Bioglass/ carbonate apatite/ collagen composite scaffold dissolution products promote human osteoblast differentiation

Silvia A. Ferreira[1*], Gloria Young[2], Julian R. Jones[2], Sara Rankin[1]

[1]National Heart & Lung Institute, Imperial College London, London, UK
[2]Department of Materials, Imperial College London, London, UK

*To whom correspondence should be addressed
Correspondence and requests for materials should be addressed to **S.A.F** (s.ferreira@imperial.ac.uk)

Silvia A. Ferreira
National Heart & Lung Institute
Faculty of Medicine
Imperial College London
South Kensington Campus
London SW7 2AZ, UK
+44 (0)7423664272

S.A.F. ORCID: 0000-0001-7837-3040*,* **G.Y.** (gloria.young14@imperial.ac.uk, ORCID: 0000-0002-8045-5990), **J.R.J.** (julian.r.jones@imperial.ac.uk, ORCID: 0000-0002-2647-8024) **S.R.** (s.rankin@imperial.ac.uk, ORCID: 0000-0003-2194-4089)

Abstract

OssiMend® Bioactive (Collagen Matrix Inc., NJ) is a three-component porous composite bone graft device of 45S5 Bioglass/ carbonate apatite/ collagen. Our *in vitro* studies showed that conditioned media of the dissolution products of OssiMend Bioactive stimulated primary human osteoblasts to form mineralised bone-like nodules *in vitro* in one week, in basal culture media (no osteogenic supplements). Osteoblast differentiation was followed by gene expression analysis and a mineralization assay. In contrast, the dissolution products from commercial OssiMend (Bioglass-free carbonate apatite/ collagen scaffolds), or from 45S5 Bioglass particulate alone, did not induce the mineralisation of the extracellular matrix, but did induce osteoblast differentiation to mature osteoblasts, evidenced by the strong upregulation of *BGLAP* and *IBSP* mRNA levels. The calcium ions and soluble silicon species released from 45S5 Bioglass particles and additional phosphorus release from OssiMend mediated the osteostimulatory effects. Medium conditioned with OssiMend Bioactive dissolution had a much higher concentration of phosphorus and silicon than media conditioned with OssiMend and 45S5 Bioglass alone. While OssiMend and OssiMend Bioactive led to calcium precipitation in cell culture media, OssiMend Bioactive produced a higher concentration of soluble silicon than 45S5 Bioglass and higher dissolution of phosphorus than OssiMend. These *in vitro* results suggest that adding 45S5 Bioglass to OssiMend produces a synergistic osteostimulation effect on primary human osteoblasts.
In summary, dissolution products of a Bioglass/carbonate apatite/collagen composite scaffold (OssiMend® Bioactive) stimulate human osteoblast differentiation and mineralization of extracellular matrix *in vitro* without any osteogenic supplements. The mineralization was faster than for dissolution products of ordinary Bioglass.

Highlights

- The dissolution products of OssiMend Bioactive induce osteoblasts mineralization.
- The bioactive ions released from OssiMend or 45S5 Bioglass drive osteoblast maturation.
- OssiMend Bioactive combines Si release with P dissolution and Ca deposition.
- Adding 45S5 Bioglass to a collagen/hydroxyapatite foam produces a synergistic osteostimulation effect.

Keywords: Human osteoblasts, Osteogenic differentiation, Bioglass, Bioactive ions, Bone tissue engineering

1. Introduction

The original bioactive glass composition 45S5 (Bioglass), has been in clinical use in the form of a particulate since 1994, when the medical device Perioglas (NovaBone Products LLC, Jacksonville FL) was launched, consisting of particles in the range 90 - 710 μm [1]. Perioglas is a synthetic bone graft approved for use in dental and maxillofacial bone regeneration. It is generally applied by the clinician mixing the particulate with the patient's blood and filling a bone defect. *In vivo* studies comparing synthetic hydroxyapatite (sHA) to Bioglass particles of the same size, in a rabbit femoral chondyle model, found Bioglass to stimulate more bone in-growth and higher quality bone in-growth than sHA [2-4]. While *in vivo* studies can show enhanced bone growth, understanding the mechanisms underlying the observations require *in vitro* studies. For example, the reason for the improved bone regeneration of 45S5 Bioglass was attributed to the ions that are released during bioactive glass dissolution stimulating osteogenic cells [5], according to *in vitro* gene expression studies with primary human osteoblasts by Xynos *et al.* [6]. Importantly, the *in vitro* studies focused on the ions released from the glass, rather than cells in contact with the glass itself. The Bioglass particles were soaked in the cell culture media, for 24 h without cells, at which point the Bioglass particles were filtered out and the filtrate used for cell culture. Gene expression studies, including use of gene arrays, found that seven families of genes involved in osteogenesis were upregulated [6, 7] on exposure of the cells to the dissolution products (soluble silicon, phosphate and calcium ions). Those studies used osteoblasts harvested from excised femoral heads with average donor age of 64 ± 8 years.

Hoppe *et al.* has provided a comprehensive overview of bioactive glasses and glass-ceramics focusing on the bioactive ions-cell interactions, experimental evidence of human cell responses to ionic dissolution products, including their therapeutic effects *in vitro* [8]. Bioactive glasses can also be loaded with drugs and bioactive molecules to further improve tissue functional recovery upon injury [9], but this may unnecessarily increase the regulatory burden. Clinical use of bioactive glasses has also recently been reviewed [1, 10].

The orthopaedic product, NovaBone (NovaBone Products LLC, Jacksonville FL) followed in 2005, when approval of the Bioglass particulate was granted for orthopaedic use. NovaBone claimed the property of "Osteostimulation", which refers to the dissolution products of the Bioglass, provoking activation of the osteoblasts *in vitro*. Another device that releases Si on dissolution is Si-doped sHA, Actifuse (Baxter Healthcare, Elstree, UK). Porous Actifuse granules were compared to NovaBone particulate, in a rabbit model, wherein NovaBone remodelled more rapidly than Actifuse [11]. Several mouldable bone graft devices have also been launched, usually based on a collagen Type I matrix and a bioactive component of sHA and/or Bioglass, *e.g.* NovaBone's MacroForm™ or Vitoss BA (Stryker, NJ) [1].

Here, the aim was to evaluate the potential osteostimulation effect of a composite scaffold that contains 45S5 Bioglass particulate, using the protocols of Xynos *et al.* [6]. OssiMend (Collagen Matrix Inc., NJ) is a porous composite made of freeze-dried Type I collagen and bovine carbonate apatite. OssiMend Bioactive has the same components but also contains 45S5 Bioglass particles. The objective was to compare differentiation of primary human osteoblasts *in vitro*, in response to the dissolution products of OssiMend Bioactive against controls of 45S5 Bioglass particulate and OssiMend (Fig. 1 A). Our hypothesis was that the bioactive ions released from the new formulation, OssiMend Bioactive, would favour faster primary human osteoblasts differentiation and extracellular matrix mineralization. We used a combination of gene expression and imaging analysis to examine primary human osteoblast response without the addition of exogenous osteoinductive factors. Our observations demonstrate that primary human osteoblasts rapidly differentiate *in vitro* in response to OssiMend Bioactive ionic environment with increasing

deposition of calcium (Ca) and hydropxyapatite in bone-like nodules. However, bioactive ions from OssiMend and 45S5 Bioglass conditioned media only induce osteoblast maturation within the same period in culture. Our results suggest the synergistic effect of combining the soluble silicon (Si) release from 45S5 Bioglass with the phosphorus (P) dissolution and Ca deposition from OssiMend, in the OssiMend Bioactive formulation.

