



Improved bio-physical performance of hydroxyapatite coatings obtained by electrophoretic deposition at dynamic voltage

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Received 29 January 2014; received in revised form 15 March 2014; accepted 21 April 2014

Available online 29 April 2014

Abstract

This study investigates the effects of the electrophoretic deposition process (EPD) at dynamic voltage on the physical and biological characteristics of hydroxyapatite (HA) coatings. HA powder is synthesized and used for preparation of HA/ethanol suspension which is subsequently characterized by X-ray diffraction (XRD), energy-dispersive spectroscopy (EDS), Fourier transform infrared spectroscopy (FT-IR), zeta potential and particle size analyses. Samples are prepared from commercially pure titanium (CP-Ti) substrates and coated with HA using EPD at dynamic voltage that keeps a constant depositional rate by adjusting the current and electrical field during the process. The HA-coated samples are dried and sintered at 800 °C for 2 h to densify the coatings. The XRD, scanning electron microscopy (SEM), EDS and adhesion tests are used to characterize the coated samples. The cross-sectional SEM images indicate that the thickness of coatings enhances as the current increases from 0.07 to 0.35 mA. The coatings are more uniform, packed and non-cracked at lower currents while HA particles start to arrange in a highly porous structure and show non-uniform, cracked and non-stable coatings as the current increases. The results also demonstrate that the best adhesions for the coatings are obtained at lower currents of 0.07 and 0.15 mA. Morphological studies and cell biological experiments are conducted using MG63 cells cultured on the HA-coated sample with the best overall physical performance. The number of attached and proliferated cells on the selected HA-coated sample is higher than on the non-coated titanium sample and culture plate used as control. There are significantly higher ALP activity and better cytoskeleton organization of cells on the HA-coated sample. This study shows that the EPD process at dynamic voltage can influence the structure and morphology of the coatings; therefore, substrate engineering can be used to improve and control cell–substrate interactions.

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Keywords: Hydroxyapatite coating; Electrophoretic deposition; Dynamic voltage; Physical performance; Biological response

1. Introduction

Instability is the number one cause for bone implants' failure [1]. The key step to obtain a stable implant is a direct structural and functional connection between the bone tissue and the surface of the implant material which involves optimal cellular and molecular interaction at bone–material interface [1–4]. The initial stability and rigid fixation of the implant would secure

long-term durability which reduces the rate of revision surgeries and health care costs [5]. Much research has been performed to chemically or structurally modify the surface of an implant material to gain an optimized reaction at the interface [5,6]. Owing to close similarity in composition and structure to natural human bone, hydroxyapatite (HA) has been widely used over the years as one of the most bioactive materials [4,7]. Pure HA has poor mechanical properties [8,9], hence, it has been used as coatings on the surface of metallic materials in order to combine the strength and toughness of the substrate with the bioactivity of HA [6,10,11]. It is also suggested that the application of HA coatings will improve

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corrosion resistance of the underlying implant material and reduce metallic ion release at the same time as promoting its bone bonding ability [5,9].

Many coating techniques have been investigated in order to deposit HA coating on the surface of metallic substrates including plasma spraying [12], sol–gel [13], ion beam dynamic mixing, pulse laser deposition, biomimetic coating [14], and electrophoretic deposition [15]. However, there are many limitations in the current coating methods which include non-uniform coating formation over geometrically complex surfaces, thermal decomposition of HA, formation of non-uniform metastable phase of HA during the high temperature process [15,16], sluggishness of the process, low crystallinity and poor adhesion of the coating on the substrates [7]. Therefore, there has been an increasing interest in the application of other techniques in recent years [17].

Electrophoretic deposition (EPD) process, on the other hand, is a fairly rapid and inexpensive method that exhibits some advantages over other alternative processes. Some of these advantages are simplicity of the method, ability to form complex shapes and patterns, control over the thickness and morphology of the coating, low temperature, ability to produce a composite layer and most importantly applicability for medical purposes [15–17]. Moreover, it is now well established that the particle size and the state of agglomeration of ceramic powders are important factors for the quality of the coating. Agglomerate-free structures made from close-packed fine particles can be densified at lower sintering temperatures. An important advantage of electrophoretic processing is that agglomerates can be separated by pre-sedimentation. Owing to the insulating properties of the deposit, the electric field provides a higher deposition rate in defect regions, resulting in better packing and uniformity of the deposit [18].

During the EPD process and under an applied current, charged particles from a stable colloidal suspension are deposited onto a charged electrode [19,20]. EPD has particularly been used with ceramic particles, since a range of porous and non-porous materials can be produced that have been employed as filters, porous carriers, and bioactive scaffolds [19,21]. Once a well-dispersed suspension is prepared, the applied voltage becomes a critical parameter [20]. By the application of constant voltage during the EPD process, the thickness of the deposited coating will increase and cause a non-constant electrical field which leads to the formation of a non-uniform coating on the electrodes. In fact, the coating itself is a non-conductive material, thus, the functional electrical field will decrease as the amount of deposited particles increases. This is the main drawback in the EPD process while using a constant voltage [21].

The present study aims to control and improve the structure, porosity, and adhesion of HA coatings by the application of the EPD process at dynamic voltage. The voltage is adjusted in a way to keep a constant current and optimum electrical field during the EPD process. Nano-meter sized HA powder is synthesized, characterized and electrophoretically coated on the surface of commercially pure titanium (CP-Ti) substrates. The coated samples are dried and sintered at elevated temperature to densify the coatings followed by characterization and adhesion tests. It is known that the quality of organic–inorganic interface

is directly correlated to the interactions between the macromolecules produced by cells and structure of the substrates. Therefore, the most uniform, un-cracked coating with the highest adhesion on the substrate is selected and tested for its bio-performance. The biological performance is assessed through cell attachment, proliferation, alkaline phosphatase activity and morphological studies.

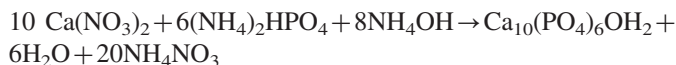
2. Materials and methods

2.1. Preparation of titanium substrates

Commercially-pure titanium (CP-Ti) sheet (McMaster-Carr Company, Los Angeles, CA) was used and cut in a way to have the same surface area ($10 \times 10 \times 1 \text{ mm}^3$). Each sample was mechanically grounded with a set of SiC papers followed by vibratory polishing with a vibrometer (Buehler, Lake Bluff, IL) to achieve a mirror surface finish. Subsequently the samples were cleaned with ethanol and distilled water, respectively, in an ultrasonic bath for 20 min. To increase surface roughness, the CP-Ti samples were etched and oxidized with a solution consisting of 10% nitric acid, 10% hydrochloric acid, 10% sulfuric acid and 10% H_2O_2 for 1 h. This treatment created small, nano-sized pits that increases the potential adherence of the HA powder on the surface of CP-Ti samples [15]. The samples were then submerged in a 5.0 mol/L NaOH aqueous solution at 50 °C for 48 h followed by washing with deionized water. Finally, before the EPD process, the samples were heated in an electric furnace at a heating rate of 1 °C/min, maintained at 600 °C for 1 h. The furnace was then cooled to room temperature.

