Greater Osteoblast and Mesenchymal Stem Cell Adhesion and Proliferation on Titanium with Hydrothermally Treated Nanocrystalline Hydroxyapatite/Magnetically Treated Carbon Nanotubes

Mian Wang, Nathan J. Castro, Jian Li, Michael Keidar, and Lijie Grace Zhang

Department of Mechanical and Aerospace Engineering, GW Institute for Biomedical Engineering and GW Institute for Nanotechnology, The George Washington University, 801, Washington DC 20052, USA

With an increasingly active and aging population, a growing number of orthopedic procedures are performed annually. However, traditional orthopedic implants face many complications such as infection, implant loosening, and poor host tissue integration leading to implant failure. Metal implant materials such as titanium and its alloys are widely used in orthopedic applications mainly based on their excellent mechanical properties and biological inertness. Since human bone extracellular matrix is nanometer in dimension comprised of rich nanostructured hydroxyapatite particles and collagen nanofibers, it is highly desirable to design a biologically-inspired nanostructured coating which renders the biocompatible titanium surface into a biomimetic and bioactive interface, thus enhancing osteoblast adhesion and promoting osseointegration. For this purpose, a biomimetic nanostructured coating based on nanocrystalline hydroxyapatite and single wall carbon nanotubes was designed. Specifically, nano hydroxyapatites with good crystallinity and biomimetic dimensions were prepared via a wet chemistry method and hydrothermal treatment. Microcrystalline hydroxyapatite with larger grain sizes can be obtained without hydrothermal treatment. The carbon nanotubes with different diameter and length were synthesized via an arc plasma method in the presence or absence of a magnetic field. Transmission electron microscopy images illustrate the regular, rod-like nanocrystalline and biomimetic nanostructure of hydrothermally treated nano hydroxyapatite. In addition, the length of carbon nanotubes can be significantly increased under external magnetic fields when compared to nanotubes produced without a magnetic field. More importantly, the in vitro study demonstrated for the first time that osteoblast and mesenchymal stem cell adhesion and proliferation were greater on titanium with hydrothermally treated nanocrystalline hydroxyapatites/magnetically treated carbon nanotubes, which suggests the potential of these novel nanostructured materials for orthopedic applications.

Keywords: Nanocrystalline Hydroxyapatite, Carbon Nanotubes, Osteoblast, Stem Cell, Nanocomposite.

1. INTRODUCTION

Orthopedic procedures have strikingly increased with an increasing aging population in the United States. The American Academy of Orthopedic Surgeons reported that in just a 4 year period, there was an 83.72% increase in the number of hip replacements performed from nearly 258,000 procedures in 2000 to 474,000 procedures in 2004. The total hospitalization costs for knee replacements doubled to $11.38 billion in 2003 compared with $5.67 billion in 1999. Traditional orthopedic implant devices are mainly composed of various metals such as titanium (Ti) and its alloys. These materials are commonly used in orthopedic and dental prostheses owing to their desirable mechanical properties to include: relatively low modulus, good fatigue strength, formability, machinability, corrosion resistance, and suitable biocompatibility. However, it is important to note that current Ti-based implant materials are not perfect. Inadequate host tissue integration between the implant and juxtaposed bone (osseointegration) and long term in vivo functionality remain to be critical clinical obstacles for long-term implant success. Therefore, a significant paradigm shift in this field is towards the design of biologically-inspired coatings that can render...
inert Ti surfaces into biomimetic and bioactive interfaces to enhance bone cell adhesion and osseointegration.

As a form of calcium phosphate that bears close chemical resemblance with the mineral component of human bone, hydroxyapatite (HA) has promising osteoconductive properties since its surface can undergo selective chemical reactivity with surrounding tissues resulting in a tight bond between native bone and the implant surface.1,2 Thus, it has been considered as one of the most popular materials for orthopedic applications. Human bone is a nanocomposite material consisting of a protein-based soft hydrogel template (i.e., collagen, non-collagenous proteins and water) and hard inorganic components (nano crystalline hydroxyapatite, nHA, Ca_{10}(PO_{4})_{6}(OH)_{2}).3 Specifically, 70% of the bone matrix is composed of nHA typically of the order of 20–80 nm in length.4 In this study, biomimetic nHA was synthesized via a wet chemistry precipitation method with subsequent hydrothermal treatment. Hydrothermal treatment allows for high control of the crystalline phase and surface morphology of synthesized nHA at the nano scale. The hydrothermally treated nHA will be used as a bioactive nano coating on Ti to improve bone cell functions for enhanced osseointegration.

