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DMEM SOLUTION DEPOSITING AMORPHOUS CaP ON PURE TITANIUM AT 37°C

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ABSTRACT

DMEM (Dulbecco's modified Eagle medium) solutions are used in performing *in vitro* cell culture experiments to assess the cell biocompatibility of synthetic biomaterials. In this study, Hepes-buffered, phenol red- and sodium pyruvate-free DMEM solutions were used, for the first time as immersion media at 37°C, to test alkali-treated (5 M NaOH, 60°C, 24 h) grade-1 titanium substrates. Such DMEM solutions were found to deposit X-ray-amorphous calcium phosphate (ACP), in one or two weeks, on the soaked grade-1 Ti substrates. An inorganic solution (free of amino acids, vitamins, glucose, sodium pyruvate and phenol red), simulating the ion concentrations of DMEM solutions, was also used for the first time in depositing ACP on grade-1 Ti substrates upon soaking at 37°C for only 24 h. Samples were analyzed by using SEM, TEM, XRD, FTIR, ICP-AES, and XPS.

INTRODUCTION

Alpha-minimum essential medium (α -MEM) and Dulbecco's modified Eagle medium (DMEM) are solutions (media) which contain amino acids, vitamins, glucose and especially the inorganic salts at concentrations similar to those present in the whole mammalian serum. Both α -MEM and DMEM solutions, the preferred media to perform *in vitro* cell culture studies, originated from the pioneering work of Eagle [1, 2], which were focused on developing synthetic media with components essential and sufficient for the survival and growth of a wide variety of animal cells. Eagle's original minimum essential medium (MEM) contained 13 amino acids, 8 vitamins, glucose and inorganic salts such as NaCl, KCl, CaCl₂, MgCl₂·6H₂O, NaH₂PO₄·2H₂O and NaHCO₃ [2]. Eagle's MEM solution had a Ca/P molar ratio of 1.64 and a HCO₃⁻ concentration of 23.8 mM. Dulbecco's modification to the Eagle medium consisted of adding 2% horse serum to it [3, 4] resulting in an increase in the number of amino acids to 15. In a cell culture study directly comparing the α -MEM and DMEM solutions by using the human osteoblastic bone marrow cells, Coelho *et al.* [5] reported that the cell proliferation was similar in cultures grown in the two media but ALP (alkaline phosphatase) activity and ability to form mineralized deposits were lower in DMEM cultures.

DMEM can be obtained either as a powder or as a solution and there happens to be a number of variants of DMEM available [6], mainly in the forms containing high, low or no glucose at all, with or without glutamine, with or without Na-pyruvate, with or without phenol red, and with or without Hepes (*4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid*). Therefore, it is important to specify the catalog number of the manufacturer of the DMEM preferred in any study. The specific DMEM solution chosen for this study was previously used in testing the biomineralization of brushite powders at the human body temperature [7]. Most of the DMEM solutions produced today, in contrast to the original Eagle's MEM, have a Ca/P molar ratio of 1.99 and a HCO₃⁻ concentration of 44.05 mM. Blood plasma's Ca/P molar ratio and HCO₃⁻ concentration are 2.50 and 27 mM, respectively. Three different SBF (synthetic/simulated body fluid) solution formulations, which do not contain amino acids, vitamins and glucose, can match the Ca/P molar ratio (2.50) and the HCO₃⁻ concentration (27 mM) of human blood plasma [8-12], but cells cannot survive and grow in SBF solutions [13, 14].

The direct comparison between DMEM and HCO₃⁻-deficient (*i.e.*, 4.2 mM), Cl-rich (148 mM) SBF [15] solutions have been the subject of a limited number of previous studies, in which bioglass [16,

17] or calcium phosphate [18] samples have been soaked in both solutions, side-by-side, at 37°C, followed by the microscopic examination of the spherulites (or globules) forming on the sample surfaces. These studies [16-18], by only reporting EDXS (energy-dispersive x-ray spectroscopy) data, proved that the DMEM solution used was able to cover the bioglass, glass-ceramic, hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$), β - and α - polymorphs of tricalcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$) surfaces with the spherulites of a calcium phosphate (CaP) phase just like the SBF solutions would do. Nevertheless, none of these reports [16-18] provided any x-ray diffraction (XRD) data to ascertain whether the CaP formed on sample surfaces upon immersion in DMEM was amorphous, cryptocrystalline or crystalline, as well as whether that CaP phase contained any octacalcium phosphate ($\text{Ca}_8(\text{HPO}_4)_2(\text{PO}_4)_4 \cdot 5\text{H}_2\text{O}$) and/or brushite ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$) or not. Declercq *et al.* [19], on the other hand, had given quite a practical example on how to use experimental XRD and FTIR data to monitor the extent of biomineralization (or calcification) on rat osteoblast cells kept at 37°C in a cell culture medium.

Immersion tests performed at 37°C by using Hepes-free DMEM solutions containing phenol red, instead of SBF, were studied only on Ti6Al4V alloy substrates but not on pure Ti samples. Faure *et al.* [20] soaked the NaOH-treated (10 M NaOH solution, 60°C for 24 h) Ti6Al4V substrates in a DMEM solution (free of Hepes buffer, but containing phenol red and Na-pyruvate) at 37°C, and reported by XRD data that well-crystallized apatitic calcium phosphate (Ap-CaP) forming on the substrates. However, the low magnification electron microscope (SEM) photomicrographs provided by Faure *et al.* [20] made the detection and differentiation of the morphology of the formed Ap-CaP particles from the texture of the underlying NaOH-treatment layer somewhat difficult. Benhayoune *et al.* [21, 22] soaked Ti6Al4V samples having an electrodeposited CaP layer on them into a DMEM solution (again, Hepes-free yet containing Na-pyruvate and phenol red) at 37°C and observed the formation of new Ap-CaP nuclei on the sample surfaces.

