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Enhanced Proliferation and Growth of Human Stem Cells on the Surface of HVOF-sprayed Nano TiO₂-HA Coatings

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Abstract

Biomedical thermal spray coatings produced via high velocity oxy-fuel (HVOF) from nanostructured titania (n-TiO₂) and 10wt% hydroxyapatite (HA) (n-TiO₂-10wt%HA) powders have been engineered as possible future alternatives to HA coatings deposited via air plasma spray (APS). This approach was chosen due to (i) the stability of TiO₂ in the human body (i.e., no dissolution) and (ii) bond strength values on Ti-6Al-4V substrates more than two times higher than those of APS HA coatings. To explore the bioperformance of these novel materials and coatings, human mesenchymal stem cells (hMSCs) were cultured from 1 to 21 days on the surface of HVOF-sprayed n-TiO₂ and n-TiO₂-10wt%HA coatings. APS HA coatings and uncoated Ti-6Al-4V substrates were employed as controls. The active profiles of the hMSCs were evaluated for (i) cell proliferation by Alamar Bleu assay, (ii) biochemical analysis of alkaline phosphatase (ALP) activity, (iii) cytoskeleton organization (fluorescent/confocal microscopy) and (iv) cell/substrate interaction via scanning electron microscopy (SEM). Cell proliferation and biochemical analysis indicated that the hMSCs cultured on n-TiO₂-10wt%HA coatings exhibited similar or superior levels of bioactivity to hMSC cultured on APS HA. The cytoskeleton organization demonstrated a higher degree of cell proliferation and attachment on the HVOF-sprayed n-TiO₂-10wt%HA coatings. These results are considered promising for engineering improved performance and increased longevity in the next generation of thermally sprayed biomedical coatings.

Introduction

Hydroxyapatite Coatings

Hydroxyapatite (HA) coatings deposited via air plasma spray (APS) have been successfully employed as biomedical coatings for medical implants (e.g., load bearing bone

implants such as hip-joints) (Ref 1). Despite the success of APS HA coatings, there are still concerns regarding their long-term performance related to the HA's physiologic post-implantation stability, i.e., dissolution and osteolysis (pathologic process involving resorption of bone surrounding the implant) (Ref 2-4). Therefore, there is need to improve the performance of the current biomedical APS HA coatings.

HVOF-sprayed n-TiO₂ as a Biomedical Coating

A possible alternative to improve the performance of APS HA coatings is based on the use of nanostructured titania (n-TiO₂). Nanostructured agglomerated TiO₂ powders produced via spray-drying of individual n-TiO₂ particles have been thermally sprayed via the high velocity oxy-fuel (HVOF) technique as an attempt to produce a new class of biomedical coating (Ref 5, 6). This coating was chosen due to the fact that it is a biocompatible material, it is not significantly absorbed/dissolved in the human body and it exhibits bond strength levels on Ti-6Al-4V substrates more than 2X higher than those of APS HA coatings (Ref 5, 6). In addition, previous preliminary studies demonstrated that the HVOF-sprayed n-TiO₂ coatings support of fetal rat calvaria osteoblasts cells growth and matrix production, in equivalent levels to those of APS HA coatings (Ref 5, 6). *In vivo* experiments were also performed. Coatings were deposited on small Ti-6Al-4V rods that were subsequently implanted in the femurs of rabbits. Grit-blasted Ti-6Al-4V rods without a coating were also implanted and served as a control. After 7 weeks of implantation the rabbits were euthanized and the contact surface between the bone and implant was measured/evaluated via optical microscopy. On average, the contact surface between the HVOF-sprayed n-TiO₂ coating and bone was 1.7 times higher than that of the uncoated Ti-6Al-4V rods (Ref 5).

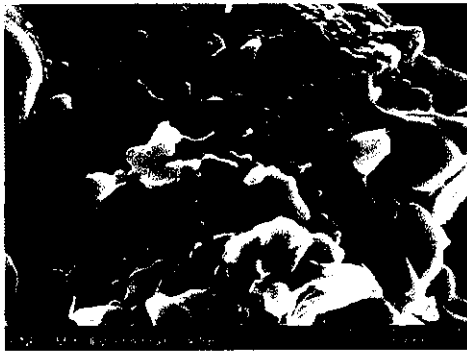


Figure 1: Nanostructured zone on the HVOF-sprayed n-TiO₂ coating surface (Ref 5).

