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Bioactivation of titanium dioxide scaffolds by ALP-functionalization

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ABSTRACT

Three dimensional TiO_2 scaffolds are receiving renewed attention for bone tissue engineering (TE) due to their biocompatibility and attractive mechanical properties. However the bioactivity of these scaffolds is comparatively lower than that of bioactive glass or hydroxyapatite (HA) scaffolds. One strategy to improve bioactivity is to functionalize the surface of the scaffolds using biomolecules. Alkaline phosphatase (ALP) was chosen in this study due to its important role in the bone mineralization process. The current study investigated the ALP functionalization of 3D titanium dioxide scaffolds using self-polymerization of dopamine. Robust titanium scaffolds (compressive strength~2.7 ± 0.3 MPa) were produced via foam replica method. Enzyme grafting was performed by dip-coating in polydopamine/ALP solution. The presence of ALP was indirectly confirmed by contact angle measurements and enzymatic activity study. The influence of the enzyme on the bioactivity, e.g. hydroxyapatite formation on the scaffold surface, was measured in simulated body fluid (SBF). After 28 days in SBF, 5 mg ALP coated titania scaffolds exhibited increased hydroxyapatite formation. It was thus confirmed that ALP enhances the bioactivity of titania scaffolds, converting an inert bioceramic in an attractive bioactive system for bone TE.

1. Introduction

The repair and reconstruction of damaged bone tissue caused by traumata, bone diseases or loss or bone tumour removal represent major clinical challenges [1,2]. Current bone restoring methods include autografts and allografts/xenografts which involve the transplantation of tissue. However, these methods have different drawbacks such as high morbidity, pain, potential infection and tissue rejection, which can lead to long term health complications [3]. Bone tissue engineering (TE) represents an alternative to treat these problems by using combination of biomaterials, biomolecules and cells to grow new tissue with the same properties and functionality of the damaged host [3,4]. A variety of natural and synthetic materials has been investigated over the last decades for bone tissue engineering including combination of various biomaterials and designs [4]. Three dimensional scaffolds have emerged as an important partner of bone TE approaches [3]. A suitable scaffold material should support cell adhesion and migration, growth, proliferation as well as differentiation to heal injured tissue [1,4–6]. Hence, scaffolds must fulfil several physiochemical and biomedical requirements [6]. The development of a suitable scaffold material is a scientific challenge. Mechanical properties matching those of bone, ability to mineralise (form calcium phosphate on the surfaces) [7], a suitable interconnected pore structure with pore of size 200–400 μm [4,8] and no toxic reaction in the body [9] are some of the very important scaffold requirements. Scaffolds for bone TE should fulfil the above-mentioned criteria, moreover, they should be osteoinductive and osteoconductive [10]. TiO_2 is one of the bioceramics being considered to develop bone tissue scaffolds [5,11]. Due to the fact that titania is bioinert but not bioactive, surface functionalization of TiO_2 scaffolds is required to impart bioactivity or bioreactivity, e.g. to induce the formation of a calcium phosphate layer on the TiO_2 surface. Biomolecule surface functionalization involves the attachment of biomolecules to a material surface which results in a change of surface properties leading to strong link with proteins and cells [11]. Biomolecule immobilization can be subdivided into covalent- and non-covalent bonding [12]. Covalent immobilization provides a robust chemical bonding between the surface and the chosen biomolecule [11].

There are various methods of surface functionalization, namely

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physical, chemical and biological. This work focuses on the immobilization of alkaline phosphatase (ALP) on TiO_2 scaffold surfaces based on a simple dip coating method using the process of self-polymerization of dopamine. This functionalization method was originally developed by Lee et al. [13], and applied also by Nijhuis et al. [14,15] to functionalize inorganic and organic materials with various biomolecules, e.g. bone morphogenetic protein 2 (BMP-2), bovine serum albumin (BSA), DNA as well as alkaline phosphatase. It has been reported that polydopamine induces a higher strength and forms reversible bonds to inorganic surfaces by developing a thin surface layer due to a self-polymerization process [14–16]. Also the immobilization of ALP/polydopamine onto the surfaces of titanium discs has been carried out [14,15].

In this work, ALP was chosen as a biomolecule because of its huge influence on the calcification of bone [15,17,18]. Alkaline phosphatase catalyzes the organic phosphate hydrolysis which leads to the formation of hydroxyapatite (HA) and the mineralization of the bone matrix [15]. Verne et al. [18] have reported the ALP grafting on bioactive glasses and glass-ceramics which was shown to enhance the mineralization processes of the materials. Groeneveld et al. [19] reported the ALP activity and its influence on the mineralization process for cellular cementum formation and in the periodontium of the rat molar, and the relationship between enzyme activity and cementum was explained. In the field of orthopedic coatings, De Jonge et al. [20] reported electro sprayed immobilization of ALP to provide local enrichment of Ca^{2+} and PO_4^{3-} ions and Piattelli et al. [21] described the ALP functionalization of titanium plasma sprayed implants showing that ALP functionalization led to improved new bone formation. To the author's knowledge ALP functionalization of TiO_2 scaffolds has not been reported previously. The overall goal of this work is thus to explore the bioactivation of titania scaffolds by ALP coatings and to evaluate the formation of HA on the titania surface upon immersion in simulated body fluid (SBF).