The novelty of this study is the formation of X-ray-amorphous CaP (ACP) at 37°C, instead of hydroxyapatite, by the Hepes-buffered, phenol red- and sodium pyruvate-free DMEM solution, and by its purely inorganic variant solution, on the immersed alkali-treated grade-1 titanium coupons. Most supersaturated calcification solutions, including all SBFs, can form hydroxyapatite on substrates (including alkali-treated titanium [23]) immersed in them at 37°C, but none of such solutions were yet reported to form only ACP. DMEM solutions are used more widely than the SBF solutions in testing the biological compatibility of synthetic biomaterials (regardless of their polymeric, ceramic or metallic nature) in the presence of cells. The hereby reported ability of such solutions in depositing amorphous CaP, at 37°C, on the soaked biomaterials may also affect the results of research using Hepes-buffered, phenol red- and Na-pyruvate-free DMEM solutions as the cell culture medium.

EXPERIMENTAL

Grade-1 titanium (Ti) is obtained in the form of a 51 x 51 x 0.5 mm sheets (ESPI Metals, Lot number Q14641, Ashland, OR) and then cut into 10 x 10 x 0.5 mm square coupons by using a guillotine-cutter, followed by cleaning in pure acetone and water. The titanium coupons were certified by the supplier to contain no more than 0.18 wt% O, 0.20% Fe, 0.08% C, 0.03% N and 0.015% H. As-received grade-1 Ti coupons had the mean Vickers hardness of 140 HV \pm 17, which did not change with soaking in solutions at 37°C.

The Hepes-buffered DMEM solution (of pH 7.4) used in this study did not contain phenol red and sodium pyruvate (Gibco, 1X, sterile, Catalog number 21063-029, Life Technologies, Grand Island, NY). The composition of the DMEM solution is given elsewhere [7]. Alkaline treatments of Ti coupons were performed in a Teflon[®] beaker placed in a Teflon-lined stainless steel pressure vessel (Model 4760,

Parr Instrument Company, Moline, IL). One hundred mL of 5 M NaOH (pellets, Catalog number 28245, Merck) solutions were prepared in the Teflon beaker by using pre-boiled deionized water, followed by placing one Ti coupon into the alkali solution [23]. The isothermal heating of the sealed pressure vessel containing the Ti coupon was performed at 60°C for 24 h in a microprocessor-controlled oven. Samples were then washed with deionized water and dried at RT for 36 h. The soaking of NaOH-treated Ti coupons at 37°C in DMEM solutions was performed in heat-sterilized (at 140°C for 12 h) and sealed glass media bottles at 37±0.1°C. Ti coupons were soaked horizontally in 100 mL portions of DMEM solutions. At the end of the prescribed soaking times of one to two weeks, Ti coupons were removed, washed with deionized water and dried at RT. DMEM solutions did not show a change in their pH values at the end of 1 and 2 week runs.

For the purposes of providing a direct morphological comparison between the CaP deposits obtained from SBF and DMEM solutions, a Tris-buffered, 27 mM HCO₃⁻-containing SBF solution was also used during the immersion tests of grade-1 Ti coupons. This unique SBF solution had a Ca/P molar ratio of 2.50 and had a HCO₃⁻ concentration (=27 mM) identical with that of blood plasma [8, 9]. The SBF soaking of alkali-treated grade-1 Ti samples was continued for 4 days at 37°C.

The following chemicals were used in preparing an inorganic salt solution (free of amino acids, vitamins, glucose and Hepes) which mimic the inorganic salts compartment of the DMEM solution; NaCl (>99.5%, Cat. No: 106404, Merck, Darmstadt, Germany), KCl (>99.9%, Cat. No: 104933, Merck), NaHCO₃ (>99.9%, Cat. No: 106329, Merck), NaH₂PO₄·H₂O (>99.5%, Cat. No: 106346, Merck), CaCl₂·2H₂O (≥99.9%, Cat. No: 102382, Merck), and MgCl₂·6H₂O (>99.5%, Cat. No: 459330, Carlo Erba Reagenti, Milano, Italy). This solution with a pH value between 7.38 and 7.43 from RT to 37°C (Table 1) was previously formulated by our group [24] as a simple aqueous medium in testing the hydrothermal transformations of calcium phosphate bioceramic powders. The Ca/P molar ratio of this inorganic solution was adjusted to 2.50 in deviation from that of the DMEM formulation [24]. The salts indicated in Table 1 are added one-by-one to 1 L of deionized water in the order they were given. Alkali-treated grade-1 Ti coupons were kept in 100 mL of this solution at 37°C for 24 h, followed by washing with deionized water and drying at RT. The pH value of the solution did not change in 24 h.

