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# Biomimetic whitlockite inorganic nanoparticles-mediated *in situ* remodeling and rapid bone regeneration

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ABSTRACT

Bone remodeling process relies on complex signaling pathway between osteoblasts and osteoclasts and control mechanisms to achieve homeostasis of their growth and differentiation. Despite previous achievements in understanding complicated signaling pathways between cells and bone extracellular matrices during bone remodeling process, a role of local ionic concentration remains to be elucidated. Here, we demonstrate that synthetic whitlockite (WH: $Ca_{18}Mg_2(HPO_4)_2(PO_4)_{12}$) nanoparticles can recapitulate early-stage of bone regeneration through stimulating osteogenic differentiation, prohibiting osteoclastic activity, and transforming into mechanically enhanced hydroxyapatite (HAP)-neo bone tissues by continuous supply of $PO_4^{3-}$ and $Mg^{2+}$ under physiological conditions. In addition, based on their structural analysis, the dynamic phase transformation from WH into HAP contributed as a key factor for rapid bone regeneration with denser hierarchical neo-bone structure. Our findings suggest a groundbreaking concept of 'living bone minerals' that actively communicate with the surrounding system to induce self-healing, while previous notions about bone minerals have been limited to passive products of cellular mineralization.



## 1. Introduction

Natural hard tissues continuously maintain a healthy state throughout life by self-regenerating their micro-damaged parts through a bone remodeling process [1–9]. At the initiation of bone remodeling process, osteoclast precursors migrate from blood vessels to the bone by chemorepulsion [1,2]. Then, osteoclasts form a sealing zone with ruffled boarders and inorganic minerals are degraded by protons supplied through $H^+$-transporting adenosine triphosphate ($H^+$-ATPase) [3,9,10]. In addition, organic bone matrices are further resorbed by proteinase, such as cathepsin K, in an acidic pH condition [4,11,12]. Recent evidences showed that these resorption processes are necessary for successful bone formation as resorbed components along with activated osteoclasts sequentially drive endogenous stem cells functions, where activated stem cells ultimately contribute to a new bone tissue [5–8].

Recently, synthetic matrices emulating the physiochemical properties of bone tissues are being developed to control stem cell fate. Biomaterials containing calcium phosphate moieties, such as hydroxyapatite (HAP: $Ca_{10}(PO_4)_6(OH)_2$), have been shown to induce osteogenic differentiation of stem/progenitor cells and bone tissue formation. However, the participation of inorganic minerals during the bone regeneration and remodeling processes has not been fully elucidated. During the bone remodeling process, the acidic environment created by osteoclasts mobilizes pre-existing minerals that have a similar phase with HAP [3,9]. HAP gets resorbed as the local pH inside the sealing zone of osteoclasts and macrophages is approximately 3–4.5 [3,10]. Recent evidences suggest that one of the main components of inorganic phase of bone is whitlockite (WH: $Ca_{18}Mg_2(HPO_4)_2(PO_4)_{12}$). Even though WH a relatively rare mineral in nature, it is the second most abundant mineral in human bone with approximately upto 20 wt%, and it is particular found in bone with elevated dynamic loading [13]. Whitlockite is a mineral

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with an unusual form of calcium phosphate with unknown biological role. Based on its physiochemical properties, WH has unusual form of calcium phosphate and it is very different than its synthetic analogue β-tricalcium phosphate (β-$Ca_3(PO_4)_2$) [14,15]. Furthermore, unlike HAP, WH is relative stable in acidic condition. The increased detection of short micro-ranged WH in bone under increased loading along with its acidic stability suggests that it may act as an inorganic template composition for further mineralization. In addition, elevated composition of WH in adolescent's bone suggest that it may be actively involved in bone remodeling process [16,17].

Here, we investigated the role of WH on bone remodeling and formation. WH nanoparticles were synthesized, and we demonstrate that the synthetic WH can recapitulate early-stage bone regeneration via elevated extracellular $PO_4^{3-}$ and $Mg^{2+}$ concentration as well as inhibition of osteoclastic differentiation. Furthermore, our studies showed that WH participates in bone formation via increased affinity with extracellular proteins. Finally, we demonstrated that the WH containing scaffold platform could stimulate through phase transformation *in vivo* bone formation. The multidisciplinary approach conducted in this study provided an organized methodology to find biological functionalities of WH and introduced a useful clinical application.

## 2. Results

To mimic the natural components of bone minerals, WH nanoparticles were synthesized by a wet precipitation method in an aqueous system. Additionally, HAP nanoparticles were prepared by a similar precipitation method for comparison. As shown in Fig. 1a, X-ray diffraction (XRD) patterns of WH and HAP nanoparticles showed that both synthesized nanoparticles were pure without the existence of any heterogeneous phases. Their peaks matched previously known XRD patterns of WH (JCPDS 70-2064) and HAP (JCPDS 84-1998). From field emission scanning electron microscopy (FESEM) analysis, WH nanoparticles had a rhombohedral-like shape with a homogenous size of approximately 50 nm (Fig. 1b). HAP nanoparticles were ellipsoidal with an average size of approximately 80 nm (Fig. 1c).

