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Is non-buffered DMEM solution suitable medium for *in vitro* bioactivity tests?

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Abstract

For decades the bioactivity of materials has been tested in laboratories by means of *in vitro*
tests under standard ISO 23317 in a TRIS-buffered simulated body fluid solution (SBF). The
TRIS buffer, as we found, reacts with the tested material and affect test results due to its
ability to support the dissolution of crystalline phases of the tested glass-ceramic scaffold and
the crystallization of HAp. Several laboratories had tested bioactivity of the materials in
commercially available solution DMEM (Dulbecco´s Modified Eagle´s Medium) that is
normally used for cultivation of cell cultures. The objective of this work was to find out
whether it is possible to replace TRIS-buffered SBF currently used for bioactivity tests with
the non-buffered DMEM solution. To understand the role of the organic part of the DMEM

solution in the process of crystallization, we have prepared non-buffered solution simulating only its inorganic part (identified as I-solution). It was found that under static-dynamic test conditions calcite ($CaCO_3$) and the amorphous phase of calcium phosphate (ACP) formed on the surface of the glass-ceramic (Bioglass® - based) scaffold exposed to both solutions. Additionally, halite (NaCl) formed at the beginning of exposure to DMEM. Hydroxyapatite phase was not detected on the surface in either non-buffered solution, only $CaCO_3$ globules which looked similar to apatite crystals were observed. Organic components contained in the DMEM solution failed to prevent formation of crystalline phases but they probably slowed down the dissolution of the tested glass-ceramic scaffold. The present results indicate that it is not recommendable to use DMEM for bioactivity tests of glass-ceramic materials due to its low concentration of Ca^{2+} ions (insufficient supersaturation of DMEM with respect to HAp), high concentration of HCO_3^- ions and the necessity to maintain sterile environment during the test.

Key words: Bioactivity; scaffold; glass-ceramics; hydroxyapatite; calcite; SBF; DMEM; *in vitro test*; static-dynamic conditions, buffer TRIS

1. Introduction

The first tests usually performed on materials intended for substitution of hard tissues are bioactivity tests, i.e. tests monitoring the formation of a layer of biologically active hydroxyl carbonate apatite formed on the biomaterial surface. [1] Several preconditions need to be met for biomineralization (oseointegration) of the tested materials. The material must be dissolvable in blood serum (it must release Ca^{2+} ions or potentially also $(PO_4)^{3-}$, Na^+ and Si ions in a controlled manner); "fresh" solution (blood serum) is supplied to the proximity of the material and bone mineral hydroxyapatite (HAp) may crystallize on it due to local

supersaturation. The so-called Simulated Body Fluid (SBF) has been used for bioactivity tests

for many years[2]. Unlike human blood serum, the original SBF[3] contains no organic

components; it has a higher content of Cl^- ions, low content of HCO_3^- ions (4 mmol.dm^{-3}

versus 27 mmol.dm^{-3} in real human serum). The pH of SBF is maintained at around 7.5 (at

37^oC) by using TRIS ($NH_2C(CH_2OH)_3$, tris-[(hydroxymethyl) aminomethane]. In blood

serum the pH is partly buffered by hydrogen carbonate ions and by partial pressure of CO_2.

The pH value is critical for the formation of HAp. According to de Aza et al.[4], the ideal pH

for precipitation and crystallization of HAp is in the alkaline region (pH = 7.5 - 8.0).

However, during static and dynamic tests of dissolution of bioactive glasses, glass-ceramics

and calcium-phosphate materials (i.e. easily dissolving ones), the pH values in TRIS-buffered

SBF do not remain in the neutral area. Several hours after the material is exposed to SBF the

pH value increases towards the alkaline region. This pH increase results in formation of an

ideal, but not realistic, environment for HAp formation. Some authors have questioned

whether bioactivity of materials can be predicted only based on development of a HAp layer

on their surface after its exposure to SBF.[5,6] This is because SBF cannot simulate

physiological conditions in a live organism completely but it only substitutes its analytical

components (inorganic part of blood serum). Certainly it is justified to question if this

information is sufficient for bioactivity assessment? Indeed for a first indication about

potential bioactivity of biomaterial it probably is. As we have found out in our previous

work[7], TRIS buffer, which is a part of SBF, reacts with the tested material (in our case with

Bioglass[®] - derived glass-ceramic). We exposed the scaffold to a series of SBF solutions –

TRIS-buffered SBF, SBF without TRIS, TRIS alone and water. TRIS buffer supported

dissolution of the crystalline phase of the glass-ceramic scaffold and it was the principal

component for crystallization of HAp phase. Moreover, Ca^{2+} ions bind with TRIS buffer to

form a soluble complex which had been described elsewhere [8,9] and which may distort the results of *in vitro* tests.

Other authors [3] have reported on preparation of SBF solutions whose concentrations of HCO_3^- are near to that in human blood serum (27 mmol.dm^{-3}) and, apart from approaching to the real biological environment, they also anticipate an increased buffering ability of the inorganic part of SBF alone. However, all revised or modified SBF solutions still contain TRIS buffer (Revised (r-SBF), corrected SBF (c-SBF) and newly improved (n-SBF)). The authors of ref.[10,11] used modified SBF27 solution (concentration of HCO_3^- was 27 mmol.dm^{-3}) and demonstrated formation of carbonate hydroxyapatite (CHAp). In another investigation[12] collagen spongious Ap-CaP whiskers and $CaSO_4$ doped with calcium hydrophosphate ($CaHPO_4$ monetite) were exposed to solution of Tris-SBF-27 mmol.dm^{-3}. All mentioned materials induced formation of nanoporous apatite in the solution, with the exception of $CaSO_4$ alone, which crumbled in the solution into powder.

In a relevant investigation involving bioactive glasses Cannillo et al.[13] exposed two types of glasses (BG45 and MG45) not only to SBF but also to commercial solutions HBSS+ and HBSS- designed for growing of tissue cultures. HBSS solutions were not buffered with TRIS but they differed in the contents of Ca and Mg ions. SBF solution was the most reactive one and it induced formation of apatite on the surface of BG45 glass in a shorter time than the other solutions. The microstructure of the crystallized apatite on the BG45 glass was identical both in HBSS+ and in SBF. However, the reactivity (in the sense of apatite formation) was higher in SBF and this was explained by the authors by the presence a higher concentration of Ca ions in SBF (1.4 mmol.dm^{-3} Ca^{2+} in HBSS+ compared to 2.5 mmol.dm^{-3} in SBF). A magnesium phosphate phase developed on the surface of MG45 glass in SBF which means that also this glass significantly dissolved in SBF and SBF was in this case supersaturated in respect to magnesium phosphate.