Moreover, besides nHA, human bone matrix is also composed of abundant collagen nanofibers which nHA can align with and assemble together into a robust nanocomposite matrix surrounding osteoblast (bone forming cells) and other cells (such as bone marrow mesenchymal stem cells) in the bone. For this purpose, single wall carbon nanotubes (SWCNTs) were investigated in this study. In fact, SWCNTs have been investigated as reinforcing materials for various bone applications due to their biomimetic nanostructure, high aspect ratio, excellent mechanical/electrical properties, and suitable biocompatibility.5 SWCNTs have the potential to strengthen and toughen nHA coating, thus, making them suitable for orthopedic applications. In this study, SWCNTs of varying diameter and length was synthesized via an arc plasma method in the presence (B-SWCNT) or absence (N-SWCNT) of a magnetic field.

More importantly, as a new approach to orthopedic implant design, this study will develop biomimetic nanocomposite coatings based on nHA and SWCNTs on Ti and investigate its effect on osteoblast and human bone marrow-derived mesenchymal stem cell (MSC) adhesion and proliferation. In order to illustrate grain size effects on bone cell functions, the current study also synthesized micron size hydroxyapatites (mHA) and evaluated osteoblast response on nHA/SWCNTs composites.

2. MATERIALS AND METHODS

2.1. Titanium Sample Preparation

Titanium (Ti, Alfa Aesar) samples of 1 cm² with a thickness of 0.05 cm were used in the evaluation of nHA/SWCNT nanostructured coatings. The samples were thoroughly cleaned by first soaking in acetone for 15 min, then sonicating for 15 min and rinsed three times with deionized water (dH₂O). They were then soaked in 70% ethanol, sonicated for 15 min and thoroughly rinsed with dH₂O. Lastly, they were soaked in dH₂O, sonicated for another 15 min and rinsed. Ti substrates were then put into a drying oven overnight and autoclaved for sterilization before coating with various SWCNTs, nHA and mHA.

2.2. Synthesis of Crystalline mHA and Hydrothermally Treated nHA

A well-established wet chemistry process was used to synthesize mHA and nHA.3 Briefly, 37.5 ml of a 0.6 M ammonium phosphate (Sigma Aldrich, St. Louis, MO) solution was added to 375 ml of dH₂O and adjusted to a pH of 10 with ammonium hydroxide (Fisher Scientific, Pittsburgh, PA). A 1 M calcium nitrate (Sigma Aldrich, St. Louis, MO) solution was slowly titrated into the above mixture while stirring. Precipitation of HA continued for 10 min at room temperature. The solution with HA precipitate was treated hydrothermally at 200 °C for 20 h in a 125 ml Teflon liner (Parr Instrument Company, Moline, IL). Hydrothermal treatment produced a small grain size of nHA at relatively low temperatures but under a higher pressure. After 20 h, the nHA was centrifuged and rinsed thoroughly with dH₂O three times, then dried at 80 °C for 12 h and crushed into a nHA powder.

Larger grain sizes of mHA can be obtained without hydrothermal treatment. Precipitation of HA was allowed to occur at room temperature for 24 h with stirring. The HA precipitate was obtained after washing, centrifugation, and drying similar to the method described above. HA powder was sintered at 1100 °C for 1 h to achieve a micracrystalline HA grain size.

