

In Vitro Biomineralization of a Novel Hydroxyapatite/Superhydrophilic Multiwalled Carbon Nanotube Nanocomposite using Simulated Body Fluids

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Nanobiomaterials based on superhydrophilic vertically-aligned multi-walled carbon nanotubes (VAMWCNT-O₂) are promising for their properties and bone tissue biocompatibility. VAMWCNT-O₂ films with nanohydroxyapatite (nHAp) aim to improve mechanical properties and biocompatibility of this new nanocomposite due to its resemblance to bone matrix structure. This study aimed to produce *in vitro* biomineralized nHAp/VAMWCNT-O₂ nanocomposites using simulated body fluid (SBF) with two different pHs (6.10 and 7.40) during 7 days to obtain a new surface design with higher crystallinity and better morphology of nHAp/VAMWCNT-O₂ nanocomposites. The objective is to obtain biomineralized nanobiomaterials to enable its applicability as “scaffold” to cellular support and consequent bone tissue formation, accelerating the osseointegration. Layer densification has been achieved due to polycrystalline nanoapatites deposition on surface and between the biomineralized nHAp/VAMWCNT-O₂ nanocomposites, without any heat treatment. Therefore, through its characteristics and properties these nanocomposite applications can be considered extremely viable for acceleration of *in vivo* regenerative processes.

Keywords: *biomineralization, carbon nanotubes, superhydrophilic, nanohydroxyapatite, SBF and characterization techniques*

1. Introduction

In nature, organisms control the nucleation and growth of crystals using interfaces such as organic templates¹. Scientists have been trying to learn from nature how to design biomimetic biomaterials, inspired by bone complex hierarchical structure and other mineralized natural tissues, and how to control the biomineralization process on biomaterials substrates to induce osteoconductive properties on implantable devices². The analysis of artificial materials bioactivity, when implanted *in vivo* can be reproduced by immersion tests in a simulated physiological solution that mimics the ion concentration of body typical fluids, known as Simulated Body Fluid (SBF)³. Biomineralization key aspects may also be important to propose new methodologies to improve the calcification on the surface of biomaterials or to develop bioactive three dimensional models that can be used in regenerative medicine¹. It has been demonstrated that some chemical groups and proteins,

as well as three dimensional matrix where calcification could take place, play a key role in nucleation and growth of nanohydroxyapatite (nHAp) and calcite gotten through biomineralization⁴.

The model of induced nHAp has broad prospects in applied fields of bone regenerative medicine, since nHAp is classified as one of the best biocompatible and bioactive materials with many biological applications, such as scaffolds for bone repair⁵. Superhydrophilic vertically aligned multi-walled carbon nanotubes (VAMWCNT-O₂) have been of current interest because their excellent mechanical properties and biomimetic characteristics⁶. Therefore, the combination of these two materials can be interesting for bone tissue engineering. The VAMWCNT-O₂ functional groups, such as carboxyl (–COOH), act as deposition and nucleation sites for nHAp⁷. Polycrystalline nanoapatites formation occurs over and between VAMWCNT-O₂ through biomineralization in SBF or other Ca/P solutions, as the one used in this study for nHAp electrodeposition process.

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The preparation of SBF solution has been made through different methodologies in the literature, which culminates in the presence of apatite crystals with different morphologies and apatite deposition processes are not sufficiently clear⁸. Despite these different methodologies, the precipitation process of bioactive calcium phosphate in SBF and the influence of the medium composition on its formation and phase transformation have attracted extensive research interest, because of their analogy to the biological mineralization process^{1,3,4,8}. For the first time, the biom mineralization process of nHAp/VAMWCNT-O₂ nanocomposites will be tested comparing two different methodologies of SBF solution aiming to improve nanocomposite features, getting higher crystallinity and better morphology of grown crystals, enabling its uses as *in vivo* grafts.