Table 1 Composition of the inorganic salt solution similar to DMEM (DMEM-i)

Chemical (g/L)	Amount (mM)	Ion concentration (mM)	Ion in DMEM (mM)
NaCl	4.7865	Na ⁺ : 126.856 *	126.856
KCl	0.3974	K ⁺ : 5.33	5.33
MgCl ₂ ·6H ₂ O	0.1655	Mg ²⁺ : 0.814	0.814
CaCl ₂ ·2H ₂ O	0.3323	Ca ²⁺ : 2.26	1.8
NaH ₂ PO ₄ ·H ₂ O	0.1250	H ₂ PO ₄ ⁻ : 0.906	0.906
NaHCO ₃	3.7005	HCO ₃ ⁻ : 44.05	44.05

Surface deposits of soaked samples was studied by using a scanning electron microscope (SEM, EVO50, Carl Zeiss AG, Dresden, Germany). To produce powder samples of CaP deposited on Ti soaked in DMEM solutions larger coupons (25 x 25 x 0.5 mm) were soaked in the solutions, and the material scraped out using a clean razor was named as the powder samples. Quantitative chemical

analyses (*i.e.*, Ca/P molar ratio) of powder samples were performed by using inductively-coupled plasma atomic emission spectroscopy (ICP-AES, Model 61E, Thermo Electron, Madison, WI). Atomic absorption spectroscopy (AAS, Model PinAAcle 900H, PerkinElmer, Waltham, MA) was used for the quantitative determination of calcium and phosphor concentrations in the immersion solutions of Ti samples, as a function of aging time at 37°C. Solution samples were collected for AAS analyses at every 24 h, from the first to the end of the 13th day at 37°C (*i.e.*, total of 13 bottles for 13 days, each containing an alkali-treated grade-1 Ti sample in it), and the solutions were filtered through a 0.22 µm filter membrane prior to measurements. X-ray photoelectron spectrometer-based (XPS, Model 5400, PerkinElmer, Waltham, MA) depth profile analysis was performed by slowly removing the CaP coating on Ti samples using an argon (Ar) ion etch gun, which was calibrated at the removal rate of 3 nm/min.

On the other hand, the scraped CaP deposits from the surfaces of the Ti coupons were also analyzed by using a powder X-ray diffractometer (XRD, Advance D8, Bruker AG, Karlsruhe, Germany). These powders were also analyzed by Fourier-transform infrared spectroscopy (FTIR, Spectrum One, Perkin Elmer, MA, USA) after mixing them with KBr powder, followed by pressing into a 1 cm diameter transparent pellet. FTIR analyses were performed at 0.5 cm⁻¹ resolution with 128 scans. A diamond ATR (attenuated total reflection) accessory was used to directly collect FTIR data of CaP deposits formed on Ti samples. For the transmission electron microscope (TEM, JEOL 2010, Tokyo, Japan) investigations of the SBF precipitates, small aliquots of respective powder samples were first dispersed in pure ethanol, and then few drops of those suspensions were dried on Cu sample holder grids prior to imaging at 200 kV. The surface area of the SBF precipitate samples was determined by applying the standard Brunauer–Emmet–Teller (BET) method to the nitrogen adsorption isotherm obtained at -196°C using a Micromeritics ASAP 2020 instrument.

RESULTS AND DISCUSSION

Alkali treatment (5 M NaOH solution, 60°C, 24 h) of grade-1 Ti coupons rendered the surface covered with a porous network of fibers of Na₂Ti₂O₄(OH)₂ (ICDD-PDF 57-0123) less than 100 nm thick, as shown in the SEM photomicrographs of Figures 1a and 1b. We have previously reported the grazing incidence-XRD data of 5 M NaOH (or 5 M KOH)-treated Ti [25].

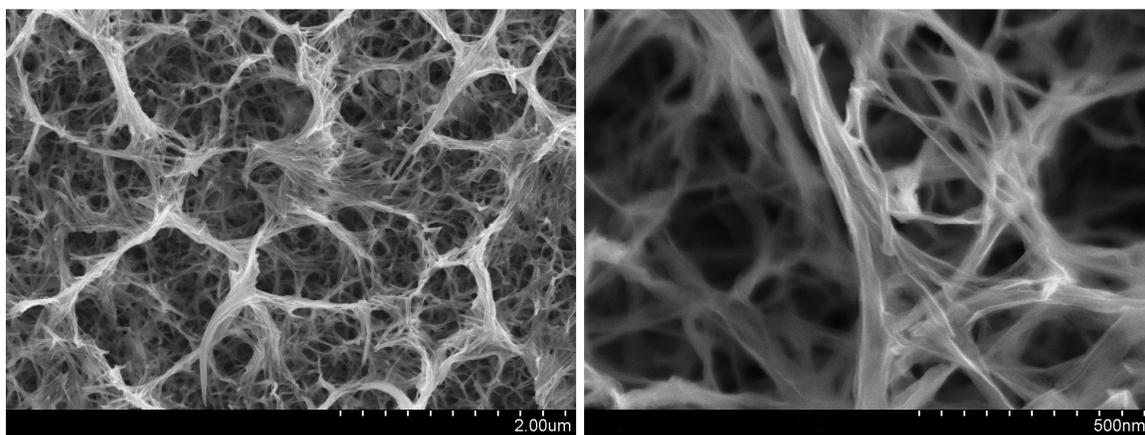


Figure 1 Typical SEM photomicrographs of the surface of grade-1 Ti soaked in 5 M NaOH solution at 60°C for 24 h (at two different magnifications)

The specific DMEM solution (of light yellow color) used in this study deposited a CaP phase of a unique morphology on grade-1 Ti coupons both in 1 week and 2 weeks of immersion runs. While the

Ti coupon-containing solutions aged at 37°C for 1 week did not display any turbidity, the solutions aged for 2 weeks started to display slight turbidity after about the 10th day. The SEM photomicrographs of Figures 2a through 2f depicted the covering of the NaOH-treated grade-1 Ti surfaces, upon 1 and 2 weeks of soaking in DMEM solutions, with a phase having a drastically different morphology than those of the typical SBF-produced globular deposits (shown in Figures 2g and 2h to provide a direct comparison).