2. Experimental procedure

2.1. Scaffold and pellet fabrication

TiO_2 scaffolds were produced via the foam replication technique [22]. The starting material was a mixture of (50 wt.-%) nanoparticles (TiO_2 P25 Aeroxide from Evonik Industries Aerosil of size 21 nm) and (50 wt.-%) microparticles of size 38 μm (Hombitan TiO_2 from Sachtleben). Prior to the scaffold production, TiO_2 microparticles were washed with 1 M NaOH in order to remove contaminants and dried in an oven at 100 °C for approximately 24 h. Afterwards, the powder was grinded with a mortar and sieved manually with a sieve of 38 μm mesh size. The slurry for scaffold fabrication was prepared as follows: 20 g of titania nanopowder and micropowder were stirred in 69 ml de-ionised(DI) water. Then 15 ml of 1 M hydrochloride acid (HCl from EMSURE) was added slowly to the solution. HCl was added to decrease the pH to a value of 1.9. The slurry was stirred at 3000 rpm for one hour at room temperature. Cubical-shaped polyurethane foams (Eurofoam Deutschland GmbH, Wiesbaden, Germany) with 60 ppi and an average side length of 1 cm were dipped in the TiO_2 slurry for 5min and were squeezed manually to remove the excess slurry. The squeezed scaffolds were dried for 2 h at room temperature. This procedure was repeated three times to get homogeneous and thick coatings. The scaffolds were then dried at room temperature in air for at least 24 h before sintering.

The polymer sponge burnout and sintering steps were carried out following a previously reported procedure [23]. Briefly, the scaffolds were placed in a furnace and slowly heated to 450 °C at a heating rate of 0.5 K/min, the holding time was set to 1 h to burn out the polymer sacrificial foam. Then the temperature was increased to 1400 °C at a heating rate of 3 K/min and the sintering time at this temperature was set to 5 h. The sintered scaffolds were cooled down to room temperature at the cooling rate of 6 K/min.

Dense TiO_2 pellets were also produced for contact angle tests and for this purpose, 0.3 g of titania powder (the same used to make the scaffolds) was filled into a die and cold-pressed with an electrohydraulic press (Mauthe Maschinenbau) with a force of 2.10^4 N. The size of the pellets was 13 mm in diameter and 1.3 mm in thickness. The pellets were sintered in the same way as the scaffolds.

2.2. Surface functionalization

2.2.1. Hydroxyl exposure

The first step in the functionalization process is the cleaning of the surface to remove contamination and to expose hydroxyl groups. The scaffolds and pellets were ultrasonically cleaned for 10 min in acetone and isopropanol, respectively, in order to remove contamination [15]. Afterwards, the samples were washed ultrasonically in DI water for 10 min. The cleaned samples were dried at 37 °C for 2 h.

2.2.2. ALP grafting

In order to covalently graft ALP to titania scaffolds and pellets, three different amounts of ALP (alkaline phosphatase from bovine intestinal mucosa, P7640 from Sigma-Aldrich) with dopamine (Dopamine hydrochloride, H8502 from Sigma Aldrich) were used and an uncoated titania scaffold was considered as reference. For the coating of scaffolds, the 1-step coating process reported by Nijhuis et al. [14] was used. ALP/dopamine solutions were prepared as follows: a buffer solution containing 2 mg/ml of dopamine in 10 mM Tris was made at pH 8.5. Out of this stock solution, three solutions with different amounts of ALP and the same amount of dopamine were prepared. The ALP contents were 1 mg/ml, 2 mg/ml and 5 mg/ml. The three samples were labelled as 1 mg ALP, 2 mg ALP and 5 mg ALP, respectively.

The scaffolds and pellets were put individually in a well plate containing 2 ml of the prepared solution and soaked for 16 h. After soaking, samples were washed 3 times in phosphate-buffered saline (PBS) and one time in DI water to stop the grafting reaction. Afterwards, the samples were dried and stored at room temperature [14,15].

2.3. In vitro bioactivity study

In order to study the bioactivity of the ALP/dopamine coated titania scaffolds, a standard *in vitro* procedure in SBF solution was carried out. SBF was prepared as explained by Kokubo et al. [24]. The scaffolds were immersed in 50 ml SBF at 37 °C and at pH 7.4. The solution was refreshed twice a week. Samples were extracted from SBF after 7, 14, 21 and 28 days, rinsed in deionized water, dried at room temperature and considered for further characterization by SEM and XRD analysis.

2.4. Characterization studies

2.4.1. Surface morphology and elemental analysis

For scanning electron microscopy (SEM) observations, samples were fixed on stubs using double sided carbon stickers and silver paint was used as an adhesive to improve the conductivity. Prior to SEM evaluations, the samples were coated with Au using an EMITECH-K550 sputtering device. The morphology of the scaffolds was observed by SEM (AURIGA-4750, Zeiss, Germany). For pore morphology, magnifications around 100× and 200× were

sufficient whereas for detecting hydroxyapatite a magnification up to 30KX had to be used. The average pore size was analyzed with the ImageJ software. Elemental analysis was performed by EDS (X-MaxN Oxford instruments, UK).

2.4.2. Compressive strength

Compressive mechanical testing of TiO_2 scaffolds was conducted using a Zwick Roell Z050 mechanical tester with a 50 N load cell at a crosshead speed of 5 mm/min. The tests were carried out at room temperature. Ten scaffolds were used for compressive strength testing. The scaffolds were placed in between two parallel plates. The cross-sectional area of scaffolds was calculated and typical stress–strain curves were recorded.

