# The role of CaO/SiO$_2$ ratio and P$_2$O$_5$ content in gel-derived bioactive glass-polymer composites in the modulation of their bioactivity and osteoinductivity in human BMSCs

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ABSTRACT

We obtained a range of PLGA-based composites containing sol-gel bioactive glasses (SBG) from the SiO$_2$–CaO and SiO$_2$–CaO–P$_2$O$_5$ systems. Eight SBGs with different CaO/SiO$_2$ ratios with and without P$_2$O$_5$ were incorporated at 50% w/w to PLGA matrix and structured into thin films suitable for cell culture. The SBG/PLGA composites were examined for their bioactivity in simulated body fluid (SBF), ion release profile in culture media with and without cells, and osteoinductivity in standard human bone marrow stromal cell (hBMSC) cultures without osteogenic growth factors. Our results indicate different surface activity of composites depending on the presence/absence of P$_2$O$_5$ in SBG composition. Furthermore, ion release profile to culture medium differed depending on the presence/absence of cells. Direct culture of hBMSC on the SiO$_2$-CaO/PLGA composite films resulted in elevated Runx-2 mRNA, opposite to low Runx-2 mRNA levels on SiO$_2$-CaO-P$_2$O$_5$/PLGA films. All studied composites increased Osx mRNA levels. Whereas some of SiO$_2$–CaO/PLGA composites did not elevate BMP-2 and -6 proteins in hBMSC cultures, high levels of these BMPs were present in all cultures on SiO$_2$–CaO–P$_2$O$_5$/PLGA composites. All composites induced BMP-related Tak1 signalling, whereas Smad1 signalling was restricted mostly to composites containing three-component SBGs. ALP activity of hBMSC and BMP-related luciferase activity of mouse BRITE cells differed depending on whether the cells were stimulated with culture medium conditioned with SBG/PLGA composites or the cells were directly cultured on the composite surfaces. Altogether, beyond bioactivity and osteoinductivity of SBG/PLGA composites, our studies show key differences in the biological response to both the bioactive material dissolution products and upon direct cell-material contacts.

## 1. Introduction

Composite materials comprised of a polymer matrix and bioactive glasses as modifiers are well recognized as promising materials for regenerative medicine and tissue engineering [1–6]. Despite the inherent brittleness of bioglasses and glass-ceramics, they provide several desired properties when introduced to the polymer matrix. Due to their degradation processes, they release ions, especially calcium (Ca$^{2+}$) and phosphate (PO$_4^{3-}$) ions, which are believed to be primary factors contributing to material osteoinductivity [7]. These ions can modulate several biological processes, including osteogenesis and angiogenesis. Besides, bioglasses and glass-ceramics convert to biologically active carbonated hydroxyapatite (HCA) that provides firm bonding with hard and soft tissues. Yet another advantage of bioactive glasses technology is the possibility to obtain them by a sol-gel process as an alternative to melting. The sol-gel processing of bioactive glasses results in

mesoporous glass texture, highly-developed surface and exposition of siloxane groups on the material surface, suitable for hydroxyapatite nucleation [8]. Thus, the use of sol-gel glasses as composite modifiers creates opportunities to produce "tailor-made" biomaterials with the properties better adapted to the functions they perform in a living organism [9].

There are numbers of factors affecting the biological properties of the biomaterial and its behaviour in the living organism. Besides ion release profile and material potential to form carbonated HCA, chemical and phase composition, topography, porosity and nanostructure have been reported to influence biological response in vitro and in vivo [10]. Giving that any change to the chemical composition of the biomaterial may affect its physical properties and biological performance, it is of crucial value to define the key biomaterial determinants contributing to its positive biological outcome [11,12].

The aim of this work was to examine poly L-lactide-co-glycolide

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(PLGA)-based composites modified by binary $SiO_2$–CaO or ternary $SiO_2$–CaO–$P_2O_5$ sol-gel derived glasses. Glasses differed in the CaO/$SiO_2$ ratio within each group and contained $P_2O_5$ or $P_2O_5$ was absent. The composite material surfaces were examined in simulated body fluid (SBF) as the SBF test results are expected to predict bioactive properties of the materials. Changes in the materials surface morphology and structure were assumed as a confirmation of their bioactive properties. However, materials bioactive properties are often understood as the ability of materials to stimulate tissues for faster regeneration. In order to refer to that statement, we examined the response of human bone marrow stromal cells (hBMSC) and mouse osteoblastic BMP-reporter cell line (BRITER) to the above-mentioned composite materials Cells were either directly stimulated on the material surface or cultured with the ionic extracts from the materials (indirect conditions). Moreover, we have addressed the question of whether the cells attached to the surface of the material affect the ion release profile. Furthermore, assuming that materials themselves may prime osteogenesis, most of the applied biological assays were carried out in standard growth media without osteogenic supplements.

## 2. Materials and methods

### 2.1. Composite components

PLGA (poly L-lactide-*co*-glycolide) was synthesized via a ring-opening process in the presence of low toxicity zirconium acetylacetonate as a copolymerization initiator [13]. The molar ratio of L-lactide to glycolide in the copolymer was 85:15 (as determined by ^{1}H nuclear magnetic resonance (NMR)), and molecular masses of PLGA were: Mn = 80 kDa and Mw = 152 kDa. Chemical compositions of sol-gel glasses used as composites modifiers are given in Table 1. Depending on the chemical composition of the glasses they were divided into two groups: group I glasses from the $SiO_2$–CaO system and group II glasses from the $SiO_2$–CaO–$P_2O_5$ one. Tetraethoxysilane (TEOS, Si$(OC_2H_5)_4$, Sigma-Aldrich, USA), triethyl phosphate (TEP, OP$(OC_2H_5)_3$, Sigma-Aldrich, USA) and calcium nitrate tetra-hydrate (Ca$(NO_3)_2 \cdot 4H_2O$, POCh, Poland) were used as base components to start the sol–gel process. The 1 M solution of hydrochloric acid (HCl, POCh, Poland) was used as a catalyst for the hydrolysis and condensation reactions. Preformed gels were dried at 40–120 °C for 7 days and then subjected to thermal treatment at 600 °C for 10 h ($SiO_2$–CaO), or 700 °C for 20 h ($SiO_2$–CaO–$P_2O_5$) [14]. Afterwards, they were milled and sieved to obtain bioactive glass powders with average particle diameter ca 45 μm. The textural characteristics of the sol-gel derived bioglasses have been examined and presented earlier [14].

### 2.2. Composite films fabrication

The sheets of PLGA-gel derived bioactive glass (SBG) composites were fabricated by mixing glass particles with 5% w/v PLGA solution in methylene chloride ($CH_2Cl_2$, POCh, Poland) on a magnetic stirrer for 24 h, followed by slip casting of the viscous mixture on glass Petri

**Table 1**
Chemical compositions of gel-derived glasses and their CaO/$SiO_2$ ratio.

| Material | Chemical composition (%mol) | | | CaO/$SiO_2$ ratio |
|---|---|---|---|---|
| | $SiO_2$ | CaO | $P_2O_5$ | |
| A1 | 40 | 60 | – | 1.50 |
| T1 | 50 | 50 | – | 1.00 |
| D1 | 60 | 40 | – | 0.67 |
| S1 | 80 | 20 | – | 0.25 |
| A2 | 40 | 54 | 6 | 1.35 |
| T2 | 47 | 47 | 6 | 1.00 |
| D2 | 60 | 36 | 4 | 0.60 |
| S2 | 80 | 16 | 4 | 0.20 |

dishes, evaporation of the solvent in air, and then drying in air and vacuum to a constant weight. The weight fraction of gel-derived bioactive glass in the composites was 50%, in contrast to previously published by us PLGA-SBG sheets and scaffolds containing 21% of SBG [15–17]. The obtained composite sheets were 0.11 mm thick. The composites studied in the present work were labelled by a SBG/PLGA notation so that they were distinguishable from the PLGA-SBG labelled materials that had been studied by in the past. [15]

### 2.3. Material surface changes in simulated body fluid (SBF)

In vitro simulated body fluid (SBF) test was applied according to the method proposed by Kokubo et al. [18]. In brief, SBF was prepared by dissolving the following chemicals (POCh, Poland) in UHQ-water: 141 mM NaCl, 4 mM KCl, 0.5 mM $MgSO_4$, 1 mM $MgCl_2$, 4.2 mM $NaHCO_3$, 2.5 mM $CaCl_2$, and 1.0 mM $KH_2PO_4$. The resulting SBF was buffered to pH 7.40 with Tris(hydroxymethyl aminomethane)/ HCl. The composite sheets weighing 20 mg were immersed in 20 ml of SBF solution in separate polypropylene containers and incubated at 37 °C for 7 and 14 days. Afterwards, the samples were washed in ethyl alcohol, air- and vacuum-dried to a constant weight.

Microstructure and chemical composition of PLGA and composite films before and after soaking in SBF were examined by scanning electron microscopy coupled with energy-dispersive x-ray spectroscopy analysis (SEM/EDS, NanoSEM, FEI, USA). The EDS spectra were averaged for the whole analyzed surface. Moreover, Ca/P molar ratios of the layer formed on the film's surface during incubation in SBF were calculated base on the EDS spectra semi-quantitative analyses. The spectra were collected from at least three different points of each sample, averaged and presented as mean ± SD. In addition, Fourier-transform infrared spectroscopy (FTIR, Bruker *V*ERTEX 70 V spectrometer, USA) was applied to get insights into structural changes of fabricated materials after 14-day incubation in SBF. The samples in the form of the potassium bromide (KBr) pellets were analyzed at the range of 400–4000 $cm^{-1}$ wavenumbers and 128 scans were collected at 4 $cm^{-1}$ resolution and averaged.

### 2.4. Ion release profiles

The obtained SBG/PLGA composite sheets were inserted into separate wells of 24-well cell culture plate and immersed for up to 3 days in culture medium composed of alpha-MEM and 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, USA). Next Ca, Si and P ion release profiles were determined at 24, 48 and 72 h. Culture media were exchanged every 24 h. The initial ion concentration in culture medium was assumed as a starting point and designated 0 h. Similarly, ion release profiles from the composite films to culture medium were evaluated upon culture with hBMSC. The ions released from the materials were evaluated in α-MEM medium only, in order to provide the most relevant results for their further comparison with the ion release profile from the materials seeded with cells. For assessment of ion concentrations in the culture media, the inductively coupled plasma atomic emission spectrometry (ICP-OES; Plasma 40, Perkin Elmer, USA) was used. All tests were performed in triplicates and results were presented as mean ± standard deviation (SD).

### 2.5. HBMSC isolation and culture expansion

Unless stated otherwise, all cell culture reagents were purchased from Thermo Fisher Scientific. HBMSCs were harvested from the iliac crest of adult patients (42–67 years old, both genders) according to the approved Institutional Review Board protocol (No. 1072.6120.254.2017). The mononuclear cell fraction was isolated using Ficoll-Paque (GE Healthcare), as described in the manufacturer's protocol. Mononuclear cells were expanded in T-75 flasks (NestBiotech) in a growth medium composed of alpha-Minimum Essential Medium

(alpha-MEM), 10% mesenchymal stem cell qualified fetal bovine serum (MSC qualified FBS, Biological Industries) and 1% antibiotics (i.e. penicillin and streptomycin). Once the primary cultures reached 80–90% confluence, cells were detached from the bottom of tissue culture flasks with 0.25% Trypsin-ethylene-diamine-tetra-acetic acid (EDTA) and either used for experiments or further expanded in T-75 flasks. All experimental cultures were established with hBMSCs at passages 3–5.

