

Calcification *in vitro* of Biom mineralized Nanohydroxyapatite/Superhydrophilic Vertically Aligned Multiwalled Carbon Nanotube Scaffolds

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Received: November 1, 2012; Revised: December 19, 2012

Nanocomposites based on superhydrophilic vertically aligned multi-walled carbon nanotubes (VAMWCNT-O₂) and nanohydroxyapatite (nHAp) are of great interest in bone regenerative medicine. The biom mineralization using simulated body fluid (SBF) has been extensively studied to evaluate the bioactivity of biomaterials. Thus, the combination of nHAp and VAMWCNT-O₂ is attractive and promising. The aim of this study was to evaluate the *in vitro* calcification of nHAp/VAMWCNT-O₂ nanocomposites before and after the period of biom mineralization in SBF. *In vitro* calcification of the extracellular matrix (ECM) of HOB cells in culture after 24 hours was investigated through the assay of alkaline phosphatase. These promising *in vitro* results validate biom mineralized nHAp/VAMWCNT-O₂ as possible scaffolds for bone tissue regeneration.

Keywords: carbon nanotubes, hydroxyapatite, superhydrophilic, osteoblastic cells, SBF, calcification, cellular adhesion

1. Introduction

Carbon nanotubes (CNTs) are tubular structures formed by hexagonal arrays of carbon, which can be classified into two types: single walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs)¹⁻⁴. The MWCNTs are of particular interest for regenerative medicine due to their biocompatibility that permits extensive cell adhesion, spreading, and growth. MWCNT has been shown to support the growth of osteoblast cells by stimulating ECM production in bone tissue formation and growth of hydroxyapatite crystals⁵. Its properties and biocompatibility are improved when MWCNT becomes aligned (VAMWCNT) and superhydrophilic (VAMWCNT-O₂)³.

The nanohydroxyapatite (nHAp) is a ceramic material that promotes bone growth due to its biocompatible and bioactive behavior. nHAp has application in bone regeneration because it can establish chemical bonds between the material and the bone tissue allowing cell proliferation due to its similarity to bone mineralized matrix⁶.

In addition to the application of nHAp/VAMWCNT-O₂ as scaffolds, the process of biom mineralization in biomaterials conducted by incubating the samples in SBF (ion concentrations similar to human extracellular fluid) assists the development of biological apatites on the surface of nHAp/VAMWCNT-O₂, improving *in vitro* adhesion and proliferation of human osteoblast cells (HOB)^{7,8}.

The osteoblasts are coated cells responsible for the synthesis of organic components of the bone matrix. They release fibronectins, which have the ability to form bone calcification and increase the release of alkaline phosphatase (ALP). ALP is an enzyme responsible for cell proliferation and increasing, which is widely distributed in human tissues, such as bone (osteoblasts), so that bone activity is confined to osteoblast and ALP assays⁹.

The objective of this study was to evaluate the ECM calcification potential of HOB cultured on this new biom mineralized nHAp/VAMWCNT-O₂ nanocomposite, to be used as scaffolds in bone tissue regeneration.

2. Methodology

The VAMWCNTs were produced as thin film, using a microwave plasma chamber (2.45 GHz). The substrates were 10 mm titanium squares, covered by a thin of Fe layer (10 nm), both deposited by an e-beam evaporator. The Fe layers were pretreated to promote nanocluster formation, which forms the catalyst for VAMWCNTs growth. The pretreatment was carried out during 5 minutes in plasma of N₂/H₂ (10/90 sccm) at a substrate temperature around 760 °C. After pretreatment, CH₄ (14 sccm) was inserted in the chamber at a substrate temperature of 760 °C for 2 minutes. The reactor was kept at a pressure of 30 Torr during the whole process.

Functionalization of the VAMWCNT tips by the incorporation of oxygen-containing groups to obtain the

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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 kHz⁶.

