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Synthesis and Vacuum Cold Spray Deposition of Biofunctionalized Nanodiamond/Hydroxyapatite Nanocomposite for Biomedical Applications

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Insufficient biological performances of titanium alloys have been the long-standing problems for their clinical applications. Here, we report synthesis of novel hydroxyapatite/nanodiamond-bone morphogenetic protein 2 (HA-ND/BMP2) composite powder and their coatings deposited by vacuum cold spray operated at room temperature. The microstructure and chemistry of the HA-ND/BMP2 powder and coatings are characterized by transmission electron microscopy, field-emission scanning electron microscopy, thin-film X-ray diffraction, Raman spectrometry, and X-ray photoelectron spectroscopy. *In vitro* growth assay of osteoblasts on the coatings showed that the biofunctionalized nanodiamonds promoted cell adhesion and proliferation. This study provides a promising technical route for constructing biofunctionalized nanocomposites coatings for potential biomedical applications.

1. Introduction

Titanium and titanium alloys have been widely used as clinical orthopedic implants due to their bioinert nature and promising mechanical properties.[1,2] However, challenges still remain as to further enhance their biological performances for desired biological functions. Surface engineering for desirable physical and chemical properties is critical in regulating bio-performances of titanium implants. To date, many techniques have been attempted to improve their bio-performances, including surface coating technology,[3] biofunctionalization,[4] and nanotechnology,[5] etc. To closely mimic natural bone tissues, hydroxyapatite (HA) coatings have been widely employed for surface modification of titanium alloys using physical vapor deposition,[6] electrochemical deposition,[7] electrostatic spray deposition,[8] and thermal spray deposition.[9] Among them, plasma spraying has been successful in constructing HA coatings for orthopedic surgery.[10] Besides surface coatings, local surface chemistry of the substrate is another key factor that contributes to cellular microenvironment, which directly regulates cell-material interactions. Loadings of drugs or biomolecules are feasible approaches to alter surface chemistry of substrates.[11–13]

Attaching drugs and biomolecules to nanomaterial is currently a topic of interest in nanomedicine.[14] Nanodiamonds (NDs) have been reported as a promising biomaterial for such applications due to their superior physiochemical properties.[15–17] In the past few years, there were a number of studies exploring the possibilities of NDs for drug delivery.[18–20] NDs were assembled on the surface of solid materials as delivery vehicles and *in situ* release of drugs and biomolecules in lesion tissues was reported.[21] However, most of the techniques previously reported are not suitable for simple fabrication of thick coatings and efficient loading of drugs/biomolecules was limited to surfaces of solid materials. Therefore, appropriate fabrication techniques are to be developed for constructing biofunctionalized NDs composite coatings for practical applications.

In order to deposit coatings from nanosized powder by traditional plasma spray method, nanoparticles are usually agglomerated into microparticles via spray-drying.[22] However, both the processes of spray-drying and plasma spray are carried out at high temperature, which is not suitable for processing temperature-sensitive biomaterials such as drugs and biomolecules. Vacuum cold spray (VCS) is a novel and promising surface coating technique to deposit powder feedstock with particle size of 0.02–2 μm at room temperature, which is of great prospect for preparing temperature-sensitive biofunctionalized nanostructured coatings.[23–27] However, there are so far few studies reporting the deposition of biofunctionalized nanocomposites by VCS for biomedical applications.

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In this study, we report for the first time the use of VCS to deposit novel HA-ND/BMP2 nanocomposites on titanium substrates for biomedical applications. Microstructural characterization and *in vitro* cell culture testing were performed to investigate the performances of the coatings. This study provides a promising technical route for constructing biofunctionalized nanocomposites materials and coatings.

2. Materials and Methods

Pristine NDs powder (Nafortis Technology Co. Ltd., China) was produced by detonation of carbon-containing explosives.[28] The NDs powder was pre-treated according to the protocols established by previous studies.[28–30] Briefly, the NDs powder was heated at 425 °C for 5 h followed by acid treatment with nitric and sulfuric acids. The NDs powder was finally washed with distilled water.