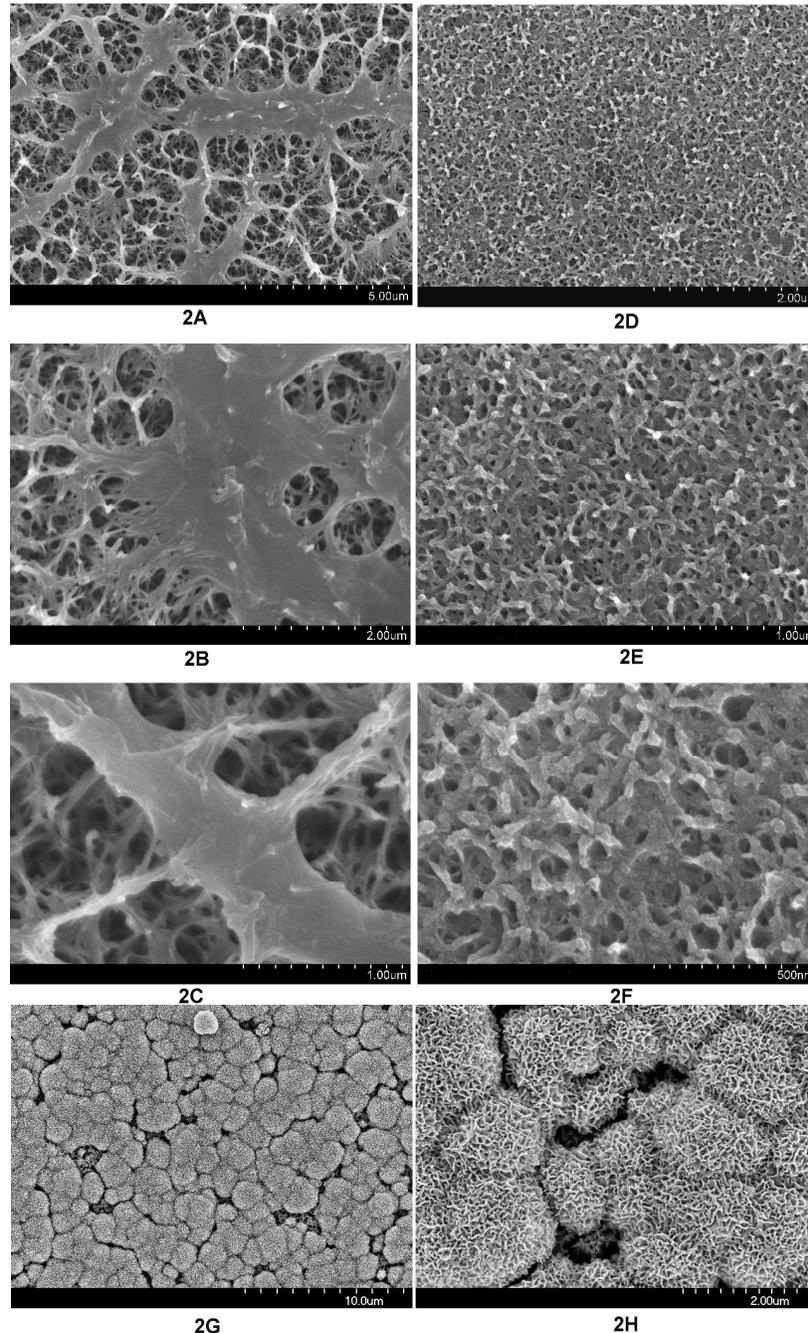


Figure 2 SEM photomicrographs of alkali-treated grade-1 Ti soaked in DMEM solution for 7 d at 37°C (a) to (c); for 14 d at 37°C (d) to (f); alkali-treated grade-1 Ti soaked in a Tris-buffered, 27 mM HCO₃⁻ containing SBF solution for 4 d at 37°C (g) to (h).

By the end of 1 week of DMEM-soaking (Figs. 2a, 2b and 2c), large sheets of relatively smooth-surfaced CaP (confirmed by numerous EDXS spot analyses) deposits seemed to connect the underlying hydroxylated sodium titanate nanofibers with one another. SBF solutions do not form such planar sheets of CaP material on Ti. However, at the end of 2 weeks of continuous soaking (Figs. 2d, 2e and 2f) in DMEM almost none of those starting hydroxylated sodium titanate nanofibers were visible, and the surface coverage with the nanoporous CaP phase was complete. It seemed like the sheets of CaP were forming first (within 1 week or so) and then these were acting as planar CaP scaffolds on which dissolution-reprecipitation processes were taking place at the nanoscale during the 2nd week. Such a mechanism was again not encountered in the SBF solution-based Ti coating practices.

ICP-AES analyses confirmed that the coated substance is Mg-doped calcium phosphate. ICP-AES analyses (5 analyses on 5 repetition samples) indicated the presence of 1568 ± 45 ppm Mg in the coated substance having a Ca/P molar ratio of 1.40 ± 0.11 . ATR-FTIR data of DMEM-coated Ti coupons are shown in Fig. 3, together with those of as received grade-1 Ti and alkali-treated grade-1 Ti.

