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Evaluation of hemocompatibility and *in vitro* immersion on microwave-assisted hydroxyapatite–alumina nanocomposites ☆

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ABSTRACT

This study reports the microwave-assisted synthesis and characterization of nHAp (nano-hydroxyapatite)–alumina composites. The crystalline phase and interaction of alumina with nHAp was analyzed using X-ray diffraction (XRD) and Raman microscopy analysis, respectively. High resolution transmission electron microscopy (HRTEM) micrographs exhibit morphological changes of nHAp composites with increasing alumina concentrations. Microhardness studies reveal the enhanced mechanical strength of nHAp10 and nHAp20 nanocomposites than pure nHAp. *In vitro* bioactivity of the nanocomposites was studied by immersing samples in simulated body fluid (Hank's solution) for 21 days. The surface of biomineralized samples were analyzed using field emission scanning electron microscopy (FESEM) and energy dispersive X-ray spectroscopy (EDX). Hemolytic assay revealed acceptable compatibility for varying concentrations of all the samples. Cell proliferation assay was systematically investigated for 1 day and 3 days on Saos-2 osteoblast-like cell lines and it was found that nHAp nanocomposites improved the proliferation.

1. Introduction

Mammalian bone is a natural nanocomposite of collagen fibrils impregnated with HAp. Organic collagen matrix provides resilience, while the inorganic HAp gives strength and stiffness to bone. The organic–inorganic combination provides mechanical support and dynamic function in the body [1]. Synthetic nHAp structure and chemical composition are similar to natural bone. HAp is responsible for biomineralization, osteoinduction, and osteointegration [2] and has good biocompatibility [3] and bioactivity [4]. However its clinical applicability is restricted due to its poor mechanical strength [5]. Several research studies are being undertaken to overcome this limitation by preparing nanocomposites with reinforcing polymers, metal oxides and strong bioceramics [6–8].

Reinforcement of polymers into HAp could provide superior mechanical strength. *In situ* synthesis of nHAp in polyvinyl alcohol (PVA) could affect its crystallization if the pH is basic. On other hand, acidic pH hampers the absorption of Ca^{2+} on hydroxyl pendant groups of PVA backbone [9]. The polymer–HAp nanocomposites such as HAp–gelatin [10], silk–HAp [11] and poly L-lactic acid (PLLA)–HAp [12] have also been investigated for bone regeneration application. Few reports on HAp-based nanocomposites with metal oxides and bioceramics prepared by various methods have been explored [13,14]. Among the bioceramics, alumina (Al_2O_3) is widely used due to its high mechanical strength, chemical inertness, toughness and high elastic modulus [15]. Al_2O_3 is a stable oxide due to its ionic and covalent bonding, but gets corroded in strong acid or alkaline environment [16].

Besides alumina coatings, efforts have also been made to reinforce alumina in nHAp to improve mechanical properties by heat treatments at higher temperature [17,18]. Highly crystallized nHAp-based ceramics exhibited poor *in vivo* resorption rate [19]. However, when alumina is used as a reinforced material in nHAp, apatite to β-TCP (β-tricalcium phosphate) phase decomposition occurs as reported earlier [20]. The β-TCP is one of the secondary phases, as well as decomposition phase of nHAp. In another report, the large interfacial reactions that exist between nHAp and alumina influence the secondary phase formation [21]; hence, that results into poor densification and low mechanical strength of bioceramics [22,23]. Alternatively, HAp calcinated at higher temperature transformed directly into β-TCP phase which significantly improved solubility [24,25] and rapidly resorbed from the implanted sites before the complete new bone regeneration.

Mechanical properties of nHAp–alumina based composites are enhanced by sintering at 1200 °C [26]. HAp–alumina composites densified at 1275 °C hot isostatic press with varying HAp contents showed improved properties for higher HAp content and lower alumina content [27]. Tensile strength of HAp composites with ceria-stabilized alumina and zirconia implants through biomimetic coatings is evaluated in rat tibia [28]. Bioceramics developed for biomedical applications should essentially possess both biocompatibility and mechanical strength. Synthetic nano-hydroxyapatite is a highly biocompatible nano-ceramic but it lacks mechanical strength which reduces its utility in load bearing

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applications. Therefore, to address this issue, this work explores reinforcing of alumina with nHAp as an effective strategy.

Several synthesis routes have been reported for the preparation of nHAp and nHAp-based nanocomposites [29–32]. These conventional synthesis methods are time-consuming processes. Microwave synthesis is a simple, fast and efficient method for nHAp synthesis [33,34]. Here, we report a microwave-assisted method to prepare pure nHAp and nHAp–alumina nanocomposites with enhanced mechanical strength by varying the Al_2O_3 concentration.

2. Experimental

2.1. Chemical synthesis

Chemicals such as calcium nitrate tetrahydrate ($Ca(NO_3)_2 \cdot 4H_2O$), orthophosphoric acid (H_3PO_4) from Fisher Scientific, ammonium hydroxide (NH_4OH) from Sisco Research Laboratories and Hank's balanced salt obtained from Sigma-Aldrich were procured.

For synthesis of pure nHAp, 0.5 M concentration of $Ca(NO_3)_2 \cdot 4H_2O$ and 0.3 M concentration of H_3PO_4 were taken with the Ca/P ratio of 1.67. This solution was stirred for 15 min and the pH was adjusted to 10 with addition of NH_4OH. Then, this reaction mixture was subjected to microwave irradiation with operating power of 800 W for 20 min. The obtained precipitate was washed several times with distilled water to remove unwanted ions such as NO_3^{2-} and NH^{4+} and then calcined at 500 °C. The nHAp–alumina composites was also prepared by adopting the same procedure as discussed above with addition of 10 to 20 wt.% alumina to $Ca(NO_3)_2 \cdot 4H_2O$. Henceforth, the nHAp with 10 wt.% and 20 wt.% of alumina incorporation was coded as nHAp10 and nHAp20, respectively. The Al_2O_3 NPs used in this study was synthesized following the procedure published in literature [35,36]. The synthesis flow chart of nHAp–alumina nanocomposites was given in Fig. 1.

