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Original

A novel synthesis and characterization of bio-based hydroxyapatite obtained from a residue of eggshells

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Abstract: Eggshells are waste generated in enormous quantities worldwide and can be utilized, as they are a rich source of Ca and can be converted into CaO through a thermal process. This is an efficient precursor for the synthesis of hydroxyapatite (Ha), it is an effective precursor for the synthesis of hydroxyapatite (Ha), which is a biomaterial. The aim of this work was to synthesize bio-based Ha from eggshells. The precipitation method was used, in which the addition of NH4OH, which is used in traditional synthesis, is omitted and the washes are conducted with water and citric acid. The eggshells, CaO obtained, and bio-based Ha were characterized by FTIR-(ATR), XRD and TGA, which allowed the functional groups, crystalline structure, and thermal stability of each material to be detected. The MTT assay on the NIH-3T3 fibroblast cell line showed that the viability exhibited 110.7% at a concentration of 200 µg/mL.

Keywords: Eggshell, bio-based material, biomedicine, hydroxyapatite, MTT and LDH assay

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1. Introduction

In recent years, the future of humanity from the point of view of environmental protection and human health has aroused great interest (Gómez Ayala & Yory Sanabria, 2018). For this reason, the improvement and development of existing materials is increasing due to their multiple uses, trying to reduce their impact on the environment. Bio-based materials are an example of this type of materials, which are partially or completely derived from natural sources such as animals, plants, minerals, microorganisms, etc. (Hairon Azhar et al., 2022).

The food industry generates thousands of tons of waste every year due to the increase in population and its economic activity (Ravindran & Jaiswal, 2016), so several researchers have focused on the use of waste to obtain bio-based materials. Chicken eggs are a food used in different dishes around the world, due to which there has been interest in the waste generated by this food. Currently, eggshell is widely studied for being a natural source of $CaCO_3$, which represents about 93% by weight of the shell, in addition to containing a smaller percentage of water (1.6%), magnesium (0.86%), phosphates (0.86%), and organic matter such as glycoproteins and proteoglycans (3.3%) (Arias et al., 1991; Hamidi et al., 2017; Patel et al., 2019). According to available information, more than 65 million tons of eggs are produced each year. The five main world producers are China, United States of America, India, Mexico, and Indonesia. In Mexico, in 2019 there was an increase of 18% in egg production, therefore, the generation of eggshell waste also increased (Awogbemi et al., 2020; Ramesh et al., 2016).

Eggshell has been studied in recent years for its high calcium content, since it can be used in a wide variety of applications in different areas such as pharmaceuticals, water treatment, medicine for the development of biomaterials, etc.

A biomaterial that has been synthesized from eggshells is hydroxyapatite (Ha) (FENAVI, 2023; Waheed et al., 2020). Ha is a hydroxylated salt of calcium phosphate whose chemical formula is $Ca_{10}(PO_4)_6(OH)_2$, and is the main component of human bones, and teeth (Polat & Sayan, 2020). Ha can be obtained from different natural sources that provide Ca, such as eggshells from different birds, corals, mollusk shells, or bovine, porcine and fish bones (Coelho et al., 2020). There are various methods by which it can be synthesized; the most used are mechanochemistry, sol-gel, alkaline hydrolysis, chemical precipitation, electrodeposition, sonochemistry, and microwaves (Agbeboh et al., 2020).