2.2. Synthesis and characterization of HA powder

The HA powder was synthesized by the metathesis method (proposed by Hayek and Stadlman) [16] in a solution containing tetra hydrated calcium nitrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$) and di-ammonium hydrogen phosphate ($(\text{NH}_4)_2\text{HPO}_4$) with Ca/P ratio of 1.667. The reaction was as follows:



To obtain the HA powder, NH_4^+ and NO_3^- ions were removed by washing the precipitate repeatedly with water followed by drying in an oven at 100 °C for 24 h. The powder was calcinated at 1000 °C (heating rate of 5 °C/min) for 1 h in air atmosphere. HA powder was obtained by grinding with an agate mortar and pestle for 1 h and characterized by XRD (EQuinox 3000, INEL, France) and Fourier transform infrared spectroscopy (FT-IR-Bruker IFS 48, Germany).

2.3. Dynamic voltage EPD process

A suspension was made by adding 5 g of HA powder into 500 mL of ethanol (99.99%, Merck, Germany) and dispersed by adding 0.25% of carboxy-methyl cellulose (CMC, $[\text{C}_6\text{H}_7\text{O}_2(\text{OH})_2\text{OCH}_2\text{COONa}]_n$). The suspension was

stirred and sonicated in an ultrasonic bath for 30 min to make a homogeneous suspension [9,11]. The suspension was left for 30 min to eliminate the agglomerated particles through sedimentation. The mean particle size of the HA suspension was analyzed by Zeta-sizer Nano ZS (Malvern Instruments Ltd., Malvern, Worcestershire, UK). Additionally, the zeta potential of the suspension was measured with the same instrument at 25 °C by electrophoretic mobility.

EPD process was performed in a glass beaker equipped with a holder to fix the electrodes [6]. The platinum counter electrode and titanium samples were used as anode and cathode, respectively. Since HA particles acquire positive charge and their deposition occurs at pH=4, the suspension was adjusted to this pH by addition of small quantities of NaOH (0.1 M) and HNO₃ (0.1 M) [22]. It is known that during the EPD process, the current will continuously decrease and reach to a damping value as the deposition of particles onto the cathode increases. Here, a set of voltages was applied to the samples and the related damping currents were obtained from the current–time diagrams. For the EPD process, the voltages were set dynamically to keep these currents steady during the deposition. Each current was applied for 150 s at 25 °C using a power supply (HBPS-1A600V Hobang Electronics Co. Ltd., Korea). After the EPD process, samples were dried at room temperature for 24 h and sintered at 800 °C for 2 h to densify the coatings.

2.4. Characterization of HA-coated samples

The HA-coated titanium samples were characterized by XRD, scanning electron microscopy (SEM) and water contact angle measurements. The grazing-angle XRD (EQuniox 3000, INEL, France) was performed using a Philips X'pert MPD System (Philips, Eindhoven, Netherlands) with a copper anode ($K\alpha=0.154$ nm) and scanning rate of $0.75^\circ \text{ s}^{-1}$. Data were acquired between $2\theta=20^\circ$ and 80° at 0.01° increments. The experiments were carried out at room temperature (25 °C) at 1° takeoff angle and operating at 50 kV and 40 mA. The coatings morphologies and the cross-sectional views of HA-coated samples before and after sintering were examined by SEM (AIS2100, Seron Technology). Energy-dispersive spectroscopy (EDS; Thermo Noran) was used to determine the elements present in the deposit and estimate the Ca/P ratio. The wettability of HA-coated and non-coated titanium samples were determined by deposition of a distilled water droplet on the surface of samples and measuring the contact angle after 10 s, using a light microscope (OCA 15 plus, Data-physics) equipped with two CCD color cameras and an image analyzer system.

2.5. The coating adhesion test

The adhesion of HA coatings to the substrates was assessed using ASTM D3359. The test covers procedures for assessing the adhesion of a coating on a metallic substrate by applying and removing pressure-sensitive tape over cuts which were made in film. The test is used to establish whether the adhesion

of a coating to a substrate is at an adequate level or not. Based on the procedure, a lattice pattern with several cuts in each direction was made in the coating using a sharp razor blade. A pressure-sensitive tape was applied over the lattice and removed. The adhesion is evaluated by comparison of the removed part with descriptions and illustrations. The test was performed at least five times on separate samples.

Based on the characterization experiments, the best coating in terms of morphology, adhesion, and porosity, was selected for further biological experiments.

2.6. Cell culture

Human osteoblast-like cell (MG63) provided by American Type Culture Collection (ATCC, Manassas, VA, USA) was used for this study. The cells were cultured in T25 plastic flasks (Nunc, Germany) in alpha minimum essential medium (α -MEM, Invitrogen, Corporation, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. The growth medium was changed every 48 h. Cultured cells were detached by trypsinization, suspended in fresh culture medium and used for the experiments. Conventional culture well plates were used as control for each set of experiments.

2.7. Cell attachment, proliferation and alkaline phosphatase activity

Cell attachment experiments were performed on the selected HA-coated (0.150 mA current) and non-coated titanium samples by counting the number of attached MG63 cells after 60, 120, and 240 min of incubation. The samples were placed into a 24-well culture plate and seeded with a density of 5×10^4 cells/ml. After each time point, the samples were washed with a phosphate buffer solution (PBS) to remove unattached cells. Adherent cells were removed from the samples by incubation with 0.25% trypsin in EDTA (Invitrogen Corporation, USA). Trypsin was removed by centrifugation and the cells were re-suspended in fresh growth medium. The number of cells in the solution was counted with a hemocytometer. The proliferation of MG63 cells on titanium samples and plastic culture plates (controls) was investigated by the same method as attachment after 2, 5, and 7 days of culture. The cells were seeded onto the samples with a density of 5×10^3 cells/ml. The alkaline phosphatase activity (ALP) was assayed by the hydrolysis of *p*-nitrophenyl phosphate (Sigma, St. Louis, MO) as the release of *p*-nitrophenyl from *p*-nitrophenolphosphate. The color changes of the products were measured via Autoanalyzer-902 Hitachi (Germany) at 405 nm. The enzyme activity was expressed as unit $\text{mg}^{-1} \text{ min}^{-1}$ of protein.

2.8. Cell morphology

Morphological characteristics of cells on the surface of HA-coated and non-coated samples were investigated with a Philips XL30 field emission SEM (FE-SEM). Cells grown on

the samples for 3 days were first washed with PBS, fixed with 2.5% glutaraldehyde and dehydrated in graded series of ethanol–water baths. The surface of the samples was sputter coated with a 15 nm layer of Pt/Pd and examined in FE-SEM.

2.9. Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) with Tukey's Multiple Comparison Test to a confidence level of $p < 0.05$.

3. Results

3.1. Characterizations

Fig. 1 shows the FT-IR spectrum and XRD pattern of the synthesized HA powder as well as particle size and zeta potential analyses for the HA/ethanol suspension. The OH⁻ bands at 3555 cm^{-1} and 622 cm^{-1} as well as bands at 561 cm^{-1} , 603 cm^{-1} , and 1040 cm^{-1} identified in the FT-IR spectra correspond to PO_4^{3-} . The XRD pattern also indicates the (211), (112), (202), and (002) peaks which are the main characteristic peaks of HA. There was no sign of any other calcium phosphates present in the powder.

