Perspectivas Online: Exatas & Engenharias
Dezembro/2020, v. 10, n. 30, p. 1-14
ISSN: 2236-885X (Online)
DOI: 10.25242/885X102920202078

ESTUDO DO BIOCOMPÓSITO DE P3HB/NANODIAMENTE PARA APLICAÇÕES EM DISPOSITIVOS MÉDICOS, PARTE 2: CITOTOXICIDADE *IN VITRO* E TESTES DE BIOCOMPATIBILIDADE

Lucivan Pereira Barros Junior[1,*], Fabrício Moreira Almeida[2], Yan Yucif Vieira Azevedo Maia[1], Príscila Pinheiro Pereira[1]

RESUMO

BARROS JUNIOR, L. P. Study of P3HB/nanodiamond biocomposite for medical devices application, part 2: in vitro cytotoxicity and biocompatibily tests. **Perspectivas Online: Exatas & Engenharias**, v. 10, n. 30, p. 1-14, 2020.

Na Parte 1 deste trabalho, foi apresentado um novo nanocompósito biodegradável e biocompatível com matriz polimérica de poli-hidroxibutirato (PHB) carregado com nanopartículas de diamante (ND) obtido, pela primeira vez, sob conhecimento dos autores, a partir da técnica de vaporização de solvente (técnica sob pedido de patente) para aplicação em acessórios médicos. As propriedades mecânicas, térmicas e morfológicas, como tambem a eficiência da técnica na homogeneidade e dispersão da carga na matriz polimérica confirmada através de modelos mecânicos foram investigadas na Parte 1 do artigo. Na Parte 2, testes *in vitro* foram feitos para testar a citotoxicidade e biocompatibilidade do nanocompósito. Os testes resultaram na ausência de resposta inflamatória em culturas de fibroblastos e macrófagos para todas as composições do nanocompósitos estudados.

Palavras-chave: Poli-3-hidroxibutirato; nanodiamantes; nanocompositos, citotoxicidade, biocompatibility.

[1]Northern State University Fluminense Darcy Ribeiro - UENF – Advanced Materials Laboratory LAMAV/CCT - Alberto Lamego Avenue, 2000, Parque Califórnia, Campos dos Goytacazes, RJ, CEP: 28013-602, Brazil.
[2]Northern State University Fluminense Darcy Ribeiro - UENF – *Reconhecer* Biology Laboratory LBR/CBB - Alberto Lamego Avenue, 2000, Parque Califórnia, Campos dos Goytacazes, RJ, CEP: 28013-602, Brazil.
(*) e-mail: lpb24@case.edu

Data de recebimento: 29/06/2020 Aceito para publicação: 30/07/2020 Publicado online: 31/12/2020

Revista Perspectivas Online: Exatas & Engenharias
December/2020, v. 10, n. 30, p. 1-14
ISSN: 2236-885X (Online)
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STUDY OF P3HB/NANODIAMOND BIOCOMPOSITE FOR MEDICAL DEVICES APPLICATION, PART 2: *IN VITRO* CYTOTOXICITY AND BIOCOMPATIBILY TESTS

Lucivan Pereira Barros Junior[1,*], Fabrício Moreira Almeida[2], Yan Yucif Vieira Azevedo Maia[1], Príscila Pinheiro Pereira[1]

ABSTRACT

BARROS JUNIOR, L. P. Study of P3HB/nanodiamond biocomposite for medical devices application, part 2: in vitro cytotoxicity and biocompatibily tests. **Perspectivas Online: Exatas & Engenharias**, v. 10, n. 30, p. 1-14, 2020.

In Part 1 of this work, we presented a novel biodegradable and biocompatible nanocomposite with polymer matrix of poly-hydroxybutyrate (PHB) loaded with nanoparticles of diamonds (ND) obtained by a solvent vaporization technique for the first time, to the authors knowledge (the technique is being patented by the group) for medical devices application, its mechanical, thermal, and morphological properties were investigated. The efficiency of the technique in the homogeneity and dispersion of the nanofiller in the polymer matrix was confirmed by applying mechanical models that predict these characteristics in nanocomposites. In Part 2, we tested *in vitro* the cytotoxicity and biocompatibility of the nanocomposite. The tests resulted in no inflammatory response of the nanocomposite in fibroblast and macrophage cell cultures for all the composition.

Keywords: Poly-3-hydroxybutirate; nanodiamonds; nanocomposites, cytotoxicity, biocompatibility.

[1]Northern State University Fluminense Darcy Ribeiro - UENF – Advanced Materials Laboratory LAMAV/CCT - Alberto Lamego Avenue, 2000, Parque Califórnia, Campos dos Goytacazes, RJ, CEP: 28013-602, Brazil.
[2]Northern State University Fluminense Darcy Ribeiro - UENF – *Reconhecer* Biology Laboratory LBR/CBB - Alberto Lamego Avenue, 2000, Parque Califórnia, Campos dos Goytacazes, RJ, CEP: 28013-602, Brazil.
(*) e-mail: lpb24@case.edu