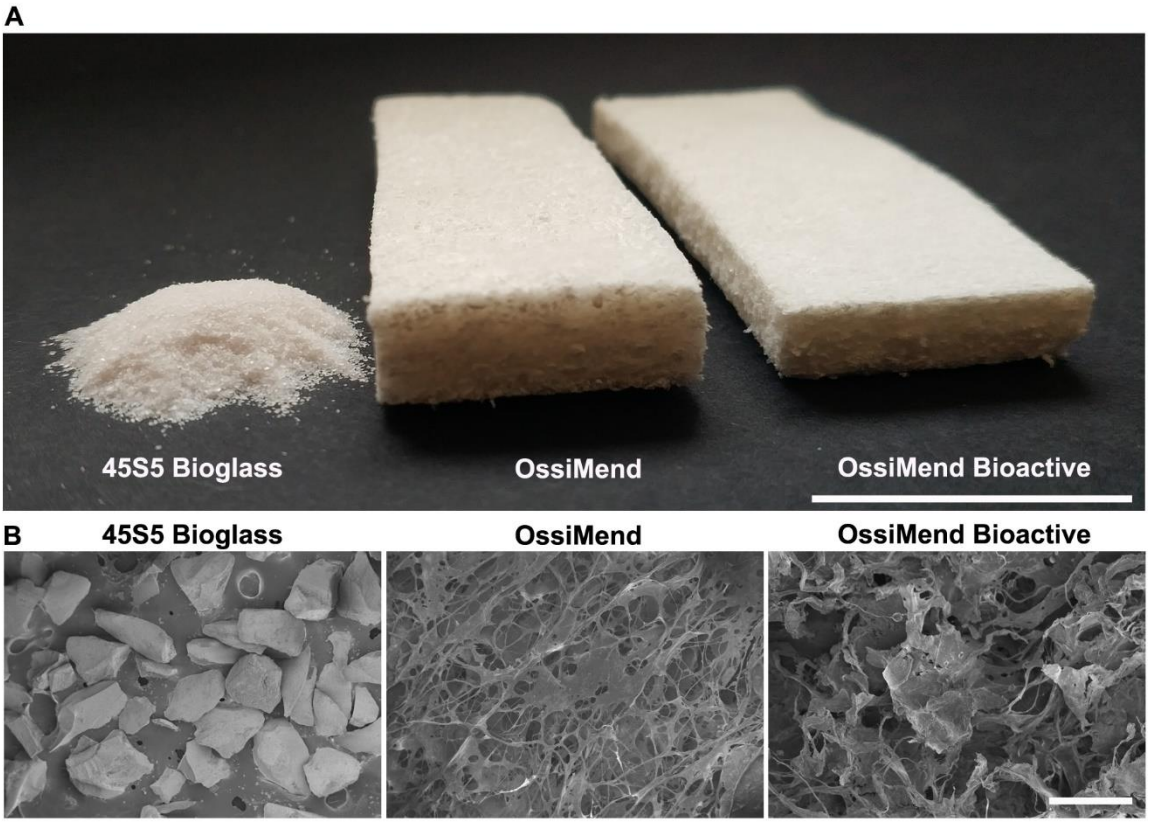

Figure 1: **45S5 Bioglass, OssiMend and OssiMend Bioactive** (A) pictures and (B) SEM micrographs. Scale bar in (A) is 2 cm and in (B) is 500 µm.

2. Materials and Methods

2.1. Materials

OssiMend Bioactive, a mouldable bone graft matrix strip (20 w/w% bovine collagen, 30 w/w% Bioglass 45S5, 50 w/w% carbonate apatite), OssiMend, a bone graft matrix strip (20 w/w% bovine collagen, 80 w/w% carbonate apatite) and 45S5 Bioglass particulate (45 wt% SiO_2, 24.5 wt% CaO, 24.5 wt% Na_2O, and 6 wt% P_2O_5) of the same particle size (approximately 100 – 300 μm) as used in the OssiMend Bioactive, were supplied from Collagen Matrix Inc. (NJ). The 45S5 Bioglass particle size distribution was confirmed via laser particle size analysis (Malvern Panalytical, UK).

2.2. Imaging

Scanning Electron Microscopy (SEM) images were taken using an Auriga dual-beam field emission gun SEM workstation (Zeiss, Germany) using an InLens detector at 2.5 kV with a working distance of 5 mm. Samples were coated with 25 nm chromium for imaging of the top surfaces of OssiMend, OssiMend Bioactive blocks and of dispersed 45S5 Bioglass particles.

2.3. Conditioned media

Conditioned medium containing the ionic dissolution products in Dulbecco's Modified Eagle Medium (DMEM, #D5671, Sigma) was prepared using 1% w/v 45S5 Bioglass (i.e. 0.8 g of 45S5 Bioglass in 80 mL of DMEM), as used by Xynos *et al.* [6]. To ensure the same amount of 45S5 Bioglass was immersed in the media for OssiMend Bioactive, 2.66 g of OssiMend Bioactive was immersed in 80 mL of DMEM. OssiMend control condition medium was prepared by adjusting medium volume to give the same w/v ratio of the strip as for OssiMend Bioactive (i.e. 2.66 g in 80 mL of medium). DMEM alone was used as negative control and each condition was tested in triplicate.

Samples were sealed and incubated for 6 h or 24 h at 37 °C without agitation in a 5% CO_2 atmosphere. Aliquots of the supernatant were collected during incubation (1 mL) for measuring elemental concentrations. The pH was measured, and the supernatant was filtered using a 0.2 µm non-pyrogenic sterile surfactant-free cellulose acetate filter (Corning). Conditioned media were stored at 4 °C.

2.4. Elemental analysis of the conditioned media

Concentrations of Ca, P, and Si in solution were measured with Thermo Scientific iCAP 6300 Duo inductively coupled plasma – optical emission spectrometer (ICP-OES) with auto sampler. Mixed standards of Ca (#19051, Sigma), P (#13862, Alfa Aesar), Si (#1.70365.0100, Merck) were prepared at 0, 1, 2, 5, 10, 20 and 40 µg mL^{-1} for the calibration curve. Samples - aliquots obtained during incubation, conditioned media with or without 10% (v/v) FBS (Gibco), 2 mM L-glutamine (Gibco), 1% (v/v) antibiotic antimycotic solution (Sigma) given to cells, and cell supernatant - were diluted by a factor of 10 with sterile deionised water. Calibration was carried out at the beginning of each sequence. P was measured in the axial direction of the plasma flame whereas Ca, and Si were measured in the radial direction. Each treatment was tested in triplicate and each sample was measured three times in ICP. ICP results were averaged over three repeat measurements of three replicates of each treatment. Results are shown as mean ± standard deviation.

2.5. Human osteoblasts culture and treatment with conditioned media

Primary human osteoblasts (NHOst, #CC-2538, LONZA) were routinely expanded in basal medium composed of DMEM with 10% (v/v) FBS, 2 mM L-glutamine, 1% (v/v) antibiotic antimycotic solution at 37 °C, in 95% air humidity and 5% CO_2 (using 0.05% Trypsin-EDTA, Thermo Fisher Scientific) and used prior to passage 5, at population doubling level (PDL) 5.61. Human osteoblasts were tested and devoid mycoplasma contamination before being used in each experiment.

Human osteoblasts were seeded 1 x 10^4 cells/cm^2 in 24-well tissue culture plates (TCP) or coverslips (Sarstedt). 24 h later, cells were treated with conditioned media with 10% (v/v) FBS, 2 mM L-glutamine, 1% (v/v) antibiotic antimycotic solution. Media were exchanged every other day. Cell supernatant was collected from treated cells (cell supernatant). Treated cells in culture were imaged with bright field microscopy over time using Real-Time Automated Cell Imaging System - JuLI™ Stage (Cambridge Bioscience).

2.6. Human osteoblast metabolic activity

Human osteoblast metabolic activity over time was evaluated by conducting an alamarBlue™ HS assay (Thermo Fisher Scientific), according to the manufacturer's instructions. At each time point (n = 9), alamarBlue reagent 10% (v/v) in basal culture medium replaced cell supernatant and was incubated for 2 h under standard culture conditions. The fluorescence was read at 535 ± 25 nm excitation and 595 ± 35 nm emission in a Spark Multimode Microplate Reader (Tecan) using black 96 micro well-plates with clear bottom (Corning). Results are shown as box plots expressing the median, first and third quartiles, and the highest and lowest values.