In other published articles[14,15,16] attempts to substitute SBF with the commercially available DMEM solution, which is primarily designed for growing and maintaining tissue cultures, have been reported. The solution is available in many variants - non-buffered or buffered with TRIS, and with another buffer - HEPES ($C_8H_{18}N_2O_4S$), 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid), (Sigma Aldrich, Invitrogen GIBCO USA). Apart from the inorganic part of blood serum, this solution also contains the organic component. For example, Theodorou et al.[17] have found that as early as after three days of immersion in DMEM solution, amorphous apatite phase developed at certain locations on silicate glass (*Bioactive glass 45S5)* and carbonated crystalline apatite (CHAp) phase was found on *58S* glass. It was discussed that development of the CHAp crystalline phase is inhibited by the organic part of DMEM solution and the authors explained it by the effect of the adsorption of proteins on an amorphous Ca-P layer[17]. However, it is not clear whether they used buffered or non-buffered DMEM solution which is critical for interpretation of the results. Miller et al.[18] studied the transformation of brushite into OCP (octacalcium phosphate) in various DMEM derived solutions. Solutions without organic phase had not any SO_4^{2-} ions and solutions with organic phases as lactic acid and TRIS buffer contained SO_4^{2-} ions. Any of the solutions were not able to maintain the pH at 7.4, even during the first 24h. Brushite crystals were transformed into a biphasic mixture of OCP and CDHA (Ca-Deficient HA), when soaked for 1 week in different biomineralization solutions at $37^{o}C$. Authors also noticed, that the extent of any hydrothermal transformation of DCPD into OCP and CDHA strongly depend on the overall dimensions or thickness (sizes) of the samples. Temizel et al.[19] used derived solutions DMEM buffered with HEPES and solution marked as BM-3 non-buffered with HEPES. When HEPES was eliminated in BM-3, it became possible for the first time to completely convert the DCPD crystals to OCP in less than 72 h at $36.5^{o}C$. The effect of the presence of HEPES could be due to the complexation of some of the Ca^{2+} ions of the solution by the

HEPES buffer at the Ca/P molar ratio of 1.99 and reduce the concentration of free Ca^{2+} ions available for the DCPD to OCP transformation. The experimental results showed that even increasing the temperature from 55 ^{o}C to 60 ^{o}C in a 1 h stirred experiment increased the changes of obtaining Apatite-CaP mainly due to the high solubility of DCPD. Optimum temperature to achieve completely transformation to OCP crystals was at 75 ^{o}C - 80 ^{o}C. Evidently, the type of used solution, buffering system and the arrangement of the test (e.g. S/V) and temperature is very important for the interpretation of obtained results.

The purpose of this research was to study the suitability of non-buffered DMEM solution for bioactivity testing, considering its reduced concentration of Ca^{2+} and high concentration of HCO_3^- ions, the fact that it contains organic components of blood serum and, unlike SBF; it is not buffered with TRIS.

2. Materials and methods

2.1. Materials

The material used for testing was silicate glass-ceramic in the form of a highly porous structure (scaffold) prepared by the foam replica technology[20]. The initial material for preparation of a glass suspension was 45S5 Bioglass® powder with mean particle size < 5μm. For scaffold preparation polyurethane (PUR) foam was immersed into the prepared suspension, it was taken out 15 minutes later and excessive suspension was squeezed out. The created porous precursors, the so-called *green bodies*, were left to dry for 12 hours at room temperature and were then subjected to a thermal treatment consisted of firing at 400°C/1 hour, to burn-out the PUR template and further sintering at 1100°C for 5 hours[20]. The scaffold had an open porous structure with pore size in the range 510 - 720 μm and porosity of approximately 90%. The material contained crystalline and residual glass phases. Their

contents before and after the crystallization, according to a previous publication [20], are shown in Table 1.

2.2. Solutions for in vitro test

Modified simulated body fluid with reduced concentration of Ca^{2+} ions and increased concentration of HCO_3^- ions to 44 $mmol.dm^{-3}$ (I-solution), was prepared by mixing solutions of the following reagents: KCl, NaCl, $NaHCO_3$, $MgCl_2.6H_2O$, $CaCl_2$, Na_2SO_4 and KH_2PO_4 in respective ratios. Azide (NaN_3) was added to prevent bacteria growth in the solution [7, 21]. I-solution was not buffered and the pH value was not adjusted.

Dulbecco´s Modified Eagle´s Medium (DMEM) is normally used to grow tissue culture and it simulates the environment of the human organism because, in addition to inorganic ions, it also contains organic substances, such as amino acids, glucose and vitamins. A modified DMEM (mod-DMEM) was prepared for testing in this study by enriching the standard DMEM (D1145, Sigma-Aldrich) with fetal bovine serum (FBS, Invitrogen), vitamins (MEM, Invitrogen) and antibiotics (Sigma-Aldrich). A5955 Sigma Antibiotic Antimycotic Solution (100×), Stabilized with 10,000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B per mL, sterile-filtered, BioReagent.

The reason for the FBS and vitamins addition was to enrich the organic part of the DMEM. Antibiotics protect prepared solution against degradation during the test. Table 2 presents the ion composition of I-solution and the inorganic part of mod-DMEM in comparison with blood plasma (BP), as published in the literature[22], Ca/P molar ratio is presented.

2.3. Static-dynamic conditions of in vitro test

To prevent the effect of exhaustion of ions from the solutions, the so-called static–dynamic test was carried out in which, although the testing solutions did now flow continually around the sample as in dynamic tests, the solution was replaced on daily basis (50 ml.day^{-1}), i.e. every 24 hours. Scaffolds used for the static-dynamic arrangement of the *in vitro* test had average weight in the range 0.045 - 0.055 g, they were placed in platinum spirals and suspended in 50 ml plastic bottles filled with I-solution or mod-DMEM. The bottles with the samples were placed into a thermostat maintaining the temperature at 36.5 ± 0.5 °C. The interaction time was 15 days and two samples were collected in selected time intervals (after 1, 3, 7, 11 and 15 days), rinsed with demineralized water and left to dry at laboratory temperature. In order to maintain sterile environment the replacement of the mod-DMEM solution was performed in a "flow box".

2.4. Analysis of the materials

2.4.1. Scanning electron microscopy / energy-dispersive spectroscopy (SEM/EDS)

The surface of tested materials before and after the immersion tests were inspected with an Hitachi S-4700 scanning electron microscope (SEM) equipped with EDS analyzer (NORAN D-6823) working at an accelerating voltage of 15 kV. The samples were powder coated with an Au-Pd layer during 80-100 s for SEM observations.

2.4.2. X-ray powder diffraction analysis

Samples were ground in an agate mortar in a suspension with cyclohexane. The suspension was then placed on a mylar film to a transmission sample holder. After solvent

evaporation a thin layer of the prepared sample was covered with another mylar film. Diffraction patterns were collected using a PANalytical X´Pert PRO diffractometer equipped with a conventional X-ray tube ($Cu_{K\alpha}$ 40 kV, 30 mA, line focus) working in transmission mode. An elliptic focusing mirror with divergence slit 0.5°, an anti-scatter slit 0.5° and Soller slit of 0.02 rad were used in the primary beam. A fast linear position sensitive detector PIXcel with anti-scatter shield and Soller slit of 0.02 rad were used in the diffracted beam. All patterns were collected in the range of 3 to 88 deg. 2 theta with the step of 0.013 deg and 600 sec/step producing a scan of about 4.5 hours. Qualitative analysis was performed with HighScorePlus software package (PANalytical, The Netherlands, version 3.0e), Diffrac-Plus software package (Bruker AXS, Germany, version 8.0) and JCPDS-ICDD PDF-2 database [23].

2.5. Leachate analysis

All tests and analyses were measured using two parallel series of samples.