Received: 29/06/2020 Accepted: 30/07/2020 Published online: 31/12/2020

1. INTRODUTION

Biocompatibility is the ability of a material to perform a beneficial and/or appropriate response on the host in a given application. The appropriate response involves acceptance of the artificial implant by both the tissues surrounding the prosthesis and the organism as a whole (ANDERSON, 2009). Biocompatibility is associated with the success of the implant in the body, and in addition to the material properties, it depends on the context in which the material will be used, the surgical techniques and the patient health conditions (BOUILLAGUET at al., 2006). Thus, the biocompatible material, mostly called as a biomaterial, must not provoke an inflammatory response, cannot be cytotoxic or immunogenic, and it must provide adequate mechanical properties so that it does not fail during use and in the normal activities of the host (ANDERSON, 2009). The mechanical properties were studied in Part 1 on this work (BARROS JUNIOR et al., 2020). According to Williams (1987), biomaterials are any substances and/or combinations of substances, with synthetic or natural origins, that can be used for a long and/or indeterminate period of time, replacing part or all of the damaged biological system, being an organ, tissue or function of the organism in question (WILLIAMS, 1987). Therefore, these materials must be biocompatible, which means that they must meet the requirement of the application for which they were designed, that is, they must not stimulate or cause any type of allergic and/or inflammatory reaction. That is why testing biomaterial cytotoxicity and biocompatibility is a requeriment for a biomaterial to be used.

Poly-3-hydroxybutyrate (P3HB) or polyhydroxybutyrate (PHB) (ANDRADE, 2006), was the first biopolymer in the family of PHAs to be synthesized through Bacillus megaterium, in 1923 by the microbiologist Maurice Lemoigne. However, it was only in 1958 that its true function as an energy and carbon storae was discovered by Macre and Wilkinson (CARMINATTI et al., 2006; TELLES et al., 2011) Polyhydroxyalkanoate (PHA) belongs to the family of microbiological polyesters, they are biodegradable polymers produced from microorganisms, which need to storage energy in carbon form (SCHIMIDT, 2011; SILVA, 2009). More than 300 microorganisms are capable of producing PHAs, however for industrial production only a few have an appropriate yield (RODRÍGUEZ et al., 2003; SCHIMIDT, 2011; SILVA et al., 2000). At least 100 different monomers have already been identified as constituents of PHAs synthesized by bacteria, demonstrating the great diversity of the material, increasing the range of applications of PHAs (SCHIMIDT, 2011; LIMA et al., 2001).

PHB has several properties in common with polypropylene (PP), including its melting point of 177ºC that approaches PP. However, the biopolymer is more fragile rigid (BRAUNEGG et al., 1998), mainly due to its high crystallinity. To overcome this fact and decrease the crystalline percentage of PHB comonomers are synthesized to form copolymers, 3-hydroxyvalerate (3-HV), 3-hydroxypropionic (3-HP) and 4-hydroxybutyrate (4-HB) comonomers, are generally used. The lower crystallinity is related to the higher molar masses of these comonomers, which slows down the crystallization process. Studies find that the crystallinity of the copolymers decreases with higher fractions of monomeric units of 3-HP and HV (BRAUNEGG et al., 1998; MACHADO et al., 2010, ALMEIDA NETO et al., 2017; BARCELOS et al., 2017). P3HB was initially proposed for typical medical applications such as sutures, bone plates, splints, microcapsules, gauze, and in powder form as a lubricant for gloves (HOLMES, 1985; MILLER; WILLIAMS, 1986).

The external/foreign body, implants and/or PHB prostheses produces an exceptionally smooth response and its biodegradability is very slow. The main objective of the researchers,

in fact, is to obtain a material that replaces the damaged part of the organism and at the same time serves as a substrate for the growth of natural tissue, while it is degraded. A practical example would be the vascular graft or blood vessel made up of very thin PHB fibers arranged in the form of water-impermeable tubes with an adequate diameter. This tube would act as a temporary "skeleton" for the growth of new tissues, and as a consequence, from the degradation of the biomaterial, it would be completely replaced by natural tissue. This would prevent the formation of blockages in synthetic arteries, which arise in response to the non-degrading foreign organism in the vessel wall (HOLMES, 1985). The development of PHB medical devices is already a reality regarding dental, orthopedic, craniomaxillofacial and skin surgeries (BONARTSEV et al., 2007).

This work aimed the use of PHB for orthopedic devices, in bone fracture pins, associated with diamond nanoparticles forming a nanocomposite material. The use of PHB as a matrix for biocomposites has been proved to be promising by the research group (LEILA, 2009). However, the incorporation of PHB and nanodiamonds to form a nanocomposite biodegradable and biocompatible was done for the first time by Barros et al (BARROS, 2014), when poly-3-hydroxybutirate (P3HB) was used as a matrix. The part 1 of this work has developed a methodology to produce the nanocomposite from the dissolution of PHB in chloroform and posterior addition of ND particles during mixing with the formation of microcapsules of nanodiamonds particles encapsulated by the P3HB polymer matrix. This methodology was proved to provide a better distribution and dispersion of the ND in PHB matrix regarding the nanocomposite mechanical and thermal properties (BARROS JUNIOR et al., 2020).

After the investigation of the mechanical properties, its biological evaluation was necessary as well (ALVES et al., 2010). The biological tests were carried out to evaluate cytotoxicity and induce an inflammatory response of the material, placing the studied nanobiocomposites in direct contact with fibroblast and macrophage cell cultures, respectively (RARE, 1985). All tests were performed at (LBR/CBB/UENF).

