Endothelialization and the bioactivity of Ca-P coatings of different Ca/P stoichiometry electrodeposited on the Nitinol superelastic alloy

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Abstract

An alternative approach to improve the cardiovascular stents with less restenosis than drug eluting stents, involves an improvement in endothelialization of implants. In this study, the bio compatibility of the modified Ti-50.9Ni alloy was investigated. At the first step, a thermo-chemical surface modification process was used to control the Ni release of the alloy. XPS and Raman analysis revealed that the surface of the alloy contains titanium dioxide after the modification process. According to the Ni release test, this surface condition has a good durability in Ringer's solution and offers a standard range to the leached Ni. At the next step, porous Ca-P films were electrodeposited on the modified surface. The results of endothelial cell culture on the coated samples revealed that the Ca-P coating, which has the highest value of Ca/P ratio shows the best result. The coating revealed a moderately wettable surface with a water contact angle of 53.3°. According to Ca content analysis of the cell culture medium, this coating has the lowest amount of Ca as a result of minimum solubility of the coating. In the other Ca-P coatings with lower Ca/P ratios, the solubility of coatings results in the detachment of the cells. Also nano-indentation and SEM studies revealed that the low

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stiffness in the calcium deficient coating can result in the failure of the coating as a result of the tensions created by the cells.

Keywords: Nitinol; Electrocrystallization; Calcium Phosphates; Ca/P stoichiometry; Endothelialization.

1. Introduction:

NiTi alloys have considered as an important biomaterial since several years ago [1, 2]. In most other metallic implants, the elastic module mismatch between the implant and surrounding tissues results in the implantation failure [3]. The low difference between the elastic modulus of NiTi alloys and that of the human tissues makes the alloys a suitable candidate for implant materials [4]. Also the superelastic property of the alloys makes them exclusive for especial applications such as self expanding cardiovascular stents [5].

There are some problems in the application of these alloys as implants especially because of the Ni release and the bio inert surface. Not only does the nickel ion lead to allergic symptoms, but it also weakens the proliferation of cells in the NiTi surface [6, 7]. Also the bio inert surface leads to the formation of a nonadherent layer at the surface of implants and failing of the implantation [8-11].

Although calcium-phosphate (Ca-P) based compounds offer a good medium for living body cells growth, they cannot be used as bulk materials in implants because of the poor mechanical properties. Using Ca-P based coatings on the surface of metallic implants is an effective method for achieving the both advantages of metallic implants and Ca-P compounds [12, 13].

Different methods have been introduced for the deposition of Ca-P compounds [12-18]. Electrocrystallization is one of the effective methods for the deposition of these compounds in low temperature with controlled thickness, crystallinity and morphology [18].

Calcium phosphate coatings have demonstrated to favor the hard tissue integration in orthopedic implants [19, 20]. On the other hand, there are some ideas for the application of these coatings on the cardiovascular stents. These types of coatings can be used in drug eluting stents. In spite of the polymer coated stents, Ca-P coatings do not increase the platelet activation in circulating human blood [21].

In drug eluting stents, the growth of smooth muscle cells is inhibited inside the vessels by a controlled drug releasing technique. Although this technique would be helpful at the initial stages of stenting, preventing endothelial cells growth can result in other problems such as restenosis over time. Accordingly, as an alternative approach, improving the endothelialization of the stents is the subject of recent researches [22, 23].

Despite the progress made towards Ca-P deposition, the influence of the coating properties in controlling the adhesion and proliferation of endothelial cells is still poorly understood. There are some reports on the bioactivity of calcium phosphates which have characterized some other kinds of cells and deposition methods. For example, it was shown that the Ca/P molar ratio of bioglasses has a significant effect on the fibroblast cells growth [24]. In the field of bone implants, because of the structural similarity between bone and calcium deficient hydroxyapatite, most studies have been focused on the calcium deficient hydroxyapatite [25]. Even though, there are some reports which show the best growth condition for osteoblasts occurs at Ca/P ratio around 1.7 in bulk Ca-P materials [26, 27].

The main objective of this study is investigating the potential of Ca-P coatings in endothelialization of superelastic Nitinol alloys. For this purpose, the effect of Ca/P ratio on the biological properties of the coated samples was investigated. The chemical and

nanomechanical tests were carried out on the coatings to justify the observed in-vitro behaviors.