Titanium (Ti) plates with the dimension of 10 mm × 10 mm were used as substrates. Three types of powder and corresponding coatings were fabricated. The HA powder was synthesized according to a previous report.[31] To obtain the HA-ND composite powder, the HA powder was dispersed in phosphate buffered saline (PBS) buffer and then the ND powder was added under ultrasound condition, followed by freeze-drying for 24 h. The HA-ND/BMP2 composite powder was synthesized as per the following steps: BMP2 (Sinopharm Chemical Reagent Co. Ltd., China) was added into the ND PBS suspension under ultrasound condition; the mixture was then gently mixed before added into the HA suspension and continued stirring was maintained for 2 h at 4 °C, followed by freeze-drying for 24 h. The HA, HA-ND, and HA-ND/BMP2 powder were deposited on Ti–6Al–4V (Ti) substrates at room temperature by VCS 2000 system (developed by Xi'an Jiaotong University, China), respectively, according to a previous study.[27] For the spraying, helium was used as the carrier gas with the flow rate of 5 L min^{-1}, and the gun-scanning speed was 10 mm s^{-1} with the spray distance of 10 mm.

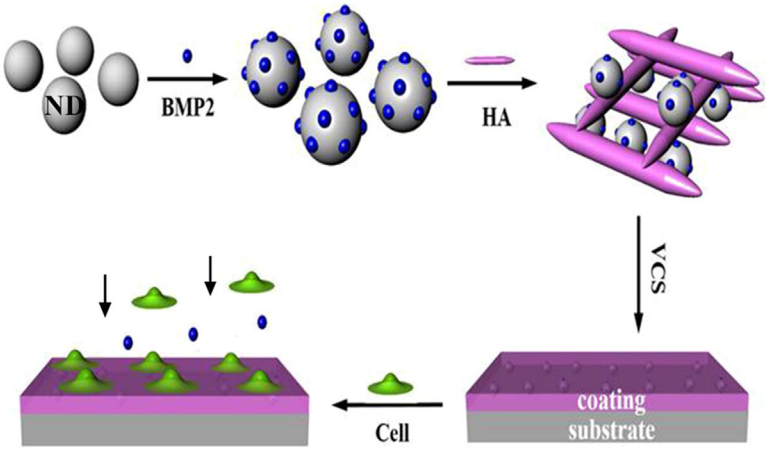
Morphology and microstructure of the powder and the coatings were characterized by field emission scanning electron microscopy (FESEM, FEI Quanta FEG250, the Netherlands, and Hitachi S4800, Japan) and transmission electron microscopy (TEM, FEI Tecnai F20, the Netherlands) and high resolution transmission electron microscopy (HR-TEM). Chemistry of the samples was analyzed by X-ray diffraction (XRD, D8 Advance, Bruker AXS, Germany) using Cu Kα radiation ($\lambda = 1.5406$ nm) operated at 40 kV and 40 mA with a scan rate of 0.02° s^{-1} over a 2θ in the range of 20–60°. The structure of ND was further examined by Raman spectrometry (RenishawinVia Reflex, Renishaw, UK) operated with the laser wavelength of 325 nm. Chemical composition of the samples was determined using X-ray photoelectron spectroscopy (XPS, AXIS ULTRA DLD, Japan). Microhardness of the coatings was determined using a Vickers hardness tester (HV-1000, Shanghai Lianer Testing Equipment Co., China). A load of 100 g was applied and ten measurements were collected for each sample for an average value.