For synthesizing Ha from eggshells, these must be calcined to decompose $CaCO_3$ into CaO, then reacted with H_3PO_4 (see reactions). The Ha obtained from natural sources presents trace elements which allow the final material to present better biocompatibility, similarity to human bone, mineralization, osteogenic differentiation potential, and osteoconduction (Adeogun et al., 2018; Arokiasamy et al., 2022; Maqsood & Eddie, 2022; Waheed et al., 2020).

$$CaCO_3 \xrightarrow{\Delta} CaO + CO_2 \quad (1)$$

$$CaO + H_2O \rightarrow Ca(OH)_2 \quad (2)$$

$$10Ca(OH)_2 + 6H_3PO_4 \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 18H_2O \quad (3)$$

Rivera et al. (1999) presented a novel procedure for obtaining Ha from eggshells by wet chemical precipitation; these authors described the production of Ha as the only product obtained from the reaction. They also report that the procedure used is an environmentally friendly method (Rivera et al., 1999). Kamalanathan et al. (2014) successfully reported the production of Ha by a wet process using eggshell as a calcium precursor. They performed different heat treatments by varying the temperature when transforming $CaCO_3$ to CaO, reporting that optimum temperatures occur at 850 °C and above; they also obtained pure Ha and it remained stable after sintering at 1250 °C (Kamalanathan et al., 2014). Other studies conducted in Turkey by Yilmaz et al. (2019) reported obtaining a bio-based scaffold composed of chitosan, Ha synthesized from eggshells, and graphene oxide, finding that the highest cell viability was obtained with the scaffold containing 60% bio-based Ha, reporting Ha as an excellent biocompatible and osteoconductive material so they are proposed as a promising candidate for use in bone tissue regeneration.

The main objective of this paper was to obtain a bio-based biomaterial, Ha synthesized from eggshells, modifying the traditional synthesis by wet precipitation method. Relevant characterizations were performed by FTIR-(ATR), TGA, and XRD. In addition, the in vitro viability and cytotoxicity of the biomaterial Ha at different concentrations were examined using NIH-3T3 fibroblast cell lines by MTT and LDH assays. Images of cultured cells have shown confluent growth in the presence of bio-based Ha.

2. Materials and methods

The reagents used were H_3PO_4 with 65% purity from Analitika, Ha from Sigma Aldrich with 97% purity, 200 nm and 502.31 g/mol molecular weight, MTT assay kit from Merck, LDH assay kit from Cayman Chemical. And the equipment of laboratory drying oven of RIOSSA model H-82, Thermolyne muffle model F48055-60 Thermo Scientific, and photometer reader of microplates Miltiskan FC for plates of 96 and 384 wells with incubation, wavelength 340-850 nm Thermo Scientific. Figure 1 represents the flow chart conducted for the synthesis of Ha from eggshells.

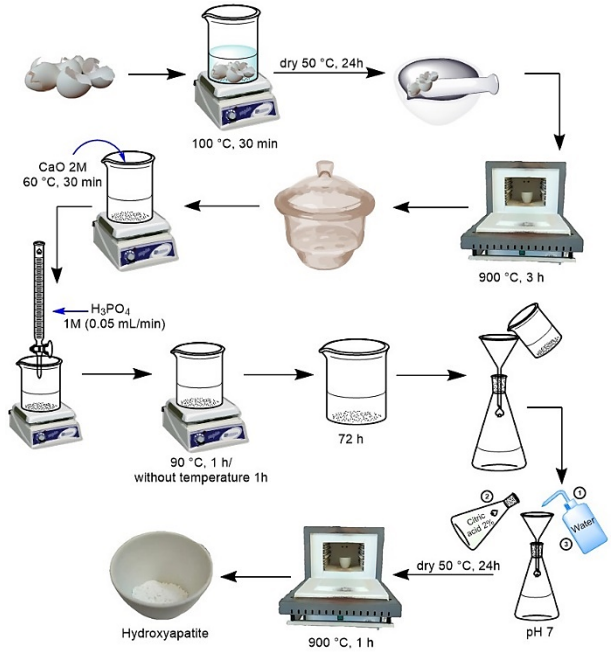

Figure 1. Flow chart of Ha synthesis.

2.1. Hydroxyapatite synthesis

White eggshells were collected from household waste and washed with boiling distilled water for 30 min to remove the inner membranes and avoid contamination during storage. They were dried at 50 °C for 24 h and then ground to a homogeneous powder using an agate mortar. Then 36 g were weighed and calcined at 900 °C for 3 h. The CaO was stored in a desiccator to avoid the absorption of moisture.