2.7. Quantitative polymerase chain reaction

After two and seven days in culture, cell lysates were collected using RLT buffer from the RNeasy Mini Kit, snap-frozen in liquid nitrogen, stored at -80 °C. RNA was isolated from cell lysates using QIAshredder, RNeasy Mini Kit and RNase-Free DNase Set, according to the manufacturer's instructions (all from Qiagen). RNA quality and concentration in each sample were calculated using Nanodrop ND-1000. RNA was reverse transcribed into cDNA in two steps: (1) 0.1 µg of RNA, 2 µL 500 µg mL^{-1} random primers, and 2 µL of RNase-free water was incubated at 70 °C for 5 min: (2) the reaction mixture was mixed with 1.25 µL of 1 mM PCR Nucleotide Mix, 5 µL of M-MLV reverse transcriptase 5x Buffer, 1 µL of 200 U $µL^{-1}$ M-MLV reverse transcriptase (all from Promega), and 3.75 µL RNase free water and incubated for 1 h at 42 °C. cDNA was stored at -20 °C. Real-time quantitative polymerase chain reaction (qPCR) was performed on an Applied Biosystems ViiA 7 real-time PCR (Thermo Fisher Scientific) detection system using 384-well PCR plates. Each primer pair (Supplementary Table 1) concentration was optimized to the maximum efficiency. Reaction mixtures were prepared with 0.5 µL of cDNA, 3 µL of primer mix (forward and reverse), 5 µL of Power SYBR™ Green PCR Master Mix (Thermo Fisher Scientific), and 1.5 µL of RNase-free water. A three-step cycle was employed: (1) AmpErase Uracil N-glycosylase inactivation at 50 °C for 2 min and denaturation at 95 °C for 10 min; (2) annealing/extension at 60 °C for 1 min; and (3) melting from 60 °C to 95 °C at 0.5 °C/step.

Samples ($n \geq 5$) were run in triplicate and water was used in place of cDNA as a control. The ΔΔCQ method was used to quantify fold changes in the expression for each gene of interest (GOI) and normalised to the expression of undifferentiated human osteoblasts (control treated with basal medium), using *Eukaryotic translation elongation factor 1 alpha 1* (*EEF1A1)*, *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), and *Ribosomal protein L13a* (*RPL13A*) as the reference genes (RG): fold change in expression = $2^{-\Delta\Delta Cq}$, $\Delta\Delta Cq = [\text{treatment}(Cq_{GOI,t(x)} - Cq_{RG,t(x)}) - \text{control}(Cq_{GOI,t(x)} - Cq_{RG,t(x)}]$. Fold change in expression normalized to expression in human osteoblasts cultured with basal medium (set to 1) for each time point is shown as column graphs with scatter dot plots showing mean ± standard deviation.

2.8. Mineralization assay

At day seven and day fourteen, human osteoblasts on coverslips were fixed in 4% w/v paraformaldehyde in PBS for 20 min. The mineralization was evaluated using the OsteoImage™ assay (#PA-1503, LONZA), according to the manufacturer's instructions. The fluorescent OsteoImage™ staining reagent specifically binds to the hydroxyapatite portion of the bone-like nodules deposited by cells. Nuclei were counterstained with 10 µg mL^{-1} Hoechst 33342, Trihydrochloride, Trihydrate (ThermoFisher Scientific) for 15 min. After washing, cells were coverslipped and imaged in Leica SP8 inverted confocal laser scanning microscope. Sequential acquisition and Kalman filter mode, 20× dry objective with numerical aperture of 0.75, and 1024 × 1024 pixel size. Detector gains were set to be constant between samples to facilitate comparison. Quantification of bone nodules area and integrated intensity of stained hydroxyapatite per bone-like nodule (> 10 $µm^2$) was evaluate after thresholding Otsu's method using Analysis Particles plugin in Image J. Results are shown as violin plots expressing the median, first and third quartiles, and the highest and lowest values.

2.9. Statistical analyses

Ion concentration profile plot is shown as mean ± standard deviation. Metabolic activity is shown as box plots expressing the median, first and third quartiles, and the highest and lowest values. Gene expression analysis is shown as column graphs with scatter dot plots showing mean ± standard deviation. Measurements of mineralization (after removal of outliers with ROUT test Q = 1%) are shown as violin plots expressing the median, first and third quartiles, and the highest and lowest values. Statistical analyses were carried out using a non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test for multiple comparisons, or Mann-Whitney test (two-tailed) for comparisons between two groups. Statistical analyses were carried out using GraphPad Prism

version 8 for Windows (GraphPad Software, USA). *p* values are indicated in figure captions or in Supplementary Tables.

3. Results

3.1. OssiMend Bioactive conditioned medium combines greater P and Si content and favours Ca deposition

OssiMend and OssiMend Bioactive porous structure was imaged using SEM (Fig. 1B). The 45S5 Bioglass particles were imaged using SEM (Fig. 1B) and their size distribution was confirmed via Laser particle size analysis ($d_{0.1}$ = 138 ± 6 μm, $d_{0.5}$ = 261 ± 4 μm, $d_{0.9}$ = 462 ± 5 μm, *n* = 4). Conditioned media were produced by ion exchange and dissolution of the devices in DMEM. Elemental analysis of the conditioned media by ICP showed the elemental changes in the DMEM over 24 h (Table 1). The DMEM conditioned with the 45S5 Bioglass for 6 h contained 81 ± 2 μg mL^{-1} Ca; 25 ± 1 μg mL^{-1} P, 21 ± 1 μg mL^{-1} Si; which was a similar Si concentration to the media used by Xynos *et al.* [6, 7, 12], although they used 24 h incubation. An incubation time of 6 h was therefore chosen to produce all conditioned media for the cell culture experiments.

Table 1. Elemental content (μg mL^{-1}) of conditioned media of Basal medium negative control, 45S5 Bioglass, OssiMend, and OssiMend Bioactive, measured by ICP-OES.

		Basal medium	45S5 Bioglass	OssiMend	OssiMend Bioactive
Calcium	**6 h**	70 ± 1	81 ± 2	27 ± 4	25 ± 4
	24 h	72 ± 1	70 ± 2	8 ± 2	8 ± 1
Phosphorus	**6 h**	33 ± 6	25 ± 1	190 ± 21	477 ± 25
	24 h	28 ± 1	19 ± 1	206 ± 73	475 ± 86
Silicon	**6 h**	0	21 ± 1	3 ± 1	28 ± 4
	24 h	0	32 ± 1	4 ± 1	54 ± 7

Figure 2 shows the elemental content of the conditioned media prepared with the test devices over 6 h incubation and then after filtration and storage at 4 °C. OssiMend Bioactive and OssiMend conditioned media showed a reduction in Ca ion concentration relative to the basal medium over the 6 h extraction period (to 27 ± 4 and 25 ± 4 μg mL^{-1}, respectively), indicating Ca precipitation, whereas the 45S5 Bioglass showed Ca release (81 ± 2 μg mL^{-1} at 6 h, Fig. 2A, Table 1, Supplementary Table 2). P was released from OssiMend Bioactive and OssiMend (477 ± 25 and 190 ± 21 μg mL^{-1}, respectively, Fig. 2B), which was significantly higher than for the 45S5 Bioglass conditioned medium (Supplementary Table 2). This was due to residual phosphate present in the OssiMend matrix due to the processing method. The release of Si was greater from OssiMend Bioactive than the 45S5 Bioglass (28 ± 4 compared to 21 ± 1 μg mL^{-1} at 6 h), perhaps due to the geometry of the block giving a greater area in contact with medium (Fig. 2C, Table 1). The Si content for the OssiMend group were at trace levels, as it did not contain Bioglass. All conditioned media showed similar pH to the basal medium control.
OssiMend Bioactive and OssiMend conditioned media differ significantly in the content of Si and both show significantly lower Ca and higher P levels than 45S5 Bioglass conditioned medium (Fig. 2, Table 1 and Supplementary Table 2). The elemental content in the extracts was stable when

kept at 4 °C (Fig. 2, Supplementary Table 3). This ensures that the conditioned media given to the cells over the subsequent 14 days were consistent at each time point.