The electrodeposition of the nHAp crystals on the superhydrophilic VAMCNT (VAMWCNT-O₂) films was performed using 0.042 mol.L⁻¹Ca(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 made using a three-electrode cell coupled to an Autolab PGSTAT 128N equipment. VAMWCNT-O₂ films were used as working electrode, and the geometric area in contact with electrolytic solution was 0.27 cm². A platinum coil wire served as auxiliary electrode, and an Ag/AgCl electrode was used as reference electrode. The nHAp crystals were produced 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-O₂⁶.

Biom mineralization of the nHAp/VAMWCNT-O₂ samples occurred in SBF (5×)^{10,11} of the concentration proposed by Kokubo¹² and shown in Table 1. The pH of the solution was adjusted to 6.10 using HCl addition. Each nHAp/ VAMWCNT-O₂ sample was placed in a corning tube with 15 mL of SBF. Samples were shaken at 36.5 °C for 7, 14, and 21 days, and the SBF (5×) solution was changed every 3 days of experimentation. After the biom mineralization period, samples were removed from SBF, washed in hot deionized water, dried in a laboratory stove at 50 °C for 24 hours and put in laminar air flux with UV light for 24 hours to be sterilized. 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 1,000-100,000×.

HOB cells were grown in cell culture bottles in 25 cm³ in low glucose DMEM supplemented with 10% fetal bovine serum and 1% antibiotic antimycotic, incubated in an incubator (Forma Scientific Model 3110), in a controlled atmosphere of 5% CO₂ at 37 °C. The HOB cells were replicated from the initial count and the cell culture medium was replaced every 48 hours for better conditions for cell proliferation and viability.

The HOB cells were trypsinized for 3 minutes, centrifuged for 5 minutes, and resuspended in DMEM low glucose (Gibco) supplemented with 10% fetal bovine serum and 1% antibiotic antimycotic. The volume of 10 µL was applied to the chamber and brought to Newbauer HOB cell count at inverted microscope (Olympus CK 40) and determined concentration of 4.0 × 10⁵ cells/mL.

The samples were placed in individual wells of three plates and 24 culture wells, seeded on each group at a concentration of 4.0 × 10⁵ cells/mL and dried at 37 °C with 5% CO₂ for 30 minutes. The samples were overlaid with DMEM low glucose (Gibco) until a total volume of 500 µL and incubated in the laboratory stove for 24 hours and 48 hours. Both the cellular adhesion test and ALP assay were performed in triplicate.

The nHAp/VAMWCNT-O₂ nanocomposites were sterilized for 24 hours under UV irradiation and placed in individual wells of 24-well culture plates. The cells were maintained as sub-confluent monolayers in minimum

Table 1. Quantity of Reagents used to prepare SBF.

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

essential medium (MEM) with 1.5 mM L-glutamine adjusted to contain 2.2 g/L sodium bicarbonate 85%; fetal bovine serum (FBS) 15% (Gibco, BRL), 100 units/mL penicillin-streptomycin (Sigma), and 25 mg/mL L-ascorbic acid (Sigma). The incubation occurred within a CO₂ (5%) atmosphere at 37 °C.

The HOB cells adhesion on nHAp/VAMWCNT-O₂ nanocomposites were monitored up to 48 hours. The cells were seeded in each well at 5 × 10⁵ cells/mL concentration, supplemented with 10% PBS. The incubation was performed under a CO₂ atmosphere (5%) at 37 °C. The substrate attached cells were fixed with 3% glutaraldehyde/0.1 M sodium cacodylate buffer for 1 hour and dehydrated in a graded ethanol solution series (30%, 50%, 70%, 95%, and 100%) for 10 minutes. each. The drying stage used a 1:1 solution of ethanol with hexamethyldisilazane (HMDS) and the samples were dried with pure HMDS at room temperature. After deposition of a thin gold layer, the specimens were examined with a SEM (model: JEOL-JSM 5610 VPI). Images were recorded at magnification of 1,000-4,000×.