Enhanced Biocompatibility of Nanomaterials

On the surface of these HVOF-sprayed n-TiO₂ coatings it was observed via field-emission scanning electron microscopy (FE-SEM) nanostructured zones (Fig. 1), which were formed when semi-molten nanostructured agglomerated TiO₂ particles impinged and solidified on the previously deposited layers during thermal spraying (Ref 5, 6). The importance of the presence of nanozones on the coating surface comes from the widely accepted hypothesis that nanostructures enhance the biocompatibility of biomaterials (Ref 7). For example, nanozones may improve the adsorption of the adhesion proteins like vitronectin and fibronectin. These types of proteins mediate the adhesion of anchorage-dependent cells (such as osteoblasts) on substrates and coatings (Ref 7). These adhesion proteins spread on the surface of an implant almost immediately upon its implantation in the human body. However, only a fraction of them stick or adsorb onto it. Shi et al. (Ref 8) and Lee et al. (Ref 9) observed that nano and submicron textured surfaces tend to exhibit a higher likelihood to retain proteins as a result of the interlocking between the proteins and nano-submicron asperities. When the osteoblast cells arrive at the implant surface they “see” a protein-covered surface that will connect with the transmembrane proteins (integrins) of the osteoblast cells, thereby promoting cell adhesion on the surface. It should be noted that these proteins, such as fibronectin, exhibit nanosized lengths and structures. For example, the average size of fibronectin is about 150 nm (Ref 10).

It is important to point out that Webster et al. (Ref 7, 11, 12) observed higher osteoblast proliferation levels on nanostructured ceramics (including TiO₂) when compared to those of conventional ceramics. This higher osteoblast proliferation on nanostructured ceramics was attributed to the higher probability of protein adsorption caused by nano and submicron asperities on the nanomaterials’ surfaces.

HVOF-sprayed n-TiO₂-10wt%HA Coatings

Based on these promising results, it was speculated that this “good” biological performance would be enhanced by adding

HA on the coating structure. Therefore, n-TiO₂-HA powder mixtures were produced and subsequently thermally sprayed via HVOF (Ref 13) (Figs. 2a-b). In spite of the HA addition, the bond strength levels of these coatings were shown to be still more than 2X higher than those of APS HA coatings (Ref 13). These coatings have been engineered to exhibit nano and submicron structured surface texture (as those of the pure n-TiO₂ coating), in addition to the chemical character of HA, in an attempt to improve the coating possessing biocompatibility. Therefore, it seems logical as the next step, to carry out biological experiments with these coatings.

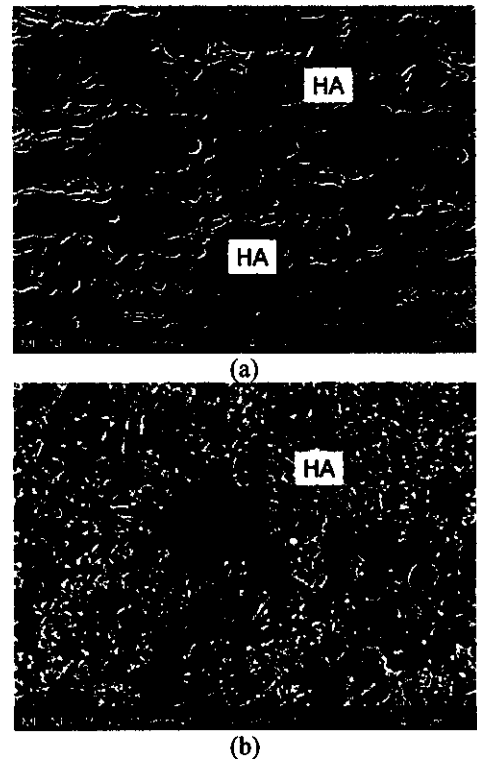


Figure 2: (a) Cross-section and (b) surface of an HVOF-sprayed n-TiO₂-10wt%HA (Ref 13).