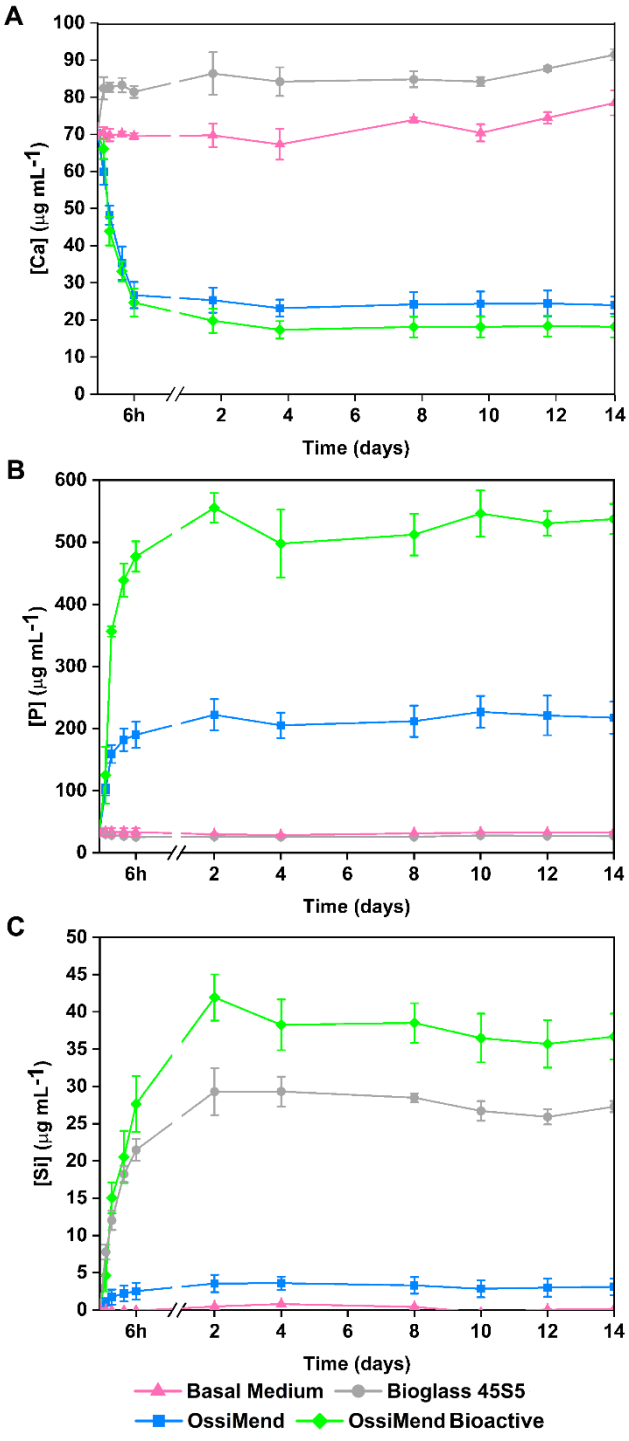

Figure 2: **OssiMend Bioactive, OssiMend and 45S5 Bioglass conditioned media differ in bioactive ions content**. Elemental content of the conditioned media prepared with basal medium control, 45S5 Bioglass, OssiMend, and OssiMend Bioactive, over 6 h incubation at 37 °C, filtered, and stored at 4 °C for 14 days, without addition of serum and antibiotics: (A) calcium, (B) phosphorus, and (C) silicon. Plots show mean values ± s.d, *n* = 3. A non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test was used to test both if each ion content was

different in conditioned media prepared after 6 h incubation at 37°C (Supplementary Table 2) and if each ion content was stable during storage over 14 days (Supplementary Table 3).

3.2. OssiMend Bioactive conditioned medium leads to reduced metabolic activity of human osteoblasts and mediates changes in cell morphology

We first evaluated if the elemental content of the conditioned media led to changes in cell behaviour. Osteoblast differentiation is marked with reduction in metabolic activity, changes in cell morphology and consequential apoptosis (programmed cell death) during development of bone-like tissue *in vitro* [13, 14]. Here, human osteoblasts treated with OssiMend Bioactive conditioned medium showed a significant decrease in metabolic activity after two days in culture (Fig. 3A), which could suggest either toxicity; or differentiation into a less metabolic active stage [15]; or apoptosis [13, 14, 16], which is typical of onset of mineralisation. The other cells treated with OssiMend or 45S5 Bioglass generally showed similar metabolic activity to those cultured in the basal medium control (Fig. 3A). Cell number also decreased when human osteoblasts were treated with OssiMend Bioactive conditioned medium; particularly on day eleven and by day fourteen very few cells were observed (Fig. 3B). To determine how cells are responding, the cell metabolic activity should not be considered in isolation but with other assays of the cellular response to the dissolution products.

Bone-like nodules appeared to start forming in the cultures treated with OssiMend Bioactive after three days in culture. In the other treatments, human osteoblasts proliferated slowly over time, similarly to basal medium control and cell morphology was spread and typical of osteoblasts seeded on tissue culture plates (Fig. 3B). These observations suggest OssiMend Bioactive conditioned medium stimulated osteogenenic differentiation *in vitro*.

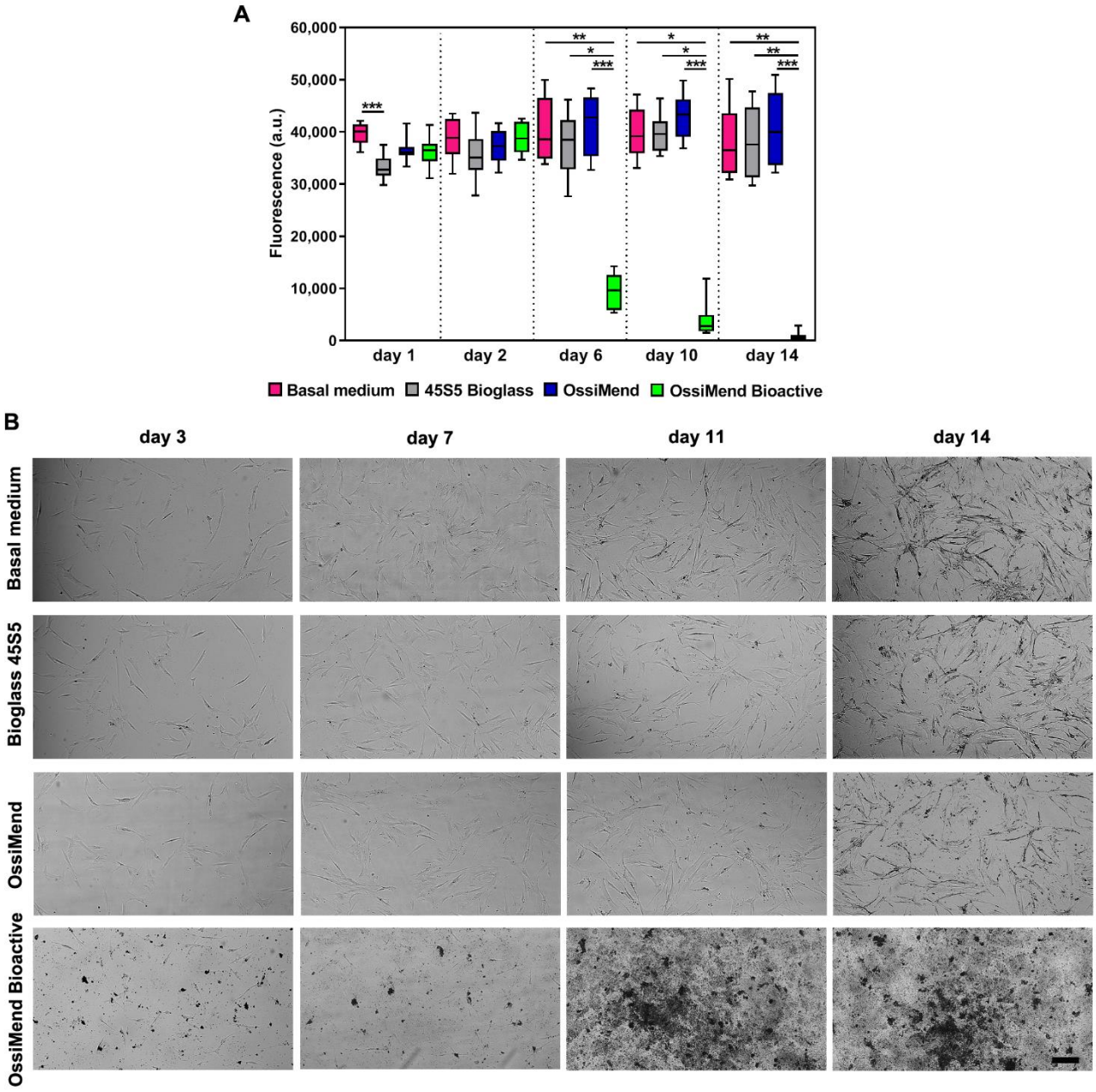

Figure 3: **Human osteoblasts show low metabolic activity and form bone-like nodules upon treatment with OssiMend Bioactive conditioned medium.** (A) Cell metabolic activity measured by alamarBlue™ HS assay and (B) Bright field micrographs of human osteoblasts (1 x 10^4 cells/cm^2 on TCP, $n \geq 9$) treated for 14 days with conditioned media prepared with basal medium control, 45S5 Bioglass, OssiMend, and OssiMend Bioactive. In (A) box plots show $1^{st}/3^{rd}$ quartiles, high/low values and median of cell metabolic activity. A Kruskal-Wallis and Dunn's Multiple Comparison Test was used to detect statistical significance, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Scale bar in (B) is 100 µm.