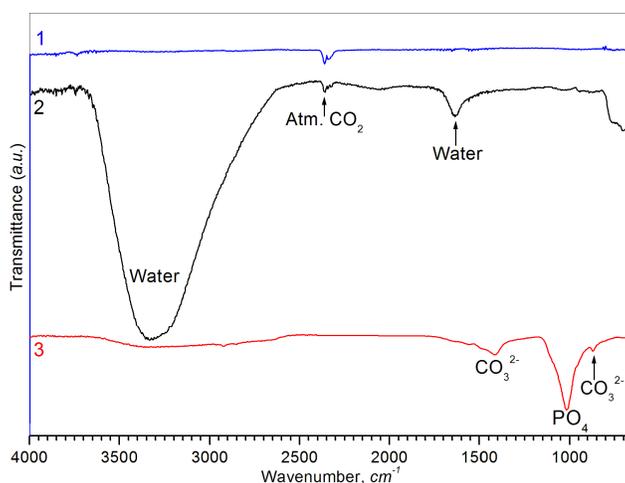


Figure 3 ATR-FTIR data of grade-1 Ti (1), alkali-treated Ti (2), and DMEM-ACP-coated Ti samples (3)

The inorganic salt solution (with a Ca/P molar ratio of 2.5) of Table 1 was much faster, in comparison to the DMEM solutions, in depositing X-ray amorphous CaP globules with extremely smooth surfaces on the alkali-treated grade-1 Ti samples at 37°C (Figs. 4a and 4b).

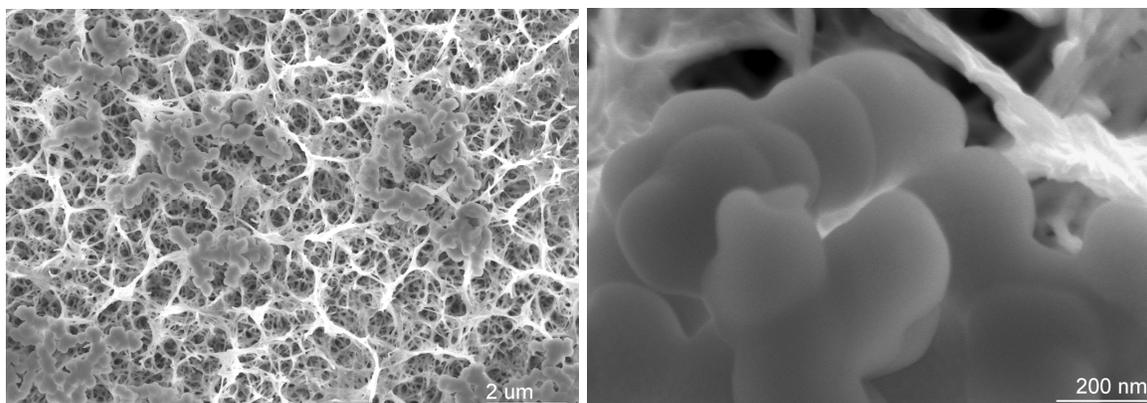


Figure 4 SEM photomicrographs (at two different magnifications) of ACP deposits forming in 24 h on the alkali-treated grade-1 Ti coupons soaked at 37°C in the solution of Table 1

The ICP-AES analyses (5 analyses on 5 repetition samples) of the scraped powdery deposits gave the Ca/P molar ratio as 1.45 ± 0.19 . The powders scraped off from the surfaces of Ti coupons soaked in the inorganic salt solution contained 1544 ± 53 ppm magnesium, according to the ICP-AES analyses. The powder XRD and FTIR data of the scraped ACP deposits were given in Figs. 5a and 5b. Both of Fig. 5a and Fig. 5a data correspond to ACP. To facilitate direct comparison with Hepes-buffered DMEM and SBF solutions, a characteristic XRD data of cryptocrystalline CaP deposited on Ti, by using a 27 mM HCO_3^- -containing Tris/HCl-buffered SBF solution [9], is given in Fig. 5c, in which broad peaks of apatitic CaP (labeled as *Ap*) are visible.