To test the ALP activity, solutions 1 and 2 of the assay Kit Acid Alkaline Phosphatase (Millipore) were prepared according to the product manual. At 24 and 48 hours, the plates were removed from the incubator and 100 µL of solution 1 and 25 µL of solution 2 was added to each well. Then, the plate was covered with foil and kept for 15 minutes at room temperature. The samples were taken to the immunohistochemistry analysis apparatus in a microplate reader (Synergy HT Multi-Detection, Bio-Tek, Winooski, VT) at 620 nm absorbance. The blank reference was taken from wells without cells, also incubated with the ALP solution. The ALP activity was calculated by the normalization of optical density (OD) of the samples (VAMWCNT-O₂ nHAp/VAMWCNT-O₂ nHAp/ VAMWCNT-O₂-7D nHAp/VAMWCNT-O₂-14D nHAp/ VAMWCNT-O₂-21D) to the cell culture and expressed in percentage. The Equation 1 shows the normalization applied:

$$\% \text{ ALP} = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{cell}} - \text{OD}_{\text{blank}}} \times 100 \quad (1)$$

To carry out the statistical analyzes, four different readings were collected, with N = 4, and expressed as the average. The ALP test was used for ECM calcification. Statistical differences were analyzed by Two-way Anova using the GraphPad Prisma® program. The population of the groups was analyzed with independent normal distribution for each experiment. P values less than 0.005 (P <0.005) were considered to indicate statistical differences of the groups.

3. Results and Discussion

SEM examination revealed morphologic differences in the nHAp deposition layer under different processes. Figure 1a shows details of nHAp crystals formed on the VAMWCNT-O₂ surface after electrodeposition process. The plate-like crystal structure covered the entire nanobiomaterial surface, giving the nanobiomaterial a roughness aspect. SEM micrographs, shown in Figure 1b, are VAMWCNT-O₂ details of electrodeposition process before biomineralization, while Figure 1c shows nHAp/VAMWCNT-O₂ details after 7 days of biomineralization. This image shows that there are nHAp not only above, but also between

nHAp/VAMWCNT-O₂ nanocomposite, and it also reveals morphologic differences in formed nHAp, which now has a globular shape. The micrographs indicate a crystallographic structure change from plate-like to a globular morphology of deposited nHAp layer (c, d, e). Furthermore, after 14 days (1d), this layer became denser; however, as this density increases, the roughness decreases as seen after 21 days of biomineralization (1e).

Figure 2 shows HOB cells adhesion on nHAp/VAMWCNT-O₂ before biomineralization (2a) and after 7 days (2b), 14 days (2c), and 21 days (2d) in SBF. SEM examination in Figure 2a shows an HOB adhered on nHAp/VAMWCNT-O₂ surface with short filaments

