Fabrication of nanohydroxyapatite-chitosan coatings by pulse electro deposition method

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Research Article

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Fabrication of nanohydroxyapatite-chitosan coatings by pulse electro deposition method

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Abstract

In this study, Mg-2wt% Zn scaffolds were fabricated by powder metallurgy method and the effects of the porosity content on the microstructure and the mechanical properties of the scaffolds were studied. The nano hydroxyapatite-chitosan (nHA-CS) coatings were developed using pulse electro deposition (PED) method to improve corrosion resistance and biocompatibility of scaffolds in the solution body fluids (SBF). The effect of PED parameters (voltage and time) and nHA/CS ratio were studied to achieve optimal nanocomposite coating. The results showed that optimal coating was obtained at voltage 40 V, 10 minutes of coating time and nHA/CS ratio =10. The pulse-peak current density (CD), the pulse duty cycles (DC), pH and temperature for the fabrication of the coatings were conducted at 10 mA cm^{-2}, 0.2 ,7 and 37^{o}C, respectively. In optimal coating, the nHA atomic ratio was obtained Ca/P=1.57, similar to the value of bone hydroxyapatite. The in vitro biocompatibility of the optimal coating was evaluated by cell adhesion, cytotoxicity and alkaline phosphatase (ALP) assays using MG63 cells. The results indicate that the optimal nanocomposite coating was highly biocompatible, making this material more suitable for applications in bone tissue engineering and to repair bone defects caused by sports injuries.

Keywords: Mg-2wt% Zn scaffold, Chitosan, Nano hydroxyapatite, Pulse electro deposition

1. Introduction

Mg-alloys reveal mechanical properties, specifically density and strength, similar to human cortical bone. The essential advantages using Mg-alloys for bone implants are connected to their biocompatibility, bio-absorbability, the lack of surgical removal, osteoconductivity and antibacterial activity. On the contrary, the main limitation of Mg-alloys is due to the poor mechanical resistance of small scaffolds that lack of adequate strength to tolerate high forces. Therefore, an important future prospect could rely in the development of innovative hybrid systems aimed at fixing high load-bearing fractures, as well as in regenerative medicine by developing new Mg-based engineered scaffolds [1-5]. Generally, the surface morphology, microstructure, and composition, which play critical roles in the efficacy of scaffolds, can change protein absorption which intercede adhesion of favorable and unfavorable cells [6]. In order to successfully the development of Mg scaffolds, various kinds of hindrance coatings have been investigated [7]. Calcium and phosphorus are the principal elements in bone tissues and Ca–P coatings, mainly osteoconductive minerals such as HA and tricalciumphosphate (TCP), and have been widely used to make new bones and promote osteointegration on biomedical scaffolds [8-10]. Histological and pathological examinations indicated that the Ca-def HA coating had good osteoconductivity and successful new bone formation on the surface of the Mg–Zn–Ca alloy implant [10]. Fascinatingly, the application of nanostructured CaP to implant surfaces has been investigated as a possible means to increase osseointegration. HA and nHA are mostly fabricated by electrochemical deposition (EPD) [8, 13-15], electrophoretic deposition [16], sol-gel [17-19], cold spray [20] and micro-arc oxidation [21]. Between these technologies, EPD is an absorbing method for the deposition of HA/CS composite coatings for scaffolds. In general, EPD has unique characteristics due to the formation of uniform coating on porous substrates, controlling

the thickness, chemical composition of the coating and low deposition temperature [22]. However, electrodeposition process may increase loose and low adhesive coatings due to the non-favorable effect of concentration polarization and manufacture of hydrogen. This non-adhesive coating will crust during biodegradation and prevents the fixation of implanted material to the host's tissue. Therefore, to solve above problems, it is suggested that PED method is used to deposit uniform and adhesive coating along with optimal morphology [23]. In this research, PED method was applied to fabricate of nHA/CS coatings on Mg-2Zn scaffolds. Biocompatible polymers approved for human clinical applications constitute a favorable option to modify the initial corrosion resistance and cell compatibility on Mg-based materials to meet the healing requirement [6].

