Materials Science and Engineering C 36 (2014) 84–94

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Grade-1 titanium soaked in a DMEM solution at 37 °C

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ARTICLE INFO

Article history:
Received 12 July 2013
Received in revised form 27 October 2013
Accepted 28 November 2013
Available online 7 December 2013

Keywords:
DMEM
Amorphous
Calcium phosphate
Titanium

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. A limited number of previous studies focusing on the biomimetic coating of alkali-treated Ti6Al4V coupons in DMEM have actually used different DMEM solutions, which were not Hepes-buffered and containing phenol red and sodium pyruvate. The previous studies with such DMEM solutions reported the deposition of cryptocrystalline apatitic calcium phosphate (Ap-CaP) on Ti6Al4V substrates, but not ACP. An inorganic solution (free of amino acids, vitamins, glucose, sodium pyruvate and phenol red), simulating the ion concentrations of the 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. The solutions and deposits of this study were analyzed by AAS, ICP-AES, FTIR, XRD, XPS, and surface profilometry.

1. 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, $CaCl_2$, $MgCl_2 \cdot 6H_2O$, $NaH_2PO_4 \cdot 2H_2O$ and $NaHCO_3$ [2]. Eagle's MEM solution had a Ca/P molar ratio of 1.64 and a HCO_3^- 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, the physiological medium of interest in this study, 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 HCO_3^- concentration of 44.05 mM. Blood plasma's Ca/P molar ratio and HCO_3^- 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 HCO_3^- 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 HCO_3-deficient (*i.e.*, 4.2 mM), Cl-rich (148 mM) SBF [15] solutions has 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 ($Ca_{10}(PO_4)_6(OH)_2$), β- and α-polymorphs of tricalcium phosphate ($Ca_3(PO_4)_2$) 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

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http://dx.doi.org/10.1016/j.msec.2013.11.045

on sample surfaces upon immersion in DMEM was amorphous, cryptocrystalline (*i.e., yielding poor crystallinity XRD patterns incapable of resolving the apatite's quartet of peaks, namely (211), (112), (300) and (202) reflections, over the Cu K_α-radiation 2θ range of 30 to 35°*) or crystalline, as well as whether that CaP phase contained any octacalcium phosphate ($Ca_8(HPO_4)_2(PO_4)_4 \cdot 5H_2O$) and/or brushite ($CaHPO_4 \cdot 2H_2O$) or not. Declercq et al. [19], on the other hand, had given quite a proficient example on how to use experimental XRD and FTIR data to monitor the extent of biomineralization (or calcification) on rat osteoblast cells kept 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, to the best of our knowledge, not on pure Ti samples. This was why this study examined the surfaces of pure Ti (grade-1) when soaked in a Hepes-buffered DMEM solution at the human body temperature. 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 observation of X-ray-amorphous CaP (ACP) formation 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 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 forming a nano layer of amorphous CaP, at 37 °C, on the soaked biomaterials should affect the results of research using Hepes-buffered DMEM as a cell culture medium.

The current morphological examination study is originally aimed at finding answers to the following questions;

(i) will alkali-treated grade-1 Ti coupons soaked in Hepes-buffered, glucose-containing, but sodium pyruvate- and phenol red-free DMEM solutions at 37 °C form spherulites/globules of cryptocrystalline apatitic CaP (similar to those encountered in the SBF immersion tests) on their surfaces as previously reported by Faure [20] and Benhayoune et al. [21,22] for a different DMEM solution as mentioned above?

(ii) will alkali-treated grade-1 Ti coupons soaked (at 37 °C) in an aqueous inorganic salt solution (free of amino acids, vitamins, glucose and Hepes) only resembling the inorganic salts compartment of a DMEM solution form cryptocrystalline apatitic CaP on their surfaces?

2. Materials and methods

2.1. Materials, chemicals and solution treatments

Grade-1 titanium (Ti) is obtained in the form of a 51 × 51 × 0.5 mm sheets (ESPI Metals, Lot number Q14641, Ashland, Oregon, USA) and then cut into 10 × 10 × 0.5 mm square coupons by using a guillotine-cutter, followed by cleaning in glass beakers containing pure acetone placed in an ultrasonic bath for 5 min, rinsing with freshly boiled (and cooled) deionized water and drying at room temperature (RT, 22 ± 1 °C) in a desiccator. 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 Ti coupons had the mean Vickers hardness of 140 HV ± 17, which did not change with soaking in solutions at 37 °C. The micro-roughness of *as is* and coated Ti coupons were experimentally determined by using a surface profiler (Model Dektak 3030, Veeco, Plainview, NY).

The Hepes-buffered DMEM solution (of pH 7.4) used in this study contained no phenol red and no sodium pyruvate (Gibco, 1×, sterile, Catalog number 21063-029, Life Technologies Corp., Grand Island, NY, USA). The composition of the DMEM solution is given in Table 1. Although no bacterial growth was observed (by scanning electron microscope) on the Ti samples soaked in the sterile DMEM solutions of this study, the addition of NaN_3 (sodium azide) at the concentration of 15 mg/L, if deemed to be necessary, may also be considered to circumvent any bacterial activity in such amino acids-, vitamins- and glucose-containing solutions when they are used for the purpose of producing CaP deposits on the surfaces of synthetic biomaterials.

Alkaline treatments of Ti coupons were performed in a 150 mL-capacity Teflon® beaker placed in a Teflon-lined stainless steel pressure vessel (Model 4760, Parr Instrument Company, Moline, Illinois). One hundred mL of 5 M NaOH (pellets, Catalog number 28245, Merck) solutions was 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 an ample volume of deionized water and dried overnight at RT. 5 M NaOH-60 °C treatments

Table 1
Composition of the DMEM solution used (*Gibco, 21063-029*).

