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Preparation and characterization of amine functional nano-hydroxyapatite/chitosan bionanocomposite for bone tissue engineering applications

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ABSTRACT

In this study, three different types of scaffolds including a uniquely modified composite scaffold – namely chitosan (CTS), nano-hydroxyapatite/chitosan composite (CTS + nHAP), and amine group (NH_2) modified nano-hydroxyapatite/chitosan composite (CTS + nHAP-NH_2) scaffolds – were synthesized for bone tissue engineering (BTE) purposes. As results of the study, it was found that all scaffold types were biodegradable with CTS and CTS + nHAP scaffolds losing up to 15% of their initial weight, while the CTS + nHAP-NH_2 scaffold showing 10% of weight loss after six weeks of lysozyme treatment. In addition, all three types of scaffolds were shown to be biocompatible, and amongst them CTS + nHAP-NH_2 scaffolds supported the most cell proliferation in WST-1 assay and expressed the least and acceptable level of cytotoxicity in lactate dehydrogenase (LDH) test for human bone mesenchymal stem cells (hBM-MSCs). Finally, during osteoinductivity assessment, CTS + nHAP-NH_2 nearly tripled initial alkaline phosphatase (ALP) activity when whereas both CTS and CTS + nHAP scaffolds only doubled. These results indicate that all synthesized scaffold types under investigation have certain potential to be used in bone tissue engineering approaches with CTS + nHAP-NH_2 scaffold being the most promising and applicable one. In the future, we plan to intensify our studies on osteogenic differentiation on our scaffolds on a detailed molecular level and to include *in vivo* studies for pre-clinical purposes.

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1. Introduction

As an alternative to autologous or allogeneic bone grafts, scaffolds made of synthetic or natural biomaterials supporting the migration, proliferation, and differentiation of allogenic bone cells might be used in bone tissue engineering (BTE). Several types of porous scaffolds have been shown to support *in vitro* bone formation by human cells, including those made of ceramics, native and synthetic polymers and composite materials (Black et al., 2015). The design of scaffolds using composite materials offers optimized patterns in terms of biodegradability and bioactivity. It is possible to materialize scaffolds with tailored physical, biological and mechanical properties by combining biopolymers and bioactive ceramics (Puppi, Federica, Piras, & Chiellini, 2010).

An ideal cellular source for BTE approaches should be non-immunogenic, non-tumorigenic, possess off-the-shelf availability,

Abbreviations: ALP, alkaline phosphatase; APTES, 3-aminopropyltriethoxysilane; BM, bone marrow; BTE, bone tissue engineering; CTS, chitosan; DMEM-LG, Dulbecco's Modified Eagle's medium low glucose; ELISA, enzyme-linked immune sorbent assay; FT-IR, Fourier transform infrared spectroscopy; hBM-MSCs, human bone mesenchymal stem cells; HMDS, hexamethyldisilazane; LDH, lactate dehydrogenase; MSC-FBS, mesenchymal Stem Cell – Qualified Fetal Bovine Serum; nHAP, nano-hydroxyapatite; PBS, phosphate buffered saline; RGD, arginylglycylaspartic acid; SEM, scanning electron microscopy; TGA, thermogravimetric analysis; WST-1, Water Soluble Tetrazolium Salt-1; XRD, X-Ray diffractometry; β-GP, β-glycerophosphate.

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and potent proliferative and osteogenic potential (Gong et al., 2015; Logeart-Avramoglou, Anagnostou, Bizios, & Petite, 2005). Up to now, studies demonstrate that hBM-MSCs have the ability to differentiate into osteogenic, chondrogenic and adipogenic lineages, when incubated under appropriate *in vitro* or *in vivo* conditions (Kemp, Hows, & Donaldson, 2005; Zigdon-Giladi, Rudich, Geller, & Evron, 2015).

A scaffold for the purpose of BTE should have microstructure with interconnected and dispersed pores throughout almost its entire volume supporting cellular ingrowth and sufficient supply. Furthermore, it should exhibit sufficient mechanical stability for bearing loads after implanation. Finally, an ideal scaffold should support the formation of new bone matrix by the cells (Bessa, Casal, & Reis, 2008; Neman, Hambrecht, Cadry, & Jandialü, 2012; Sundelacruz & Kaplan, 2009). Numerous different natural materials have been studied as scaffold preparation materials and evaluated in BTE. Their biodegradability, low toxicity, renewability, and low production and disposal costs contributed the interest on natural polymers to grow in economic and environmental aspects (Shogren & Bagley, 1999). Collagen and silk fibroin are known examples of natural polymers of protein origin (Puppi et al., 2010). Hyaluronic acid, alginate, starch-based materials, bacterial cellulose, dextran and chitosan are good examples for natural polymers of polysaccharide origin (Puppi et al., 2010).

Chitosan has numerous advantages due to its properties which make it ideal as a bone graft substituent to be used in orthopedic applications (Di Martino, Sittinger, & Risbud, 2005). Chitosan is a biodegradable (Seol et al., 2004), non-toxic, biologically compatible polymer (Thano, Verhoef, & Junginger, 2001), and can be formulated in a variety of forms including powders, gels and films. In addition, it exhibits ahydrophilic surface enhancing cellular adhesion and proliferation. It was shown to promote cell growth and mineral rich matrix deposition by osteoblasts *in vitro* (Seol et al., 2004). It is highly biocompatible and inducing minimal host response due to its low immunogenity. Also, it is shown to have antibacterial activity (Ding, Deng, Du, Shi, & Wang, 2014; Younes & Rinaudo, 2015). All these reasons make CTS one of the most preferred natural polymers for BTE.

