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Preparation and Physicochemical Evaluation of Hydroxyapatite-gelatin Nanoparticles

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Abstract: One of the promising fields of research is tissue engineering that can facilitate available therapeutic methods. Nanosized hydroxyapatite (HA) is one of the key elements in mineral bone. It is possible to affect the surrounding osteoprogenitor cells using nano-sized HA and through this improve bone repair through changing paracrine signaling. The present paper is an attempt to prepare and evaluate physicochemical properties of hydroxyapatite-gelatin nanoparticles. To this end, two sizes were prepared including S100 and S150 using standard chemical precipitation. To characterize the size and morphology of the synthesized powders, X-ray diffraction and Brunauer-Emmett-Teller (BET) surface area were determined using Autosrob-IQ2-MP. To measure the calcium ions released by HANPs, an inductively coupled plasma optical emission spectrometer (ICP-OES) was used. The collected data was analyzed in SPSS 19.0. In the case of S100, the hydrodynamic diameter based on DLS analyses was equal to 626.10±14.95 nm; this figure for S150 was equal to 262.33±46.5. There was a larger specific surface area in S100 compared to S150; in addition, S100 had wider diffraction peaks, which is in agreement with small and poorly crystalline crystals. On the other hand, the diffraction peaks of S150 were sharper, which means that the crystallinity was higher in S150. In addition, HANPs of all sizes had degradability and HANPs with smaller sizes (S100) degraded faster compared to larger-sized S150. The pH level of the control, S100, and S150 was 7.24±0.01, and 7.26±0.02 so that there was no significant difference between them. Nanoparticle size is a key factor in the biological environment, which provides a reference for HANPs in biomedical uses.

Keyword: hydroxyapatite-gelatin; Nanoparticles; Nanotechnology

1. Introduction

Tissue engineering is A promising research field to lower the complication of therapeutic methods. It is an interdisciplinary field that uses the principles of life sciences and engineering to create biological substitutes to preserve, improve, and restore tissue functions[1-3].

Over the past few years, engineered nanoparticles and nanotechnology have emerged as a new field in material science. It is possible to use nanotechnology to alter matter at the atom level and create new nanoproducts containing new properties[4,5]. The new properties of these materials have been extensively studied in cosmetics, medicine, and environment and technology. A highly complicated field of material science is biomaterials so researchers have tried to develop improved biomaterials with specific uses in medicine.

Nanosized hydroxyapatite (HA) is the key component in mineral bone. Living bones permanently experience a formative-resorptive process known as bone remodeling. The process features bone replacement and removal that happens at the same time through the respective activities of osteoclasts and osteoblasts along with vascular supply and a network of lacunae and canaliculi[6-8]. HA demonstrates exceptional bioactivity and biocompatibility properties as to bone tissues and cells, mostly because of similarity with the body's hard tissues. So far, the most common clinically used biomaterial is calcium phosphate, which is used as granules, powder, dense and porous blocks and different composites Calcium phosphate materials are the key mineral part of calcified tissues. Still, calcium phosphate is mostly found in the bone as nanometer-sized needle-like[9-11].

One of the mainstreams of research works is new HA formulations to develop more effective and better biomedical applications and produce materials as similar as possible to living bones like monolithic and nanosized structures[12,13]. In comparison to standard ceramic formulation, the properties of nanophase HA like pore size, surface grain size, wettability and so on can control protein interactions (e.g. configuration, adsorption, and bioactivity). Thus. Long-term functionality and osteoblast adhesion improve modulating subsequent[14,15].

