Electrochemical and bioactive evaluation of polycaprolactone/multi-walled carbon nanotubes coatings on titanium alloy

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Abstract. Composite materials of polymer – carbon nanotubes have been widely studied for biomedical applications due to the versatility of polymers in this field and the excellent mechanical, thermal and electrical properties of carbon nanotubes. However, carbon nanotubes are materials which can provide some toxicity in biological systems, since they have low solubility in water and organic solvents, tend to form aggregates in solution, are bioincompatible and limited compatibility with polymeric matrices. For this reason, carbon nanotubes considered for biomedical applications must comply with this basic requirement of solubility. To improve the compatibility of the carbon nanotubes, they were exposed to treatment with HNO₃, NaOH and HCl to eliminate impurities and improve the solubility in other solvents. Polycaprolactone coatings at 2.5% w/v and multi-wall carbon nanotubes (0.5, 1.0 and 1.5 g/L) were developed to evaluate the electrochemical and bioactive properties. Techniques such as infrared spectroscopy, contact angle, scanning electron microscopy, atomic force microscopy, electrochemical impedance spectroscopy using simulated body fluid as electrolyte and the bioactivity of human osteosarcoma cells (HOS ATCC® CRL-1543) by alkaline phosphatase were used. From the reported characterizations, it was observed that the resistance to polarization increases according to the degree of dispersion of carbon nanotubes present in the polymer matrix, decreasing the degradation rate of the material.

1. Introduction
The alloy titanium–6 aluminium–4 vanadium (Ti6Al4V), one of the main ones since world war II with 6% aluminium and 4% vanadium [1] is very useful in the biomedical industry because aluminium and vanadium act as stabilizers of the two allotropic phases of titanium (α and β phases), improving the corrosion resistance of an implant. However, this material presents some drawbacks, its elastic modulus is higher than that of human bone, this can cause stress and implant failure due to bone resorption. In addition, this alloy is biologically inert, the organism recognizes the implants as strangers, isolating them, for which osseointegration is hindered by inducing the occurrence of faults in the prosthesis. To improve the life of the implants, its surface can be modified by applying a biocompatible coating [2] such as polycaprolactone (PCL) which is a biodegradable polymer of great interest used in tissue engineering for various organs, including bone, cartilage, tendon, ligament, blood vessel, skin and nerve. However, because it exhibits low mechanical properties, it cannot be applied in situations of high load, limiting its use in the engineering of bone tissues [3,4].

Since the year 2000, carbon nanotubes have taken relevance in biomedical applications, with 2004 being the beginning of the use of compounds based on these for bone engineering [5]. The high tensile
strength, excellent flexibility and low density of carbon nanotubes make them ideal to produce lightweight high-strength materials such as bone [6]. However, it is necessary to purify them by acid treatments in order to remove deposits of remaining metals inside and outside the tubes. On the other hand, the low dispersion of nanotubes in aqueous and organic solvents due to the high Van der Waals forces and the π-π bonds, limits their use in medicine. To correct this, mechanical functionalization can be implemented, such as the use of ultrasound or grinding, or physical and chemical functionalization, which are based on the adsorption, hydrophobic interaction or covalent binding of different molecules that make the nanotubes more hydrophilic [7].

In recent studies [4,8], the interaction between carbon nanotubes (CNT) and polycaprolactone was evaluated for its application in osteosynthesis, the formation of nanocomposites that facilitate the proliferation of osteoblasts and model cell morphology, as well as their interaction when being in contact with stem cells [3], in turn, analysis of the relaxation behaviour of the material before the applied stresses and the mechanical properties of the same, to identify an optimal composition applicable in the bone tissue engineering, making sure that the addition of carbon nanotubes in polymer matrices increase the mechanical properties of the matrix [9]. We propose a coating of PCL with multi-walled carbon nanotubes (MWCNT) made by spin coating as a possible coating for the improvement of the titanium alloy Ti6Al4V surface, evaluating the electrochemical and bioactive properties by electrochemical impedance spectroscopy and activity of HOS cells by phosphatase alkaline respectively, in addition to the physicochemical properties.

