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Mechanical and biological properties of electrodeposited calcium phosphate coatings

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ABSTRACT

Calcium phosphate (Ca–P) coatings were electrochemically deposited on titanium substrates. By increasing the electrodeposition time (from 1 to 30 min), the coating thickness increases but also the surface morphology of the Ca–P coatings is greatly affected going from smooth to plate-like, featuring elongated plates, ribbon-like and finally sharp needle structures. Micro-stretch tests reveal that, regardless of the coating morphology and thickness, the electrodeposited Ca–P coatings have strong adhesion with the titanium substrates and their failure mode is cohesive failure. The effects of different morphologies on cellular behavior such as adhesion, viability, proliferation, and osteogenic gene expression were studied. The surface morphology of Ca–P coatings has a remarkable effect on cell attachment, proliferation, and viability. A smooth surface results in better adhesion of the cells, whereas the presence of sharp needles and ribbons on rough surfaces restricts cell adhesion and consequently cell proliferation and viability. The improved cell adhesion and viability on the smoother surface can be attributed to the higher contact area between the cell and the coating, while the needle-like morphology inflicts damage to the cells by physically disrupting the cell wall. There is no significant difference in the level of osteoblast gene expression when osteosarcoma cells are cultured on coatings with different morphologies. Our study provides crucial insights into the optimum electrodeposition procedures for Ca–P coating formation leading to both good cell-material interaction and sufficient mechanical properties. This can be achieved with relatively thin coatings produced by short electrodeposition times.

1. Introduction

Titanium (Ti) and its alloys have been extensively used in dentistry and orthopedics for many decades as a medical implant, interacting with bone tissue. Titanium and titanium alloys are used because of their desirable properties, such as reasonably high mechanical strength, high corrosion resistance, desired elastic modulus, high biocompatibility with bone tissue, and low toxicity [1,2]. However, the surface of Ti implants exhibits rather poor bioactivity and osseointegration resulting in insufficient bone growth and long-term stability [3]. Enhanced osseointegration of Ti implants can be achieved by applying bioactive ceramic coatings such as calcium phosphate (Ca–P) compounds [4]. Ca–P ceramics are able to promote direct chemical bonding at the implant/bone interface and, because of their compositional similarity to natural bone mineral, excellent biocompatibility, and low toxicity, they can also stimulate the formation of bone tissue [4–6]. Various

Ca–P coating methods such as plasma spraying [7], hydrothermal [8], pulsed laser and electron depositions [9,10], magnetron sputtering [11], sol-gel process [12], electrophoretic deposition [13], and electrochemical deposition (ECD) [14] have been used to deposit Ca–P coatings on metal substrates. Among these techniques, electrochemical deposition has received more attention because of its advantages such as sufficiently low cost, simplicity of performance, low processing temperature, and rapid deposition. This method allows highly irregularly shaped objects, including porous implants, to be coated uniformly [15,16]. Therefore, depositing Ca–P coatings via electrochemical deposition on Ti is a very promising strategy to enhance the biocompatibility of implants.

The success of a biomedical implant in vivo depends on several factors, such as implant bulk properties, as well as the physical and chemical characteristics of the implant surface. Particularly, implants coated with bioactive materials are of utmost importance as these will

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positively influence the adsorption of proteins, cell attachment, cell spreading, proliferation and differentiation and thereby greatly improve the biomaterial performance [17–20]. Several studies demonstrated that cellular behavior is promoted by altering the roughness [21], morphology and topography [22–26], chemical composition [26–28], and surface charge [29] of the coating. Among these factors, the morphology and topography of the surface is a critical issue since it strongly affects the interaction with tissue cells. It was shown that surface features of anodized Ti such as topography and morphology have a strong influence on the osseointegration processes of osteoblast cells in terms of the attachment and quantity of growing tissue. Morphological changes in the surface improved cell adhesion and enhanced the contact area between the cells and the Ti surface [30]. Another factor that determines the successful implantation and long-term stability of the coated implant is the adhesion strength between the coating and the metallic substrate and it must be taken into consideration when the coated implant is employed [31].

