due to its corrosion resistance, excellent mechanical properties[2] and low cost[3]. It is frequently employed for temporary devices in orthopedic surgery because of its low cost and acceptable biocompatibility[1]. But, the biocompatibility of the permanent and temporary implants has always been a concern due to the known adverse biological effects

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of individual metal components of stainless Steel[4,5]. Cr (VI) is toxic and carcinogenic; nickel is accepted to be carcinogenic and provokes contact dermatitis[5,6]. Hence, it is extremely necessary to modify the surface or to develop a new kind of biomedical surface-coated materials to improve the biocompatibility of these 316L SS implants[5].

Progress in research on materials has allowed the development of new materials such as alloys[1], polymers[2] and films[3] for application in biomedicine. Sol–gel is the method of making glasses and ceramics at low temperature. The evolution of a sol–gel system from a colloidal solution (the 'sol') into a solid 'gel' phase involves hydrolysis and polycondensation reactions of a metal alkoxide precursor, which proceed through a second order nucleophilic substitution[4,6]. This technology has been widely applied in different fields of materials science, from purely inorganic materials to organic and biological systems allowing the development of biosensor for medical diagnostic[7], studies for superhydrophobic and superoleophobic surfaces[8], superconductors[9,10], fuel cells[11,12], ceramic composites[13], dental ceramics[14] and other materials as 316L SS, under a wide range of compositions[15,20].

In this article, the biocompatibility bismuth-titanium coatings are evaluated. The thin films were obtained by the sol-gel method in different concentrations of the precursors, and deposited by spin-coating onto substrates AISI 316 L. The biocompatibility testing as cell adhesion and cytotoxicity as MTT were performed; by osteoblast cells and fibroblasts cells for establishing biocompatibility levels in both cell types and therefore, establish the possible applications in the field of medicine

## EXPERIMENTAL

### Materials and reagents

The chemicals used in this study were titanium tetrabutoxide $C_{16}H_{36}O_4Ti$ (Aldrich, 98%) and bismuth nitrate (III) pentahydrate $Bi(NO_3)_3{\cdot}5H_2O$ (Alfa Aeser, 98%) as the precursors, glacial acetic acid (Aldrich, 99.7 + %) as the solvents and ethanolamine (Aldrich, 98%) as the complex agent.

### Preparation of BTO composite

For the deposition of the BTO films, the sol were prepared by modifying the method proposed by Gu et al.[14] The Bi-Ti solution was prepared by mixing bismuth nitrate and titanium butoxideen the presence of acetic acid. Sols synthesis was established in the following sequence: First, the 98% pure $Bi(NO_3)_3{\cdot}5H_2O$ was dissolved in the acetic acid (25 mL) under constant stirring for 2 hr at room temperature. Later, the 97% titanium tetrabutoxide (TBT) was added in different concentrations, as shown in Table 1, under

constant stirring for 3 hr at room temperature. Finally, the ethanolamine was added to pH = 4 and to protect the bismuth ions from hydrolysis. Due to the large amount of water in $Bi(NO_3)_3 \cdot 5H_2O$ was vacuum dried by 96 hr at 70°C. Fig. 1 shows the scheme followed in the synthesis.

**Table 1: Quantities of precursors**

| Concentration Bi/Ti | Bismuth nitrate (g) | TBT (mL) |
|---|---|---|
| 20/80 | 9.078 | 12.61 |
| 40/60 | 12.18 | 6.35 |
| 50/50 | 13.06 | 4.54 |
| 60/40 | 13.73 | 3.18 |
| 80/20 | 14.67 | 1.27 |