2.5.1. Atomic absorption spectrophotometry

Concentrations of Ca^{2+} ions were analyzed in leachates from both types of solutions with VARIAN-SpectrAA 300. The so-called release agent (KCl) was added to each sample to determine the quantity of Ca. The leachate was atomized in acetylene-N_2O flame. The wavelength used for absorbance measurements was 422.7 nm. Concentrations of Si were analyzed in leachates from both types of solutions with VARIAN-SpectrAA 880. The leachates were atomized in acetylene-N_2O flame. The wavelength used for absorbance measurements was 251.6 nm.

2.5.2. Spectrophotometry

Concentrations of $(PO_4)^{3-}$ ions were analyzed in I-solution leachates with UV-VIS spectrophotometer UV1601 at wavelength 830 nm (ČSN 830540). Ion concentrations were calculated using a calibration line method from the measured absorbance values.

2.5.3. Inductively coupled plasma - optical emission spectroscopy (ICP-OES)

Concentrations of P in mod-DMEM leachates were measured by ICP-OES with a Perkin Elmer-Optima 2000DV instrument. The solution was vaporized with GemConeTM nebulizer and the flow rate of the solution through the nebulizer was 2.2 $ml.min^{-1}$. The produced fine aerosol was carried out with argon stream into a plasmatic burner (1300 W). Concentrations were measured at wavelengths 231.620, 214.917 and 178.221 nm.

2.5.4. pH measurement

pH values in I-solution and mod-DMEM leachates were measured with inoLab pH-meter with a combined glass electrode at laboratory temperature.

3. Results

3.1. Interaction of scaffolds with mod-DMEM

3.1.1. Leachate analysis of mod-DMEM during interaction with scaffold

Figures 1a, b show concentrations of Ca^{2+}, $(PO_4)^{3-}$ and pH in mod-DMEM leachates after the scaffold exposure. The leachate analysis suggests that potential processes of scaffold dissolution and precipitation of new phases occurred at a stable rate practically throughout the entire duration of the experiment. Concentrations of $(PO_4)^{3-}$ ions after 7 days of interaction slightly increased which means that the process of scaffold dissolution started prevailing over the precipitation of new phases. ICP could not be used to determine concentrations of Si

(which is a "marker" for scaffold dissolution) in mod-DMEM leachates, because of the high error of the measurement of the samples with low concentration of Si (1 - 10 mg.dm^{-3}). The mod-DMEM solution was not buffered and the pH value increased from 7.45 to 8.90 practically immediately after the beginning of the exposure and it oscillated at around pH 8.90 until the end of the test (Fig. 1b). The pH changes can be considered an indicator of ongoing processes of dissolution and precipitation during scaffold exposure to mod-DMEM.

3.1.2. X-ray powder diffraction analysis of scaffolds before and after interaction with mod-DMEM

Records from XRD powder diffraction analysis of the scaffold before and after 3, 7, 11 and 15 days of interaction with mod-DMEM are shown in Fig. 2.

XRD analysis before the exposure confirmed the presence of the main crystalline phase $Na_2O.2CaO.3SiO_2$ (combeite) and two minority structurally isomorphic phases $2CaO.SiO_2$ and $NaCaPO_4$ (buchwaldite) in the original scaffold (Fig. 2). Crystalline phases of NaCl (halite) and $CaCO_3$ (calcite) developed after three days of exposure (3D). The intensity of diffraction lines for the halite phase decreased with increasing interaction time (7 - 15 D) and, on the contrary, it increased for the calcite phase. The XRD diffractograms indicate the growth of a nanocrystalline phase (indicated in the diffractogram as a broad diffusion maximum) approximately from the 7th day of the test. It is probably a phase consisting of a mixture of nanocrystalline – amorphous calcium phosphate (ACP), which has been also discussed in the literature for immersion in SBF[7]. The intensity of the original crystalline phases of the scaffold remained practically unchanged which means that they did not significantly dissolve in the mod-DMEM solution.

3.1.3. SEM/EDS characterization of scaffolds before and after interaction with mod-DMEM

Changes on the surface of the scaffolds after 3, 7, 11, 15 days of interaction with mod-DMEM can be seen in SEM images (Fig. 3b-e) and EDS analyses (indicative measurement) are provided in Table 4. The original morphology of the scaffold surface is shown in Fig. 3a.

Figure 3a shows the surface of the original scaffold with the small tabular crystals of combeite and needle like crystals, likely corresponding to buchwaldite as well as the structural isomorphic phase $CaO.SiO_2$. The SEM/EDS analysis confirmed that as early as after three days (3D) the scaffold surface was covered with ACP (Amorphous Calcium Phosphate) (growing content of Ca and P) and NaCl (Table 3, Fig.3b, white crystal, 1µm in size), which is in agreement with the XRD analysis (Fig. 2). A phase with a high content of Ca developed after one week (7D) (by XRD diffraction detected as $CaCO_3$) and it can be seen in images from SEM/EDS as white 1µm globules (Fig. 3c). After 11 days (11D) the phase with a high Ca content started prevailing and quantities of Na and Cl on the scaffold surface gradually decreased. The content of P remained unchanged after the third day of interaction. At the end of the test (15D) the scaffold surface was mostly covered with a phase with a high content of Ca (according to XRD - $CaCO_3$, Fig. 2) with a cauliflower-like structure (Fig. 3 e, f) and also ACP was detected on scaffold surfaces. Results of SEM/EDS measurements were therefore in good agreement with the XRD analysis.

3.2. Interaction of scaffolds with I-solution

3.2.1. Leachate analysis of I-solution during interaction with scaffold

Analyses of the I-solution leachates showed that the scaffold significantly dissolved immediately after the exposure to the medium, which was well-documented by the presence

of Si in the leachates (Fig. 4a). 48 hours after the beginning of exposure the rates of scaffold dissolution and precipitation of Ca-P phase stabilized, as documented by concentrations of Ca^{2+} and $(PO_4)^{3-}$ ions (Fig. 4b). The decrease of Ca^{2+} and $(PO_4)^{3-}$ concentrations indicates the precipitation of the Ca-P phase. The pH value of non-buffered I-solution was pH 8.50 at the beginning and, due to scaffold dissolution and formation of the new phase; it increased to pH 9.20 and remained around that value until the end of the test (Fig. 4c).

3.2.2. Si as a dissolution "marker"

Concentrations of Si in I-solution leachates made it possible to calculate quantities of the dissolved scaffold. The calculation was possible also thanks to the conditions of the static-dynamic test as it was possible to use the current concentration of Si in a known volume of leachate (every day precisely 50 ml) to calculate the quantity of leached SiO_2. The total quantity of leached SiO_2 was subsequently converted to the weight of dissolved scaffold, based on an assumption that SiO_2 represents 45 weight % of the scaffold. The fact that the leached SiO_2 originated both from the crystalline and glass phases was neglected for reasons of simplification (the crystalline phase contains ca. 7x more SiO_2 than the residual glass phase). Thus assuming that the dissolution rates are identical for both scaffold phases then our calculations indicate that 7.2 mg SiO_2 leached in total from ca. 55 mg of the exposed sample. This means that ca. 16 mg of scaffold dissolved in 15 days of exposure in the I-solution, i.e. approximately 1/3 of the initial weight of the tested scaffold. If the dissolved phase had been only the residual glass phase it would have dissolved entirely. We also tried to calculate quantities of dissolved Ca and P, however due to their re-precipitation and formation of

several different phases on the scaffold surface the calculation would be very complicated and indeed quantitatively inaccurate.