### 2.6. Cultures on the experimental surfaces and in the presence of condition medium

Before cell seeding, the material sheets were cut into round disks fitting the bottom of either 24- or 6-well tissue culture plates. The disks were soaked in 70% ethanol (water solution), washed with phosphate-buffered saline (PBS) to remove ethanol traces, exposed to UV light (10 min each side) and then left overnight under the laminar chamber to dry. HBMSC were seeded on the material disks at the density of $2 \times 10^4$ cells/cm$^2$ in the standard growth medium and maintained in culture for 2–3 days without any media change. HBMSC at the same density were also seeded on tissue culture plastic (TCP) and treated with condition medium (CM) harvested from the material incubated in growth media. Specifically, the materials were soaked separately in growth media for 24 h. The CM's were collected and replaced with fresh media for a further 3 days. The procedure was repeated up to day 19 with CM collection and replacement every 3 days. The collected CMs were used to treat cells seeded on TCP starting at 4 h post cell seeding and followed by CM changes every 3 days up to culture day 21st. The effects of materials surface or condition medium collected from the materials on bone morphogenetic protein (BMP) response of cells was evaluated in BRITER mouse osteoblasts, the BMP-responsive reporter osteoblast cell line [19]. Briefly, in this cell line, the reporter construct contains BMP responsive element (BRE) driven Firefly Luciferase gene (FFLuc) and SV40 promoter/enhancer driven Renilla luciferase (RRLuc) gene. The latter serves as an internal normalization control for cell number as well as non-specific transcription activation. Upon BMP stimulation, cells increase Firefly Luciferase activity in a robust and sensitive manner. BRITER cells were expanded in a growth medium composed of alpha-MEM, 10% FBS and antibiotics and then they were seeded directly onto material surfaces or into 24-well TCP plates, at the density of $2 \times 10^4$ cells/cm$^2$. The cells seeded onto material surfaces were cultured for 3 days without any medium changes. A separate set of materials was soaked in a growth medium for 3 days and this CM was used to treat cells seeded on tissue culture plates. Cells were stimulated with CM for 3 days. At culture day 3, either on material surface or in the presence of CM, cells received 100 ng/ml recombinant human BMP-2 for 3 h, followed by dual luciferase assay.

### 2.7. Evaluation of cell cultures

The messenger RNA (mRNA) levels of runt-related transcription factor-2 (Runx-2) and Osterix, Sp7 transcription factor (Osx) were evaluated 2 days post cell seeding onto material surfaces. Briefly, total RNA was isolated with Gene MATRIX Universal RNA Purification Kit (EurX®) and 0.1 μg RNA from each culture was reverse-transcribed to complementary DNA (cDNA) with High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems). The cDNA samples were used for real-time PCR (polymerase chain reaction) analyses with TaqMan probes for Runx-2 (Hs01047973) and Osx (Hs01866874). The reaction mixtures for quantitative PCR (qPCR) consisted of 50 ng cDNA, 7,5ul TaqMan Universal PCR Master Mix and 0.75 ul TaqMan probe. The PCR reactions were performed for 40 cycles with denaturation step at 95 ˚C for 15 s, annealing at 60 ˚C for 1 min, and elongation at 60 ˚C for 1 min. Relative quantification (i.e. ddCT method) was used to analyze the results with cells cultured on PLGA as a reference. BMP-2 and BMP-6 protein levels in culture medium were analyzed 3 days post cell-seeding onto material surfaces using Elisa kits (Thermo Scientific and FineTest,

respectively), according to manufacturers' instructions. The concentrations of proteins in the culture medium were calculated based on respective standard curves and the amounts were normalized to total protein levels present in the culture medium. The levels of phospho-Smad1, 5, 8 and total Smad were analyzed 3 days post cell-seeding onto material surfaces by Elisa kit (Abcam) and Western blot. Elisa was performed on whole-cell extracts according to the manufacturer's protocol. For Western blot, whole-cell extracts were prepared with the use of cell lysis buffer (Cell Signaling Technology). Protein concentrations in the extracts were determined with the MicroBCA protein assay kit. Equal amounts of protein samples were separated on NuPAGE 4–12% Bis-Tris gels under reducing conditions, transferred to polyvinylidene fluoride (PVDF) membranes and then probed overnight with primary anti-human Smad or anti-human phospho-Smad 1/5/9 antibodies (Cell Signaling Technology, #6944 and #13820, respectively). The horse-radish peroxidase-linked secondary antibodies (GE Healthcare) were then applied and the peroxidase-based signal was detected using Western Lightning Chemiluminescence Reagent Plus (GE Healthcare). The signal was captured on Hyperfilm ECL chemiluminescent films (Perkin-Elmer). As with Smads, the levels of phospho-transforming growth factor beta-activated kinase 1 (Tak1) and total Tak were 5a., in whole-cell extracts obtained 3 days post-cell seeding onto material surfaces. Western blot analyses were performed as described above with anti-human TAK and anti-human phospho-Tak1 antibodies (Cell Signaling Technology, #4505 and #4505, respectively). Alkaline phosphatase activity (ALP) was assessed in 7-day hBMSC cultures plated directly onto material surfaces or seeded into 24-well TCP plates and stimulated by (culture media CMs) harvested from the materials. At culture day 7, the culture medium from individual wells was replaced with 0.2 ml solution of 10% MTS reagent (CellTiter96Aqueous One Solution Cell Proliferation Assay; Promega) in phenol-free alpha-MEM and the development of colorimetric reaction was captured by measuring the absorbance at 490 nm. Afterwards, cells were washed with phosphate-buffered saline (PBS) and ALP activity was measured kinetically as originally described by Osyczka and Leboy [20]. Briefly, protein extracts were obtained with cell digestion buffer and used for reactions with the ALP substrate p-nitrophenol phosphate (pNPP) (SigmaAldrich). The changes in the absorbance at 405 nm were measured at 1 min intervals for up to 45 min. ALP activity was expressed as nmol pNPP/min/total volume of the protein extract and normalized to the number of viable cells estimated from the MTS assay. For the evaluation of mineralization of extracellular matrix, hBMSCs were plated in tissue culture plates (TCP) and stimulated for 21 days with CMs harvested from the materials. At culture day 21, cells were assayed for viability with MTS reagents, and then the cultures were extensively washed with PBS, fixed in cold methanol and stained for mineral with Alizarin Red S (Sigma Aldrich). The dye was then extracted with 5% perchloric acid (water solution) and samples measured calorimetrically at 410 nm. For the evaluation of BRITER mouse osteoblasts response to BMP-2, dual luciferase assay (Promega) was carried out as recommended by manufacturer.

### 2.8. Statistical analyses

All biological data were collected in triplicates and expressed as mean ± SD. Statistical Analyses were performed in SigmaPlot software. One-way or multiple comparisons ANOVA with *post-hoc* Tukey's test was applied to calculate statistically significant differences at $p < 0.05$.

## 3. Results

### 3.1. Material surface changes upon incubation in SBF

The SEM/EDS analyses were applied to assess the surface morphology changes of composite sheets and PLGA before and after 7- and
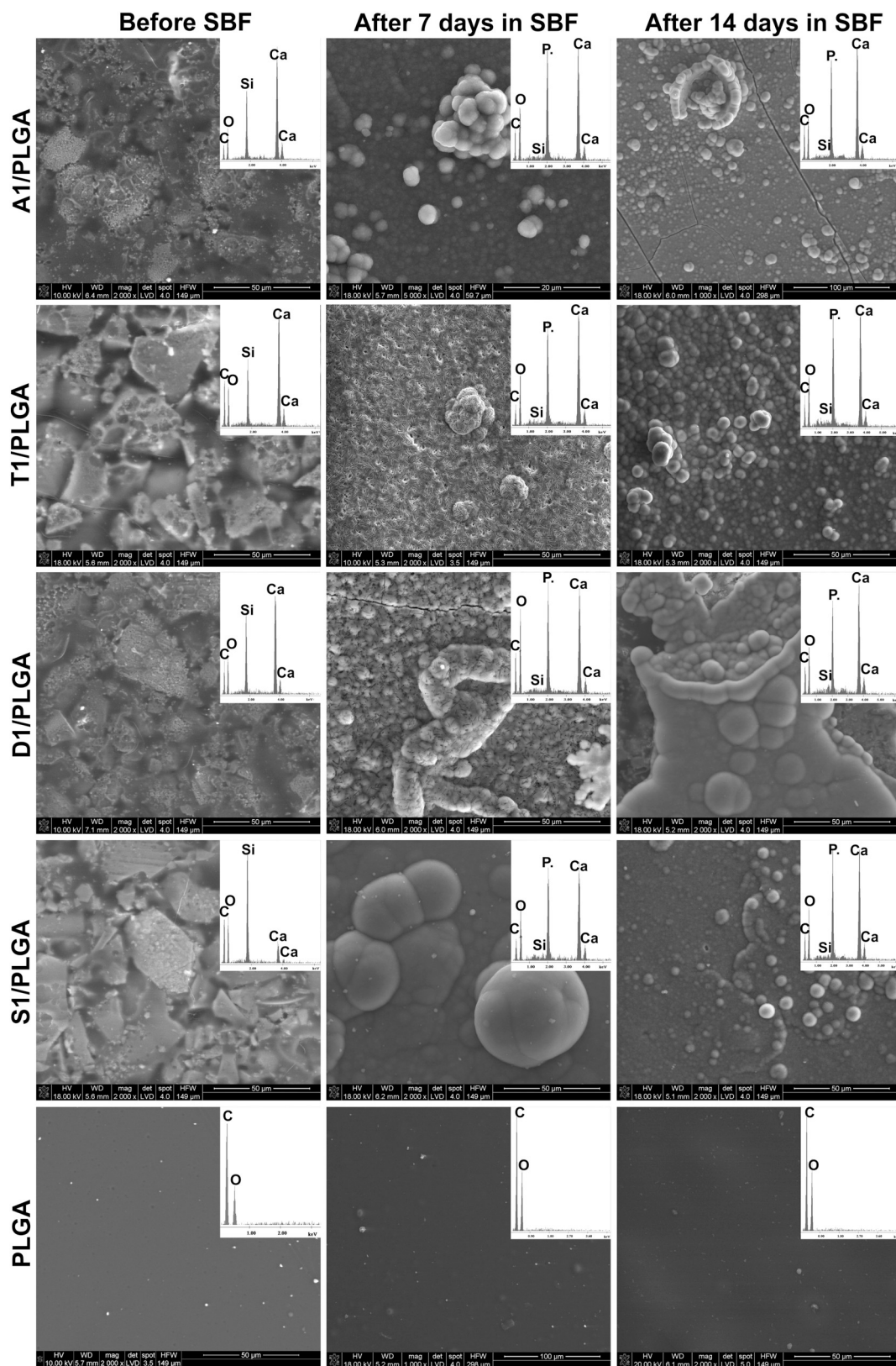
**Fig. 1.** SEM and EDS results for PLGA and PLGA based composites containing gel-derived SiO$_2$–CaO bioactive glasses before and after incubation in SBF for 7 and 14 days.

14-day incubation in SBF. As presented in Fig. 1, all PLGA based composites containing SiO$_2$–CaO sol-gel bioactive glasses (SBGs) showed altered surface morphology after 7-day incubation in SBF. Numerous "cauliflower" precipitates forming a thick, locally cracked layer were observed. The surface forms created on contact with SBF were typical for the apatite responsive on the surface [21–24]. That was further verified with EDS analyses, which indicated elevated levels of calcium and phosphorus after contact with SBF. Extension SBF
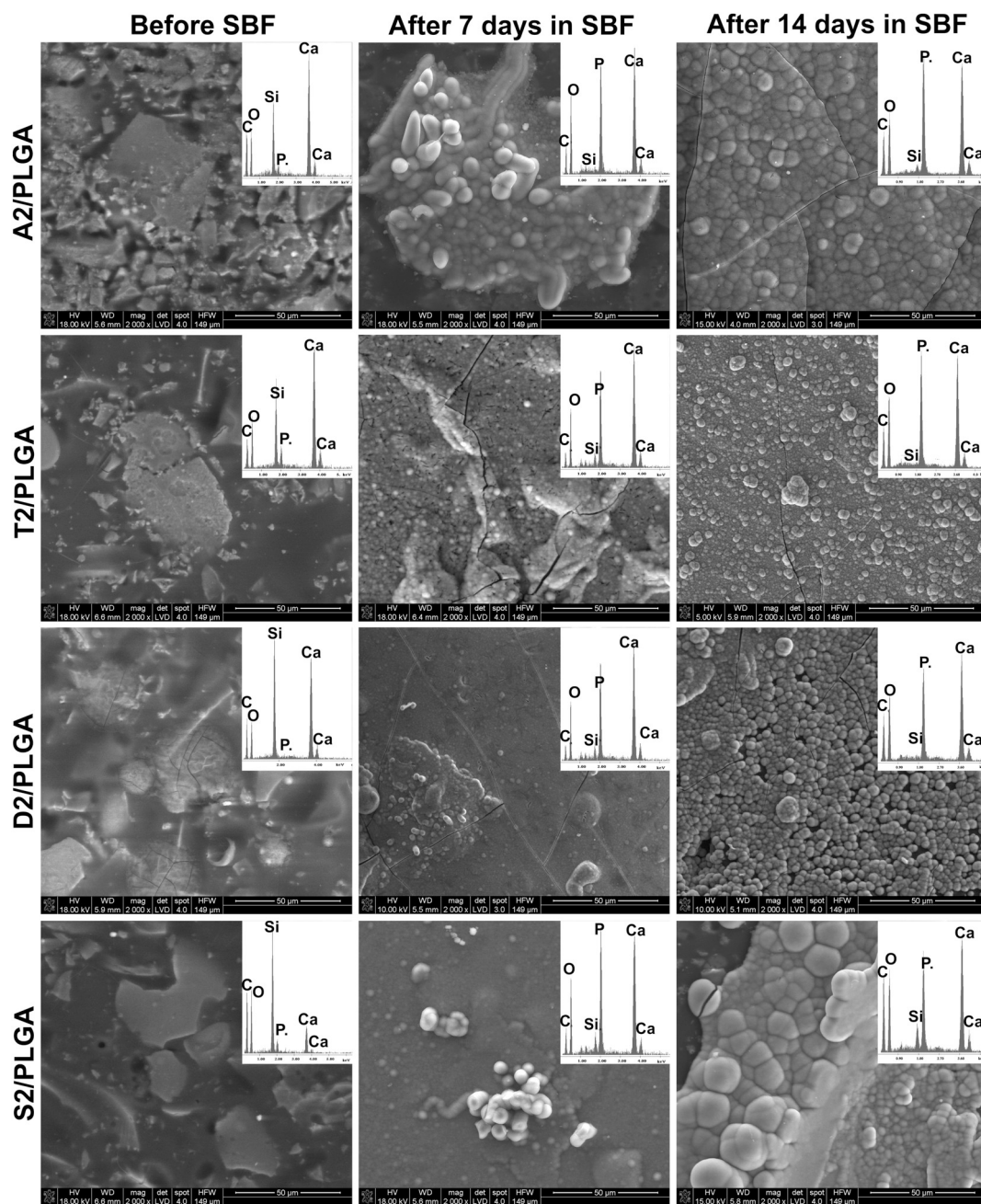
**Fig. 2.** SEM and EDS results for PLGA based composites containing gel-derived SiO$_2$–CaO–P$_2$O$_5$ bioactive glasses before and after incubation in SBF for 7 and 14 days.