3.3. OssiMend Bioactive favours Ca and Si deposition and P release by human osteoblasts

The changes in human osteoblast behaviour prompted the measurement of the elemental content of the conditioned media upon incubation with cells in culture to determine whether ions were removed from the media, which could be due to mineralisation, precipitation or internalisation by

the cells. The conditioned media given to the cells at each time point was compared directly with the cell supernatant collected (Fig. 4, Supplementary Table 4).

For cells that were given media conditioned with OssiMend Bioactive, ~20 μg mL^{-1} of Ca was removed from solution every two days (as seen in the difference between the Ca levels in the conditioned media given to the cells and the cell supernatant collected two days later). This repeated removal indicates continued Ca deposition over the fourteen days (Fig. 4A, Supplementary Table 4). In contrast, results obtained for OssiMend or 45S5 Bioglass showed cell supernatant with similar levels of Ca as the conditioned medium added to the cells from the second day of culture (Fig. 4A, Supplementary Table 4).

We detected higher levels of P in cell supernatant than in conditioned media for the first 10 days in culture in the OssiMend Bioactive or OssiMend groups (Fig. 4B, Supplementary Table 4), and significantly higher levels of P in cell supernatant than in conditioned media were observed for the 45S5 Bioglass particulate, at certain time points. Still, P levels were always lower for 45S5 Bioglass conditioned medium than for OssiMend and OssiMend Bioactive.

Si levels in the cell supernatant showed a significant drop with respect to the conditioned media at day 2 for OssiMend Bioactive and 45S5 Bioglass, of 5 μg mL^{-1} and 4 μg mL^{-1}, respectively (Fig. 4C, Supplementary Table 4). This result suggests Si incorporation by the cells.

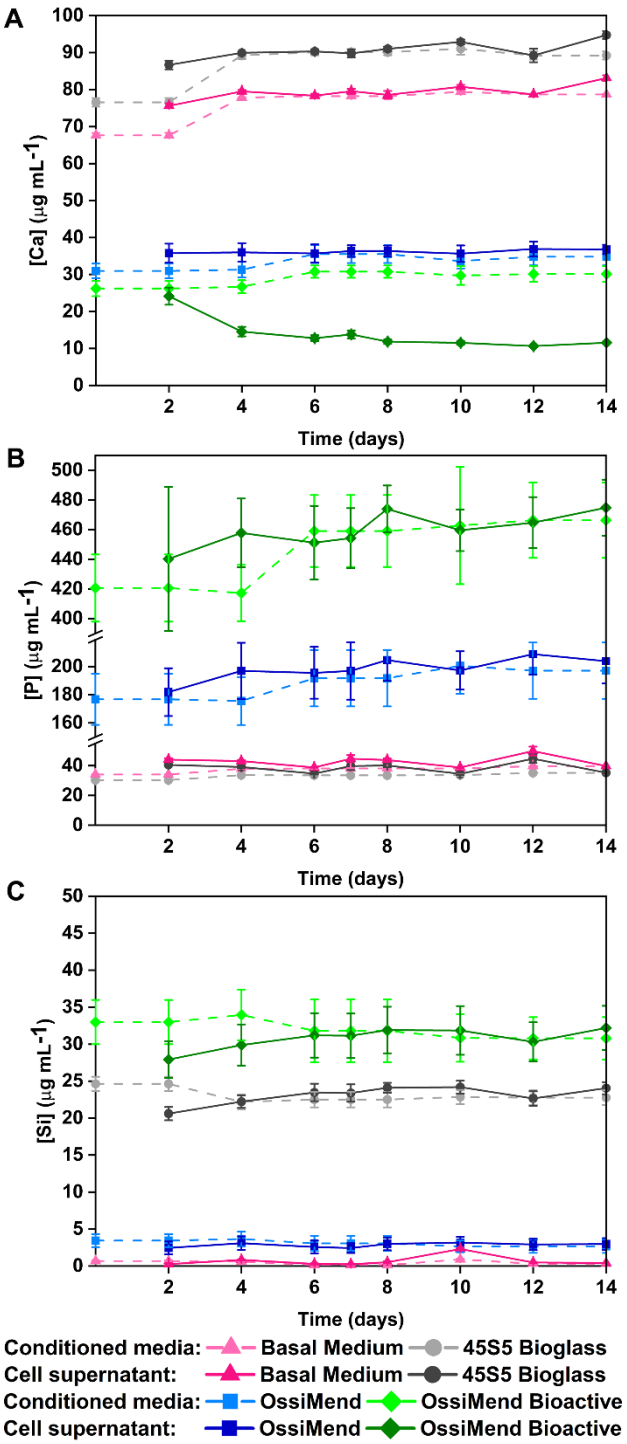

Figure 4: **Human osteoblasts adjust bioactive ion levels in culture upon treatment with conditioned media.** Elemental content of the conditioned media prepared with basal medium control, 45S5 Bioglass, OssiMend, and OssiMend Bioactive and of the supernatant collected after treating human osteoblasts (cell supernatant): (A) calcium, (B) phosphorus, and (C) silicon. Plots show means ± s.d, *n* = 3. A Mann-Whitney test (two-tailed) was used to compare the elemental content for each ion in supplemented media versus the cell supernatant for each time point (Supplementary Table 4).

3.4. OssiMend Bioactive reveals higher osteostimulatory potential than OssiMend and 45S5 Bioglass

Osteoblast differentiation occurs in three distinct stages: proliferation, matrix maturation, and mineralization [17, 18]. Here, we assessed the osteostimulation mediated by dissolution products of OssiMend Bioactive *in vitro,* compared to 45S5 Bioglass and OssiMend, by quantifying the mRNA expression of osteogenic-related markers that are highly expressed in specific periods of time during differentiation [19-22].

Vascular endothelial growth factor (*VEGF*), which stimulates angiogenesis, osteoblastic differentiation and bone repair [23, 24], was highly expressed by human osteoblasts treated with OssiMend Bioactive dissolution products (Fig. 5A) at both day two and day seven. OssiMend Bioactive conditioned media led to a significant upregulation of matrix metalloproteinase 14 (*MMP14*) at day seven (Fig. 5B). MAP kinase-activated protein kinase 2 (*MAPKAPK2*) expression was induced by OssiMend Bioactive conditioned medium after seven days in culture (Fig. 5C). MAPKAPK2 has calcium-dependent protein serine/threonine kinase activity and has transferase activity, transferring phosphorus-containing groups. Insulin-like growth factor type II (*IGF2*), which is a potent osteoblast mitogenic growth factor [6] and a key regulator of osteoblast homeostasis [6, 25] that is well known to be upregulated during osteoblastic proliferation [26, 27] and differentiation [28], was significantly downregulated by day seven in cultures treated with OssiMend Bioactive conditioned medium (Fig. 5D).

In contrast, when human osteoblasts were treated with OssiMend and 45S5 Bioglass conditioned media, the expression of *VEGF, MMP14, MAPKAPK2*, and *IGF2* did not change significantly (Fig. 5) suggesting absence of further osteostimulation.