The highest zeta potential of the suspension was obtained at pH=4. In this pH, the zeta potential was 45.80 mV (Table 1) which indicates a high surface positive charge and stability for the suspension [18]. The synthesized HA powder showed

Table 1

The zeta potential and particle size analysis of the synthesized powder after dispersion in ethanol.

Hydroxyapatite powder characteristics	Zeta potential (mV)	Effective diameter (nm)	Polydispersity
Run 1	43.91	35.5	0.202
Run 2	45.73	33.3	0.248
Run 3	46.48	30.6	0.264
Run 4	48.07	32.3	0.278
Run 5	44.82	34.3	0.294
Mean	45.80 ± 1.59	33.2 ± 1.9	0.257 ± 0.035

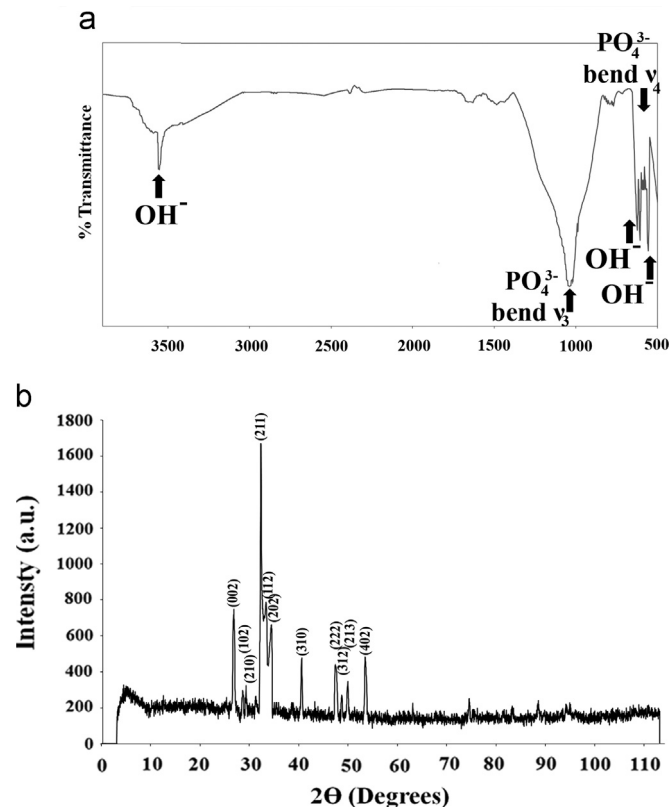


Fig. 1. (a) FT-IR spectra and (b) XRD pattern of nano-hydroxyapatite powder.

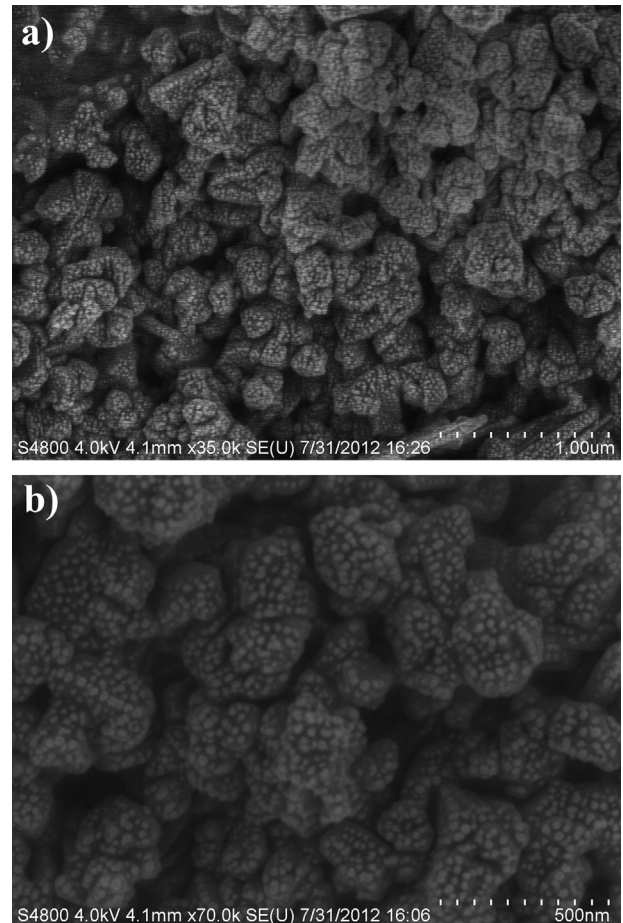


Fig. 2. High-resolution SEM images of HA coatings after the EPD process (a) 35,000 \times and (b) 70,000 \times .

an average particle size of $\sim 33.2\text{ nm}$ in the suspension as revealed by particle size analyzer (Table 1). This was in agreement with the results obtained from SEM images of HA-coated samples which were analyzed by ImageJ software (Fig. 2).

After the EPD and sintering processes, the HA-coated titanium samples were characterized by SEM, XRD, contact angle measurements and EDS. Fig. 3 shows SEM observations of nano-sized pits on the surface of titanium samples after etching and oxidizing with a mixed solution of $\text{HNO}_3\text{--HF--H}_2\text{SO}_4\text{--H}_2\text{O}_2$

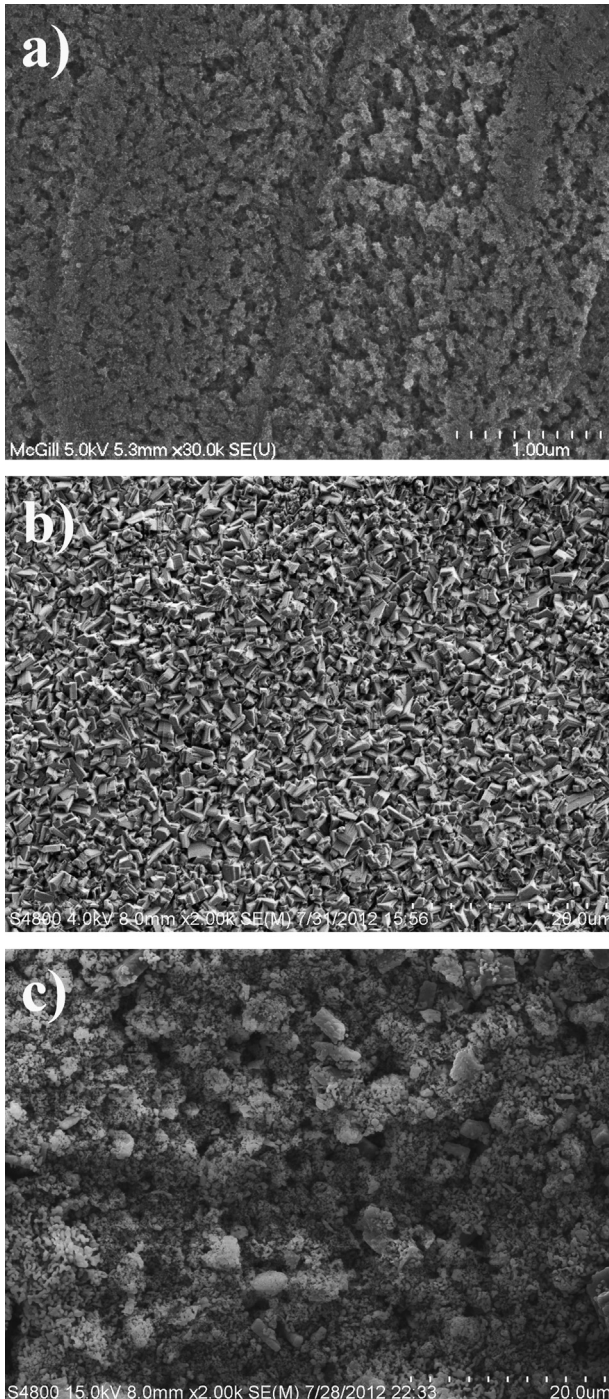


Fig. 3. SEM images of (a) nano-sized pits on the surface of titanium samples after etching and oxidizing, (b) after the EPD process, and (c) after sintering process for 2 h at 800 °C.