2. Experimental details:

2.1. Preparation of specimens

In this work, NiTi rod with nominal composition of 50.9% Ni was used as substrates. The rods with the diameter of 13mm were sliced into 1mm disks. The surface of the samples was abraded with different grades of SiC papers from P80 to P600 grit and then was etched in an acid solution of 1 HF-4 HNO_3 -5 H_2O for 4 min, and finally was soaked in distilled boiling water for 20 min. After each step, specimens were cleaned in acetone and then rinsed with deionized water. For the heat treatment process, the specimens were encapsulated in a glass tube after purging and vacuuming the high purity Ar gas inside the tube for several times. The heat treatment carried out at 470 °C for 30 min.

2.2. Electrochemical deposition

The electrodeposition was performed in an individual cell using a regular three electrode configuration in which NiTi alloy served as the cathode and a platinum mesh act as the anode. Mechanical stirring of the electrolyte was controlled at a speed of 150 rpm for all depositions. Fig.1 shows a schematic representation of the electrochemical cell which demonstrates the relative position of each component in the cell. The selected positions result in a uniform stream of the electrolyte from the anode to the cathode surface.

Three different conditions were used for the electrodeposition of calcium-phosphate based coatings. In the (A) condition, an electrolyte solution of 0.042M $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and 0.025M $\text{NH}_4\text{H}_2\text{PO}_4$ and 6 ml/l H_2O_2 was prepared and used. The pH of the electrolyte was 4.3 and the temperature of the electrolyte was maintained at $70 \pm 1^\circ\text{C}$ using Bain-Marie method. The

deposition was carried out in a galvanostatic mode and the current density was maintained at -0.6 mA/cm^2 .

In (B) and (C) conditions an electrolyte solution of $0.0084 \text{ M Ca(NO}_3)_2 \cdot 4\text{H}_2\text{O}$, $0.005 \text{ M NH}_4\text{H}_2\text{PO}_4$, 0.1 M NaNO_3 , and $6 \text{ ml/l H}_2\text{O}_2$ was prepared and used. The pH of the electrolyte was 6.0 and the temperature of the electrolyte was maintained at $65 \pm 1^\circ\text{C}$ using Bain-Marie method. An inert gas was purging during electrodeposition to decrease the carbonate contamination. In the (B) condition the deposition was carried out in the galvanostatic mode at the current density of -0.5 mA/cm^2 . In the (C) condition, a pulse current with a direct current (ON) time of 1s and reverse current time of 2s was used. The direct and reverse current densities were -3 and 0.1 mA/cm^2 respectively.

2.3.Characterization of modified samples and coatings

The micro-Raman spectra were recorded on a LabRam confocal Raman microscope with a 300 mm focal length. The spectrometer was equipped with a Ventus LP 532, 50 mW, diode pumped solid-state laser operator at 20 mW, with a polarization ratio of 100:1, a wavelength of 532.1 nm, and a 1024x256 element CCD camera.

X-ray photoelectron spectroscopy (XPS) analysis was performed with a PHI 5000 Versa Probe spectrometer (F-ULVACPHI Inc., Japan/USA) using monochromatized Al $K\alpha$ radiation (1486.6 eV) as an X-ray source with a 45° electron collection angle. The high resolution spectra of the surface constituents were acquired from three spots with 58.7 eV pass energy.

For the Ni release test, four samples from each group were immersed in a plastic bottle containing Ringer's solution with the composition of 8.6 g NaCl, 0.6 g KCl, and 0.66 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in 1L H_2O . The surface area of each sample was fixed at 1 cm^2 and the prepared electrolyte pH was 5.9 at room temperature. The bottles were closed tightly and incubated at

37±0.1°C for four weeks. The Ni concentration of the solutions was analyzed regularly by Graphite Furnace Atomic Absorption Spectroscopy (PerkinElmer, Germany). Prior to each sampling the bottles were shaken gently.

For the electrodeposited samples on Nitinol substrates, Attenuated Total Reflectance Fourier Transform Infrared spectroscopy (ATR-FTIR) analysis were carried out on Bruker alpha (Germany) platinum ATR equipped with a diamond crystal. The spectra were recorded between 400-4000 cm⁻¹ and resolution of 4.0 cm⁻¹ by accumulation of 130 scan.

Contact angles were measured with deionized water drop at ambient condition using a CMOS camera (BEL Products, Italy) attached to a microscope (TZM-2). Samples were immersed in pure ethanol and then dried completely before the measurement. Analyses of the results were carried out by image processor software (ImageJ-DropSnake analysis). At least measurements on three similar samples were made and averaged.

The surface morphology and composition of the deposited films was examined by a Cam Scan MV2300-Czech scanning electron microscope (SEM) equipped with an energy dispersive spectroscopy (EDS) analyzer. The operating voltage in the microscope was 20.0kV.