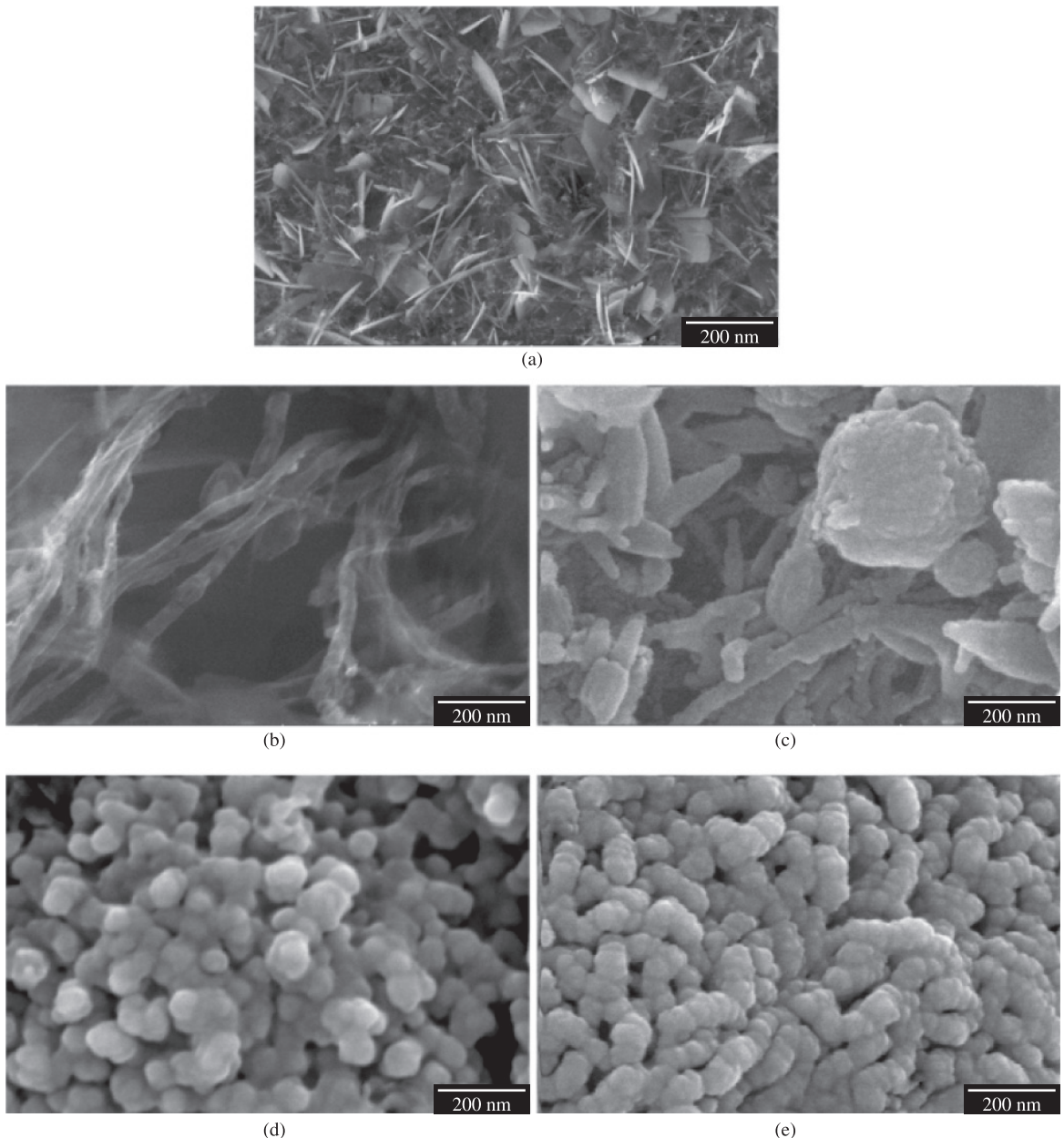


Figure 1. SEM of nHAp/VAMWCNT-O₂ before biomineralization process (a, b), nHAp/VAMWCNT after biomineralization for 7 days (c), 14 days (d), and 21 days (e).

attached to nHAp crystals. At the same magnification (4,000 \times), Figure 2b shows an increased number of HOB cells adhered with longer cytoplasmic projections, already proliferated and in a good spreading process, not only on nHAp/VAMWCNT-O₂ surface, but also between nHAp plate-like crystals clusters. The cell spreading is also evident in Figure 2c, but the presence of HOB is not significant. The same aspect can be found in Figure 2c, but this examination shows another important aspect: an HOB cell well adhered to a nHAp crystal cluster with a long cytoplasmic projection attached it to another cluster of nHAp crystals between nHAp/VAMWCNT-O₂.

Both the nHAp and VAMWCNT-O₂ have physicochemical properties (surface tension, surface free energy, ionic charge, and wettability) suitable for cell adhesion and proliferation⁶; however, surface topography and cell protein production activity also interfere with the cell adhesion process¹³. In relation to surface topography, geometric structure is related to material roughness and porosity. The aspects found on SEM examination shown in Figure 2 could be proved through the results found in Figure 3.

