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# Hybrid materials for bone tissue engineering from biomimetic growth of hydroxiapatite on cellulose nanowhiskers

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ABSTRACT

Cellulose nanowhiskers (CNWs) with different surface composition were used to generate the biomimetic growth hydroxyapatite (HAp). Hybrids materials primarily consist of CNWs with HAp content below 24%. CNWs were produced by different inorganic acid hydrolyses to generate cellulose particles with surface groups to induce HAp mineralization. In the present study, we evaluate the use of CNWs prepared from hydrochloric acid, sulfuric acid and phosphoric acid. HAp growth was obtained from the biomimetic method using a simulated body fluid concentration of 1.5 M (SBF). The sulfonate and phosphonate groups on the CNW surface have a direct impact on the nucleation and growth of HAp. HAp/CNW were also compared with the physical mixture method using HAp nanoparticles prepared by chemical precipitation. The bioactivity and biocompatibility of the hybrid materials were assessed by cell viability studies using fibroblast cells (L929). The materials obtained from the biomimetic method have superior biocompatibility/bioactivity compared to the material synthesized by the wet chemical precipitation method with an incubation period of 24 h.

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## 1. Introduction

With the continuous increase in the life expectancy and higher incidence of diseases that cause bone loss, such as infections, tumors, and trauma, medical procedures for bone grafts have become even more requested for repairing bone defects (Zadegan, Hosainalipour, Rezaie, Ghassai, & Shokrgozar, 2011). Additionally, bone regeneration is a constant process throughout life, but the regeneration speeds decrease as a function of the age. The speed of bone regeneration is detrimental to the recovery of artificial implants or in the healing of small bone fractures. The introduction of chemical species to promote and/or burst bone regeneration has been thought crucial for improving healthcare and allowing for the development of less invasive medical procedures to treat bones (Govindan, Kumar, & Girija, 2015; Qi et al., 2014).

In fact, the development of materials for bone repair has been an ongoing research field for a long time, and the current challenge resides in finding materials that can stimulate the bone regenerative process (Stevens, 2008). Therefore, the material is expected to promote cell differentiation to favor the osteoblastic lineage, guiding bone growth to desired areas and stimulating the integration of the new growing bone into the surrounding bone (Kim et al., 2014). Among all tested materials for bone repair, hydroxyapatite (HAp) stands out because it is the main calcium phosphate phase constituent of natural bones and teeth (Kumar, Negi, Choudhary, & Bhardwaj, 2014). Due to its excellent biocompatibility; bioactivity; and non-inflammatory, non-toxic and osteoconductive properties (He, Chang, Peng, & Zhang, 2012), synthetic HAp is widely used in orthopedic, dental implants, spinal fusion and treatment of bone defects (Othmani, Aissa, Bac, Rachdi, & Debbabi, 2013; Yang, Liu, Liang, Lin, & Wu, 2013; Si, Cui, Wang, Liu, & Liu, 2016). HAp stimulates osteoconduction without causing rejection (Singh, 2012). Nonetheless, the direct use of hydroxyapatite has some drawbacks associated with the mechanical and chemical stability of synthetic crystals (Fang, Wan, Tang, Gao, & Dai, 2009). Hence, one possible

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approach for developing advanced biomaterials for bone repair is the use of HAp in composites, but also, it could also result in new materials with superior bioactivity compared to the pure components. The preparation of inorganic/organic hybrids materials is a promising alternative because the organic phase can be explored to improve the stability of the inorganic phase (Cellet, Pereira, Muniz, Silva, & Rubira, 2015; Silva, Pereira, Muniz, & Rubira, 2009). HAps have already been combined with polymeric materials, such as collagen, polyamide, poly(lactic acid), poly(lactide-*co*-glycolide) (Liuyun, Yubao, Li, & Jianguo, 2008) and heparin (Goonasekera, Jack, Cooper-White, & Grondahl, 2016) to produce nanocomposites. Natural biodegradable polymers are typically used because they have better biocompatibility than synthetic polymers (Liuyun, Yubao, & Chengdong, 2009).

Cellulose, the most abundant natural polysaccharide in the world, has many meaningful features that would make it helpful as a component in hybrid materials for bone repair because it is a renewable homopolymer that is biodegradable, sustainable, non-toxic and highly biocompatible (Gu & Huang, 2013; Joshi et al., 2016; Zhou, Wu, Yue, & Zhang, 2011). Importantly, additional features are introduced when cellulose is used as a nanocellulose material (Korhonen et al., 2011). Sugarcane bagasse is an abundant agro-industrial residue; 280 kg of bagasse is produced per ton of processed sugarcane. Therefore, this agro-industrial is promptly available at no cost, mainly in countries like Brazil and India, which are the major sugarcane producers in the world. In addition, sugar cane is a very robust plant that is cultivated in many countries, such as the USA, Australia, China, South Africa and many others (Saelee, Yingkamhaeng, Nimchua, & Sukyai, 2016). Natural cellulose is a semicrystalline material with alternating crystalline and amorphous regions in the cellulose fibers (Credou & Berthelot, 2014; Ishikawa et al., 2015; Silva, Haraguchi, Muniz, & Rubira, 2009). Once exposed to strong acid medium, amorphous regions become hydrolyzed faster than the crystalline regions due the higher permeability of the amorphous phase. The difference in the hydrolysis speed allows for degradation of the amorphous phase and the recovery of the crystalline phase in the form of isolated nanocrystals. The cellulose nanocrystals have a high aspect ratio, and they are known as cellulose nanowhiskers (CNWs) (Eichhorn et al., 2010). CNWs have extraordinary mechanical and chemical stability, and the surface composition of CNWs depends on the synthetic method used or it can be further modified to generate special interaction sites (Peng, Dhar, Liu, & Tam, 2011). In addition, the biocompatibility of cellulose nanowhiskers has been already demonstrated in cell culture assays (Jinga et al., 2014; Rodríguez, Renneckar, & Gatenholm, 2011).