Cell adhesion and proliferation on the Ti substrates, the HA coatings, the HA-ND coatings, and the HA-ND/BMP2 coatings were examined using human osteoblast cells (HFOB 1.19 SV40 transfected osteoblasts). Cells were cultured in α-minimum essential medium (α-MEM) (SH30265.01B, HyClone, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 U–mL^{-1} penicillin and 100 μg mL^{-1} streptomycin under 5% CO_2 at 37 °C. Cells were seeded with an initial density of 1×10^4 cells mL^1. For SEM observation of the cells attached on the surfaces of the samples, cells were fixed with 2.5% glutaraldehyde, followed by gradually dehydrated and coated with platinum. Cell viability was evaluated by methyl thiazole tetrazodium (MTT) assay. Briefly, cells were cultured on different samples for 3 and 7 days, respectively. Then, 70 μL of MTT (Sigma, St Louis, MO, USA) with the concentration of 5 mg mL^{-1} was added into each well and incubated at 37 °C for 4 h. The MTT-containing medium was removed and 700 μL dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystal. The absorbance at 490 nm was measured by a microplate reader (Spectra Max 190, MD, USA). All data were expressed as mean ± standard deviation (SD) for $n = 3$. Statistical analysis was performed with OriginPro (version 6.1) at confidence levels of 95 and 99%.

3. Results and Discussion

3.1. Sample Fabrication and Characterization

Scheme 1 shows the schematic illustration of the synthesis and VCS deposition of the biofunctionalized ND/HA nanocomposites on titanium substrates. NDs were pretreated with acids to generate carboxylic functional groups (−COOH) on the surfaces. The carboxylic functional groups were then utilized to conjugate BMP2 onto ND particle.[32] The obtained ND/BMP2 was thoroughly mixed with HA to produce HA-ND/BMP2 composite powder. Finally, the HA-ND/BMP2 composite powder was deposited on



Scheme 1. Schematic illustration of the fabrication of the HA-ND/BMP2 powder and coatings.

titanium substrates via a VCS coating technique at room temperature. The immobilization of BMP2 on ND was expected to improve the biocompatibility of titanium substrates, and the continuous releasing of BMP2 from the HA-ND/BMP2 composite coatings could promote cells growth for a long-term period.

The as-received ND particles show well-dispersed state with an average diameter of ≈5 nm (**Figure 1**a). Aggregation of the particles is seen, which is probably due to the unstable nature of the detonation NDs.[33] Further HRTEM characterization confirms the aggregation. Lattice fringes with interlayer spacing of 0.206 and 0.34 nm are clearly seen, and they correspond to the lattice plane of ND and graphite, respectively (Figure 1a-2, inset),[34] evidencing that the particle is diamond. The as-synthesized nano-HA particles show a rod-like shape with the size of ≈20–100 nm in length and ≈10 nm in diameter (Figure 1b). The HA-ND/BMP2 composite powder shows both rod-like and round shapes, which could be attributed to the co-existence of HA and NDs (Figure 1c).

To comparatively investigate the influence of the addition of NDs and BMP2 on biological behaviors of the cultured cells, the HA coatings, the HA-ND coatings, and the HA-ND/BMP2 coatings were successfully deposited on titanium substrates by the VCS technique (**Figure 2**). The coatings exhibit similar microstructural features with a relatively rough topographical morphology (Figure 2a–c, -1 and -2). This structural feature is normal for cold sprayed coatings, since the coatings are fabricated by accumulation by tamping effect of individual particles.[25] It was reported that adhesion and proliferation of osteoblasts were higher on rough surfaces than on smooth surfaces.[35] In this case, thickness of the coatings is ≈40 μm and their cross-sectional views suggest a dense structure (Figure 2a–c, -3), which indicates plastic deformation of the particles during coating formation stage, in turn leading to strong adhesion and cohesion of the coatings.

Apart from microstructural effects, local chemistry of surface coating is another important factor of cellular microenvironment. As shown in **Figure 3**, XRD patterns and Raman spectra of the powder and the coatings are also acquired. Different from other spray techniques such as plasma spray, the VCS processing is performed at room temperature. It is therefore not surprising that there is no phase change detected between the coatings and the starting powder (Figure 3). Almost identical XRD and Raman peaks are seen for the coatings and corresponding powder. Furthermore, XPS measurement was employed to confirm the successful fabrication of the HA-ND/BMP2 composite coatings with successful retention of BMP2. The representative XPS spectra of the HA coating, the HA-ND coating, and the HA-ND/BMP2 coating are shown in **Figure 4** and their surface chemical compositions are listed in **Table 1**. The HA coating contains three elements of O, Ca, and C. After the addition of NDs, the C content slightly increases to 22.25%, which is mainly derived from NDs. After being biofunctionalized with BMP2, an additional peak at 399 eV is observed. The presence of N element is attributed to the amino groups of BMP2. The high content of N at 1.93% suggests the loading of BMP2 into the coating, which is consistent with a previous study.[36] These results indicate the successful fabrication of the HA-ND/BMP2 coating with perfectly retained NDs and BMP2.

