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Development and Characterization of Hydroxyapatite-Functionalized Bacterial Nanocellulose Spheres for Bone Regeneration

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ABSTRACT

The reduction of bone defects occurs with the use of a barrier scaffold that prevents the invasion of soft tissues, as it creates a new space to guide bone growth in the defective area. The present research aimed to create and develop a new biomaterial for regeneration applications. Specifically, the study focused on the use of bacterial nanocellulose (BNC) spheres functionalized with hydroxyapatite (HAp). The spheres were synthesized *in situ* and characterized by scanning electron microscopy (SEM) to verify the distribution of HAp among the BNC fibers. The cells were injected inside the sphere and characterized for metabolic activity using the MTS assay. The cell distribution inside the spheres was analyzed by SEM, and the morphology of the cell nucleus and cytoskeleton was examined using a confocal microscope. It was confirmed that the addition of the osteoinductive element did not interfere with cell growth and did not present cytotoxicity. In the field of applications for artificial bones and scaffolds for tissue engineering, nanocomposites containing hydroxyapatite are esteemed.

Keywords: Biomaterials. Materials Engineering. Hydroxyapatite.

1 INTRODUCTION

Bone defects remain a significant challenge in medicine. Therefore, tissue engineering aims to identify materials that exhibit biocompatibility and can serve as scaffolds for their development[1]. Traditionally, autografts and allografts are used in bone regeneration. Although effective, allografts have limitations such as the risk of disease transmission and high cost[2]. On the other hand, autografts, although commonly more utilized due to their high therapeutic efficiency, face barriers such as limited tissue availability, the need for reoperation, and infection, which complicate their clinical application to some extent[3]. Therefore, many bone substitutes derived from calcium phosphate cements and polymers are being explored[4].

The need for polymers and their composites with unique properties has garnered significant attention with recent advances in nanotechnology. Polymers mixed with nanoparticles have attracted interest due to their ability to undergo economical processing, along with distinctive physicochemical properties such as enhanced electrical/thermal conductivity, improved mechanical properties (rigidity and strength), and better replicability[5,6].

Produced by certain microbial genera, bacterial nanocellulose (BNC) is a biopolymer that offers a broad range of applications in tissue engineering, medicine, biomedicine, and chronic wound therapy[7]. Its production can occur in static or agitated medium. In this project, it was produced in a static medium, where a gelatinous membrane accumulates on the surface, taking the shape of the flask in which the inoculum grows[8,9]. The BNC has been widely investigated due to its water retention, high porosity, biocompatibility, high crystallinity, and biodegradability[9,10].

Amid osteoinductive materials, hydroxyapatite (HAp) has been utilized for the development of biomaterials for bone regeneration, as it is a calcium phosphate similar to human hard tissues in morphology and composition[11]. BNC membranes have proven beneficial in bone tissue regeneration, both *in vitro* and *in vivo.* Studies indicate that *in vitro* degradability occurs through three different methods by incorporating HAp into BNC membranes: physical mixing, *in situ* formation, and biomineralization[11]. Most methods proposed in the literature to incorporate HAp into BNC fibers retain the HAp particles on the surface of the membranes[12]. Therefore, considering the current objective, it is evident that the *in situ* insertion method of hydroxyapatite into BNC is a relevant alternative due to its ease of application, high efficiency, low cost, and good reproducibility[13,14].

Thus, the objective of the present study was to produce BNC spheres with uniformly distributed HAp nanoparticles inside the biomaterial, as it possesses free hydroxyl functional groups allowing interaction with HAp in its matrix. This facilitates the creation of structures with high symmetry, and fibroblast culture inside was conducted to assess cytotoxicity and potentially enable osteoblast cell application.