2. EXPERIMENTAL

2.1. Materials

Poly-3-hydroxybutirate (P3HB) with M_W = 600,00 D, T_M = 175 ºC, T_g = 19.5 ºC and density between 1.20-1.24 g/m^3 suplied by PHB Industria, syntethic nanoparticle diamonds (ND) with a diameter of 125 nm obtained by Diambra Diamates, Chloroform (99,8%) and Ethyl alcoholl purchased by Sigma-aldrich. For nanocompostites (denoted 1 to 4) containing 9/91, 12/88. 14/86 and 20/80 (P3HB/ND, w%/w%), respectively, were prepared according to the methodology already described in Part 1 of this work (BARROS JUNIOR et al., 2020).

2.2 Cell cultures

Culture of L929 murine fibroblasts

Murine fibroblast cells L929 (ATCC CLL-1; American Type Culture Collection) previously stored in liquid nitrogen were thawed and cultured in DMEM-F12 medium supplemented with 10% fetal bovine serum and kept in an oven at 37 ° C with 5% CO2 until they form an adherent monolayer at the bottom of the bottle.

Culture of raw macrophages264.7

The raw264.7 macrophages (ATCC TIB-71; American Type Culture Collection), also thawed and cultured in the same medium described in the previous item, added with the antibiotic solution Penicillin 50u/mL and Streptomycin 50µg/mL. The cells were cultured in complete medium at 37°C in humidified atmosphere containing 95% of air and a 5% of CO2 until the formation of the monolayer at the bottom of the bottle before the beginning of the tests.

2.3 Preparation of material for biological tests

The cells were collected from the bottles for counting and later testing *in vitro* with the nanocomposites. Trypsin 0.05% and EDTA 0.05% in PBS (trypsination) were used for the culture of fibroblasts, mainly due to the good adhesion of the cells to the bottom of the bottle, whereas for the culture of macrophages, a blasting with a pipette with the medium itself so that the cells at the bottom of the bottle could detach. A 50 µL sample of each cell culture was stained with Tryplan-blue dye (0.1% in PBS) and taken to the Newbawer chamber for counting live cells, using the ZEISS Germany Axiovert 135M microscope , in the 4 quadrants for the determination of cell concentration in the medium of 2 x 105 cells per ml. Samples of the biocomposites were cut in cubic form with dimensions of approximately 3 mm on the sides to be put in contact with the cell cultures. The samples were sterilized in a vertical autoclave of the Phoenix Luferco model AV-75 at a temperature of 121 ° C for 15 minutes. All samples, in duplicate, were divided into different wells of the 24-well cell culture plate numbered 1 to 4 to identify the different ND concentrations (see item 2.1) and 5 for the neat PHB sample. 60,000 cells were placed in each well with 1 ml of culture so that samples are covered. Wells containing cells were kept without material to serve as a control for the absence of cytotoxicity. Positive controls for cytotoxicity were made using a 10% Triton 100x solution for each day of the experiment.

2.4 Inflammatory induction test

In the inflammatory response induction test, macrophages and fibroblasts were plated on the plates containing the biocomposites and kept in the oven at 37 ° C during the ten days of experiment, at an atmosphere of 5% CO2. During the experiment, samples of supernatants were collected from wells containing biocomposites and from control wells. The collection points for the macrophages were after the 1st, 2nd, 3rd, 4th, 7th and 9th days of contact and the supernatant medium was changed on the 4th day of the experiment. For fibroblasts, collections were made after the 1st, 3rd, 4th, 7th and 10th day of contact and the supernatant medium was changed on the 4th day of the experiment. The assessment of NO production by macrophages and fibroblasts was performed using the Griess method. In this way, the Griess reagent p-aminobenzenesulfonamide 1% + 0.1% naphthylenediamine dihydrochloride in 5% phosphoric acid, freshly prepared, was added to 50 µL of the supernatants collected from the wells containing the samples of the biocomposite. After 10 minutes, the absorbance at the wavelength of 570 nm was measured in the plate spectrophotometer (Dynatech MR 5000) present in the LBR / CBB / UENF. To calculate the NO concentration in the collected supernatants, a sodium nitrite curve subtracted from the additive values without cells was used as reference.

On the 10th day of contact, the samples were stained with trypan blue dye (0.02% PBS) in order to reveal the dead cells in blue for counting, by optical microscopy, evaluating the proportion of cells typed with trypan (dead) and unmarked (live) in three different fields.

2.5 Cytotoxicity test

For cytotoxicity tests, both types of cell cultures were used. The points of collection of supernatants from cells plated with macrophages and fibroblasts, which were collected as described in the previous item, were used to monitor the presence of a cytotoxicity marker enzyme after the contact of the cells with the biocomposite. LDH (lactic dehydrogenase) was used as a marker of cell damage/death. Since any damage to the cell membrane will cause the release of the enzyme, this can be seen in the fraction collected from the supernatant where it will be dissolved (RARE, 1985). For the L929 fibroblast cultures on the 3rd and 7th day, the cell morphology of the cells was analyzed. wells containing the biocomposite compared to the control wells by optical microscopy. For the monitoring of cell death, both of fibroblasts and macrophages, at the end of the experiment the Tryplan-blue dye (0.02% PBS) was used to identify dead cells stained in blue. The cell morphology of the macrophages was also analyzed by optical microscopy during the days of contact.

3. RESULTS AND DISCUSSION

3.1 Evaluation of the induction of nitric oxide production by nanocomposites

For the tests of inflammatory induction, the measurement of the production of nitric oxide (NO) by macrophages and fibroblasts was performed in the supernatants of cells in contact with biocomposites in the wells of cell culture plates. NO is a soluble gas produced by macrophages, fibroblasts and other types of cells such as endothelial cells and neurons. This chemical mediator plays an important role in leukocyte recruitment during the inflammatory process (ROBBINS et al., 2001; CINELLI et al., 2020). As NO is a product of these cells when activated by cytokine, microbial compounds or both (MACMICKING et al., 1997) any inflammatory stimulus on fibroblasts and macrophages will be released doses of NO by the cells.

