Plasma-sprayed carbon nanotube reinforced hydroxyapatite coatings and their interaction with human osteoblasts in vitro

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Abstract

Carbon nanotubes (CNT) possess excellent mechanical properties to play the role as reinforcement for imparting strength and toughness to brittle hydroxyapatite (HA) bioceramic coating. However, lack of processing technique to uniformly distribute multiwalled CNTs in HA coating and limited studies and sparse knowledge evincing toxicity of CNTs has kept researchers in dispute for long. In the current work, we have addressed these issues by (i) successfully distributing multiwalled CNT reinforcement in HA coating using plasma spraying to improve the fracture toughness (by 56\%) and enhance crystallinity (by 27\%), and (ii) culturing human osteoblast hFOB 1.19 cells onto CNT reinforced HA coating to elicit its biocompatibility with living cells. Unrestricted growth of human osteoblast hFOB 1.19 cells has been observed near CNT regions claiming assistance by CNT surfaces to promote cell growth and proliferation.

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1. Introduction

Hydroxyapatite (HA), \(\text{Ca}_{10}(\text{PO}_4)_{6}(\text{OH})_{2}\), is an attractive biomaterial owing to its close chemical resemblance (Ca/P = 1.67) with bone and teeth \cite{1-3}. Osteoblasts proliferate onto HA owing to its bioactivity and biocompatibility \cite{3,4} and therefore HA coatings have long been applied to dental implants, bone repair scaffolds, skeletal implants, and body/bioinsert material \cite{5}. Microstructure, crystallinity, and phase composition of HA coating is critical in deciding its cell response and mechanical performance. Plasma sprayed HA coating often result in the generation of secondary phases such as tricalcium phosphate (TCP), tetracalcium phosphate (TTCP), calcium oxide (CaO), and amorphous calcium phosphates (ACPs) \cite{6}. Though HA is very stable in the body environment, presence of secondary phases causes dissolution leading to degradation of the implant in vivo. Hence, higher crystallinity content is required for the increased implant life. In addition, researchers have used carbon nanotubes (CNT), Ti-alloys, yttria stabilized zirconia (YSZ), Ni\textsubscript{3}Al, and alumina (Al\textsubscript{2}O\textsubscript{3}) reinforcements to HA coating \cite{7-9} for improving its fracture toughness and wear resistance \cite{1,5,10-13}.

Chen et al. investigated mechanical properties of laser processed HA-multiwalled CNT coating showing strong improvement in the fracture toughness and marginal improvement in the elastic modulus \cite{1,14}. However, laser synthesized coating result in the formation of undesired TiC phase \cite{1}. The purpose of addition of CNT was to enhance the mechanical performance of the coating without deteriorating the biocompatible properties of HA \cite{15-17}. On the contrary, CNTs have also been debated as toxic \cite{18} under organic environment \cite{1,19}. Though in a recent study, osteosarcoma cell growth has been observed on functionalized CNT \cite{20}, researchers have also depicted micro patterning of CNTs to result directed growth of osteoblasts \cite{21}, or surface modification with DNA- and HA-nanostructured films for enhanced detection and biosensitivity \cite{22}. Haddon et al. has concluded that CNT...
surface also acts as crystallization site and scaffolding substrate for the growth of needle like nano-apatite crystals \cite{19,20,23}. Hence, the debate on the use of CNT for biomedical applications is still ongoing among researchers. In the current work, we have synthesized HA–4 wt% multiwalled CNT coating via plasma spraying onto Ti-6Al-4V (orthopedic implant material) and performed human-osteoblast biocompatibility studies.

Plasma spraying is an existing commercially viable technique and has been used by researchers to economically coat HA for the real life implants \cite{5,24}. Though new materials processing and coating techniques are emerging \cite{3,12,14,25-28}, their applicability is limited due to generation of non-biocompatible secondary phases \cite{1}, weight loss and residue \cite{26}, degradation, non-homogeneity, amorphous and more carbonated HA coating \cite{3} and very limited functional scalability \cite{29}. In the present work, our research group has improvised the conventional plasma spray technique in a novel manner for multiwalled CNT addition and dispersion to HA coating, aiding bone growth to act as scaffolding and nucleation for apatite crystals.