CS, a naturally cationic polysaccharide, has also established perfect applications in tissue engineering and drug delivery systems [24-26]. This is essentially because of its cell compatibility biodegradability, non-toxic specifications and antibacterial properties [27-31]. The most attractive characteristics of CS in the fabrication of nanocomposites and coatings are due to its ideal film-forming properties and its cationic nature (Positive surface charges) in aqueous solutions [32]. HA/CS coatings on Mg alloys have been used on preparing improving corrosion resistance, inductivity, and biocompatibility. Hahn et al. [33] prepared a dense and well-adherent HA/CS composite coating on an AZ31 Mg alloy. All the coatings indicated high adhesion strength and better corrosion resistance than the uncoated Mg alloy. Moreover, the biocompatibility of the coated alloy was improved extremely. Zhang et al. indicated that the nHA and chitosan can be got by the electrophoretic deposition. The composite coating had a good protection effect on AZ91D magnesium alloy when the thickness of nHA/chitosan coating was 0.40~ 0.61 mm and the effect of coating thickness on degradation rate of AZ91D magnesium alloy in SBF was elaborated [34]. A few studies have been conducted on improvement of

corrosion resistance and biocompatibility of nHA and nanocomposite coatings on Mg-2Zn scaffold by PED process. This method is often used to prepare coatings on implants since it could control the chemical composition, coating thickness, and suppress the reduction of H_2 [10,23]. Seyedraoufi et al. [35] investigated the effects of the PED parameters and alkali treatment on the properties of the nHA coating on the Mg-2Zn porous scaffold for bone tissue engineering application. Montazeri et al. [36] investigated the effect Graphene oxide contents and PED process parameters, including peak current density and duty cycle on fabrication of CS/GO nanocomposite coatings with Mg–2Zn scaffold. The obtained results showed that the optimal conditions to fabricate a uniform CS/GO coating on the scaffolds were 2wt% GO**,** CD=20 mA cm^{-2}, and DC=0.5. The novelty of the present work is to prepare nHA/ CS coatings on Mg-2Zn scaffold in order to control of coatings morphology by PED parameters.

For this purpose, first the effects of the porosity content on the microstructure and mechanical properties of the Mg-2Zn fabricated scaffolds were studied. Then, the deposition of nHA/CS nanocomposite coatings on the Mg-2Zn scaffold was carried out by a PED method. The effects of pulse voltage, coating time and nHA/CS ratios were evaluated for determination of optimal coating condition. Additionally, the biological responses of scaffolds were evaluated using MTT assay, ALP production and cells attachment.

2. Material and Methods

2.1. Materials

The Mg powder (purity of 97%, Merck) and Zn (purity of 99%, Merck) were used as the raw materials for the fabrication of the scaffolds. Carbamide ($CO(NH_2)_2$) particles purchased from Merck were employed as the space-holder agent. The CS with medium molecular

weight (MW=161,000 g/mol, degree of deacetylation (DD) of 75.6% and viscosity of 1406 m.Pas) was supplied from Aldrich Chemical Co. HA nano powder was synthesized according to Feng et al. method [37].

2.2. Methods

2.2.1. Fabrication of Scaffolds

The Mg and Zn powder mixtures were prepared based on 2wt% Zn, and the urea particles were thoroughly added to the former specimens with different volume contents of (5,10,15 and 25%). After mixing the raw materials with urea particles, porous scaffolds were fabricated by powder metallurgy, applying a press pressure of 200 MPa using a cylindrical mold with an inner diameter and height of 1 cm. The heat treatment cycle consisted of two steps:1) heating to 250 °C at a rate of 1°/min and preserving this temperature for 2 h: 2) heating up to 550 °C at a rate of 10°/min and preserving the final temperature for 2 h. Pore sizes and morphologies of the Mg-2wt% Zn porous scaffolds were observed using Scan Electron Microscopies (SEM).

