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MECHANICAL AND BIOLOGICAL EVALUATION OF THE GRAPHENE / NANOHYDROXYAPATITE COMPOSITE FOR APPLICATION IN BONE TISSUE ENGINEERING

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Abstract. *Graphene-based materials have also led to new approaches to regenerative medicine and tissue engineering. Loss of tissue or organs caused by injury or disease is a serious health problem that negatively affects the patient's life. Bone tissue engineering, which aims at regenerating bone, is a promising solution for faster healing and for reconstruction of large bone defects, and graphene and its derivatives have a wide range of applications in bone tissue engineering in obtaining ceramic scaffolds. The nanohydroxyapatite used was the nanoXIM-CarePaste produced and marketed by FLUIDINOVA® which is characterized by high purity nonparticles below 50 nm particle size. To form the composite, graphene was added at 5% by weight supplied by the company PHOSTER® as a reinforcing agent. The addition of graphene increased the mechanical strength values of the composite when compression and Vickers microhardness tests were performed. The scaffolds presented porosity of 60- wt. (%) and pore diameters of 100-300 μm with interconnected structure in SEM analysis. In vitro, results showed that the composite can provide a satisfactory environment for human primary fibroblasts.*

Keywords: *biomaterials, graphene, nanohydroxyapatite, bone tissue.*

1. INTRODUCTION

The human body has a limited ability to correctly self-regenerate most, if not all, of its tissues and organs that have eventually lost their integrity due to severe damage as a result of medical disorders involving some dysfunction in its tissues or severe damage to their function. Facing this scenario where an increasing number of traumas, degenerative diseases, and congenital abnormalities can be found tissue engineering and medicine promise to find new methods for a range of various diseases that are currently untreatable. Moreover, in most cases, this type of research seeks to aid and accelerate the regenerative process by stimulating the patient's inherent healing potential or, alternatively, to create replacement biological tissues (or, more defiantly, whole organs) to replaced, deteriorated, or lost body parts. (Chen and Liu, 2016)

Several factors have contributed to the creation of new biomaterials with superior behavior. Among these factors are the poor performance of the materials currently used together with the lifetime for a maximum of 10 years (influenced by the increase in the average lifespan of the population, making it necessary to create biomaterials with superior properties and able to perform their activities for longer periods); the need to decrease the number of revision surgeries where damaged implants are replaced; the need, in Brazil, to meet a growing domestic demand for the product and to decrease the cost of the materials involved; the lack of donors for transplants.

Often, only simple material does not have all the desired properties, requiring the formation of a composite, in order to guarantee adequate characteristics. The present research aims to evaluate the mechanical properties of a ceramic graphene/hydroxyapatite/alumina composite developed for application in craniomaxillofacial prosthesis, as well as its

cytotoxicity in human fibroblasts. Tests will be made of resistance to compression, fracture toughness, scanning electron microscopy analyze and particle size measurement.

Tissue engineering consists in the use of biomaterials to give support to cells cultured and implanted to restore, maintain, or improve tissue function. In bone tissue engineering, scaffolds used as matrices for tissue formation plays a pivotal role, and has to fulfill a few basic requirements, that is, high porosity and proper pore size (minimum of 100 μm) required surface properties permitting cell adhesion, differentiation and proliferation, desirable mechanical integrity to maintain the pre-designed tissue structure, non-cytotoxicity and osteoconductivity. The selection of the most appropriate material to produce a scaffold to be used in bone tissue engineering application is a important step towards the construction of a tissue-engineered product, since its properties will determine the properties of the scaffold.

Hydroxyapatite has been extensively studied, as it is the most important mineral component of natural bone and teeth, and its synthetic form is able to form a direct chemical bond with bone. HA has properties such as osteoconductivity and bioactivity towards cells, which are essential features to promote bone formation and biological attachment of bone tissues 4,5). Unfortunately, the mechanical properties of HA are low, which seriously inhibits its clinical applications for conditions that require high load application.

The nano-hydroxyapatite is a bioceramic that has been used in the manufacture of scaffolds for use in bone recovery, since it is part of the composition of bone. Thus, this material becomes an alternative, because besides presenting excellent mechanical properties and zero cytotoxicity, it can mimic the extracellular matrix of several types of human body tissue.