In the electrochemical deposition, applying different process parameters significantly influences the properties of the coating such as chemical composition, thickness, and morphology, which are characteristics that strongly affect cellular behavior [15,32–34]. It was shown that these parameters can determine the mechanical properties of a coating such as its adhesion strength [35,36]. However, it lacks a deep understanding of the relation between coating characteristics and biological properties and also their effects on modulating cellular response. Moreover, the relationship between the deposition parameters and mechanical properties of the coatings is not clearly understood. Nevertheless, a few of studies coupled both biological and mechanical properties of the coatings as a function of deposition parameters. The present work aims at making a deeper insight into the correlation between deposition parameters and mechanical/biological properties of the coatings, which provides a great opportunity to improve the performance of biomedical implants.

The main objective of the present work is to identify the influence of deposition process parameters, namely the deposition time, on the mechanical and biological properties of the electrodeposited Ca–P coatings. Coatings with different morphologies were obtained by altering the electrodeposition time. The surface of the coatings was characterized, and the micro-scratch test was employed to evaluate the adhesion strength of Ca–P coatings on titanium substrates. Furthermore, the response of osteosarcoma cells (SaOs), an osteoblast cell line, cultured on Ca–P coatings was studied to investigate the effect of the surface morphology on cell adhesion, spreading, viability, proliferation, and osteogenic gene expression.

2. Materials and methods

2.1. Electrodeposition and characterization of Ca–P coatings

The deposition of Ca–P coatings on Ti substrates was conducted according to our previous report [14]. Commercially pure Ti discs (Alfa Aesar) with diameter of 14 mm and thickness of 0.7 mm was used as a substrate. In order to achieve a uniform surface roughness, the surface of the Ti substrate was mechanically ground with SiC papers from P120 to P800 grit. Subsequently, it was rinsed with distilled water, ultrasonically cleaned in acetone for 15 min, rinsed with distilled water again, and dried with a drying apparatus. An electrolyte solution containing 0.042 M $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (Alfa Aesar), 0.025 M $\text{NH}_4\text{H}_2\text{PO}_4$ (Alfa Aesar) and 1.5 wt% of H_2O_2 was prepared in distilled water. The pH of the electrolyte was 4.3 and the temperature of the electrolyte was fixed at $65 \pm 1^\circ\text{C}$.

Pulsed electrodeposition was conducted in a regular two electrode cell in which the prepared Ti substrate was used as the cathode. A platinum sheet with the dimensions of 15×15 mm was used as the anode. The distance between the cathode and anode was fixed at 20 mm. Pulsed deposition was carried out with fixed frequency (1 Hz)

in potentiostat mode at -1.4 V cathodic potential. Both the pulse on and off times were set to $t = 0.5$ s. Coatings with different morphologies were deposited by altering the deposition times from 1, 2, 3, 5 to 30 min and the samples are denoted as CaP-1, CaP-2, CaP-3, CaP-5, and CaP-30, respectively. After deposition, the specimens were removed from the electrolyte solution, rinsed with distilled water, and dried under ambient conditions without any further treatment.

The surface morphology and cross section of the coatings were examined using a Philips ESEM-XL30 environmental scanning electron microscope (ESEM). Prior to SEM examination, specimens were sputtered with gold. The cross section of the coatings was prepared by a cryogenic ion beam cross-section polisher (JEOL, IB-1952CCP). A slice of about $100\ \mu\text{m}$ was milled away with ion beam energy of 6 keV and the stage swing was set to $\pm 30^\circ$. SEM images were taken from the ion-beam milled cross sections and the thickness of coatings measured by ImageJ software. To measure the thickness of the coatings, the interface between the coating and the Ti substrate was chosen as starting point and the top layer of the coating was chosen as the end point (three different points were chosen across the milled area).