3.2.3. X-ray powder diffraction analysis of scaffolds before and after interaction with I-solution

Records from XRD powder diffraction analysis of the scaffold before and after 1, 3, 7, 11 and 15 days of interaction with I-solution are shown in Fig. 5.

XRD diffraction confirmed formation of a calcite crystalline phase ($CaCO_3$) as early as after the first day (1D) of exposure to I-solution (Fig. 5). After three days of exposure (3D), an increase of content of amorphous phase ACP (Amorphous Calcium Phosphate) was recorded. No formation of HAp phase was observed, not even after 15 days of exposure to I-solution. No halite (NaCl) was formed either, probably due to lower concentrations of Na^+ and Cl^- ions in the I-solution in comparison to mod-DMEM. The original phases of the scaffold are visible in the XRD patterns until the end of the test (0-15D). We assume that the main dissolved phase of glass-ceramic was the residual glass phase of the scaffold.

3.2.4. SEM characterization of scaffolds before and after interaction with I-solution

Changes on the scaffold surface after 1, 3, 7, 11, 15 days of interaction with I-solution are shown in Fig. 6b-f. The scaffold surface before the interaction is shown in Fig. 6a.

Figures 6 b – f and results of EDS analyses in Table 6 confirm conclusions from XRD analysis. Well-developed globules of $CaCO_3$ (Fig.6b) with the diameter of ca. 1 μm were visible from the very beginning of the interaction (1D). It was the same phase that developed on the scaffold surface exposed to mod-DMEM. Apart from the growth and agglomeration of

the globules (3D) (Fig.6c), there was a significant growth of the ACP phase (7-15D) (Fig 6d), which has been also detected for immersion in SBF[7], NaCl was not detected, neither with XRD nor with SEM/EDS. $CaCO_3$ globules covered by ACP phase formed in I-solution looked like hydroxyapatite at the first sight but XRD analysis failed to confirm the presence of the HAp crystalline phase.

4. Discussion

Analyses of leachates from Bioglass[®] - based glass-ceramic scaffolds immersed in non-buffered mod-DMEM and non-buffered I-solution shown that pH values in those systems (thanks to enormous dissolution of the tested scaffold) increased far into the alkaline area (up to pH 9). We assume that at such pH values the residual glass phase, which functions as a "binding agent" in the glass-ceramic material, will significantly dissolve. This fact has been confirmed by practical experience from the test as the glass-ceramic material in non-buffered I-solution (but also in mod-DMEM) disintegrated. XRD measurements after 15 days of exposure in both cases confirmed the presence of the original crystalline phases of the scaffold. The results indicate therefore that high concentration of HCO_3^- ions alone (DMEM contains aprox. 160 % HCO_3^- (s) of that in human plasma) in couple with CO_2 (g) from air do not have sufficient buffering capacity in the tested solutions. The DMEM solution fails to contain sufficient quantity of Ca^{2+} ions (only 70% of that in human serum) and Ca/P molar ratio is near 2 on the contrary to human plasma (2.5). Such environment is not supersaturated in respect to HAp but, it is preferentially supersaturated in respect to amorphous calcium phosphate phase and $CaCO_3$.

After being exposed to both investigated solutions the newly formed crystalline phase on the surface of scaffolds was mainly calcite ($CaCO_3$) (shaped into globules). In our previous study[7], we discussed that a visually identical phase that formed in SBF without TRIS buffer

was amorphous ACP, and even by conducting XRD analysis we were not able to prove the presence $CaCO_3$ after the scaffold exposure to SBF without TRIS. The quantity of $CaCO_3$ was very low and the most intense lines for $CaCO_3$ overlapped with diffraction lines of the main crystalline phase of the as fabricated scaffold; i.e. combeite. However, we have to take into account, that solution SBF without TRIS from our previous experiment had the concentration of HCO_3^- ions more than 10-times lower opposite to I-solution or mod-DMEM used here. Based on the findings described above it is possible to expand our knowledge on the *in vitro* behavior of these particular Bioglass[®] - derived scaffolds. In this study we found that the amorphous phase containing Ca and P (ACP) precipitated in both tested solutions on the scaffold surface, as it was anticipated on the grounds of consumed Ca and P in the non-buffered I-solution leachates, and in both cases the phase was detected thanks to the increased quantity of phosphorus on the sample surface as detected by EDS. The presence of ACP was confirmed also by the XRD results (the share of amorphous phase increased with the time of scaffold exposure to the solutions). ACP phase is probably in the form of nano-spheres covering the $CaCO_3$ globules. Later amorphous phase can transform into OCP crystals[24]. As shown also in our recent study[25], crystallization of $CaCO_3$ on the glass-ceramic surface in non-buffered DMEM derived solution with concentration of HCO_3^- ions near to those of human plasma ($27 mM.dm^{-3}$) will prevail. Moreover, ACP was also found in 27-SBF solution (without buffer TRIS).[25]

We have not found evidence that the organic part of non-buffered mod-DMEM suppresses crystallization of phases, as calcite crystallized on the scaffold surface in both solutions. Another phase, halite, crystallized at the beginning of the immersion in mod-DMEM thanks to higher concentrations of Na^+ and Cl^- ions (not only in comparison with I-solution but also with blood serum) and thanks the presence of Na^+ ions released from the tested glass-ceramics scaffold. Has to be noticed, that halite is not the residue of mod-DMEM or I-

solution. The same treatment was applied on the each glass-ceramics scaffold taken out from

testing solutions (see 2.3.). The transmission geometry of the XRD measurement enabled to

find the NaCl phase covered by later precipitated of $CaCO_3$ phase. Probably, halite crystals

served advantageous places for $CaCO_3$ and ACP nucleation.

The presence of the organic components in mod-DMEM solution affects the rate of

glass-ceramic dissolution; it is possible that the tested scaffold dissolved more slowly in mod-

DMEM compared to I-solution: we estimated this based on small changes of Ca and P

concentrations in the mod-DMEM leachates.

After completion of the test in I-solution the inert Pt spirals, in which the scaffold

samples were suspended, were covered with a very thin layer. XRD analysis of the precipitate

showed that the layer consisted mainly of NaCl phase and Ca-defective carbonate apatite

(CDHA). This phenomenon had no influence on the conclusions of the present experiments

but it should be discussed in the context of establishing suitable setup and experimental

conditions of bioactivity tests which is the matter of current work.

5. Conclusions

1. Non-buffered mod-DMEM solution is not the ideal environment for the formation of
    hydroxyapatite phase in bioactivity tests of glass-ceramics material.

2. Exposure to non-buffered mod-DMEM and non-buffered I-solution resulted in
    crystallization of calcite and precipitation of amorphous calcium phosphate (ACP) on
    the scaffold surface.

3. The organic part of mod-DMEM does not suppress crystallization of calcite, halite or
    precipitation of ACP, probably it suppress the glass-ceramics dissolution.

4. Considering the general goal of monitoring the growth of hydroxyapatite phase (HAp)
    on the surface of materials as one of important factors determining the so-called

"bioactivity", a highly critical question remains to be answered related to the selection of the suitable buffering system for SBF commonly used and to the determination of appropriate *in vitro* test conditions.

