Investigating Helical Rosette Nanotubes and Nanocrystalline Hydroxyapatite as Novel Bone-like Biomaterials for Orthopedic Applications

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ABSTRACT

The objective of this study was to design a novel biocompatible and biomimetic composite based on the self-assembly of helical rosette nanotubes (HRNs) and nanocrystalline hydroxyapatite (HA) for improved orthopedic applications. Previous studies have shown that biologically-inspired HRNs can greatly enhance osteoblast (bone-forming cell) adhesion when coating titanium or embedding in hydrogels. In this study, HRNs were combined with hydrothermally treated nano HA as new nanocomposites. The nanocrystalline HA/HRNs have osteoconductive properties originating from nano HA, and also exhibit excellent biocompatibility by mimicking the nano HA/collagen structure. In addition, 2% nanocrystalline HA were well-dispersed in HRN hydrogels to serve as bone tissue engineering scaffolds with potentially improved mechanical and biocompatible properties. In short, the biomimetic HA/HRNs showed excellent cytocompatibility properties which makes them promising for various orthopedic applications (such as coatings on titanium, embedding into hydrogels as synthetic scaffolds, etc.)

Keywords: helical rosette nanotubes, nanocrystalline hydroxyapatie, hydrogels, biomimetic, bone.

1 INTRODUCTION

With the remarkably increasing number of patients who need various new and revised orthopedic implants, it is urgent to design better biomaterials to efficiently improve new bone growth and satisfy the high expectation of patients. Specifically, an important reason that necessitates orthopedic revision surgeries is insufficient osseointegration between the implant and bone. Although conventional metal implant materials (such as titanium) have excellent mechanical properties, sometimes they fail to promote sufficient osseointegration at the tissue-implant interface and even cause implant failures. One popular way to improve osseointegration is to design a biomimic implant material. Therefore, the objective of this in vitro study was to create a novel biocompatible and bone-like nanocomposite based on helical rosette nanotubes (HRNs) and nanocrystalline hydroxyapatite (HA) for various orthopedic implants.

HRNs are newly-developed self-assembled nanomaterials with DNA base pair building blocks (Figure 1) [1]. HRNs not only mimic the helical nanostructure of collagen in bone but they can also be functionalized to possess flexible surface chemistries by conjugating various amino acids (such as lysine, RGD, etc.). Previous studies have shown that HRNs with lysine side chains (HRN-K1) can greatly enhance osteoblast adhesion when coating on titanium [2-3] or embedding in biocompatible hydrogels (specifically, poly(2-hydroxyethyl methacrylate)) [4]. In addition, HRNs increase in viscosity when heating [3], which makes them suitable as injectable bone substitutes. Since HA is a main component in the bone and has been used in orthopedic applications, it will be combined with HRNs in order to increase cytocompatibility and mechanical properties. In the present study, nanocrystalline HA were synthesized by wet chemistry method and hydrothermal treatments [5-6]. Then the efficacy and potentials of nanocrystalline HA/HRNs were investigated as biomimetic coatings on titanium and as bone tissue engineering scaffolds with hydrogels.

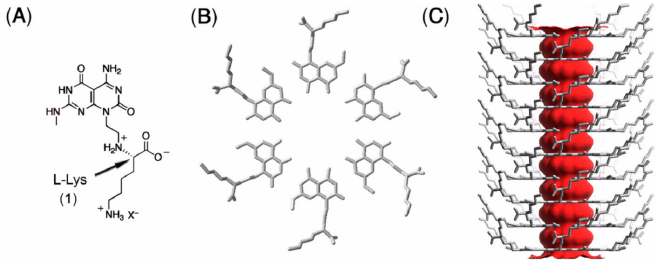

Figure 1. Helical rosette nanotubes self-assembling in water. (A) DNA base pair building blocks (Guanine^Cytosine), (B) a rosette assembled by six building blocks, and (C) a stacking helical rosette nanotube 3.5 nm in diameter.