For the measurement of Ca content in the cell culture medium (Dulbecco's Modified Eagle's Medium-DMEM), coated samples were soaked inside DMEM at 37 °C for 48h. The amount of calcium was measured using an Agilent (200 AA, USA) atomic absorption spectrometer. For this purpose the calcium (Ca) single element hollow cathode lamp (Agilent, USA) was used in the experiment.

Nanoindentation measurements were performed on the coated samples using a CSM nanoindenter (MF 33049, Switzerland) with a Berkovich diamond indenter. The indents were all performed in a load-control mode with a loading rate of 2 mN/min and a dwell time of

20s. The analyzing of obtained data was performed by Oliver–Pharr method. At least the mean value of five indentations was used for reporting the data.

2.4. Cell culture

Human Umbilical Vein Endothelial Cells (HUVECs, Pasteur Institute, Tehran, Iran) were cultured in DMEM culture medium (Cat. Number: F4815- Biochrom company, Germany) supplemented with 10% fetal calf serum (FBS, Cat. Number: S0115-Biochrom company, Germany) and 1% penicillin/streptomycin antibiotic solution (Cat. Number: A2213-Biochrom company, Germany) and incubated at 37°C in a humidified incubator with 5% CO₂ till 70% confluency. After that, cells were passaged using Trypsin/EDTA solution. The supernatant was taken away and cells were washed twice with sterile Phosphate-Buffered Saline (PBS) for neutralizing FBS. The second-passage cells were harvested by trypsinization and condensed to a concentration of 100.000/10µl.

Before being used, the Ca-P coated samples were sterilized by 70% ethanol for 20 minutes, and then prewetted in DMEM medium. Samples were then placed in a 24-well culture plate (1 sample per well). Cell suspension of 10 µl was dropped on the top of the scaffold and then 1 ml of DMEM/F12 medium containing 10% FBS (supplemented as described above) was added carefully to each well. The constructs were incubated at 37°C/5%CO₂. Samples were undertaken for evaluating with scanning electron microscope (SEM) on 2 days after seeding. After 48h incubation of samples in the cell culture medium, cytotoxicity was assessed using the cell counting Kit-8 assay (Dojindo, USA).

For investigation of constructs using SEM, supernatant was removed; cell/scaffold constructs were washed with PBS and then fixed with 5% glutaraldehyde (Sigma–Aldrich, Taufkirchen, Germany) overnight and washed again with PBS. Subsequently, the cells were dehydrated in

an ethanol series (30%, 50%, 70%, 80%, 90%, 96% and 100%) for 5 min each. After drying in still air, the samples were coated twice with gold for SEM analysis.

3. Results and discussion:

For the examination of the oxide types on the heat treated samples, Raman spectra were gathered on the chemically modified and unmodified samples after the heat treatment at 470°C for 30 min. According to Fig. 2, Raman spectrum of the chemically modified alloy indicates rutile specific peaks after the heat treatment process. While for the abraded substrate, the oxide film is mainly composed of anatase that is less stable than rutile phase [28]. It seems that the formation of some metastable titanium oxides during the chemical modification process promotes the formation of rutile phase during subsequent heat treatment process.

The high resolution XPS spectra of the chemically etched, boiled in distilled water and age hardened specimen are presented in Fig. 3. Beside to these spectra, the high resolution spectrum in the binding energies between 840-890 eV was also gathered. According to the data, neither Ni oxides nor metallic Ni were detected by XPS. As a result, after the surface treatment process the outermost surface of the alloy is completely depleted from Ni. As the location of Ti 2p_{1/2} and 2p_{3/2} spectrum indicates, Ti is in the oxidized +4state [29-31]. According to the spectrum, there is no evidence for the presence of metallic titanium, titanium monoxide, and titanium carbide in the outermost layer of the film [32, 33]. The 1s spectrum of oxygen shows a double peak configuration. The lower binding energy peak around 530.0 eV originates from titanium oxide and the higher binding energy peak around 531.5 is for hydroxyl groups (–OH). The result indicates the formation of Ti-OH groups in the oxidized surface of the modified alloy [34].

The results of the Ni release test on the chemically treated and untreated samples after the age hardening process are presented in the graph of Fig.4. In the chemically treated and age hardened sample, the results show a good protection ability of the oxide layer all along the test. Although in both conditions the standard range of the released Ni is satisfied (200 µg/L) [35], the lower value of the leached Ni in the chemically treated substrate makes it possible using an implant with the higher surface area in comparison with that of the only aged sample.