2. Materials and methods

2.1. Processing

Irregular HA powder (particle size 10–50 μm), Fig. 1a, was blended with 4 wt% multiwalled CNTs (95% + pure, OD = 40–70 nm, \( l = 0.5–2 \) μm) in a jar mill for 18 hours to result in powder feedstock. CNTs are well dispersed and spread over HA particles as seen in the SEM image of the blended powder feedstock, Fig. 1b. From now on, multiwalled CNTs are referred to as CNTs in the entire manuscript. Plasma spraying of HA with and without CNTs was carried out using Praxair SG-100 gun with optimized spray parameters presented in Table 1. Powders were internally injected with Ar as the carrier gas to result coatings onto Ti-6Al-4V bioimplant substrate (50 mm × 50 mm × 2 mm).

2.2. Microstructural and mechanical characterization

X-ray diffraction spectrum was obtained using Siemens D-500 operating at 40 kV and 20 mA with CuK\( \lambda \) peak of 1.54 Å along with unsuppressed CuK\( \beta \) peaks. Field Emission FESEM JEOL JSM 6330 F was used for microstructural imaging and Shanghai Taiming Optical Instruments HXD-100 TMC Lenovo Vickers microhardness tester was used for estimating hardness and fracture toughness of the plasma sprayed HA coatings. Indentation toughness of HA coatings was calculated for load of 100 g for dwell time of 15 s using the following formula \cite{30} (assuming the Young’s modulus of HA to be 100 GPa): \( K_{IC} = 0.016(E/H)^{0.5} Pa^{-1.5} \), where \( E \) is the Young’s modulus, \( H \) is the hardness, \( P \) is the applied load and \( a \) is the crack length from the center of indent.

2.3. Cell culture

Human osteoblasts (ATCC, CRL-11372) were cultured in Dulbecco’s Modified Eagle Media (DMEM) without phenol red (Gibco, 31053-028) supplemented with 10% FBS (Hyclone, SH30071) and 1% penicillin/streptomycin/glutamine (PGS) (Gibco, 15140-122). The fibroblasts were seeded at a density of ~300 cells/cm\(^2\) onto coated HA samples (with and without CNT) and were grown under standard culture conditions (37 °C and 5% CO\(_2\)–95% air) for 3 days. Prior to seeding, the coated samples were UV sterilized for 30 min on each side. The substrates were then prepared for SEM analysis. The substrates were washed with PBS (Gibco, 14040-133) to remove any residual serum and were then incubated in a 25% formalin solution (1 part formalin/3 parts PBS) (Thermo—Shandon, 9990244) for 30 min. The samples were washed three times with fresh PBS. The samples were incubated again in a 25% formalin/PBS solution for 30 min followed by washing three times with fresh PBS and placing in a lyophilizer to dry for approximately 24 hours.

3. Results

HA coating with and without 4 wt% CNT has been plasma sprayed onto real-life bioimplant Ti-6Al-4V material. Similar plasma-spray processing conditions with optimized parameters were used for fabrication of both the coatings for comparison purpose. This representation depicts the ease of HA–CNT coating fabrication for applications with scalability to useful size. Plasma sprayed

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Optimized plasma spray parameters</th>
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<tbody>
<tr>
<td>Gun current</td>
<td>500–700 A</td>
</tr>
<tr>
<td>Power</td>
<td>20–25 kW</td>
</tr>
<tr>
<td>Primary gas</td>
<td>Argon (0.20–0.25 N/m(^2))</td>
</tr>
<tr>
<td>Secondary gas</td>
<td>Helium (0.50–0.55 N/m(^2))</td>
</tr>
<tr>
<td>Standoff distance</td>
<td>0.08–0.12 m</td>
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Fig. 1. SEM image of (a) as-received HA powder with inset showing corresponding EDS, and (b) blended HA–CNT powder feedstock showing CNT distribution over HA particles.
HA with CNT appears more grayish due to the presence of CNTs.

3.1. Microstructural features of plasma sprayed HA and HA–CNT coatings

Plasma sprayed coatings, Fig. 2a (HA without CNT) and Fig. 2b (HA with CNT), depict typical lamellar structure with sound interface showing no delamination from the Ti-6Al-4V substrate. Coatings are uniformly thick and appear microstructurally homogeneous at lower magnifications. Thickness of the HA coating is observed as ~150 μm (Fig. 2a) whereas thickness of HA–CNT coating is ~110 μm (Fig. 2b). Both coatings are similar in nature exhibiting transverse cracks and distributed micron-size porosity. Image processing analysis evaluated density of more than 92% for plasma sprayed coatings.