2.2.2. Coating Process

For preparing the electrolyte solution, about 1g of CS was dissolved in 1wt% Acetic acid (100 ml) at 1100 rpm for 20 hours. The nHA with mean particle size of 50 nm was poured in mold containing ethanol and stirred for 1 hour with magnetic mixer. Finally, these two compounds were mixed to obtain an electrolyte solution. The nHA/CS ratio was 1, 5 and 10. After preparing the electrolyte solution, the coating was applied to the scaffolds. The graphite electrodes were attached to the anode pole, and uncoated scaffolds (Mg-2Zn) were attached

to the cathode poles and they were in parallel with each other. After the completion of the coating, the fabricated scaffolds were washed with distilled water and then dried.

It is worth noting that the coating process was carried at CD =10 mA cm^{-2}, DC= 0.2, T= 37 °C and pH= 7. The voltages of 20, 40 and 60V and coating time of 5, 10 and 20 min were selected as pulsed parameters for coating process.

2.3. Characterization

The compressive strength and modulus of the scaffolds was measured using compression testing of samples with dimensions of Φ 10mm×10mm. The tests were performed with a SANTAM (STM-20, Iran) testing machine at room temperature at a rate of 0.3 mm/s. Each result was taken as the mean value of testing on five samples**.** A Broker-based D8-Advance device detector was utilized for the XRD measurements. The analyses were carried out at all angles of 2θ (from 5° to 45°) with steps of 0.1° and stop time of 0.1 seconds per step at the room temperature and pressure of 1 atm. In this investigation, a SEM (the TeScan-Mira III model) equipped with an energy dispersive X-ray spectrometer (EDS). In order to evaluate the corrosion behavior of coatings applied under different conditions, the dynamic potential test of A263 EG&G device from Artisan Technology Group, made in USA, was performed in SBF at 37 ° C. Prior to test, the samples were first kept in solution for 60 min to stabilize the open circuit potential. All polarization tests were performed from -2.1 to -1.1 volts with a scan rate of +1 mV/s. The reference electrode used was the standard calomel electrode and the platinum auxiliary electrode was used for all electrochemical tests.

The quantitative toxicity MG63 cells culture tests were performed on the scaffolds for 1, 5 and 10 days to study biocompatibility. Human osteosarcoma cell line (MG-63) was obtained from

the national cell bank (The Iran Pasteur Institute, Tehran, Iran). After defrosting the cells, they were transferred to a flask containing DMEM (Dulbecco's Modified Eagle Medium) culture medium with 10% FBS and then the flask was incubated at 37°C, 90% humidity and 5% carbon dioxide concentration. It should be noted that the culture medium was changed every 3-4 days. In order to investigate the toxicity of the samples and their effect on cell growth and proliferation, the extraction process was performed according to ISO 10993-5 standard [38]. To each sterilized sample, 1mm of culture medium was added for every 3 cm^2 of surface. Then, after 1, 5 and 10 days, the medium was removed and added to the cells. A certain amount of culture medium (DMEM) was also considered as a control.

To evaluate the cell adhesion, first 1×10^4 cells with 100 µl of culture medium were poured into each well of 96-well culture plate and then incubated at 37°C for 24 h to allow the cells to adhere to the bottom of the plate. Then, the culture medium was removed from the cells as much as possible and 90 µl of the extract of each sample as well as the extracts diluted with the culture medium along with 10 µl of FBS were added to each culture well. The culture medium was then removed and 100 µl of MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) at a concentration of 0.5 mg/ml was poured into each well and incubated for 4 hours. After 4 h, the solution was removed from the cells and isopropanol was added to dissolve the dark blue crystals. To better dissolve the MTT precipitate, the plate was placed on a shaker for 15 minutes. Then the concentration of the substance dissolved in isopropanol was calculated using an ELISAR device (BioTek ELx808, USA) at a wavelength of 570 nm. Wells with more cells show higher optical density (OD) than wells with fewer cells. Therefore, it is possible to determine the amount of more cells from the well below the comparison and compare it with the control sample. Viability of cells was obtained from Equation 1.