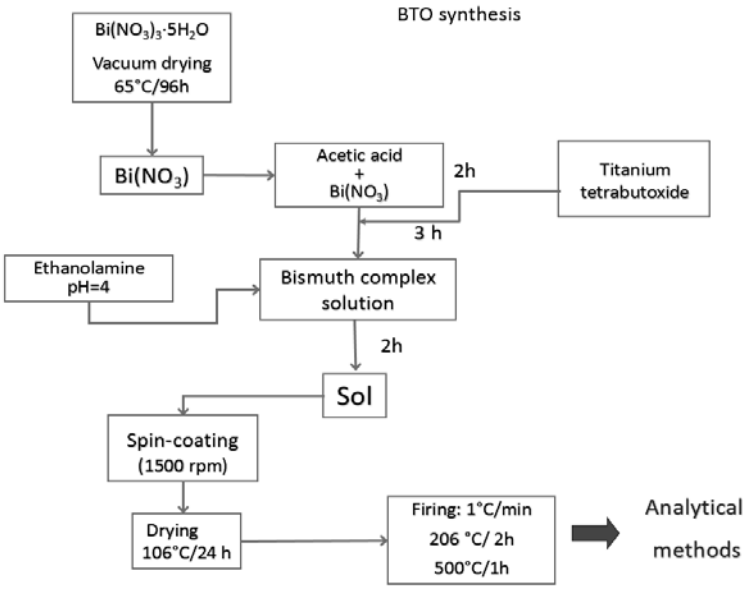


**Fig. 1: BTO synthesis soles**

## BTO thin films

Once a sol is obtained, the films were deposited directly on steel substrates AISI/SAE 316L of size 3.5 cm x 2.5 cm x 0.32 cm in this case by Spin-coating. Before deposition, the substrates were polished to metallographic brightness and were cleaned off unwanted residue (oil, dust, and residual metals) with acetone in ultrasonic bath for 10 min

and then dried using a hot air blower for 3 min before the deposition of coating on the substrate. All the depositions were performed keeping the substrates at room temperature. To form the films by spin-coating, it has been used a speed of 1500 rpm for 10s.

The films in the different molar concentrations were conformed to a period of aging of 500 hours of sol, approximately. They were dried in oven for 24 hr at 65°C. The sintering of the films was carried out at a speed of heating of 1°C/min, so: the temperature rises from 65°C to 215°C staying constant during two hour, soon it is taken to 500°C and it is stabilized by one hour and finally, it is cooled until getting room temperature at the speed of cooling of the furnace (Fig. 1).

## Preparation of subcultures

To perform the subculture of fibroblast cells and osteoblast cells was performed using petri dishes with line $SaOS_2$ cell lines (American Type Culture Collection, ATCC Manassas), ready to be re-incubated. Table 2 and 3 the culture medium composition are presented.

**Table 2: Composition of the serum-free culture**

| Compound | Concentration | Quantity |
|---|---|---|
| DMEM/F12 | | 12 (g) |
| Bicarbonate ($CO_3$) | 1 (g/L) | 1 (g) |
| Gentamicin | 40 (mg/L) | 40 (mg) |

**Table 3: Composition of the culture with serum**

| Compound | Concentration | Quantity |
|---|---|---|
| Fetal Bovine Serum | 2.5% | 5 mL |
| Adult Bovine Serum | 10% | 20 mL |
| Ketoconazole | 5 (µL/mL) | 200 µL |

Cells were incubated at 37°C and pH 7.4 in a concentration of 5% $CO_2$ to simulate the conditions of the human body.

## Cell adhesion test

The cells were incubated at 24, 48, 120, 144 and 168 hours. They are aired daily and are adding 500 mL of antibody. After each period, the cells were incubated with 60 µL of

MTT at 37°C for 4 hours with a pH of 7.4. Then, the supernatant was removed and added 250 μL of dimethyl sulfoxide (DMSO). After 30 min of incubation, the absorbance at 570 nm was measured.

To set mitochondrial activity, cells are deposited on the coatings according to the different concentrations, stored in Petri dishes and put into incubator where periodically evaluated at 24, 48, 120, 144 and 168 hours to establish mitochondrial activity fibroblast cells and osteoblast cells.

### Cell viability Live/Dead test

The cultivate medium is added Calcein AM, which enters the cell through the membrane and through staining is identified by green cells. Then propidium iodide (PI) that enters cells with damaged membrane is added, the reagent nucleic acids, which marks cell death through red is fixed.