For the synthesis of Ha, 50 mL of 2 M CaO was prepared and kept at 60 °C for 60 minutes with magnetic stirring. Then 50 mL of 1 M H_3PO_4 was added at a rate of 0.05 mL/min while maintaining magnetic stirring. After the addition was complete, the solution was left at 90 °C while stirring for 1 hour. After this time, the temperature was removed, and magnetic stirring was maintained for a further 1 hour.

The solution was then allowed to stand for 72 hours. After this time, it was washed with distilled water and a 2% citric acid solution until a neutral pH was reached. Finally, it was heat treated at 900 °C for 1 hour to promote crystallization of the Ha. This methodology was conducted in three replicates to confirm reproducibility.

2.2. Characterization techniques

The bio-based materials obtained were analyzed by infrared spectroscopy IR spectrum spectrophotometer, GX-PerkinElmer using the attenuated total reflection (ATR) technique with a diamond tip attachment, FTIR-(ATR). For thermogravimetric analysis (TGA), a TA instruments Q500 thermal analyzer with a heating rate of 10 °C/min and a temperature range of 30-800 °C was used. X-ray diffraction was performed with a PANalytical device at 40 kV and 30 mA in a measuring range of 2θ = 10 – 80° and using a silicon single crystal as a sample holder.

2.2.1. Viability assay

Viability tests were MTT and LDH. The NIH 3T3 cell line was used, and three different concentrations (50, 100 and 200 µg/mL) were evaluated for the biological and control base materials (eggshell, CaO, Ha and synthetic Ha). They were dispersed in 1 mL of medium, then 100 µL was added to the cells and incubated at 37 °C and 5% CO_2 for 24 hours. The supernatant was recovered to perform the LDH assay with the Cayman kit (LDH Cytotoxicity Assay Kit, n.d.). To assess the morphology of the cells, images were captured using an inverted microscope with a dino-eye lite camera and a 10x objective.

The MTT assay was performed by adding 10 µL of tetrazolium blue salt and incubating for 5 hours. Subsequently, the crystals were dissolved with 100 µL DMSO and read on a plate reader at 595 nm (MTT Assay Protocol for Cell Viability and Proliferation, n.d.). Three independent experiments were performed with three replicates for each concentration used. The results were analyzed with the prims graph program using ANOVA with a value of $p<0.05$.

3. Results and discussions

Before obtaining the bio-based Ha, the raw material was analyzed to elucidate its chemical composition and then proceed with the proposed methodology.

First, it was analyzed by eggshell, the FTIR-(ATR) analysis is shown in Figure 2(a), for which important bands were observed at 1399 cm^{-1} corresponding to the asymmetric stretching of the C-O bond, at 873 and 711 cm^{-1} attributed to the internal and external deformations exclusive to $CaCO_3$ (Lanzón et al., 2023; Lopes et al., 2023; Paruthi et al., 2023).

XRD analysis is presented in Figure 2(b) observing the characteristic peaks of calcite with a rhombohedral crystalline system (database ICSD:169931), presenting as the main intense peak with a 2θ value of 29. 51°, this is like that reported in different investigations (Castro et al., 2022; Ferreira et al., 2016).

The characterization by TGA Figure 2(c) presents two weight losses, the first of approximately 3.52 % in a temperature range of 168-488 °C, attributed to water loss and decomposition of organic matter since the eggshell has membranes containing collagen, hyaluronic acid, chondroitin, glucosamine, keratin, and lysozyme (Correia et al., 2014). The second weight loss of 40.15 % occurs at 693 °C due to the beginning of the decomposition of $CaCO_3$ to CaO (Paruthi et al., 2023; Yamaguchi et al., 2015). Considering these results, it can be elucidated that the eggshell is composed of $CaCO_3$.