$$Viability\% = (\frac{mean\ \text{OD of sample}}{mean\ \text{OD of control}}) \times 100 \quad (1)$$

To investigate the osteoblastic activity, ALP activity was measured for 1, 5 and 10 days in culture medium. Tissue culture polystyrene (TPS) was used as a negative control group for all tests. The samples were treated with lysis buffer containing
1% Triton X-100. The pellets and supernatants of the cell lysates were separated out via centrifugation at 12000 rpm for 10 min at 4°C, and then the supernatant was used to measure ALP activity. Cell degradation materials were mixed with 0.5 ml of alkaline buffer solution. The mixture was incubated at 37 °C for 15 min in an incubator. After incubation, the reaction was finally stopped by adding 1mL of 0.5 M NaOH as stopping solution to each well. The amount of released p-nitrophenylphosphate (PNP) was estimated by measuring the absorbance at 405 nm by spectrophotometric method. Alkaline phosphatase activity was calculated in terms of the ratio of PNP nanomols converted per minute to mg of total protein. Finally, the amount of total protein was measured using ELISA Kit (Pars Azmoun, Iran).

Statistical analysis

The data were expressed as the mean or the standard deviation of one or more individual experiments wherever applicable. The analysis of data was performed with Graph Pad Prism 6.0, and Origin Pro 8.0 for various statistical analyses ($p < 0.05$).

3. Results and discussion

3.1. The characterization of nHA and Mg-2wt% Zn scaffold

Fig.1 shows the SEM image and the particle size distribution analysis of synthesized nHA powder. As indicated, the nHA structure has a spherical type morphology (Fig.1 (a)). The result of particle size distribution by image analyzer software indicated that the mean size of spherical particles is estimated about 50 nm (Fig.1 (b)).

Fig. 2 illustrates porosity distribution in Mg-2Zn synthesized scaffold by SEM. Additionally, the porosity of the scaffolds was measured by method of Archimedes [39]. Two types of porosity can be distinguished: 1) continuous macro porosity that are determined based on the percentage and size of porous material; 2) closed micro porosity that have formed by the volume contraction of Mg and Zn powders. According to researches of Čapek [40], there are very tiny micro pores even in the scaffolds without porous factor. This is caused by the non-uniform of the powders and not filling the space between powders during pressing the scaffolds.

As indicated in Fig. 2, the pores distribution is similar and the size of macro pores is between 100 to 200 and micro pores are 1 to 10 µm. Since the optimal size of pores to grow and osteoblast cell proliferation is about 100-500 µm [41], therefore, these porosities can be suitable for adhesion and cell proliferation as well as blood circulation and vascularization. Another important characteristic for a sufficient scaffold is compressive strength. The compressive strength of the scaffold must be such that it can withstand the mechanical load during the operation of the body and does not collapse. The percent of porosities, compressive strength and Young's modulus of the fabricated scaffolds given in Table 1. The compressive strength of scaffolds has decreased by increasing porosity; because of the concentration of stress around the porosities. Gibson has studied the mechanical characteristic of sponge bone [42]. They reported that compressive strength of bone is 0.2-80 MPa and its Young's modulus is 0.01-2GPa. In the present research, the compressive strength and Young's modulus of the scaffold with 25% porosity were 25MPa and 1.8 GPa respectively. It is necessary to state, the porosity more than 25% reduces the strength, on the other hand, low porosity prevents the growth of bone cells, and it seems that 25% porosity is suitable for fabricating the scaffolds. Some studies have shown that the porous Mg–Zn specimens with a porosity and pore size of approximately 21–36% vol. % and 150–400 mm, respectively,

could have enhanced mechanical properties comparable with those of cancellous bone. In addition, the compressive strength and Young's modulus of the porous samples decreased with an increase in the volume fraction of porosities at Mg-Zn alloys. Seyedraoufi et al. indicated that the best porosity for improvement of biocompatibility and corrosion properties of Mg-2Zn scaffold coated by nHA, was obtained about 20 vol. % [43].