Regeneration of bone essentially happens along with the invasion of neovessels. The inner lining of the vascular system is formed by an endothelial cell (EC) that passively delivers blood and also has a role in specifying, inducing, and guiding organ regeneration. It also helps maintain homeostasis and metabolism[16-18]. Mesenchymal stem cell (MSC) plays a role in the periendothelial niche and has a self-renewal and multi-differentiation capability when induced by biochemical microenvironments and particularly physiological in their resident niches. HA nanoparticles (HANPs) in bone tissue engineering might contact with neovessels and become endocytosed by EC, which can change the physiological functions of the cells[19-21]. This may also affect the surrounding osteoprogenitor cells and bone repair through changing paracrine signaling. Still, we know a lot about the direct effect of HANPs on MSCs while our knowledge of HANPs role in inducing osteogenic differentiation of MSCs through EC is very limited. This insight is needed to have a clearer picture of the effects of HANPs on bone repair and the first step for this goal is understanding physiochemical characteristics of HANPS. The present study is an attempt to prepare and evaluate physicochemically hydroxyapatite-gelatin nanoparticles as a nanoparticle for Mesenchymal Stem Cell bone damage.

2. Material and Methods

2.1 Conventional chemical precipitation of HANPs

HANPs was prepared at two sizes assigned with S100 and S150 using standard chemical precipitation. In short, drops of calcium chloride ($CaCl_2$) solution were added to diammonium hydrogen phosphate ($(NH_4)_2HPO_4$) solution while the solution was gently stirred and the molar ratio of Ca/P was at 1.67. Along with the precipitation, aqueous ammonia was used to modify pH values up to 8 and 10 for S100 and S150 respectively. In addition, the temperature for S100 and S150 was stabilized at 50°C and 90°C respectively. Following precipitation, the obtained suspension was kept at ambient temperature for 16 h. Eventually, the obtained powder was collected, washed using deionized water, and dried using vacuum freeze drying.

2.1.1 Precipitation of gelatin solution

Afterward, 0.1% solution of gelatin in double deionized water was developed and then, nanohydroxyapatite powder was added to it to obtain a mixture with a final weight of 61% gel and 0.1% hydroxyapatite. The obtained solution was stirred using a magnetic stirrer (Hidofl-Germany) for 10 min at 10°C to achieve a uniform solution. The obtained solution was poured into a petri dish with a plastic thickness of 2mm. The obtained sample was stored in a freezer for 20 h until it was dry. However, only HANPs were used

in the tests.

2.1.2 Morphology and size of HANPs

The size and morphology of the synthesized powder were determined using transmission electron microscope. The crystalline phases of the powders examined using Xray diffraction. To measure Brunauer-Emmet-Teller (BET) surface area, Autosorb-IQ2-MP was used. The hydrodynamic diameter of S150 and S100, dispersed in basal medium using 10% fetal bovine serum and measured through dynamic light scattering.

2.1.3 The effect of HANPs on the pH

To examine the effect of HANPs on the pH of the culture medium, 10 µg/mL of HA NPs was immersed in the osteogenic induction medium at 37°C in humid air containing 5% CO_2. The osteogenic induction medium was renewed once every two days, so that the pH of the medium with and without HANPs was measured following two days of incubation.

2.2 Calcium ions released by HANPs

The experiment to examine degradation *in vitro* was carried out through immersing 10 µg/mL of HANPs in DPBS for 14 days at 37°C. To measure the concentration of calcium ions discharged by HANPs, inductively coupled plasma optical emission spectrometer (ICP-OES) was used.

2.3 Data analysis

The results were reported as mean ± SD and data analyses were done in SPSS19.0 ($p < 0.05$) using one-way ANOVA and Tukey tests.

3. Results

3.1 Size and hydrodynamic diameter of HANPs

The S100 and S150 were about 100 and 150 nm in length respectively and the width was 15 and 20 nm respectively. The hydrodynamic diameter according to DLS analysis for S100 and S150 were 626.10 ± 14.95 nm and 262.33 ± 46.5 nm respectively. The surface area of S100 was higher than S150 (**Figure 1**).