2. Experimental

Multi-walled carbon nanotubes (MWCNT) from 6 to 13 nm of diameter and from 2.5 to 20 μm length (Sigma Aldrich CAS number 308068-56-6) and polycaprolactone (Sigma Aldrich CAS number 24980-41-4 Mn 80000) were used to make the coatings. The carbon nanotubes (CNT) were exposed to an acid - base treatment with HNO₃, NaOH and HCl for elimination of impurities. It was started with a reflux of 9 M HNO₃ for 3 hours at 130°C, then the MWCNT cooled and then introduced in a 0.1 M NaOH solution for 1 hour at room temperature. After this time, another reflux was carried out with hydrochloric acid 5M for 6 hours at 110°C, then rinsed with d-H₂O until neutral pH and filtered under vacuum in order to remove excess liquid. Finally, they were exposed to a drying for 8 hours at 80°C.

The coatings were obtained using as substrate a Ti6Al4V alloy of 13 mm diameter and 3 mm thickness with a surface prepared with silicon carbide paper number 180, 240, 360, 400 and 600 and chemically treated in accordance with the ASTM E407-07 standard (2015) [10] for 20 seconds. Finally, the samples were washed and dried with hot air. PCL was dissolved in acetic acid (AA) obtaining a solution 2.5% w/v by mechanical stirring for 4 hours at room temperature. Three additional solutions were prepared, adding MWCNT in concentrations of 0.5 g/L, 1.0 g/L and 1.5 g/L. Each of these 3 solutions were taken to an ultrasound equipment in order to improve the dispersion using 45% amplitude and 2:1 pulse for 60 minutes. Finally, the solutions were subjected to mechanical agitation and ultrasonic bath for 30 minutes at 25°C.

The spin coating technique was used to obtain the coatings, 5 layers were deposited, each with a time of 60 seconds and a drop volume of 30 μL. The speed used for the first layer was 3000 rpm, while the other 4 were performed at 4,000 rpm. In order to improve the adhesion of the coating, the substrates were exposed to a heat treatment at 50°C for 10 minutes between layers. The FT-IR spectroscopy technique was used to study the presence of the functional groups in the coatings, using the attenuated total reflection technique (ATR) in a range of 4,000 to 400 cm⁻¹. The wettability of the coatings was evaluated by contact angle in an OCA 15EC software SCA 20 Module (Data Physics Instruments GmbH Germany) using a drop volume of 1 μL. Images were taken at 120 seconds after the drop was deposited. The morphology of the coatings was analysed by scanning electron microscopy (SEM Evo Ma 10 Carl Zeiss Detector X-act oxford instruments) with a working distance of 8.5 mm and a voltage of 15 kV, coupled with the energy dispersion spectrometry technique of X-rays (EDS). Atomic surface microscopy (AFM equipment NX10 Park Systems) was used to evaluate surface roughness.
Electrochemical impedance spectroscopy using SBF as electrolyte [11] was used to evaluate the degradation of the coating when it is in contact with the physiological medium. The software Zview v3.2b was used in order to analyse the resistance to the degradation of the different coatings. The samples were left immersed for 0, 4 and 8 days in 15 mL of SBF at a temperature of 37°C ± 1.5°C. A Gamry 600 potentiostat was used, using a graphite bar, a calomel electrode as a reference electrode, and a cell with exchangers that allowed temperature stability at 37°C during the test, circulating hot water from a thermostatic bath. The exposure area was 1 cm². The frequency used was 100,000 Hz at 0.01 Hz with 10 mV amplitude and a potential stabilization time of 300 seconds.