2 MATERIALS AND METHODS

2.1 Preparation of HRNs and nano HA

HRN-K1 building blocks were synthesized by using a synthetic method described in [1]. The building blocks were dissolved in deionized water (dH_2O) to achieve 0.1, 0.01, and 0.001 mg/ml solution. Solutions were sterilized with a 0.22 µm syringe filter [3].

Nanocrystalline HA was synthesized (Equation 1) through

a conventional wet chemistry method [5-6]. Generally, 1M calcium nitrate (Sigma) and 0.6M ammonium phosphate (Sigma) solutions were prepared. Then ammonium hydroxide (Fisher) was used to keep pH value of the reaction solutions around 10. The ammonium phosphate was firstly mixed with 375 ml dH_2O and 1M calcium nitrate was slowly dripped into the above mixture. HA precipitates occurred immediately and continued to react for 10 min. Then the solution with HA was treated hydrothermally at 200°C for 20 h in order to grow good crystalline small grain size nano HA [7]. After 20 h, the nanocrystalline HA was centrifuged and washed sequentially with water and dried in the oven overnight. Lastly, the nanocrystalline HA and HRN-K1 were characterized by transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

$$10\ Ca(NO_3)_2 + 6\ (NH4)_2HPO_4 + 8\ NH_4OH = \mathbf{Ca_{10}(PO_4)_6(OH)_2} + 6\ H_2O + 20\ NH_4NO_3 \quad (1)$$

2.2 Nanocrystalline HA/HRNs coated on titanium and embedded in hydrogels

1×1cm^2 titanium was cleaned according to standard procedures [2] and served as model substrates for nano HA/HRN coatings. Before coating, nano HA was mixed with 70% ethanol and well dispersed by sonicating. Then, 5 ml of 0.001 mg/ml HRN-K1 and 250 mg HA homogeneous solutions were co-coated on the titanium by absorption for 45 min and dried overnight. In addition, 0.001 mg/ml HRN-K1 without HA was coated on titanium. Uncoated titanium served as controls.

2% (wt) nanocrystalline HA/HRNs were embedded in hydrogels as bone tissue engineering scaffolds. 2-hydroxyethylmethacrylate monomer (Polysciences) was mixed with nanocrystalline HA. A well dispersed nano HA in hydrogels were achieved by ultra-sonicating for 12 min. 0.01 mg/ml HRN-K1 and 2, 2'- azobisisobutyronitrile (free radical initiators) (Sigma) were added into the above mixture and heated at 78°C around 1 hour to polymerize [3]. Agglomerated 2% nano HA/HRNs hydrogels and hydrogels without any HA/HRNs served as controls. All of these samples were characterized under SEM.

2.3 Osteoblast adhesion on nanocrystalline HA/HRN coated titanium

0.001 mg/ml HRN-K1 with nano HA coated on titanium, 0.001 mg/ml HRN-K1 coated on titanium and uncoated conventional titanium were sterilized in a 70% ethanol for 15 minutes and were washed by PBS before cell culture. Human fetal osteoblasts (ATCC) at a density of 3500 cells/cm^2 were seeded on three substrates and were cultured in DMEM (Gibco) supplemented with 10% FBS (Hyclone) and 1% penicillin/ streptomycin (Hyclone) at 37°C with a humidified and 5% CO_2/95% air environment for 4 h. Then non-adherent cells were removed by PBS washing and adherent cells were fixed with 10% normal buffered formalin (Fisher), stained with DAPI (Invitrogen) and counted on five different areas of each substrate by a fluorescence microscope.

The cellular experiment was conducted in triplicate and repeated at three different times. Results were analyzed using Student t-test with statistical significance considered at $p < 0.05$.

3 RESULTS AND DISCUSSION

3.1 Characterization of nanocomposites

In the present study, morphology of nanocrystalline HA, HRN-K1 and their combination were characterized by TEM and SEM. Figure 2A showed nanocrystalline HA has regular rod shapes with nano sizes. Extremely long HRN network was imaged in Figure 2B. In addition, Figure 2C and 2D revealed that HRNs intimately interacted with nanocrystalline HA rods and formed a special nanostructured and biomimetic nanocomposite surface similar to bone.