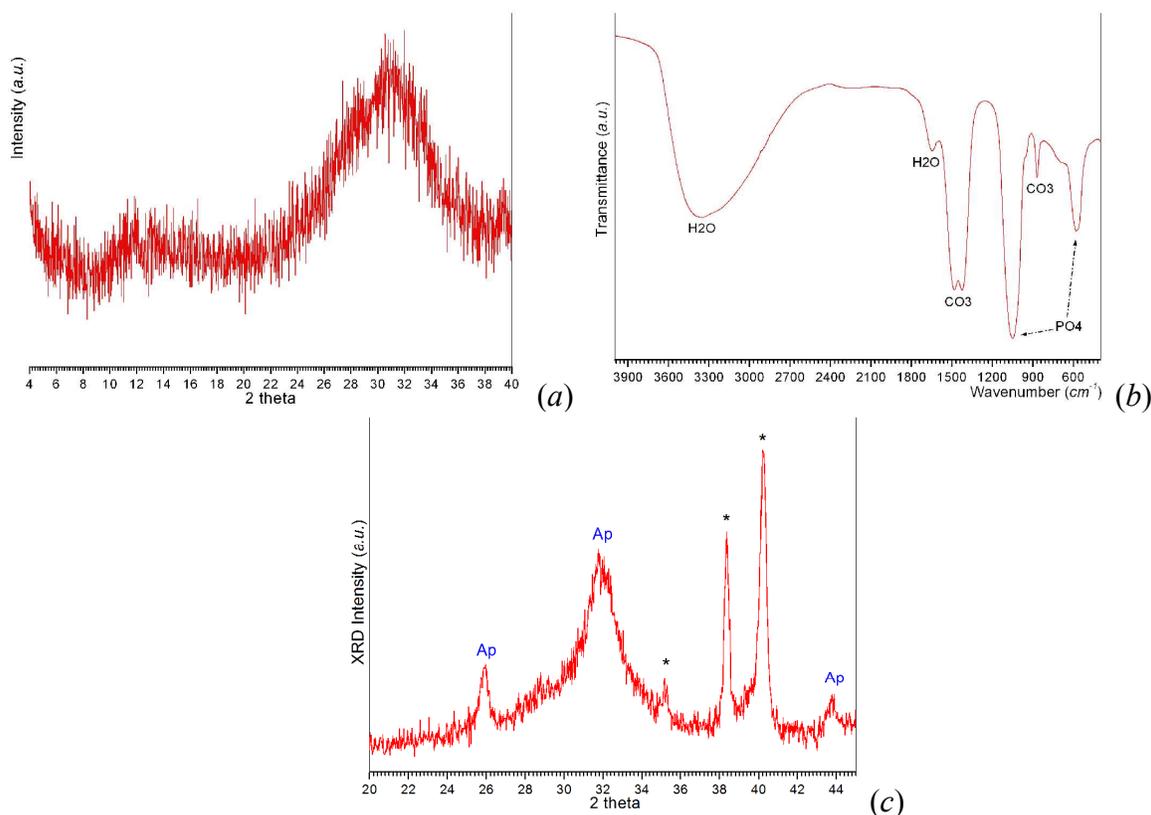


Figure 5 (a) XRD data of ACP deposits scraped from the surface of coupons shown in Fig. 4; (b) FTIR data of the same ACP deposits; (c) XRD data of Ti coated in Tris-SBF of 27 mM HCO_3^- for 4 d at 37°C

The XRD and FTIR data (Figs. 5a and 5b) obtained from the scraped deposits were decisive in confirming the amorphous nature of the CaP deposits. CaP deposited from a physiological solution with such an extraordinary smooth surface (Fig. 4b) was not reported prior to this study.

The XPS depth profile analysis of grade-1 Ti immersed in an Hepes-buffered DMEM solution at 37°C for two weeks is shown in Fig. 6a. The SEM photomicrographs of this sample is shown in Figs. 2d through 2f. The XPS depth profiling showed that the Mg-doped CaP coating had a thickness of about 220 nm (Fig. 6a). Fig. 6b shows the AAS data of solutions kept at 37°C from 1 through 13 days. Calcium and phosphor concentrations of the solutions showed a slight yet noticeable decrease with aging time. Phosphor concentrations, as a function of aging time, of DMEM and the inorganic solution (Table 1) were found to be identical with one another.

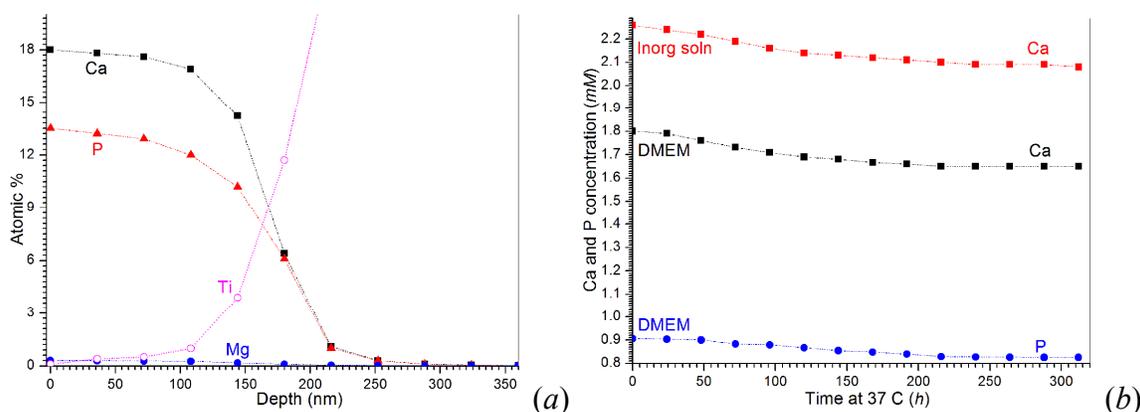


Figure 6 (a) XPS depth profile of DMEM-soaked Ti sample for 14 d at 37°C; (b) AAS data of the DMEM solution and the inorganic solution (Table 1) as a function of aging time at 37°C

Synthetic biomaterials, whether they are metallic, ceramic or polymeric, shall be thoroughly tested for their *in vivo* biocompatibility. *In vivo* animal tests apparently place a burden on the shoulders of many materials-based research groups since such animal tests require tedious histological examinations and careful interpretation. *In vitro* osteoblast cell culture tests, mainly reporting the live/dead cell numbers and the popular ALP (alkaline phosphatase) activity data, have thus been the most routine tests performed by the materials-based research groups which lack collaborative partnerships with the external veterinarians and skilled histologists. DMEM (or α -MEM) solutions are the media of choice in such *in vitro* cell culture tests.

One may underline several issues here; (1) the SBF solutions commonly deposit micron-sized spherulites/globules of cryptocrystalline apatitic CaP at 37°C even if the samples to be soaked in it were selected from a set as diverse as bioglass, glass-ceramic, titanium, or polymers, (2) since the SBF solution is a supersaturated (with respect to apatitic CaP formation) and metastable calcification solution, its CaP deposition rate on the sample surfaces will be accelerated if the soaked material is soluble in that solution (*i.e.*, leaching out of calcium and/or phosphate ions from the substrate) or has a slightly basic surface to trigger the nucleation of the apatitic and Ca-deficient CaP in the form of spherulites/globules, (3) as a practical example, if one keeps a liter of freshly prepared SBF solution (with no substrate whatsoever to test in it) in a tightly sealed sterile bottle in a refrigerator at 4°C for 4 months, it will autogenously precipitate high surface area (approx. 900 m²/g) carbonated, cryptocrystalline apatitic CaP (Figs. 7a through 7d); if a similar bottle of SBF, with no sample in it, is heated at 37°C for 1 week the rate of precipitate formation (with the observation of excessive solution turbidity) is drastically increased, (4) in case of planning an *in vitro* cell culture study for a given synthetic biomaterial, one can easily incorporate into that study, as a control sample, the “biomaterial + DMEM (or α -MEM) + no cells” compartment. Moreover, at the end of a cell culture study it may always be a good practice to examine the extent of any biomineralization (by using SEM, XRD and FTIR) taking place on the samples in the presence of the cells (or in the absence of cells) in the culture medium, as exemplified by Declercq *et al.* [19].