3.2. The formation mechanism of nHA-CS coating

The weight or thickness of nHA-CS coatings is function of voltage, coating time and nHA/CS ratio. The weight of the obtained precipitates by PED can be described by the following formula [32]:

$$W = \frac{C\mu Ut}{d} \tag{2}$$

Where, C and μ indicate the particle concentration and mobility ions, respectively. (U) is a voltage the deposition process, (t) is deposition time and (d) is a distance between two electrodes. The mobility ions can be expressed by the Smoluchowski equation:

$$\mu = \frac{\varepsilon\zeta}{4\pi\eta} \tag{3}$$

Where, ς is a Zeta potential and ε is electrolyte constant, η is the electrolyte viscosity. According to the equation (2), thickness of deposit increases with the addition of deposition time and voltage. In order to create the higher ς during process of PED, consequently the enhancement of mobility ions, nHA particles was first positively charged in electrolyte solution, and CS-NH_2 was protonated and positively charged in the acetic acid aqueous solution. The reaction formula are as follows:

$$nHA + H^+ \rightarrow nHA - H^+ \quad \text{(Protonated nHA)} \tag{4}$$

$$CS - NH_2 + H_3O^+ \rightarrow CS - NH_3^+ + H_2O \quad \text{(Protonated CS)} \tag{5}$$

$$2H^+ + 2e^- \rightarrow H_2 \quad (6)$$

$$2H_2O \rightarrow 2H^+ + 2OH^- \quad (7)$$

$$nHA - H^+ + e^- \rightarrow nHA \quad \text{(nHA Deposition)} \quad (8)$$

$$nHA - H^+ + OH^- \rightarrow nHA + H_2O \quad \text{(nHA Deposition)} \quad (9)$$

$$CS - NH_3^+ + e^- \rightarrow CS - NH_2 \quad \text{(CS Deposition)} \quad (10)$$

$$CS - NH_3^+ + OH^- \rightarrow CS - NH_2 + H_2O \quad \text{(CS Deposition)} \quad (11)$$

The PED included two processes: the charged particles under the electric field force moved to the cathode and were reduced at the cathode surface. After H^+ was reduced to H_2 on the surface of cathode, the OH^- concentration at the cathode area increased. Under the electric field force, as the positively charged nHA-H^+ and CS-NH_3^+ moved to the cathode, they neutralized with OH^-, accompanying with the cathodic reduction, and then deposited on the surface of cathode (Fig.3). In the pulse current method, the duty cycle (γ) is given by the following equation [36]:

$$\text{Duty cycle}(\gamma) = \frac{T_{on}}{(T_{on} + T_{off})} \quad (12)$$

During the T_{on} period, deposition process initiates and the concentration of ions and species in the adjacency of working electrode is reduced. T_{off} in which the deposition is stopped for a given period, enables the present ions in the bulk electrolyte to move toward the electrode. This may recover the concentration of the electrolyte. Hence, at higher duty cycles, there is a limited time and chance for recovery of the electrolyte concentration so that the nuclei cannot grow sufficiently. The scheme of the deposition mechanism of nanocomposite coating is given in Fig.3.