Component	Concentration (mM)
Amino acids	
Glycine	0.4
L-Arginine hydrochloride	0.398
L-Cystine·2HCl	0.201
L-Glutamine	4
L-Histidine hydrochloride–H_2O	0.2
L-Isoleucine	0.802
L-Leucine	0.802
L-Lysine hydrochloride	0.798
L-Methionine	0.201
L-Phenylalanine	0.4
L-Serine	0.4
L-Threonine	0.798
L-Tryptophan	0.0784
L-Tyrosine disodium salt dihydrate	0.398
L-Valine	0.803
Vitamins	
Choline chloride	0.0286
D-Calcium pantothenate	0.00839
Folic acid	0.00907
Niacinamide	0.0328
Pyridoxine hydrochloride	0.0194
Riboflavin	0.00106
Thiamine hydrochloride	0.0119
i-Inositol	0.04
Inorganic salts	
Calcium chloride ($CaCl_2$) anhydrous	1.8
Ferric nitrate ($Fe(NO_3)_3 \cdot 9H_2O$)	0.000248
Magnesium sulfate ($MgSO_4$) anhydrous	0.814
Potassium chloride (KCl)	5.33
Sodium bicarbonate ($NaHCO_3$)	44.05
Sodium chloride (NaCl)	81.9
Sodium phosphate monobasic ($NaH_2PO_4 \cdot H_2O$)	0.906
Other components	
D-Glucose (Dextrose)	25
HEPES	25.03

were not performed in silicate-based glassware since elemental silicon contamination would be detected afterwards (by EDXS, for instance) on the surfaces of samples. 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 tightly sealed glass media bottles (100 mL-capacity) at 37 ± 0.1 °C in a microprocessor-controlled oven. 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 HCO_3-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 HCO_3^- 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) resembling the inorganic salts compartment of the DMEM solution; NaCl (>99.5%, Cat. No: 106404, Merck, Darmstadt, Germany), KCl (>99.9%, Cat. No: 104933, Merck), $NaHCO_3$ (>99.9%, Cat. No: 106329, Merck), $NaH_2PO_4 \cdot H_2O$ (>99.5%, Cat. No: 106346, Merck), $CaCl_2 \cdot 2H_2O$ (≥99.9%, Cat. No: 102382, Merck), and $MgCl_2 \cdot 6H_2O$ (>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 2) was previously formulated by us [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 [24] was adjusted to 2.50 in deviation from that of the DMEM formulation of Table 1. The salts indicated in Table 2 are added one-by-one to 1 L of pre-boiled (*i.e.*, dissolved carbonate-free) deionized water in the order they were given and the addition of the next salt must be performed immediately after the preceding salt was stirred well to dissolve. Alkali-treated grade-1 Ti coupons were kept in 100 mL of this solution (in 100 mL-capacity sealed glass media bottles) at 37 °C for 24 h, followed by washing with deionized water and drying at RT. The pH value of the solution was again in the close vicinity of 7.4 at 37 °C at the end of 24 h soaking tests.

2.2. Sample characterization

Surface deposits of soaked samples was studied by using a scanning electron microscope (SEM, EVO50, Carl Zeiss AG, Dresden, Germany). Energy dispersive X-ray spectroscopy (EDXS) was used to perform qualitative chemical analyses on all samples. SEM and EDXS samples were sputter-coated with a thin (approx. 5 nm) layer of Au–Pd alloy prior to imaging.

To produce powder samples of CaP deposited on Ti soaked in DMEM solutions larger coupons (25 × 25 × 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). For the ICP-AES analyses, 20 mg portions of powder samples were dissolved in 3 mL of concentrated HNO_3 solution. 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.

Thin film X-ray diffraction data of the CaP-deposited Ti samples were collected in the 2θ-omega scan mode using a Philips X'Pert X-ray diffractometer with parallel beam optics. A one mm divergence height limiting slit was used, and the divergence, scattering, and receiving slits were open. The source to sample fixed angle was 1°. X-ray tube (Cu) settings of 45 kV, 40 mA, and 3 kW were used for these measurements. Data were collected in 0.02° steps for 5 s per step. 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, powdery 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). Powder was placed onto a zero-background quartz single crystal sample holder. X-ray tube (Cu) settings of 40 kV, 30 mA, and 1.7 kW were used for the powder XRD measurements. Data were collected in 0.02° steps for 5 s per step. These powders were also analyzed by Fourier-transform infrared spectroscopy (FTIR, Spectrum One, PerkinElmer, 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 Brunnauer–Emmet–Teller (BET) method to the nitrogen adsorption isotherm obtained at −196 °C using a Micromeritics ASAP 2020 instrument.

3. Results

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 $Na_2Ti_2O_4(OH)_2$ (ICDD-PDF 57-0123) less than 100 nm thick, as shown in the SEM photomicrographs of Fig. 1A and B. We have previously reported the grazing incidence-XRD data of 5 M NaOH-treated Ti [25]. EDXS data of *as is* Ti and NaOH-treated Ti of the current study are given in Fig. 1C. Since the nanofibers, forming a web-like network, on Ti coupons were composed of $Na_2Ti_2O_4(OH)_2$, EDXS analyses (Fig. 1C) can be used to detect Na on the surface. Our previous study focused more on the alkali treatment (either with NaOH or KOH) of Ti [25].

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 suspended particles causing this turbidity should be colloidal since they did not settle to the bottom of the glass bottles. The high resolution SEM photomicrographs of Fig. 2A through f 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

Table 2
Composition of the inorganic salt solution similar to DMEM.

Chemical	Amount (g/L)	Ion concentration (mM)	Ion in DMEM (mM)
NaCl	4.7865	Na^+: 126.856*	126.856
KCl	0.3974	K^+: 5.33	5.33
$MgCl_2 \cdot 6H_2O$	0.1655	Mg^{2+}: 0.814	0.814
$CaCl_2 \cdot 2H_2O$	0.3323	Ca^{2+}: 2.26	1.8
$NaH_2PO_4 \cdot H_2O$	0.1250	$H_2PO_4^-$: 0.906	0.906
$NaHCO_3$	3.7005	HCO_3^-: 44.05	44.05
		Ca/P molar: 2.50	Ca/P molar: 1.99

*Na concentration shown was the sum of three contributing sources; 81.9 (from NaCl), 0.906 (from $NaH_2PO_4 \cdot H_2O$), and 44.05 mM (from $NaHCO_3$).