Calcination was conducted to obtain CaO after verifying that the eggshell was composed of $CaCO_3$. Figure 3(a) shows its FTIR-(ATR) spectrum, where important bands are observed at 1440 and 1063 cm^{-1} associated with the stretching of the Ca-O bond, in addition, at 3640 cm^{-1} there is a weak band due to the presence of -OH. This is since CaO is highly hygroscopic, therefore it can absorb humidity from the environment. Different investigations report similar bands for CaO obtained from eggshells (Lanzón et al., 2023; Nadeem et al., 2021; Yılmaz et al., 2019).

Figure 3(b) shows the diffractogram of calcined eggshell, observing a peak of higher intensity with a value of 2θ=37.5°, which according to the ICSD:75786 database, is due to the presence of CaO with a cubic crystalline system (Nadeem et al., 2021; Nagabhushana et al., 2017). Finally, thermogravimetric analysis (Figure 3(c)) was performed to determine the thermal stability of the material obtained. Two weight losses are observed in the thermograms, the first at 450 °C with 18.7 %, attributed to evaporation of moisture and decomposition of $Ca(OH)_2$ (Khoshraftar & Ghaemi, 2023), the second weight loss is at 663 °C with 5.52 % due to decarboxylation of $CaCO_3$ still present in the sample (Hossain et al., 2023). In 2019 Jitjamnong reported obtaining CaO from eggshells, having two weight losses at 450 and 750 °C, attributed to $Ca(OH)_2$ decomposition and decarboxylation of $CaCO_3$, respectively (Jitjamnong et al., 2019). Due to the results, it is possible to transform $CaCO_3$ from eggshells to CaO by heat treatment at 900 °C for 3 h.

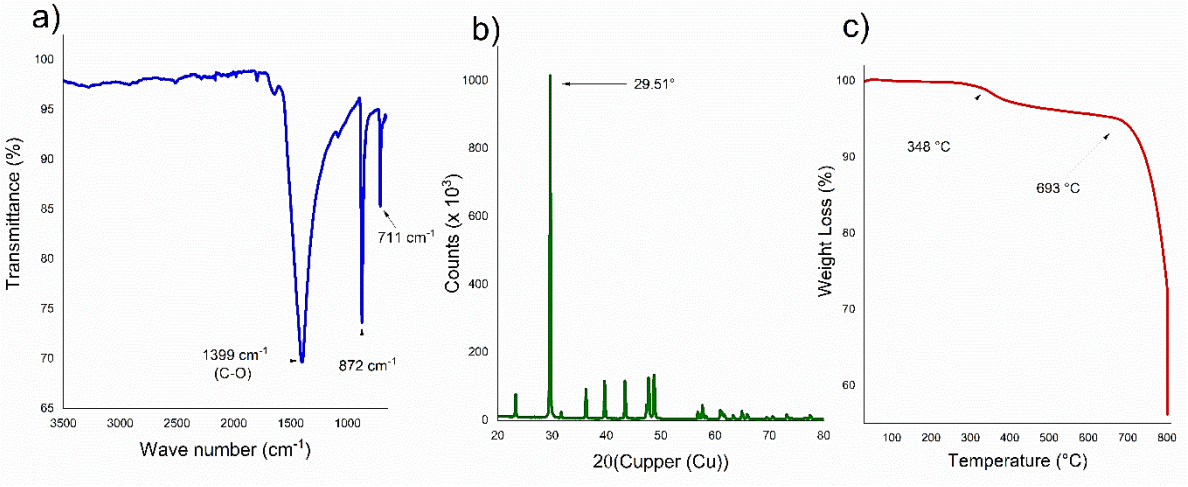

Figure 2. FTIR spectra (a), XRD diffractogram (b), and thermogram TGA (c) for eggshell powder.