2.3. Synthesis and Characterization of N-SWCNT and B-SWCNT

Magnetically (N-SWCNT) and non-magnetically (B-SWCNT) treated SWCNTs were both fabricated by arc discharge method.5 The synthesis apparatus consists of a cylindrical reaction chamber made from stainless steel of 254-mm length and 152-mm diameter containing the cathode-anode assembly installed within the chamber. The cathode is a solid graphite rod, and anode is a hollow graphite rod. A mixture of graphite powder and NiY catalyst powder was loaded into the anode while maintaining a total molar radio of C:Ni:Y of 94.8:4:2:1. After pumping down the chamber to vacuum, helium was introduced and the pressure was kept close to 500 Torr by an Omega CN-8502 controller. All experiments were conducted with a fixed arc current of ~75 A and discharge voltage of 30 V. In regard to the sample of B-SWCNT, a permanent magnet was placed inside the chamber at...
25-mm distance from the central axis of electrodes producing a 0.06-Tesla magnetic field within the electrode gap. Samples of N-SWCNT and B-SWCNT were sonicated for 60 min (Fisher Scientific 150T dismembrator) and morphologically examined via transmission electron microscopy (TEM, JEOL 1200 EX). SWCNTs were also characterized by a micro-Raman spectroscopy system (Horiba, HR) using a 50 mW He/Ne laser at a wavelength of 632.8 nm with a thermo-electric cooled CCD detector.

2.4. Various HA and SWCNT Coatings on Titanium

Magnetically and non-magnetically treated SWCNTs were examined in our study at a concentration of 0.5 mg/mL. Hydroxyapatite (nano and micro) solutions (10% w/v) were prepared via ultra-sonication (Ultrasonicator, QSonica, Newtown, CT). Sterile titanium was coated with the following combinations of SWCNTs and HA: (1) mHA; (2) nHA; (3) N-SWCNT; (4) B-SWCNT; (5) nHA+N-SWCNT; (6) mHA+B-SWCNT; (7) nHA+N-SWCNT; and (8) nHA+B-SWCNT for 1 hr at room temperature. All the samples were removed and left to dry aseptically overnight, and then sterilized under the UV light for 2 hrs in a biosafety cabinet. Uncoated Ti and sterile glass coverslips served as controls. Surface topography of the various coatings was observed via focused ion beam operating in SEM mode (Zeiss NVision 40 FIB, National Institute of Standards and Technology, Gaithersburg, MD).

2.5. Osteoblast and Mesenchymal Stem Cell (MSC) Culture

A human fetal osteoblast cell line (ATCC, CRL-11372, Manassas, VA, passage #5–8) was cultured in Dulbecco’s modified eagle’s medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT) and 1% penicillin/streptomycin (P/S, Hyclone, Logan, UT) under standard cell culture conditions (37°C, a humidified, 5% CO2/95% air environment).

Primary human bone marrow MSCs were derived from healthy consenting donors from the Texas A and M Health Science Center, Institute for Regenerative Medicine and thoroughly characterized.6 It will be used to evaluate the cytocompatibility properties of the nanocomposite coatings. MSCs (passage #3-6) was cultured in a complete media comprised of Alpha Minimum Essential medium (α-MEM, Gibco, Grand Island, NY) supplemented with 16.5% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 1% (v/v) L-Glutamine (Invitrogen, Carlsbad, CA), and 1% penicillin/streptomycin solution (Invitrogen, Carlsbad, CA) and cultured under the same conditions as previously described.

2.6. Osteoblast Adhesion Study

Osteoblasts were seeded at a cell density of 3500 cells/cm² on various nHA, nHA and B-SWCNT and N-SWCNT nanocoatings and cultured under standard cell culture conditions (37 °C, 5% CO₂) for 4 hrs. Then, the substrates were then washed three times with phosphate-buffered saline (PBS) to remove non-adherent cells. The remaining cells were then washed three times with phosphate-buffered formaldehyde solution and cell nuclei were stained with DAPI (Invitrogen, Carlsbad, CA) and five different fields were counted for each substrate by a fluorescence microscopy (Nikon Eclipse TE300, Linthicum, MD).

2.7. Osteoblast and MSC Proliferation Studies

For osteoblast proliferation, the experimental groups include:

(1) Ti controls;
(2) nHA coated Ti;
(3) B-SWCNT coated Ti;
(4) nHA+N-SWCNT coated Ti;
(5) nHA+B-SWCNT coated Ti and
(6) Glass references.