Figure 3 illustrates the results of ALP activity in percentage for 24 and 48 hours of the control (HOB), nHAp/VAMWCNT-O₂, and biomaterialized nHAp/VAMWCNT-O₂ nanocomposites for 7, 14, and 21 days. Two culture times (24 and 48 hours) were used to compare calcification

of the ECM, to validate which biomaterialized nHAp/VAMWCNT-O₂ nanobiomaterials expressed ECM calcification through percentage presented in the thymolphthalein micromol/h/mg (normalized by the Equation 1).

When comparing both groups (24 and 48 hours), ALP showed higher values for nHAp/VAMWCNT-O₂ and nHAp/VAMWCNT-O₂ nanocomposites biomaterialized for 7 days in SBF (nHAp/VAMWCNT-O₂7D) compared to HOB cells. The difference is more significant at 48 hours (nHAp/VAMWCNT-O₂7D). The remaining samples showed no potential for calcification of the ECM as intense as nHAp/VAMWCNT-O₂7D.

The ECM released in the first 24 hours, in rougher and less dense surfaces, is more significant, in this case nHAp/VAMWCNT-O₂ and nHAp/VAMWCNT-O₂7D (Figure 2a, b), being less significant in the thickest surfaces-like nHAp/VAMWCNT-O₂14D and nHAp/VAMWCNT-O₂21D nanocomposites.

In the first 6 hours of contact with the biomaterial, cells begin the surface recognition process⁶. The biomimetic nHAp/VAMWCNT-O₂ nanocomposite surface, before and after 7 days of biomaterialization, characterizes nanocomposite bioactivity. However, after 24 hours the rough surface is replaced by the presence of Ca⁺⁺, as shown in Figure 3. Now, the rougher material with a greater concentration of Ca⁺⁺ (nHAp/VAMWCNT-O₂7D)