2.4.3. Wettability study

For measuring the wettability of the samples, contact angle tests were performed on the produced pellets using the sessile drop method employing a contact angle-measuring instrument (DSA30-E, Krüss, Germany). After the adjustment of the base line, 3 μl distilled water was dropped on the surface of the pellets and the contact angle was measured. The measurement was taken five times for each pellet and then the average contact angle was calculated.

2.4.4. Structural analysis

In order to investigate the crystalline structure of the scaffolds, X-ray diffraction (XRD) analysis (Siemens Kristalloflex D500, Siemens AG, München, Germany) was carried out with a current of 30 mA, voltage of 30 KV, step size of 0.02° and step time of 3s, in the measurement range of 25–75° (2θ).

2.4.5. Enzymatic activity study

To verify the biochemical activity of ALP immobilized onto the scaffolds, the transformation of para-Nitrophenylphospate (pNPP) to 4-nitrophenol was measured [15]. ALP hydrolyses p-nitro phenol phosphate releasing p-nitrophenol which has a distinct yellow color. This can be measured using UV spectroscopy. Scaffolds which were functionalized in the Tris-Dop solution containing 1 mg, 2 mg and 5 mg ALP were measured. Therefore an ALP-buffer with a pH around 9.8 was produced containing 0.1 M Tris, 2 mM $MgCl_2$ and 9 mM 4-nitrophenyl phosphate as well. The scaffolds were stored in well plates with 1.5 ml of the reactive mixture for 60 min and placed in an incubator at 37 °C. In order to get values of the transformed para-Nitrophenylphospate (pNPP) per minute, the reactive mixture was diluted with DI in the ratio of 1/60. Afterwards the scaffolds were taken out and the reaction was stopped by adding 0.5 ml of 1 M sodium hydroxide into the solution. Accordingly, 1 ml of the mixture was filled in a cuvette and the intensity of the yellow color produced was evaluated with an UV-spectrometer (AnalytikJena, Specord 200) by measuring the absolute UV absorbance at 405 nm.

3. Results

3.1. Surface morphology

Fig. 1 shows a typical SEM image of a sintered TiO_2 scaffold. The average pore size of the scaffolds was around 300–400 μm, which was considered to be in the range of interest for bone tissue engineering applications [5,8]. An interconnected pore structure and open pores were observed. The porosity, p, was calculated using the formula:

$$p = \left(1 - \rho_{foam} / \rho_{solid}\right)$$

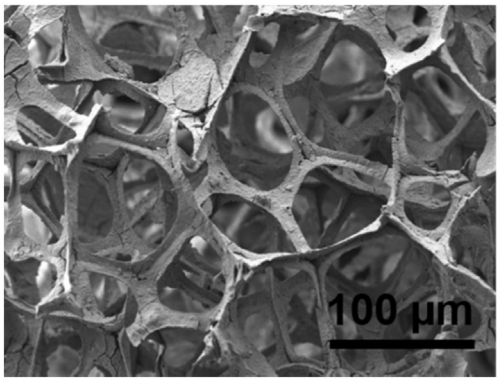

Fig. 1. SEM image of a TiO_2 scaffold produced by the foam replica method showing the typical open pore structure.

The mass and dimensions of the sintered foams were measured to calculate the density of the foam, ρ_{foam}, while ρ_{solid} was considered to be the theoretical density of rutile titania [23], which is 4.27 g cm^{-3} considering that rutile was the crystalline phase detected by XRD (see section 3.2). The porosity of the scaffold was calculated to be around 97% and the average strut thickness was in the range of 51–63 μm. Similar morphologies have been reported previously for various sintered TiO_2 foams synthesized by the foam replica method [5,23]. From SEM observations, the pore size and high porosity of the ceramic foam could be confirmed, making it a suitable material for osteoblast cell migration, transport of nutrients and removal of waste, as reported by Haugen et al. [25].

3.2. Crystal structure

The used titanium dioxide microparticles were in the anatase modification while the as-received nanoparticles exhibited a mixture of rutile and anatase phase (the weight ratio of anatase and rutile being approximately 80/20 as stated by the supplier). Since the sintering temperature was 1400 °C, which is clearly higher than the conversion temperature of anatase, the anatase phase was fully transformed into rutile phase [23]. Fig. 2 shows the XRD data of the sintered TiO_2 scaffolds. The indicated peaks were all from the rutile form, which was confirmed by comparison with literature values [2,26] by the presence of strong diffraction peaks at 27.4, 36.1, 39.2, 41.2, 44.0, 54.4, 56.7, 63, 64, 68.9 and 70°, indexed to the (110), (101), (200), (111), (210), (211), (220), (002), (310), (301) and (112)

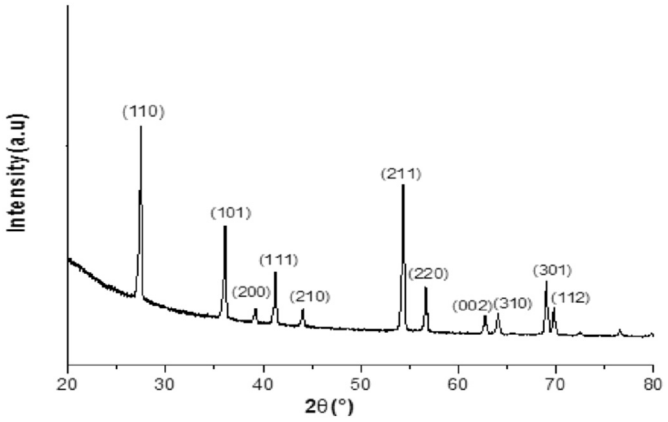

Fig. 2. XRD pattern of sintered TiO_2 scaffold showing the rutile phase characteristic peaks [27].

crystal planes of rutile TiO_2 (PDF card 75-1755, JCPDS) [27]. The fact that scaffolds are highly crystalline with rutile as predominant phase indicates the bioinert character of the scaffolds [26], as discussed further below.