2. Methodology

2.1. Synthesis of vertically aligned multi-walled carbon nanotubes (VAMWCNT)

The VAMWCNT were produced as thin films using a microwave plasma chamber equipped with a 2.45GHz microwave generator (MWCVD). The substrates were 10 mm titanium squares covered by a thin Fe layer (10 nm) deposited by an e-beam evaporator. The Fe layers were pre-treated to promote nanocluster formation, which forms the catalyst for VAMWCNT films growth. The pre-treatment was carried out for 5 minutes in plasma atmosphere of N₂/H₂ (10/90 sccm) at a substrate temperature around 760 °C. After pre-treatment, CH₄ (14 sccm) was inserted into the chamber at a substrate temperature of 760 °C for 2 minutes. The reactor was kept at a pressure of 30 Torr during the entire process⁹.

2.2. VAMWCNT functionalized by polar groups

Functionalization of the VAMWCNT tips by the incorporation of oxygen-containing groups to obtain the superhydrophilic character was performed in a pulsed-direct current plasma reactor with an oxygen flow rate of 1 sccm, at a pressure of 85 mTorr, -700 V, and with a frequency of 20 kHz. The total time of the plasma etching was 120 seconds. Detailed descriptions of the superhydrophilic properties to produced VAMWCNT-O₂ are given elsewhere⁹.

2.3. nHAp/VAMWCNT-O₂ nanocomposites fabrication

The electrodeposition of the nHAp crystals on the VAMWCNT-O₂ films was performed using 0.042 mol L⁻¹ Ca(NO₃)₂·4H₂O + 0.025 mol L⁻¹ (NH₄)₂HPO₄ electrolytes (pH 4.8). These concentrations were chosen according to the 1.67 Ca/P ratio. The electrochemical measurements were carried out using a three-electrode cell coupled to Autolab PGSTAT128N equipment. VAMWCNT-O₂ films were used as the working electrode and the geometric area in contact with electrolytic solution was 0.27 cm². A platinum coil wire served as the auxiliary electrode and an Ag/AgCl electrode was used as the reference electrode. The nHAp crystals were produced by applying a constant potential of -2.0 V for

30 minutes and the solution temperature was maintained at 70 °C and constant agitation for 30 minutes. These samples were named as nHAp/VAMWCNT-O₂¹⁹.

2.4. nHAp/VAMWCNT-O₂ nanocomposites biom mineralization

The SBF (5×) solution was prepared by dissolving ions in distilled water at a stir plate. The SBF (5×) compositions used were proposed by Barrere et al.^{10,11} (Table 1), whose components concentration are 5× higher than the original solution proposed by Kokubo¹² and solutions pH was adjusted at pH meter (Metrohm), so that values of 6.10 (HCl) and 7.40 (NaOH) were obtained.

The nHAp/VAMWCNT-O₂ samples were placed in a corning tube with 15 mL of each SBF (5×) pH solution (adjusted to: 6.10 and 7.40) during 7 days and placed in refrigerated bench top incubator (Cientec CT-712-R), shaken at 75 rpm at a temperature around 36.5 °C. After the biom mineralization period, the samples were removed from the SBF (5×) solution, washed with hot distilled water and dried in a laboratory stove for 24 hours at 50 °C. The objective of this methodology was obtained different apatites formations on nHAp/VAMWCNT-O₂ nanocomposites.

2.5. nHAp/VAMWCNT-O₂ nanocomposites characterization

To carry out the morphological analysis of all types of samples analyzed in this study, a scanning electron microscope was used (SEM, model: JEOL-JSM 5610 VPI). Images were recorded at magnification of 1000-100,000×.

Semi-quantitative elemental analyses of calcium and phosphorus were carried out by micro energy-dispersive X-ray fluorescence (μ-EDXRF) (1300, Shimadzu, Kyoto, Japan), equipped with a rhodium X-ray tube and a Si (Li) detector cooled by liquid nitrogen (N₂). The energy range of scans was from 0.0 to 40.0 eV. The voltage in the tube was set at 15 kV.