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Table 1

Composition of 45S5 Bioglass[®], crystalline and glass phases [20]

Oxides	45S5 Bioglass[®] (100 wt%)	$Na_2Ca_2Si_3O_9$ (77.4 wt% scaffold)*	Residual glass phase (22.6 wt% scaffold)
SiO_2	45.0	50.9	24.8
Na_2O	24.5	17.4	48.5
CaO	24.5	31.7	-
P_2O_5	6.0	-	26.5

*The minority crystalline phases $2CaO.SiO_2$ and $NaCaPO_4$ (buchwaldite) are included

Table 2

Ion composition ($mmol.dm^{-3}$) of mod-DMEM, I-solution BP[22], SBF[22] and Ca/P molar ratio

	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	HCO_3^-	HPO_4^{2-}	SO_4^{2-}	Ca/P
mod-DMEM	154.5	5.4	1.8	0.8	118.5	44.0	0.9	0.8	1.97
I-solution	142.0	5.0	1.8	1.0	91.6	44.0	1.0	0.5	1.80
BP	142.0	5.0	2.5	1.5	103.0	27.0	1.0	0.5	2.50
SBF Orig	142.0	5.0	2.5	1.0	148.8	4.2	1.0	0.0	2.50

Table 3

Chemical and crystalline composition of the original scaffold surface and the surface after its exposure to mod-DMEM [wt.%] (by SEM/EDS)

Time [days]	O	Na	Mg	Si	P	Cl	K	Ca	Probable Phases*
ORIGIN	39.4	20.3	-	20.9	1.6	-	-	17.9	$Na_2Ca_2Si_3O_9$

3D	45.2	10.9	2.1	2.6	6.4	6.6	1.0	25.1	ACP, NaCl
3D - cube	49.3	14.0	2.6	-	-	7.9	0.8	19.6	NaCl
7D	37.3	9.8	1.3	5.8	3.7	7.3	0.7	34.2	NaCl, ACP, CaCO$_3$
11D	40.9	3.8	0.9	8.7	4.2	3.3	1.0	37.2	CaCO$_3$, ACP, NaCl
15D	45.5	2.4	1.5	3.5	5.5	1.5	0.3	39.9	CaCO$_3$, ACP

[*]Combeite: Na$_2$Ca$_2$Si$_3$O$_9$, Halite: NaCl, Calcite: CaCO$_3$, ACP: Amorphous Calcium

Phosphate

Table 4

Chemical and crystalline composition of the original scaffold surface and the surface after the

interaction with I-solution [wt. %] (SEM/EDS)

| Time [day]	O	Na	Mg	Si	P	Ca	Probable Phase[*]
ORIGIN	43.5	22.8		17.1	0	16.6	Na$_2$Ca$_2$Si$_3$O$_9$
1D	44.2	4.9	2.0	8.2	5.2	35.4	Na$_2$Ca$_2$Si$_3$O$_9$, ACP, CaCO$_3$
3D	36.1	1.6	-	2.0	4.4	56.0	ACP, CaCO$_3$
7D	49.8	2.5	2.8	3.1	6.9	34.9	ACP, CaCO$_3$
11D	45.5	2.6	2.7	-	7.0	42.2	ACP, CaCO$_3$
15D	41.6	2.6	3.5	3.0	5.5	43.7	ACP, CaCO$_3$