(Fig. 3a), after the EPD process and sintering. After EPD, a porous HA coating that contains water molecules in its structure was formed that subsequently was dehydrated to create a homogeneous coating with no observable cracks on the surface (Fig. 3c). It can be seen that before the sintering process the samples present uniform and non-cracked coatings. The thermal process fused the HA particles and increased the grain size of the HA powders significantly. The XRD pattern of HA-coated

substrates revealed that HA deposition and calcination processes caused no phase transformation in the HA coatings; hence, all of HA peaks are identifiable. Also, the sharpness of the XRD peaks is an indication of a high degree of HA crystallinity (Fig. 4). EDS analysis of the HA-coated samples indicated a mole mass ratio of Ca and P, $n_{(Ca)}/n_{(P)}$ of ~ 1.667 in the HA-coated samples that confirms the presence of HA with no decomposition during the EPD process (data not shown). The surface hydrophilicity of the HA-coated and non-coated samples showed significant differences (Fig. 5). The surface of non-coated titanium sample had a higher aqueous contact angle of $67.39^\circ \pm 2.95^\circ$ indicating a less hydrophilic surface compared to the HA-coated sample that presented a water contact angle of $17.41^\circ \pm 1.17^\circ$ (Table 2).

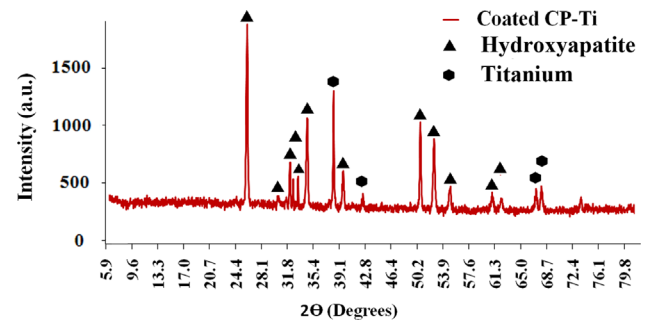


Fig. 4. Low-angle XRD patterns of HA-coated titanium sample.

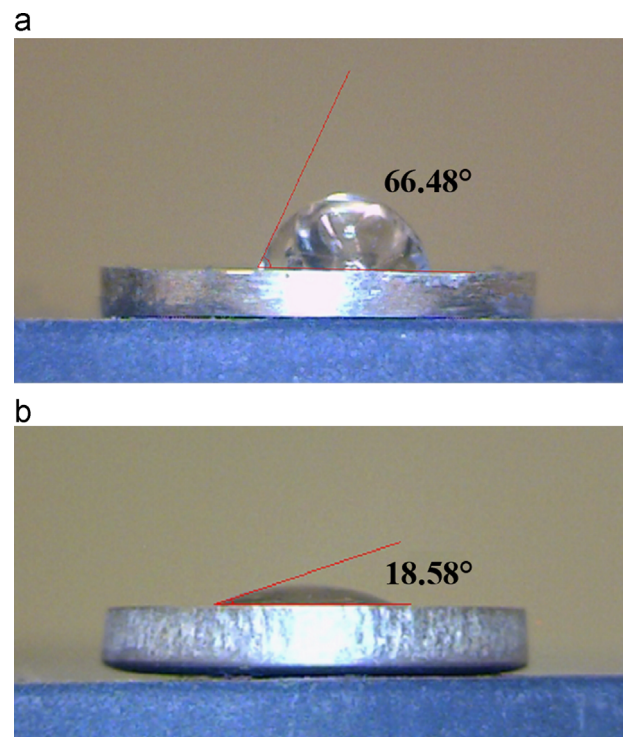


Fig. 5. Water contact angle measurements of (a) non-coated and (b) HA-coated titanium samples.

Table 2

Water contact angle of HA-coated and non-coated titanium substrates. The values were calculated by image analyzer software and reported as mean \pm SD ($n=5$). * $p < 0.01$ compared to the coated sample.

Material	Contact angle (degree)
Non-coated CP-Ti	$67.39^\circ \pm 2.95^{**}$
Coated CP-Ti	$17.41^\circ \pm 1.17^\circ$

3.2. Dynamic voltage EPD process

The voltages of 10, 20, 40 and 80 V were applied to the samples and the related damping currents were obtained as 0.07, 0.15, 0.25, and 0.35 mA. The voltage was then set dynamically in order to keep these currents steady during the EPD process. Fig. 6 shows SEM images of coatings and related cross-sectional views of HA-coated substrates after