The bioactivity of human osteosarcoma cells (HOS ATCC® CRL-1543) exposed to the coatings was evaluated by the production of alkaline phosphatase (ALP). For this, HOS cells in exponential growth phase (4 days) at a concentration of 1.5×10⁴ cells/mL were seeded on the coatings previously placed on 24-well plates and incubated for 15 days on RPMI culture medium 10% of SFBi at 37°C, 5% CO₂ and 95% humidity. The amount of ALP produced by the cells was determined in the supernatant medium after 7 and 15 days of incubation and in cell lysate (ALP contained inside the cells) obtained by addition of Triton X-100 for 10 minutes with centrifugation at 3,000 rpm after 15 days of incubation. All samples were stored at -20°C until used. Cells seeded in wells without coating were used as control and coatings were sterilized by a stream of ethylene oxide. The concentration of ALP was determined by visible ultraviolet spectroscopy in a microplate reader Synergy H1 (BioTeK) software Gen5 V3.03v by modification of the technique described in the BioSystems kit for alkaline phosphatase. Briefly, in a 96-well culture plate, 6 μL of the sample plus 300 μL of p-nitrophenyl phosphate disodium salt (p-NPP) 0.1M is added and the reading was made at a wavelength of 405 nm.

3. Results and discussion

From the acid - base treatment that was made to the multi-walled carbon nanotubes, it was found through EDS that the treatment served to purify the nanotubes and decrease the percentage of elements such as cobalt, coming from the same manufacturing process of the nanotubes. It was found for pristine nanotubes in %At (C-88.12%, O-5.32%, Mg-4.65%, Al-0.26% and Co-1.66%) while for treated nanotubes (C-93.64%, O-6.35% and Co-0.01%).

3.1. Chemical and morphological characterization of coatings

In the infrared spectrum, the presence of C = O, C–C, C–O, asymmetric C–O–C and symmetrical C–O–C stretching is detected, as well as the asymmetric and symmetric C–H stretching of the bonds present in PCL [12-14]. Table 1 shows the positions in the wavelengths for each assigned band.

| Sample        | Asymmetric stretch C-H₂ | Symmetric stretch C-H₂ | Stretching C=O | Stretch C-C and C-O | Symmetric stretch C-O-C | Symmetric stretch C-O-C |
|---------------|-------------------------|------------------------|----------------|---------------------|-------------------------|-------------------------|
| PCL - 0.0 g/L CNT | 2.941.4                 | 2.856.1                | 1.727.9        | 1.297.4             | 1.193.4                 | 1.193.4                 |
| PCL - 0.5 g/L CNT | 2.937.1                 | 2.849.3                | 1.723.6        | 1.294.5             | 1.188.0                 | 1.188.0                 |
| PCL - 1.0 g/L CNT | -                      | -                      | 1.723.1        | 1.296.4             | 1.170.4                 | 1.170.4                 |
| PCL - 1.5 g/L CNT | -                      | -                      | 1.722.0        | -                   | -                       | -                       |

The presence of polycaprolactone in the coatings is perceptible as soon as the concentration of the carbon nanotubes is low as visualized in the spectra shown in Figure 1, where the high order or symmetrical link of them interferes in the evaluation of the vibrations of the chemical bonds and in the signalling of the functional groups present [4].
The SEM micrographs of the coatings obtained by spin coating on Ti6Al4V are shown in Figures from 2 to 5. It is observed that at a concentration of MWCNT 0.5 g/L the dispersion is little, causing spaces not occupied by the nanotubes, while the coatings at 1.0 g/L and 1.5 g/L are more homogeneous, however, at a concentration of 1.5 g/L, the presence of a greater number of agglomerates is evidenced.
3.2. Hydrophilicity and roughness of PCL and PCL MWCNT coatings

Table 2 shows the values for the contact angle and the roughness of the different surfaces. The hydrophobic character of the polycaprolactone is evidenced by increasing the value of the angle with respect to the uncoated sample. Likewise, carbon nanotubes have intrinsic hydrophobicity, which causes the hydrophobicity to be increased proportionally to the increase in MWCNT concentration when the carbon nanotubes are added to the polymer matrix.

| Sample          | Contact angle | Roughness (nm) |
|-----------------|---------------|----------------|
| Ti6Al4V-ELI     | 61.30°        | 20.7 ± 1.1     |
| PCL - 0.0 g/L CNT| 62.45°        | 27.7 ± 5.5     |
| PCL - 0.5 g/L CNT| 70.75°        | 187 ± 111.2    |
| PCL - 1.0 g/L CNT| 72.45°        | 138.4 ± 14.9   |
| PCL - 1.5 g/L CNT| 80.45°        | 181.4 ± 6.5    |

On the other hand, higher rugosities are found for PCL – 1.5 g/L CNT, typical of the morphology observed by SEM. However, the standard deviations of the roughness for PCL - 0.5 g/L CNT are greater, because in this coating there are spaces not occupied by carbon nanotubes, since the dispersion is not as efficient in an acid solvent as the AA and at low concentrations of MWCNT.