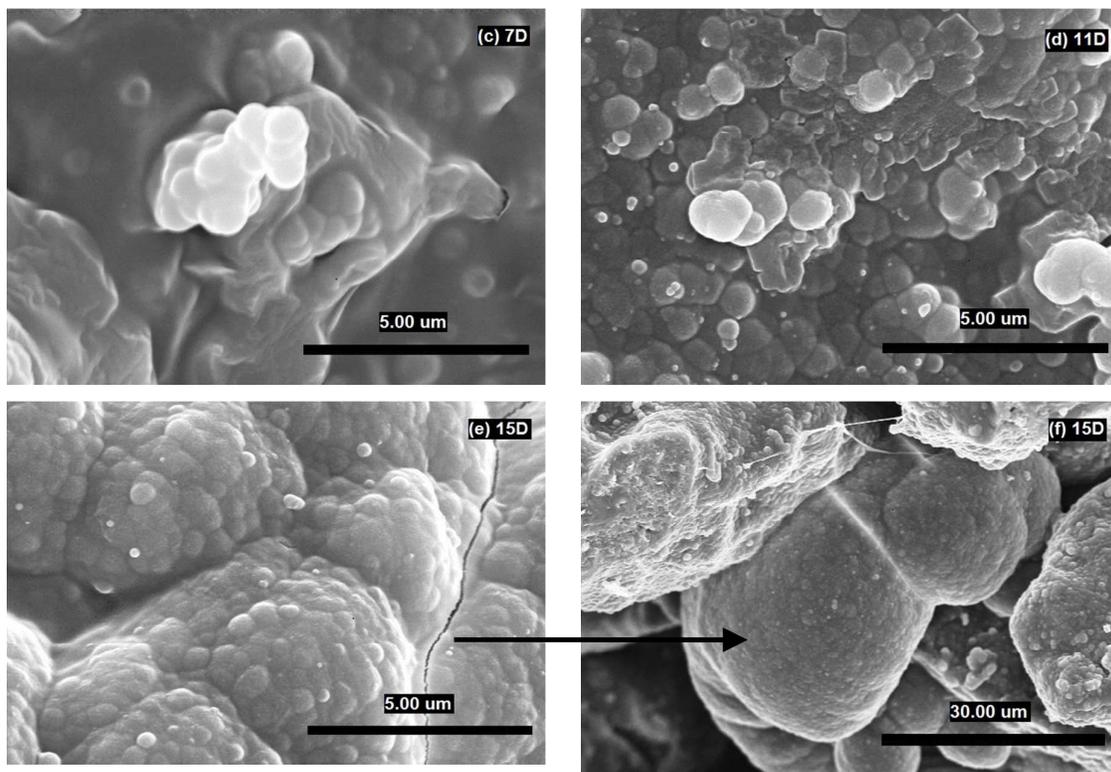*Combeite: Na$_2$Ca$_2$Si$_3$O$_9$, Calcite: CaCO$_3$, ACP: Amorphous Calcium Phosphate
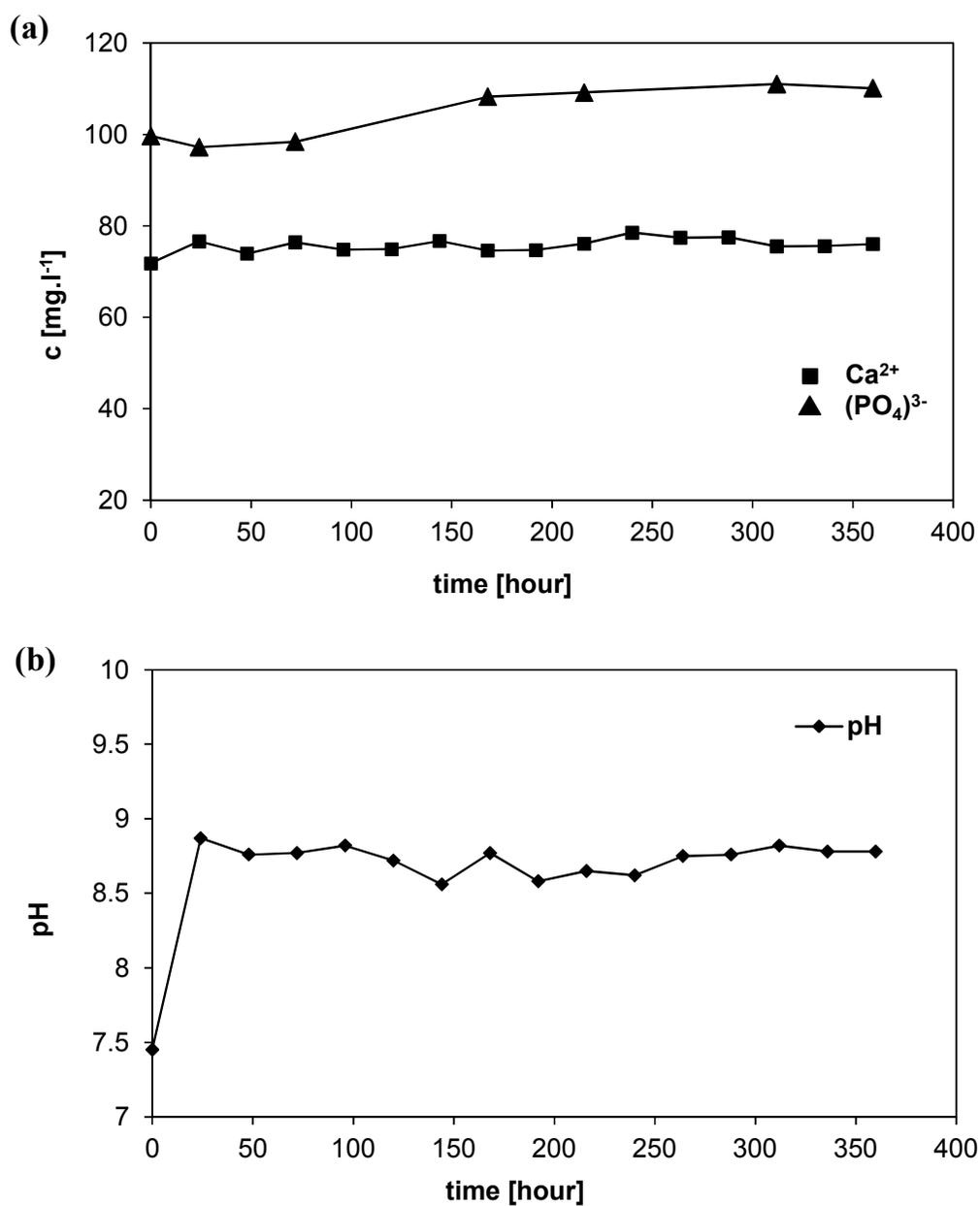
Fig. 1. Concentrations of (a) Ca^{2+}, $(PO_4)^{3-}$ ions and (b) values of pH in mod-DMEM during interaction with scaffolds
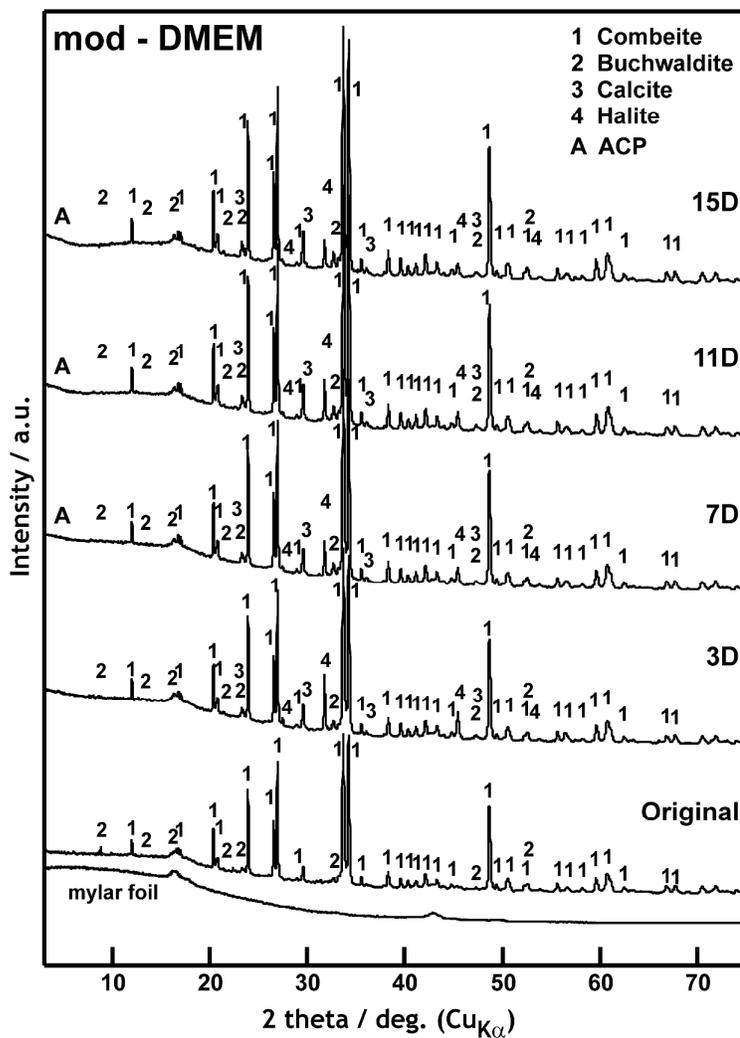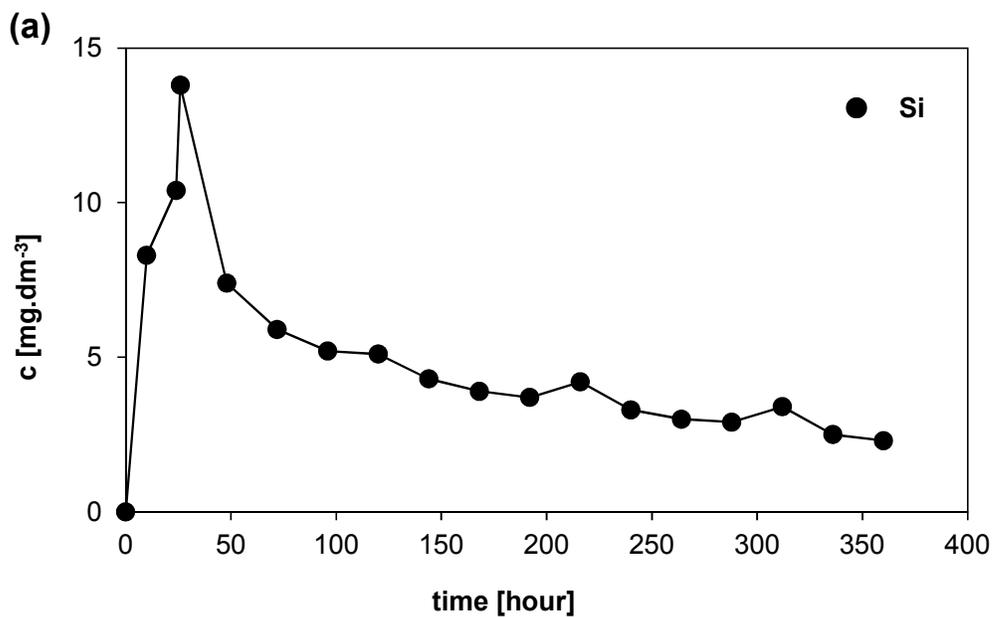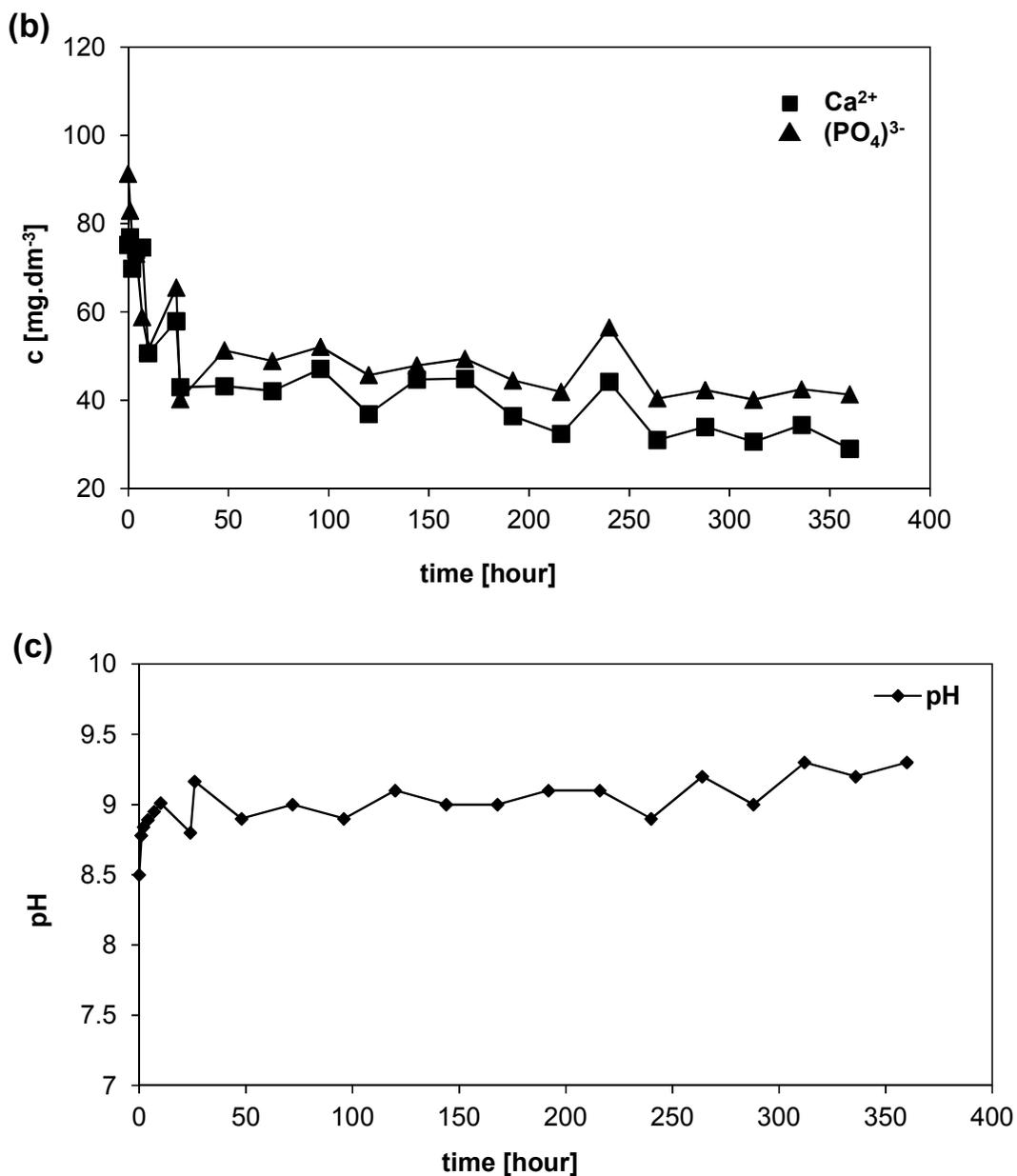