Our results demonstrated that there was no NO production in the 10 days that the cells were in contact with the nanocomposite. The same occurs for macrophages and can be seen the absence of production of nitric oxide (NO) by them in the 9 days of contact of the cells with the fragments.

Given the synthesis of NO, an important indication of stress or aggressive state of the cells. This indicates that the contact of the cells with the biocomposite did not generate any inflammatory stimulus in the cells in any concentration of ND for the period of the 10 days of test. The same occurs for macrophages, and can be seen in figure 1 the absence of production of nitric oxide (NO) by them in the 9 days of contact of the cells with the fragments. To confirm that the macrophages used were capable of producing NO, they were stimulated with 1 µg / mL of LPS. Figure 1 shows the NO concentration produced by the positive control wells for inflammation in the wells in which the cells were stimulated by LPS and, consequently, their death was 100%. For samples 1, 2, 3 and 4 with different percentages of ND particles and sample 5 for neat PHB, 0% NO production and no cell death were observed. This means that biocomposites are inert to macrophages regardless of the compositions of the formulations.

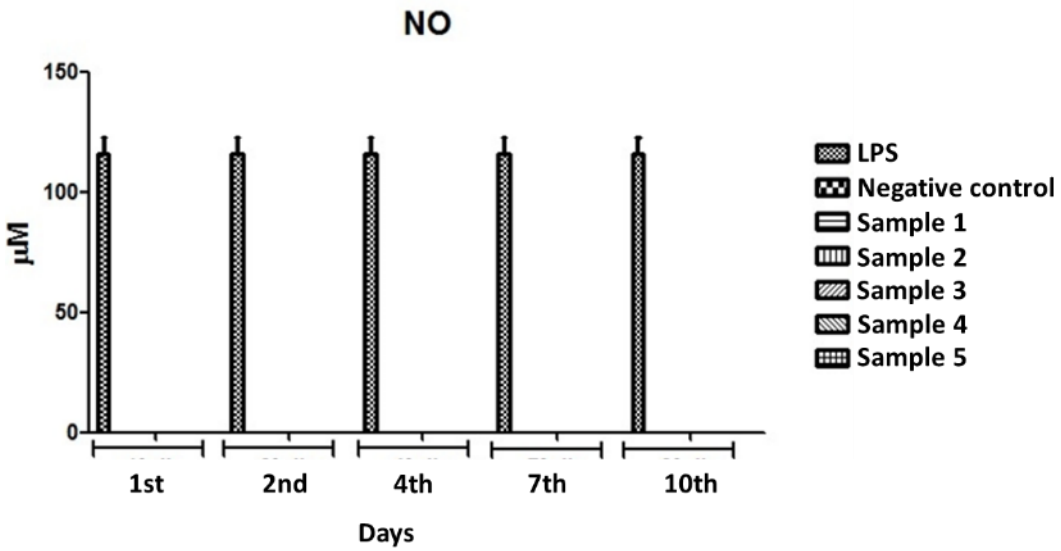

Figure 1: Graph of [NO] production by Raw macrophages 264.7 during contact with biocomposites. Raw 264.7 macrophages were plated at a concentration of 2,105 cells per well of 24-well plate in the presence or not of the nanocomposite. Culture medium samples were collected after 1, 2, 4, 7 and 10 days after counting the cells with the samples for the evaluation of nitric oxide (NO) production using the Griess method. Macrophage cultures of stimulated with 1 µg / mL of LPS for 24 hours were used as positive controls of NO production in the reaction. Negative control: Culture of macrophages that had no contact with the nanocomposite of P3HB/ND.

3.2 Evaluation of the biocompatibility of nanocomposites

For the biocompatibility tests, the presence of lactic dehydrogenase (LDH) in the culture supernatant in the presence of the nanocomposites was evaluated. LDH is a mitochondrial enzyme that is released into the culture medium when the cell dies, so we can evaluate the cytotoxicity of nanocomposites for cell culture. Cell morphology for the verification of adverse effects on cell appearance (RARE, 1985). Figure 2 shows the percentage of LDH released by L929 fibroblasts. It can be seen that the release of the LDH enzyme, in the wells where the fibroblasts were in contact with the samples of the nanocomposites (samples 1-4) and for neat PHB (sampl 5) was similar to the well where the fibroblasts were not in contact with any material, around 10%. This result indicates that the release of LDH was not stimulated by the presence of the fragments, and the 10% of LDH present in the culture medium might be related to the natural cell death that occurs in the culture. Once again, the percentage of ND particles present in the different formulations (samples 1, 2, 3 and 4) does not influence the biocompatibility of the material. Thus, biocomposites have a non-cytotoxic behavior for fibroblast cultures, which can also be seen in the Figure 3 showing a regular and healthy cellular appearance (RARE, 1985) on the third (left picture) and seventh (rigth picture) day of contact for all formulations of biocomposites studied. The cell development/growth can be visualized, on the third day of contact there is still some space at the bottom of the cell culture bottle for the proliferation of the cells, on the seventh day of contact this space is fully covered by fibroblasts, indicating that the presence of the biocomposite did not interfere with cell growth.