For MSC proliferation study, the experimental groups include:

(1) Ti controls;
(2) nHA coated Ti;
(3) B-SWCNT coated Ti;
(4) nHA+B-SWCNT coated Ti and
(5) Glass references.

All experiments were run in triplicate and repeated three times for each substrate. Data are presented as the mean value ± standard error of the mean (SEM) and were analyzed with student’s t-test for pair-wise comparison. Statistical significance was considered at p < 0.05.

3. RESULTS

3.1. Characterization of Hydrothermally Synthesized nHA and Magnetically/Non-Magnetically Treated SWCNT

TEM and SEM were employed to investigate the structures of the two different SWCNTs and hydrothermally synthesized nHA. Magnetically and non-magnetically treated SWCNT morphologies are shown in Figure 1. These images revealed that the CNT can form a dense nanostructured network. Figures 1(A) and (B) compare the different morphologies of N-SWCNT and B-SWCNT with a
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**Fig. 1.** TEM images of (A) single-walled carbon nanotubes without magnetic field (N-SWCNT); (B) single-walled carbon nanotubes with magnetic field of 0.06 Tesla (B-SWCNT); (C) graphene flakes with magnetic field of 0.06 (B-SWCNT); and (D) hydrothermally treated nHA.

Non-uniformed 0.06 T magnetic field. B-SWCNTs appear to be more closely-packed into bundles which can be attributed to Van der Waals interaction between individual SWCNT resulting in bundle diameters ranging from 2 to 20 nm. Single-wall carbon nanotubes synthesized in the absence of a magnetic field (N-SWCNT) produce a sample exhibiting a larger diameter of bundles and larger individual tube diameters which has been validated via Raman spectroscopy (Fig. 2). In addition to producing carbon nanotubes with decreased diameter size, B-SWCNT synthesis produced trace amounts of graphene sheets (Fig. 1(C)) which may increase the electrical conductivity of B-SWCNT composite materials. Previous studies demonstrated the effects of magnetically-enhanced arc discharge with respect to decreased diameter distribution of metallic catalyst particles and carbon nanotubes with increased SWCNT length, as well as alterations to the ratio of metallic and semiconducting carbon nanotubes. The hexagonal electron diffraction dot pattern (Inset of Fig. 1(C)) illustrates evidence of the well-ordered crystal structure of graphene. Transmission electron microscopy analysis of hydrothermally treated nHA (Fig. 1(D)) illustrate the regular rod-like nanocrystalline (as confirmed by X-ray diffraction, not shown here) morphology comprised of grain sizes within the range of 50–100 nm in length and 20–30 nm in width validating the biomimetic nanostucture of nHA.

Raman spectroscopy (Fig. 2) was taken from 100 to 500 cm⁻¹ were measured on the surface of synthesized deposition. The radial breathing mode (RBM) between 120 and 350 cm⁻¹ can be used to indirectly measure the nanotube diameter through the coherent vibration frequency (ω_{RBM}) of the carbon atoms in the radial direction. The experimental relation between the frequency and SWCNT diameter is determined by the following equation:

\[
\omega_{RBM} = \frac{A}{d} + B
\]

where \(A\) and \(B\) were measured to be 234 and 10 cm⁻¹ respectively, for the typical SWCNT formed in bundles. The RBM frequency of 163.8 cm⁻¹ for N-SWCNT (Fig. 2) was measured. Raman spectra of B-SWCNT (Fig. 2) displayed two major RBM peaks at 190.5 and 215.2 cm⁻¹, respectively. Therefore, the averaged individual diameter

**Fig. 2.** Raman spectra of SWCNT sample synthesized without magnetic field (blue) and composite sample containing SWCNT and GF synthesized with non-uniform magnetic field (red).
3.2. Surface Morphology of nHA/SWCNT Coatings

SEM images of surface topography alterations from the various surface treatments can be seen in Figures 3(A)–(H). Untreated, pure Ti substrates exhibit a relatively smooth surface (Fig. 3(A)) as compared to all treatment conditions. Uniform biomimetic nano-texturization of Ti substrates coated with crystalline nHA, SWCNT, and the respective mixtures (Figs. 3(B, C, E, G) is clearly evident. Relatively uniform crystalline mHA surface coating can be seen in Figures 3(D), (F), and (H), with large aggregate formation. SEM images of SWCNTs (Fig. 3(C)) illustrate the nanotubular networks of coated SWCNTs.