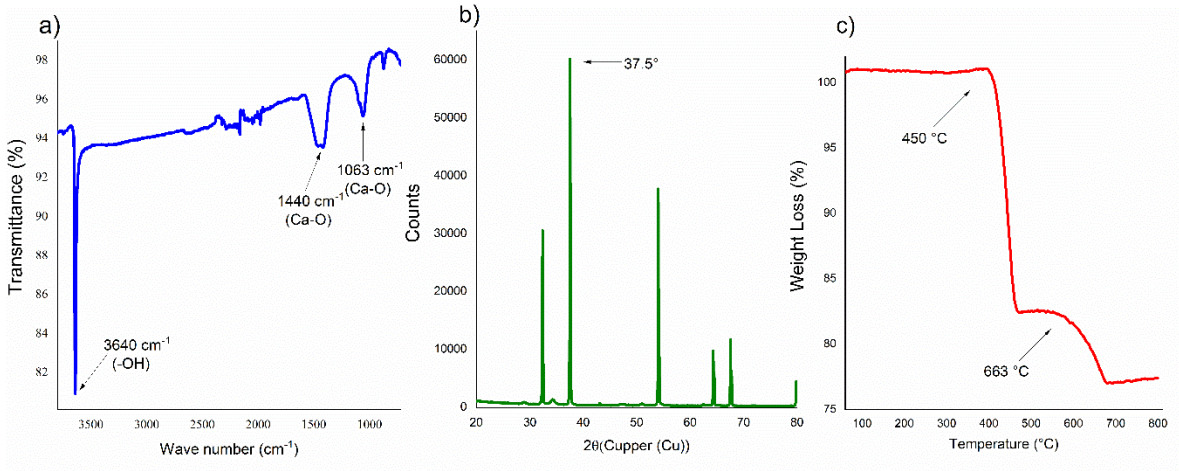

Figure 3. FTIR spectra (a), XRD diffractogram (b), and thermogram TGA (c) for calcined eggshell powder.

The synthesis of Ha by the precipitation method was continued after obtaining CaO from eggshells. Figure 4 shows the FTIR- (ATR) spectra for the bio-based Ha and the synthetic (control) Ha. They show significant bands for both at wavenumbers 3577 cm^{-1}, which are assigned to the vibrations and stretching of the -OH groups, and 1021 and 628 cm^{-1}, which represent the vibrations of the PO_4^{3-} ion. In addition, the presence of CO_3^{2-} was detected at wavenumbers 1424 and 872 cm^{-1}. Several authors point to these bands and peaks as specific signals of Has from eggshells (Mobarak et al., 2023; Das Lala et al., 2019; Muñoz et al., 2023).

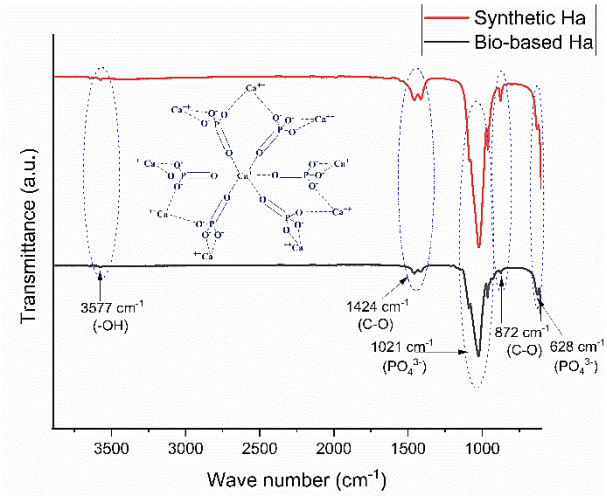

Figure 4. FTIR-(ATR) spectra of bio-based and synthetic Ha.