For test of cell death, the cells deposited on the coatings are incubated for 12 hours at 37°C in a 5% concentration of $CO_2$. Subsequent to this, observations are made on the stereo-microscope and the microscope identifying red coloration.

For each of the tests was required averaged data obtained for each sample and standard deviation between different experimental data is performed; analyzes were performed by Tukey statistical analysis to see significant differences between the different samples and control, and between samples, if necessary.

## RESULTS AND DISCUSSION

The results of biocompatibility associated with fibroblasts cells and osteoblasts cells to the five coatings studied and AISI 316L substrate are presented.

### Cell adhesion assays

Fig. 2 shows absorbance levels of fibroblasts cells, related to the growth and adherence of cells to different coating and substrate. According to Fig. 2, the Bi/Ti: 20/80 coating stands out because they have the highest cellular adhesion values. The coating [Bi/Ti: 80/20] has lower cell adhesion values, but high compared with the substrate. The Bonferroni test setting a reliability index of 90% was used to determine significance indexes in the study. Similarly, in Fig. 2 it was found that the coatings in different concentrations allow cell adhesion if these results are compared with those obtained for the stainless steel substrate uncoated.

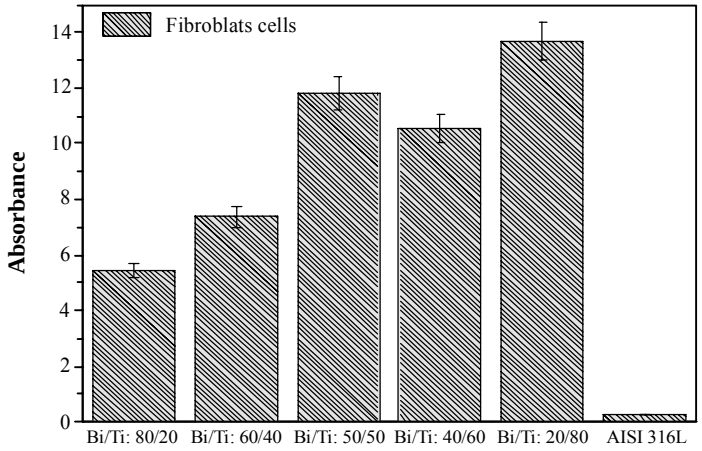


**Fig. 2: Absorbance for fibroblast cells at 24 hours incubation**

In Fig. 3, the results osteoblast cells adhesion depending on the various coatings and the stainless steel substrate are presented. The results of the coatings with higher content of titanium compound are highlighted, which establish that higher values in the titanium compound are directly related to a high value of cell adhesion. The best results are established cell adhesion to the [Bi/Ti: 20/80] coating. The least efficient is the [Bi/Ti: 60/40] coating.

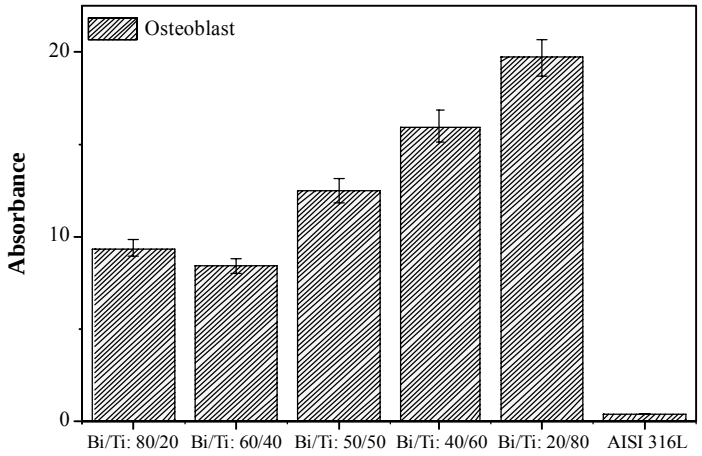


**Fig. 3: Absorbance for osteoblast cells at 24 hours incubation**

The results of cell adhesion of osteoblasts cells, Fig. 3, reflect that all coatings studied show good results compared to the uncoated substrate. The reliability levels found are 80% because the observation was established after 4 hrs of incubation.