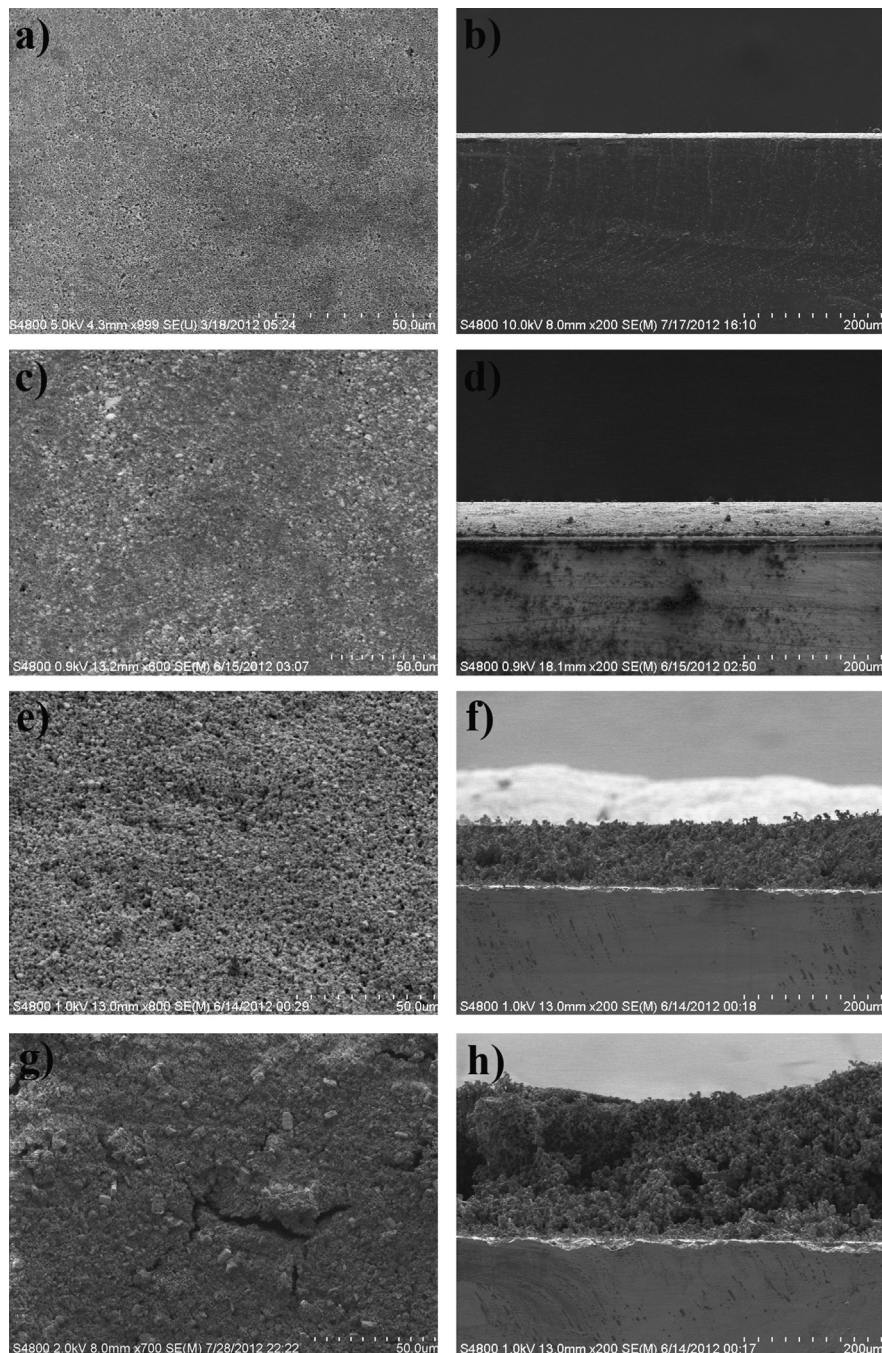


Fig. 6. SEM images and cross-sectional views of HA-coated samples using dynamic voltage-constant current EPD process, (a, b) 0.07 mA, (c, d) 0.15 mA, (e, f) 0.25 mA, and (g, h) 0.35 mA.

EPD at different currents and sintering processes. It can be seen that the thickness of coatings increased as the current increased from 0.07 to 0.35 mA. The sample obtained at the current of 0.07 mA, shows a thickness of 14 μm having a uniform and non-porous structure (Fig. 6a and b). At 0.15 mA, samples were partially dehydrated after the sintering process and homogeneous coatings having the thickness of 45 μm were formed with no cracks (Fig. 6c and d). In higher currents, the HA particles started to arrange in a more porous structure. The samples obtained using the currents of 0.25 and 0.35 mA, showed highly porous, non-uniform, cracked and non-stable coatings with the average thicknesses of 104 μm and 192 μm , respectively (Fig. 6e–h). Table 3 presents the average thickness and adhesion of the coatings obtained at different currents.

3.3. The coating adhesion test

The adhesion of the coatings obtained by the dynamic voltage EPD process was evaluated according to ASTM D3359 procedure on at least five samples. The results demonstrate that the best adhesion for the samples was obtained at 0.07 and 0.15 mA having a mean value of 4.4 and 4.5, respectively. As an example, the removed part was less than 5% ($n=3$) and zero percent ($n=2$) of the whole coating area for the sample obtained at current of 0.15 mA (Fig. 7). Therefore, according to the ASTM D3359, a mean score value of 4.5 ± 0.4 out of five is assigned to the coating which indicates an adequate adhesion to the underlying substrate (Table 1).

Table 3
Thickness and adhesion of HA coatings obtained by EPD process at different currents.

Applied current (mA)	Coating thickness (μm)	Coating adhesion
0.07	14 ± 2	4.4 ± 0.2
0.15	63 ± 5	4.5 ± 0.4
0.25	104 ± 9	2.8 ± 0.2
0.35	192 ± 14	1.2 ± 0.6

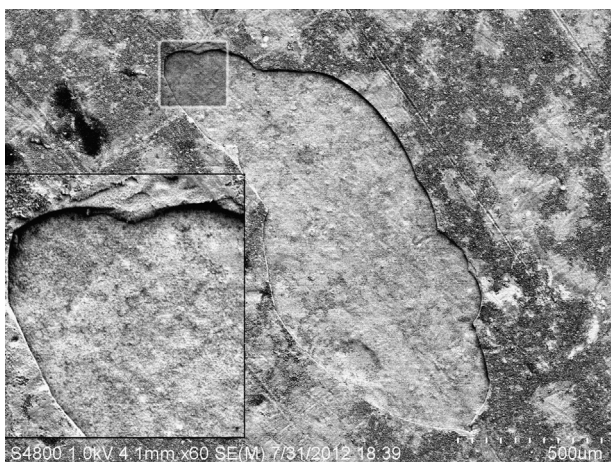


Fig. 7. SEM image of the coating adhesion test for sample obtained at 0.15 mA using ASTM D3359 procedure.

The 0.15 mA was selected as the final current to be used for the coating of titanium substrates in the EPD process, as it provided a non-cracked and uniform morphology as well as the highest coating adhesion. This sample was subsequently tested for its bioperformance in cell culture experiments.

3.4. Cell attachment, proliferation and alkaline phosphatase activity

Experiments of MG63 cell attachment showed that the number of attached cells was affected by the surface chemistry of the samples (Fig. 8a). After the first hour, the amount of MG63 cells adhered on the non-coated substrate was significantly higher compared to the coated sample and control. There was, however, a significantly higher amount of cells attached on the HA-coated samples compared to the control within the first hour. The amount of cells attached on the HA-coated sample increased with longer incubation time being significantly the highest after four hours compared to the non-coated sample and control.

The number of grown cells and alkaline phosphatase (ALP) activity were significantly higher on the HA-coated sample