Sufficient mechanical strength is required for metallic implants to achieve long-term functional services. To enhance the mechanical properties of HA coatings deposited on metallic implants, various methods have been attempted, for instance the fabrication of composite structures through addition of titanium, zirconium, or other components.[37] The VCS HA coating shows an average hardness of HV30.78 (**Figure 5**). The hardness of the HA-ND and the HA-ND/BMP2 coatings dramatically increased to HV61.44 and HV56.41, respectively. The enhanced hardness could be explained by the diffusion of ND particles among HA particles in the coatings. These results are in close agreement with a previous study.[38] No statistically significant difference is observed between the HA-ND and the HA-ND/BMP2 coatings.

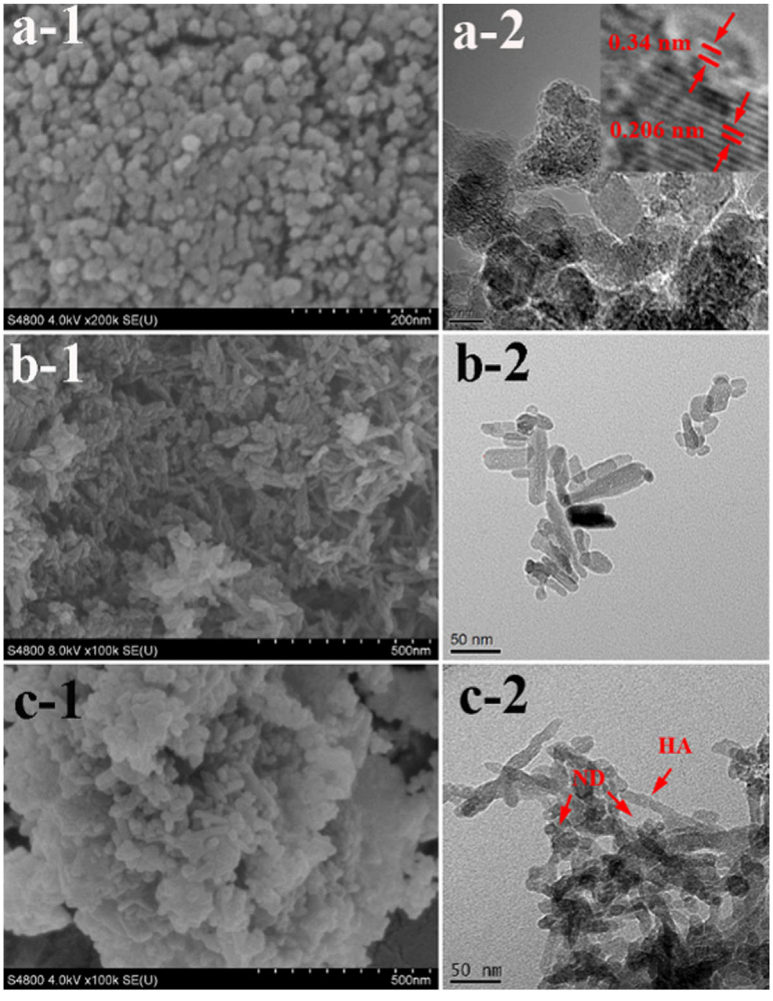


Figure 1. SEM (-1) and TEM (-2) images of the a) ND/BMP2, b) HA, and c) HA-ND/BMP2 composite powder. The inset in (a-2) is enlarged view of selected area.