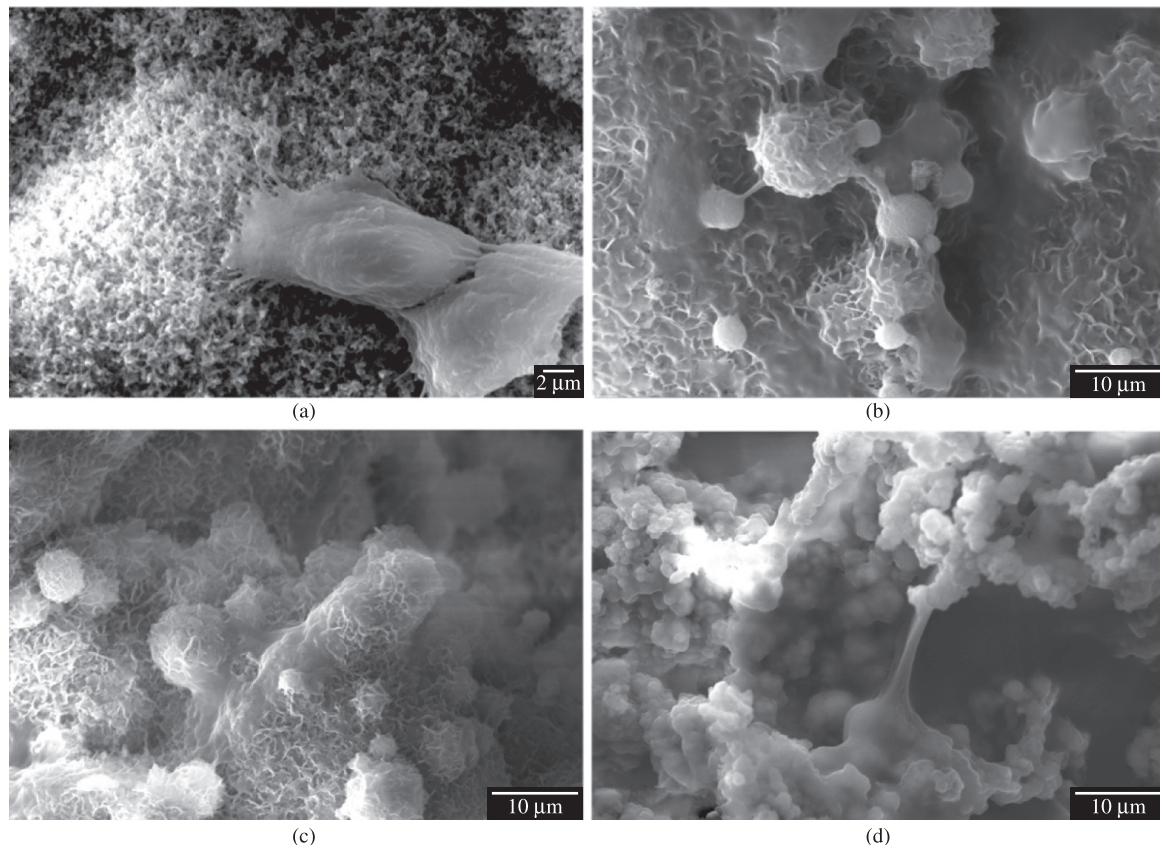


Figure 2. SEM images of HOB adhesion before biomaterialization (a) and on biomaterialized nanocomposites at 7 days (b), 14 days (c), and 21 days (d).

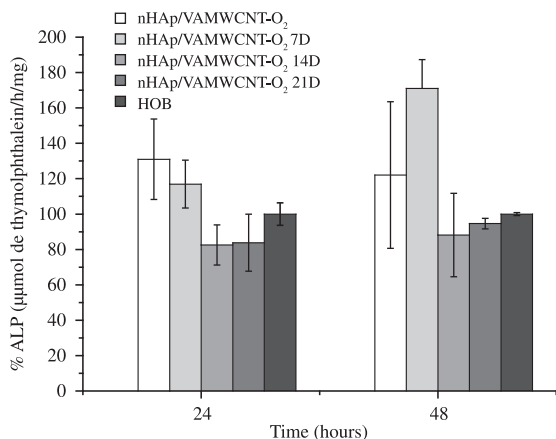


Figure 3. Comparison of calcification of the ECM as a percentage through ALP assays.

is attracting more HOB cells and starting significant ECM calcification. Less significant increase of calcification can be noticed in nHAp/VAMWCNT-O₂14D and nHAp/VAMWCNT-O₂21D.

These results are complementary to those presented in literature^{7,14,15}, which performed ALP test in CNTs and

observed that HOB cells showed higher proliferation in samples of CNTs than in another biomaterial, which was similar to the result shown in Figures 2 and 3.

4. Conclusion

This study indicates that biomineralized nHAp/VAMWCNT-O₂7D nanocomposites showed higher ALP assay in HOB cell culture, indicating the potential of *in vitro* biomineralization. The ALP expression confirms nHAp/VAMWCNT-O₂7D nanocomposite bioactivity through ECM calcification, as shown by SEM analysis of HOB cell adhesion, proliferation, and spreading. These results also prove that nHAp/VAMWCNT-O₂ nanocomposites biomineralized for 7 days in 5× SBF appear to be privileged candidates as scaffolds for inducing *in vivo* regenerative bone tissues process.

Acknowledgements

The authors thank the Fundacao de Amparo a Pesquisa do Estado de Sao Paulo (2011/17877-7), (2011/20345-7), (2012/02159-4) and FVE for financial support, and to everyone from *Laboratory of Biomedical Nanotechnology* for all support in the procedures. Special thanks to Priscila Leite for scanning electron microscopy images and Alene Alder-Rangel for reviewing the English.

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