2.2. Adhesion strength test

The adhesion strength between the Ca–P coating and the Ti substrate was evaluated with a CSEM Instruments Micro-Scratch Tester, using a Rockwell C diamond spheroconical indenter ($200\ \mu\text{m}$ radius). The tests were conducted in the progressive load mode with starting and final indenter loads set at 0.5 and 35.0 N, respectively. The scratch length was 4.0 mm with an indenter speed of 1.7 mm/min and a vertical loading rate of 15 N/mm. The scratch track was observed using SEM, energy dispersive spectroscopy (EDS), and focus ion beam (FIB). Additionally, the scratch test was evaluated through determining the friction force and friction coefficient as a function of load.

2.3. Cell culture and characterization

2.3.1. Osteoblast cell line

Osteosarcoma cells (SaOs), a human osteoblast cell line, were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco), 1% penicillin-streptomycin (Gibco), and 0.1% (v/v) ascorbic acid 2-phosphate (AA2P, Sigma) at 37°C in an atmosphere of 5% CO_2 . The cells were expanded until approximately 80–90% confluence and then harvested from T75 culture flask by treatment with trypsin (Gibco) for 5 min at 37°C . Before the cell experiments, the coated substrates were sterilized with 70% ethanol and placed individually in 24-well plates. For all cell experiments except for gene expression, SaOs cells were seeded at a concentration of 4.0×10^4 cells/ml and cultured for 2 and 7 days. For gene expression, cells were seeded at a concentration of 8.0×10^4 cells/ml and cultured for 7 and 14 days. The medium was exchanged every 2 days.

2.3.2. Cell fixation and sample preparation for cell imaging

To study cell adhesion and spreading, after 2 and 7 days, the cultures were washed with phosphate-buffered saline (PBS), and then fixed with 3.7% paraformaldehyde (PFA, Sigma-Aldrich) in PBS for 20 min at room temperature. Subsequently, they were rinsed three times with PBS. For cell observation by SEM, all samples were dehydrated in a graded ethanol series (25, 50, 75, 98 and 100 vol%) and after that washed with hexamethyldisilazane (HMDS) followed by air drying in a desiccator. Finally, the samples were sputtered with gold.

2.3.3. Live/dead staining

In order to determine the viable cells on the Ca–P coatings, the cultured cells were stained with a Live/Dead BacLight kit (Molecular Probes Inc.). For this, cell culture medium was removed from the well plates and the samples were rinsed with PBS. The live/dead staining

solution was prepared by mixing components A (SYTO 9) and B (propidium Iodide) at 1:4 ratio and then added to the PBS. After adding 250 µl of the staining solution to each well plate, samples were incubated for 15 min in the dark at room temperature. At the end of the staining time, samples were rinsed with and stored in PBS. The cells were imaged by fluorescence microscopy (Leica DFC350 FX). Coatings were scanned at randomly selected positions and the live/dead data were recorded from 3 different locations. Experiments were performed in triplicate.

2.3.4. XTT assay

Cell metabolic activity was analyzed by 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay (Applchem A8088). After 2 days of culture, each sample was rinsed with PBS and transferred to a new well plate. Then, the fresh medium was added to each well along with 250 µl of XTT reaction mixture (5 ml XTT reagent and 100 µl activation reagent for one plate). After 3 h of incubation at 37 °C in an atmosphere of 5% CO₂, 200 µl of the solution from each well were transferred to a 96-well plate and the absorbance at 485 and 690 nm was recorded on a FLUOStar OPTIMAL microplate reader (BMG LABTECH). Experiments were performed in triplicate.