The Interest on Human Mesenchymal Stem Cells (hMSCs)

Testing materials with hMSCs is becoming a regular practice; however, to the author’s best knowledge this is the first work in which interactions of human primary bone marrow derived cells with biomedical thermal spray coatings have been studied. The hMSCs can be differentiated into a specific group of human cell types, e.g., osteoblasts (bone), chondrocytes (cartilage), myocytes (muscle), fibroblasts (connective tissue) and adipocytes (fat) (Ref 14). The use of hMSCs in the research and development of thermally sprayed biomedical coatings represents a very important alternative and a complement to the studies in which human osteoblasts cells are used (Ref 15). For example, once a prosthetic device is implanted in the human body (e.g., acetabular cup and hip-

joint), hMSCs produced by the bone marrow cover the surface of the implant. Once the hMSCs are well-adhered to the surface of the implant, they begin the process of proliferation and differentiation into osteoblast cells (Ref 14). Therefore, studying the behaviour of hMSCs on the surface of these coatings provides important information and data about the onset of the interaction between the human cells and the implant, which affects both long-term biocompatibility and longevity of the implant. It is important to point out the major difference between mesenchymal stem cells and human embryonic stem cells (hESCs). The hESCs can be differentiated into any type of cells of the human body; however, hESCs are obtained from human embryos, which can lead to important ethical issues. hMSCs can be readily obtained from human bone marrow, which can be available during hip-joint replacement surgery, such as total hip replacement surgeries. This type of new data will provide valuable information to materials scientists, engineers and biologists on how to engineer the next generation of high performance implants.

Experimental Procedure

Powders, Coatings and Substrates for Thermal Spraying

n-TiO₂: The n-TiO₂ powder (VHP-DCS (5-20 μm), Altair Nanomaterials Inc., Reno, NV, USA) was HVOF-sprayed (DJ2700-hybrid, Sulzer Metco, Westbury, NY, USA) using propylene as fuel. The average particle temperature and velocity of the powder particles were 1881±162°C and 686±93 m/s, respectively. The average coating surface roughness (R_a) was 2.2 ± 0.3 μm (n = 10). More details about this coating can be found elsewhere (Ref 5, 6).

HA: The HA powder (Captal 30 SD, Plasma Biotol, Tideswell, North Derbyshire, England) was pure and crystalline. It exhibited a nominal particle size of 15-50 μm. This powder was sprayed via APS (SG100, Praxair, Concord, NH, USA) using only Ar as plasma gas. The average particle temperature and velocity of the powder particles were 2659±234°C and 189±19 m/s, respectively. The coatings produced from the HA powder were used as a control. Crystalline HA and amorphous calcium phosphate were the main phases of the coating, with a minor content of tetracalcium phosphate. The average coating surface roughness (R_a) was 4.1 ± 0.5 μm (n = 10). More details about this coating can be found in the paper of Auclair-Daigle et al. (Ref 16).

n-TiO₂-10wt%HA: The feedstock powder mixture was composed of 90wt%n-TiO₂-10wt%HA. The starting n-TiO₂ and HA powders were the same materials employed for producing n-TiO₂ and HA coatings. The n-TiO₂-10wt%HA powder was prepared through a mechanical-blending process in a planetary mill. This powder was HVOF-sprayed (DJ2700-hybrid, Sulzer Metco, Westbury, NY, USA) using propylene as fuel. The average particle temperature and velocity of the powder particles were 1875±162°C and 651±88 m/s,

respectively. The n-TiO₂-10wt%HA coating exhibited rutile as major phase with anatase and HA as minor phases. No significant degradation of HA was observed by means of X-ray diffraction (XRD). The average coating surface roughness (R_a) was 3.0 ± 0.5 μm (n = 10). More details about the coating can be found elsewhere (Ref 13).

Ti-6Al-4V: Ti-6Al-4V medical grade alloys were used as substrates and control. The substrates were cut into disk-shaped samples (pucks) of 12.7 mm diameter by 2 mm thickness. The Ti-6Al-4V samples for substrates (coating deposition) and controls were grit-blasted with alumina grit #24, as a standard procedure of surface preparation (impurity removal and roughening) for thermal spraying. The average surface roughness (R_a) was 4.6 ± 0.7 μm (n = 10).