Graphene is attracting increasing interest from researchers due to its exceptional combination of mechanical, thermal, chemical, optical, and electrical properties.

The unique structure of graphene provides several intriguing properties such as high electron mobility (Novoselov etal., 2005; Novoselov et al., 2004), exceptional thermal and electrical conductivity (Balandin et al., 2008), good transparency, inherent flexibility, huge surface area, and superior mechanical properties (Lee et al., 2008) compared to other compounds.

Graphene and its derivatives, such as graphene oxide, currently represent the greatest potential in terms of biomaterials due to their excellent physicochemical and biocompatibility properties. Thus, the present study was premised on conducting an integrative literature review in order to verify the ability of graphene oxide to replace metallic biomaterials.

The incorporation of graphene into ceramic scaffolds has been an area of still preliminary evaluations regarding research and development. The main focus of interest has been the mechanical strength and biocompatibility of these materials for use as biomaterials. The creation of different graphene-based matrices has been used in various engineering fields. Among these, the area of biomaterials is booming.

2. METHODS

2.1 Ceramic Processing

Mixtures containing 5% by weight of graphene in nanohydroxyapatite were weighed. These materials are in the form of a very fine nanometer-sized powder that is made up of an agglomerate of particles in simple juxtaposition, held together by very weak bonds. Under these conditions, the material does not exhibit good mechanical properties, so sample preparation is necessary. For the partial solution to the problem a ball mill was used, where the composite remained for approximately 12 hours. The coarser particles, obtained from the grinding, probably come from the agglomeration of the powder that occurs inside the mill. This powder was homogenized with distilled water and 1% ascorbic acid in the appropriate proportions to form a slip sheet. In the preparation of the barbotine to form the hydroxyapatite/graphene composite, a mixture of 50 g of each of the materials by weight was made with 100 ml of distilled water. This mixture was inserted into a 500 ml Nalgene brand jar, best suited for homogenization, containing 600 g of zirconia balls (grinding elements) in a ball mill. A combination of the use of deflocculant associated with mechanical mixing is required to ensure adequate homogenization of the mixture. (Gomide, 2005)

The polymeric sponge used was commercial polyurethane and its pore structure. Sodium bicarbonate NaHCO3 Merck, commercial alcohol Cooperalcool INPM 92.8º and distilled water were used. (Zavaglia and Galdino, 2012)

Sintering of the material was performed at a temperature rise rate of 10°C per minute and the material was held at a temperature of 1200°C for 2 hours.

2.2 Compressing Test

For the mechanical compression test, a sample of each composition was used where the maximum force, thickness and diameter of each sample were tabulated. The equipment used was the TestStar 2 MTS and the test speed was 0.01 mm/s, using cylindrical specimens of 10 mm in diameter and 18 mm in height, following the ASTM C1424 - 99 standard (Standard test method for monotonic compressive strength of advanced ceramics at ambient temperatures). This test determined the mechanical compressive strength modulus of the material studied, with load cells of 100 kgf. The result was obtained by the arithmetic mean of 7 specimens.

2.3 Vickers microhardness testing

The Vickers microhardness test was performed in order to determine the hardness of the composite. Seven indentations were made in a Shimadzu Corporation microdurometer model HMV - 2T for hardness measurement. The application time of each indentation was 15 seconds with a load of 0.3 kgf. After measurement, the arithmetic mean with standard deviation of the penetrations was taken. The tests were made according to ASTM C 1327-99.

2.4 SEM

The arrays were analyzed and coated with gold (Sputter Coater - SPI Supplies) for 90 seconds, 13mA. Images were acquired using a scanning electron microscope (JEOL JSM 6301F/ Oxford INCA Energy 350 / Gatan Alto 25), for qualitative evaluation of interconnectivity and pore size.