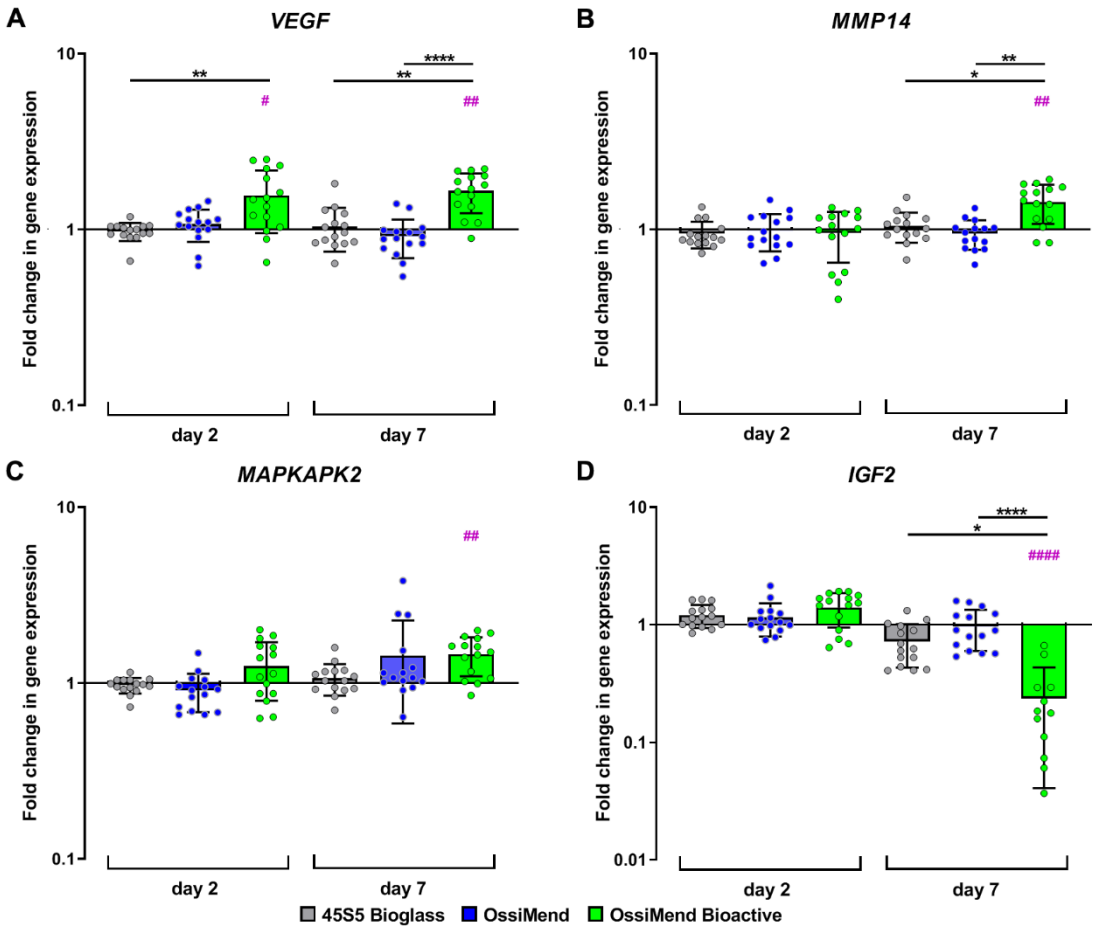

Figure 5: **OssiMend Bioactive soluble cues reveal superior inherent osteostimulation than OssiMend and 45S5 Bioglass.** Gene expression analyses for (A) *VEGF,* (B) *MMP14,* (C) *MAPKAPK2* and (D) *IGF2,* by human osteoblasts (1 x 10^4 cells/cm^2 on TCP, $n \geq 5$) treated for 2 or 7 days with conditioned media prepared with 45S5 Bioglass, OssiMend, and OssiMend Bioactive. Expression levels are shown as fold change normalized to expression in human osteoblasts cultured with basal medium control (set to 1). Plots show means ± s.d. A Kruskal-Wallis and Dunn's Multiple Comparison Test was used to detect statistical significance among treatments, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, and for each treatment versus the control, cultured with basal medium, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$.

3.5. Human osteoblasts form bone-like nodules when treated with OssiMend Bioactive extract

Expression of alkaline phosphatase *(ALPL),* an early marker of osteoblastic differentiation [29], did not change significantly with any of the conditioned media, after two days in culture (Fig. 6A). In contrast, OssiMend Bioactive conditioned medium caused a significant downregulation of *ALPL* (Fig. 6A) by day seven in culture, which was not seen for the 45S5 Bioglass or OssiMend conditioned media. Osterix (*OSX*, also known as *SP7*), an osteoblast-specific transcription factor, that governs the transition of pre-osteoblasts into functional osteoblasts and then into osteocytes [30-32], was significantly upregulated by day seven in culture, when human osteoblasts were treated with OssiMend Bioactive and OssiMend conditioned media, but not for the 45S5 Bioglass conditioned medium (Fig. 6B). Osteocalcin *(BGLAP*) was upregulated by day seven in culture with OssiMend and 45S5 Bioglass conditioned media, but was significantly downregulated for the OssiMend Bioactive conditioned medium (Fig. 6C). Expression of osteopontin *(SPP1)*, which is

involved in terminal differentiation and mineralization, was unchanged when cells were cultured in 45S5 Bioglass conditioned medium, while it was strongly upregulated by OssiMend Bioactive and OssiMend conditioned media by day two in culture (Fig. 6D). This upregulation was sustained until day seven in culture for OssiMend Bioactive. Expression of the integrin binding sialoprotein *(IBSP),* a marker of terminal differentiation and related to mineralization, was significantly upregulated on day seven in culture with all treatments in comparison with basal medium control (Fig. 6E). Decorin (*DCN*) is an essential component of the osteoid that modulates matrix mineralization [33], and decreases in the calcified bone matrix [34]. *DCN* (Fig. 6F), at day seven, was downregulated by the OssiMend Bioactive conditioned medium, but not by the other treatments.

The gene expression patterns seen here are consistent with OssiMend and 45S5 Bioglass conditioned media stimulating the maturation of osteoblasts, while OssiMend Bioactive conditioned medium stimulated terminal differentiation.