Fig. 2. XRD patterns of scaffold before and after 3, 7, 11 and 15 days of interaction with mod-DMEM
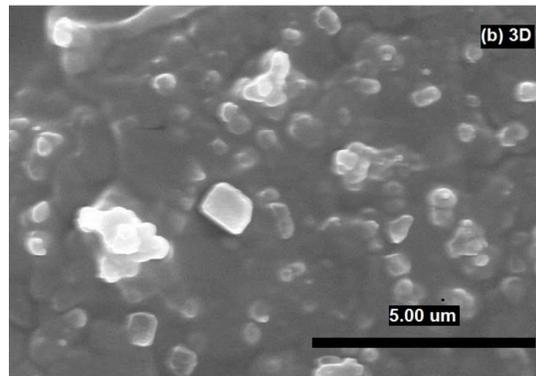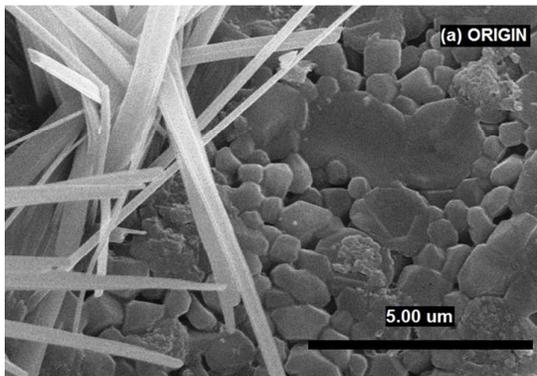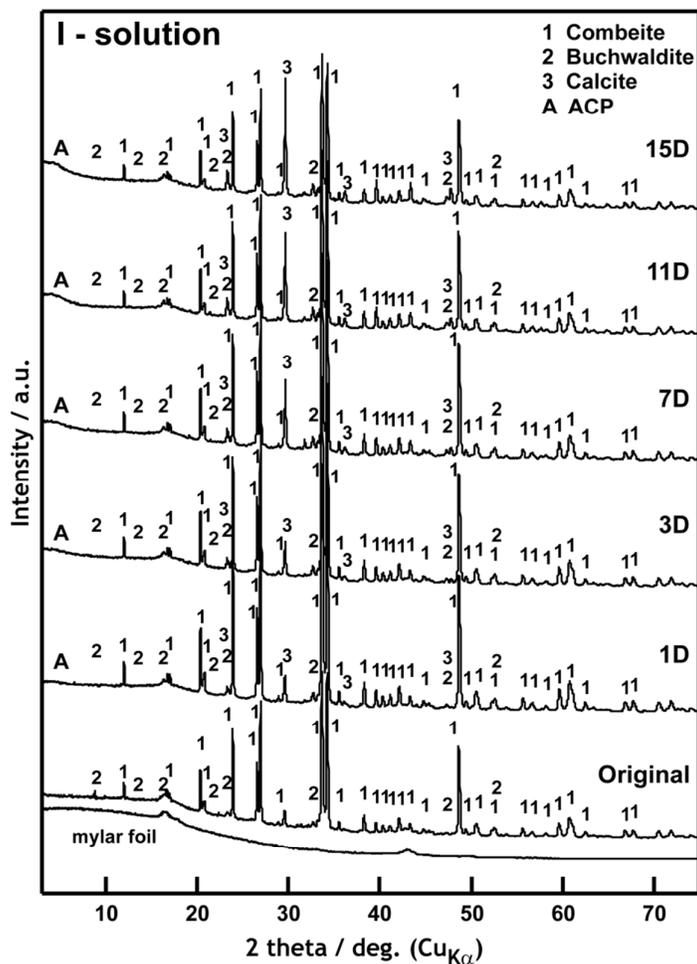
Fig. 3. SEM images of scaffolds (a) before (combeite - the small tabular crystals, buchwaldite and structurally isomorphic phase CaO.SiO$_2$ - needle-like crystals) and after (b) 3, (c) 7, (d) 11, (e, f) 15 days of interaction with mod-DMEM
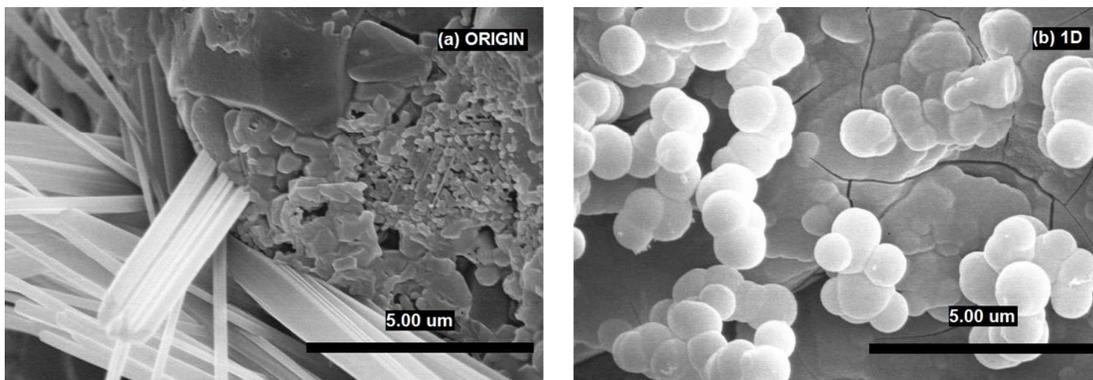
(b)