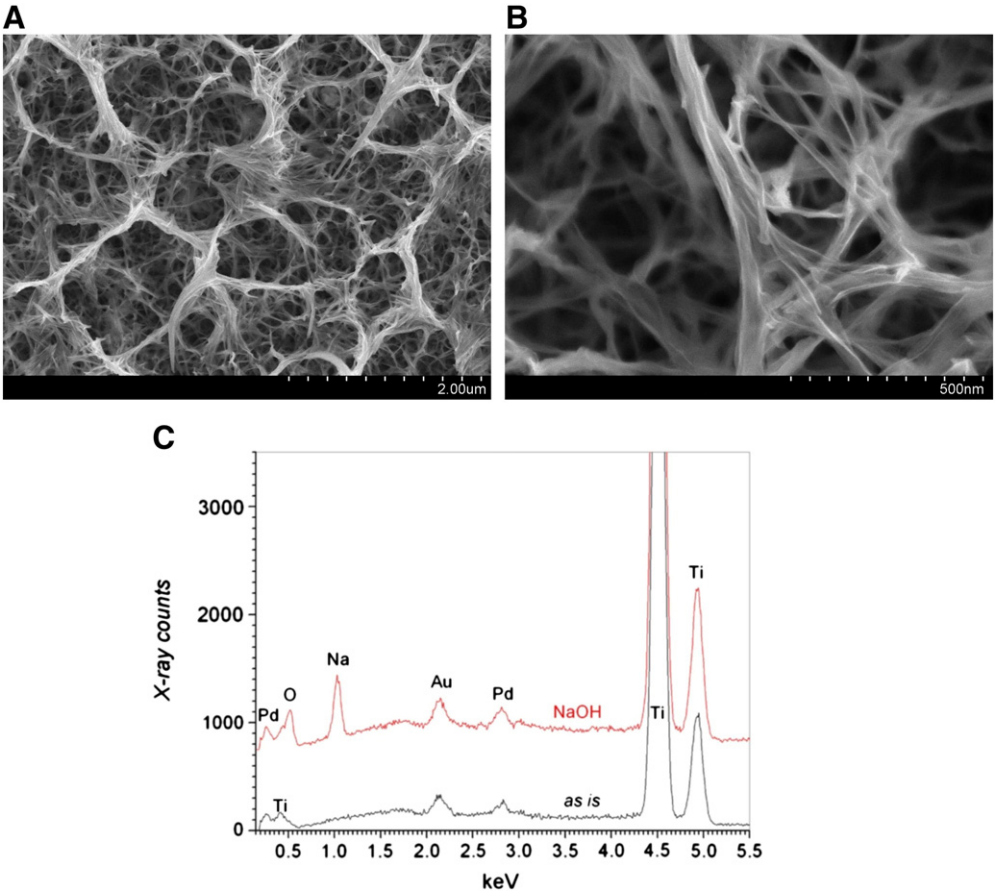

Fig. 1. (A) and (B): SEM photomicrographs of the surface of grade-1 Ti soaked in 5 M NaOH solution at 60 °C for 24 h, showing the morphology of sodium titanate hydroxide nanofibers, (C) EDXS data of *as is* and alkali-treated grade-1 Ti.

drastically different morphology than those of the typical SBF-produced globular deposits (shown in Fig. 2G and H to provide a direct comparison). By the end of 1 week of DMEM-soaking (Fig. 2A, B and C), 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 never formed such planar sheets of material on Ti. However, at the end of 2 weeks of continuous soaking (Fig. 2D, E and F) 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. The EDXS spot analyses performed (at 20 kV and 150 s of acquisition time) throughout the entire surface showed the simultaneous presence of Ca and P without any Na. We decided not to rely only on qualitative EDXS analyses and thus scraped the deposited material (2 week sample) off of the Ti surface and analyzed those with ICP-AES as well.

EDXS (Fig. 3A) and 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 1.43 ± 0.13. The thin film XRD analysis of the coating on the 2-week sample showed that it is X-ray amorphous (Fig. 3B), *i.e.*, amorphous calcium phosphate (ACP). The sharp peaks with their respective Miller indices in Fig. 3B belong to hexagonal close-packed (ICDD PDF 44-1294) α-Ti. 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. 3C. Broad apatitic CaP peaks (labeled as *Ap*) are visible in Fig. 3C.

SBF solutions deposit cryptocrystalline (poorly crystalline) apatitic CaP on Ti, but they do not form X-ray amorphous CaP deposits [23,26]. The typical XRD charts (Fig. 3C) of SBF-deposited Ap-CaP on Ti contain a rather sharp peak at 25.9° 2θ (apatite's (*002*) reflection) and a higher intensity broad peak located over the 2θ range of 30 to 34° [23,26]. The DMEM solution of this study formed ACP, without any such XRD peaks, on the alkali treated grade-1 Ti coupons in stark contrast to SBF solutions. ATR-FTIR data of non-treated grade-1 Ti, alkali-treated Ti and DMEM-ACP-coated Ti samples are given in Fig. 4. Upon direct comparison between Fig. 2G–H and Fig. 2D–F it is apparent that while the spherulites/globules formed in SBF solutions were in the micron-size range, the formed surface features in DMEM solutions were all in the nano-size range. The first question posed at the end of Chapter 1 is thus answered, and the DMEM solutions used in this study were actively depositing amorphous CaP (ACP) on grade-1 Ti coupons.

The inorganic salt solution (with a Ca/P molar ratio of 2.5) shown in Table 2 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 (Fig. 5A and B). Thin film XRD data of these deposits were identical with the one shown in Fig. 3B. 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 Fig. 5C and D. Both of Fig. 5C and D data correspond to ACP. The weak shoulder seen at around