3.3. Compressive strength testing

The average compressive strength of TiO_2 scaffolds was calculated as 2.7 ± 0.3 MPa. The slurry for the preparation of TiO_2 scaffolds was prepared with 50 wt% of TiO_2 microparticles and 50 wt% of TiO_2 nanoparticles and this particular mixture of particle sizes contributed to the adequate densification of the scaffold struts leading to better compression strength of scaffolds compared to the results of previous studies [2,23]. The multilayer dip coatings [5] of PU foams is likely another reason for the increased compressive strength value even if scaffolds exhibited a very high porosity (97%). It should be noted that ALP coating does not have a remarkable effect on the compressive strength of TiO_2 scaffolds. Indeed the main purpose of applying ALP coating is to improve the bioactivity of TiO_2 scaffolds and not the mechanical properties, however the compressive strength values of the present scaffolds are higher than those of similar TiO_2 scaffolds reported in literature [23].

3.4. Surface functionalization

3.4.1. Contact angle measurement test

To prove the effect of the polydopamine layer used to functionalize the scaffold surfaces, the wettability of the samples was measured by the sessile drop technique. The results for both the coated and uncoated titania pellets are reported in Fig. 3. In all measurements, it was observed that the wettability was high as the water drop did not remain on the surface for a long time and spread quickly. The uncoated titania pellets exhibited high hydrophilicity so the contact angle was around zero. This result proves the relatively high surface energy of rutile titanium dioxide, which was reported by Lee et al. [16] It is a well known fact that surfaces with contact angles smaller than 90° are called hydrophilic which corresponds to high wettability, while surfaces with contact angles greater than 90° can be classified as hydrophobic, which corresponds to low wettability [28,29]. The coated titania pellets showed a higher contact angle than the uncoated ones. For TiO_2+Dop+1 mg ALP the contact angle was 10 ± 3, for TiO_2+Dop+2 mg ALP it was 16° ± 2° and for TiO_2+Dop+5 mg ALP it was 23° ± 3°, confirming that all surfaces were hydrophilic. There is an additive effect of ALP concentration on contact angle which is likely related to the extent of coverage of the TiO_2 surface with increasing ALP concentration in the coating solution.

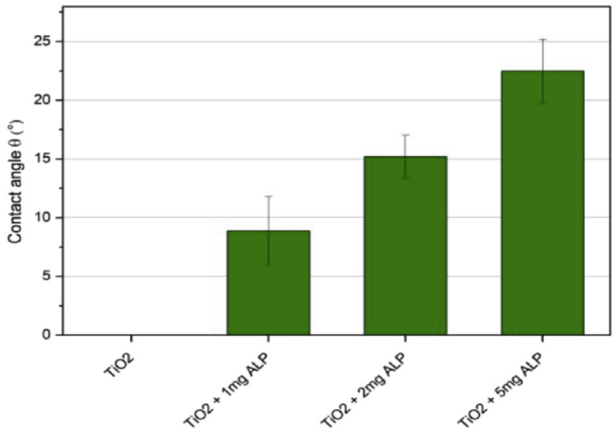

Fig. 3. Results of contact angle measurements of uncoated TiO_2 pellets and TiO_2 pellets coated with dopamine and different concentrations of ALP.

3.4.2. Enzymatic activity test

To confirm the grafting quality and quantity of the alkaline phosphatase, an enzymatic activity study was performed and results are shown in Fig. 4(A) and (B). The color change induced from the para -nitrophenol during the 60 min enzymatic test could also be detected and it was quantified by uv–vis spectrometry. The absorbance values at 405 nm showed that the samples dipped in the solution containing 5 mg ALP transformed considerably more pNPP than the samples with 1 mg and 2 mg.

The conversion into activity units (1U = 1 μg/ml) per volume indicated that the enzyme quantity was around four times higher on the 5 mg coated samples than on the samples with 2 mg. In comparison to 1 mg, the 2 mg coated scaffolds showed nearly the same proportion as the enzyme concentration in the grafting solutions. Overall, it was shown that the dopamine-based ALP anchoring was successfull while the enzyme retained its activity. Furthermore, it was confirmed that the amount of ALP in the coating solutions corresponded to the quantity of grafted enzyme. However, a further measurement, not shown here, led to the conclusion that the enzyme activity decreased with increasing storage time at room temperature. Hence, storage conditions should be considered since they influence the enzyme activity and therefore also the bioactivity of the functionalized surface.

3.5. In vitro bioactivity study

After 7 and 14 days of immersion in SBF, there were no significant changes observed on the surface morphology of scaffolds. The surface was smoother but the presence of HA like structures was not detected. Further, there were no visible differences between the coated and the uncoated samples.