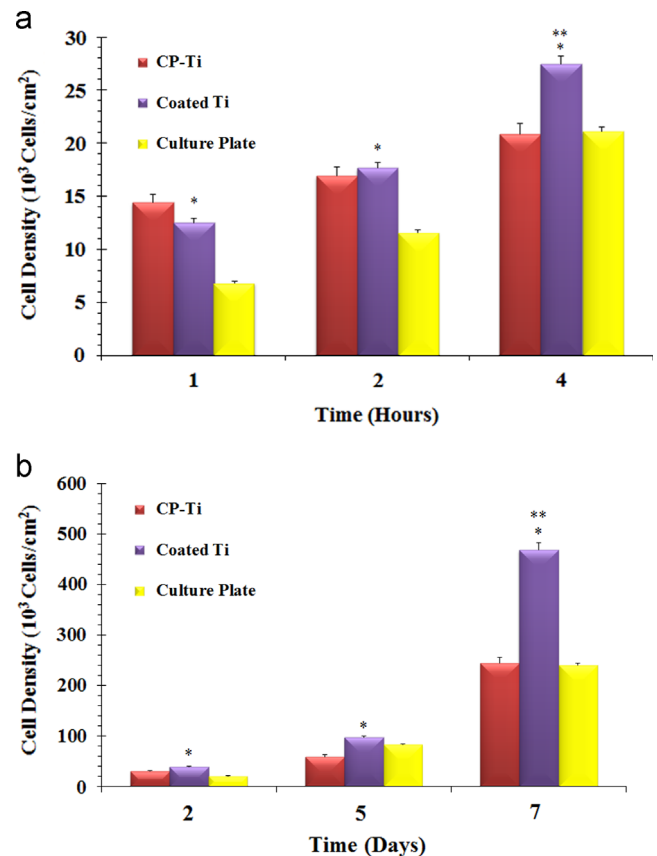


Fig. 8. Histogram showing the osteoblasts (a) attached (b) proliferated on the HA-coated and non-coated titanium samples. Each bar represents the mean of attached osteoblasts \pm SD ($n=3$ in each group) in one of three identical experiments. * $p < 0.05$ compared to control; ** $p < 0.05$ compared to the non-coated sample.

compared to the non-coated sample and control at all times points tested (Figs. 8b and 9).

3.5. Cell morphology

SEM observations showed differences in cell density and spreading patterns among the MG63 cells grown on the HA-coated and non-coated titanium samples after 72 h. The majority of cells, however, exhibited their phenotypic morphology. They were flat with a polygonal configuration and attached to the substrate by cellular extensions (Fig. 10). The cells on the non-coated sample displayed a flattened, osteoblast-like morphology while on the HA-coated sample the cells spread over the coating material and showed more

distinct filopodia to surrounding areas of the substrate and other cells (Fig. 10b and d).

4. Discussion

Among chemical modifications, application of HA coatings on metallic materials has been widely used in order to improve the surface bioactivity and bone bonding ability of substrates. However, there are many limitations in the coatings morphology, uniformity, composition, and most importantly crystallinity and adhesion, owing to the methods of coating. In the present study, an innovative method was used that comprises the application of EPD process at dynamic voltage which aimed to improve the coating morphology, crystallinity and adhesion on the titanium substrates. Application of a constant voltage in the EPD process creates an unstable electrical field on the surface of the electrodes due to the increase in size, thickness and amount of deposited particles. As a result, porous and non-uniform coatings will form on the surface of substrates having poor physical properties. Here, by application of dynamic voltage that keeps the current constant during the EPD process, a uniform and steady electrical field was obtained leading to a uniform and densely packed HA coating. The optimized coated sample was then tested for its bio-performance through cell culture experiments.

The results showed that the low currents of 0.07 and 0.15 mA decreased efficient electrical field and tendency of crack initiation in the coatings, leading to a uniform and non-cracked coating. This is in agreement with previous studies which have revealed that the lower the electrical field, the denser the coating with less observable cracks [12,17]. The

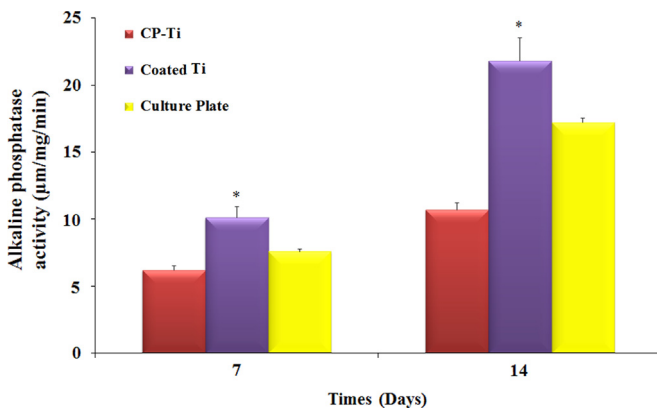


Fig. 9. Histogram showing alkaline phosphatase activity of cells after 7 and 14 days of incubation. * $p < 0.05$ compared to the non-coated titanium sample.

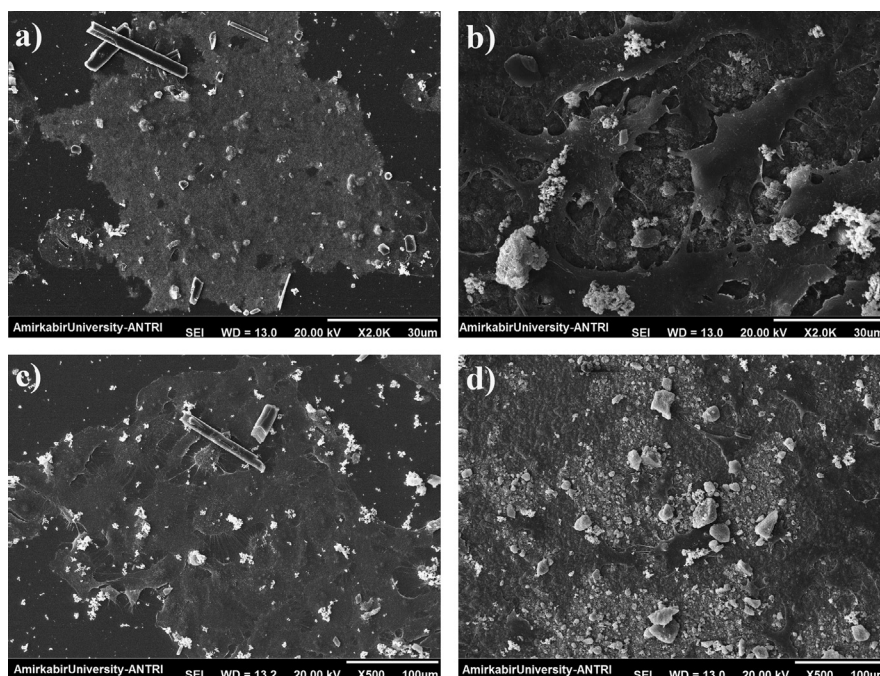


Fig. 10. SEM images of osteoblasts cultured directly on (a, c) non-coated and (b, d) HA-coated titanium samples after 3 days incubation.

higher currents created higher voltages and caused agglomeration of HA nano-particles. Due to the increase in particle size and more rapid deposition in higher voltages, the particles have less time to rearrange [17]. Therefore, the porosity of the deposit increases whereas the large pores in the particles matrix will worsen the mechanical properties of the coating [13,14]. It is essential for the voltage to be set at an optimum value in order to balance out a porous surface for cell growth along with acceptable mechanical properties, simultaneously. Meng et al. demonstrated that HA coatings prepared at dynamic voltage consist of particles with continuous gradient and their morphology is distinct from coatings prepared by constant voltage [23,24]. Although they showed that increasing the dynamic voltage through the EPD process is useful for obtaining a uniform and stable coating, their voltage set up was stepwise. In this study by keeping the constant current, the electrical field near the surface remained stable which resulted in a uniform thickness and morphology for the coating. The SEM images revealed that the HA coating prepared at the current of 0.07 mA, consisted of fine HA particles which were densely packed with low thickness. On the other hand, the HA coatings obtained at currents of 0.25 and 0.35 mA consisted of larger HA particles which were porous due to the higher force of deposition imposed by the larger electrical field. The mechanical properties of these coatings were obviously poor as revealed by their morphology and adhesion tests. Indeed, at the current of 0.15 mA, a much uniform, non-cracked, dense and stable coating that presented good adhesion on the substrate was obtained.