2 MATERIAL & METHODS

The membranes were prepared using the *in situ* method, sintered by the bacterium *Gluconacetobacter xylinus*[3] The medium used for BNC production consists of: glucose, bacteriological peptone, mannitol, and powdered yeast extract. For the synthesis of hydroxyapatite, the following reagents were used: calcium nitrate ($Ca(NO_3)2{\cdot}4H_2O$, Synth), ammonium hydrogen phosphate ($(NH_4)_2{\cdot}HPO_4$, Neon), and ammonium hydroxide (NH4OH, Neon). The bacterial culture medium without hydroxyapatite was used

for the production of BNC spheres, which served as the control. Subsequently, the produced spheres were sterilized in an autoclave to eliminate potential contaminations. After the sterilization period, the spheres were transferred to the biological safety cabinet, with 5 spheres per well in two 6-well plates, and 4 mL of supplemented DMEM medium was added to each well. They were then placed in the incubator at 37 °C for 24 hours. The BNC-HAp spheres were added to a Petri dish for visualization.

For the characterization of the BNC-HAp spheres, scanning electron microscopy (SEM) and confocal microscopy were used. The distribution, orientation, fiber diameter porosity, and distribution of hydroxyapatite among the bacterial nanocellulose fibers were asses[7].

For the injection of fibroblasts into the interior of each sphere, L929 fibroblast cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and 1% Pen/Strep. Using a "BD u100 Insulin" syringe, 100 µL of the cell solution ($1 \cdot 10^5$ cells) was injected. Subsequently, the spheres containing the cells were incubated at 37°C for 1, 3, and 7 days.

For the analysis of cell viability, after 1, 3, and 7 days, the BNC and BNC-HAp spheres were removed and added to a 48-well plate. Then, 300 µL of DMEM medium and 60 µL of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] were added to each well, and the plate was incubated at 37°C for 2 hours, protected from light. Following this, using a 200 µL pipette tip, manual centrifugal force was applied to each sphere to extract the resulting solution from the reaction. Subsequently, 100 µL of this solution was added to a 96-well ELISA plate for absorbance analysis at 490 nm[7].

For SEM and Confocal analysis, the BNC and BNC-HAp spheres incubated for 1, 3, and 7 days were washed with PBS and fixed with glutaraldehyde for SEM analysis and paraformaldehyde for Confocal analysis. After the fixation period, the reagents were removed, the spheres were washed with PBS, and stored in the refrigerator. For the analysis of nucleus and cytoskeleton morphology using confocal microscopy, the spheres were halved using a razor blade, left in a solution of 0.1% PBS and Triton X-100 for 5 minutes, and then washed with PBS. Then, a solution of 200 µL BSA and 5 µL Phalloidin was added, allowing it to react for 20 minutes for cell staining and subsequent microscopic analysis. Following that, the spheres were washed with PBS, followed by Tween 20, and finally left in PBS until the time of analysis.

3 RESULTS & DISCUSSION

NC was produced by adding hydroxyapatite nanoparticles to the cellulose sphere production medium. Through SEM, we can analyze the spheres' morphology (Fig. 1A) before the drying process, which varies from 2 to 6 mm. It is possible to observe the internal structure of the spheres after the critical point drying process (Fig. 1B), and upon closer examination, we can see the distribution of hydroxyapatite nanoparticles inside the BNC spheres (Fig. 1C).

One of the project's objectives was to verify if adding hydroxyapatite would influence fibroblast cells' growth inside the bacterial nanocellulose sphere. Based on the MTS analysis (Fig. 1D), it can be observed that the cell growth did not vary significantly between the two types of spheres. Therefore, we can perceive that adding this osteoinductive element would create a potential scaffold for bone regeneration containing cells inside without hindering metabolic activity over time.