In comparison to 7 and 14 days, the morphology of coated scaffolds showed visibly clear changes after immersion in SBF for 21 days. Fig. 5 (A–I) shows the SEM morphology of the surfaces of uncoated and ALP-coated (2 mg, 5 mg) TiO_2 scaffolds after immersion in SBF. Especially, on 2 mg and 5 mg ALP coated scaffolds a deposit of particles, (presumably crystals) is observed which could be calcium phosphate deposits [30] detected after 28 days in SBF (Fig. 5-H,I). Fig. 6 (A,B) shows high magnification SEM images of CaP agglomerates on 2 mg and 5 mg ALP coated titania scaffolds after 28 days in SBF. On the uncoated titania scaffolds, such structures were not observed. This result proved that bioactivity appears on the ALP-functionalized scaffolds and it depends on the amount of active ALP grafted onto the surface even though the substrate (rutile titania phase) shows unfavorable bioactivity [23].

From Fig. 7, which shows the EDS scans of the uncoated and ALP-coated (2 mg, 5 mg) TiO_2 scaffolds, the presence of Ca and P (as well as sodium and chloride as they are the main ion ingredients in SBF [24]) can be detected. The higher amount of ALP likely enhances HA formation. In the uncoated scaffolds, no Ca and P were detected, as expected. The presence of Na and Cl from SBF solution indicates that salt precipitates could not be removed by ultrasonic cleaning of the scaffolds after their immersion in SBF. The presence of Al is possibly the result of the sintering process where aluminium oxide was used to protect the scaffolds from sticking to the alumina plate in the furnace, while the presence of Au is due to the coating required for SEM sample preparation. The presence of Ca and P is more pronounced in scaffolds treated with 5 mg ALP than in those with 2 mg ALP, confirming, at least qualitatively, that ALP concentration affects the extent of CaP formation on the TiO_2 surfaces. It is likely that with higher ALP concentration in the coating solution the extent of coverage of the TiO_2 surfaces has increased resulting

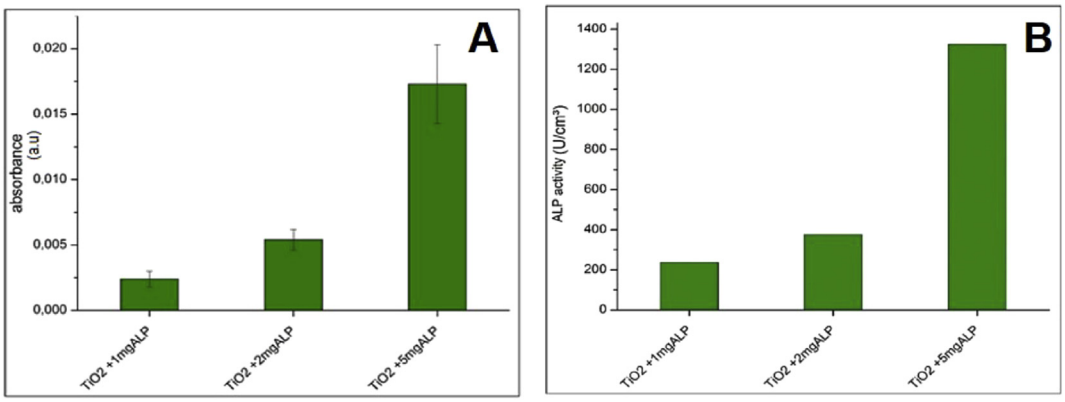

Fig. 4. (A) Specific absorption from 4 -nitrophenol at 405 nm and (B) enzymatic activity of ALP/dopamine - coated titania scaffolds.