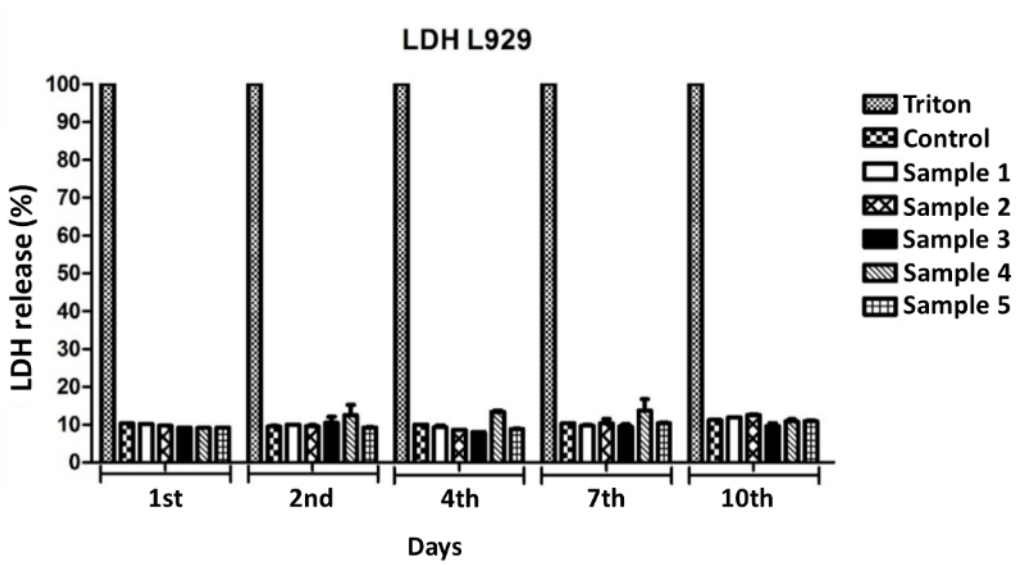

Figure 2: Graph of % LDH release by fibroblasts during contact with biocomposites. L929 fibroblasts were plated at a concentration of 2,105 cells per 24-well plate well in the presence or not of biocomposites. Fibroblast cultures were lysed with 1% Triton solution to be the positive control of LDH release.

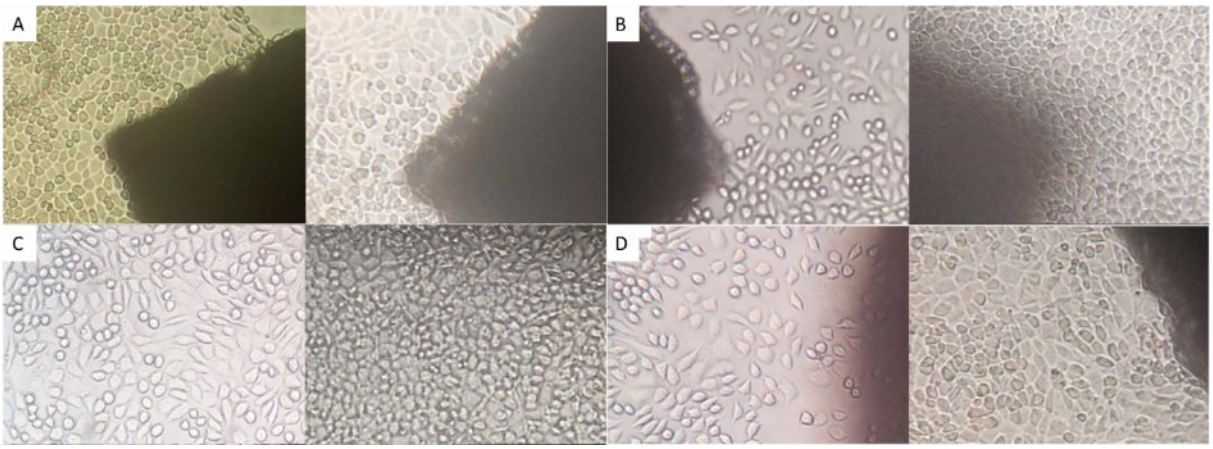

Figure 3: Optical micrographies showing the fibroblast growth in the wells containing samples (A) 1, (B) 2, (C) 3 and (D) 4 on the third (left picture) and on the seventh day (rigth picture). The shadows in the pictures are the nanocomposite fragments.

The same LDH release test was carried out for macrophage culture. Figure 4 shows the percentage of LDH released by macrophages in contact with the nanocomposites of different compositions in the 9 days of biological experiment. It is possible to notice that there was a significant release of LDH by the macrophages on the first day after contacting the cell culture with the fragments of the biocomposites. The LDH concentrations found in the wells containing the fragments were slightly higher than the LDH concentrations observed in the negative control wells on the first and second days after contact with the biocomposites, this difference on the seventh and ninth day was greater, mainly due to the enzyme LDH is not volatile and accumulates in the supernatant of the culture medium. Despite some LDH release observed by the macrophages (figure 4), it is possible to observe that there was no cellular cytotoxic stimulus in the macrophages, as can be seen in Figure 5. The figure shows a normal growth of cell culture