The XRD pattern after the sintering process did not show any peaks relevant to other calcium phosphate phases which indicates the temperature of 800 °C was low enough to avoid decomposition of HA. Many literatures report that the underlying titanium substrate could result in partial transformation of the structure and cause decomposition of HA at temperatures as low as 950 °C [12,25]. Furthermore, the sharpness of XRD peaks indicated a high crystallinity for HA coatings deposited by the EPD process. It is important to note that there was no sign of amorphous calcium phosphate in the coatings and both XRD and EDS analyses confirm the crystalline structure of HA. It is known that crystallinity of a coating is one of the most important factors influencing the bone bonding ability of an implant. It has been shown that non-homogeneous formation of hydroxyapatite crystals along with amorphous calcium phosphate in the surface coatings, leads to instability of the implant which accounts as the main cause of implants failure [26,27]. The amorphous hydroxyapatite is not stable in human body and will dissolve within days after implantation. In contrast, the crystalline phase of hydroxyapatite is believed to remain stable and its crystal structure is similar to that of natural human bone. It is therefore essential to achieve a crystalline phase of HA in order to enhance the bio-performance of an implant material [26]. The hydroxyapatite layers obtained by current coating methods such as plasma spraying, magnetron sputtering and ion beam deposition have been shown to present low crystallinity [27]. It is clear that the application of the EPD process particularly at dynamic voltage

could be an attractive strategy to overcome this issue. It has also been reported that the biomaterial surface crystallinity affects specific cell responses such as cell proliferation and organization of cytoskeleton filaments. The spreading of osteoblasts, for example, is reported to be faster on more crystalline surfaces, mainly due to the development of a more organized cytoskeleton [28]. This would legitimize the different cell morphology, and more lamellipodia and filopodia existence which is observed on the surface of HA-coated samples.

Cell attachment and proliferation on the surface of the HA-coated sample obtained at 0.15 mA showed that osteoblast cells can attach and grow more efficiently on the coated sample compared to the non-coated one. It is believed that the cell–substrate interactions are directly corresponded to the sequential events happening at the organic–inorganic interface. Development of this interface is complex and involves sophisticated mechanisms of interacting water molecules, proteins, and ultimately cells with inorganic substrates. The surface water contact angle was significantly lower in the HA-coated samples compared to the non-coated titanium samples suggesting the higher surface wettability. This is directly related to the difference in the surface chemistry of HA-coated and non-coated samples. Water molecules that attach differently to the surfaces enable the interaction of specific molecules (proteins) involved in the cell substrate interactions [29,30]. Increased surface wettability or hydrophilicity has also been related to enhanced protein adsorption [31,32] and cell spreading [33,34] on biomaterials. Moreover it has been reported that calcium ions in HA coatings form sites of positive charges which aid the adsorption of fibronectin and vitronectin [35] which are the two main proteins affecting cell attachment and spreading [32]. These could clarify the increased cell attachment and proliferation observed on the HA-coated samples.

Finally, our results showed that alkaline phosphatase (ALP) activity, which is conventionally used as the initial marker for osteoblast differentiation [36], was significantly higher on the surface of HA-coated samples compared to the non-coated samples at all time-points being tested. It is however, generally accepted that ALP transcription and activity would increase on any calcium–phosphate coating or in the presence of Ca^{2+} ions which is reflected in improved osteoblast activity and mineralization [37,38]. Therefore, the enhanced bioactivity of the HA-coated samples compared to the non-coated samples could be explained by the combined effects of crystallinity and Ca^{2+} content of the surface [3].

5. Conclusions

In this study, for the first time, the EPD process at dynamic voltage was utilized as a tool to control and improve the physical and biological performance of HA coatings. It is demonstrated that the adhesion, morphology and structure of a coating could be positively affected by alterations in the parameters of the EPD process. It is believed that the physical

and structural alterations in the coatings could improve the biological characteristics of the coatings. These findings have implications for enhancing the quality of HA-coated biomaterials including dental and other bone related implants. Future studies however, should be directed towards the investigation of molecular mechanisms involved in the cell–substrate interactions including the ultra-structure at organic (proteins) and inorganic (crystal structure of substrate) interfaces.

Acknowledgments

This work was supported by Tissue Engineering and Biomaterials Division, National Institute of Genetic Engineering and Biotechnology (NIGEB) and High Impact Research Grant UM.C/HIR/MOHE/ENG/10 D000010-16001 from the University of Malaya. We wish to thank Dr. Dayoush Vashae (Oklahoma State University), Dr. Lobat Tayebi (Oklahoma State University) and Leila Daneshmandi (Amirkabir University) for their guidance and assistance.