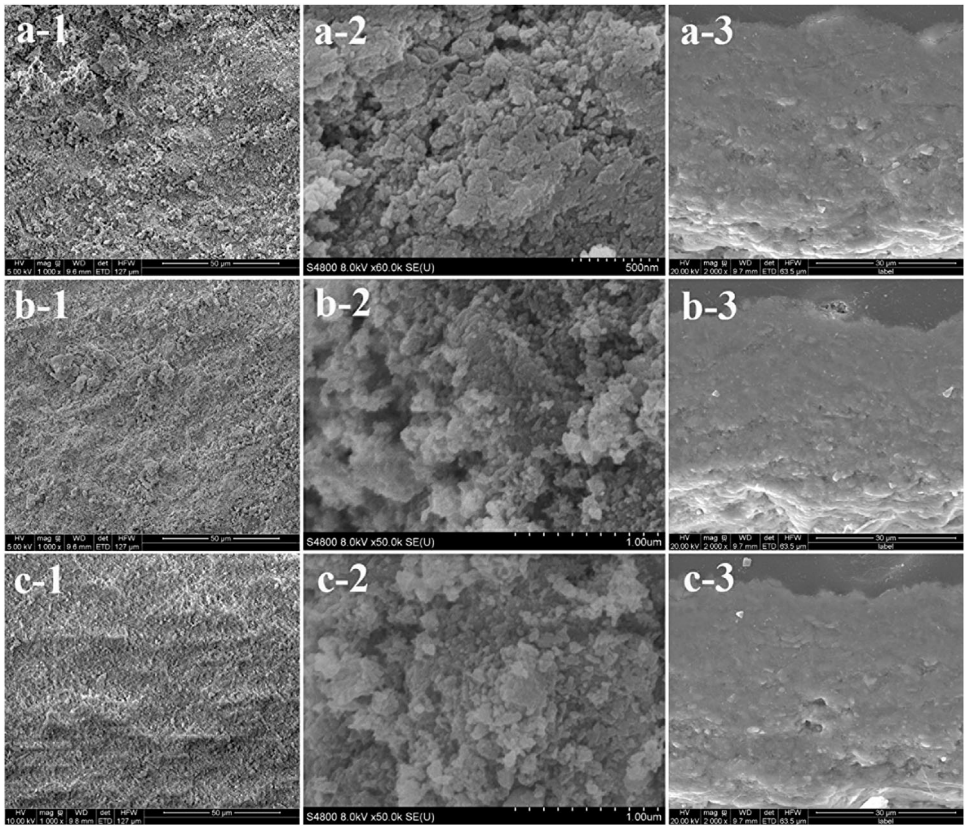


Figure 2. SEM images of the a) HA coating, b) HA-ND coating, and c) HA-ND/BMP2 coating. (-1: surface view, -2: enlarged view of selected area in -1, and -3: cross-sectional view).

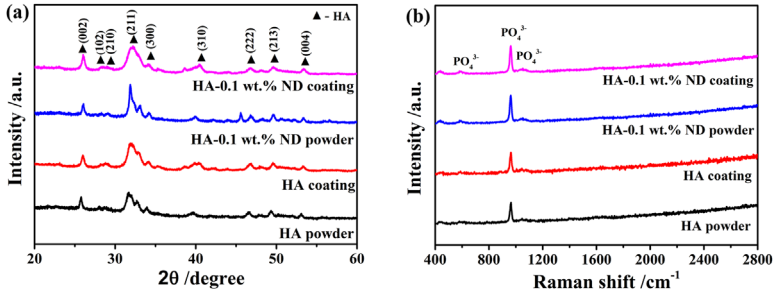


Figure 3. XRD patterns a) and Raman spectra b) of the powder and the coatings.