The direct visualization of a single WH nanoparticle was also performed by high-resolution transmission electron microscopy (TEM). Diffraction pattern analysis of an enlarged single WH nanoparticle was well matched with the crystal information of naturally occurring WH crystals (Fig. S1). Based on morphology analysis, the exposed surface plane of the synthesized WH nanoparticle was similar to that of naturally occurring WH crystal (Fig. 1d) [18]. In Fig. 1e, the {012} plane of the enlarged WH nanoparticle has its edge angle at approximately 77.5°, which corresponded well to the theoretical angle between $(\bar{1}02)$ and $(1\bar{1}2)$ in a hexagonal setting. Moreover, developments of {104} small planes were distinctly observed. In Fig. 1f, the growth of the (001) plane among {012} planes and {104} planes was found. Fig. 1g presents the atomic arrangement in the WH crystal structure, which depicts $(10\bar{2})$, (012) and $(1\bar{1}2)$ planes at the side, front and bottom, respectively. Supplementary Movie 1 provides the three-dimensional (3D) morphology of a WH nanoparticle monitored by field emission transmission electron microscope (FE-TEM) tomography.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2016.10.009.

We further investigated the surface characteristics of WH and HAP nanoparticles by X-ray photoelectron spectroscopy (XPS). Due to the presence of $HPO_4^{2-}$ in WH, the position of the P2p peak was shifted with higher peak intensity compared to that of HAP (Fig. 1h, j, and Fig. S2). Additionally, while the Mg KLL auger peak was clearly shown in WH (Fig. 1j), there was none in HAP (Fig. 1k). In addition, the atomic ratios on the surfaces of WH and HAP nanoparticles were calculated from the number of detected electrons in the $Ca_{2s}$, $Mg_{2s}$ and $P_{2s}$ peaks. From the XPS analysis results, the ratios between cat- and anions of WH ((Ca + Mg)/P) and HAP (Ca/P) were 1.44 and 1.63, respectively, while their theoretical values were 1.43 and 1.67, respectively. Therefore, for both WH and HAP nanoparticles in this research, we concluded that the surface characteristics were practically indistinguishable to the bulk.

For the resorption stability and ion release kinetics, WH and HAP nanoparticles were dispersed in distilled water, filtrates were collected through a membrane with 200 nm-sized pores, and an inductively coupled plasma atomic emission spectrometer (ICP-AES) was used to analyze the amounts of Ca, Mg and P ions. Ions release kinetic from both WH and HAP nanoparticles were evaluated up to 4 weeks in neutral pH condition (Fig. 2a–c). WH displayed faster P and Mg ions release kinetics compared to the HAP. However, elevated Ca ions were detected with HAP nanoparticles. In addition, ICP data (Fig. 2) showed reduced Ca ions in WH sample. This can be explained as Ca ions precipitated at the expense of P ions released from WH to form HAP crystals in a neutral pH condition (i.e., phase change from WH to HAP). Furthermore, highly elevated molar amount of P ions were detected with WH nanoparticles.

Next, we examined the interaction between the WH and bone-related extracellular matrix (ECM) proteins. Bone ECM proteins play a vital role in facilitating the bone formation by assembling with inorganic subunits [19,20]. Since the charged surface can strengthen the electrostatic interactions with proteins by changing the protonation state through a charge regulation mechanism [21,22], we evaluated the surface charge of WH and compared it with HAP in various pH conditions (from pH 2 to 9) by addition of HCl and NaOH. As shown in Fig. 2d, HAP reached its isoelectric point (IEP) at around pH 7, which corresponded well with the previously reported [23,24]. Notably, the IEP of WH was located between pH 2 and 3 and thus, the surface charge of WH was highly negative compared to HAP at a neutral pH. To examine whether the WH surface provides a favorable environment for protein binding, we measured the absorbed amount of bovine serum albumin (BSA) and type I collagen on WH nanoparticles (Fig. 2e, f and Fig. S3). Serum albumin was selected as a general protein because it does not include complex tryptophan, carbohydrates, and prostatic groups in its structure and thus is involved less in specific biochemical reactions [25,26]. In addition, type I collagen was chosen, as it is one of the major bone ECM components. The results showed that the adsorbed amount of BSA was higher on WH cylindrical scaffold than HAP throughout the period of 120 min. Furthermore, the absorbed amount of type I collagen was also higher in the WH than the HAP cylindrical scaffolds, which were fabricated by pressing in a mold and sintering at 700 °C (Fig. 2e and f).

For the *in vitro* cellular response comparisons of WH and HAP, we fabricated cylindrically shaped discs with same roughness by high-temperature pressand cellular morphology, cytotoxicity, and proliferation were performed (Fig. 3a and S4). In addition, we compared the mineral microenvironment-dependent osteogenic differentiation of human tonsil-derived mesenchymal stem cells (hTMSCs). hTMSCs (passage 6) cultured with osteogenic medium (OM) on the surface of WH and HAP subsequently showed osteogenic differentiation behavior. Gene expression analysis of bone-related markers showed that the WH microenvironment particularly activated genes involved in early bone formation. On day 14 of hTMSCs differentiation, quantitative real time-PCR analysis of bone-related genes, such as Alkaline phosphatase (*ALP*), osteocalcin (*OCN*), runt-related transcription factor 2 (*RunX2*), and type I