References

- [1] J. Duyck, I. Naert, Failure of oral implants: aetiology, symptoms and influencing factors, *Clin. Oral Invest.* 2 (1998) 102–114.
- [2] Z. Schwartz, B. Boyan, Underlying mechanisms at the bone–biomaterial interface, *J. Cell. Biochem.* 56 (1994) 340–347.
- [3] N. Eliaz, S. Shmueli, I. Shur, D. Benayahu, D. Aronov, G. Rosenman, The effect of surface treatment on the surface texture and contact angle of electrochemically deposited hydroxyapatite coating and on its interaction with bone-forming cells, *Acta Biomater.* 5 (2009) 3178–3191.
- [4] O. Albayrak, O. El-Atwani, S. Altintas, Hydroxyapatite coating on titanium substrate by electrophoretic deposition method: effects of titanium dioxide inner layer on adhesion strength and hydroxyapatite decomposition, *Surf. Coat. Technol.* 202 (2008) 2482–2487.
- [5] A. Stoch, A. Brozek, G. Kmita, J. Stoch, W. Jastrzebski, A. Rakowska, Electrophoretic coating of hydroxyapatite on titanium implants, *J. Mol. Struct.* 596 (2001) 191–200.
- [6] M. Javidi, S. Javadpour, M. Bahrololoom, J. Ma, Electrophoretic deposition of natural hydroxyapatite on medical grade 316L stainless steel, *Mater. Sci. Eng. C* 28 (2008) 1509–1515.
- [7] M. Ogiso, Bone formation on HA implants: a commentary, *J. Long-Term Effects Med. Implants* 8 (1998) 193.
- [8] F. Garcia-Sanz, M. Mayor, J. Arias, J. Pou, B. Leon, M. Perez-Amor, Hydroxyapatite coatings: a comparative study between plasma-spray and pulsed laser deposition techniques, *J. Mater. Sci.: Mater. Med.* 8 (1997) 861–865.
- [9] C. Kwok, P. Wong, F. Cheng, H. Man, Characterization and corrosion behavior of hydroxyapatite coatings on Ti_6Al_4V fabricated by electrophoretic deposition, *Appl. Surf. Sci.* 255 (2009) 6736–6744.
- [10] A.T. Rad, M. Novin, M. Solati-Hashjin, H. Vali, S. Faghihi, The effect of crystallographic orientation of titanium substrate on the structure and bioperformance of hydroxyapatite coatings, *Coll. Surf. B: Biointerfaces* (2012).
- [11] M. We, A. Ruys, M. Swain, B. Milthorpe, C. Sorrell, Hydroxyapatite-coated metals: interfacial reactions during sintering, *J. Mater. Sci.: Mater. Med.* 16 (2005) 101–106.
- [12] J. Huang, S. Best, W. Bonfield, T. Buckland, Development and characterization of titanium-containing hydroxyapatite for medical applications, *Acta Biomater.* 6 (2010) 241–249.
- [13] T. Peltola, M. Päätsi, H. Rahiala, I. Kangasniemi, A. Yli-Urpo, Calcium phosphate induction by sol–gel-derived titania coatings on titanium substrates in vitro, *J. Biomed. Mater. Res.* 41 (1998) 504–510.
- [14] J. Ma, H. Wong, L. Kong, K. Peng, Biomimetic processing of nanocrystalline bioactive apatite coating on titanium, *Nanotechnology* 14 (2003) 619.
- [15] F. Chen, W. Lam, C. Lin, G. Qiu, Z. Wu, K. Luk, W. Lu, Biocompatibility of electrophoretic deposition of nanostructured hydroxyapatite coating on roughen titanium surface: in vitro evaluation using mesenchymal stem cells, *J. Biomed. Mater. Res. Part B: Appl. Biomater.* 82 (2007) 183–191.
- [16] N. Eliaz, T. Sridhar, Electrocrystallization of hydroxyapatite and its dependence on solution conditions, *Cryst. Growth Des.* 8 (2008) 3965–3977.
- [17] P. Mondragon-Cortez, G. Vargas-Gutierrez, Electrophoretic deposition of hydroxyapatite submicron particles at high voltages, *Mater. Lett.* 58 (2004) 1336–1339.
- [18] I. Zhitomirsky, L. Gal-Or, Electrophoretic deposition of hydroxyapatite, *J. Mater. Sci.: Mater. Med.* 8 (1997) 213–219.
- [19] L. Besra, M. Liu, A review on fundamentals and applications of electrophoretic deposition (EPD), *Prog. Mater. Sci.* 52 (2007) 1–61.
- [20] A. Boccaccini, S. Keim, R. Ma, Y. Li, I. Zhitomirsky, Electrophoretic deposition of biomaterials, *J. R. Soc. Interface* 7 (2010) S581–S613.
- [21] B. Ferrari, R. Moreno, EPD kinetics: a review, *J. Eur. Ceram. Soc.* 30 (2010) 1069–1078.
- [22] O. Albayrak, C. Oncel, M. Tefek, S. Altintas, Effects of calcination on electrophoretic deposition of naturally derived and chemically synthesized hydroxyapatite, *Rev. Adv. Mater. Sci.* 15 (2007) 10–15.
- [23] X. Meng, T.Y. Kwon, Y. Yang, J.L. Ong, K.H. Kim, Effects of applied voltages on hydroxyapatite coating of titanium by electrophoretic deposition, *J. Biomed. Mater. Res. Part B: Appl. Biomater.* 78 (2006) 373–377.
- [24] X. Meng, T.Y. Kwon, K.H. Kim, Hydroxyapatite coating by electrophoretic deposition at dynamic voltage, *Dent. Mater. J.* 27 (2008) 666–671.
- [25] P. Ducheyne, W. Van Raemdonck, J. Heughebaert, M. Heughebaert, Structural analysis of hydroxyapatite coatings on titanium, *Biomaterials* 7 (1986) 97–103.
- [26] L. Sun, C.C. Berndt, K.A. Gross, A. Kucuk, Material fundamentals and clinical performance of plasma-sprayed hydroxyapatite coatings: a review, *J. Biomed. Mater. Res.* 58 (2001) 570–592.
- [27] A. Mello, Z. Hong, A. Rossi, L. Luan, M. Farina, W. Querido, J. Eon, J. Terra, G. Balasundaram, T. Webster, Osteoblast proliferation on hydroxyapatite thin coatings produced by right angle magnetron sputtering, *Biomed. Mater.* 2 (2007) 67.
- [28] M. Ball, S. Downes, C. Scotchford, E. Antonov, V. Bagratashvili, V. Popov, W.J. Lo, D. Grant, S. Howdle, Osteoblast growth on titanium foils coated with hydroxyapatite by pulsed laser ablation, *Biomaterials* 22 (2001) 337–347.
- [29] B. Kasemo, J. Gold, Implant surfaces and interface processes, *Adv. Dent. Res.* 13 (1999) 8–20.
- [30] E. Zimmerman, L. Addadi, B. Geiger, Effects of surface-bound water and surface stereochemistry on cell adhesion to crystal surfaces, *J. Struct. Biol.* 125 (1999) 25–38.
- [31] J.G. Steele, C. McFarland, B. Dalton, G. Johnson, M.D.M. Evans, C. Rolfe Howlett, P.A. Underwood, Attachment of human bone cells to tissue culture polystyrene and to unmodified polystyrene: the effect of surface chemistry upon initial cell attachment, *J. Biomater. Sci. Polym. Ed.* 5 (1994) 245–257.
- [32] T.J. Webster, C. Ergun, R.H. Doremus, R.W. Siegel, R. Bizios, Specific proteins mediate enhanced osteoblast adhesion on nanophase ceramics, *J. Biomed. Mater. Res.* 51 (2000) 475–483.
- [33] G. Altankov, F. Grinnell, T. Groth, Studies on the biocompatibility of materials: fibroblast reorganization of substratum-bound fibronectin on surfaces varying in wettability, *J. Biomed. Mater. Res.* 30 (1998) 385–391.
- [34] J. Schakenraad, H. Busscher, C.R.H. Wildevuur, J. Arends, The influence of substratum surface free energy on growth and spreading of human fibroblasts in the presence and absence of serum proteins, *J. Biomed. Mater. Res.* 20 (1986) 773–784.
- [35] B. Feng, J. Weng, B. Yang, S. Qu, X. Zhang, Characterization of titanium surfaces with calcium and phosphate and osteoblast adhesion, *Biomaterials* 25 (2004) 3421–3428.

- [36] D. Wang, K. Christensen, K. Chawla, G. Xiao, P.H. Krebsbach, R.T. Franceschi, Isolation and characterization of MC3T3-E1 preosteoblast subclones with distinct in vitro and in vivo differentiation/mineralization potential, *J. Bone Miner. Res.* 14 (1999) 893–903.
- [37] M. Wiens, X. Wang, U. Schloßmacher, I. Lieberwirth, G. Glasser, H. Ushijima, H.C. Schröder, W.E.G. Müller, Osteogenic potential of biosilica on human osteoblast-like (SaOS-2) cells, *Calcif. Tissue Int.* (2010) 1–12.
- [38] S. Maeno, Y. Niki, H. Matsumoto, H. Morioka, T. Yatabe, A. Funayama, Y. Toyama, T. Taguchi, J. Tanaka, The effect of calcium ion concentration on osteoblast viability, proliferation and differentiation in monolayer and 3D culture, *Biomaterials* 26 (2005) 4847–4855.