The XRD analysis presented in Figure 5 shows that both the bio-based material and the synthetic Ha exhibit the characteristic peaks of Ha with a hexagonal crystal system, according to database 01-086-0740. In addition, the presence of CaO and $Ca(OH)_2$ is observed in the bio-based Ha, confirming the results of the FTIR analysis. Numerous studies in which Ha was synthesized from eggshells report the presence of these peaks and crystalline structure (Mobarak et al., 2023; Vinayagam et al., 2023; Wu et al., 2023).

Figure 6 shows the TGA analysis for bio-based Ha and synthetic Ha. For bio-based Ha, two weight losses are observed, the first at 444 °C with 7.515 %, which is due to the decomposition of $Ca(OH)_2$ present in the sample, and the second loss occurs at 636 °C with 1.48 %, which is due to the decarboxylation of $CaCO_3$ from eggshells. Something similar is reported by several authors who obtain bio-based Ha from eggshells and mention that these two weight losses are due to the presence of $Ca(OH)_2$ and $CaCO_3$ (Nasr et al., 2023; Ramesh et al., 2016; van Niekerk & Langmi, 2023). In contrast, no weight loss is observed for synthetic Ha, as it is stoichiometric, and its thermal stability remains constant above 800 °C.

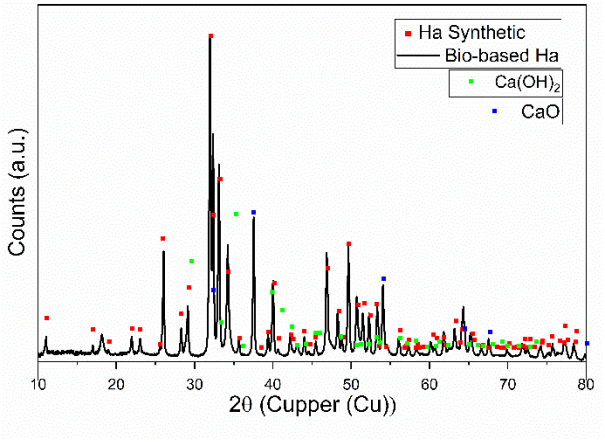

Figure 5. XRD diffractogram of bio-based and synthetic Ha.

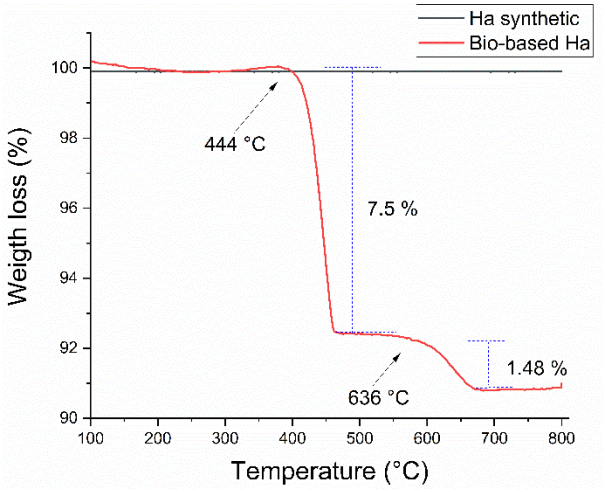

Figure 6. Thermogram of bio-based and synthetic Ha.

The results of the analysis of the synthesized bio-based material show that with the proposed method it is possible to obtain Ha with a purity of about 90% and with a hexagonal crystal system.

The viability and cellular cytotoxicity (MTT and LDH, respectively) were evaluated in vitro on the NIH 3T3 cell line to determine the behavior of the material in contact with living cells. Eggshells, CaO and synthesized Ha were used for these tests; synthetic Ha was used as a control. Each of these materials was analyzed at three different concentrations: 50, 100 and 200 µg/mL.

Figure 7 shows the photos of the cells after 24 h in contact with the material. In both the control cells (+) and the analyzed materials, a monolayer can be observed formed by the cells that continue to adhere to the plate, indicating that the cells do not show pronounced cell lysis as in the cells in contact with the Triton (control -).