incubation time to 14 days resulted in an intensification of surface changes, the surface layers had visible cauliflower-like forms and appeared to be thicker and tighter packed in comparison to the samples incubated in SBF for 7 days. PLGA-based composites with SiO$_2$–CaO–P$_2$O$_5$ SBGs showed similar surface changes to those described for SiO$_2$–CaO SBGs (Fig. 2). However, for these composite groups, surface changes were less intense, as evidenced by the presence of small amounts of Si in the EDS spectrum. Nevertheless, the EDS surface analyses in the region of cauliflower-like forms showed increased amounts of calcium and phosphorus indicating calcium phosphates crystallization [25–27]. Surface morphology of PLGA (Fig. 1, bottom panel), serving as a reference material had not changed after incubation in SBF. EDS analyses of PLGA before and after incubation in SBF showed similar elements.

### 3.1.1. Ca/P ratio analysis

The Ca/P molar ratios for the layers formed during the 3, 7, and 14 days incubation in SBF were evaluated using the EDS semi-quantitative analysis (Fig. 3, Table 2). Analyses were carried out to specify the differences in bioactive in vitro response between these materials, for which data provided with SEM microphotographs were insufficient to diversify the bioactivity potential of these materials. Moreover, such analyses allowed us for the determination of a stage of development of the layer on the surface of the material, at a particular time point. For all tested materials the increase of Ca/P value was observed throughout the whole incubation period, however for individual materials that process presented different patterns. For the materials containing binary system glasses (SiO$_2$ – CaO) after 3 days of incubation in SBF the layer consisted of calcium-deficient hydroxyapatite (CDHA, Ca/P: 1.50–1.67) [28]. After 7 days incubation in SBF for all materials from that group the Ca/P ratio exceeded 1.67 level, characteristic for HAp
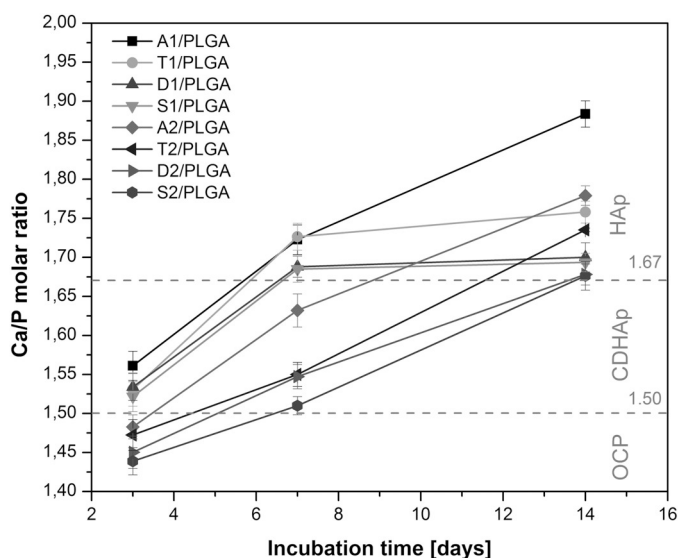
**Fig. 3.** Variations of the Ca/P molar ratio of the formed layer after incubation in SBF.

**Table 2**
Ca/P molar ratio in the formed layer after various SBF incubation times according to the EDS quantitative study (mean ± SD).

| Material | Ca/P ratio | | |
|---|---|---|---|
| | 3 SBF | 7 SBF | 14 SBF |
| A1/PLGA | 1561 ± 0,019 | 1723 ± 0,019 | 1884 ± 0,017 |
| T1/PLGA | 1531 ± 0,021 | 1726 ± 0,017 | 1758 ± 0,014 |
| D1/PLGA | 1534 ± 0,017 | 1688 ± 0,014 | 1700 ± 0,019 |
| S1/PLGA | 1522 ± 0,019 | 1685 ± 0,017 | 1693 ± 0,012 |
| A2/PLGA | 1483 ± 0,015 | 1632 ± 0,021 | 1779 ± 0,012 |
| T2/PLGA | 1472 ± 0,020 | 1550 ± 0,016 | 1753 ± 0,002 |
| D2/PLGA | 1450 ± 0,021 | 1547 ± 0,015 | 1678 ± 0,013 |
| S2/PLGA | 1439 ± 0,017 | 1510 ± 0,012 | 1676 ± 0,018 |

[29]. Between days 7 and 14, for T1/PLGA, D1/PLGA and S1/PLGA only a slight Ca/P growth was observed, in contrast to A1/PLGA for which a continuous, increase of Ca/P was observed. Notably higher $CaO/SiO_2$ ratio in binary system glasses resulted in the Ca/P ratio increase for the surface layer after contact with SBF in particular incubation period.

For all the composite materials containing ternary system glasses $(SiO_2 – CaO – P_2O_5)$, a similar increase of Ca/P ratio at the initial stage of incubation could be observed. On day 3, for all of the studied materials Ca/P ratio was below 1.50, indicating the formation of octacalcium phosphate (OCP). After 7 days of incubation, the Ca/P ratio increased above 1.50 and indicated the formation of calcium-deficient hydroxyapatite (CDHA). After 14 incubation day the 1.67 level was exceeded for all these materials, as a result of further dynamic increase of Ca/P ratio. For A2/PLGA and T2/PLGA further increase of Ca/P ratio above stoichiometric value for hydroxyapatite (1.67) was observed. That can indicate the formation of a carbonated HAp of B-type HCA (i.e. HAp with carbonate ions $CO_3^{2-}$ substituting for phosphate ions $PO_4^{3-}$) [30]. For the materials containing ternary glasses the higher the $CaO/SiO_2$ value was in the initial bioglass composition the faster transformation into stoichiometric HAp and further into HCA was observed.

### 3.2. FTIR analyses of the material surface after incubation in SBF

The FTIR spectroscopy was applied to determine the structure of composite surfaces after incubation in SBF. Since EDS analysis showed the formation of surface layers enriched in calcium and phosphorus on

both groups of composites, it was plausible to expect surface crystallization of apatite, indicative of the bioactive material properties. Thus phosphate structures on the surfaces were expected to be identified by a spectroscopic method. Their presence could be evidenced by the appearance of the double band about 564–604 $cm^{-1}$ occurring in the FTIR spectra of the apatite [31–35].

As shown in Fig. 4 there were no bands characteristic for phosphate structure in the spectra of PLGA-based composites containing $SiO_2$–CaO SBGs before incubation in SBF. That could be explained by the lack of phosphorus in the composition of glasses used for the preparation of these composites. However, after 14-days incubation in SBF a distinct band at 563–603 $cm^{-1}$ appeared, indicating the apatite crystallization. For PLGA-based composites containing $SiO_2$–CaO–$P_2O_5$ SBGs the IR absorption in the range 560–609 $cm^{-1}$ before incubation in SBF related to the presence of phosphorus in bioactive glasses, which formed phosphate structures. After SBF incubation, the band at about 560–609 $cm^{-1}$ had clearly a doublet shape and became more intense compared to the spectra of materials before SBF incubation. This indicated the surface crystallization of apatite upon materials contact with SBF. Although FTIR analyses do not allow for the quantitative assessment of the phosphate layer being formed, taking into account the highest intensity of the 560–609 $cm^{-1}$ band for T2/PLGA composite, we assume that for that composites the phosphate structures were most firmly shaped.

The tests conducted in SBF showed that all examined composite sheets display potential to form apatite-like layers on their surface and thus all could be considered to display bioactive properties. This surface activity appeared practically independent of the chemical composition of SBG used as a PLGA modifier. The only noticeable difference was a slightly slower formation of the calcium phosphate layers for the composites modified by SBGs with the lowest CaO and the highest $SiO_2$ content (i.e. S2). The formation of apatite-like layers on the studied composite surfaces implies that such materials should integrate well with bone tissue in a human body by a stable interface between the implant and bone. In fact, our previous studies with selected PLGA–SBG composites and SBGs alone showed their good integration with hard and soft tissues [36,37]. However, SBF tests do not provide insights into osteoinductive material properties and their potential to support osteogenesis. Thus, we followed with the biological tests assessing to what extent do the changes in the chemical composition of SBGs affect ion release profile and cell response to SBG/PLGA composites.

### 3.3. Characteristics of SBG/PLGA composites as the substrates for cell cultures

Any substrate can interact with cells in a chemical and physical manner. Chemical interaction is determined by the chemical composition of the base, the chemical groups exposed to the biological environment and by ions or other material degradation products released from the material to the medium [38]. Giving the SBG chemical compositions used for the present study, we focused on the ion release profile of calcium, silicon, and phosphorus to the plain culture medium (i.e. no cell culture) and to the culture medium upon culture with hBMSC.