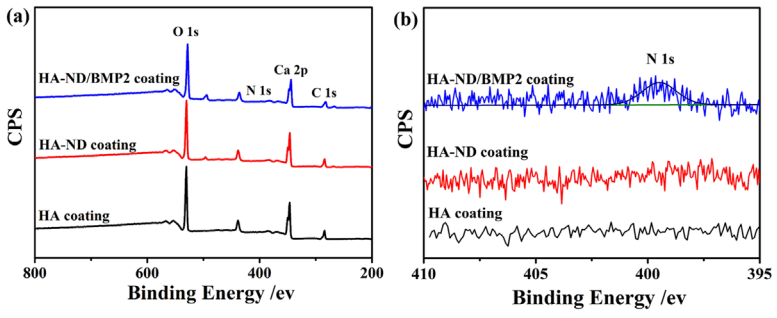


Figure 4. XPS spectra of the coatings a) and high resolution XPS spectra of N1s b).

3.2. *In Vitro* Biocompatibility of the Coatings

Favorable biocompatibility of biomaterials is essential for potential bio-applications. Insufficient biocompatibility can lead to failure of orthopedic and dental implants. Novel biofunctional implants with outstanding biological performances are urgently needed. Bone morphogenetic proteins (BMPs) have been reported to play an important role in cell growth of osteoblasts and consequent bone formation.[11,12] Among the members of BMP family, BMP2 has been shown to promote multiple cellular processes including adhesion and proliferation of cells, thus induce osteo-differentiation and other processes in new bone formation.[11,13] In this study, biocompatibility of the coatings was evaluated by cell adhesion and MTT assays. SEM images clearly suggest that the cells have better adhesion on the HA coating, the HA-ND coating, and the HA-ND/BMP2 coating than on the Ti substrate (**Figure 6**). Moreover, the best cell adhesion is observed on the HA-ND/BMP2 coating, suggesting further enhanced cell adhesion by presence of BMP2 in the coating. In addition, MTT assay shows no statistically significant difference among the Ti substrates, the HA coatings, and the HA-ND

Table 1. Chemical compositions of each sample measured by XPS.

Samples	O [at%]	N [at%]	Ca [at%]	C [at%]
HA coating	61.69	0	17.61	20.70
HA-ND Coating	60.25	0	17.49	22.25
HA-ND/BMP2 coating	55.50	1.93	15.84	26.73

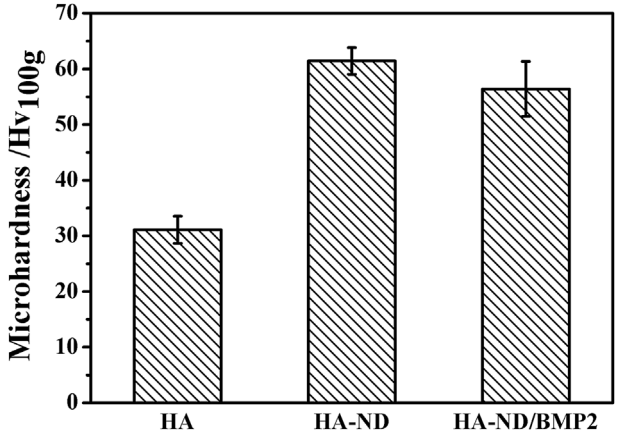


Figure 5. Vickers hardness of the coatings.

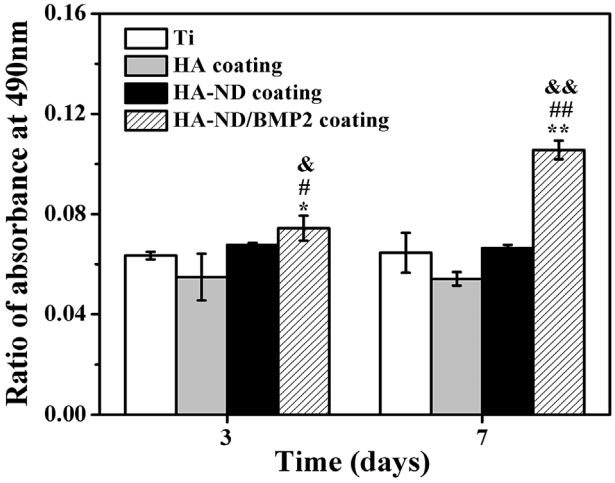


Figure 7. Cell viability of the osteoblasts cultured on the surfaces of the Ti substrate, the HA coating, the HA-ND coating, and the HA-ND/BMP2 coating. Error bars represent mean ± SD for $n = 3$, $^{*}p < 0.05$, and $^{**}p < 0.01$ compared with the Ti substrate, $^{\#}p < 0.05$, and $^{\#\#}p < 0.01$ compared with the HA coating, $^{\&}p < 0.05$, and $^{\&\&}p < 0.01$ compared with the HA-ND coating.