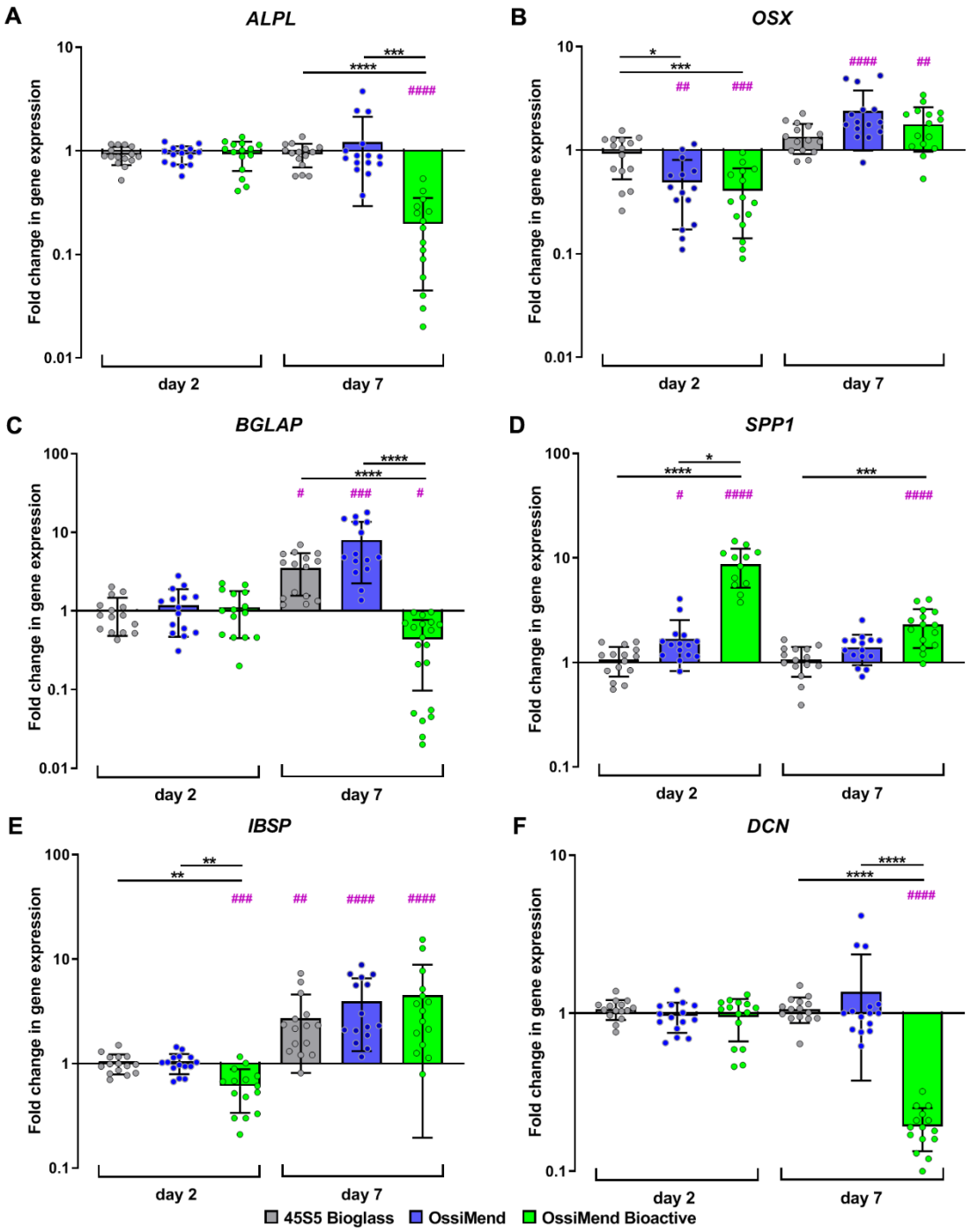

Figure 6: **OssiMend and 45S5 Bioglass bioactive ions coax human osteoblasts maturation whilst late mineralization stage is attained with OssiMend Bioactive condition medium.** Gene expression analyses for markers of osteogenesis: (A) *ALPL,* (B) *OSX,* (C) *BGALP,* (D) *SPP1,* (E) *IBSP,* and (F) *DCN* in human osteoblasts (1 x 10^4 cells/cm^2 on TCP, $n \geq 5$) treated for 2 or 7 days with conditioned media prepared with 45S5 Bioglass, OssiMend, and OssiMend Bioactive. Expression levels are shown as fold change normalized to expression in human osteoblasts cultured with basal medium control (set to 1). Plots show means ± s.d. A Kruskal-Wallis and Dunn's Multiple Comparison Test was used to detect statistical significance among treatments, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, and for each treatment versus the control, cultured with basal medium, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$.

3.6. OssiMend Bioactive extractable induces matrix mineralization

Matrix mineralization takes place when: extracellular osteocalcin binds Ca (calcium carbonate and calcium phosphate) and hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) [35-37]; the secreted osteopontin binds Ca, phosphate and mineral [38]; and the bone sialoprotein binds to collagen Type I and nucleates hydroxyapatite crystal formation [39-41].

To quantify mineralized extracellular matrix (ECM), we stained the hydroxyapatite portion deposited in the bone-like nodules using the fluorescent OsteoImage™ Staining Reagent. Matrix mineralization was solely detected when human osteoblasts were treated with OssiMend Bioactive conditioned medium (Fig. 7A). Bone-like nodules size and hydroxyapatite incorporated increased significantly from day seven up to day fourteen in culture (Fig. 7B), reinforcing that the late mineralization stage was attained.

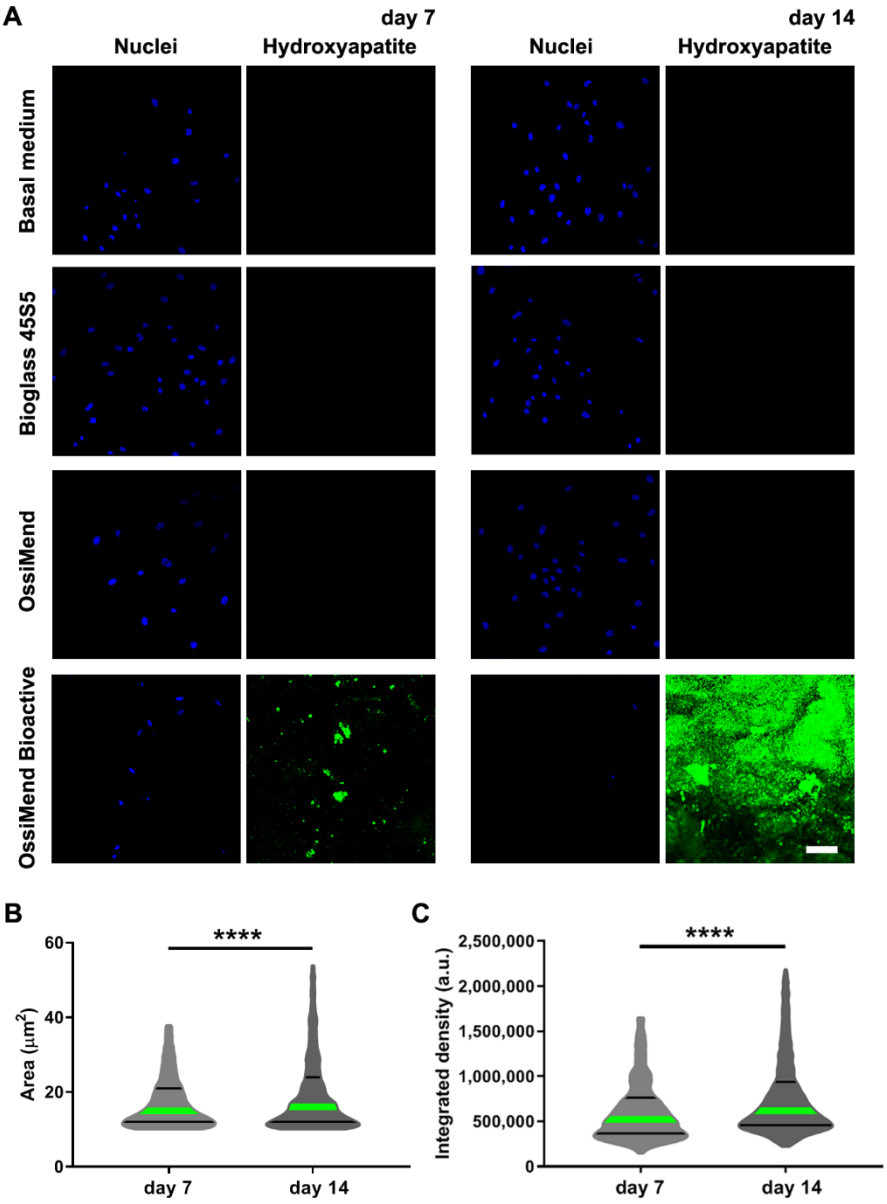

Figure 7: **Bone-like nodules matrix is enriched over time with hydroxyapatite in presence of OssiMend Bioactive condition medium.** (A) Representative confocal micrographs of human osteoblasts (1 x 10^4 cells/cm^2 on TCP, *n* = 6) treated for 7 or 14 days with conditioned media prepared with basal medium control, 45S5 Bioglass, OssiMend, and OssiMend Bioactive, showing

the deposited hydroxyapatite portion of the bone-like nodules stained by specific binding of the fluorescent OsteoImage™ Staining Reagent (green), and nuclei counterstained with Hoechst (blue). Scale bar is 100 µm. (B) Bone-like nodules area (μm^2) and (C) Integrated density (a.u.) of deposited hydroxyapatite per bone-like nodule formed after 7 and 14 days of treatment with OssiMend Bioactive condition medium (green line represents the median). In (B, C) a Mann-Whitney test (two-tailed) was used to detect statistical significance, ****$p < 0.0001$.