2.3.5. Osteoblast-relevant gene expression

Real-time quantitative polymerase chain reaction (qPCR) (CFX 96, Bio-Rad) was performed after 7 and 14 days of culture to evaluate the expression of 3 osteoblast-relevant genes, namely Alkaline phosphatase (ALP), Type I collagen (COL I), and Osteopontin (OPN) (Sigma). After the desired incubation time, cells were lysed and RNA was extracted using Trizol reagent following the manufacturer's protocol (STRATEC Molecular CmbH, D-13125 Berlin). Total RNA was quantified by spectrophotometer (Nanodrop, Thermo Scientific) and was reverse-transcribed to cDNA. The resulting cDNAs were used for real-time polymerase chain reaction (PCR). The PCR cycling consisted of 40 cycles of amplification of the template DNA with primer annealing at 60 °C. For statistical analysis, all experiments were performed in triplicate. Primers used to amplify specific targets are shown in Table 1.

2.4. Statistical analysis

All data points were expressed as mean values $\pm$ standard deviations with $n = 3$. Statistical analysis was performed using Origin 8.0 software by one-way ANOVA followed by Tukey's test. Statistical significance was considered at a value of $P < 0.05$.

3. Results and discussion

3.1. The surface morphology and the cross section of electrodeposited Ca–P coatings

Electrodeposited Ca–P coatings can obtain distinct morphologies by altering the deposition time. Detailed characterization of the chemical phase, morphology, and crystal growth mechanism of electrodeposited Ca–P coatings has been described previously [14]. Transmission and scanning electron microscopy observations revealed that the growth mechanism of electrodeposited Ca–P coatings was a time-dependent process. The X-ray diffraction patterns of Ca–P coatings deposited at

different deposition times reveals that Ca–P coatings consisted of hydroxyapatite and octa-calcium phosphate (OCP) (see Fig. S11 in the supplementary information).

The surface morphology, cross section and thickness of the Ca–P coatings deposited at different deposition times (1, 2, 3, 5 and 30 min) are shown in Fig. 1. After 1 min of deposition (CaP-1), a relatively dense and flat coating is formed on the substrate (Fig. 1a), which consists of randomly oriented nano-plates; the thickness of the coating is $3.1 \pm 0.2 \mu\text{m}$. The morphology of the CaP-1 coating is described here as 'smooth' morphology. As shown in Fig. 1b, with 2 min deposition time (CaP-2), some plates are formed on the coating surface and pore sizes increase in the cross-section view. The thickness of the CaP-2 coating increases to $5.1 \pm 0.2 \mu\text{m}$. This coating is described as having 'plate-like' morphology. After 3 min of deposition time (Fig. 1c), the length of the plates as well as the coating thickness increases ($7.9 \pm 0.3 \mu\text{m}$). The coating deposited during 5 min (CaP-5) has more elongated plates that can be clearly seen in the cross-section view (Fig. 1d). The thickness of the CaP-5 coating increases to $14.9 \pm 0.4 \mu\text{m}$. Finally, after 30 min of electrodeposition time (CaP-30), the coating morphology displays elongated ribbons and sharp needles with total thickness of $18.7 \pm 0.3 \mu\text{m}$. The morphology of the CaP-5 and CaP-30 coatings are denoted as 'ribbon-like' morphology. Ribbons propagate along the c-axis, which is the preferred orientation for synthetic Ca–P crystals [37,38]. According to the crosssection images, the morphology of all coatings at the interface is dense without any delamination and/or crack formation. In summary, by increasing the deposition time, the surface morphology of Ca–P coatings changes according to nucleation and growth theory and the thickness and surface roughness of the coatings increase. However, increase in layer thickness is not continuous as the growth over time shows a very regular increase during the initial 5 min of layer deposition up to $14.9 \mu\text{m}$ but increases marginally after that with only a $3.8 \mu\text{m}$ increase to $18.7 \mu\text{m}$ between 5 min and 30 min deposition time. This indicates that much thicker layers, $> 20 \mu\text{m}$, will be challenging (Fig. 1f).