Considering Fig.4, an in-vitro test for several days cannot show real differences between the chemically treated and untreated substrates. It seems that the oxide layer on the only age hardened sample can protect the Ni release into Ringer's solution at the early stages of the test; however breakdown of this layer after one week can result in dissolution of Ni from Ni-rich compounds.

Based on the results of the Ni-release test, the chemically modified and heat treated sample was used for the post deposition of calcium phosphates. The surface morphologies of deposited Ca-P coatings in three different electrochemical conditions are shown in Fig. 5. In (A) condition the coating shows a needle like morphology while in (B) and (C) conditions the coatings are composed of Ca-P plates. The Ca/P ratio of the coatings in (A), (B) and (C) conditions were measured with EDS analysis and the values were 1.4, 1.5, and 1.7 respectively. A typical EDS spectrum for the coating of the C condition is shown in Fig. 5d.

The FTIR spectra of the coatings are shown in Fig. 6. In these spectra the infrared bands at 525, 915, 1055, 1075, 1192 cm^{-1} are assigned to biphosphate groups (HPO_4^{2-}). Also the bands at 630 and 870 cm^{-1} are related to OH^- and CO_3^{2-} respectively. The rest bands correspond to PO_4^{3-} group. According to EDS and FTIR analysis, the coating of the (C) condition is mainly composed of HA while in the coating of the other conditions there are some structures

containing biphosphate groups. The presence of biphosphate groups can be related to the co-presence of brushite and octa calcium phosphate in the coatings.

The SEM micrographs of endothelial cells adhered to the prepared Ca-P coatings are shown in Fig. 7. Measurement of cytotoxicity revealed that there was no cytotoxicity symptom caused by any of the specimens. It can be seen that more cells grew and adhered to the specimen of the (C) condition. In the (A) condition, although there are some connections between cells and the coating, the cells are not highly spread out over the material surface. From (A) to (C) condition, cells become more flattened on the coating and at the (C) condition the cells are well connected to the coating through propagating on the fine structure of the coating.

The wettability of Ca-P coatings were quantified by the drop contact angle method (Fig. 8). Significantly different contact angles were observed for the Ca-P coatings at different electrodeposition conditions. The (A) electrodeposition condition was found to be the most hydrophilic coating with a contact angle of 4.1° . The highest contact angle was observed for the (C) electrodeposition condition (53.3°). It has been shown that endothelial cells reveal optimal adhesion to moderately wettable surfaces with the contact angle around 55° and both more hydrophilic and more hydrophobic substances demonstrate poor adhesion of human endothelial cells [36, 37].

Although the contact angle is an important factor in biomaterials, there are some other factors that influence the bioactivity of a material. While the water contact angle value of the modified bare Nitinol alloy was something between that of the (B) and (C) coatings (39.2°), after endothelial cell culture test the surface was almost free of cells and there were only a limited number of rounded shape cells on the alloy (Fig. 9). This result shows that there are some other specifications in Ca-P coatings that have positive effect on the bioactivity.

The calcium analyzing of the cell culture medium after incubating the coated samples inside DMEM are listed in Table 1. The Ca content in the (C) condition shows the lowest value in comparison with that of the other conditions. Because HA is the most stable phase between calcium phosphates, during soaking in DMEM the coating acts as nucleation sites for precipitation of Ca rich compounds. While in the coatings containing brushite or other metastable compounds, dissolution of coatings happens beside the precipitation process. The precipitation of Ca-rich compounds at the surface of HA is accompanied by the diffusion of calcium ions to the surface of the coating for producing a supersaturated state there.

Table 1- Ca content of DMEM after incubation samples at 37 °C for 48h

	Empty well	(A) condition	(B) condition	(C) condition
Ca content (ppm)	77.48±0.001	66.80±0.001	62.02±0.001	52.52±0.001

It was shown that the calcium ions have an important role in cell to cell junctions and the generation of cadherins in endothelial cells [38-40]. Also calcium ions in apatite coatings can act as active sites for proteins adhesion, thereby cells can adhere to the calcium rich coatings [41]. It seems that, a super saturation condition at the initial stages of cell culture test and the stable calcium ions site in the apatite coating can result in better adhesion of cells to the coating of the **C** condition in comparison with those of the other conditions. Furthermore it must be considered that, if dissolution of the coatings happens in the **A** and **B** conditions, the cells binding sites to the specimens will be lost that will result in lower cell density after the test.