2.5 Cell lines and Maintenance

The human primary fibroblasts (Fibs) used in the present study were obtained from healthy volunteers undergoing plastic surgery, following informed consent obtention, as approved by the Ethics Committee of the University of Brasília (protocol: 30175020.00000.5558). Fibs were cultured in DMEM (Dulbecco's Modified Eagle's Medium, Gibco, USA) supplemented with 10% v.v. fetal bovine serum (FBS) (Gibco, USA), and 1% v.v. of penicillin / streptomycin solution (1,000 U.mL -1) (Invitrogen, Grand Island, NY), and kept in a 5% CO2, 37oC and 95% humidity, as described by Lanza, 1997.

2.6 Preparation graphene/nanohydroxypatite composite of ionic product

Half a gram of biomaterial was added in 50 mL of DMEM medium, which was kept under agitation at 37oC. Then, the medium was filtered with a 0.22 μm membrane. The medium containing graphene / nanohydroxyapatite composite ionic product had its pH adjusted to 7.34, supplemented with 10% fetal bovine serum, and used for experiments, as described by Hench, 2002.

2.7 Cell viability/proliferation assay

Third passage Fibs were trypsinized and plated at 10,000 cells/cm2 per well in 96-well plates and maintained in complete medium for overnight attachment. After, the medium was aspirated and exchanged for fresh medium containing the ionic product of the dissolution of composite with 10% fetal bovine serum (FBS).

After 72 hours, medium was aspirated and replaced by DMEM/FBS supplemented with 5 mg/ml of MTT. The cell culture plates were then incubated for 4 hours at 37oC and 5% CO2, protected from light. After this period, the plates were observed under an optical microscope to visualize the formazan crystals. Then, DMSO was added to solubilize the crystals. After 10 minutes, 100 μL of the supernatant were removed from each well and transferred to another 96-well plate and quantification was performed in a spectrophotometer (ADAP 1.6, Anthos Labtec Instruments) using a 595 nm filter. Cells maintained in complete cell culture medium were considered as positive controls showing 100% viability, 100% proliferation. Cells maintained in DMEM lacking FBS supplementation, were considered as negative proliferation controls. The experiments were carried out with n=3.

2.8 Data Analysis

Data were analyzed using the PRISM software (GraphPad, San Diego, CA), using the One-Way ANOVA followed by Bonferroni post test.

3. RESULTS

3.1 Ceramic Processing

The first step of the methodology of this present work focused on the full range of ceramic processing. Therefore, the first result presented should be to ensure that the composite studied is fundamentally, presenting the required characteristics, through simple ceramic processing, as proposed.

In Figure 1, the two initial states of the powdered materials prior to material processing are demonstrated.

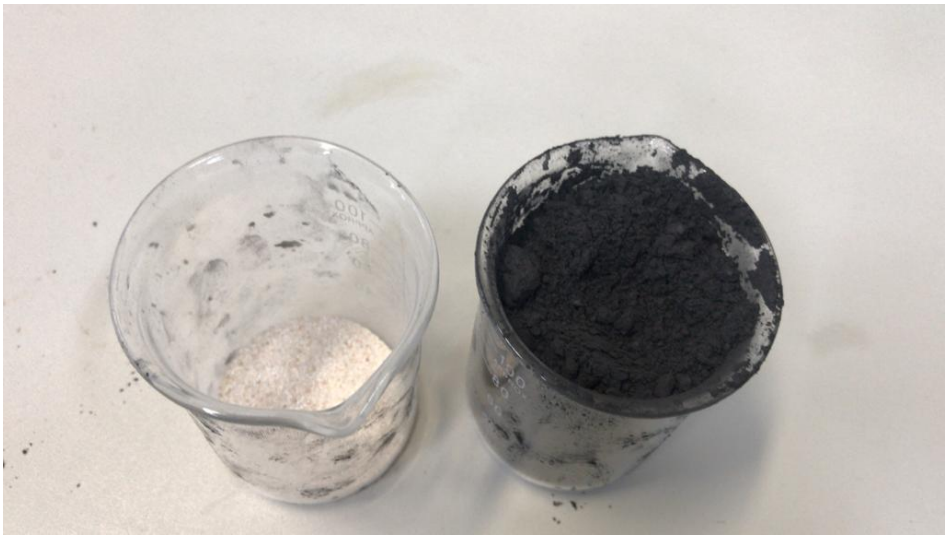

Figure 1. Nanohydroxyapatite and Graphene Powders.