3.2. Mechanical properties

So far, a number of methods have been developed to evaluate the adhesion strength of Ca–P coatings, such as micro-scratch tests, the indentation method, and the tensile adhesion test (pull-out test) [39–41]. However, considering the porous structure and high surface roughness of electrodeposited Ca–P coatings, indentation is not a suitable technique. Likewise, tensile tests have some disadvantages that restrict their ability to evaluate the exact value of adhesion strength, for instance, given the possibility of penetration of adhesive glue into the coating or even down to the interface in the case of thin coatings (e.g. $50 \mu\text{m}$). For this reason, the obtained results may represent the strength of the glue rather than the adhesion strength of the coating [40,42]. Cheng et al. [43] studied the adhesion strength of fluoridated hydroxyapatite coatings on Ti alloy through both the pull-out method and the scratch test. They claimed that the pull-out results were a combination of adhesion failure, cohesive failure, and glue failure, while the results of the scratch test only represent the coating-substrate adhesion strength. Consequently, the micro-scratch test may be a better approach to evaluate the adhesion strength of electrodeposited Ca–P coatings.

SEM images of scratch tests for three different coatings (CaP-1, CaP-

Table 1
Sequences of forward and reverse primers.

Primer	Forward 5'-3'	Reverse 5'-3'
Alkaline phosphatase (ALP)	CCACGTCTTCACATTGTGGTG	AGACTGCGCTGGTAGTTGT
Type I collagen (COL I)	GGACACAGAGGTTTCAGTGGT	GCACCATCATTTCAGGAGC
Osteopontin (OPN)	ACTCGAACGACTCTGATGATGT	GTCAGGTCTGCGAACTTCTTA
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	AACGGGTACAAACGAGTC	AGATGGATCAGCCAAGAAG

Fig. 1. SEM micrographs showing the cross section (top) and the surface morphology (bottom) of Ca–P coatings deposited for (a) 1, (b) 2, (c) 3, (d) 5 and (e) 30 min, (f) the thickness of coatings at different deposition times.

3 and CaP-30) are shown in Fig. 2. As shown in Fig. 2a1 and a2, after the scratch test on the CaP-1 coating, some layers of coating remain on the substrate with visible cracks. The EDS elemental analyses also illustrate the existence of calcium and phosphate on the scratch track, meaning that the coating does not peel off from the substrate completely. The CaP-3 and CaP-30 coatings fail in a similar manner to the scratch test (Fig. 2b and c). To demonstrate the presence of coatings after the scratch test, FIB sectioning was performed on the CaP-1 and CaP-30 coatings (Fig. 3). Although the structure of the coating is damaged severely, no delamination occurs along the interface. Instead, a thin layer of the coating remains on the substrate. According to these results, spallation or delamination of the coating, which are the characteristics of adhesive failure, are not observed along the scratch, but rather cohesive failure is detected under loading forces. Although in many coatings the thickness is associated with decreasing adhesive strength, from our results, it can be concluded that, regardless of the coating thickness, the failure mode of the electrodeposited Ca–P coatings is cohesive failure.

Typical scratch curves, plotting friction force as a function of load, and the corresponding friction coefficient, are shown in Fig. 4 for CaP-1, CaP-3 and CaP-30 coatings and also for the Ti substrate used as a control. As illustrated in Fig. 4, the scratch curves in all cases can be divided into three different regions. In the first region, the curves are relatively smooth and linear without any fluctuations, demonstrating that, in this region, the tip of the indenter only “slides” on the surface [43]. In the second region, some fluctuations are recorded in both the friction force and the friction coefficient curves, which indicate the penetration of the indenter inside the surface. The third region starts after a sudden increase in both curves, where the plastic deformation of titanium starts. According to Fig. 4a and b, the small fluctuations in the friction force curve start at the loads of 4.6 N and 7.6 N on the titanium substrate and the CaP-1 coating, respectively. Thus, the first region of the CaP-1 coating is larger than that of the titanium substrate. In