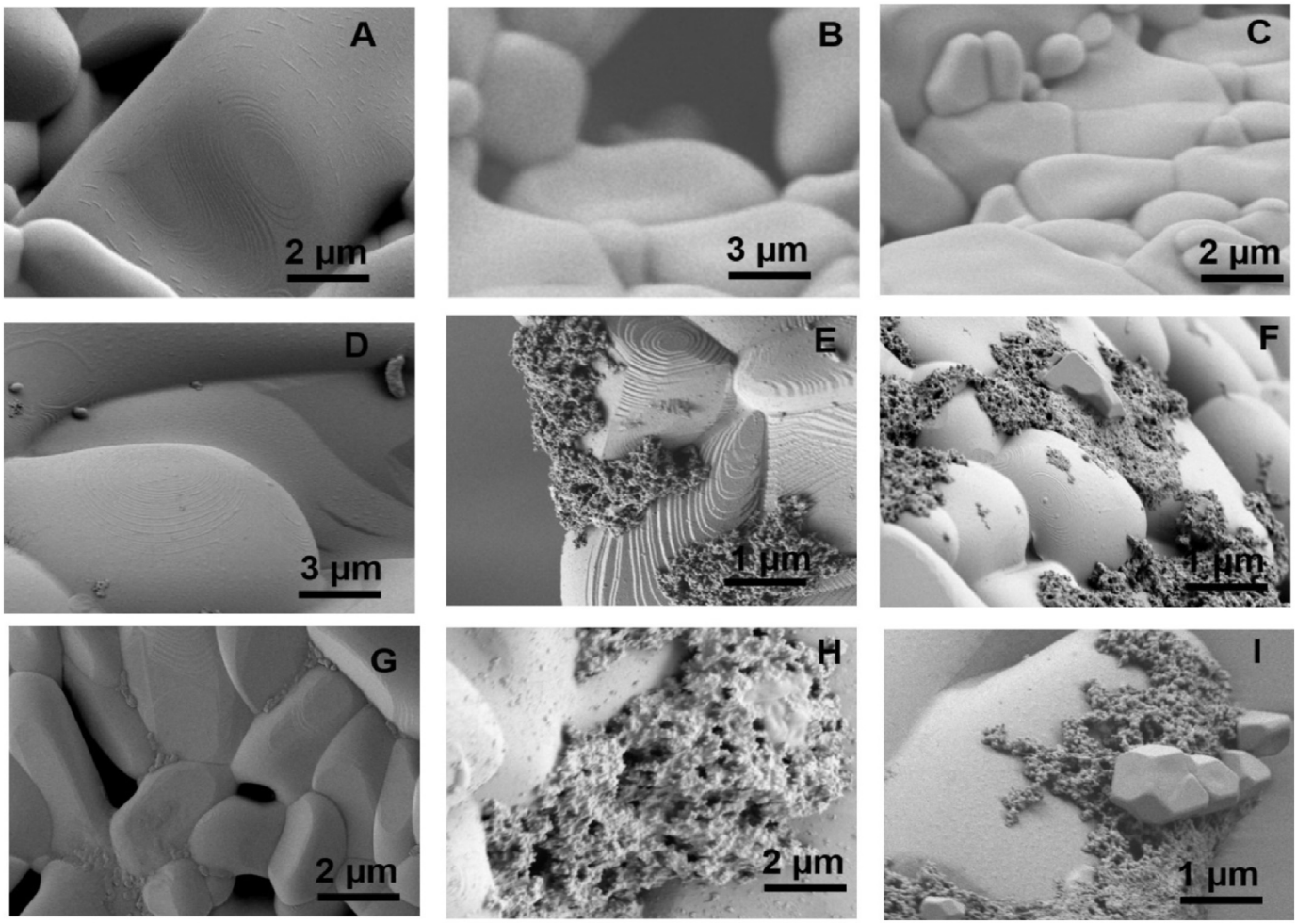

Fig. 5. SEM images of high magnification: (A) uncoated titania scaffold, (B) 2 mg and (C) 5 mg ALP coated titania after immersion for 7 days in SBF; (D) uncoated titania scaffold, (E) 2 mg and (F) 5 mg ALP coated titania scaffolds after immersion for 21 days in SBF; G) uncoated titania scaffold, (H) 2 mg and (I) 5 mg ALP coated titania scaffolds after immersion for 28 days in SBF.

in enhanced HA formation. The Ca/P ratio of the crystals was found to be ~1.1, which is lower than the value of stoichiometric HA (= 1.67). Liu et al. [31] have discussed the effect of different Ca/Pa ratios of different calcium phosphate ceramics showing that Ca/P ratios of ~1 enhance alkaline phosphatase activity in osteoblasts and, in general, Ca/P ratios <2 improve osteoblast viability.

Fig. 8 shows the XRD patterns of 1 mg, 2 mg and 5 mg ALP coated titania scaffolds after 28 days in SBF. In all the obtained spectra, a peak corresponding to hydroxyapatite (JCPDS file No: 09-0432) was observed. This result correlates with already reported values in the literature [23]. There is an increase in the relative intensity of HA peaks with increasing ALP concentration,

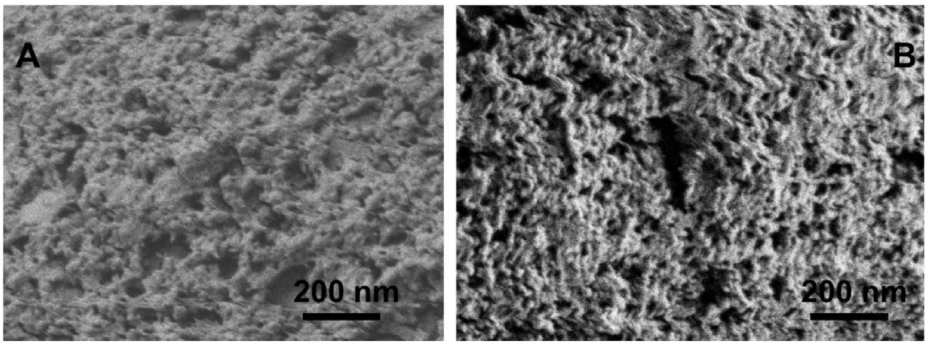

Fig. 6. High magnification SEM images of CaP agglomerates on the surface of scaffolds: (A) 2 mg and (B) 5 mg ALP coated titania scaffolds after immersion for 28 days in SBF.