(c)

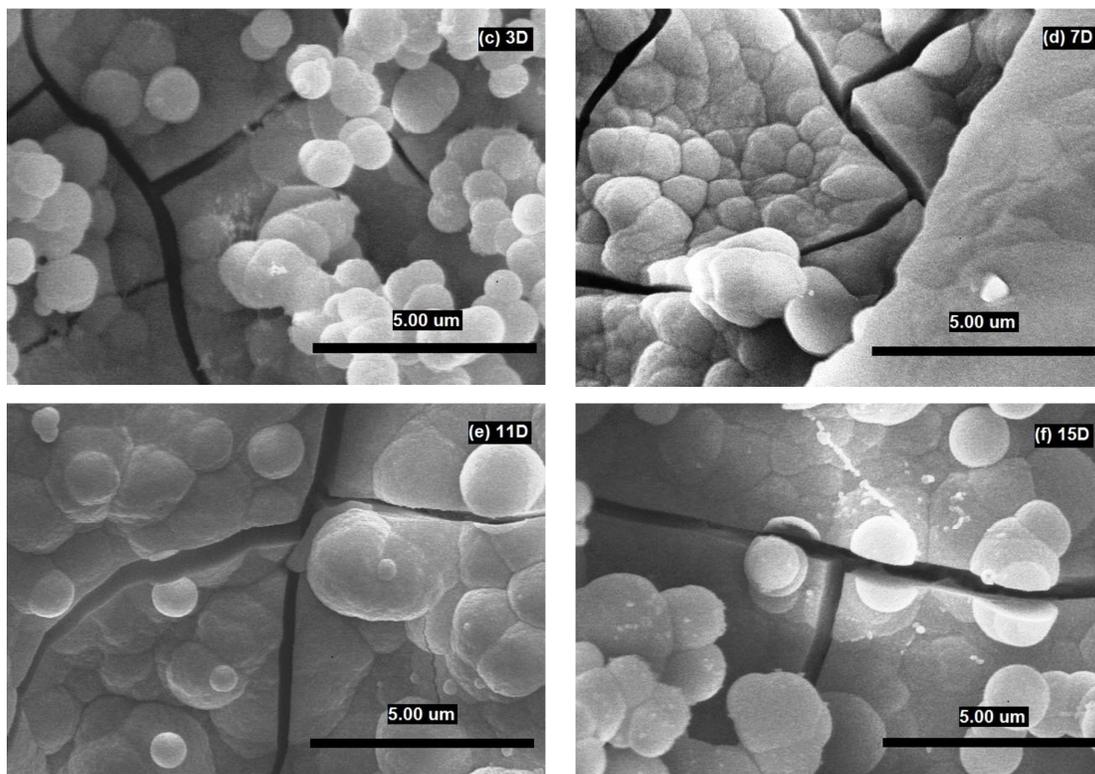
Fig. 4. Concentrations of (a) Si, (b) Ca^{2+}, $(PO_4)^{3-}$ ions and (c) values of pH in I-solution during interaction with scaffolds

Fig. 5. XRD patterns of scaffolds before and after 1, 3, 7, 11 and 15 days of interaction with I-solution

Fig. 6. SEM images of scaffolds (a) before (combeite - the small tabular crystals, buchwaldite and structurally isomorphic phase $CaO.SiO_2$ - needle-like crystals) and after (b) 1, (c) 3, (d) 7, (e) 11 and (f) 15 days of interaction with I-solution. The fractures in the newly developed layers were caused by drying of samples.

For decades the bioactivity of materials has been tested in laboratories by means of *in vitro* tests under standard ISO 23317 in a TRIS-buffered simulated body fluid solution (SBF). The TRIS buffer, as we found, reacts with the tested material and affect test results due to its ability to support the dissolution of crystalline phases of the tested glass-ceramic scaffold and the crystallization of HAp. Several laboratories had tested bioactivity of the materials in commercially available solution DMEM (Dulbecco´s Modified Eagle´s Medium) that is normally used for cultivation of cell cultures. The objective of this work was to find out whether it is possible to replace TRIS-buffered SBF currently used for bioactivity tests with the non-buffered DMEM solution. To understand the role of the organic part of the DMEM solution in the process of crystallization, we have prepared non-buffered solution simulating only its inorganic part (identified as I-solution). It was found that under static-dynamic test conditions calcite ($CaCO_3$) and the amorphous phase of calcium phosphate (ACP) formed on the surface of the glass-ceramic (Bioglass® - based) scaffold exposed to both solutions. Additionally, halite (NaCl) formed at the beginning of exposure to DMEM. Hydroxyapatite phase was not detected on the surface in either non-buffered solution, only $CaCO_3$ globules which looked similar to apatite crystals were observed. Organic components contained in the DMEM solution failed to prevent formation of crystalline phases but they probably slowed down the dissolution of the tested glass-ceramic scaffold. The present results indicate that it is not recommendable to use DMEM for bioactivity tests of glass-ceramic materials due to its low concentration of Ca^{2+} ions (insufficient supersaturation of DMEM with respect to HAp), high concentration of HCO_3^- ions and the necessity to maintain sterile environment during the test.