Cell Isolation, Expansion and Culture on Samples

Human Mesenchymal Stem Cells (hMSCs): Bone marrow samples were obtained from 15-mL aspirates from the intramedullary canal of osteoarthritis patients undergoing total hip replacement surgery (3 three patients: 1 woman and 2 men, aged 52–76 years). Only tissue that would have been discarded was used, with the approval of the Research Ethics Committee of the Jewish General Hospital. This research also received the approval of the ethics standards of the National Research Council of Canada. The hMSCs were isolated and expanded as previously described (Ref 17). The medium was changed every 3 days, with all cultures maintained at 37°C with 5% CO₂. Culture-expanded hMSCs were trypsinized, counted and seeded separately onto ethylene oxide (EtO) sterilized n-TiO₂-10wt%HA, n-TiO₂ and HA coated pucks at a density of 2×10⁴ cells/cm. Ti-6Al-4V samples and tissue culture seeded in the same manner as the test samples were used as references.

hMSC Proliferation on Samples

Alamar Bleu: The n-TiO₂-10wt%HA, n-TiO₂ and HA coated pucks seeded with the hMSCs were incubated for 1, 7 and 21 days. At each time point, the cell proliferation was monitored using the Alamar Blue™ assay according to the manufacturer (Biosource, Nivelles, Belgium). The assay is based on a fluorometric/colorimetric growth indicator that detects metabolic activity. Numerical data were analyzed statistically using independent Student *t*-tests.

Biochemical Analysis

Alkaline phosphatase activity (ALP): ALP activity of the conditioned media was evaluated as a determinant of osteoblast differentiation. The ALP activity, the driver of bone matrix mineralization, was determined in the hMSC lysates at 1, 7 and 21 days using a commercially available kit (AnaSpec, San Jose, CA, USA) in accordance with the provided instructions. Briefly, the measurements were performed using 100 μl supernatants which 100 μl of p-nitrophenolphosphate dye was added and the absorbance subsequently measured at 410 nm using a spectrophotometer. For each time point the

activities in three samples were normalized as μmol per mg protein per min.

Cytoskeleton Organization (confocal microscopy)

Immunocytochemistry (fluorescence): In order to visualize the cytoskeleton and nuclei of the hMSCs, after 1 and 7 days of hMSC seeding, two types of dyes were employed: propidium iodide (PI) for the nuclei and F-actin for the cytoskeleton. Briefly, cells were fixed in formaldehyde (Sigma, St. Louis, MO, USA), permeabilized in 0.1% buffered Triton X-100 (Sigma, St. Louis, MO, USA) and stained for cell cytoskeletal F-actin with 5 U/mL Alexa Fluor 488 phalloidin (Molecular Probes, Burlington, ON, Canada) for 1 h. Cell nuclei were counterstained with 15 M propidium iodide (Molecular Probes, Burlington, ON, Canada) for 20 min. Samples were mounted (Vectashield, Vector Laboratories, Burlington, ON, Canada) and examined optically (Cell Observer System, Carl Zeiss, Gottingen, Germany).

hMSC/Substrate Interaction (SEM)

The morphologies of hMSCs were evaluated using FE-SEM (S-4700, Hitachi, Tokyo, Japan). The specimens were sputter coated with palladium and observed at 500X magnification.

Results and Discussion

Cell Proliferation

The Alamar Bleu assay, which is based on the detection of metabolic activity of living cells, demonstrated that HVOF-sprayed n-TiO₂-10wt%HA coatings supported the growth and proliferation of hMSCs (Fig. 3). The performance levels are equivalent or superior to those of standard APS HA coatings (Fig. 3) throughout the time of the study. It is important to point out that the hMSCs are anchorage-dependent cells, i.e., they will die if they do not become well-adhered to the surface of the coatings. Therefore, the results of Fig. 3 show strong evidence that the surface of the HVOF-sprayed n-TiO₂-10wt%HA coating is creating favourable conditions for hMSCs growth and proliferation.

At this point, the dominant factor giving rise to the excellent behaviour concerning the proliferation of hMSCs on the n-TiO₂-10wt%HA coatings has not been precisely identified. However, some hypotheses can be examined. The observations of Shi et al. (Ref 8) and Lee et al. (Ref 9) on the effect of nano and submicron textured surfaces (like that of Fig. 1) on the likelihood of protein retention (interlocking), as well as, the higher osteoblast proliferation on nanostructured ceramics observed by Webster et al. (Ref 7), should be taken into account in explaining the experimental results reported in this paper. In addition, the overall surface roughness of the samples probably played a role in these results. Comparing the information provided in Experimental Procedure, the HVOF-sprayed n-TiO₂-10wt%HA exhibited roughness (R_a) levels 27% lower than those of APS HA. This difference was caused mainly by the higher average velocity of the HVOF-sprayed

particles when compared to that of APS HA particles (651 versus 189 m/s). Anselme et al. (Ref 18) evaluated the adhesion of human osteoblast cells on polished and sandblasted Ti-6Al-4V surfaces. A higher degree of cell adhesion was observed on the polished surfaces. In this present study, the uncoated Ti-6Al-4V substrates exhibited the highest roughness and the poorest performance of all samples tested (Fig. 3). Therefore, surfaces with lower degree of submicron and micron asperities (i.e., lower R_a values) seem to facilitate hMSC adhesion.