3.3. Osteoblast Responses to nHA/SWCNT Nanocoatings

3.3.1. Osteoblast Adhesion

All nanoscale surface modifications of the Ti substrate significantly enhanced osteoblast adhesion as compared to uncoated Ti after 4 hr incubation (p < 0.05) (Fig. 4), illustrating the excellent cytocompatibility properties of both SWCNTs and nHA as promising orthopedic implant nanocoating materials. Specifically, our results demonstrated that biomimetic nHA-coated Ti can greatly increase osteoblast attachment when compared to mHA which exhibit slightly less uniform surface coverage, larger individual grain size, and aggregate formation, which is consistent with previous work. In addition, the results illustrate that both B-SWCNT and N-SWCNT coated titanium had excellent cytocompatibility properties exhibiting increased osteoblast adhesion on SWCNT-coated Ti. Furthermore, B-SWCNT-coated Ti substrates produced better osteoblast adhesion when compared to N-SWCNT-coated Ti which may be attributed to the smaller tube diameter and increased aspect ratio of B-SWCNT's leading to a more biomimetic nanoscale architecture. This trend was further enhanced with the combination of nHA with SWCNTs yielding a more biomimetic bone-like environment composed of materials exhibiting dimensions and composition similar to those of native collagen and hydroxyapatite. Nanometer scale surface textures have been shown to elicit specific cell behavior. The current work provides more evidence that the size of these coating materials may play a critical role in improving osteoblast adhesion on Ti implants.

3.3.2. Osteoblast Cell Proliferation

Based on the results of the above adhesion study, nHA and SWCNTs were employed as the optimal constituents to examine the effects of nano-scale surface treatments for osteoblast proliferation studies. The results of 1, 3, and 5 day osteoblast proliferation study (Fig. 5) exhibit similar trends with regards to the synergic effects of nHA/SWCNT coatings on improving bone cell functions. For example, the nHA combined with B-SWCNTs or N-SWCNTs can achieve the highest osteoblast proliferation density after 1, 3, and 5 days when compared to all of other nanocoatings and controls. Figure 6 illustrates osteoblast proliferations on various substrates examined in this study. Fluorescence microscopy images of B-SWCNT-coated Ti substrates (Figs. 6(E1–E5)) illustrate the favorable surface topography for cell spreading where extended cellular processes and cytoplasm can be seen by the transient fluorescence signal.
3.4. MSC Responses to nHA/SWCNT Nanocoatings

A human bone marrow-derived MSC line was also investigated and characterized for cell proliferation under the same Ti-coating conditions (Figures 7 and 8). Based on the results obtained from the aforementioned experiments, MSC proliferation was characterized in response to two biomimetic nanomaterials: nHA and B-SWCNTs. Figure 7 demonstrated that after one day proliferation, all of the nanocoatings (nHA, B-SWCNT and nHA+B-SWCNTs) can form a MSC favorable surface which attracted more MSC growth than uncoated Ti controls. More importantly, the result that the highest density of MSCs occurred on the nHA/B-SWCNT nanocomposite coating with significant increases at all three time points (1, 3, and 5 days). Fluorescence microscopy images (Figs. 8, 9) show MSC proliferation density changes on different substrates.

Fig. 4. Significantly enhanced osteoblast adhesion on nHA and B-SWCNTs coated on Ti. Data are mean ± SEM; n = 9. *p < 0.05 when compared to all other substrates; **p < 0.05 when compared to uncoated Ti control, nHA or mHA coated Ti, ***p < 0.05 when compared to uncoated Ti controls and mHA coated Ti.