Comparing Figs. 2 and 3, it can be inferred that cell adhesion is best for osteoblast cells to fibroblast cells. However, fibroblasts grow on coatings with equal concentrations of Bi and Ti compounds, have a stable growth and adhesion, on incubation period as shown in the graph of Fig. 4.

## Cytotoxicity and proliferation cell with MTT test

This test determines the cytotoxic effect of the compound (coatings) on tumor cell lines or cultures of normal cells. It is based on the metabolic reduction of bromide 3 (4,5-dimethyl-2-thiazoyl)-2,5 diphenyltetrazolium (MTT), which has a yellow color, in insoluble form formazan (compound purple). This action is performed by the mitochondrial enzyme succinate dehydrogenase mitochondrial allowing to determine functionality of the treated cells. The amount of formazan formed is an indicator of cell viability in this test.

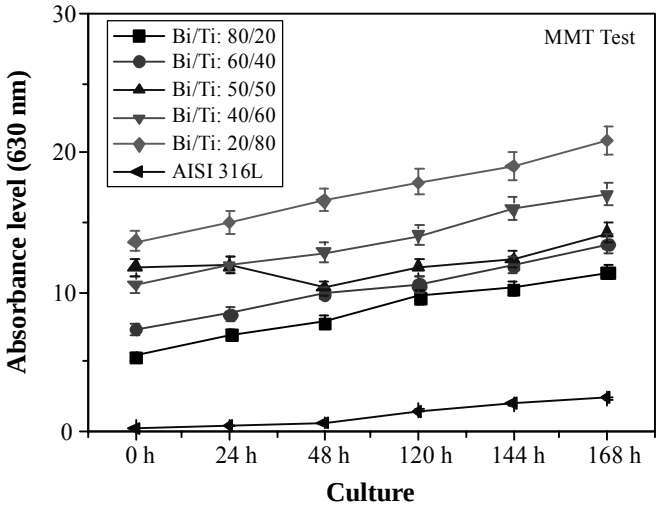


**Fig. 4: Cell Proliferation fibroblasts to 24, 48, 120, 144 and 168 hrs**

The formazan crystals are retained by the cell and is dissolved in isopropanol and is then quantified by measuring the absorption.

In Fig. 4, the cellular proliferation of fibroblasts cells, on the coatings under study is presented to an incubation period of 168 hrs. It is inferred that the growth of fibroblasts is homogeneous in the surface of the films indicated that the components of the coatings studied have no active cytotoxicity and exceed, in number, the tendency of cell growth compared to the results obtained for stainless steel. This behavior is determined by applying the Bonferroni test.

As for cytotoxicity curves for each of the coatings shows that the coating with increased cell efficiency is [Bi/Ti: 20/80], where in the absorbance values begin with 13.67 and reaches 20.87 to 168 hrs incubation. With absorbance values determined between 11.85 and 14.3 for the entire incubation treatment: coating [50/50 Bi/Ti]. The coating shows little efficiency by absorbance data is composed of [Bi/Ti: 80/20] with values from 5.46 to 11.45. It is important to note that regardless of the amount of Bi and Ti films forming the absorbance values are much higher compared to the results obtained for the uncoated substrate.

Fig. 5, allows to observe the behavior of cell growth of fibroblasts when incubated in the coatings and the substrate 316L SS. It is observed that cell growth after 24 hours incubation is substantial in each of the AISI coatings. In assessing the results at 168 hrs of incubation is homogeneous growth with little fluctuation of cellular activity. When comparing cell growth trends by type of coating and substrate is evidence that the coatings, in varying degrees, effectively support cellular life. By applying the Bonferroni test reliability is obtained is 90%.