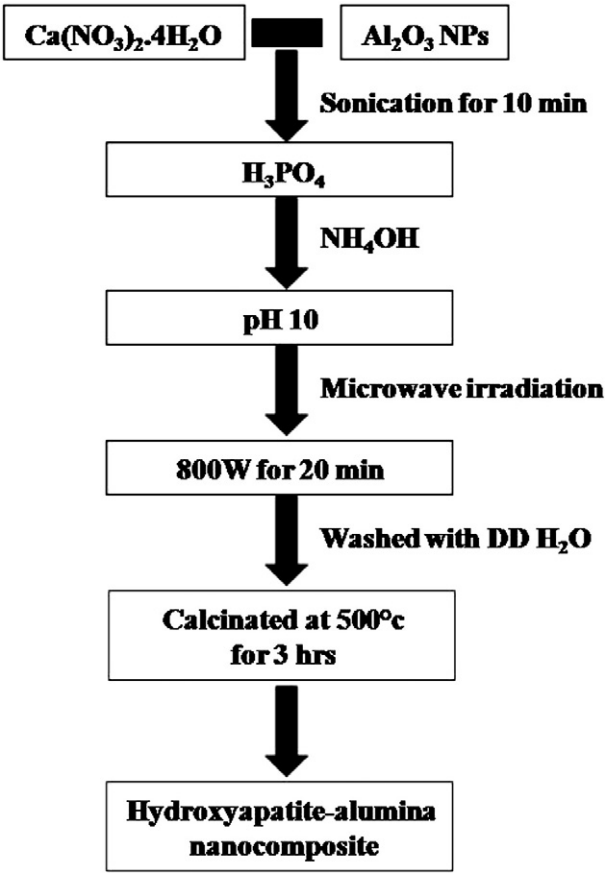

Fig. 1. Synthesis flow chart of nHAp–alumina nanocomposites.

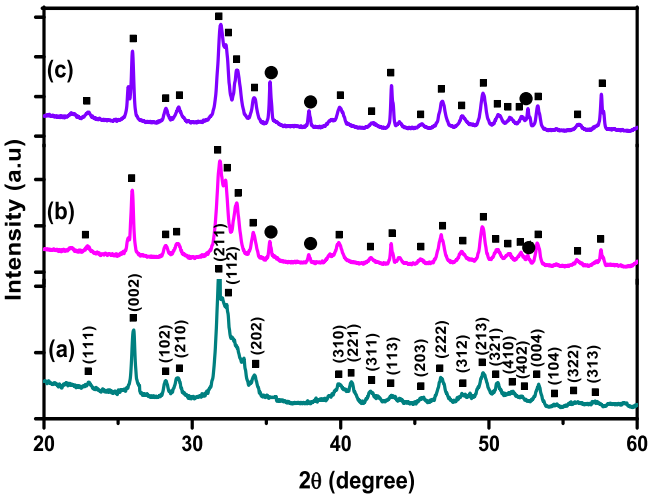

Fig. 2. XRD patterns of (a) nHAp, (b) nHAp10, and (c) nHAp20; the peaks of nHAp marked as (■) in addition with small peaks of Al_2O_3 marked as (●).

2.2. Characterization

The crystalline nature and phase of synthesized samples were studied by XRD. The diffraction patterns were recorded in Rigaku X-ray diffractometer with Ni-filtered Cu Kα radiation (λ = 1.54 Å) over the 2θ range of 20–60°. The interaction of pure nHAp and its nanocomposites were studied using Laser Raman microscope—RAMAN 11i from Nanophoton Corp., Japan, by using excitation wavelength of 532 nm. For morphological analysis, samples were dispersed in ethanol by ultrasonication for 10 min, then coated on carbon coated copper grid and examined in HRTEM, FEI-TECNAI G^2 instrument. For hardness measurements, 150 mg quantity of samples were compressed by applied 88 bar pressure to make 8 mm diameter discs. The compressed discs were analyzed using micro Vickers hardness tester, Wilson Wolpert, Germany. Loads of 2 N and 5 N forces are applied on the surface of the compressed discs for 15 s through a pyramidal diamond indenter.

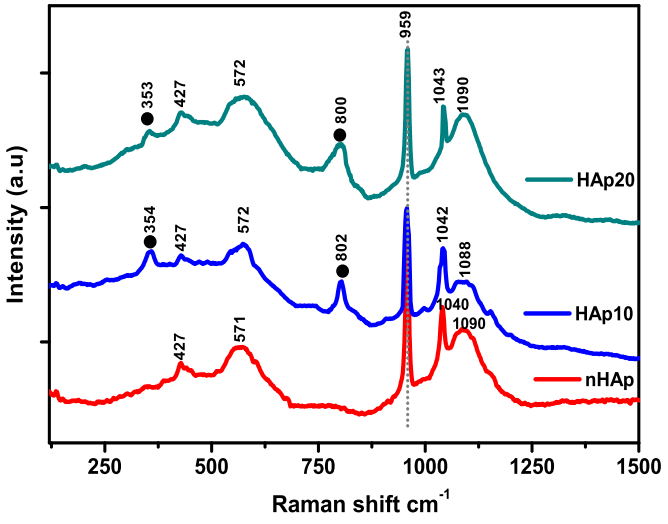

Fig. 3. Raman spectra of nHAp, nHAp10 and nHAp20, where Al_2O_3 peaks are indicated as (●) in addition to nHAp peaks.