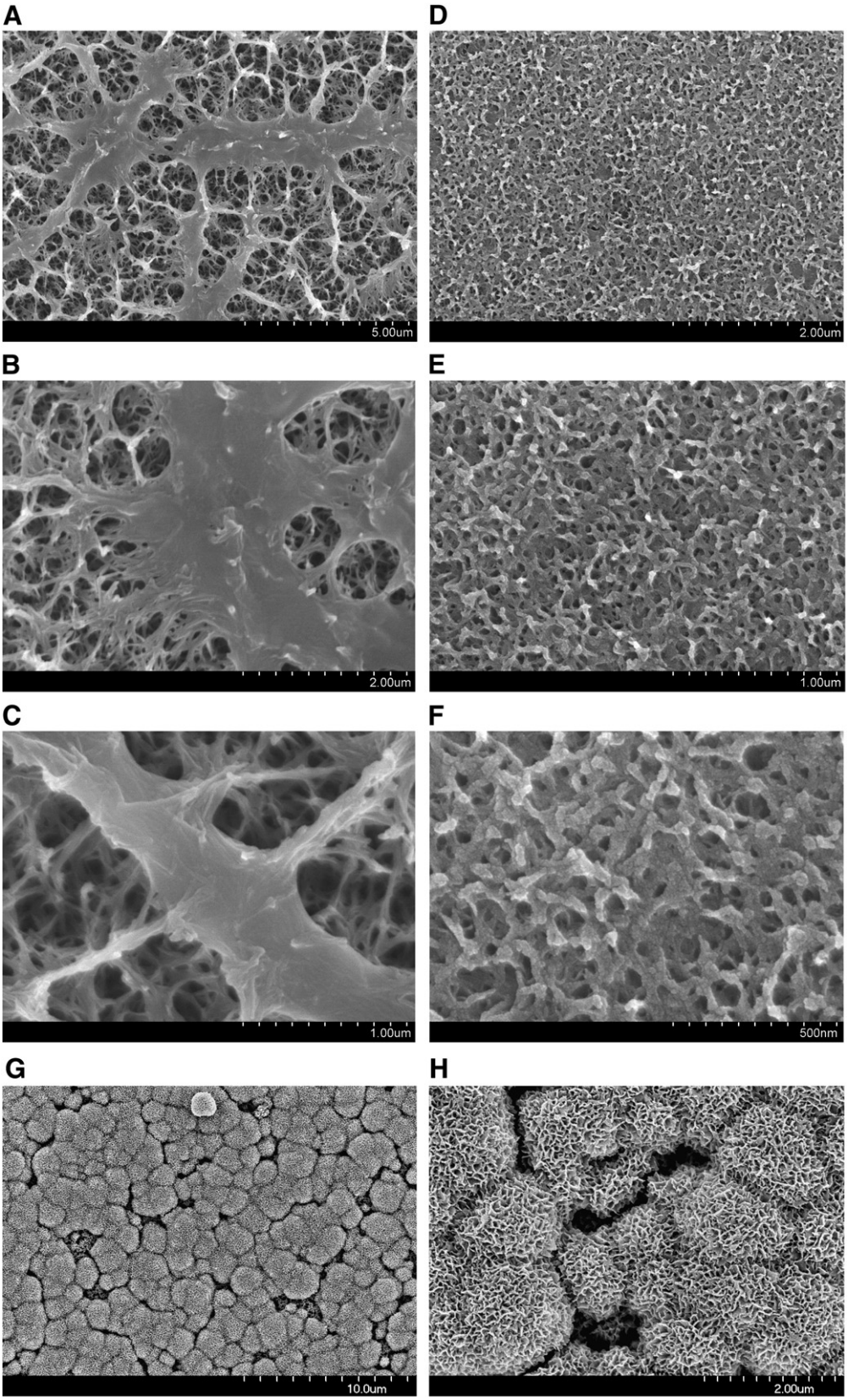

Fig. 2. (A) to (C): SEM photomicrographs of alkali-treated grade-1 Ti soaked in DMEM solution (*Gibco, 21063-029*) for 1 week at 37 °C, (D) to (F): SEM photomicrographs of alkali-treated grade-1 Ti soaked in DMEM solution for 2 weeks at 37 °C, (G) and (H): SEM photomicrographs of alkali-treated grade-1 Ti soaked in a Tris-buffered, 27 mM HCO_3^- containing SBF solution for 4 days at 37 °C.

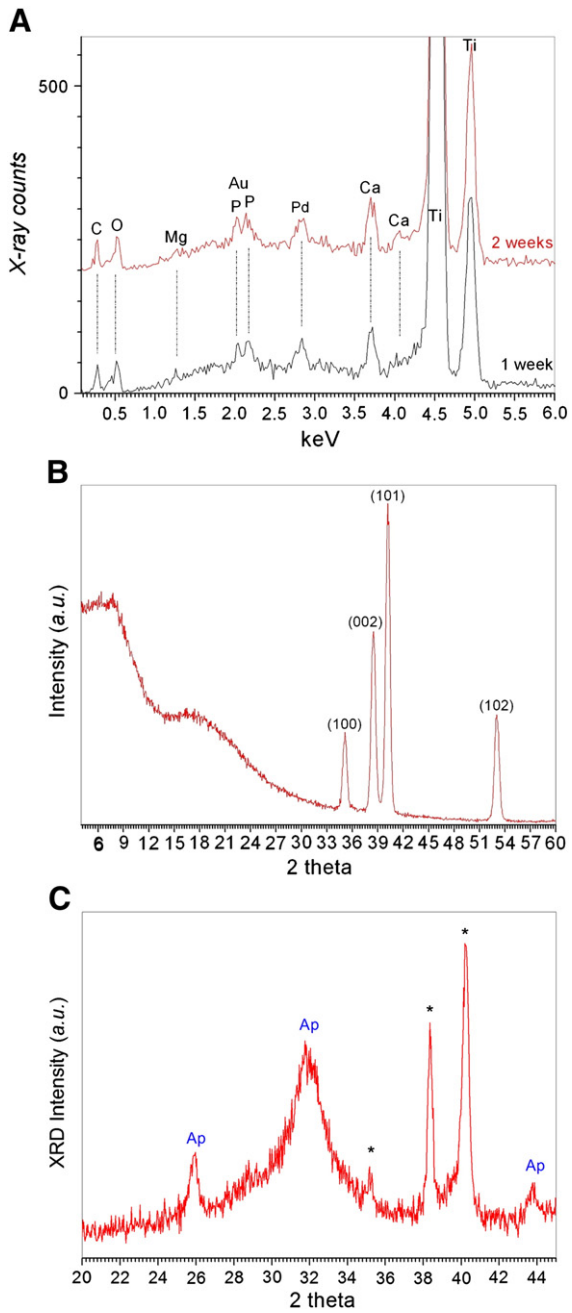

Fig. 3. (A) EDXS data of alkali-treated grade-1 Ti coupons soaked in the DMEM solution (*Gibco, 21063-029*) for 1 and 2 weeks at 37 °C, Au and Pd peaks were due to sputter coating; (B) thin film X-ray diffraction data of grade-1 Ti soaked at 37 °C in the DMEM solution for 2 weeks (the data for the 1 week samples were virtually the same, therefore, not shown); (C) X-ray diffraction data of Ti soaked for 4 days at 37 °C in a Tris/HCl-buffered SBF solution having 27 mM HCO_3^-.

700 cm^{-1} in the IR data of Fig. 5D can be ascribed to C–O, $\nu 4$ in-plane bending mode. Amorphous calcium carbonate (ACC) is known to exhibit the C–O, $\nu 3$ asymmetric stretching (1410 and 1470 cm^{-1}) and C–O, $\nu 2$ out-of-plane bending mode (865 cm^{-1}), as well as the above-mentioned C–O, $\nu 4$ mode [27]. This band at 700 cm^{-1} is usually confused with that of calcite at 712 cm^{-1}.