The structural analysis of apatites formed on the superhydrophilic nHAp/VAMWCNT-O₂ surface were performed at room temperature by X-ray diffractometry (XRD) with CuK-α radiation (α = 0.154056 nm) from 10 to 70° in 2-theta with the condition voltage of 40 kV, current of 30 mA, step size of 0.02°, and a counting time of 2 seconds per step (Model: X'Pert MRD, Philips).

An Optical profilometer was used to measure the topography and to estimate the thickness of the material. The nHAp/VAMWCNT-O₂ nanocomposites roughnesses were measured by a profilometer (Model: WYKO NT 1100 series Optical Profiling System).

Table 1. SBF (5×) components and concentration.

Reagents	Quantity (mM)
NaCl	733.5
MgCl ₂ ·6H ₂ O	7.5
CaCl ₂ ·2H ₂ O	12.5
Na ₂ HPO ₄ ·2H ₂ O	5.0
NaHCO ₃	21.0

3. Results and Discussion

The literature identifies different methodologies for preparing biomimetic SBF solution^{10,11}. It was initially proposed by Kokubo¹², but in order to minimize the reactants and make it less expensive, small changes were made⁸. In addition to the various types of reagents, it had also been proposed different pHs due to improvements on Ca and P precipitation process. All procedures were performed in two dimensional surfaces using, in most cases, Ti alloys¹³. VAMWCNT-O₂ can be considered candidate to SBF biomineralization process too due to the presence of calcium carbonate⁷.

After electrodeposition process, nHAp/VAMWCNT-O₂ surface is negatively charged and selectively combined with Ca ions positively charged, forming polycrystalline nanoapatites¹⁴. As Ca ions accumulate on the biomaterial surface, it becomes positively charged and these ions combine with phosphate ions negatively charged, forming calcium phosphates¹⁵. The calcium phosphate apatite spontaneously turns, simulating the many phases of the chemical calcium phosphate present in biological tissues such as bone, which justifies its use as nanobiomaterial¹³.

When immersed in SBF (5×) pH 7.40, nHAp/VAMWCNT-O₂ nanocomposite surface keeps its charge features, and aside from nHAp crystals, calcite is also formed, likely because of carbonate ions present in SBF (5×) solution, and start replacing released phosphate groups in the nHAp¹⁶. These crystals can be identified as the deposited ones observed in Figures 1, 2 and 3.

Figure 1 shows SEM micrographs of nHAp/VAMWCNT-O₂ nanocomposites surfaces before (Figure 1a – control group) and after immersion in SBF (5×) at the two used pH: 6.10 (Figure 1b) and 7.40 (Figure 1c). Figure 1a shows nHAp crystals homogeneously electrodeposited over the VAMWCNT-O₂. Figures 1b, c refer to nHAp/VAMWCNT-O₂ nanocomposites immersed in SBF (5×) at pH 6.10 and 7.40, respectively. The samples immersed in pH 6.10 (Figure 1b) showed plate-like crystals and clusters of nHAp, while in pH 7.40 (Figure 1c) there is a morphologic change to polycrystalline and globular nanoapatites more homogeneously deposited, appearing more spherulitic, with a complete nanoapatite layer densification, that loosened up of the VAMWCNT-O₂ surface.

μEDXRF analysis shows lower Ca/P ratio in control sample (1.13, Table 2) than in the samples immersed in

solution at pH 6.10 (Figure 1b), which had surface coated with similar apatite nanocrystals formations, but with different sizes. The samples submitted to pH 7.40 were more homogenous and the polycrystalline nanoapatites formed presented Ca/P ratios of 2.44, indicating that the calcium concentration is increasing while phosphorus is decreasing, supposing that the phosphate is being replaced by carbonate. It was found a homogeneous Ca and P deposition on the nHAp/VAMWCNT-O₂ nanocomposite before and after the process of biomineralization (distribution data not shown); however it was decided to use only the Ca/P ratio for comparison (Table 2). We used electrodeposited nHAp on the VAMWCNT-O₂ as control analysis.