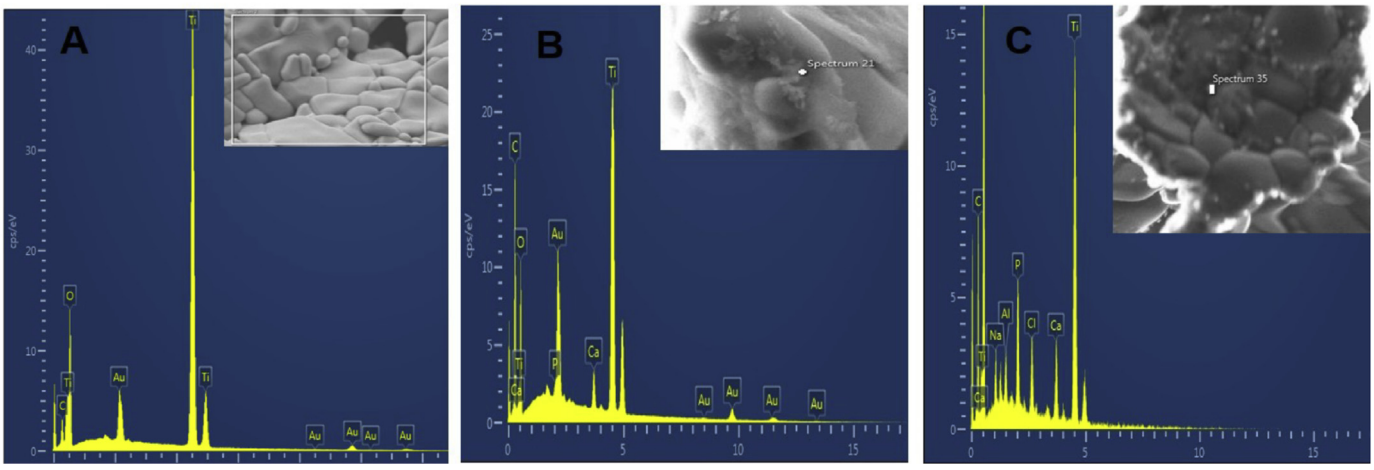

Fig. 7. EDS spectra of (A) uncoated scaffolds, (B) 2 mg and (C) 5 mg ALP coated titania scaffolds after immersion in SBF for 28 days.

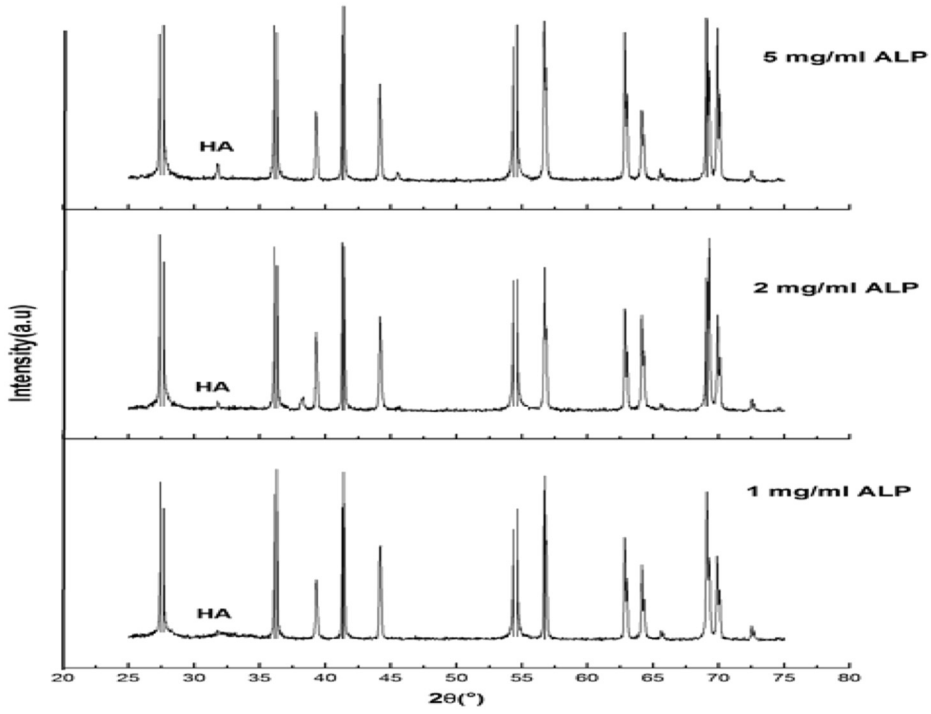

Fig. 8. XRD patterns of 1 mg, 2 mg and 5 mg coated ALP titania scaffolds after 28 days in SBF, the peak at 2θ-32° is attributed to HA and other sharp peaks represents the rutile phase.

suggesting higher content of HA with increasing ALP concentration.

4. Discussion

Bone is a highly complex and vascularized tissue structure exhibiting hierarchical levels of organization with a variety of structural and mechanical functions. Several requirements are critical for scaffolds in bone TE, such as an appropriate pore structure and porosity for cell migration, blood vessel formation and tissue ingrowth, a competent mechanical stability comparable to that of natural bone in order to be suitable for load-bearing applications and good bioactivity to deposit HA on the surface for bone bonding. In the current work, the scaffold pore size was calculated to be in the range of 300–400 μm which, according to Murphy et al. [32], is optimal for osteoblast migration and bone-tissue infiltration. The morphology of the titania scaffolds showed a high porosity of 97% and an open pore structure which should favor cell migration, mass transport of nutrients and waste removal [33]. The interconnective pore structure will allow effective cell seeding and cell migration throughout the complete structure of the scaffolds [34]. Scaffolds with porosity greater than 90% and a large pore size of 300 μm should enable bone formation in the entire scaffold [5,8,35]. The stability of aqueous ceramic slurries is influenced by the zeta potential. Fostad et al. [2] reported that a pH of 1.9 results in the maximum zeta potential of TiO_2 suspensions used to make scaffolds and the same pH was used in the current work. The slurry was prepared with equal proportions (wt%) of micro and nano TiO_2 particles, which significantly improved the compression strength of the scaffolds in comparison to previous studies [23]. The scaffolds showed compressive strength values of ~2.7 MPa. The compression strength of trabecular bone is typically 2–12 MPa [36]. The fabricated titania constructs exhibit thus suitable compressive strength and have high potential as load bearing scaffolds for bone TE applications. However, the major disadvantage of Ti-based biomaterials is that they lack bioactivity [23,37,38]. Bioactivity is considered one of the most important requirements for bone regeneration and it should be enhanced by the nucleation of HA on the scaffold surfaces enabling direct bonding of bone [39]. Therefore, the goal of the current study was to improve the bioactivity of titania scaffolds by ALP immobilization using a single step procedure that involves self-polymerization of dopamine. It has been proven already that dopamine forms high-strength, reversible bonds to inorganic surfaces by forming a thin layer on a surface due to the self-polymerization process [14,16]. Three different concentrations of ALP solutions, namely 1 mg/ml, 2 mg/ml and 5 mg/ml ALP with 20 mg dopamine were used to coat TiO_2 scaffolds.