The second question posed at the end of Chapter 1 is thus answered, *i.e.*, the inorganic salt solution having similar inorganic ion concentrations to those of DMEM was also depositing amorphous CaP on the surface of grade-1 Ti coupons. The XRD and FTIR data (Fig. 5C and D) obtained from the scraped deposits were decisive in confirming the amorphous nature of the CaP deposits. CaP deposited from a solution

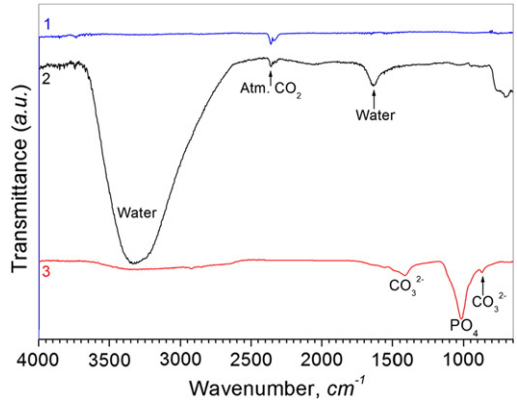

Fig. 4. ATR-FTIR data of non-treated grade-1 Ti (*1*), alkali-treated Ti (*2*), and DMEM-ACP-coated Ti samples (*3*).

with such an extraordinary smooth surface (Fig. 5B) was not reported prior to this study.

The XPS depth profile analysis of grade-1 Ti immersed in a Hepes-buffered DMEM solution at 37 °C for two weeks is shown in Fig. 6A. The SEM photomicrographs of this sample is shown in Fig. 2D through F. 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 (Table 1) and the inorganic solution (Table 2) were found to be identical with one another.

The micro-roughness of the surfaces of as-received grade-1 Ti, alkali-treated Ti, DMEM-soaked Ti as well as the alkali-treated Ti kept in the inorganic solution was compared in the surface profilometry data shown in Fig. 7. In these roughness measurements, a diamond stylus is first placed vertically on the sample surface and then moved laterally over a distance of 3000 μm, with the contact force of 15 mg. The as-received grade-1 Ti specimens possessed a metallic luster and had the smoothest surface, with the mean protrusions (peaks) and valleys (dips) not exceeding 200 nm (Fig. 7A). In the case of alkali-treated Ti (as shown in the SEM photos of Fig. 1A and B), the mean value of protrusions and dips increased to 1.5 μm (Fig. 7B). The alkali-treated Ti soaked at 37 °C in DMEM solution for two weeks (see the SEM photos of Fig. 2D through F) displayed peaks and valleys in the vicinity of 300 nm (Fig. 7C). On the other hand, the alkali-treated Ti samples immersed in the inorganic solution of Table 2 had a relatively rough surface as shown in Fig. 7D, since the ACP globules were not able to cover the entire surface in 24 h (Fig. 5A and B).

4. Discussion

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. Over the recent years, numerous research articles started to appear which simultaneously report the data of *in vitro* cell culture study (performed in DMEM or α-MEM media) and the so-called *in vitro* bioactivity testing performed by soaking the samples in a HCO_3-deficient (4.2 mM) and Cl-rich (148 mM) SBF solution. We hereby cite three exemplary and randomly selected articles, all