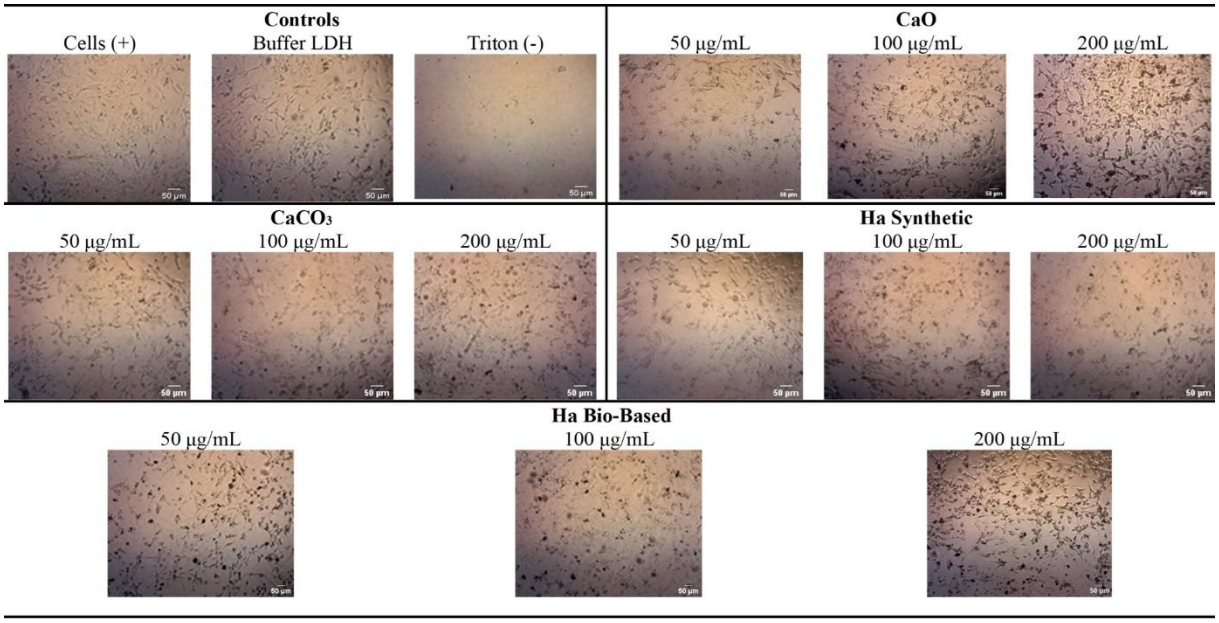

Figure 7. Photographs of NIH-3T3 cells after 24 h in contact with the varied materials. Images were captured using an inverted microscope with a dino-eye lite camera and a 10x objective, the scale bar represents 50 µm.

The graph presented in Figure 8 shows the analysis of the data obtained for the MTT assay. It can be observed that the cell control (+) has a viability of 99 %, while all the materials analyzed present a viability higher than 90 %, finding significant difference ($p>0.05$) only for Triton, CaO 200 µg/mL and $CaCO_3$ 200 µg/mL. Of note is the bio-based Ha, which has a viability of 108.72, 103.27 and 110.73% for concentrations of 50, 100 and 200 µg/mL, respectively, however, there is no significant difference ($p>0.05$) among the different concentrations. An increase in viability was obtained when compared to synthetic Ha is due to the presence of oligoelements such as Na, K, Mg and CO_3^{2-} in Ha obtained from natural resources, which promotes biocompatibility and osteoconduction (Arokiasamy et al., 2022; Ramesh et al., 2016).

The graph of the analysis of the data obtained in the LDH assay is shown in Figure 9. It can be observed that Triton (control -) shows the highest cell lysis with almost 100 %, while the cell control (+) presents a lysis of 6.04 % ($p<0.05$). Among the materials analyzed, with respect to Triton, in all concentrations, showed a significant difference ($p<0.05$).