Figure 2 shows the composites roughness before (Figure 2b) and after biomineralization (Figure 2b, c), using an optical profilometer. To calculate the average roughness (Ra), three measurements were made on each sample (Table 3). These analyzes were made because we observed a variation in the samples microstructures dimensions (formation of polycrystalline nanoapatites) at both pHs studied. When compared to the control sample (Figure 2b), all other samples showed higher roughness. The samples immersed in pH 7.40 showed a lower Ra when compared to ones immersed in pH 6.10 (Figures 2c and 2b, respectively).

These surface morphological changes culminate in differences in roughness analysis shown in Figure 2 and Table 3. It was observed that there was no uniform growth of samples surface layer due to the presence of both crystals and globular apatites. In samples immersed in pH 7.40 with a globular morphology and dense layer, the Ra was found superior to the samples under pH 6.10 with crystalline morphology, due to densification of spherulitic nanoapatites from samples under pH 7.40 (Figure 1c), when compared to crystals from samples under pH 6.10 (Figure 1b). Surface roughness can deform the cell membrane and hinder the motion vesicles inside the cell, affecting the distribution and diffusion of adhesion proteins. Roughness also generates

Table 2. Mean of calcium and phosphorus percentages and Ca/P weight ratios obtained by X-ray fluorescence.

Samples	Calcium	Phosphorus	Ca/P ratio
Control	53.16	46.83	1.13
pH = 6.10	63.55	36.45	1.74
pH = 7.40	70.95	29.04	2.44

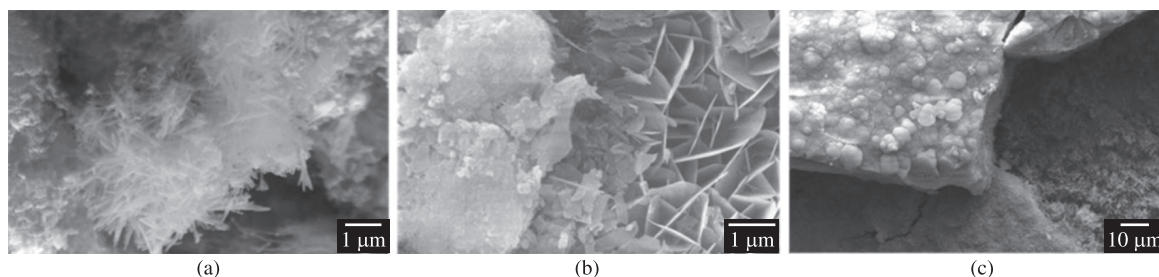


Figure 1. SEM images of nHAp/VAMWCNT-O₂ nanocomposite before (a) and after biomineralization for 7 days in SBF pH 6.10 (b) and pH 7.40 (c).

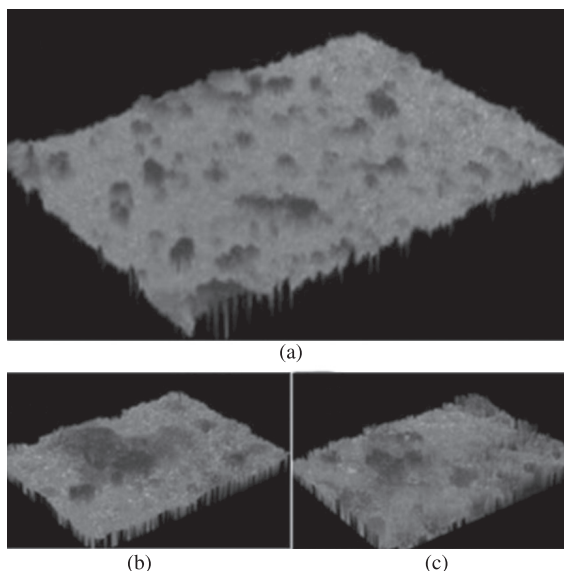


Figure 2. Images of optical profilometry of nHAp/VAMWCNT-O₂ nanocomposites immersed for 7 days in SBF at pH 6.10 (b) and pH 7.40 (c). The images were obtained in the range of 300 μm².