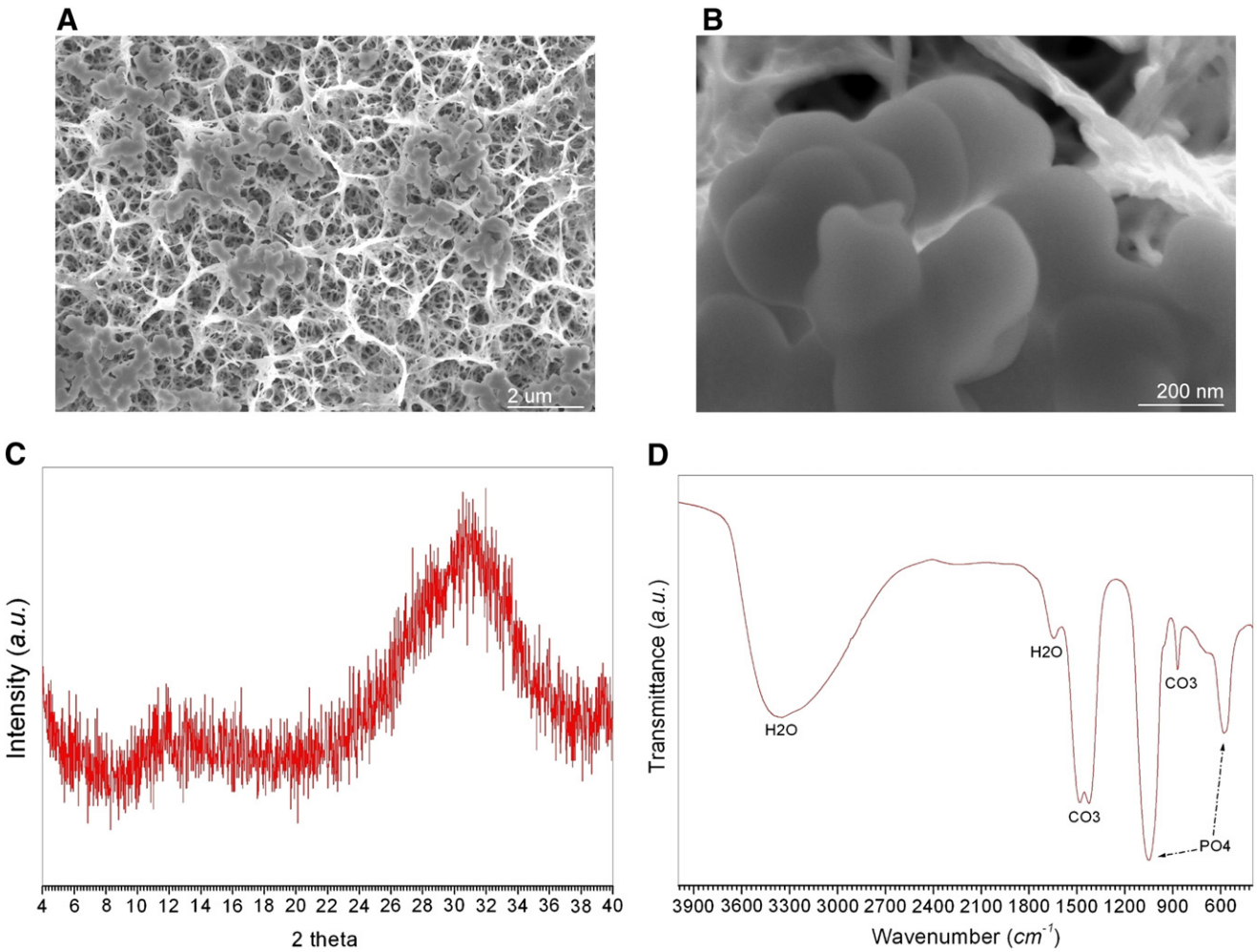

Fig. 5. (A) and (B): SEM photomicrographs of ACP deposits forming in 24 h on the alkali-treated grade-1 Ti coupons soaked at 37 °C in an inorganic (amino acid-, vitamin-, Hepes- and glucose-free) solution mimicking DMEM; (C) XRD data of ACP deposits scraped from the surface of coupons shown in (A) and (B); (D) FTIR data of the ACP deposits after removal from the surfaces shown in (A) and (B).

published in 2012, which performed the cell culture study in a 44.05 mM HCO_3-containing DMEM or α-MEM medium and then separately performed an SBF study in a 4.2 mM HCO_3-containing SBF solution for the same experimental materials [28–30]. It shall be quite difficult to correlate the results coming from an inorganic solution which possesses an 11-fold deficiency in its HCO_3^- concentration with those obtained from DMEM, remembering the fact that CaP formation in aqueous solutions is strongly influenced by the HCO_3^- concentration [31].

There are several issues need to be underlined here; (*1*) the SBF solution [32] always deposits 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 [16], glass-ceramic [17], titanium [26], Teflon [33], cellulose [34], alumina-zirconia [35], polycaprolactone [29,30] or poly-lactic-glycolic-acid [36], (*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 m^2/g) carbonated, cryptocrystalline apatitic CaP (Fig. 8A through D); 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]. The researchers who were including an SBF soaking compartment in their cell culture studies probably were not aware of the DMEM studies cited in Chapter 1 and of the ability of DMEM solutions to form CaP on the immersed samples.

As shown in Figs. 4, 5D and 8A, the FTIR data can be used as a rapid and powerful tool in differentiating between ACP and cryptocrystalline Ap-CaP. ACP samples (Fig. 5D) 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 [37]. The XRD data of Figs. 3C, 5C and 8C also help to differentiate between X-ray-amorphous CaP (Fig. 5C) and poorly crystalline Ap-CaP samples.

Since both solutions of Table 1 (DMEM) and Table 2 (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. Ciobanu and Ciobanu [38] added collagen and vitamins A and D_3 to a supersaturated

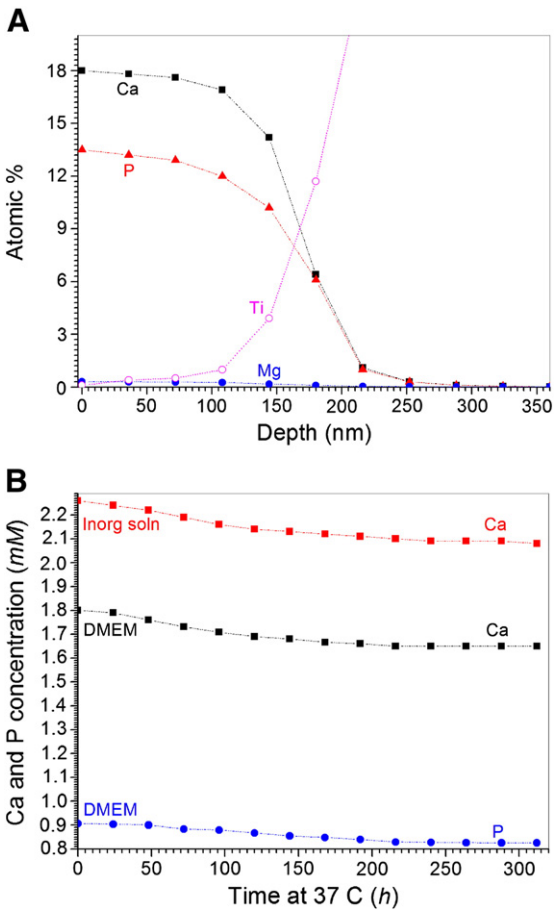

Fig. 6. (A) XPS depth profile of DMEM-soaked Ti sample for two weeks at 37 °C; (B) AAS data of the DMEM solution (Table 1) and the inorganic solution (Table 2) as a function of aging time at 37 °C.

calcification solution having 10 mM Ca^{2+}, 5 mM $H_2PO_4^-$, 1.5 mM HCO_3^-, 6.5 mM Na^+ and 20 mM Cl^- and reported the formation of well-crystallized Ap-CaP at 37 °C on the immersed *c.p.* Ti coupons in about 12 h. Although their solution (quite deficient in HCO_3^- concentration) was far from mimicking the inorganic salt concentrations of physiological solutions, the high crystallinity of the deposited CaP was also evident from their FTIR data. Drevet et al. [39] tested the influence of two amino acids (glutamic and aspartic acid) separately added to electrolysis solutions free of HCO_3^- ions in altering the morphology of the electrodeposited CaP on Ti6Al4V. These two amino acids, according to Drevet et al. [39], significantly reduced the particle size of the deposited cryptocrystalline Ap-CaP to the nano size range.

Fetal bovine serum (FBS) is usually added to DMEM solutions when performing cell culture studies, and FBS itself contains amino acids, glucose, proteins, polypeptides and hormones. Liu et al. [40] reported that a physiological medium, namely RPMI1640 (similar in composition to DMEM solutions but with a significantly lower HCO_3^- concentration of 23.81 mM and when Hepes-free), was able to form micron-size cryptocrystalline Ap-CaP particles (but not ACP) when heated at 37 °C for 24 h. However, upon adding 10 vol.% FBS to RPMI1640, the CaP precipitates forming were less than 100 nm in diameter and X-ray-amorphous, and their XRD and FTIR data resembled Figs. 3B, 5C and D of our results. The significance, for the ongoing discussion, of the data of Liu et al. [40] is that it showed the cryptocrystalline Ap-CaP forming tendency of a Hepes-free, low in HCO_3^- concentration (wrt DMEM), amino acids-, vitamins- and glucose-containing cell culture medium essentially similar to the DMEM solutions previous researchers used in coating Ti alloy substrates [20–22].