since the day of contact (day 0), the first 24 hours (day 1) and after the 2nd, 4th, 7th and 9th day of contact where the supernatants were collected and the levels of LDH concentration in the cell media were measured. Macrophages, by optical microscopy, had normal growth and no cell deformation, or phagocytic response to fragments of biocomposites present in the same medium as cells. Thus, the concentration of LDH present in supernatants may have other origins, other than the induction of cytotoxicity by macrophages, such as the stress of cells caused by the movement of fragments during tests and lack of space for cells to grow freely. As the fragments of the biocomposites were loose inside the wells, the movement of the plate during its handling caused the fragments to move from the place by dragging and detaching the cells from the growth sites and overlapping them over each other (Figure 6), this might have caused a stress on the macrophages, causing the release of LDH. The lack of space for the high number of cells and for cells to proliferate is also a stress factor that can be caused to macrophages, this is another indication that the biocomposite does not generate an inflammatory response, since for the micrographs of negative control wells for inflammation, that is, with the absence of fragments of the biocomposites, significant concentrations of LDH were also released, as well as by the macrophages of the wells containing the fragments with the formulations of the biocomposites, this can be seen in Figures 5. This means that the presence of the biomaterial did not influence the release of the LDH enzyme by macrophages, since the same levels of LDH were found in the control wells without the fragments.

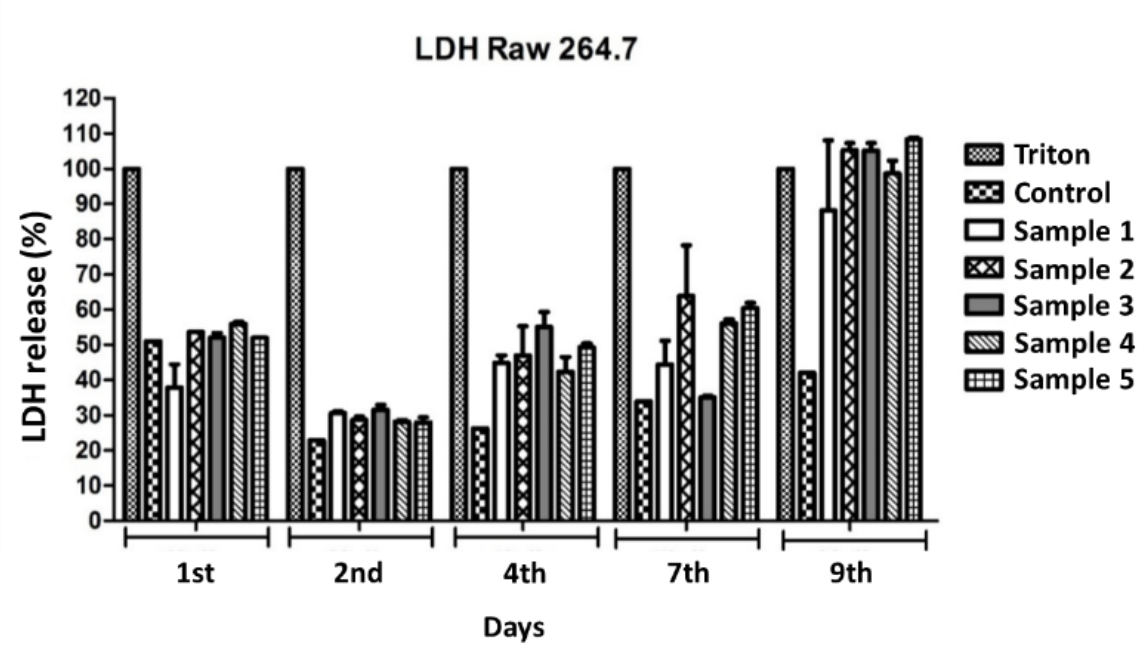

Figure 4: Graph of % LDH release by macrophages during contact with biocomposites.