It is important to point out that the addition of 10wt%HA to pure n-TiO₂ caused a noticeable improvement in the bioperformance of the n-TiO₂-10wt%HA coating (Fig. 3). Consequently, not only topographical effects but also chemical effects probably played an important role in the improved cell proliferation.

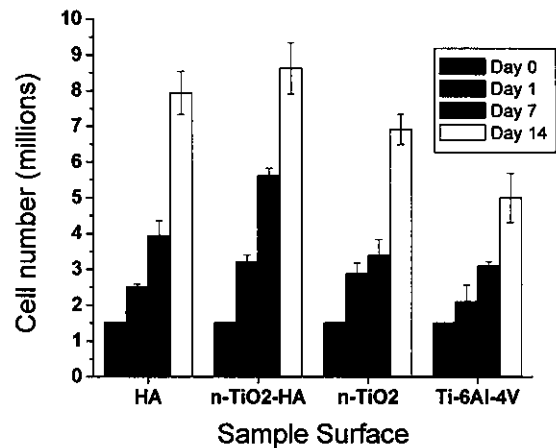


Figure 3: hMSC proliferation profiles during 14 days on HA, n-TiO₂-10wt%HA and n-TiO₂ coatings and uncoated Ti-6Al-4V substrates investigated by Alamar Bleu.

Biochemical Analysis

The early marker of osteoblastic activity measured in this study, ALP, demonstrated clear differences of hMSC behaviour among the different coatings (Fig. 4). At day 7, the activity of ALP was significantly higher relative ALP levels when plated on the n-TiO₂-10wt%HA than HA and n-TiO₂ reference coatings and uncoated Ti-6Al-4V substrates. The increase in ALP activity is a marker of the commitment towards osteoblastic lineage, i.e., the trend of the hMSCs to differentiate into osteoblast cells. The subsequent temporal decrease may correlate with advanced matrix mineralization and a more mature phenotype.

Confocal Microscopy

Confocal microscopy was used to investigate the cytoskeleton organization and nuclei morphology of the hMSCs from 1 to 7 days (Fig. 5). Cells stained with PI for nuclei (red) and F-actin for cytoskeleton (green) were clearly identified.

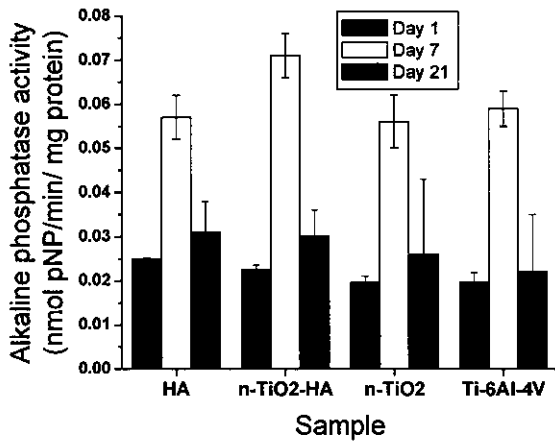


Figure 4: Normalized alkaline phosphatase activity (ALP), levels in medium of hMSCs cultured on HA, n-TiO₂-10wt%HA and n-TiO₂ coatings and uncoated Ti-6Al-4V substrates as a function of time.

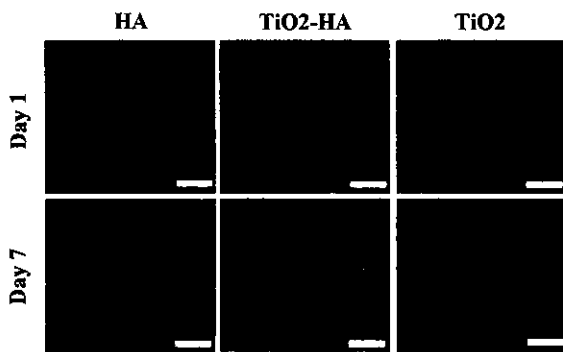


Figure 5: Cytoskeleton organization and nuclei morphology of hMSCs at day 1 and 7 cultured on HA, n-TiO₂-10wt%HA and n-TiO₂ coatings. The scale bar length is 200 μ m.