Therefore, the materials evaluated at the different concentrations show no toxicity in the NIH- 3T3 cell line. In addition, according to the parameters of ISO 10993-5, a cell viability of more than 70 should be achieved when evaluating a material for biomedical applications. Therefore, the cell viability of the NIH-3T3 cell line, eggshell, CaO obtained from the eggshells and synthesized Ha, are within the acceptable parameters of the standard. It should be noted that the presence of CaO and $Ca(OH)_2$ in the final material did not affect the biocompatibility of the material, in addition, the non-use of NH_4OH to stabilize the pH during Ha synthesis and the use of citric acid in the Ha washes, did not affect the biocompatibility and structure of the material.

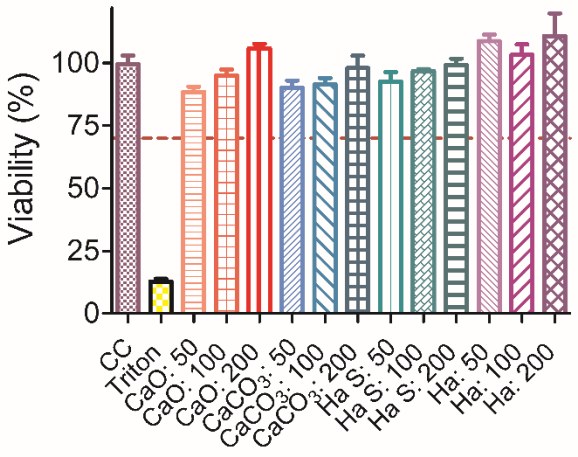

Figure 8. Graphical analysis of the MTT assay against the NIH-3T3 cell line. Where Ha S: Synthetic Ha and Ha: Bio-based Ha.

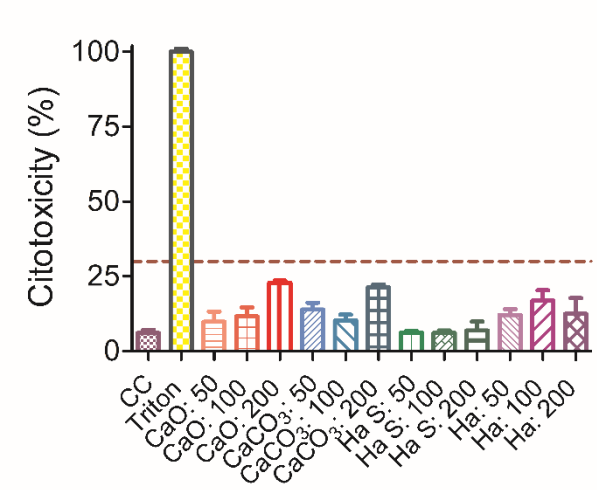

Figure 9. Graphical analysis of the LDH assay against the NIH-3T3 cell line. Where Ha S: Synthetic Ha and Ha: Bio-based Ha.

4. Conclusions

The methodology proposed for the synthesis of Ha, eliminating NH_4OH as a pH stabilizer, as well as the addition of citric acid for the washes, has been successful, obtaining bio-based Ha with a purity of approximately 90 % and with a hexagonal crystalline system.

The cell viability and cytotoxicity assays showed that the materials analyzed, eggshells, CaO obtained from eggshells and the synthesized Ha, had a cell viability of over 90%. Therefore, according to the ISO 10993-5 standard, they are within the parameters established to be considered suitable for biomedical applications.

The synthesis proposes to counteract environmental damage through the reduction of reagents and the use of agri-food waste, transforming them into high value-added products for different biomedical applications, without generating environmentally hazardous waste. Therefore, since it is a simpler methodology, with a reduction of steps and reagents, a future task of this work could be to scale up this process to a pilot plant. As well as to evaluate the cost-benefit of the use of this novel proposed method.

Conflict of interest

The authors have no conflict of interest to declare.

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