As presented in Fig. 5a, a1 calcium was released rapidly to the culture medium and Ca levels in culture medium were the highest at 24 h incubation for all studied composites. As expected, the highest Ca levels in culture medium were observed for composites containing SBGs with high CaO content (i.e. A1 and A2). Moreover, higher amounts of calcium released into culture medium were observed for composites modified with two-component SBGs (group I) than for three-component SBGs (group II) except for SBG type D. Upon culture with hBMSC (Fig. 6a, a1) higher Ca amounts were released from A1/ and A2/PLGA than during incubation in culture medium itself. This may suggest a specific cell activity on these composite types leading to faster material degradation. Depending on SBG type (i.e. A, T, D, S) the amounts of
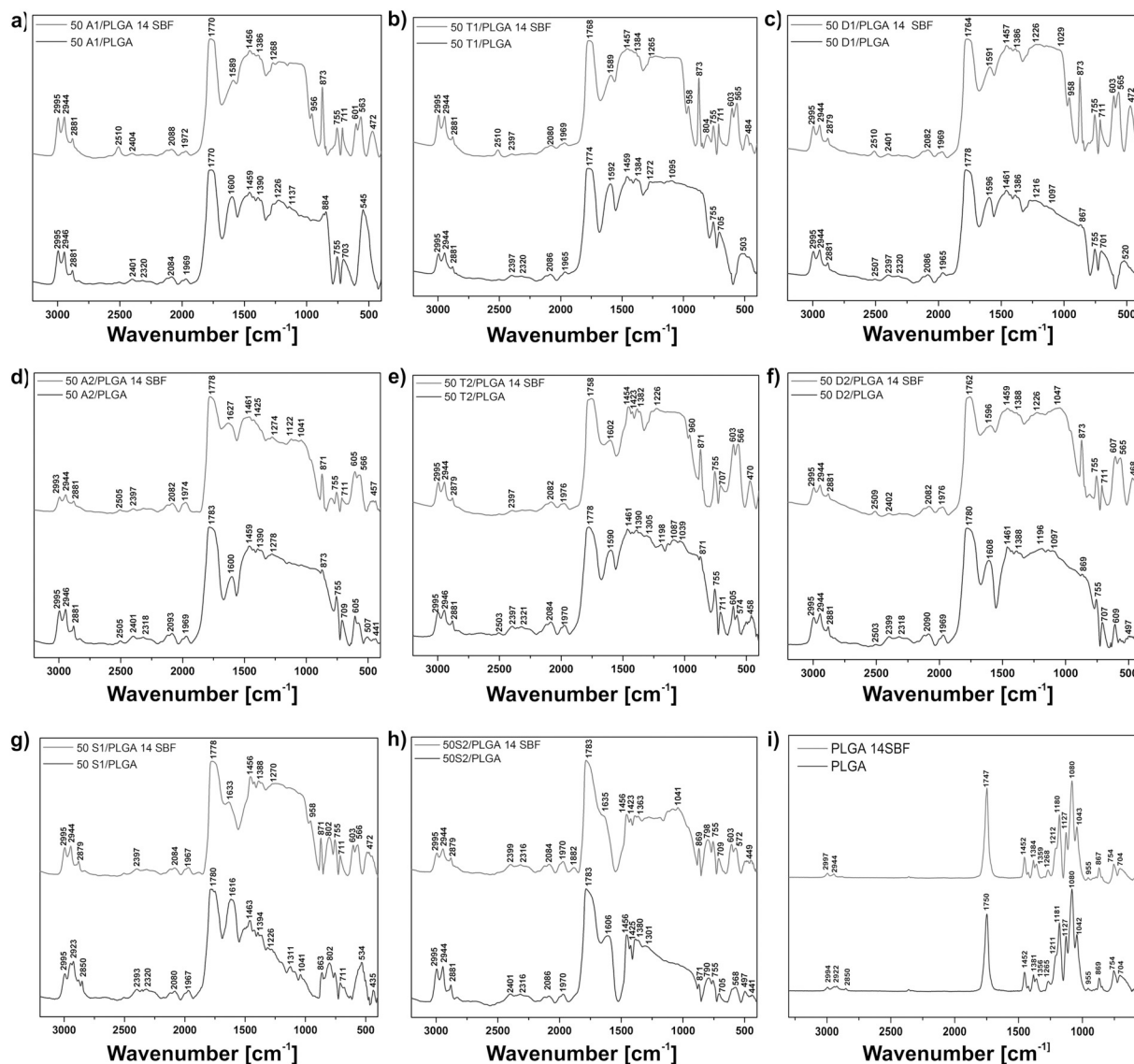
**Fig. 4.** FTIR spectra of PLGA and SBG/PLGA composites before and after incubation in SBF for 14 days.

calcium released to culture medium were decreasing accordingly with decreasing amounts of Ca in the SBG chemical composition. This was more obvious for the group I composites (i.e. 2-component SBGs, (Fig. 5a) than for group II composites (i.e. 3-component SBGs, Fig. 5a1). Also, the release of Ca to culture medium at 48- and 72 h incubation was much lower compared to 24-h incubation point and the released Ca amounts no longer correlated with the chemical compositions of SBGs. Upon hBMSC culture, the dependence of Ca release to culture medium on SBG composite component was less obvious (Fig. 6a, a1) than upon composites incubation in culture medium without cells (Fig. 5a, a1). However, at 48 h this was still noticeable upon cell culture, in contrast to culture medium itself.

There was no clear relationship between the amounts of Si released to the culture medium and $SiO_2$ content in the studied composites (Fig. 5b, b1). Notably, at 24 h incubation in culture medium less Si was released for composites enriched with high-$SiO_2$ SBGs (i.e. S1 and S2) than for low-$SiO_2$ SBGs (i.e. A1 and A2). This may indicate that Si-enriched composites have a more compact structure composed of $Q^4(Si)$ units connected by oxygen bridges from which silicon ions are harder to break free [14]. Nevertheless, increasing amounts of Si were released from all studied composites to the culture medium at 72-h incubation vs. 24-h incubation point, despite the medium was exchanged daily.

This may indicate the extensive surface changes by 72-h incubation time leading to the formation of Si-OH groups and their dissolution in the physiological fluids. The Si release profile upon cell culture differed from that obtained for culture medium without cells (Fig. 6b, b1). However, as in culture medium without cells, increased amounts of released Si could be observed with increased composites incubation time. Also, at 48- and 72-h incubation points there was significantly less Si released from composites enriched with $SiO_2$. Thus, a direct culture of cells on silica-enriched materials may block material surface activity.

Opposite to Ca and Si, it was apparent that the phosphate content in culture medium was clearly reduced in the presence of SBG/PLGA composites and this reduction was the highest for composites containing the highest Ca levels in SBG chemical composition (i.e. A1 and A2, Fig. 5c, c1). This reduction in P concentration in culture medium was even more apparent upon hBMSC cell culture (Fig. 6c, c1). This suggests that the cells were participating in the process of scavenging P ions from the incubation medium. Reduced Ca amounts in the materials resulted in smaller changes in P content in the culture medium, both without and with cells, although this was more or less apparent depending on the incubation time. Nevertheless, it is plausible that the more calcium in the materials, the more phosphate is attracted to the material surface and this may facilitate calcium-phosphate nucleation
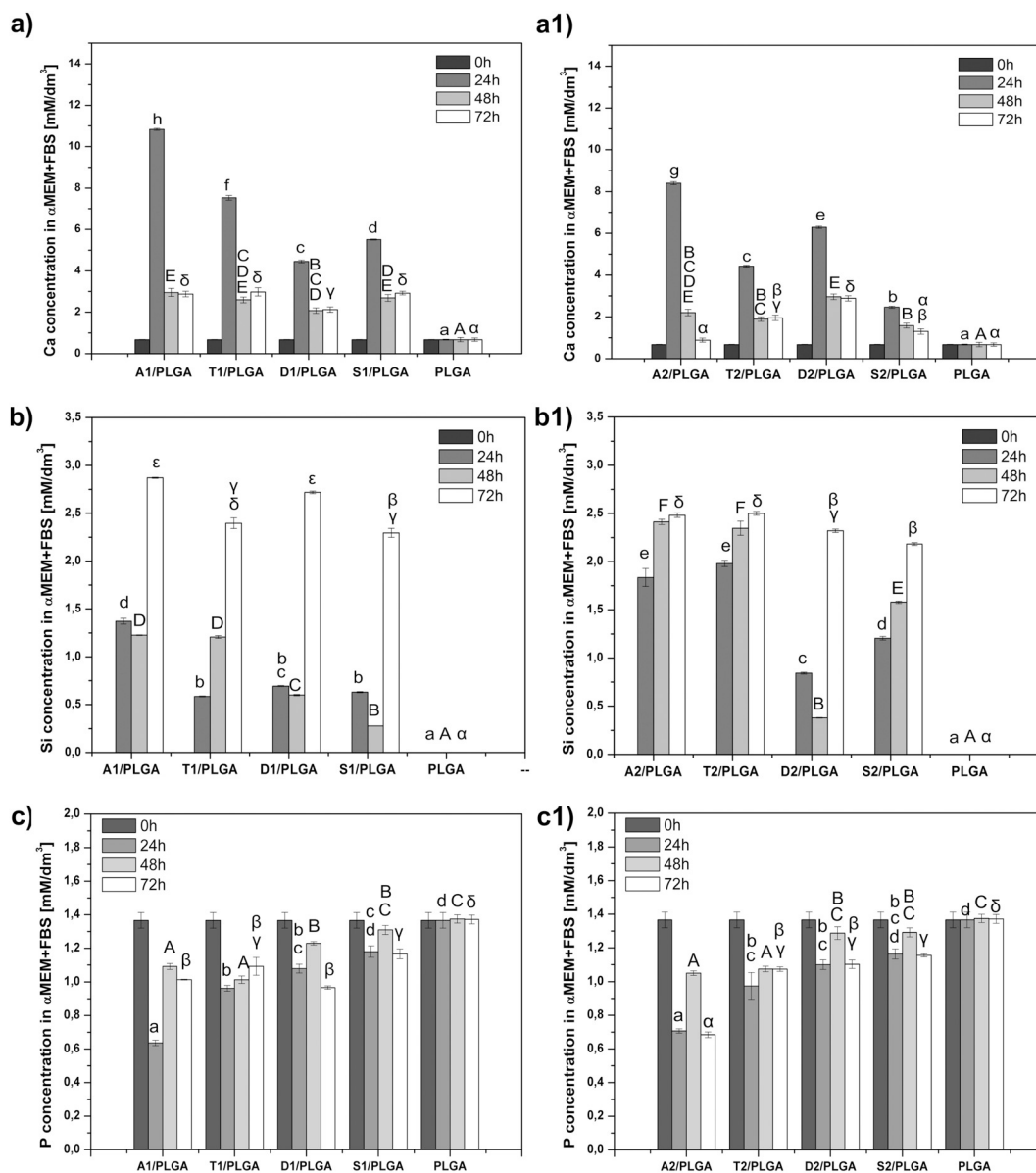
**Fig. 5.** Ca (a, a1) Si (b, b1), and P (c, c1) concentration in the culture medium after incubation of PLGA and SBG/PLGA composites up to 72 h of soaking. Day 0 stands for the ion composition of plain culture medium before material soaking. Statistically significant differences (p < 0.05) between each SBG/PLGA composite and PLGA after different soaking periods are indicated by upper and lower case, as well as Greek lettering, respectively.

and crystallization.

The presented experimental profiles of Ca, Si and P content in the culture medium find justification in the model of bioactive surface changes in the simulated body fluid proposed by Kokubo [18,21]. There are two phenomena characteristic for these changes; 1) the formation of Si-OH groups on the material surface, which reflects the depolymerization of the silicon-oxygen network and 2) the saturation of Ca and P near the discrete material surface sites resulting in the surface crystallization of calcium phosphates. At 24 h incubation time, the release of Ca from materials was the highest and this depended on the content of calcium in SBG/PLGA composites. This can be explained by the exchange of Ca ions between the material and the culture medium and such exchange of calcium decreased accordingly to the chemical composition of SBGs, i.e. A > T > D > S. This is in agreement with the trend of decreased Ca ions release to culture medium A > D, although there was no such correlation for T and D materials. At later incubation time points (i.e. 48 and 72 h) calcium release was much smaller. It is though plausible that, as the culture medium saturates with calcium

ions, the Ca concentration gradient between material surfaces and culture medium decreases and consequently less calcium is released from the bioactive composites. In fact, we observed the highest Ca release upon first few hours of materials immersion in culture medium and Ca release decreased after the medium change (data are not shown), probably due to its lower accessibility from bioactive glasses.

In parallel with the release of calcium from the composites, the surface is enriched with silicon with simultaneous hydration of the surface layer due to the formation of the Si-OH groups. The partial depolymerization of the surface layer resulting from Si-OH groups formation promotes the release of silicon ions from it. We observed greater Si ions release after longer incubation times (i.e. 48 and 72 h) compared to 24 h incubation point, which indicates network depolymerisation and Si-OH groups formation.