FTIR data can be used as a rapid and powerful tool in differentiating between ACP and cryptocrystalline Ap-CaP. ACP samples (Fig. 5b) do not exhibit that peak splitting observed in that broad 600 to 500 cm⁻¹ phosphate band (Fig. 8a) in the case of cryptocrystalline Ap-CaP samples. The XRD data also help to differentiate between X-ray-amorphous CaP (Fig. 5a) and poorly crystalline Ap-CaP (Fig. 8c) samples.

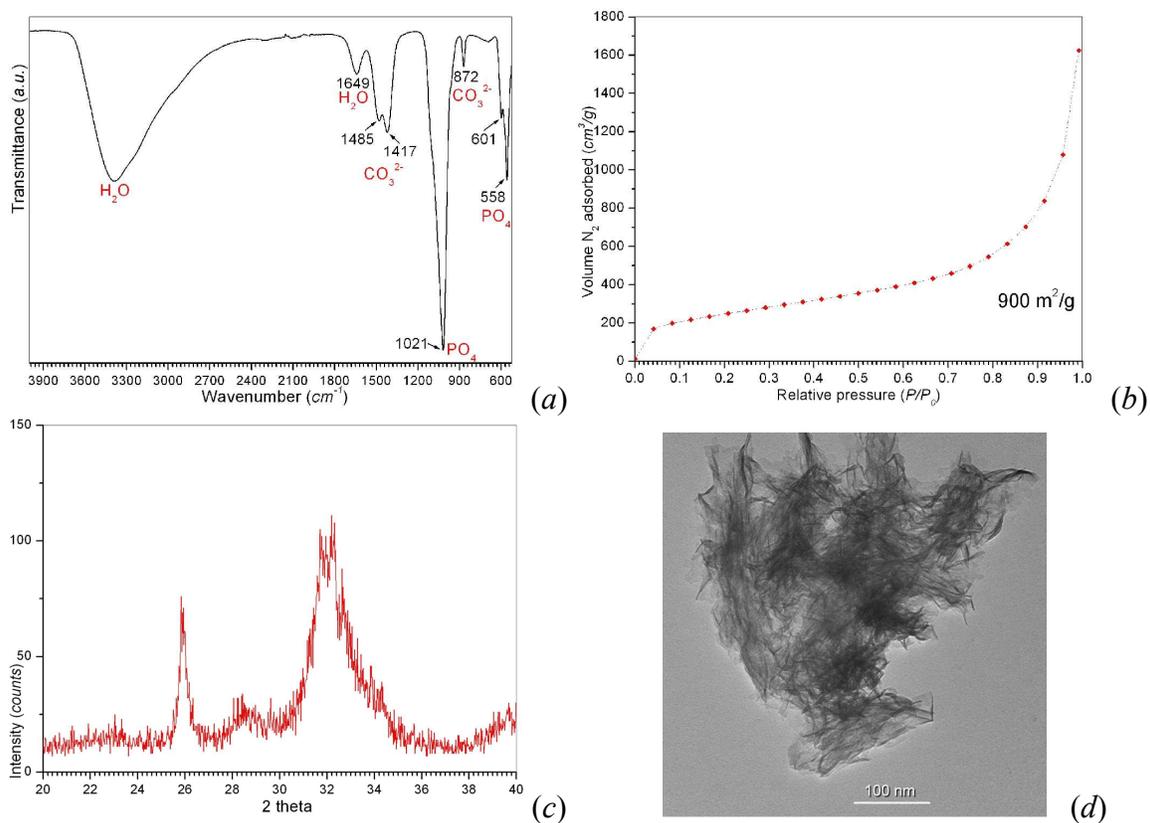


Figure 7 FTIR (a), BET surface area (b), XRD (c) and TEM (d) of *in situ* forming cryptocrystalline apatitic calcium phosphate (Ap-CaP) precipitates recovered from an SBF solution kept undisturbed, in a sealed glass bottle, in a refrigerator at 4°C for 120 days

Since both solutions of DMEM and Table 1 (inorganic) resulted in the formation of X-ray-amorphous CaP (*i.e.*, ACP) on the surface of grade-1 Ti, one may argue that the formation of ACP in the DMEM solutions cannot be ascribed solely to the presence of amino acids, vitamins, glucose and Hepes buffer present in them. The presence of Na-pyruvate ($C_3H_3NaO_3$, pyruvic acid sodium salt) and phenol red, and the absence of Hepes in the DMEM solutions of the previous studies [20-22] in coating Ti6Al4V substrates with cryptocrystalline Ap-CaP (but not with ACP) actually presents deviations from the experimental conditions of the current study in four points; (1) Na-pyruvate, (2) phenol red, (3) Hepes, and (4) use of grade-1 Ti versus Ti6Al4V. Na-pyruvate is added to cell culture solutions as an additional source of energy (besides glucose) for the cells. Since neither the previous studies [20-22] nor the present one had cells in the solutions, the inclusion of a chemical to provide additional energy to the cells is omitted in this study. If the DMEM solution selected is of the low glucose (e.g., 1000 mg/L) specification, then the manufacturers add Na-pyruvate at a concentration of about 110 mg/L (=1 mM) [6].