addition, the friction coefficient of the CaP-1 coating in this region is slightly lower than that of titanium, suggesting that the CaP-1 coating can act as a solid lubricant on the titanium substrate [44]. However, the first region of the CaP-3 and CaP-30 coatings is much smaller due to their structure. The morphology of these coatings consists of plates and ribbons, which break very easily even at very low loads resulting in small fluctuations in the scratch curve. In the second region of all coatings, although some small fluctuations are recorded, no abrupt change is observed in the curves. The absence of sudden changes indicates that there is no brittle delamination of the coating from the substrate and good adhesion strength is maintained (cohesive failure). The starting point of the third region (plastic deformation of titanium) is at 19.8, 22.6, 28.8, and 29.0 N for the titanium substrate, CaP-1, CaP-3 and CaP-30 coatings, respectively. Consequently, coating the titanium substrate with Ca–P can delay the mechanical deformation of titanium. As the thickness of the coating increases, the plastic deformation of the Ti substrates occurs at higher loads. Therefore, a thicker coating is beneficial in order to avoid damage to the titanium substrate.

3.3. Effect of surface morphology on cell adhesion and spreading

Different morphologies with varying roughness significantly affect the cellular response [22–24,26,45]. Fig. 5 shows the SEM images of SaOs osteoblast-like cells cultured for 2 and 7 days on Ca–P coatings of different deposition times. Fig. 5a1 illustrates that SaOs cells on the CaP-1 coating have excellent attachment with numerous filopodia extended beyond the edge of lamellipodia. On this smooth coating, SaOs cells are very much spread and the predominant shape of the cells is polygonal (Fig. 5a1–a3). After 7 days of culture (Fig. 5a3), SaOs cells almost cover the entire surface of the coating and form a uniform layer. Observing an individual cell on the CaP-2 coating (Fig. 5b1) reveals that, on plate-like morphology, the number of extended filopodia decrease compared to that of the smooth coating. As seen in Fig. 5b2 and

Fig. 2. SEM images of the scratch tests on the Ca–P coatings deposited at a) 1, b) 3, and c) 30 min (a1–c1) low magnification and (a2–c2) high magnification. The higher magnification was obtained from area enclosed by a dotted rectangular. EDS mapping spectrums from high magnification SEM image of 1 min-deposited coating are shown in the right columns.

Fig. 3. FIB/SEM images of the cross section after scratch test on the Ca–P coatings deposited at a) 1 and b) 30 min (a1 and b1) low magnification and (a2 and b2) high magnification. The higher magnification was obtained from area enclosed by a dotted rectangular.

Fig. 4. Scratch curve, friction force as a function of load and corresponding friction coefficient for a) titanium substrate and for the Ca–P coatings deposited at b) 1, c) 3, and d) 30 min.

b3, the number of cells on the CaP-2 coating declines as well and the attached cells have a polygonal, extremely extended or rounded shape. On the CaP-3 coating, the number of well-grown cells with polygonal shape decreases (Fig. 5c1–c3). Finally, on the highest surface roughness with ribbon-like morphology (CaP-5 and CaP-30 coatings), the attachment of cells is much lower. Fig. 5d1 and e1 reveal that cells, in their attempt to bind to the tips of the ribbons, are being physically damaged by the high aspect ratio structures. The lack of adhesion is additionally attributed to the low available attachable surface area for the cell, resulting in weaker adhesion [23]. In comparison to the highly spread polygonal shaped cells on smooth coatings, cells on these rough coatings are more elongated or rounded in a poorly attached condition, strongly indicating that most of the cells are non-viable. These results show that the surface morphology of Ca–P coatings has a remarkable effect on cell attachment. Smooth surfaces result in better adhesion of the cells because of the higher contact area. Okada et al. [23] reported that the existence of nano-sized needles and fibers restricts the elongation and growth of osteoblast-like cells because the formation of the actin stress fibers were limited to contact domains on small crystalline planes. Huang et al. [46] studied the osteoblast-like cells behavior on micro/nano topographies. They reported that the adherent cells show different morphologies and shapes on surfaces with different topographies. Numerous filopodia and lamellipodia formed on nanoleaf surfaces. In addition, cell proliferation and differentiation were enhanced on nanoleaf surface compared to that on micro-sized topography. These behaviors of cells are related to the domain size of the contact with nanostructures. The adhesion of the cells to the implant surface is crucial because it is an essential stage for the other cellular activities such as viability, proliferation, differentiation as well as rapid tissue repair, and morphology is herein an important factor [19,23].