As a result of the processing demonstrated in the methodology, the making of samples of a Nanohydroxyapatite and Graphene composite was successful.

3.2 Compressing Test

For the compression tests of the material, shown in table 1, seven different samples were chosen and submitted to a load with constant variation to define the breaking point of this material.

Table 1. Compressing Test.

Sample	Compressive strength (kN)	Average of the tests (kN)
1	9.05	
2	9.2	
3	9.1	
4	9.07	9.07 ± 0.05
5	9.03	
6	10.01	
7	8.9	

3.3 Vickers microhardness testing

Table 2 shows the Vickers Microhardness values obtained with the application of a 0.3 kgf load for the developed ceramic composite.

Table 2. Vickers Microhardness values.

Indentation	Vickers' Microhardness (HV)	Average of the tests (HV)
1(1)	1764	
2(1)	1623	
3(1)	1763	
4(1)	1589	1503.86 ± 40,6
5(1)	989	
6(1)	1256	
7(1)	1543	

(1) applied load 0.3 kgf

With the addition of graphene to nano-hydroxyapatite, the hardness values had an increase, if compared to hydroxyapatite only sintered at 1200 ºC (1465 HV) according to data obtained in the work developed by Zavaglia and Galdino in 2012.

3.4 SEM

The scaffold produced showed a pore size in the range of 60 to 300m and qualitative observation indicates an interconnected pore structure, as shown in the SEM image analysis, Figure 2.

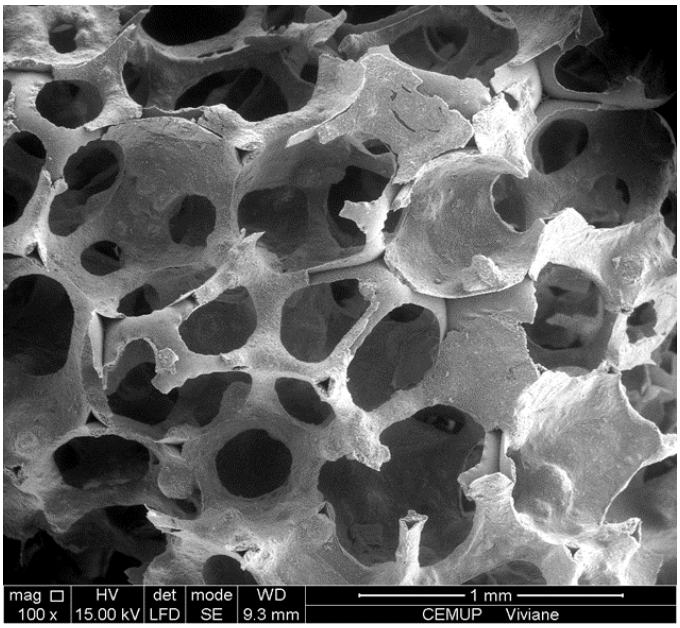

Figure 2. SEM of graphene/HA composite (100X magnification)

3.5 Cell viability/proliferation assay

When incubated with the ionic product of composite, the fibroblasts maintained regular morphology of elongated, spindle-shaped cells. After 24 hours incubation with the ionic product, no toxicity was observed and, at 72 hs, the MTT assay indicated that not only the fibroblasts treated with ionic product were viable, but also presented similar proliferation rates, compared to the positive viability/proliferation control. The cells maintained for 72 hours in the absence of FBS nor ionic product, presented lower MTT metabolization, indicative of lower proliferation rates.

4. CONCLUSION

By surviving exposure to the ionic medium, human fibroblasts showed that the material is potentially cytocompatible. It can be stated, therefore, that the graphene/nanohydroxyapatite composite may be a safe biomaterial, justifying further investigations.

The compression and Vickers microhardness tests proved, qualitatively, that the evaluated mechanical properties are suitable for application of the composite when compared to other works found in the literature regarding the mechanical properties of other calcium phosphate-based scaffolds.

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6. RESPONSIBILITY NOTICE

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