3.3. Effects of PED parameters

3.3.1. Effects of coating voltage

Fig. 4 indicates the coated scaffolds at 20, 40 and 60V voltages with t = 10 min, DC = 0.2, CD=10 $mAcm^{-2}$ and nHA/CS = 5. As seen, by increasing the voltage, the morphology of the coating changed and became denser. In voltage of 20V, the coating has not formed uniformly on the scaffold (Fig.4 (a)). Indeed, increasing voltage to 40V provides more deposit the particles on the Mg-2Zn (Fig.4 (b)). Park et al. investigated PED of HA-CS coatings on titanium substrate for dental implant. The coatings were composed of 5 to 20% CS by volume. The morphology of Fig.4 (b) is similar to HA-CS coating of Park et al. at voltage 4V, DC=0.5 and 15 vol.% CS [22]. Increasing voltage to 60V can close the pores and prevents the vascularization (Fig. 4(c)). Therefore, it seems the optimal voltage for coating is 40V.

Fig.5 presents EDS analysis of the coated samples with optimal voltage. As indicated, the coating consists of nHA-CS shows calcium, phosphorous and oxygen, that relate to nHA. Besides, the presence of nitrogen can confirm that CS exists at the coating. Ca/P atomic ratio is 1.48 that is lower than the theoretical amount calculated for HA.

3.3.2. Effects of coating time

To study the effect of coating time, following optimal conditions were studied for coating: V=40V at DC=0.2, CD=10 $mAcm^{-2}$, nHA/CS=5 and t = 5, 10 and 20 min. Fig. 6 shows the morphology of the coated scaffolds at t = 5, 10 and 20 min. At t =5 min, the coating is not uniformly formed on the Mg-2Zn and agglomerates is observed (Fig.6 (a)). By increasing the coating time, more uniform coating deposits on the Mg-2Zn scaffold (Fig.6 (b)). Although an increase in the coating time reduces the required porosity for vascularization (Fig.6(c)). The

increment at the time (20 min), leads to penetration of more deposited layer to the pores (according Equation 2) and finally, the pores become closed. It seems that the optimal coating time for Mg-2Zn scaffold is 10 min.

3.3.3. Effects of nHA/CS ratio

nHA/CS ratio was used for coating after determining the optimal voltage and coating time. Fig.7 to 8 show the morphology of coatings with different nHA/CS ratios (1, 5 and 10). As indicated, by increasing the concentration of nHA (Fig.7 (a) to (c)), the coating become denser. According to Fig 8(a), the microstructure includes strings with 100 nm diameter that are similar to nano rods-like. Fig.8 (b) presents that by increasing the concentration of nHA, the microstructure of the coating changes considerably. Increasing nHA/CS ratio from 1 to 5, leads to the formation of agglomerates that are formed of tiny particles. The size of these particles is 50-100 nm. By increasing nHA/CS to 10 (Fig.8(c))), the size of the particles reaches to 100-200 μm. As indicated, the morphology of the particle's changes from nano rod-like to nano spherical-like. Yang et al. showed that cellular behavior can be regulated by HA particles, and the morphology of the particles had a significant influence on cellular behavior. Although both the microsphere and micro rod particles inhibited the proliferation of the cells at a high dose, the inhibition effect decreased or even HA microspheres performed better than the micro rod particles for promoting osteogenic differentiation of marrow mesenchymal stem cells (mBMSCs) [44].

Kalia et al. investigated the biocompatibility and ultra structural effects of two differently shaped hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] nanoparticles (HA NPs), round (aspect ratio 2.12, AR_2) and rice-shaped (aspect ratio 3.79, AR_4). The results showed that, AR_2 had a greater osteogenic effect when compared with AR_4, as measured, suggesting that HA NPs of this shape and/or aspect ratio may have a positive effect upon new bone [45].

The comparison of quantitative results of EDS shows that by increasing the concentration of nHA in the coatings, the amount of nitrogen and carbon in CS has decreased. Indeed, the peak intensity of calcium and phosphorous show slight increase. The Ca/P ratio with nHA/CS =1, 5, and 10 were 3.47, 1.48, and 1.57 respectively. The Ca/P ratio of coating with nHA/CS =10 is closer to the bone. Considering the spherical morphology and Ca/P ratio, it seems coating with nHA/CS=10 ratio is optimal.