3.3. Electrochemical evaluation of PCL and PCL MWCNT coatings

In Figures from 6 to 8 the Nyquist diagrams of the coatings evaluated at 0, 4 and 8 days in SBF are presented. By increasing the number of immersion days, an increase in polarization resistance (Rp) was obtained for the samples with nanotubes, especially for those of 1.0 and 1.5 g/L of MWCNT, which leads to the non-release of the coating obtaining values higher than those reported for PCL without nanotubes (see Table 3). This implies that the permeability of the coating was reduced by adding the MWCNT, reducing in turn the degradation rate [15,16]. Additionally, by increasing the days of evaluation, the presence of more layers is recorded, evidenced by the presence of semicircles in the Nyquist diagram. These layers can be attributed to a process of adsorption of ions from the solution, especially calcium ions on the surface, which attributes a good interaction with the medium to this coating after the time of experimentation [9,16].

| Sample          | Rp - 0 days   | Rp - 4 days   | Rp - 8 days   |
|-----------------|---------------|---------------|---------------|
| PCL - 0.0 g/L CNT| 1,527 ± 13.34 | 978.6 ± 4.67  | 1,578 ± 6.30  |
| PCL - 0.5 g/L CNT| 281.8 ± 17.57 | 111 ± 4.92    | 1,473 ± 26.40 |
| PCL - 1.0 g/L CNT| 256.2 ± 34.62 | 1,812 ± 2.31  | 1,772 ± 20.55 |
| PCL - 1.5 g/L CNT| 142.2 ± 7.68  | 1,237 ± 1.23  | 1,756 ± 12.53 |
3.4. Bioactivity of the coatings by phosphatase alkaline

In the coatings analysis, the two of the surfaces with the highest amount of MWCNT increased the amount of ALP synthesized by the cells as the cell incubation time increases, while the for the surfaces PCL - 0.0 g/L CNT and PCL - 0.5 g/L CNT, the amount of ALP decrease when the incubation time was 15 days, see Figure 9. This indicates that the carbon nanotubes function as mechanical reinforcement of the polymer matrix, since there is less degradation of the coating, as observed in the electrochemical tests. The increase in ALP activity is indicative of cell differentiation towards the osteoblastic lineage [17,18].

![Figure 9. ALP activity of HOS cells culture in PCL and PCL MWCNT coatings in 7 and 15 days (supernatant) and cell lysate at 15 days. Data represents the mean ± standard deviation for n = 3, *p<0.05 and **p<0.01 compared with the controls (statistical test of Tukey-Kramer pair-wise comparison).](Image)

The best ALP response after 15 days incubation was for the surface PCL 1.0 g/L CNT and PCL 1.5 g/L CNT, suggesting that the CNT aggregation in the polymer matrix played an important role in the cell proliferation and differentiation process of HOS cells. For the cell lysate, there was no significant difference between all groups after 15 days incubation (p>0.05).

4. Conclusions

The purification treatment carried out on the carbon nanotubes served as a means for a better dispersion of these in the PCL-AA solution. Also, as the concentration of the carbon nanotubes increases, the hydrophobic character of the coating surface increases. However, this increase in hydrophobicity does not adversely affect the degradation of the PCL-MWCNT coating. The compatibility with the biological medium (SBF) was benefited by using the concentration of nanotubes studied at 1.0 g/L, due to the homogeneous dispersion of the coating, giving greater stability to it according to the electrochemical tests.

The use of multi-walled carbon nanotubes in polycaprolactone coatings by spin coating allows to increase the resistance to polarization of the material, which implies a decrease in the degradation speed of the coating, extending the useful lifetime as a bone implant. Additionally, the aggregation of MWCNT to the polymer matrix allows for a suitable microenvironment for cell differentiation according to the results of ALP.

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