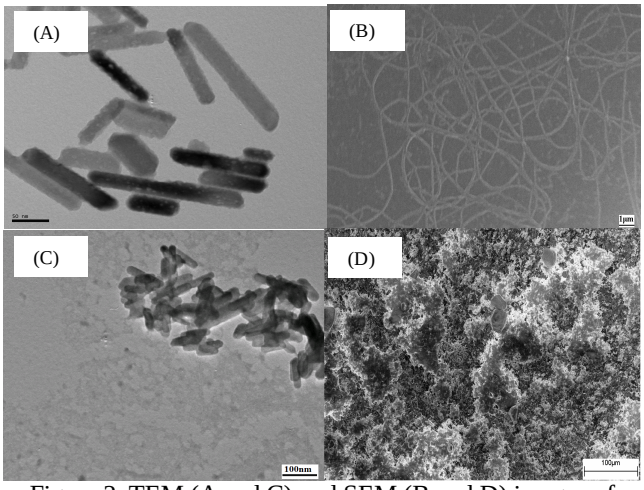

Figure 2. TEM (A and C) and SEM (B and D) images of nanocrystalline HA, HRNs, nanocrystalline HA/HRNs, and nanocrystalline HA/HRN coated titanium. (A) nanocrystalline HA; (B) 0.1 mg/ml HRN-K1 in water; (C) nanocrystalline HA with 0.001 mg/ml HRN networks; and (D) low magnification of biomimetic nanocrystalline HA/HRN (0.001 mg/ml) coatings on titanium. Scale bars are 50 nm in A, 1 μm in B, 100 nm in C and 10 μm in D.

Furthermore, it should be realized that nano HA has a tendency to agglomerate in nature, which can be observed in Figure 2C as well. But natural bone has well-organized structures and HA/collagen patterns (Figure 3). The agglomerated HA is easy to form in bulk not homogenous distributions on titanium or hydrogels. Thus, they may change the composite's mechanical properties which are important for orthopedic applications. In addition, the agglomerated HA will decrease the exposure of biologically-inspired HRNs, thus, reducing the positive effects of HRNs during osseointegration. Thus, a well-dispersed nano HA/HRN is needed.

In this study, 2% (wt) nanocrystalline HA were well-dispersed in HRN hydrogels after ultra-sonicating for 12

mins. Figure 4A showed the large bulk of agglomerated HA protruded in hydrogels without ultra-sonicating, whereas most areas of hydrogels lacked nano HA. However, a homogenous distribution of HA can be observed on the well-dispersed 2% nano HA/HRNs hydrogels (Figure 4B), which can serve as a bone-like bone tissue engineering scaffolds. Moreover, SEM images showed that a large amount of nanocrystalline HA filled into the inside pores of hydrogels after ultra-sonicating (Figure 4C), thus, potentially improving mechanical properties of the nanocomposites.

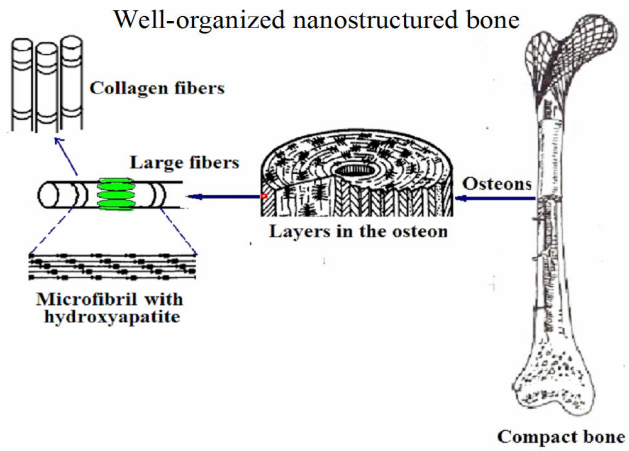

Figure 3. Schematic illustration of bone structures. Adapted and redrawn from [8-9].