The XRD analysis was also used to study constituent phases of three deposited coatings on Mg-2Zn scaffolds (Fig.9). As indicated, all coatings have both nHA and CS phases. The important peaks related to nHA are observed at angles of $2\theta=32.2^{o}$ and 47^{o} [46]. The XRD pattern of the CS exhibits the two peaks, one at 14.2^{o}, which corresponds to the hydrated crystalline structure, whereas the broadened peak at about 20.4^{o} indicates the existence of an amorphous structure [47]. Moreover, the intensity of the nHA diffraction peaks enhanced with increasing nHA/CS ratio [48].

3.3. Electrochemical corrosion of optimal coating and Mg-2Zn scaffold

In this study, polarization curves were used to compare corrosion resistance Mg-2Zn scaffold and optimal coating (see Fig.10). Table 2 lists the parameters extracted from the polarization. curves of the samples in SBF solution. Generally, cathodic polarization curve presents cathodic transformation of hydrogen by reduction of water while anodic curve indicates the dissolution of alloy Mg. As seen, anodic polarization curves of two samples have passive areas that proves the presence of the protective layer on the surface. In fact, optimal nanocomposite coating prevents the corrosion of Mg by developing a passive layer.

As can be observed, the corrosion potential of the nHA-CS coating is more positive than that of the Mg-2Zn scaffold and the corrosion current density of the nHA-CS coating was reduced

by 97.2%. Moreover, the corrosion rate (mpy) of the Mg-2Zn scaffold was 2.09 mm/year, which was very high and inappropriate in compare to the nHA/CS coating with corrosion rates of 0.58 mm/year. Therefore, nHA-CS coating can significantly improve the corrosion resistance of the Mg-2Zn scaffold. Tian et al. [49] studied the effect of nHA and PLGA (lactic-co-glycolic acid) composite coating on pure Mg. The results indicated that nano composite coating leads to more positive corrosion potential and reduces corrosion rate up to 53%. Moreover, CS coating on sheets of AZ31 alloy led to the corrosion rate of diminished from 1.45 to 0.064 mm/y in NaCl solution (3.5 wt%) [50].

Seyedraoufi et al. [35] studied the effect of nHA coating on corrosion rate of Mg-3Zn scaffold in SBF solution. Their results showed that calcium, phosphate and sulfate ions can deposit on the surface of Mg-3Zn and prevent its corrosion by formation of a passive layer. Corrosion potential illustrated significant increase in the coated sample compared to non-coated sample The current density of the non-coated and coated samples was 8×10^{-3} and 1.4×10^{-4} Acm^{-2} respectively . Therefore, nHA coating has improved the corrosion resistance of Mg-3Zn. In the present research, the current density of optimal coating is 2.68×10^{-6} A cm^{-2} (Table2), which has a significant reduction compared to the work published by Seyedraoufi et al. It means using nHA/CS nanocomposite coating on Mg-2Zn scaffolds can notably modify the corrosion resistance of Mg-2Zn.

3.4. In vitro cell behavior

The MTT assay was further used to quantify the proliferation of MG63 cells cultured onto the Mg-2Zn and optimal coating after 1, 5 and 10 days (Fig. 11(a)). After 1 day, the average cell viability for Mg-2Zn and scaffold with nHA-CS optimal coating were found to be 110, 139 % respectively compared to control substrate (TPS). Similarly, after 5 days, these scaffolds

exhibited 65 and 100% cell viability respectively. It is clear that the cells on the nanocomposite coating proliferated better than Mg-2Zn scaffold, mainly due to the simultaneous effect of nHA and CS coating. Therefore, nHA/CS composite coating can be used as a suitable coating for Mg-2Zn in order bone tissue engineering.