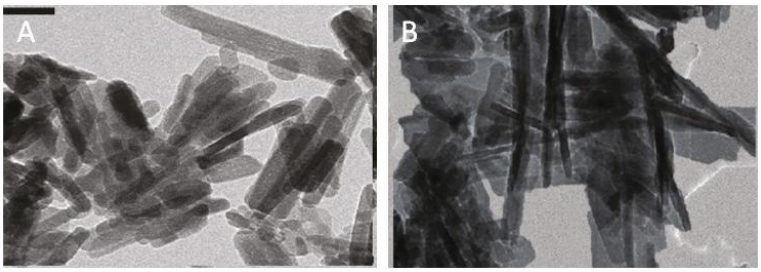

Figure 1. The surface area of S100 (A) and S150 (B) Particles.

3.2 XRD patterns of S150 and S100

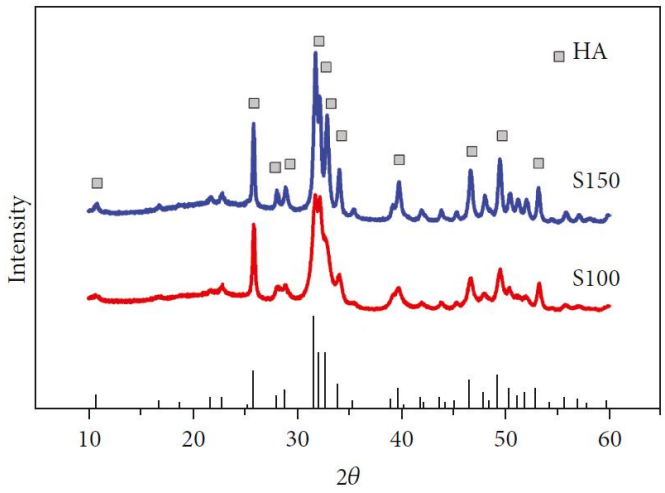

Figure 2. XRD patterns of S100 (A) and S150 (B) Particles.

As shown by the standard card of HA, the XRD pattern of S150 and S100 with typical characteristic diffraction peaks was seen in crystalline HA phase (25.87°, 31.78°, 46.71°, 49.47°, and 53.14°). In addition, the broadening diffraction peaks of S100 were higher, which indicates poor crystalline and small crystals. On the other hand, S150 had a sharper diffraction peak compared to S100, which indicates a higher crystallinity of S159 (**Figure 2**).

The ICP-OES was used to measure the release of Ca^{2+} ions from HANPs in DBPS. The concentration of Ca^{2+} of S150 and S100 groups was significantly higher than the control group (DPBS only). In addition, following 14 days of soaking, the Ca^{2+} discharge from S100 demonstrated 117% increase compared to S150, which is a significant increase. The findings also indicated that HANPs had degradability regardless of size and HANPs of smaller size (S100) had a faster degradation compared to S150 (**Figure 3**).

3.3 Calcium ion release

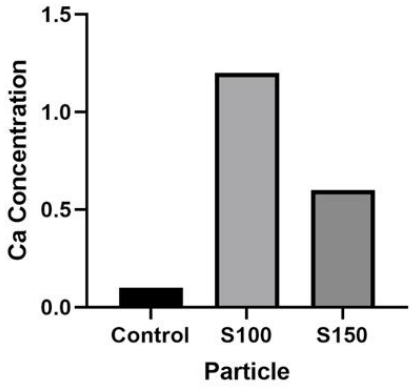

Figure 3. Calcium ion release of S100 (A) and S150 (B) Particles.

3.4 The effect of HANPs on medium pH

Following two days of incubation with standard cell culture condition, the pH in the control group (medium without HANPs) was equal to 7.23±0.02. In addition, the pH of S100 and S150 mediums was 7.24±0.01 and 7.26±0.0 respectively. All of them show no significant changes in comparison to the control group. The findings showed that adding HA NPs had no effect on the pH of the culture medium (**Figure 4**).