The decrease of phosphorus content in a culture medium upon incubation with composites could be explained by the lack of phosphorus or its small amounts in the composites. Thus, the major source of phosphorus needed for the surface formation/crystallization of calcium
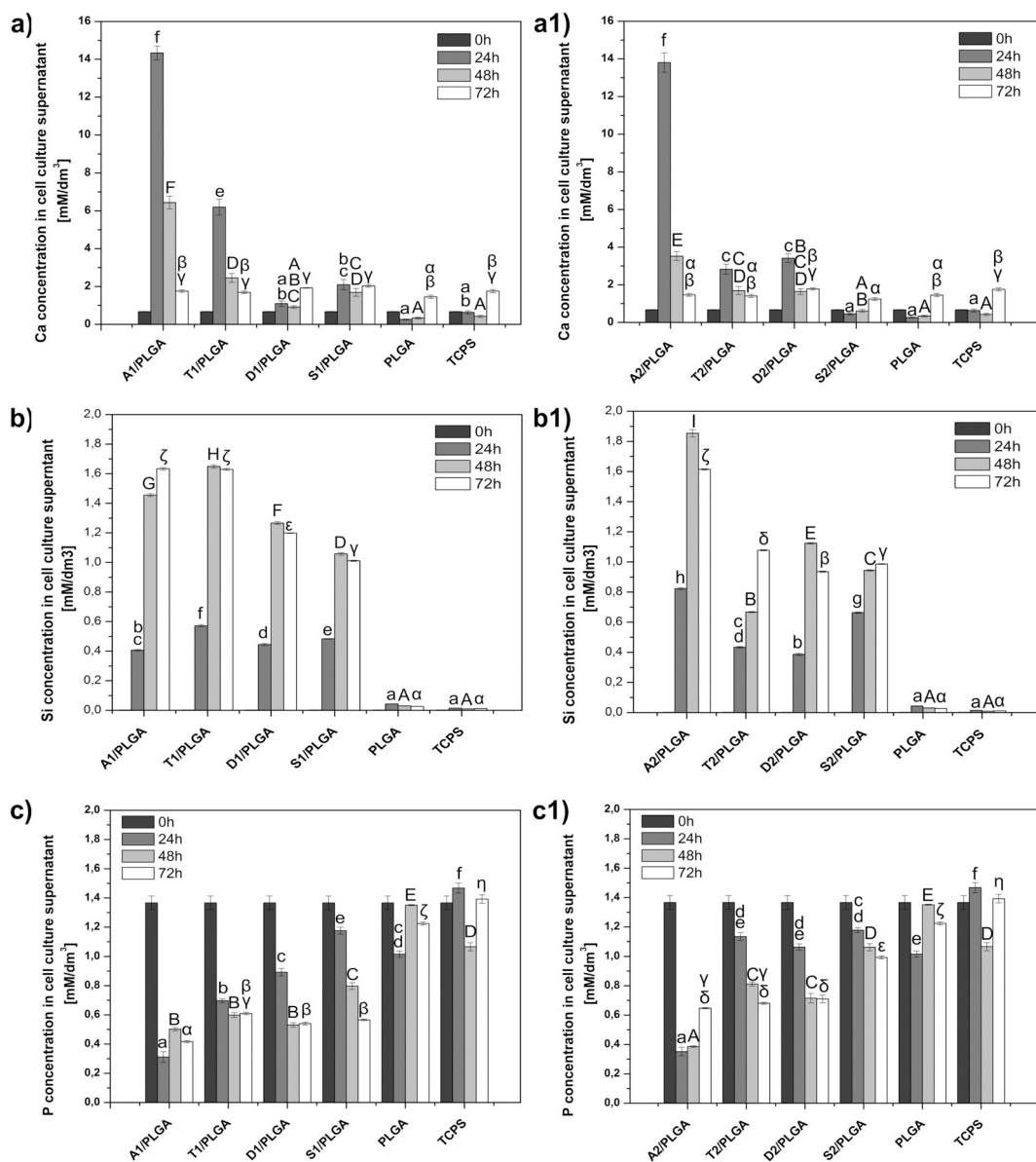
**Fig. 6.** Ca (a, a1) Si (b, b1), and P (c, c1) concentration in the medium collected from hBMSC cells cultured on PLGA and SBG/PLGA composites up to 72 h. Day 0 stands for the ion composition of culture medium before cell seeding on the material surface. Statistically significant differences (p < 0.05) between each SBG/PLGA composite and PLGA after different culture periods are indicated by upper and lower case, as well as Greek lettering, respectively.

phosphates would be provided by the culture medium and enhanced by the presence of cells. The phosphate ions would accumulate with calcium ions over the surface of bioactive materials and consequently surface crystallization of calcium phosphates would occur.

The emerging and unanticipated previously problem is the explanation for the lack of an obvious correlation between the ion release profile and the chemical composition of SBGs used by us as modifiers of PLGA. We found that the release of calcium from the composites obtained with three-component SBGs (i.e. $SiO_2$–CaO–$P_2O_5$) was lower than that for the two-component (i.e. $SiO_2$–CaO) ones. This may be due to the formation of discrete structures enriched in calcium and phosphorous in three-component glasses that are binding calcium and impeding its release. The presence of such structures was indicated in our earlier research [14]. We suggest that the above is a consequence of the complexity of both structure and microstructure of presently studied composites and particularly, the varied crystallinity of SBG/PLGA composites.

### 3.4. Studies of osteoinductivity using cell cultures

Cell culture studies revealed elevated Runx-2 mRNA expression for human BMSC cultured for 48 h on the group I composites (Fig. 7a). The highest Runx-2 levels were observed for A1/PLGA and D1/PLGA sheets, but the ion release profile for these materials differed. Whereas A1/PLGA released high levels of Ca and Si to the culture medium, D1/PLGA showed low Ca and moderate Si releases (Fig. 5a, b). Thus, the Runx-2 expression profile could not be explained simply by ion release profile from the materials and suggested the cell-material surface interactions play an important role in cell response to studied materials. Noteworthy, none of group II SBG/PLGA films elevated Runx-2 mRNA in hBMSC, despite all these surfaces showed elevated Si release (Fig. 5b). A2/, T2/ and D2/PLGA also showed increased Ca release compared to control PLGA (Fig. 5a). Thus, the presence of $P_2O_5$ in the composites could be an important factor in regulating Runx-2 transcription in BMSC. In contrast, the examination of Osterix mRNA levels (Fig. 7b) revealed elevated expression of this transcription factor in hBMSC
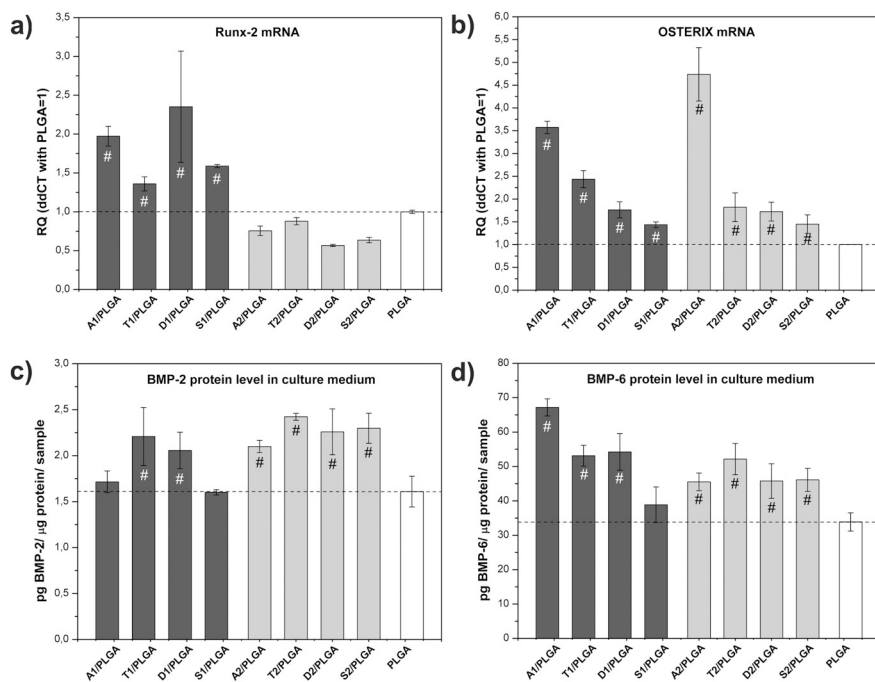
**Fig. 7.** Expression of osteogenic transcription factors and osteogenic BMPs in hBMSC cultured on composite surfaces vs. control PLGA; Runx-2 **(a)** and Osterix **(b)** mRNA levels after 48-h culture; BMP-2 **(c)** and BMP-6 **(d)** protein levels in culture medium 72-h post cell seeding on the material surfaces. The respective levels of mRNA or protein for cells cultured on PLGA are marked with a dashed line. Significantly higher expressions vs. PLGA are marked with #.

cultures on either SBG/PLGA groups with the highest Osx mRNA expression for A1/PLGA and A2/PLGA. In both material groups we could observe decreased Osx expression with decreased Ca release to culture medium. Low Runx-2 expression on SBG/PLGA films of group II and elevated Osx mRNA levels in hBMSC cultured on these materials may either suggest faster progression of osteogenesis for these surfaces or osteogenic differentiation that does not involve Runx-2 [39].

Given those biological examinations of hBMSC cultures were performed in standard growth medium without any osteogenic supplements, the elevation of Runx-2 and Osx transcription factors suggested the studied films may prime osteogenesis. We hypothesized that might be partly due to endogenous BMP production and BMP pathway autoregulation. Thus, we examined BMP-2 and BMP-6 protein levels after 72-hour contact with the composite surfaces (Fig. 7c, d). BMP-2 was elevated for all films from group II and also for T1/ and D1/PLGA from group I. BMP-6 levels were generally higher than BMP-2 and they were elevated for all materials except for S1/PLGA. Interestingly, the levels of BMP-2 and BMP-6 decreased with decreased CaO content in SBG/PLGA films of group I, but they remained comparable for SBG/PLGA films of group II. Thus, SBG/PLGA films that are supplemented with $P_2O_5$ may be better promoters of hBMSC to secrete BMPs compared to SBG/PLGA films of the group I. In the latter group BMPs secretion seemed to depend more on the SBG composition.

To determine if detected levels of BMPs are sufficient to initiate intracellular signalling we examined activation of canonical Smad and non-canonical Tak1 signalling pathways (Fig. 8a–d). We observed increased activation of BMP-specific Smads in hBMSC cultured on all SBG/PLGA films of group II which was in agreement with increased BMP levels for all materials from this group. In the material group I phospho-Smad1 was significantly elevated only for S1/PLGA, despite BMP level for cells cultured on S1/PLGA was comparable to control PLGA. It is thus plausible that some other than studied here BMPs contribute to activation of the Smad pathway in hBMSC cultured on S1/PLGA films. Notably, all studied composite surfaces except for D1/ and S2/PLGA activated Tak1 kinease. For the group I materials, we have noticed a trend of increased Smad and decreased Tak1 activation with increased $SiO_2$ content. This trend for phospho-Tak1 was even more evident for group II materials. Altogether, these data suggest that surfaces of group II composites are more efficient in activating BMP-dependent signalling pathways, whereas group I composites induce

mostly non-canonical Tak1. Moreover, the Tak1 activation may depend on silica content in the growth surface.

Finally, to distinguish between the biological effects of different ionic compositions of culture media collected from the materials and direct cell-material interactions we performed analyses in both hBMSC and BREITER mouse osteoblastic cell line. As shown in Fig. 9a condition media collected from the materials did not activate ALP of hBMSC despite significant differences in the ion release profile to the culture medium (Fig. 5). However, condition media from the group I composites were sufficient to induce calcium-phosphate deposits in 21-day cultures (Fig. 9e). In group II materials increased mineral vs. PLGA was observed only for T2/ and S2/PLGA. These data may suggest better osteogenic progression of cells on the group I composites. Importantly, when hBMSC were grown directly on material surfaces, marked activation of ALP activity could be observed for A1/PLGA and D2/PLGA surfaces (Fig. 9b) suggesting that the cell-material interactions are necessary to stimulate this enzyme activity, whereas ions released from the materials are insufficient (Fig. 9a). To explore further the possibility of different cell response upon cell treatment by materials condition media or direct culture on material surfaces, we analyzed the response of BRITER cells to exogenous rhBMP-2. As shown in Fig. 9c, condition media collected from A1/ and D1/PLGA markedly enhanced BMP response. In contrast, when cells were cultured directly on material surfaces, the BMP response was enhanced by S1/, S2/, D2/PLGA surfaces. These data, together with ALP results, indicate that materials bioactivity, understood here as ion exchange between material surfaces and the surrounding fluids, may influence osteogenic processes in vitro, but the biological effects may be distinctly different if cells come into direct contact with material surface.