In the present work, HAp and CNW hybrid materials are prepared by two different methods. A chemical method that mimics the natural process of HAp growth on the surface of CNW and a physical method based on the nanoparticle suspension and aggregation of CNWs with HAp nanoparticles. CNWs used in the present work were prepared from sugarcane bagasse. The acid hydrolysis was performed with different inorganic acids to obtain CNWs with specific surface composition. The influence of the CNW surface groups on the interaction with HAp is studied. To evaluate the bioactivity in the proliferation of connective tissue, the HAp/CNW hybrid materials prepared in the present work were tested with L929 fibroblast cells.

## 2. Materials and methods

### 2.1. Materials

Acetic Acid (Nuclear), Calcium Chloride (F. Maia), Calcium Hydroxide (Nuclear), Dimethyl Sulfoxide (Nuclear), Disodium Phosphate (Nuclear), Dulbecco's modified Eagle's medium (DMEM), (Gibco®), Ethylenediamine Tetraacetic Acid (EDTA) (Sigma-Aldrich), Fetal Bovine Serum (FBS) (Gibco®), Fibroblasts (ATCC-L929), Hydrochloric Acid 37% (Nuclear), Hydrogen Peroxide 37% (F. Maia), Magnesium Chloride Hexahydrate (Amidol), Penicillin (Sigma-Aldrich), Phosphoric Acid (Nuclear), Phosphate Buffered Saline (PBS) (Gibco®), Potassium Chloride (Anidrol), Potassium Phosphate Dibasic Trihydrate (Sigma-Aldrich), Sodium Bicarbonate (Nuclear), Sodium Chloride (Nuclear), Sodium Dodecyl Sulfate (SDS) (Sigma Aldrich), Sodium Hydroxide (Synth), Sodium Sulfate (Nuclear), Sulfuric Acid 98% (Nuclear), Streptomycin (Gibco®), Trypsin (Sigma Aldrich), Tris (Hydroxymethyl) aminomethane (Synth) 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) (Sigma Aldrich).

### 2.2. Cellulose nanowhisker preparation from sugarcane bagasse

#### 2.2.1. Cellulose hydrolysis with hydrochloric acid or sulfuric acid

The hydrolysis using hydrochloric acid (37% HCl) was performed with a cellulose to HCl ratio of 1.0 g of cellulose in 25 mL of acid solution at 45 °C for 60 min under vigorous stirring (Spagnol et al., 2012). Afterwards, the suspension was centrifuged for 30 min (9500 rpm) and rinsed with distilled water until pH 3 was obtained. Then, the material was dispersed in water and purified by dialysis until a neutral pH was reached. The resulting nanowhiskers were lyophilized at −55 °C for 48 h. The notation used for the obtained sample is CNW(HCl). The same hydrolysis procedure was used for hydrolysis with sulfuric acid; however, a 50% $H_2SO_4$ solution was used (Hamad & Hu, 2010). The notation for the nanowhiskers is CNW($H_2SO_4$).

#### 2.2.2. Cellulose hydrolysis with phosphoric acid

Cellulose (1.0 g) was sonicated in 50 mL of distilled water. Then, the suspension was placed in an ice bath at 15 min. Afterwards, 25 mL of $H_3PO_4$ (10.7 mol/L) was added. The mixture was stirred for 90 min at 100 °C (Camarero Espinosa, Kuhnt, Foster, & Weder, 2013). The suspension was centrifuged for 30 min (9500 rpm) and rinsed with water to reach pH 3. Then, the dispersion was dialyzed to neutral pH. The obtained nanowhiskers were lyophilized at −55 °C for 48 h. The notation used for the nanowhiskers is CNW($H_3PO_4$).

Pre-treatment and cellulose purification from sugarcane bagasse is shown in ESI†.

### 2.3. In situ synthesis of HAp on cellulose nanowhiskers by the biomimetic method

CNW(HCl), CNW($H_2SO_4$) or CNW($H_3PO_4$) (200 mg) was immersed in 0.1 M $CaCl_2$ solution at 37 °C with mechanical stirring for 3 days. The $CaCl_2$ solution was renewed every day by filtration. Afterwards, the sample was immersed in 50 mL of 1.5 SBF (Simulated Body Fluid) solution (Hong et al., 2006), at 37 °C with mechanical stirring for 14 days. The simulated body fluid (SBF) was prepared according to a previous report (Kokubo & Takadama, 2006). The solution was renewed every 48 h. Then, the sample was filtered and rinsed with distilled water and dried at room temperature. The notation used for the material was *b*-CNW(HCl)-HAp, *b*-CNW($H_2SO_4$)-HAp and *b*-CNW($H_3PO_4$)-Hap, respectively, where *b* means hybrid materials obtained by the biomimetic method.

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