For the (**A**) electrodeposition condition, some extended cells can be found on the coating which are very rare in the whole surface of the specimens in this group (Fig. 10). According to Fig.10a, while the cell is well stretched on the surface of the (**A**) condition coating, the cell

connection to the coating was lost in some areas which resulted in the splitting of the cell from the coating. This can occur as a result of the coating dissolution as mentioned earlier.

Another important factor in the attachment of cells to biomaterials is compatibility of mechanical properties with cells [42, 43]. According to Fig. 10b, although there is a connection between the cell and the specimen of the (A) condition, the coating was cracked completely as a result of the stresses that were introduced by the propagated cell.

Obtained results of the nanoindentation test on the coated samples are listed in Table 2. The coating of (A) condition shows the lowest value of stiffness and hardness which can be resulted from the specific morphology in this type of coating. It seems that the vertical plates of calcium phosphates in the (B) and (C) condition can withstand better against the indentation loads in comparison with the ultrafine whiskers.

Table 2- Nanoindentatio results for the Ca-P coatings electrodeposited at different electrochemical conditions

Electrodeposition conditions	(A)	(B)	(C)
Module of Elasticity (GPa)	6.58	26.92	11.68
Hardness (GPa)	1.54	7.21	6.17
Stiffness (mN/nm)	0.11	0.21	0.12

According to the literature an intermediate value for stiffness and hardness is required for an appropriate interaction between biomaterials and cells. It was shown that a proper connection cannot be seen in soft biomaterials. Also cells spreading and the kinetic of cell adhesion are not appreciable in completely rigid and hard materials [44]. According to SEM micrographs, the coating in (C) condition has enough stiffness for withstanding against the stresses which arise during the cell spreading. In this condition the stress fibers of the cells can produce

enough force for the spreading of cells. Probably the highest value of stiffness in the (B) condition can be another reason for the lower attached cells to the (B) coating in comparison with that of the (C) condition.

Conclusion:

Based on the results of this work, the heat treatment of the chemically modified Nitinol alloy at 470 °C for 30 min results in the formation of titanium dioxide at the surface of the alloy and completely removing Ni from the outermost layer of the film. According to the Ni release test, this surface condition has a good durability in Ringer's solution. Although the heat treatment process of the abraded sample results in the formation of anatase at the surface, this treatment cannot offer a reliable condition against the Ni release. Results of endothelial cell culture on the treated samples with a Ca-P coating show the best cell attachment on the pure hydroxyapatite film. Considering the Ca content of the cell culture medium after 48h, the lowest calcium content is related to the coating with the highest Ca/P stoichiometry. Also the coating has a moderate wettable surface with a water contact angle of 53.3° which offers a suitable condition for the cells growth. In the coating with the lowest value of stoichiometry, the stiffness and hardness of the coating are not much enough to resist against the stresses that are produced by cells. The findings demonstrate that the hydroxyapatite film offers a better condition for endothelialization in comparison with the other calcium deficient coatings.

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Figure Captions:

Fig.1- Schematic representation of the electrochemical cell arrangement.

Fig.2- Raman spectra of the Nitinol alloy after the heat treatment process for a) abraded and b) chemically treated samples.

Fig.3- The high resolution XPS spectra for Ti2p and O1s of the modified NiTi alloy.

Fig.4- The leached Ni concentration in Ringer's solution after different incubation time for the chemically modified and Heat treated sample (**C.H.**), and the abraded sample after the heat treatment process (**A.H.**).

Fig.5- The surface morphology of Ca-P coatings deposited at a) **A**, b) **B**, and c) **C** electrochemical conditions. (d) Typical EDS spectrum for the coating of the **C** condition.

Fig.6- FTIR spectra of the calcium phosphate coatings electrodeposited at a) **A**, b) **B**, and c) **C** conditions.

Fig.7– SEM micrographs of the attached cells on the Ca-P coatings of (a) and (d) **A** condition, (b) and (e) **B** condition, (c) and (f) **C** condition.

Fig.8- Contact angle of water drop placed on Ca-P coatings of different electrodeposition conditions.

Fig. 9- SEM micrographs of the attached cells to the bare Nitinol alloy.

Fig.10- SEM micrographs of the flattened endothelial cells on to the Ca-P coatings of the (**A**) electrodeposition condition showing connection lost of the cell from the coating (a), and cracking of the coating in the cell-coating interface (b).

SEM MAG: 2.50 kx
HV: 20.0 kV
VAC: HiVac

DET: SE Detector
DATE: 09/13/15
Device: TS5136MM

20 um

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