Fig. 5. Significantly improved bone cell proliferation on the biomimetic nanocoatings after 3 and 5 day proliferation. Data are mean ± SEM; n = 9. *p < 0.01, **p < 0.01 and ***p < 0.05 when compared to all other substrates at respective days; "p < 0.05 when compared to uncoated Ti and nHA coated Ti at 5 days; ""p < 0.05, """"p < 0.05 and """"""p < 0.05 when compared to Ti controls at respective days.
Fig. 6. Fluorescence microscopy images of DAPI stained human fetal osteoblast proliferation after 1, 3, and 5 days: (A) Glass references; (B) Ti controls; (C) nHA; (D) B-SWCNT; (E) nHA + N-SWCNT; (F) nHA + B-SWCNT.
Fig. 7. Enhanced bone marrow-derived human MSC proliferation on the hydrothermally treated nHA and magnetically prepared carbon nanotubes. Data are mean ± SEM; n = 9. *p < 0.05 when compared to all other substrates at respective days (1, 3 and 5 days); & p < 0.05 when compared to Ti controls at 5 day. **p < 0.05 when compared to uncoated Ti and nHA coated Ti at respective1 and 3 days; and ***p < 0.05 when compared to Ti controls at 1 day.

4. DISCUSSION

In vivo bone remodeling is a complicated process involving various types of cells activities. Different cell types such as osteoblast, osteoclast and bone marrow mesenchymal stem cells (which can be differentiated into bone cells) can be activated for bone regeneration. For orthopedic applications, sufficient bone remodeling at the implant-tissue interface is very important for mediating the long-term success of implants. Insufficient bone remodeling around implant materials or excessive remodeling coupled with massive bone resorption and osteolysis will lead to implant failure. Since the surface properties of implant materials intimately affect the initial protein adsorption from biological fluids and further selective recruitment as well as activation of favorable cell (such as osteoblast) functions, surface modifications (such as altering the implant surface topography, chemistry and wettablity) have become popular methods. The current work extended this understanding by illustrating the effects of a novel biomimetic synthetic Ti nanocoating material as a potential tool for better osseointegration resulting in increased efficiency of traditional orthopedic implants. It is well established that nano-scale surface morphologies alter cell behavior. The current work extended this understanding by illustrating the effects of more biomimetic synthetic structures on cell adhesion and proliferation. Comparative studies using B-SWCNTs and N-SWCNTs emphasize that smaller tube diameters and increased aspect ratios enhanced osteoblast adhesion and proliferation. In addition, our study also showed that hydrothermally treated nHA performed much better to improve bone cell functions than mHA, which is consistent with previous work investigating hydroxyapatite grain size. Impressively, our results show that 10% nHA + B-SWCNT promoted the greatest osteoblast and stem cell densities on Ti. Results also exhibit a significantly higher osteoblast cell density on Ti substrates coated with nHA in the presence of SWCNTs uncoated Ti and nHA-coated Ti, but it is still lower than nHA with SWCNs nanocoatings. These results demonstrate that surface coatings comprised of nHA and SWCNTs together may produce a synergic effect which greatly promotes osteoblast and MSC functions thus rendering it a promising improvement in developing more efficient Ti implants.
In addition to exhibiting a more biomimetic composition, alterations to the surface properties (roughness and surface chemistry) were noted through the coating method. Although B-SWCNT only can enhance more bone cell adhesion than N-SWCNTs, no significant difference was observed between nHA+B-SWCNT and nHA+N-SWCNT coatings. This may be due, in part, to dilution of the SWCNT suspension by the nHA solution upon coating leading to lessened direct nanotube exposure to seeded cells in these coatings. However, B-SWCNTs with
materials for promoting osteoblast and MSC adhesion owing to their excellent cytocompatibility and biomimetic nanoscale dimensions. Specifically, nHA or B-SWCNT coated on Ti can significantly improve osteoblast adhesion than larger constituent materials. The combination of 10% nHA and B-SWCNT coated on Ti achieved the greatest osteoblast and MSC growth densities, thus meriting further exploration.

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References and Notes


5. CONCLUSIONS

In summary, the potential of different nHA/mHA and B-SWCNT/N-SWCNT coated on the surface of Ti as novel coating materials for orthopedic applications was investigated here. We have shown the osseointegrative potential of hydrothermally synthesized nHA and magnetically treated B-SWCNT as novel biomimetic coating materials for metallic orthopedic implants. This current work demonstrates that nanocomposite coatings are promising

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