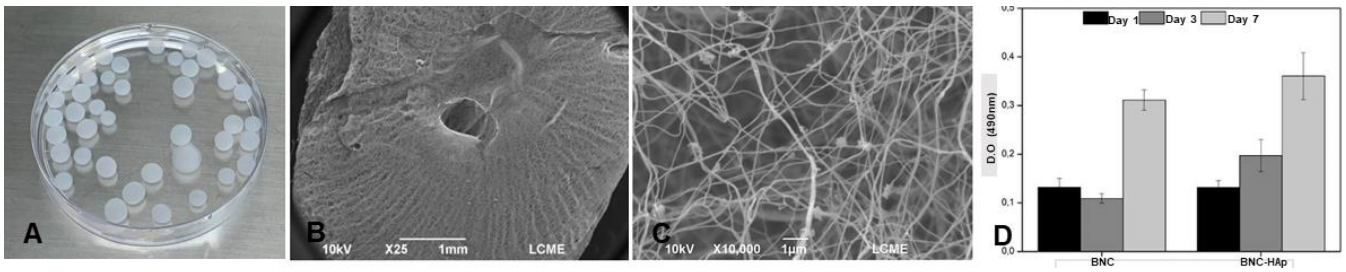

Figure 1. Morphology and Cytotoxicity of Bacterial Nanocellulose. Hydrated BNC-HAp spheres **(A)**. Sphere morphology by SEM, 25X magnification **(B)**. Distribution of hydroxyapatite inside BNC spheres by SEM, 10000X magnification **(C)**. Analysis of metabolic activity by MTS **(D).**

Through confocal microscopy, it was observed that after 24 hours of cultivation inside the BNC spheres with hydroxyapatite, the cells showed a rounded shape on top (Fig. 2). Comparatively, after 7 days of cultivation (Fig. 2), it was evident that there was cell spreading, indicating cell-cell and cell-biomaterial interaction.

Consequently, when comparing the matrices and cultivation times, we can perceive that adding hydroxyapatite to bacterial nanocellulose spheres did not influence fibroblasts' growth, meaning it did not present cytotoxic effects. Through scanning electron microscopy, it was possible to observe that after 7 days of fibroblast culture inside BNC spheres with hydroxyapatite, there was interaction between cells and between cells and the biomaterial (Fig. 2). The lack of pores when compared to the SEM image of BNC-HAp spheres without cells indicates once again that the presence of hydroxyapatite did not cause any cytotoxic effects to the cells.

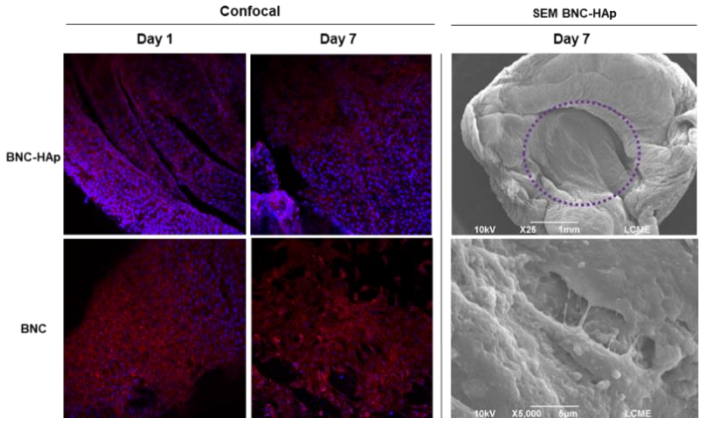

Figure 2. Confocal and SEM Analysis. Morphology of the nucleus and cytoskeleton of cultured cells in BNC-HAp spheres through confocal microscopy after 1 and 7 days. Cell distribution after 7 days of cultivation inside BNC-HAp spheres through SEM at 25X and 5050X magnifications.

4 CONCLUSION

Based on the results obtained, it was found that the presence of Hydroxyapatite inside the spheres did not affect cell adhesion and proliferation, not presenting cytotoxicity and cell death. This opens up further research possibilities for their direct application as a 3D scaffold or for applications involving other molecules in BNC. Importantly, it enables the study of adding osteoblast cells in place of the fibroblasts used. Bone regeneration through biomaterials is a field of study that should be continuously explored and developed. The material obtained holds great promise for the future of tissue engineering, given its unique characteristics such as high porosity, excellent fluid exchange capability, non-toxicity, water absorption capacity, protection against external agents, and most importantly, its ability to promote bone regeneration.

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