coatings (**Figure 7**), indicating remarkable biocompatibility of HA and NDs. However, osteoblasts grown onto the HA-ND/BMP2 display significantly higher cell viabilities than those proliferated on the Ti substrates, the HA coatings, and the HA-ND coatings after 3 days ($p < 0.05$) and 7 days ($p < 0.01$) of culturing. The result once again suggests that BMP2 has been successfully introduced into the coatings and enhances cell proliferation. Taken together, these results indicate that the HA/ND-BMP2 nanocomposite coatings offer significantly promoted biological properties for the titanium substrates. However, it should be noted that further comprehensive investigation on biocompatibility of the novel biomaterials is required. Nevertheless, the results presented here already shed light on future synthesis and coating deposition of biofunctionalized nanocomposites for clinical applications.

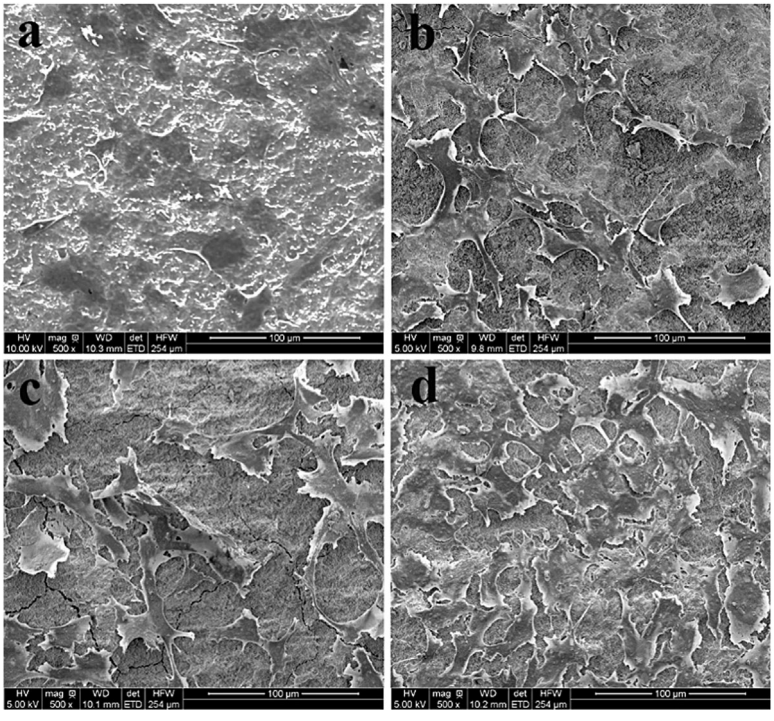


Figure 6. SEM images of the osteoblasts cultured for 1 day on the surfaces of a) the Ti substrate, b) the HA coating, c) the HA-ND coating, and(d) the HA-ND/BMP2 coating.

4. Conclusions

Novel HA-ND/BMP2 coatings were successfully fabricated on titanium substrates by VCS processed at room temperature. The addition of NDs gave rise to significantly increased microhardness of the coatings. *In vitro* cell growth assay suggests remarkably promoted osteoblast behaviors by the presence of NDs and BMP2. This study would open a new window for developing biofunctionalized nanocomposites coatings for biomedical applications.

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Conflict of Interest

None.

Keywords

biofunctionalized nanodiamond/hydroxyapatite nanocomposite; biomedical coating; bone morphogenetic proteins 2; cell behavior; vacuum cold spray

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