Osteoblastic activity of the MG-63 cells seeded on scaffolds determined by measuring ALP activity in culture media for 1, 5 and 10 days. It is observed that was significantly higher for all scaffolds in comparison to control sample (TPS) after 1 day. However, by increasing the incubation time, ALP expression was decreased. Meanwhile, the relative ALP activity of the nHA/CS optimal coating was significantly greater than the Mg-2Zn scaffold. The highest value of relative ALP activity was observed for the nHA/CS optimal coating at 1 day. Dhivya et al. demonstrated that the presence of nHAp in the Zn-CS/nHAp/β-GP hydrogel increased swelling, protein adsorption, and exogenous biomineralization. Moreover, the addition of nHAp to the hydrogel develop osteoblast differentiation under osteogenic conditions in vitro and accelerated bone formation in vivo in respect to the depositions of apatite and collagen [51]. Suo at al. investigated the enhancement of osseointegration using HA, CS and GO/CS/HA composite coatings on titanium fabricated by electrophoretic deposition. The results show that HA and CS coating considerably enhanced the bone marrow stromal cells (BMSCs) interactions in vitro. Moreover, this GO/CS/HA coating could increase osseointegration in vivo. As a result, GO/CS/HA may have potential applications in the field of dental implants [52].

The cell morphology of the MG63 cultured cells on Mg-2Zn and nHA/CS optimal coating was illustrated after 5 h in Fig.11 (c and d). The spindle shaped cells could be seen on the Mg-2Zn and nHA/CS optimal coating, but the MG63 cells on nHA/CS surface exhibited better stretch with more Filopodia extensions (Zones with red circles) compared with Mg-2Zn scaffold. The existence of multiple Filopodia extended on coating provides strong

evidence that the cells are well attached to the nHA/CS optimal coating. Therefore, the nHA/CS coating help the initial attachment, proliferation and growth of MG63 cells.

4. Conclusions

The nHA/CS nanocomposite coating with PED method was obtained to improve the biocompatibility of porous Mg-2Zn scaffolds. The results showed that changing PED parameters influences the morphology of the coating. Optimal coating conditions in PED process obtained V=40V, T= 10min, nHA/CS=10 at DC=0.2 and CD =10 Acm^{-2}. The ratio of calcium to phosphorus in the optimal coating composition was 1.57. The study of the effect of optimal coating on Mg-Zn corrosion resistance with 25% porosity indicated that corrosion rate (mm/year) decreased 72.2%. In vitro biocompatibility assay using human osteosarcoma cell line (MG-63 osteoblast) revealed that the nHA-CS optimal nanocomposite coating can provide adequate support for cell growth, proliferation and differentiation of MG-63 cells during in vitro cytological examination. Finally, the research results suggest that the PED technique provides an alternative method for manufacturing of the nHA-CS optimal nanocomposite coating on Mg-2Zn alloy and this coating may be applied in the field of scaffold to repair bone defects caused by driving accidents and sports injuries.

5. Acknowledgements

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6. Declaration of Conflicting Interests

The authors declare no conflict of interests

7. References

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Tables:

Table 1: Porosity percentage, compressive strength and Young's modulus of Mg-2Zn scaffolds

Sample	Urea (Volume percent)	Porosity (%)	Compressive Strength (MPa)	Young's modulus (MPa)
M_1	0	10	90±5	8.9±0.4
M_2	5	18	49±3	3.5±0.2
M_3	15	25	30±2	1.8±0.2
M_4	25	32	9±2	0.3±0.01

Table 2: Electrochemical parameters of the obtained from the polarization curves of Mg-2Zn scaffold and optimal nanocomposite coating

Scaffold	Corrosion potential (V)	Corrosion current density (Acm^{-2})	Corrosion rate (mm/year)
Mg-2Zn	-1.64	9.76×10^{-5}	2.09
Optimal coating	-1.52	2.68×10^{-6}	0.58

Figures

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