Mg concentration in DMEM solutions is fixed at 0.814 mM. If the pyruvate molecules (CH_3O_3) in the solution do have a higher affinity to Mg^{2+} than they have to Na^+ (Mg-pyruvate: $C_6H_6MgO_6$), then there are more pyruvate groups in such DMEM solutions than there is Mg^{2+} (1 *versus* 0.814 mM). If a significant portion of the Mg^{2+} ions in DMEM [20-22] are bound to pyruvates, then there may be not enough Mg^{2+} left necessary for the stabilization of ACP. Stabilization of ACP by Mg was meticulously described by Boskey and Posner [26]. Both DMEM solution and the inorganic solution of Table 1

contains the ACP-stabilizer Mg^{2+} ions. Phenol red ($\text{C}_{19}\text{H}_{14}\text{O}_5\text{S}$, phenolsulfonphthalein) is added at a concentration of 15 mg/L to the DMEM solutions to serve as a pH indicator, if and when the pH of a DMEM solution drops below 6.8 (typically observed when the solution is contaminated with bacteria), the solution will lose its red color and turn yellow [6]. Likewise, if and when the pH of a DMEM solution rises beyond 8.2, the solution's color will change to bright pink. The pH values of the DMEM solutions used here (phenol red-free) did not change after 1 or 2 weeks experiments at 37°C. Since there would be no cells in the DMEM solutions, it has been decided at the start to avoid both phenol red and Na-pyruvate.

The previous researchers working with titanium alloys [20-22], however, did select DMEM solutions which are not Hepes-buffered. Hepes is an essential component which shall help keeping most of the HCO_3^- ions (44.05 mM initially) of DMEM in solution especially during the long soaking times of 1 or 2 weeks at 37°C. Cell culture studies are performed in basically open (although loosely covered, i.e., not gas-tight) plastic well plates in 5% CO_2 -supplemented incubators, therefore even a Hepes-free DMEM solution can get some HCO_3^- from the CO_2 gas constantly present in such incubators. Since the experiments of previous researchers [20-22] and those of this study were not performed in such CO_2 incubators, but in sealed or capped bottles, the only HCO_3^- source available was the bicarbonate ion initially present in the DMEM solution. We considered minimizing the loss of HCO_3^- (aq) at the liquid-gas interface in the glass media bottles over long soaking times with the help of Hepes present in the starting solution. However, the presence or the absence of Hepes in DMEM may well not be the only reason for the preferential deposition of either Ap-CaP or ACP on Ti. On the other hand, the issue of using Ti6Al4V *versus* grade-1 Ti in DMEM, to come up with a plausible explanation for the formation of Ap-CaP or ACP, still needs to be tackled experimentally. The alkali treatment of Ti6Al4V *versus* the alkali treatment of grade-1 Ti may produce chemically different surfaces, which would then react differently upon soaking in DMEM.

Synthetic ACP, according to Posner and Boskey [26], consisted of roughly spherical $3\text{Ca}_3(\text{PO}_4)_2$ clusters (Ca/P=1.50), which formed in water and were then aggregated randomly to produce the larger globular particles of ACP with the inter-cluster space being filled with water. Mg^{2+} ions act as an ACP stabilizer, as mentioned above [26]. HCO_3^- ions, which are present at a high concentration of 44.05 mM (in comparison to the blood plasma value of 27 mM) in DMEM solutions, are also known to stabilize ACP [27]. Therefore, two of the most significant ACP stabilizers, namely, Mg^{2+} and HCO_3^- , were present in both the DMEM and the inorganic solution (of Table 1) used in this study. It would thus be unusual to form hydroxyapatite, instead of ACP, in both solutions.

The human venous plasma and whole blood contain amino acids and the successful identification and quantification of those amino have been previously reported [28, 29]. Conconi *et al.* [30] reported that the amino acids (lysine, threonine, methionine, tryptophan, arginine, which are all present in the DMEM solutions) increased both the osteoblast proliferation and alkaline phosphatase activity of rat osteoblasts cultured *in vitro*. Imamura *et al.* [31] and Tentorio and Canova [32] separately showed that the amino acid lysine adsorbs itself on pure metallic Ti and on amorphous Ti hydrous oxide surfaces, respectively, at neutral pH values. While the inorganic SBF solutions cannot provide any practical means of producing synthetic biomaterials with some amino acids adsorbed on their surfaces, DMEM solutions can provide unique biomaterial surfaces already containing adsorbed amino acids.

CONCLUSIONS

Hepes-buffered, phenol red- and sodium pyruvate-free DMEM solutions were found to deposit X-ray amorphous calcium phosphate (ACP) on alkali-treated grade-1 titanium coupons soaked for one and two weeks at 37°C. Previous studies performed with other DMEM solutions and with Ti6Al4V

substrates reported the deposition of cryptocrystalline apatitic calcium phosphate, these reports contrasted with the findings of this study. An inorganic and amino acid-, vitamins-, glucose-free solution mimicking the inorganic ion concentrations of the DMEM solutions was also found to deposit ACP, but not hydroxyapatite, at 37°C on alkali-treated grade-1 Ti coupons soaked for only 24 h. A cryptocrystalline apatitic calcium phosphate with an extraordinary surface area of 900 m²/g is reported to form in a Tris-buffered SBF solution of 27 mM HCO₃⁻ upon refrigerating at +4°C for prolonged times.

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