## 4. Discussion

In this work we investigated bioactivity and osteoinductivity of PLGA-based composites enriched with sol-gel derived bioactive glasses of $SiO_2$–CaO system with and without $P_2O_5$. Although the evaluation of in vitro materials bioactivity in simulated body fluid (SBF) can offer some insights for calcium-phosphate precipitation and eventually bone-like hydroxyapatite formation, we extended the bioactivity studies to examine ion release profiles in either culture medium alone or in the presence of cells. SBF tests showed that all studied composite surfaces
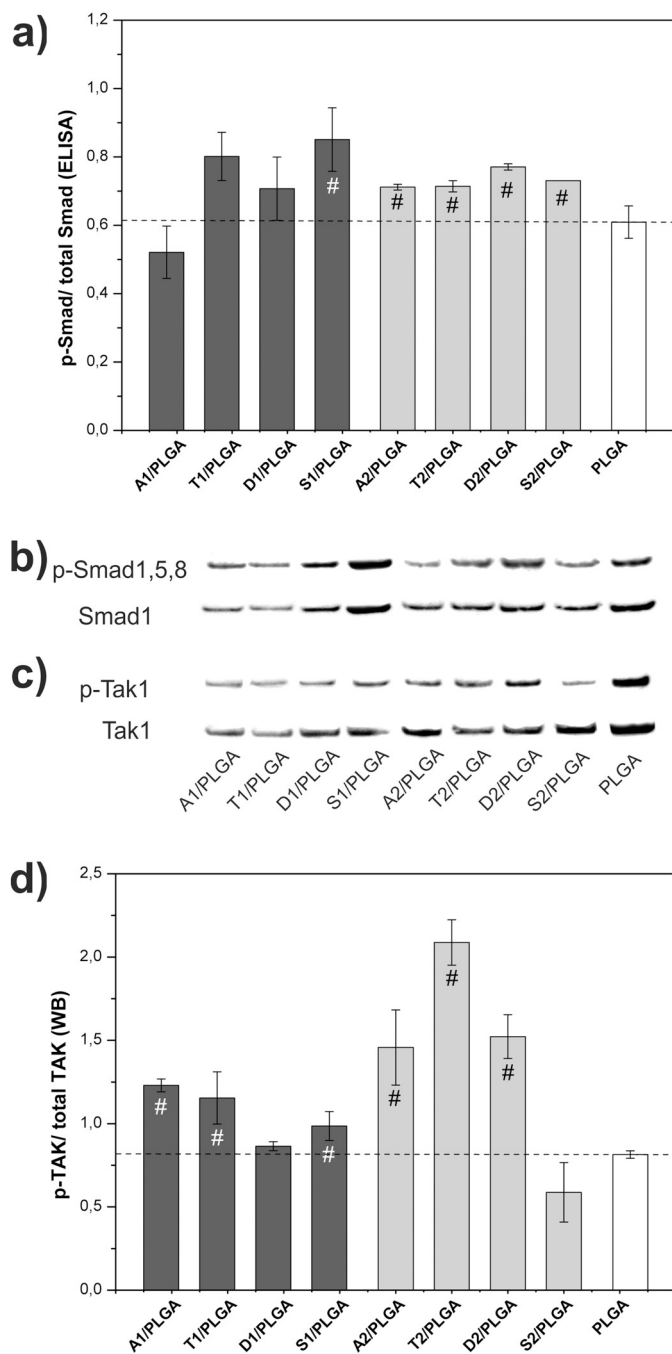
**Fig. 8.** Activation of canonical (Smad-dependent) and non-canonical (Tak1) BMP signalling in whole-cell extracts after 72-h hBMSC culture on the material surfaces vs. PLGA. Activation of Smads was analysed by Elisa (a) and Western blot (b) whereas Tak1 was analysed by Western blot (c) followed by densitometric analyses (d). Significantly higher expressions vs. PLGA are marked with #.

materials with binary $SiO_2$-CaO glasses. Since there is no phosphorus oxide in the binary system glasses composition, all the phosphorus needed to form the calcium phosphate layer derives only from the incubation medium (i.e. SBF solution). Thus, due to the lower initial P content at the material surface, composites with binary glasses exhibited higher Ca/P ratios beginning at 3 days of incubation (data not published). This was also confirmed by the results of ICP measurements, which indicated much faster chemical adsorption of phosphorus ions from SBF for the materials containing binary system glasses.

Differences in surface morphology and Ca/P ratio observed in SEM/EDS analyses indicate that differences in the kinetics of HAp layer formation depend on the chemical composition of glasses. Notably, after 14 days of incubation in SBF further increases of Ca/P ratios above stoichiometric value for hydroxyapatite (1.67) could be associated with the formation of a carbonated HAp.

Further examination of surfaces by FTIR spectroscopy showed similar changes for all studied surfaces with the only noticeable delay in a calcium-phosphate deposition for high-silica S2 composites. Although the positive role of silica and phosphate-enriched materials have been well documented in the literature [40–43], we report for the first time some discrete changes in material surface activity depending on the presence/absence of $P_2O_5$ and the $CaO/SiO_2$ ratio. Furthermore, our results suggest that the presence of $P_2O_5$ in SBG may slow-down surface activity of SBG/PLGA composites, probably due to the differences in molecular structure (i.e. formation of the phosphorus-rich clusters) [14,44,45].
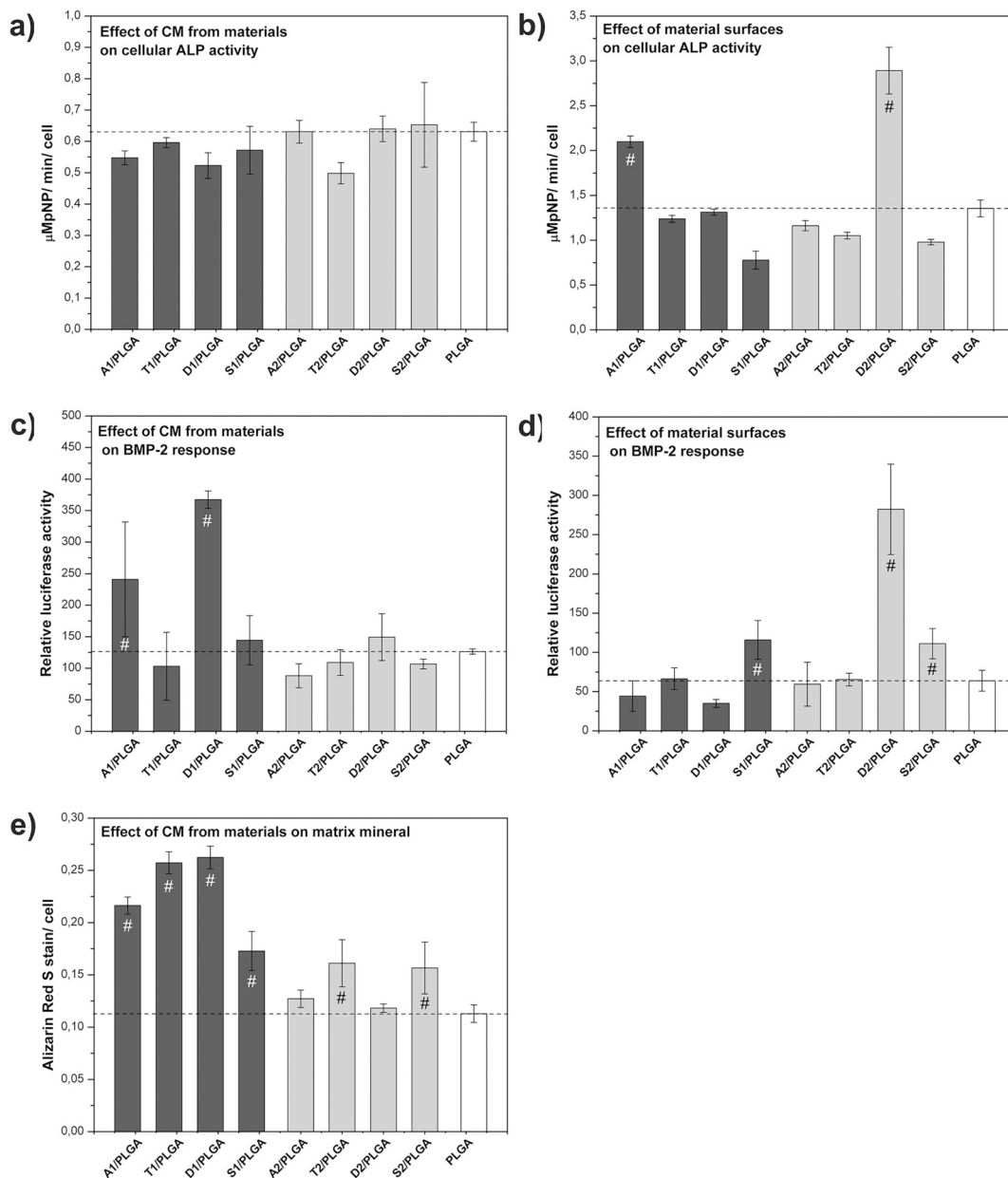
Examination of composite materials in culture medium alone or in the presence of cells indicated their different surface reactivity depending on the presence/absence of cells. In general, the calcium content in culture medium was corresponding to the calcium levels in SBGs of composites and the group I composites released more calcium than group II ones. Notably, the release of calcium to culture medium from composites containing high-calcium SBGs (i.e. A2 and A2) was higher in the presence of cells than without, which was not observed for any other studied SBG compositions. Thus, the 50-60 mol% CaO content in the SBG that is subsequently used as a modifier of PLGA matrix may be a threshold value to induce specific cell activity toward the material surface, leading to higher calcium release from such material and/or faster surface degradation. Given that the cells used in this study were of bone marrow origin; they were selected only for mononuclear fraction and used at a relatively low passage, it is possible that the cultures still contained some osteoclasts or osteoclast progenitors capable to actively resorb the material surface [46]. We have not found any obvious correlation between SBG chemical composition and silica or phosphate content in the culture medium.

Though, composites with high-silica SBG released lower amounts of Si to the culture medium with and without cells and this suggests that high silica content in the bioactive glasses may block its surface reactivity, by maintaining their high structural stability within the silicate network and thus consequently block the reactivity of such SBG/PLGA composites [47].

Moreover, a direct culture of cells on composite surface contributes to the above, as generally lower levels of Si were found in the culture medium upon hBMSC cultures. Similarly, culturing cells directly on composite surfaces contributed to the reduction of phosphate in the culture medium, which was less apparent in medium alone. Nevertheless, in both cases (i.e. with and without cells) phosphate was most utilized by composites containing calcium-enriched bioactive glasses and its uptake was reduced with the reduced calcium content.

Increased phosphorus adsorption is directly related to the high level of Ca released from the materials into the medium. The process leads to faster supersaturation in medium and thus it probably accelerates the formation of the calcium phosphate layer [47].

Finally, our studies provided new insights into the role of ions released from the bioactive surface and the surface itself in the early biological response. Since the development of Bioglass by Hench et al

were bioactive, although SEM/EDS analysis indicated that group I composites (i.e. $SiO_2$–CaO/PLGA) seemed to display faster surface changes as well as indicate formation of larger and more morphologically developed cauliflower-like forms on their surface than group II composites (i.e. $SiO_2$–CaO–$P_2O_5$/PLGA) with smoother and less morphologically developed cauliflower-like forms.

The calculated (i.e. from EDS data) Ca/P ratios revealed that a higher $CaO/SiO_2$ initial ratio in glass results in higher Ca/P ratio in the layer formed on the materials surface in SBF at particular incubation periods. Moreover, the formation of stoichiometric HAp occurs faster on

**Fig. 9.** Biological effects of condition media (CMs) collected from the materials vs. direct culture on the material surfaces. Alkaline phosphatase (ALP) activity of hBMSC after 7-d culture in the presence of CM (a) or upon direct culture on material surfaces (b), respectively; BMP-2 response of mouse osteoblasts upon stimulation with CM (c) or direct culture on the material surfaces (d); Matrix mineralization levels (Alizarin Red S staining) after 21-d culture of hBMSC in the presence of CM (e). The respective levels of ALP activity, mineral and luciferase activity for cells cultured on PLGA are marked with a dashed line. Significantly higher expressions vs. PLGA are marked with #. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

[48] the ions released from bioactive surface are believed to play primary role in attracting mesenchymal stem cells and osteoblasts to the material surface and inducing or enhancing their osteogenic differentiation, respectively. Recent studies provide several examples of bioactive ion contribution to the above-mention biological processes [49–52]. First and foremost, we verified osteoinductive properties of the SBG/PLGA composites by studying osteogenic cell response in standard growth medium without addition of any other osteogenic inducers. Such approach, although not new, is still quite rarely applied in the studies of bioactive components and the recent examples include both bioactive glasses [53] and calcium-phosphate substrates [54]. Our studies showed that culturing hBMSC on SBG/PLGA composites induces Runx-2 and Osterix expression in the cells and prompts them to release BMP-2 and -6 to culture medium. We hypothesized the osteoinductive effects of SBG/PLGA composites may be partly due to their induction of

BMP release and BMP-related signalling and indeed practically all studied composite surfaces induced BMP-related non-canonical Tak1 signalling, whereas induction of canonical Smad signalling was attributed mostly to group II composited containing three-component SBG. To our knowledge, very few reports looked so far into the cellular activation of BMP-related signalling pathways on contact with bioactive glasses [55,56]. We believe the results obtained in this work may prompt further research regarding BMP pathways activation by bioactive glasses of different chemical composition as well as prepared by different techniques.