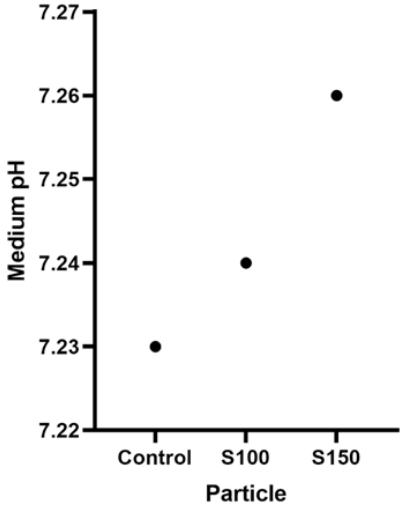

Figure 4. Effect of S100 (A) and S150 (B) Particles on Medium pH

4. Discussion

Regeneration of bone is a physiological phenomenon involving complicated and diverse cellular actions like differentiation and migration of mesenchymal stem cells (MSCs). The HANPs is used as an artificial bone material and can be taken up by cells because of their

length scale which is comparable to proteins and discrete elements of cells. Therefore, it can influence the functions of the cell, which in turn affects bone repair. The MSCs demonstrate the potential of self-renewal and multi-differentiation so that they can be used in reparative and regenerative medicine[22-24]. In terms of structure, HANPs are similar to the main mineral component of the bone and teeth and the products that have nanoparticles have improved the precipitation of calcium and phosphate ions in the tooth structure[24,25]. Studies have shown that HANPs 10% suspension (10-20 nm in diameter) improved remineralization of the outer layer in initial caries lesions to a depth of 20-40 μm. Still, the body of lesion demonstrated a small level of remineralization[26,27]. Another study showed that nano-HA (20 nm in size and 100-150 mm dimension) improved remineralization in the subsurface of initial lesions mostly in dentin compared to enamel[28].

Compared to standard HANPs that have a flat surface, the nano-sized HANPs have underlying crystals; they can cause a higher level of stress concentration at the cell-crystal interface along with higher Ca^{2+} exchange because of a higher cell extension on the surface. These features benefit osteoblast differentiation of MSCs[29]. In addition, the HANPs can act as a mediator of adhesion of specific anchorage-dependent cells through adsorbing extracellular matrix proteins such as fibronectin and growth factors such as osteonectin[30]. It is notable that studies have indicated that autophagy in osteoblasts has a role in mineralization and bone homeostasis and considered nHAP as a new class of autophagy inducer. In addition, what matters the most are the size or shape that stimulate autophagy. The role of needles and spherical shaped HA particles was examined by Xu *et al.*[31] on protein expression profiles in osteoblasts. They showed that, compared to spherical particles, phase pure needle shaped HA nanoparticles improved differentiation of osteoblasts. According to Xu *et al*. the volume of Ca^{2+} ions release was responsible for diverse behaviors of the spherical and needle shaped particles. In the case of clinical uses, nanoparticles must be incorporated or coated on 3D scaffolds, and HA nanoparticles might demonstrate different behaviors compared to pure particles when exposed to other materials with different degradation capabilities. Here, the BCP porous scaffolds were coated with diverse sizes and shapes of HA nanoparticles when combined with PCL and their effects on HOB behavior were examined. These applications in HANPS need different sizes and shapes. The HANPs used in this study were two sizes and prepared using chemical precipitation methods through changing the temperature and pH of the reaction solution. First, phosphate anions and calcium ions yielded amorphous calcium phosphate (CaP) or hydrated orthophosphates, which led to transformation into HA through phase transformation under the right condition[32]. Therefore, HA had a slow and prolonged growth process. The higher precipitation temperature and higher energy supply accelerated HANPs growth. There were wide and low diffraction peaks of S100, while S150 demonstrated well-differentiated peaks, which can be due to a higher c-axis of the unit cells of S150 with higher precipitation temperature. Different-size hexagonal CdS nanoparticles were prepared by other researchers and it is possible to control the growth rate of the OH-absorbed crystallite facets using the shielding effect of OH- on the interface In general, the different aspect ratios and sizes of S150 and S100 were due to changes in pH values. In conclusion, nanoparticle size is a key factor in the biological environment that gives us a reference for HANPs in biomedical application.

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