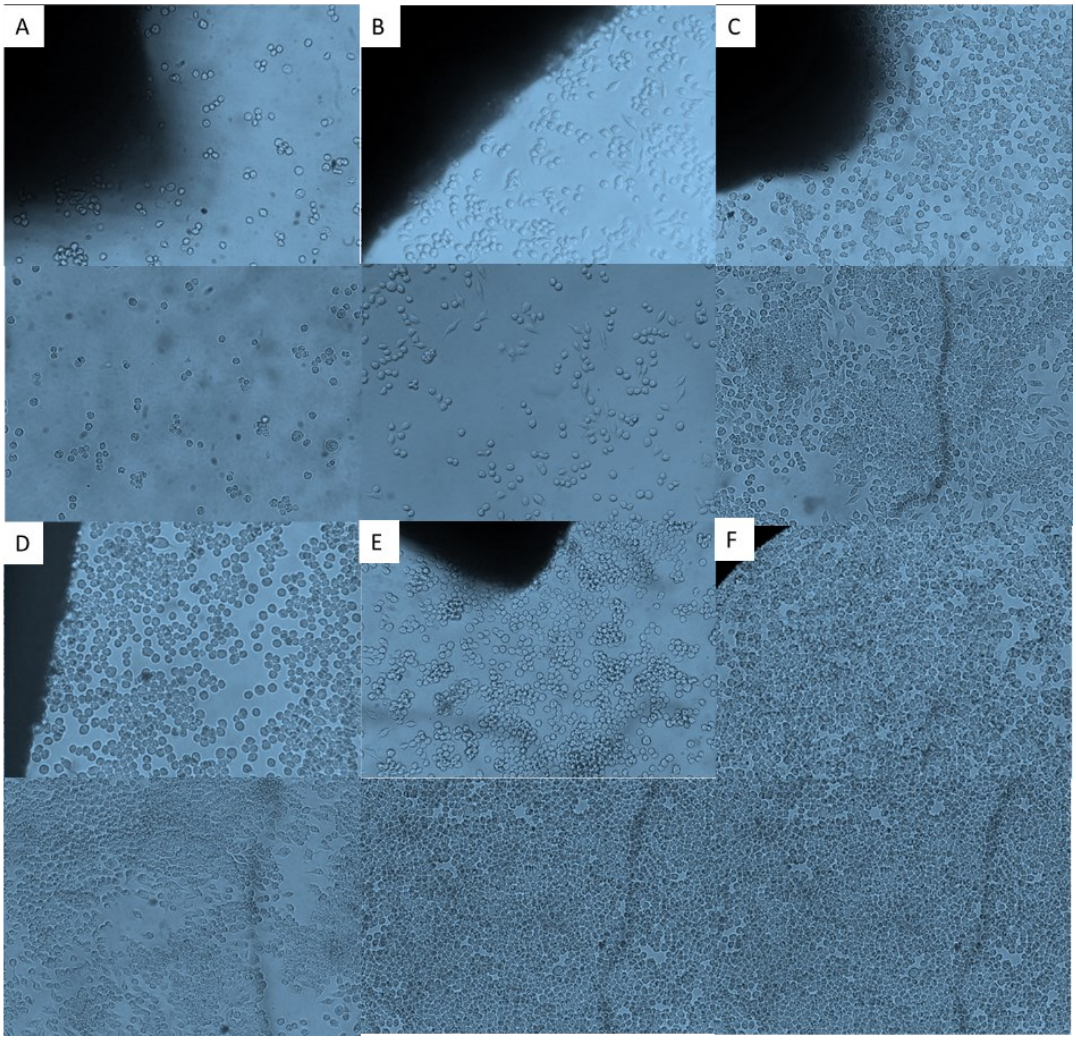

Figure 5: Optical micrographies showing the macrophage growth for the (A) day of contact (day 0), (B) the first 24 hours (day 1) and after the (C) 2nd, (D) 4th, (E) 7th and (F) 9th day of experiment (top picture) with and (bottom picture) without nanocomposite samples in contact with the supernatants . The shadows in the pictures are the nanocomposite fragments.

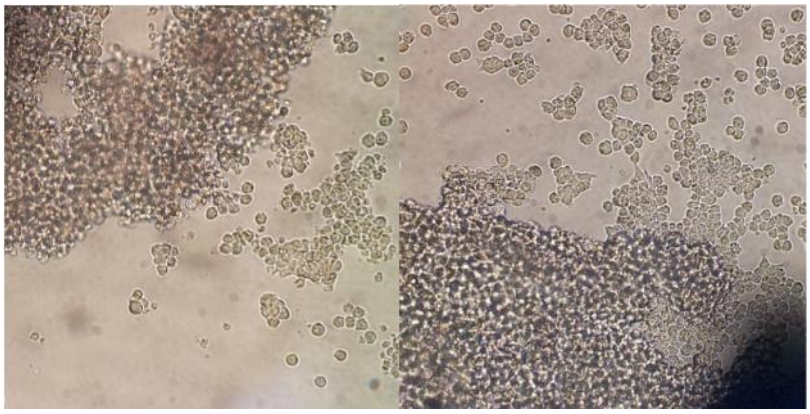

Figure 6: Optical micrographies showing the detachment of the macrophage cells at the bottom at the bottom by moving the fragments.