Microstructure of top surface of the plasma sprayed HA coatings without CNTs (Fig. 3a) and with CNTs (Fig. 3b) shows various contrasting microstructural features. Increased surface microcracks are typical in the plasma sprayed HA coatings owing to brittle nature of HA coatings undergoing thermal contraction upon cooling [5]. Top surface of as-sprayed HA coating, Fig. 3a, elicits layered structure. Various inter-splat cracks, flat appearing melt surface, and partially fused particles are also observed in the microstructure of plasma sprayed HA without CNTs, Fig. 3a. On the other hand, plasma sprayed HA with CNTs, Fig. 3b, demonstrates presence of melt-resolidified fine and nodular HA particles. HA–CNT coating surface appears rough owing to presence of nodular surface particles. It must be mentioned that surface roughness is critical in allowing the growth of cells because surfaces influence the protein interaction leading to subsequent cell adhesion [31]. Increased surface area implies higher number of atoms and higher surface defects at the delocalized surfaces open for cell adhesion. Melt surface is more wavy when compared to that of plasma sprayed HA without CNTs. In addition, the splats are more spread out and flooded with uniformly embedded spherical HA particles indicating higher surface undulations in the HA–CNT coating. Retention and distribution of CNTs after plasma spraying and the effect of CNTs towards increasing crystallinity, enhancing fracture toughness and assisting cell-growth is illustrated in later section.

3.2. Effect of CNTs on the crystallinity of as-sprayed coatings

X-ray diffraction (XRD) spectrum of the plasma sprayed coatings, Fig. 4, shows lower degree of crystallinity of plasma sprayed HA coating without CNTs as observed by peak broadening between the 2-theta values of 25–35°. Absence of CaO, TiC, or other oxides (such as V₂O₅) peaks...
affirm the non-reactivity of substrate towards degrading the HA coating during plasma spraying. HA coating without CNTs depicted crystallinity of 53.7% whereas HA–CNT coating has a crystallinity of 80.4% as calculated from the differential HA peak degradation.

Generation of secondary phases such as calcium phosphates, CaOs and ACPs [6] impair the mechanical properties and deteriorate the biocompatibility of HA [32]. Reactivity of an implant material decides the resorption and time of bonding with bone, which is believed to be induced by the presence of TCP and HA via formation of apatite layer [33]. Hence achieving higher degree of crystallinity has always been one of the foremost concerns in attaining lower dissolution of scaffold structures.

3.3. CNT distribution and subsequent fracture toughness evaluation

Plasma sprayed HA coating without CNT is presented in Fig. 5a for comparison purpose. Coated top surface, Fig. 5b of plasma sprayed HA–CNT nanocomposite coating display retention and uniform distribution of CNTs, indicating that CNTs have survived the harsh temperatures and high velocity impact observed during plasma spraying. CNTs are uniformly distributed in the coating without agglomeration. Retention and distribution of CNTs is critical since CNTs provide enhanced strength and fracture toughness [33] reinforcing the poor mechanical properties of HA [2]. Vicker’s indentation toughness improvement of upto 56% is observed for HA–CNT coating (0.61 ± 0.09 MPa m^1/2 when compared to 0.39 ± 0.09 MPa m^1/2 for the plasma sprayed HA coating). T-test statistical analysis confirmed significant difference between the fracture toughness mean values of coatings associated with greater than 95% confidence level.

3.4. Cell growth

Cell culture studies with human osteoblasts hFOB 1.19 cells were performed onto HA–4 wt% CNT plasma sprayed coating. Differential cell growth (called osteocytes) is unrestricted and seen embedded in the HA matrix, Fig. 6a, in the presence of CNTs. Good spreading of the hFOB 1.19 osteoblast cells, Fig. 6a, and unrestricted growth along CNT-abutting surfaces, Fig. 6b, endorse the non-toxicity of the CNTs in the body environment. Fig. 6a shows nodular cells extending thread-like long cytoplasmic prolongations [20] when cultured onto HA–CNT biocomposite coating, Fig. 6b. Human osteoblast hFOB 1.19 progenitor cells have differentiated to mature cells when coming in contact with neighbor cells. This study on the in vitro behavior of non-functionalized CNT reinforced HA coating with human osteoblasts provides a detailed insight of enhancing the performance of implant coatings.

4. Discussion

Transverse cracking observed in the coatings (Fig. 2) results because of differential thermal quenching between splats and deposited coating/substrate [2]. Thermal stresses usually build up in the plasma sprayed coatings owing to extreme cooling rates (in the order of 10^5–10^8 K/s) observed during processing. Quenching stresses developed during cooling therefore induces strain mismatches in the successively depositing splats sometimes leading to cracking in the coatings [2].