Table 3. Roughness of biomineralized nHAp/VAMWCNT-O₂ nanocomposites.

Samples	Roughness Average (RA)	Roughness Total (RT)
Control	3.11 + 0.83	33.97 + 2.95
pH = 6.10	6.63 + 1.26	71.72 + 3.40
pH = 7.40	14.74 + 0.51	117.56 + 13.26

a tension on actin filaments, enhancing cell adhesion, which makes biomineralized nHAp/VAMWCNT-O₂ nanocomposites great candidates to promote tissue attachment and bone growth¹⁷.

The Ca/P ratio shown in Table 2 could be confirmed by Grazing-Incidence X-Ray Diffraction shown in Figure 3. The nanocomposites biomineralized at pH 6.10 presented only nHAp and Ti peaks, while the ones biomineralized at pH 7.40 also showed five significant peaks of calcium carbonate, or calcite (CaCO₃).

In pH 7.40, the calcium ions are released from CaCO₃⁻ particles and react with PO₄³⁻ and HPO₄²⁻ ions to form nHAp, while calcium from nHAp also works as active sites for CO₃⁻ to form calcite. These aggregations not only produces plate-like and globular crystals, but also form different designs of nanoparticles with different sizes and a layer as active sites, where may occur more nHAp crystals and calcite nucleation, providing a higher contact area and increasing cellular adhesion. These mechanisms occur not as an exchange reaction but as a dissolution-precipitation reaction¹⁸.

At the same time, the different nanoparticles formed can create grooves which can be used as ion-transferred

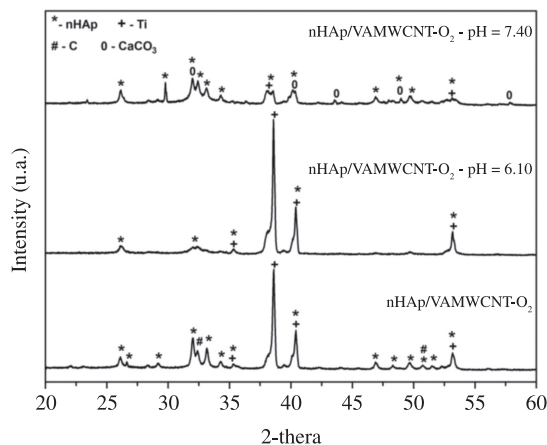


Figure 3. Grazing- Incidence X-Ray Diffraction.

channels, through which the Ca²⁺, CO₃²⁻ and PO₄³⁻ ions are released from the surfaces of CaCO₃ and nHAp nanoparticles, and ions enter into the interfaces between the CaCO₃ and nHAp, forming new apatite crystals. These processes are suggested to promote cell adhesion, adsorption of biological metabolites because its similarity to bone tissue, working as “scaffold” for bone growth and repair¹⁹.

4. Conclusion

It was obtained biomineralization of nHAp/VAMWCNT-O₂ nanocomposites through SBF (5×) immersion at both pHs studied. At pH 6.10 the nanocomposites showed a rougher surface with plate-like crystals and globular apatites and also a higher Ca/P ratio, when compared to the samples which were not immersed in SBF (5×) solution. The pH 7.40 solution promoted a denser nanoapatite layer formation, globular apatites, higher Ca/P ratio and the presence of calcite. These findings are motivating because they indicate a surface improvement and the applicability of this new biomineralized biomaterial based on nHAp/VAMWCNT-O₂ nanocomposite as “scaffold” for bone regenerative medicine. However, further *in vitro* and *in vivo* studies assays are underway to better characterize the bioactivity and calcification of biomineralized nHAp/VAMWCNT-O₂ nanocomposites and which group (pH 6.10 or 7.40) should be more appropriate to bone tissue repair.

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ERRATUM

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