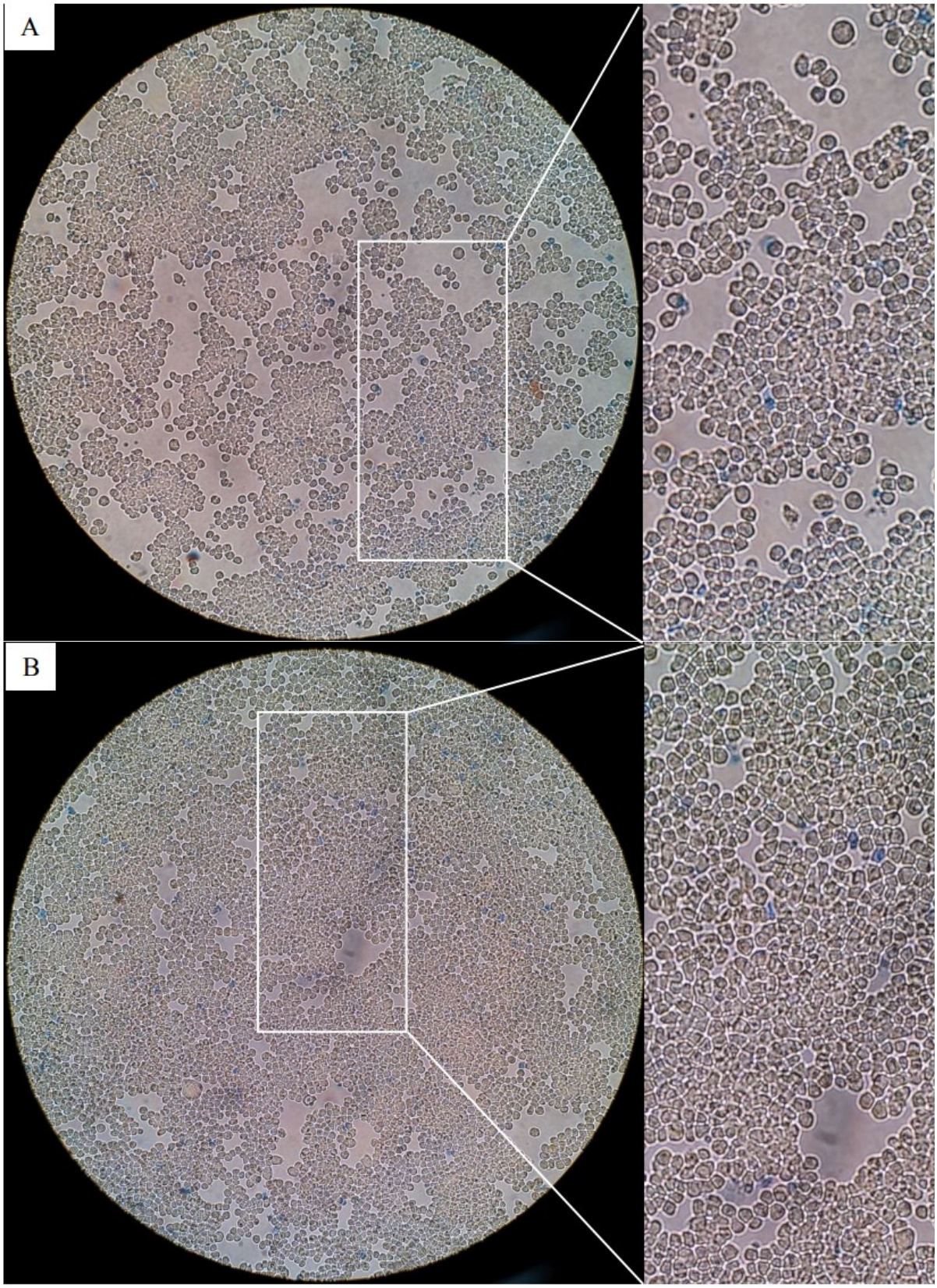

Figure 7: Wells for counting dead cells in blue by the dye Trypan-blue (A) with and (B) without nanocomposite presence.

Tests with Trypan-Blue were carried out to reveal dead cells in blue, this dye can enter dead cells by staining them in blue, thus being able to differentiate between living and dead cells. Figure 7 shows the cells stained with the dye for a well in contact with the nanocomposite and for the negative control well, respectively. The count was made in all samples of nanocomposites and pure PHB and the graph of Figure 8 was made. The figure shows practically the same percentage of cell death for the wells containing the samples and for the wells without samples (negative control), this indicates that the cells died with time, due to factors already mentioned and natural death and that they were not influenced by the contact with nanocomposites.

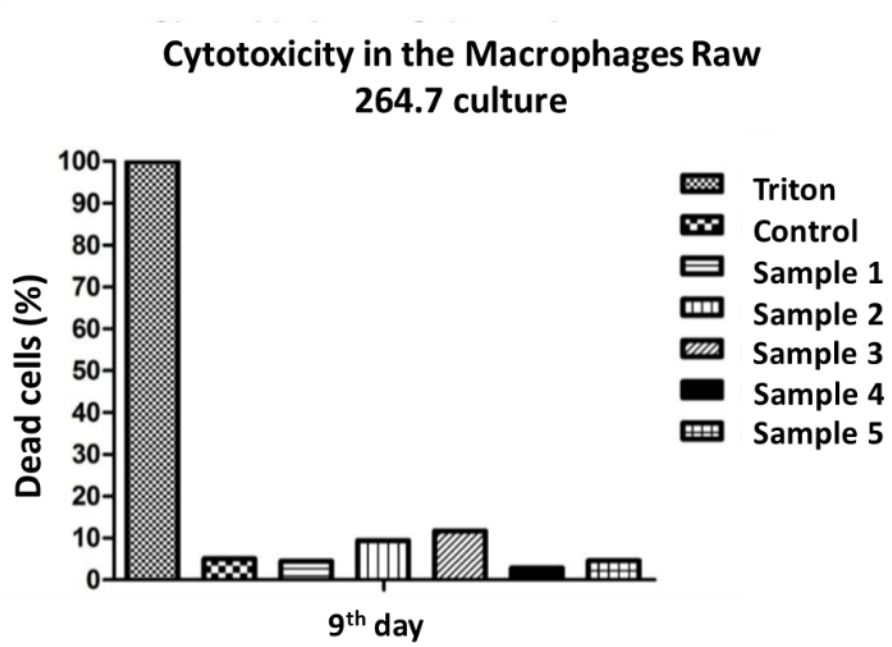

Figure 8: Graph of % of dead cells for the wells containing the nanocposite samples and negative and positive control for LDH release.

5. CONCLUSIONS

A new methodology, regarding the coating of nanodiamonds by poly-3-hydroxybutyrate, allowing the encapsulation of nanoparticles in spherical geometry capsules, was previously demonstrated in Part 1 of this work (BARROS JUNIOR et al., 2020). The results of this work allow us to conclude that nanocomposites are not cytotoxic for cultures of RAW 264.7 macrophages and fibroblasts L929 and do not induce the production of nitric oxide by these cells. In this way, these nanocomposites have great potential for medical use.

6. ACKOWLEDGMENTS

The authors would like to thank to FAPERJ and CNPQ for finacial support, the laboratories and teams of LAMAV/UENF, where the nanocomposite were produced and LBR/UENF where all the tests herein were able to be done.

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