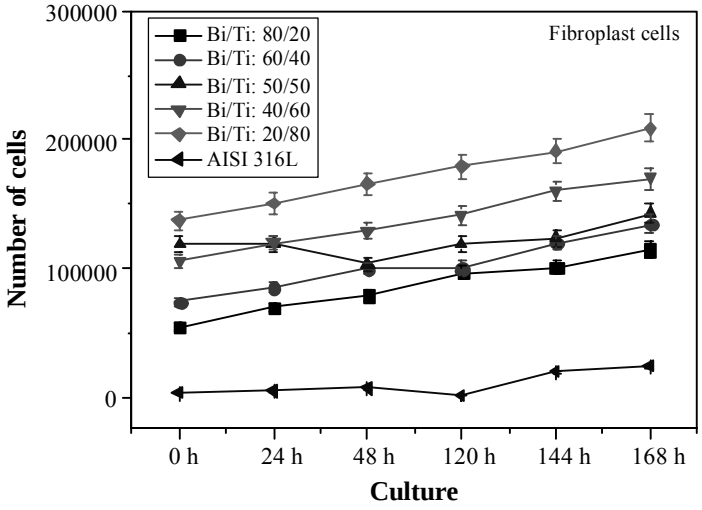


**Fig. 5: Number of fibroblast cells in the coating surface at 24, 48, 120, 144 and 168 hrs of culture**

The coating allows for greater cell growth is labeled [Bi/Ti: 80/20] followed by [Bi/Ti: 50/50], which shows a decrease in cell growth after 48 hours incubation and then stabilizes 120 hours. Finally, coatings with concentrations of [Bi/Ti:80/20] precursors presented the lowest values of cell growth. From Fig. 6, it can establish that all coatings allow a medium suitable for cell growth, with respect to the results obtained for the uncoated substrate.

The graph of Fig. 6 discloses cell proliferation of osteoblasts cells deposited on the surface of the coatings obtained with different concentrations of the precursors of Ti and Bi. The osteoblast cells cultures were balanced by fetal bovine serum. Fig. 6, a homogeneous cell growth on the surface of each film is evidenced; indicating a favorable bioactivity. Cytotoxicity was negative, because the mitotic divisions of osteoblasts cells on the material surface.

By comparing the absorbance curves according to the type of coating studied, stable behavior is observed during the incubation process. Best results are set forth in the [Bi/Ti: 20/80], coating the less favorable results are determined for coatings type [Bi/Ti: 80/20]. However, when comparing the efficiencies of the coatings with the efficiency of the substrate is clear films all provide a means for osteoblasts cells to grow. The study was developed with a reliability of 80%.

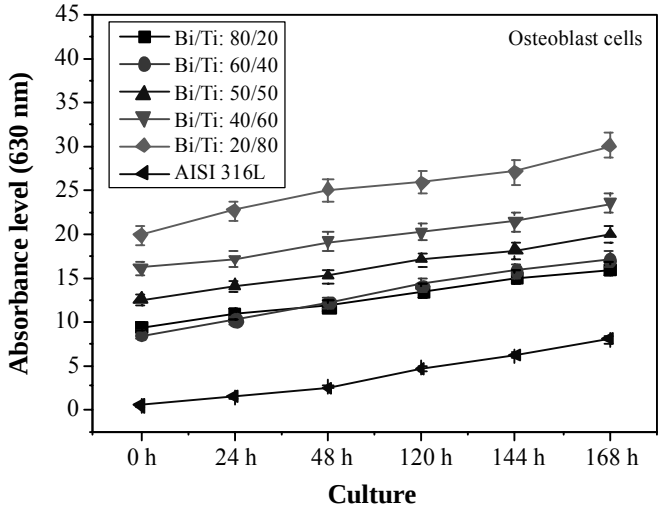


**Fig. 6: Cellular proliferation of osteoblasts to 24, 48, 120, 144 and 168 hours**

In Fig. 7, the number of osteoblast cells with incubation time is plotted. Osteoblast cell growth directly proportional to the absorbance is observed. The larger number of cells is for the [Bi/Ti: 20/80] coating and fewer cells are for [Bi/Ti: 80/20] coatings. Incidence clear that the amount of bismuth precursor, the higher the concentration of Bi in the films is less efficient support means warns cells. However, the results obtained for the different films and uncoated substrate is highlighted, regardless of the concentration of Bi or Ti precursors that form the different films are offered in medium allows the cell growth indicating that the coating more favorable for the proliferation of osteoblasts cells is the one with the highest concentration of titanium.