3.4. Effect of surface morphology on cell proliferation, viability and gene expression

To investigate the viability of the SaOs cells on the Ca–P coatings with different morphologies, live/dead cell staining tests were performed for 2 and 7 days culture time. Representative fluorescence images of the SaOs cells on Ca–P coatings and on pure HA disc as a control are shown in Fig. 6. The percentage and number of live cells per unit surface area on every coating was quantified as presented in Fig. 6g and h, respectively. As shown in the fluorescence images, most of the cells are alive on the CaP-1 coating as well as on the control (Fig. 6a and b) for both 2 and 7 days of culture. The average percentage of alive cells on the CaP-1 coating is $98 \pm 2\%$ and $99 \pm 1\%$ for the 2 and 7 days culture, respectively (Fig. 6g). As illustrated in Fig. 6c, the CaP-2 coating has fewer alive cells as compared to the CaP-1 coating, which is in agreement with the SEM results. The average percentage of alive cells on the CaP-2 coating declines to $77 \pm 12\%$ and $88 \pm 4\%$ after 2 and 7 days of culture, respectively. On the rough coatings (CaP-3, CaP-5, and CaP-30), visibly fewer alive cells are found (Fig. 6d, e and f) and the numbers of dead cells are much higher than those of the CaP-1 and CaP-2 coatings, indicating that cells are less viable on elongated plates and sharp needles. Hence, very rough surfaces are not biocompatible for the cells. After 2 days of culture, the average percentages of alive cells are $59 \pm 5\%$, $35 \pm 10\%$ and $19 \pm 5\%$ on the CaP-3, CaP-5 and CaP-30 coatings, respectively. As shown in Fig. 6h, the number of alive cells on the CaP-1 coating is almost equal to that observed on the control after both time points. However, the cell number counts on rough surfaces are obviously smaller compared to the smooth surface and the control. On the CaP-1 coating and on the control, cells show a progressive growth with increasing incubation time, indicating viability. The increases in cell number from 2 to 7 days are 3.0- and 2.9-fold on the CaP-1 coating surface and on the control, respectively. The

Fig. 5. SEM images of SaOs osteoblast-like cells on Ca–P coatings deposited at (a) 1, (b) 2, (c) 3, (d) 5 and (e) 30 min after (a1–e1 and a2–e2) 2 days and (a3–e3) 7 days of culture.

number of cells after 2 and 7 days of culture does not differ on any of the rougher surfaces. These findings reveal that any Ca–P coating deposited for longer than 1 min appears to be less biocompatible, likely because of the existence of the plates and sharp ribbons, which provide less contact surface area and may inflict physical damage to the cells. Consequently, high surface roughness restricts cell adhesion and proliferation. Earlier studies also have reported that cell proliferation was suppressed on plate-like morphologies with sharp tips (widths < 1 μm) compared to smoother surfaces without sharp features which attributed to larger contact domains on smoother surfaces [47].