Techniques proposed to improve the bioactivity of bioinert surfaces include coating by bioactive glasses [23], developing electrically active ceramic surfaces [38] or grafting biomolecules which support mineralization [20]. ALP is one such enzymes which plays a significant role in the mineralization and hard tissue formation process and it is localized to the outside of the plasma membrane of cells and of matrix vesicles [40]. ALP increases the concentration of inorganic pyrophosphate which acts as a promoter for mineralization and decreases the concentration of extracellular pyrophosphate which is an inhibitor of mineralization [41]. Tissue non-specific ALP alters the inorganic phosphate/pyrophosphate ratio in the premineralized matrix of the mineralizing tissue. ALP is also one of the first molecules expressed during the calcification process [40] and it is therefore widely used as a marker for osteoblast differentiation in *in vitro* assays [19]. It has been shown that ALP coatings on titanium implants improved new bone formation and enhanced the mineralization process [21]. In this study, the anchoring of ALP on the titania surfaces was assessed by enzymatic activity and contact angle tests. All samples showed contact angle less than 90° which corresponds to high wettability. Many studies have shown that the hydrophilicity of the surface and the surface energy play an important role in cell–material interactions such as cellular adhesion and growth [41,42]. Nijhuis et al. [14,15] have reported that the contact angle of dopamine coated surfaces was significantly lower than that of pure uncoated titania. All ALP coated titania pellets showed a high wettability which should favor cell-scaffold interactions. Enzymatic activity study after the surface functionalization with alkaline phosphatase revealed that the enzyme was grafted properly on the scaffold's surface using the self-polymerization properties of dopamine. This agress well with the studies reported by Nijhuis et al. [14] and Lee et al. [16], who have used the same strategy to graft biomolecules. The quantity of grafted enzymes depends upon the amount of ALP in the coating solution. The 5 mg ALP coated titania showed the highest enzymatic activity and this might be due to the homogeneous distribution of ALP throughout the polydopamine layer, as reported by Nijhuis et al. [14]. The 5 mg ALP coated titania scaffolds soaked for 28 days in SBF showed enhanced formation of HA in comparison to the other samples, as confirmed by XRD analysis. The results also indicated that lower concentrations of ALP do not enhance the bioactivity of rutile titania and only high concentrations of ALP could make a difference. De Jonge et al. [20] used electrospray technique for the immobilization of ALP on Ti substrates and even the sample obtained by using high deposition time (of 30 min) did not show any mineralization after 14 days in SBF. However, the samples soaked in cell culture medium (CCM, α-MEM) showed mineralization after 3 days and suggested that the α-MEM is more efficient than SBF for the process of calcium attraction in substrates containing ALP coatings [20]. Ferraris et al. [30] have reported that the chemical activation with tresyl chloride supports covalent grafting of ALP and they carried out a pre-treatment with tresyl chloride before the coatings of Ti alloy substrates [30]. An interesting aspect that remains to be investigated is the potential relative contribution of dopamine to the formation of HA in SBF, which could act by the effect of catecholamine moieties from polydopamine enriching the ceramic surface with calcium ions. Indeed Ryu et al. [43] have proposed mussel-inspired polydopamine coatings as a universal route to hydroxyapatite crystallization on virtually any type and morphology of scaffold materials, including ceramics, noble metals, semiconductors, and synthetic polymers. Possible synergic effects of polydopamine and ALP in inducing biomineralization of TiO_2 scaffolds should be considered in future research in which different contents of polydopamine in the coating solution should be applied. Overall, results indicate that ALP functionalization imparts bioactive character to bioinert surfaces and the present study has confirmed the suitability of the approach to bioactivate the surface of TiO_2 3D scaffolds, which are potentially useful for bone tissue engineering.

5. Conclusions

In this study, TiO_2 scaffolds were synthesized using the polymer foam replica method. The scaffold's characteristics such as relatively large pore size (about 300–400 μm), high porosity (around 97%) and a compressive strength of ~2.7 MPa indicate their suitability for bone TE applications. To enhance scaffold bioactivity, surface functionalization with ALP/polydopamine solution was performed. The deposition of HA was observed for the highest concentration of ALP, after immersion in SBF for up to 4 weeks. Future studies could consider methods to accelerate the bioactivation of the scaffolds. For example, bioactivity could be further improved by (i) long term immersion in cell culture medium, (ii) using even higher concentrations of ALP, or (iii) using a different

technique, such as electrospraying, to graft ALP on the surface. TiO_2 scaffolds with improved bioactivity represent an attractive novel family of scaffolds for bone TE.

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