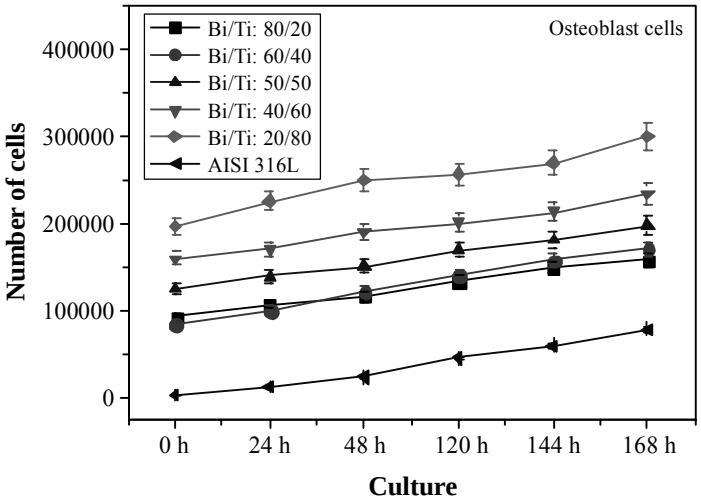


**Fig. 7: Cell proliferation for osteoblasts to 24, 48, 120, 144 and 168 hours**

## CONCLUSION

The biocompatibility tests including: cell adhesion, cell proliferation and cytotoxicity (MTT), have allowed monitor the behavior and the adhesion process of fibroblasts cells and osteoblasts cells depending on the coating and the substrate.

The cell adhesion assay follows that the power dissipation is higher in osteoblasts cells in fibroblasts, analyzed by the behavior of the cell surface adhesion of the various coatings and the substrate AISI 316L by the incubation time. Cell behavior on the material surface is modulated by the concentration, composition and conformation of proteins (fibronectin, vitronectin and fibrinogen) extracellular matrix, which help to generate cell adhesion to biomaterials. Titanium because of the equiaxed microstructure α, and martensitic promotes the protein replication fibronectin and fibrinogen, which causes adhesion of osteoblasts cells is increased in coating high titanium level.

Cytotoxicity tests allow establishing the quality of a material to be toxic to the cells. This requires that the cells adhere to the surface of study material providing a means to grow, proliferate and develop metabolic functions normally. Cytotoxicity assays of the Bi-Ti alloys have been scarce so far studies have focused on Ti alloys with Ta, Nb and Zr, which produce essentially insoluble acids that are suitable for biomedical applications.

Bismuth is known for its corrosion characteristics, which is why the form alloys with titanium can decrease the corrosion of the substrate.

During the incubation period of fibroblasts cells and osteoblasts cells were detached free radicals minimum observed behavior because metabolic processes and cellular reproduction is not affected.

In reviewing the absorbance curves and the number of cells adhered to the coatings it shows that osteoblasts have improved metabolic behavior during the period of incubation best fibroblasts cells.

Cell adhesion is a process that is performed by proteins, which are absorbed by the surface of materials and cell membrane proteins which are called intregrina. The interaction between integrin and extracellular proteins absorbed by the coating processes determine cell proliferation and differentiation. Replication of fibroblasts and osteoblasts on the surface of the coating compared to the substrate indicates that the coatings, regardless of concentration, favor cell behavior. The protein differentiation between fibroblasts cells and osteoblasts cells allow osteoblasts cells improve their adherence to the Bi-Ti coatings.

## ACKNOWLEDGMENT

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