In order to further understand the influence of surface morphology on osteoblast viability, the metabolic activity of SaOs cells cultured on Ca–P coatings with different morphologies was analyzed using the XTT

assay after 2 days of culture and the results are shown in Fig. 7. It is found that the metabolic activity of the cells on the CaP-1 coating and on the control has no marked difference, which is in agreement with the live/dead staining results and demonstrates that the CaP-1 coating is biocompatible. However, the metabolic activity of SaOs cells on the CaP-2 and CaP-3 coatings is significantly lower ($P < 0.005$) than that on the CaP-1 coating and the control. Likewise, the metabolic activity of cells on the CaP-5 and CaP-30 coatings is remarkably lower ($P < 0.001$) than that on the CaP-1 coating and the control. Based on these results, cells are not viable on rough surfaces due to the physical damages caused by sharp ribbons and needles. According to SEM images, on the surface of CaP-30 coating, the adhesion of the cells is very weak because of low contact area; additionally, the tip of the

Fig. 6. Fluorescence microscopy images of SaOs cells on (a) HA disc as control, Ca–P coatings deposited at (b) 1, (c) 2, (d) 3, (e) 5, and (f) 30 min after 2 days (a1–f1) and 7 days (a2–f2) of culture. Green and red indicate live and dead cells, respectively. g) percentage and h) number of live SaOs cells cultured on control and Ca–P coatings deposited at different deposition times after 2 days and 7 days culture. * $P \leq 0.05$, ** $P \leq 0.005$, *** $P \leq 0.001$, and **** $P \leq 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 7. Viability assay (XTT) displaying viability of SaOs cells on Ca–P coatings deposited at different deposition times after 2 days of culture. ** $P \leq 0.005$ and *** $P \leq 0.001$.

ribbons penetrates the cells and damages the cell wall (Fig. 5e1) which can induce a high stress to the cells and reduce their viability.

To investigate whether the Ca–P coatings with different morphologies influence the gene expression of osteoblast cells, SaOs cells were cultured for 7 and 14 days on coatings with different morphologies. The expression level of osteoblast-indicator genes such as ALP, COL I, and OPN were studied. Irrespective of the coating morphology, the gene expression was recorded on all coatings and the level of expression was comparable with the control. Therefore, it can be concluded that the morphology of the coatings does not alter the gene expression of SaOs cells for the ones selected, namely ALP, COL I, and OPN (see Fig. S12 in the supplementary information).

The development of Ca–P coatings for future applications in implants that facilitate bone tissue integration includes surface modifications such as morphological changes and roughness variations, as well as tuning of the mechanical properties. Changes in the deposition parameters can result in a variety of different thicknesses and morphologies. Our findings have important implications in regulating the deposition parameters of Ca–P coatings to achieve optimum mechanical and biological properties.

4. Conclusion

Ca–P coatings with different morphologies were obtained by altering the electrodeposition time. The surface morphology changes from smooth to plate-like, elongated plates, ribbon-like, and needle-like and the thickness of the coatings increases as well. Micro-scratch tests revealed that the CaP-1 coating not only has a strong bonding with the substrate, but also acts as a solid lubricant. Even though the morphology and thickness may generally affect the mechanical properties, here the morphology and thickness of the coatings did not have an effect on the adherence of the Ca–P to the Ti substrate and, in all the coatings, the failure mode is cohesive failure. Although it was found that the thicker coating delays plastic deformation in the titanium substrate. Cellular activities are remarkably affected by the surface morphology of Ca–P coatings. Smooth surfaces are found to induce better cell adhesion, viability, and proliferation than rough surfaces. The improved cell activity on smooth surfaces can be attributed to the larger contact area. On the rough surfaces, the presence of sharp needles and ribbons seems to inflict physical damage onto the cells and inhibit proper cell adhesion and consequently cell proliferation and viability. Differences in surface morphology do not induce significant changes in the level of gene expression. In previous studies, focus on controlling surface morphology of hydroxyapatite has demonstrated similar findings as presented here but these studies did not consider the fabrication process, mechanical stability, and biological activity as a unified study. The results of the current investigation show that cells are more viable on the thin and smooth coatings and therefore, the better cell/tissue compatibility with the still eminent mechanical properties make these coatings optimal as obtained via electrodeposition approaches.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.msec.2019.03.020>.

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