Extremely high cooling rates habitually lead to generation of non-equilibrium phases, as observed in the
cross-sectional image of HA coating without CNT, Fig. 7a. Spot analysis via energy dispersive spectroscopy (EDS), Fig. 7b, showed needle-like regions to be rich in phosphate corresponding to non-equilibrium secondary phase generation during plasma spraying. Presence of phosphate rich needle phases imply transformation of HA to secondary calcium phosphate phases ensuring reduced crystallinity. In addition, amorphous phosphate regions also generate as a result of non-thermodynamic cooling inherent to plasma spraying lowering the initial crystallinity content. Unfocused spot size in the EDS analysis also depicted Ti peak arising from the Ti substrate. Since the plasma spraying parameters for the both coatings are same, addition of CNTs is aiding the nucleation cites for the crystallization of HA preserving its inherent crystal structure.

CNTs are affecting the crystallinity of coating in a peculiar way since crystallinity of 53.7% obtained for plasma sprayed HA coating without CNTs, increased to 80.4% for the plasma sprayed HA–CNT coating. This in turn, suggests that CNTs in some way assist the nucleation/precipitation of HA. It is hypothesized that three orders of higher thermal conductivity magnitude of CNTs ($\approx 10^3$ W/m K compared to $\approx 0.7 \times 10^3$ W/m K for HA) induce higher heating of CNTs when compared to that of HA during plasma spraying. Owing to poor thermal conductivity of HA, the cooling down allows isolation of thermal energy near the CNT surfaces surrounded by HA melt. Hence enhanced time is available at such regions for HA nucleation when compared to that in the absence of CNTs. Annealing thermal experience, therefore, initiates recrystallization transformation, especially over CNT surface, increasing HA content with increased time [33].

Powder feedstock treatment and controlled plasma parameters have led to dispersion of undamaged CNTs in the HA coating. Fractured surface of HA–4 wt% CNT coating shows looped CNTs bridging the molten splats, Fig. 8, endorsing the CNT fortification holding the splats together. Fracture toughness improvement of 56% is obtained for HA–4 wt% CNT nanocomposite coating. Such remarkable improvement in the toughness of HA–CNT coatings is attributed to CNT distribution and anchoring of CNTs to form bridge structures. CNTs have high bending stiffness and can take high bending deformations making it exceptional toughening addition [34]. Moreover, CNTs possess high tensile modulus ($\approx 1$ TPa) behaving as rigid reinforcement with excellent elastic recovery properties [35].

As confirmed by high magnification SEM image, Fig. 9, mineralization of apatite is strongly observed over the CNT surface. Barbed wire apatite mineralization has also been observed by other researchers onto discrete CNT surface [23]. Cell-culture studies demonstrated growth of human osteoblast cell hFOB 1.19 alongside of CNTs. It must be mentioned that enhanced apatite precipitation
Plasma sprayed HA–4 wt% CNT coating have demonstrated uniform distribution of undamaged CNTs, improvement in fracture toughness by 56%, and increase in crystallinity from 53.7% to 80.4%. Thus, plasma spraying is a definitive tool for synthesizing HA–CNT coatings onto Ti-6AI-4V implants and bringing benefits of nanotechnology out of laboratory for real improvements in human health life. CNTs have proved to nurture precipitation and mineralization of HA over its surface. Unrestricted human osteoblast hFOB 1.19 cell growth and proliferation during cell culture studies demonstrate non-toxicity of HA–CNT coating. Plasma sprayed HA–CNT nanocomposite bio-coating opens the door for nanotechnology frontiers to tap its properties and extend the limited life of body inserts and implants.

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5. Conclusions

Plasma sprayed HA–4 wt% CNT coating allows growth and differentiation of nodular human osteoblasts. Hence CNTs are presented as agents for apatite re-precipitation, which will serve as reinforcing scaffold because of tissue ingrowth. Novelty in adding CNTs via plasma spraying demonstrated three-fold improvement by: (i) enhancing the fracture toughness of the nanocomposite coating by 56%, (ii) aiding human osteoblast cell growth by its uniform dispersion, and (ii) allowing precipitation and mineralization of apatite onto CNT surface.

This interlinked interdisciplinary work presents a classic example of synergy between materials science and engineering, surface engineering, nanotechnology and biotechnology for practical applications.

References