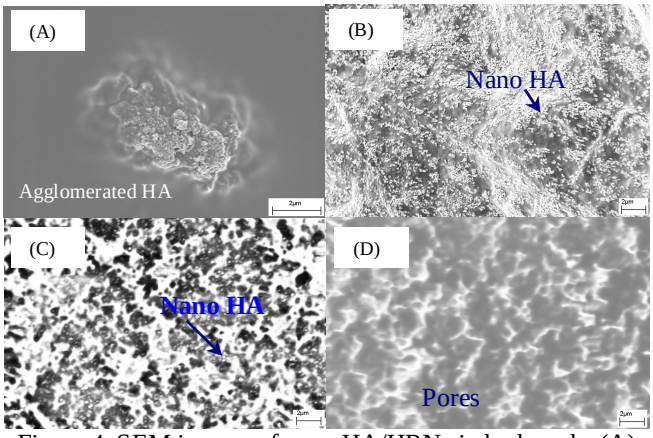

Figure 4. SEM images of nano HA/HRNs in hydrogels. (A) agglomerated 2% nano HA/HRNs hydrogels; (B) well dispersed 2% nano HA/HRNs hydrogels after ultra-sonicating 12 minutes; (C) cross section of 2% nano HA/HRNs hydrogels shows nano HA fill into the inside pores of hydrogels; and (D) cross section of hydrogel controls. Scale bars are 2 μm.

3.2 Osteoblast adhesion

Essentially, HRNs with nanocrystalline HA significantly promoted osteoblast adhesion when coated on titanium. Figure 5 showed that there were many more osteoblasts attached onto nano HA/HRN coated on titanium and HRN-K1 coated titanium compared to conventional titanium. There are no significant differences between nano HA/HRNs co-coated and HRN-K1 coated titanium. Thus, HRNs still work efficiently with nanocrystalline HA to improve osteoblast adhesion by providing a bone-like environment. Apparently, nanocrystalline HA/HRNs have excellent cytocompatibility properties which makes them suitable as biomimetic coatings on conventional implant materials. In addition, osteoblast functions on nanocrystalline HA/HRNs reinforced hydrogels will be presented for bone tissue engineering applications.

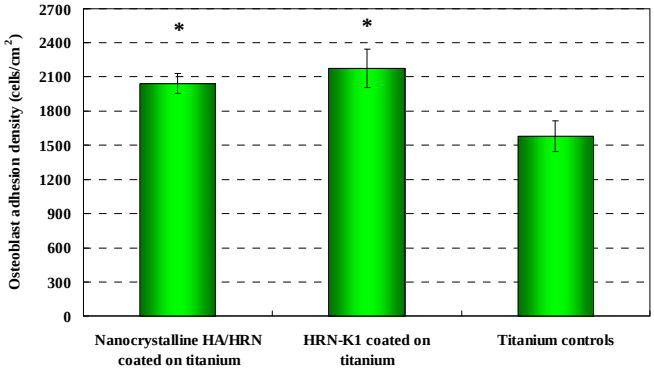

Figure 5. Better cell responses on nano HA/HRN as well as HRN coated on titanium compared with controls. Data are mean ±SEM, N=3. *p<0.05 compared to titanium controls.

4 CONCLUSIONS

In summary, the results of this study demonstrated that even at a low concentration, HRN-K1 with nano HA can enhance osteoblast adhesion on titanium. The nanocrystalline HA showed good crystallinity as well as nanostructure. HRNs with rich lysine side chains interacted closely with nano HA to form a bone-like network. Furthermore, a well-dispersed nano HA/HRNs hydrogels were prepared and solved agglomerated problems of nano HA particles, thus potentially promoted the performances of the nanocomposite for orthopedic implants. The mechanical properties of nanocrystalline HA/HRNs will be conducted in the future. Therefore, nanocrystalline HA/HRNs revealed a bone cell favorable environment and showed a wide application for orthopedic applications including coated on conventional implants as biomimetic coatings or incorporated into hydrogels as bone tissue engineering materials.

ACKNOWLEDGEMENTS

This work was supported by NIH Grant # 1R21AG027521 and the Hermann Foundation. We thank Yupeng Chen, Oluwaseun Adegbesan and the Brown University RET program for research assistance and thank Tony McCormick for assistance with SEM and TEM image collection.

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