The examinations of ALP activity in cultures of hBMSC and BMP-response in cultures of mouse BRITE cells showed marked differences in cell behaviour depending whether the cells were treated with culture medium conditioned with composite materials or they were directly seeded on the material surfaces. Although the ions released from some

studied surfaces were sufficient to induce matrix mineralization in human MSC, they were ineffective to activate ALP in hBMSC. In normal osteoblast cell culture ALP activity is required for matrix mineralization as the enzyme activity leads to increased local concentration of inorganic phosphate [57]. However, it is plausible that the exchange of ions between the bioactive surface and surrounding tissues may be sufficient to induce matrix calcification without the need for elevated ALP activity. On the other hand, elevated ALP activity is a hallmark of preosteoblasts and our results suggest that direct cell-material contact is required for bone marrow stromal cells to differentiate into osteoblasts the surface of the material, direct culture of BRITE cells on the composite surfaces showed different results for BMP-dependent luciferase activity that this for BRITE cells stimulated with condition media alone. Altogether, the results of this study point toward the need to carefully study the biological response to both the bioactive material dissolution products and upon direct cell-material contacts. They also indicate the material surface activity may markedly change upon cell culture and thus the studies of biomaterial surface or scaffolds in physiological solutions without the presence of appropriate cell population may not be sufficient to predict well their biological properties.

## 5. Conclusions

Our results indicate bioactivity of all studied composite surfaces and the possibility to modulate it by the addition/withdrawal of $P_2O_5$ to glass composition or changing $CaO/SiO_2$ ratio in a given SBG. $SiO_2$–CaO/PLGA composites seem to display faster surface changes than $SiO_2$–CaO–$P_2O_5$/PLGA, as revealed by SEM/EDS as well as calculated Ca/P ratio, whereas FTIR spectroscopy showed similar surface changes for all studied surfaces except for the composites containing high-silica S2 glass. Since the latter composites showed some delay in calcium-phosphate deposition, we postulate that the presence of $P_2O_5$ in SBG and low $CaO/SiO_2$ ratio may slow-down surface activity of SBG/PLGA composites. Furthermore, our results indicate that ion release profile from bioactive composites to culture medium varies depending on the presence or absence of cells. Upon hBMSC cultures, generally lower levels of Si and P were found in the culture medium. Surprisingly, we have not found any obvious correlation between the ion release profile from the composites to culture medium and the $CaO/SiO_2$ ratio of SBGs used as composite modifiers. Evaluation of SBG/PLGA composites as growth surfaces for hBMSC showed their osteoinductive properties, although there were some key differences between $SiO_2$–CaO/PLGA and $SiO_2$–CaO–$P_2O_5$/PLGA composites. Namely, Runx-2 remained elevated in $SiO_2$–CaO/PLGA composites but it was low in $SiO_2$–CaO–$P_2O_5$/PLGA ones. Also, some of $SiO_2$–CaO/PLGA composites did not elevate BMP-2 and -6 proteins in hBMSC cultures, opposite to high levels of BMP-2 and BMP-6 present in all cultures on $SiO_2$–CaO–$P_2O_5$/PLGA composites. Furthermore, practically all composites induced BMP-related non-canonical Tak1 signalling whereas canonical Smad signalling was restricted mostly to composites containing three-component SBGs (i.e. $SiO_2$–CaO–$P_2O_5$). The latter indicates that composite surface-induced BMP canonical pathway may depend on the composites chemistry and/or the presence of $P_2O_5$ in the SBG compositions. Considering that Osx levels were high in both composite groups, the above also suggest that the osteogenic progression of cells on $SiO_2$–CaO/PLGA composites may be delayed compared to $SiO_2$–CaO–$P_2O_5$/PLGA composites. Nevertheless, it is of significance that elevated expression of osteogenic transcription factors and activation of BMP-signaling pathways was achieved despite the absence of any additional exogenous osteogenic growth factors in culture medium. Finally, ALP activity of hBMSC and BMP-related luciferase activity of mouse BRITE cells differed depending weather the cells were stimulated only with condition medium from the composites or they were directly cultured on the composite surfaces. Although the ions released from some studied composites were sufficient to induce matrix mineralization in hBMSC, they were ineffective to activate ALP in hBMSC and direct cell-material

contact was required for the latter. Thus, although the dissolution products from bioactive materials play indisputable role in biological responses, one must consider that the overall biological properties of bioactive materials may change once they come into direct contact with cells. Altogether, beyond bioactivity and osteoinductivity of SBG/PLGA composites, our studies indicate that the examinations of materials bioactivity require the presence of cells, otherwise we may not predict well their biological properties. They also show key differences in the biological response to both the bioactive material dissolution products and upon direct cell-material contacts. Considering tissue engineering approaches and potential clinical applications of studied here and similar bioactive composites, we imply that the desired clinical outcomes may vary depending whether the bioactive material is implanted as it is or pre-seeded with cells.

## CRediT authorship contribution statement

**Krzysztof Łukowicz:**Resources, Methodology, Formal analysis, Investigation, Data curation, Visualization, Writing - original draft, Writing - review & editing.**Barbara Zagrajczuk:**Methodology, Formal analysis, Investigation, Data curation, Visualization, Writing - original draft, Writing - review & editing, Funding acquisition.**Aleksandra Nowak:**Methodology, Investigation.**Łukasz Nied**żwiedzki:Resources.**Maria Laczka:**Conceptualization, Writing - original draft, Writing - review & editing, Funding acquisition.**Katarzyna Cholewa-Kowalska:**Conceptualization, Resources, Supervision, Writing - original draft, Writing - review & editing, Funding acquisition.**Anna Maria Osyczka:**Conceptualization, Resources, Supervision, Writing - original draft, Writing - review & editing, Funding acquisition.

## Declaration of competing interest

All authors listed have contributed sufficiently to the project to be included as authors, and all those who are qualified to be authors are listed in the author byline. To the best of our knowledge, no conflict of interest, financial or other, exists.

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## References

[1] J.R. Jones, Review of bioactive glass: from Hench to hybrids, Acta Biomater. 9 (2013) 4457–4486, https://doi.org/10.1016/J.ACTBIO.2012.08.023.

[2] M.N. Rahaman, W. Xiao, W. Huang, Review - bioactive glass implants for potential application in structural bone repair, Biomed. Glas. 3 (2017) 56–66, https://doi.org/10.1515/bglass-2017-0005.

[3] M. Erol, W.J. Stark, D. Mohn, Z. Hong, Polymer/bioactive glass nanocomposites for biomedical applications: a review, Compos. Sci. Technol. 70 (2010) 1764–1776, https://doi.org/10.1016/J.COMPSCITECH.2010.06.002.

[4] A.R. Boccaccini, J.J. Blaker, Bioactive composite materials for tissue engineering scaffolds, Expert Rev. Med. Devices. 2 (2005) 303–317, https://doi.org/10.1586/17434440.2.3.303.

[5] M. Łączka, K. Cholewa-Kowalska, A.M. Osyczka, Bioactivity and osteoinductivity of glasses and glassceramics and their material determinants, Ceram. Int. 42 (2016) 14313–14325, https://doi.org/10.1016/j.ceramint.2016.07.007.

[6] F. Baino, S. Fiorilli, C. Vitale-Brovarone, Composite biomaterials based on sol-gel mesoporous silicate glasses: a review, Bioeng. (Basel, Switzerland). 4 (2017), https://doi.org/10.3390/bioengineering4010015.

[7] F. Viti, M. Landini, A. Mezzelani, L. Petecchia, L. Milanesi, S. Scaglione, Osteogenic differentiation of MSC through calcium signaling activation: transcriptomics and functional analysis, PLoS One 11 (2016) e0148173, , https://doi.org/10.1371/journal.pone.0148173.

[8] N. Sooraj Hussain, M.A. Lopes, J.D. Santos, A comparative study of CaO–P2O5–SiO2 gels prepared by a sol–gel method, Mater. Chem. Phys. 88 (2004) 5–8, https://doi.org/10.1016/J.MATCHEMPHYS.2004.06.015.

[9] F. Baino, E. Fiume, M. Miola, E. Verné, Bioactive sol-gel glasses: processing, properties, and applications, Int. J. Appl. Ceram. Technol. 15 (2018) 841–860, https://doi.org/10.1111/ijac.12873.

[10] Z. Tang, X. Li, Y. Tan, H. Fan, X. Zhang, The material and biological characteristics of osteoinductive calcium phosphate ceramics, Regen. Biomater. 5 (2018) 43–59, https://doi.org/10.1093/rb/rbx024.

[11] A. Hoppe, N.S. Güldal, A.R. Boccaccini, A review of the biological response to ionic dissolution products from bioactive glasses and glass-ceramics, Biomaterials 32 (2011) 2757–2774, https://doi.org/10.1016/j.biomaterials.2011.01.004.

[12] N. Saffarian Tousi, M.F. Velten, T.J. Bishop, K.K. Leong, N.S. Barkhordar, G.W. Marshall, P.M. Loomer, P.B. Aswath, V.G. Varanasi, Combinatorial effect of Si4+, Ca2+, and Mg2+ released from bioactive glasses on osteoblast osteocalcin expression and biomineralization, Mater. Sci. Eng. C 33 (2013) 2757–2765, https://doi.org/10.1016/j.msec.2013.02.044.

[13] P. Dobrzynski, J. Kasperczyk, H. Janeczek, M. Bero, Synthesis of biodegradable copolymers with the use of low toxic zirconium compounds. 1. copolymerization of glycolide with l-lactide initiated by Zr(Acac)4, Macromolecules 34 (2001) 5090–5098, https://doi.org/10.1021/MA0018143.

[14] B. Zagrajczuk, M. Dziadek, Z. Olejniczak, K. Cholewa-Kowalska, M. Laczka, Structural and chemical investigation of the gel-derived bioactive materials from the SiO2–CaO and SiO2–CaO–P2O5 systems, Ceram. Int. 43 (2017) 12742–12754, https://doi.org/10.1016/J.CERAMINT.2017.06.160.

[15] E. Pamula, J. Kokoszka, K. Cholewa-Kowalska, M. Laczka, L. Kantor, L. Niedzwiedzki, G.C. Reilly, J. Filipowska, W. Madej, M. Kolodziejczyk, G. Tylko, A.M. Osyczka, Degradation, bioactivity, and osteogenic potential of composites made of PLGA and two different sol–gel bioactive glasses, Ann. Biomed. Eng. 39 (2011) 2114–2129, https://doi.org/10.1007/s10439-011-0307-4.

[16] J. Filipowska, J. Pawlik, K. Cholewa-Kowalska, G. Tylko, E. Pamula, L. Niedzwiedzki, M. Szuta, M. Laczka, A.M. Osyczka, Incorporation of sol–gel bioactive glass into PLGA improves mechanical properties and bioactivity of composite scaffolds and results in their osteoinductive properties, Biomed. Mater. 9 (2014) 065001, , https://doi.org/10.1088/1748-6041/9/6/065001.

[17] J. Filipowska, G.C. Reilly, A.M. Osyczka, A single short session of media perfusion induces osteogenesis in hBMSCs cultured in porous scaffolds, dependent on cell differentiation stage, Biotechnol. Bioeng. 113 (2016) 1814–1824, https://doi.org/10.1002/bit.25937.

[18] C. Ohtsuki, H. Kushitani, T. Kokubo, S. Kotani, T. Yamamuro, Apatite formation on the surface of ceravital-type glass-ceramic in the body, J. Biomed. Mater. Res. 25 (1991) 1363–1370, https://doi.org/10.1002/jbm.820251105.

[19] P.S. Yadav, P. Prashar, A. Bandyopadhyay, BRITER: a BMP responsive osteoblast reporter cell line, PLoS One 7 (2012) e37134, , https://doi.org/10.1371/journal.pone.0037134.

[20] A.M. Osyczka, P.S. Leboy, Bone morphogenetic protein regulation of early osteoblast genes in human marrow stromal cells is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase signaling, Endocrinology 146 (2005) 3428–3437, https://doi.org/10.1210/en.2005-0303.