Hepes-buffered and phenol red-free DMEM solution (Table 1) of this study was able to coat the surfaces of alkali-treated grade-1 Ti coupons (with a coating thickness of about 220 nm as shown by the XPS depth profile data of Fig. 6A) in 2 weeks at 37 °C. Within the same period of two weeks at 37 °C, the Ca and P concentrations of the DMEM solution gradually and slightly decreased (Fig. 6B). The coverage of the surfaces of alkali-treated Ti coupons, with nano-textured ACP, was also indicated by the SEM photomicrographs (Fig. 2D through F) and the surface profilometry data (Fig. 7C). It is remarkable that the surface roughness of alkali-treated grade-1 Ti decreased from about 1.5 μm (*i.e.*, the height of the peaks and valleys in Fig. 7B) to about 0.2–0.3 μm upon soaking in the DMEM solution (Fig. 7C) for two weeks. The morphology of ACP deposits produced by the DMEM solution was not similar at all to the micron-sized globules/spherules usually encountered in SBF solutions (Fig. 2G and H). The current study reports this significant difference between the CaP deposition behavior of SBF and DMEM solutions.

One shall note that monodisperse nanoparticles of ACP can be readily formed at RT in a HCO_3-free aqueous solution of neutral pH containing 0.4 g/L bovine gelatin together with 3 mM Ca^{2+} and 3 mM HPO_4^{2-} ions [41]. Porcine, bovine or whale gelatin, being denatured collagen, is a practical source of mammalian amino acids [42]. In contrast with the above, in a gelatin- and HCO_3-free aqueous solution having NaCl, KCl, $MgCl_2 \cdot 6H_2O$, $(NH_4)_2HPO_4$ salts dissolved to yield Na^+, K^+, Mg^{2+}, HPO_4^{2-} and Cl^- concentrations identical with those of the human blood plasma, nanoparticles of ACP can be synthesized at RT upon adding calcium metal shots to achieve a nominal solution Ca/P molar ratio of 1.67 at a pH value of 11 [37]. Moreover, in a gelatin-free but 27 mM HCO_3-containing aqueous solution having NaCl, KCl, $MgCl_2 \cdot 6H_2O$, Na_2HPO_4 salts dissolved to yield Na^+, K^+, Mg^{2+}, HPO_4^{2-} and Cl^- concentrations identical with those of the human blood plasma, nanoparticles of ACP can be synthesized at RT upon adding calcium metal shots to achieve a nominal solution Ca/P molar ratio of 2.50 at a pH value of 10 [37].

Therefore, the presence of gelatin or amino acids (or any other organics) in the synthesis solutions is not a necessary condition to produce ACP on grade-1 Ti. The solution (with 44.05 mM HCO_3^-) we have described in Table 2 produced ACP in the absence of amino acids, vitamins, glucose and Hepes in the synthesis solution (Fig. 5B through D). The Tris-buffered, 27 mM HCO_3-containing SBF solutions when used as aqueous media (at 37 °C and pH 7.4) to synthesize biomimetic calcium phosphates also produced nanoparticles of ACP, but not cryptocrystalline Ap-CaP [9].

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 [43]. Both DMEM solution (Table 1) and the inorganic solution of Table 2 contain the ACP-stabilizer Mg^{2+} ions. Phenol red ($C_{19}H_{14}O_5S$, 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