The changes in phenotype and growth kinetics among the APS HA, HVOF-sprayed n-TiO₂-10wt%HA and n-TiO₂ coatings are clearly visible. After 1 day of cell seeding, hMSCs exhibited their typical spindle-shaped morphology similar on all three coatings with well formed F-actin filaments and nucleus. By day 7, the F-actin filaments deposition on the HVOF-sprayed n-TiO₂-10wt%HA coating was much more intense, denser and widespread over the entire sample surface (and not only in areas of high cell density), when compared to APS HA and HVOF-sprayed n-TiO₂ grown hMSC samples. The increased F-actin and nucleus stain of hMSC on HVOF-sprayed n-TiO₂-10wt%HA attest to the increased growth kinetics on the n-TiO₂-10wt%HA nanocomposites when compared to the reference coatings.

hMSC/Substrate Interaction (SEM)

After 2 days of culture the cells adhered on all coatings, elongated and took on a typical spindle hMSC shape (Fig. 6). However, the hMSCs cultured on the HA (Fig. 6a) appeared to have smaller cell bodies and be in a more rounded configuration when compared to the hMSC morphologies of the n-TiO₂-10wt%HA (Fig. 6b) and n-TiO₂ (not shown) coatings (the cells are highlighted by the arrows). The hMSCs culture on APS HA also appeared less spread and less flattened than those of the hMSCs cultured on the n-TiO₂-10wt%HA coating (Fig. 6b). This observation is significant as it is well known that cells in a rounded configuration divide at a lower rate than those flattened and well spread on a surface (Ref 19). Consequently, the n-TiO₂-10wt%HA (Fig. 6b) and n-TiO₂ (not shown) coatings as compared to the HA (Fig. 6a) coatings allow greater initial cell hMSC spreading. This observation agrees with the higher proliferative rates as seen with the cell proliferation assays (Figs. 3 and 5). It has to be pointed out that more information, further biomedical data and hypothesis on all the results of this paper are discussed in detail in other publication (Ref 20).

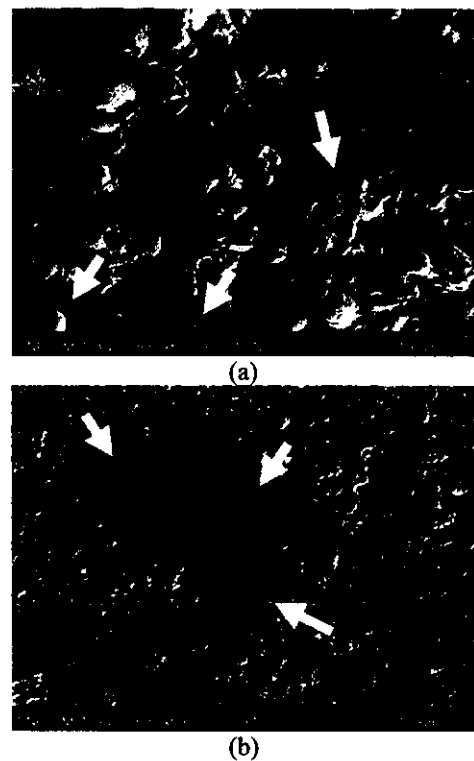


Figure 6: hMSCs on the surface of the (a) APS HA and (b) HVOF-sprayed n-TiO₂-10wt%HA coatings after 2 days of cell culture – arrows highlight the cells.

Conclusions

Previous studies have shown that HVOF-sprayed n-TiO₂-10wt%HA coatings exhibit bond strength levels at least 2X higher than those of APS HA coatings deposited on Ti-6Al-4V substrates. In addition, due to the high stability of TiO₂ in the human body, longevity related concerns of HA coatings, such as, dissolution and osteolysis, are unlikely to occur. The present results show strong evidence that hMSCs exhibit on HVOF-sprayed n-TiO₂-10wt%HA coatings (i) growth, proliferation and attachment, (ii) commitment towards osteoblastic lineage and (iii) cell/substrate interaction levels/characteristics equivalent or superior to those of APS HA coatings. There are no clear explanations regarding this favourable behaviour, but it is hypothesized that the topography and chemical composition of the surface of the HVOF-sprayed n-TiO₂-10wt%HA coating are playing important roles. These results demonstrate that surfaces engineered in this fashion have the potential to become the next generation of biomedical thermal spray coatings with enhanced performance and improved longevity.

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