[21] T. Kokubo, H. Takadama, How useful is SBF in predicting in vivo bone bioactivity? Biomaterials 27 (2006) 2907–2915, https://doi.org/10.1016/J.BIOMATERIALS.2006.01.017.

[22] M. Catauro, F. Bollino, R.A. Renella, F. Papale, Sol–gel synthesis of SiO2–CaO–P2O5 glasses: influence of the heat treatment on their bioactivity and biocompatibility, Ceram. Int. 41 (2015) 12578–12588, https://doi.org/10.1016/J.CERAMINT.2015.06.075.

[23] J. Perez-Pariente, F. Balas, J. Roman, A.J. Salinas, M. Vallet-Regi, Influence of composition and surface characteristics on thein vitro bioactivity of SiO2–CaO–P2O5–MgO sol-gel glasses, J. Biomed. Mater. Res. 47 (1999) 170–175, https://doi.org/10.1002/(SICI)1097-4636(199911)47:2<170::AID-JBM6>3.0.CO;2-J.

[24] T. Peltola, M. Jokinen, H. Rahiala, E. Levanen, J.B. Rosenholm, I. Kangasniemi, A. Yli-Urpo, Calcium phosphate formation on porous sol-gel-derived SiO2 and CaO–P2O5–SiO2 substratesin vitro, J. Biomed. Mater. Res. 44 (1999) 12–21, https://doi.org/10.1002/(SICI)1097-4636(199901)44:1<12::AID-JBM2>3.0.CO;2-E.

[25] P. Saravanapavan, J.R. Jones, R.S. Pryce, L.L. Hench, Bioactivity of gel-glass powders in the CaO–SiO2 system: a comparison with ternary (CaO–P2O5–SiO2) and quaternary glasses (SiO2–CaO–P2O5–Na2O), J. Biomed. Mater. Res. 66A (2003) 110–119, https://doi.org/10.1002/jbm.a.10532.

[26] M. Vallet-Regi, I. Izquierdo-Barba, A.J. Salinas, Influence of P2O5 on crystallinity of apatite formedin vitro on surface of bioactive glasses, J. Biomed. Mater. Res. 46 (1999) 560–565, https://doi.org/10.1002/(SICI)1097-4636(19990915)46:4<560::AID-JBM14>3.0.CO;2-M.

[27] M. Vallet-Regí, A.M. Romero, C.V. Ragel, R.Z. LeGeros, XRD, SEM-EDS, and FTIR studies ofin vitro growth of an apatite-like layer on sol-gel glasses, J. Biomed. Mater. Res. 44 (1999) 416–421, https://doi.org/10.1002/(SICI)1097-4636(19990915)44:4<416::AID-JBM7>3.0.CO;2-S.

[28] I. Izquierdo-Barba, D. Arcos, Y. Sakamoto, O. Terasaki, A. López-Noriega, M. Vallet-Regí, High-performance mesoporous bioceramics mimicking bone mineralization, Chem. Mater. 20 (2008) 3191–3198, https://doi.org/10.1021/cm800172x.

[29] M. Dziadek, B. Zagrajczuk, E. Menaszek, K. Dziadek, K. Cholewa-Kowalska, Poly(ε-caprolactone)-based membranes with tunable physicochemical, bioactive and osteoinductive properties, J. Mater. Sci. 52 (2017) 12960–12980, https://doi.org/10.1007/s10853-017-1424-8.

[30] F. Castro, A. Ferreira, F. Rocha, A. Vicente, J. António Teixeira, Characterization of intermediate stages in the precipitation of hydroxyapatite at 37 °C, Chem. Eng. Sci. 77 (2012) 150–156, https://doi.org/10.1016/J.CES.2012.01.058.

[31] S.G. Caridade, E.G. Merino, N.M. Alves, V. de Z. Bermudez, A.R. Boccaccini, J.F. Mano, Chitosan membranes containing micro or nano-size bioactive glass particles: evolution of biomineralization followed by in situ dynamic mechanical analysis, J. Mech. Behav. Biomed. Mater. 20 (2013) 173–183, https://doi.org/10.1016/J.JMBBM.2012.11.012.

[32] X. Chen, X. Chen, D.S. Brauer, R.M. Wilson, R.V. Law, R.G. Hill, N. Karpukhina, Sodium is not essential for high bioactivity of glasses, Int. J. Appl. Glas. Sci. 8 (2017) 428–437, https://doi.org/10.1111/ijag.12323.

[33] G.A. Stanciu, I. Sandulescu, B. Savu, S.G. Stanciu, K. Paraskevopoulos, X. Chatzistavrou, E. Kontonasaki, P. Koidis, Investigation of growth on bioactive glass surface, J. Biomed. Pharm. Eng. 1 (2007) 34–39.

[34] I. Notingher, J.R. Jones, S. Verrier, I. Bisson, P. Embanga, P. Edwards, J.M. Polak, L.L. Hench, Application of FTIR and Raman spectroscopy to characterisation of bioactive materials and living cells, Spectroscopy 17 (2003) 275–288, https://doi.org/10.1155/2003/893584.

[35] M. Catauro, F. Barrino, G.D. Poggetto, F. Pacifico, S. Piccolella, S. Pacifico, Chlorogenic acid/PEG-based organic-inorganic hybrids: a versatile sol-gel synthesis route for new bioactive materials, Mater. Sci. Eng. C 100 (2019) 837–844, https://doi.org/10.1016/J.MSEC.2019.03.035.

[36] K. Niedzielski, R. Sindut, K. Cholewa-Kowalska, J. Kokoszka, M. Laczka, An in vivo study of the new generation of bioactive glass-ceramics as a bone substitute, Glas. Technol. Eur. J. Glas. Sci. Technol. Part A. 52 (2011) 63–66.

[37] J. Filipowska, K. Cholewa-Kowalska, J. Wieczorek, D. Semik, Z. Dąbrowski, M. Łączka, A.M. Osyczka, Ectopic bone formation by gel-derived bioactive glass-poly-L-lactide-co-glycolide composites in a rabbit muscle model, Biomed. Mater. 12 (2017) 015015, , https://doi.org/10.1088/1748-605X/aa4eb7.

[38] S. Kargozar, F. Baino, S. Hamzehlou, R.G. Hill, M. Mozafari, Bioactive glasses entering the mainstream, Drug Discov. Today 23 (2018) 1700–1704, https://doi.org/10.1016/j.drudis.2018.05.027.

[39] Y.H. Choi, J.-H. Choi, J.-W. Oh, K.-Y. Lee, Calmodulin-dependent kinase II regulates osteoblast differentiation through regulation of Osterix, Biochem. Biophys. Res. Commun. 432 (2013) 248–255, https://doi.org/10.1016/J.BBRC.2013.02.005.

[40] M. Bohner, Silicon-substituted calcium phosphates – a critical view, Biomaterials 30 (2009) 6403–6406, https://doi.org/10.1016/j.biomaterials.2009.08.007.

[41] S. Gomes, J.-M. Nedelec, E. Jallot, D. Sheptyakov, G. Renaudin, Silicon location in silicate-substituted calcium phosphate ceramics determined by neutron diffraction, Cryst. Growth Des. 11 (2011) 4017–4026, https://doi.org/10.1021/cg200587s.

[42] A.M. Pietak, J.W. Reid, M.J. Stott, M. Sayer, Silicon substitution in the calcium phosphate bioceramics, Biomaterials 28 (2007) 4023–4032, https://doi.org/10.1016/j.biomaterials.2007.05.003.

[43] A.I. Rodrigues, R.L. Reis, C.A. van Blitterswijk, I.B. Leonor, P. Habibović, Calcium phosphates and silicon: exploring methods of incorporation, Biomater. Res. 21 (2017) 6, https://doi.org/10.1186/s40824-017-0092-8.

[44] M. Dziadek, B. Zagrajczuk, P. Jelen, Z. Olejniczak, K. Cholewa-Kowalska, Structural variations of bioactive glasses obtained by different synthesis routes, Ceram. Int. 42 (2016) 14700–14709, https://doi.org/10.1016/J.CERAMINT.2016.06.095.

[45] M.D. O'Donnell, S.J. Watts, R.V. Law, R.G. Hill, Effect of P2O5 content in two series of soda lime phosphosilicate glasses on structure and properties – part II: physical properties, J. Non-Cryst. Solids 354 (2008) 3561–3566, https://doi.org/10.1016/J.JNONCRYSOL.2008.03.035.

[46] A. Konermann, M. Staubwasser, C. Dirk, L. Keilig, C. Bourauel, W. Götz, A. Jäger, C. Reichert, Bone substitute material composition and morphology differentially modulate calcium and phosphate release through osteoclast-like cells, Int. J. Oral Maxillofac. Surg. 43 (2014) 514–521, https://doi.org/10.1016/j.ijom.2013.10.017.

[47] M. Dziadek, B. Zagrajczuk, E. Menaszek, K. Cholewa-Kowalska, A new insight into in vitro behaviour of poly(ε-caprolactone)/bioactive glass composites in biologically related fluids, J. Mater. Sci. 53 (2018) 3939–3958, https://doi.org/10.1007/s10853-017-1839-2.

[48] L.L. Hench, The story of bioglass®, J. Mater. Sci. Mater. Med. 17 (2006) 967–978, https://doi.org/10.1007/s10856-006-0432-z.

[49] S. Ali Akbari Ghavimi, B.N. Allen, J.L. Stromsdorfer, J.S. Kramer, X. Li, B.D. Ulery, Calcium and phosphate ions as simple signaling molecules with versatile osteoinductivity, Biomed. Mater. 13 (2018) 055005, , https://doi.org/10.1088/1748-605X/aac7a5.

[50] M. Yamada, H. Egusa, Current bone substitutes for implant dentistry, J. Prosthodont. Res. 62 (2018) 152–161, https://doi.org/10.1016/j.jpor.2017.08.010.

[51] S. Ali Akbari Ghavimi, R.R. Tata, A.J. Greenwald, B.N. Allen, D.A. Grant, S.A. Grant, M.W. Lee, B.D. Ulery, Controlled ion release from novel polyester/ceramic composites enhances osteoinductivity, AAPS J. 19 (2017) 1029–1044, https://doi.org/10.1208/s12248-017-0072-x.

[52] M. Shah Mohammadi, F. Chicatun, C. Stähli, N. Muja, M.N. Bureau, S.N. Nazhat, Osteoblastic differentiation under controlled bioactive ion release by silica and titania doped sodium-free calcium phosphate-based glass, Colloids Surfaces B Biointerfaces 121 (2014) 82–91, https://doi.org/10.1016/j.colsurfb.2014.05.037.

[53] A.F. Brito, B. Antunes, F. dos Santos, H.R. Fernandes, J.M.F. Ferreira, Osteogenic capacity of alkali-free bioactive glasses. In vitro studies, J. Biomed. Mater. Res. Part B Appl. Biomater. 105 (2017) 2360–2365, https://doi.org/10.1002/jbm.b.33771.

[54] S. Mechiche Alami, H. Rammal, C. Boulagnon-Rombi, F. Velard, R. Drevet, D. Laurent Maquin, S.C. Gangloff, J. Hemmerlé, J.C. Voegel, G. Francius, P. Schaaf, F. Boulmedais, H. Kerdjoudj, Harnessing Wharton's jelly stem cell differentiation into bone-like nodule on calcium phosphate substrate without osteoinductive factors, Acta Biomater. 49 (2017) 575–589, https://doi.org/10.1016/J.ACTBIO.2016.11.042.

[55] J. Zhang, J. Guan, C. Zhang, H. Wang, W. Huang, S. Guo, X. Niu, Z. Xie, Y. Wang, Bioactive borate glass promotes the repair of radius segmental bone defects by enhancing the osteogenic differentiation of BMSCs, Biomed. Mater. 10 (2015) 065011, , https://doi.org/10.1088/1748-6041/10/6/065011.

[56] G.H. Kim, Y.D. Park, S.Y. Lee, A. El-Fiqi, J.J. Kim, E.J. Lee, H.W. Kim, E.-C. Kim, Odontogenic stimulation of human dental pulp cells with bioactive nanocomposite fiber, J. Biomater. Appl. 29 (2015) 854–866, https://doi.org/10.1177/0885328214546884.

[57] E.E. Golub, K. Boesze-Battaglia, The role of alkaline phosphatase in mineralization, Curr. Opin. Orthop. 18 (2007) 444–448, https://doi.org/10.1097/BCO.0b013e3282630851.