Chitosan scaffolds are flexible and their mechanical properties are weaker than those of normal bone, as it is prone to load bearing bone implants. Chitosan scaffolds alone cannot imitate all the properties of natural bone. Its mechanical weakness and instability can easily be overcome by modifications and/or use of composite structures (Di Martino et al., 2005). The development of composite materials with CTS is shown to mimic almost all supporting properties of bone tissue according to many studies in the literature (Khan & Ahmad, 2013; Rodriguez-Vazquez, Vega- Ruiz, Ramos-Zúñiga, Saldaña-Koppel, & Quiñones-Olvera, 2015). As proven, calcium phosphate materials are osteoconductive to imitate the inorganic part of a natural bone, while CTS/nHAP composite materials show promise of mimicking the organic portion as well (Teng et al., 2009; Thein-Han & Misra, 2009; Xianmiao et al., 2009).

Hydroxyapatite is a major inorganic component of the bone (Kim & Mendis, 2006). Thus, nHAP has recently emerged as an important compound for artificial bone preparation, and therefore for BTE also. It stimulates osteoconduction by a gradual replacement by the host bone after implantation. Though, the mechanical properties of nHAP are poor because of its crystalline nature, so it cannot be used in aiming of load-bearing bone tissues. Polymers have been used as combining materials to improve the mechanical properties of nHAP including its compressive strength, Young's modulus, and fracture toughness (Nath, Dey, Mukhopadhyay, & Basu, 2009). In a previous work, nHAP was used with CTS to be able to obtain a scaffold and the resulting bio-polymer composite was showed a good results and thought to be a good imitation of a functional natural bone (Sun & Yang, 2015).

In this work, we prepared a novel CTS/nHAP composite scaffold that is composed from the amine functionalized nHAP to be able to increase cell attachment. The aim of our study was to produce a unique and efficient composite scaffold to be used in BTE applications, and to biologically evaluate the pre-clinical suitability of this novel product by indicating osteoinductive and osteocunductive properties as an assessment of osteogenic differentiation success of hBM-MSCs *in vitro*.

2. Experimental part

2.1. Materials and methods

Chitosan powder (low molecular weight, 75–85% deacetylated) was purchased from Sigma–Aldrich. Calcium nitrate tetrahydrate [$Ca(NO_3)_2{\cdot}4H_2O$], 2,4,6Trinitrobenzenesulfonic acid (TNBS), diammonium hydrogen phosphate [$(NH_4)_2HPO_4$], and 25% ammonium hydroxide (NH_4OH) were purchased from Merck Millipore (Darmstadt, Germany), 3-aminopropyltriethoxysilane (APTES) were purchased from Sigma-Aldrich (Taufkirchen, Germany). All chemicals were of analytical grade and were used without further purification.

2.2. Synthesis of hydroxyapatite

nHAP nanoparticles were synthesized according to methods described in literature (Lin, Wu, & Chang, 2014). Briefly, 500 mL of 1 M $Ca(NO_3)_2{\cdot}4H_20$ aqueous solution was adjusted with NH_4OH to pH 10. The solution was heated to 90 °C, later then 500 mL of 0.6 M $(NH_4)_2HPO_4$ at pH 10 (adjusted with NH_4OH) was added dropwise under stirring. Solid parts were maintained in the reaction solution for 5 h at 90 °C; afterwards it was centrifuged at 10,000 rpm for 10 min and repeatedly washed with distilled water. The obtained product was dried and nHAP nanoparticles were obtained.

2.3. Synthesis of hydroxyapatite

The amine content of the amine functional hydroxyapatite nanoparticles were determined by TNBS assay according to literatüre (Bagheri-Khoulenjani, Mirzadeh, Etrati-Khosroshahi, & Shokrgozar, 2016). Briefly, exact amount of dried nano-composite microspheres (10 mg) were exposed to 1 mL 4% (w/v) NaHCO3 and 1 mL 0.5 (w/v) TNBS solution for 4 h at 40 °C. To hydrolysis the microspheres, 3 mL 6N HCl were added to each sample and stirred for 2 h at 60C. After cooling at room temperature, samples were diluted using deionized water and their absorption in 345 nm against the blank sample were measured using UV–vis spectrophotometer (Shimadzo- Japan). Blank samples were prepared using the same procedure unless the HCl solution was added to the samples before TNBS solution. As the interaction of TNBS and NH2 groups take places merely in basic pHs, adding HCl prevented this reaction. This procedure was repeated for 3 times for each sample.

2.4. Modification of hydroxyapatite

The surface of the nHAP nanoparticles modified according to literature (Goonasekera, Jack, Cooper-White, & Grondahl, 2010; Russo et al., 2014). 3-aminopropyltriethoxysilane (0.221 g) was added into an aqueous alcohol solution containing 90 mL of alcohol and 10 mL of water. The solution was stirred for 30 min and then nHAP (1.0 g) was added andwas stirred for 3 h. The pH was adjusted to 9–10 with NH_4OH, and the reaction was continued for another 3 h. After filtration with fitler paper the powder was first dried at room temperature and then cured at 130 °C to strengthen the silane coating by formation of a polysiloxane network struc-

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