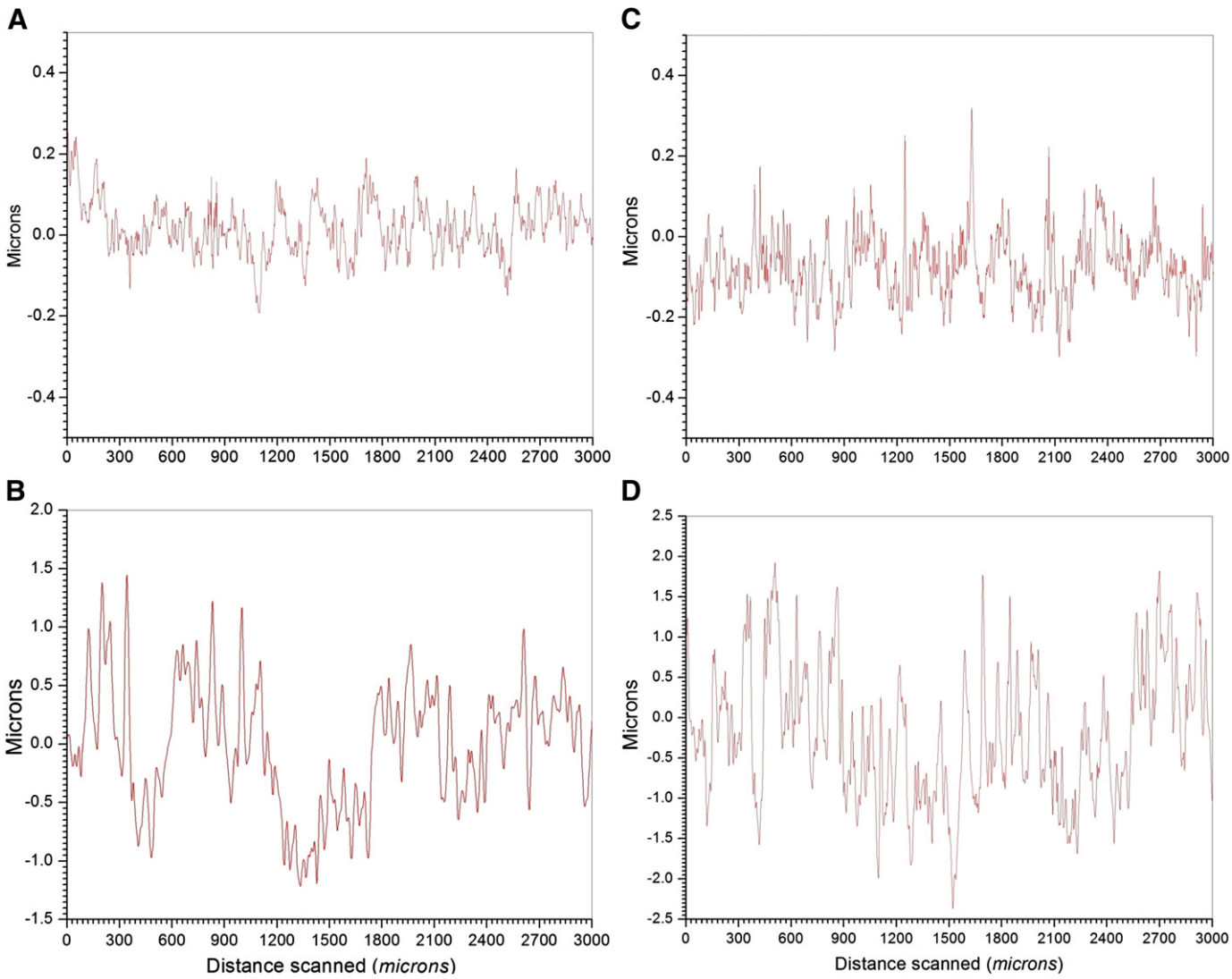

Fig. 7. Surface profilometry data of (A) non-treated (*i.e.*, as received) grade-1 Ti; (B) alkali-treated Ti (5 M NaOH, 60 °C, 24 h); (C) alkali-treated Ti soaked in DMEM for two weeks; (D) alkali-treated Ti soaked in the inorganic solution of Table 2 for 24 h.

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. We, on the other hand, deemed Hepes as 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. As mentioned above, it is possible to form ACP in virtually HCO_3-deprived aqueous solutions [37,41], therefore, 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.

The Ca/P molar ratios of the scraped ACP deposits determined by the ICP-AES analyses matched well with those reported by Brecevic et al. [41]. Synthetic ACP, according to Boskey and Posner [43], consisted of roughly spherical $3Ca_3(PO_4)_2$ 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 [43]. 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 [44]. Therefore, two of the most significant ACP stabilizers, namely, Mg^{2+} and HCO_3^-, were present in both the

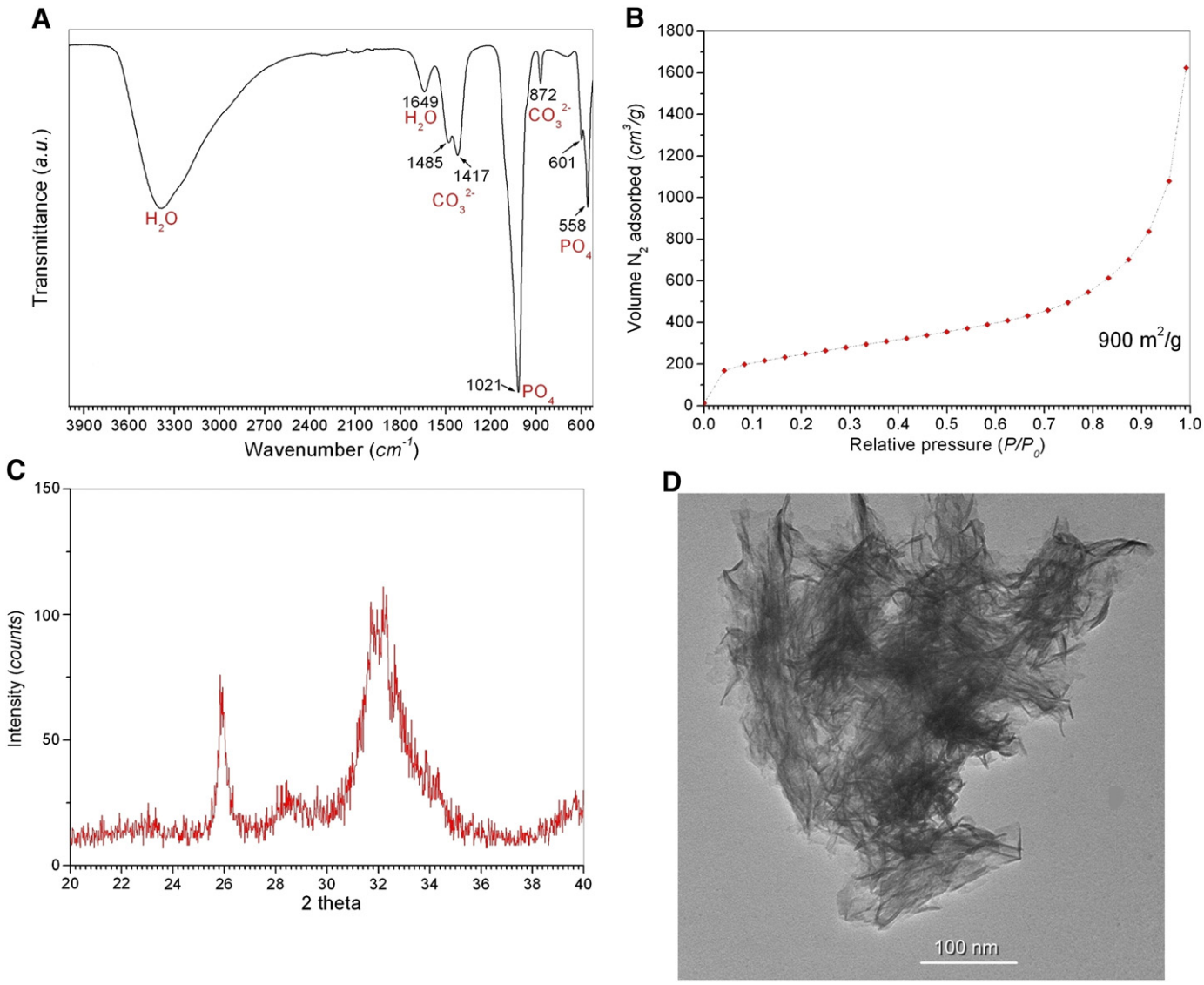

Fig. 8. FTIR (A), BET surface area (B), XRD (C) and TEM (D) of *in situ* forming cryptocrystalline apatitic calcium phosphate (Ap-CaP) precipitates recovered from an SBF solution kept undisturbed, in a sealed glass bottle, in a refrigerator at 4 °C for 120 days.

DMEM and the inorganic solution (of Table 2) 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 [45,46]. Conconi et al. [47] 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. [48] and Tentorio and Canova [49] 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.

5. 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.

Notes

Certain commercial instruments or materials are identified in this article solely to foster understanding. Such identification does not imply recommendation or endorsement by the author, nor does it imply that the instruments or materials identified are necessarily the best available for the purpose.

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