4. Discussion

We compared the "osteostimulation" properties of the dissolution products of OssiMend Bioactive, which contains 45S5 Bioglass, with those of 45S5 Bioglass and OssiMend *in vitro.* To gain insight into their osteogenic potential, we assessed the impact of their dissolution products on the differentiation of human osteoblasts without the use of osteogenic supplements, such as ascorbic acid, β-glycerophosphate and dexamethasone.

OssiMend, is a FDA approved composite, which combines carbonated apatite particles with porous bovine Type I collagen, the most abundant protein in bone. OssiMend showed 40% fusion in a posterolateral lumbar rabbit spinal fusion model by radiographic analysis after eight weeks [42] and is currently utilized to repair bone defects in orthopaedic and spinal surgery.

Our results showed that while the proliferation of human osteoblasts treated with OssiMend conditioned medium *in vitro* did not change, mRNA for *OSX*, *BGLAP* and *IBSP* was significantly upregulated by day seven as compared to the control (Fig. 6), which confirms commitment of the human osteoblast to mature osteoblasts. These results suggest that primary human osteoblasts *in vitro* respond to the OssiMend conditioned medium and its dissolution products promote their maturation. At this stage, mature osteoblasts enrich their own secreted collagenous extracellular unmineralized matrix (osteoid) with osteocalcin, osteonectin, bone sialoprotein, osteopontin, decorin and biglycan [19-21, 43].

45S5 Bioglass undergoes dissolution in aqueous media, releasing its constitutive ions into solution. Xynos *et al.* showed that the ionic products of 45S5 Bioglass dissolution (1% w/v), prepared after 24 h of incubation, stimulated human osteoblast growth, maturation and ECM deposition within two days without the addition of osteogenic supplements [6, 7]. They reported that osteoblast proliferation and differentiation stimulation by the ionic products of 45S5 Bioglass dissolution, namely Ca ions (~88.3 µg mL^{-1}) and soluble Si (~16.5 µg mL^{-1}), was due to upregulation of *IGF2* [6], *VEGF, MAPKAPK2*, *MMP14*, among other genes [7].

For our experiments we used a finer particle size (approximately 100 – 300 µm) of 45S5 Bioglass, compared with 300 – 710 µm particle size used by Xynos *et al.* [6, 7, 12], which might explain the increased dissolution rate [44], as greater surface area to volume ratio enhances its reactivity. However, we detected similar ion concentrations in our 45S5 Bioglass conditioned medium, as reported by Xynos *et al.* (Table 1, Fig. 2 and 4) [6, 7, 12]. In our studies, the 45S5 Bioglass conditioned medium had no effect on osteoblast morphology, proliferation or metabolic activity over the seven-day period. Moreover, in contrast to Xynos *et al*., we did not detect any significant upregulation of mRNA levels of *IGF2, VEGF, MMP14, MAPKAPK2* when human osteoblasts were incubated with 45S5 Bioglass conditioned medium up to seven days (Fig. 5).

There was, however evidence that the 45S5 Bioglass conditioned medium induced osteoblast maturation with highly significant upregulation of *BGLAP* and *IBSP* mRNA expression (Fig. 6). Our results for *BGLAP* with 45S5 Bioglass conditioned medium treatment align with results obtained by Xynos *et al.* when human osteoblasts were seeded on 45S5 Bioglass discs and cultured for 6 days [12]. Osteocalcin is known to inhibit both *in vitro* [45] and *in vivo* [46] apatite crystal growth, which may explain why there was no positive staining with OsteoImage™ Staining Reagent (Fig. 7).

Conditioned medium prepared with OssiMend Bioactive (OssiMend plus Bioglass) provoked a significant decrease in human osteoblast metabolic activity after two days of culture, followed by a reduction in the cell number after seven days in culture (Fig. 3), with the concomitant formation of bone-like nodules with extensive matrix mineralization (Fig. 7). This is expected once the mineralisation and gene expression results are considered. Proliferation is known to stop during osteogenesis, as osteoblasts exit the cell cycle and they start differentiate towards fully mature osteoblasts [43], which then transition to terminally differentiated stage with reduced synthetic activity [43] that are actively involved in the routine turnover of bone-like matrix [43]. Previous work have also reported a reduction in metabolic activity [15] and apoptosis [13, 14, 16] after the onset of mineralisation. Moreover, mature osteoblasts either become osteocytes surrounded by mineralized matrix, or turn into inert bone-lining cells or may undergo apoptosis[30, 47, 48].

OssiMend Bioactive dissolution products induced upregulation of *MMP14,* which suggests an increase in extracellular bone-like matrix remodelling [7, 49], and upregulation of *MAPKAPK2,* implying that the signalling cascade may be liaising with human osteoblasts stimulation. *VEGF, OSX, SPP1* and *IBSP* were significantly upregulated in human osteoblasts by day seven in culture (Fig. 5 and 6). Concomitantly, OssiMend Bioactive conditioned medium caused a significant downregulation of *IGF2*, *ALPL, BGLAP* and *DCN*, at this time, consistent with the onset of mineralization [50-56]. Indeed, we observed high hydroxyapatite deposition in this treatment group. Together, our observations confirm that human osteoblasts responded to this ionic environment, differentiating to the later stage of mineralization, forming bone-like nodules.

OssiMend Bioactive and OssiMend conditioned media showed significantly lower levels of Ca than 45S5 Bioglass conditioned medium. In addition, for the OssiMend Bioactive group, we detected a continuous reduction in the elementary content of Ca in the cell supernatant in comparison with the conditioned media given each two days to the osteoblasts that was consistent with sustained Ca deposition within the mineralized matrix that was seen in this treatment group. Extracellular Ca ions have been shown to promote osteogenic differentiation of mesenchymal stem cells (MSC) with upregulation of Ca homeostasis [57] and through L-type voltage-gated Ca channels [58, 59].

Inorganic phosphate plays a role in osteoblast proliferation, differentiation, metabolism [16, 60] and matrix maturation and mineralization [61, 62]. Phosphorus-containing compounds, such as β-glycerophosphate, are degraded by alkaline phosphatase secreted by osteoblasts, releasing phosphate ions inside the matrix vesicles, thus leading to an increased inorganic phosphate concentration in the extracellular environment [63]. Here, we did not add β-glycerophosphate to the conditioned media when treating osteoblast cultures. Still, we detected significantly higher levels of P in cell supernatant in comparison with conditioned medium for all treatments at least by day 4. This result suggests that osteoblasts were able to modulate the inorganic phosphate levels in their surroundings. Then, the phosphate and Ca ions inside the vesicles nucleate, forming the hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) [35-37]. The fibrillar phase occurs when the supersaturation of calcium and phosphate ions inside the matrix vesicles leads to the rupture of these structures and the hydroxyapatite crystals spread to the surrounding matrix [64].

OssiMend Bioactive and OssiMend conditioned media had higher levels of P than the other groups. The phosphate is thought to be from the collagen processing method, which might have led to a strong and specific signal induction of *SPP1*. *SPP1* expression is directly correlated with the enzymatic activity of alkaline phosphatase [19, 29, 65] and levels of inorganic phosphate in the medium [66]. In addition, increased intracellular inorganic phosphate increases *SPP1* expression and stimulates mineralization [67].

Osteoblasts and osteocytes express phosphate transporters, PIT1 and PIT2 [68], for active transport phosphate [69]. PIT1/2 might even sense extracellular inorganic phosphate without requiring its translocation [70]. Extracellular phosphate ions via PIT1-ATP-adenosine signalling pathway [71], regulate intramitochondrial phosphate content, ATP synthesis, secretion and

metabolism into adenosine, which then promotes osteogenic differentiation of progenitors via A2b adenosine receptors [71]. Additionally, activation of PIT1, ERK1/2 phosphorylation [72] is required for inorganic phosphate dependent stimulation of the expression of genes involved in mineralization [67]. Although, further experiments would be necessary to confirm the P mechanisms involved here, our observations suggest that P released by OssiMend Bioactive, OssiMend and 45S5 Bioglass was used by osteoblasts to drive their differentiation.

OssiMend Bioactive and OssiMend conditioned media differed significantly in the levels of Si (28 μg mL^{-1} at 6 h for OssiMend Bioactive compared to trace levels of around 3 μg mL^{-1} in OssiMend). Dissolution of the 45S5 Bioglass also released Si (21 μg mL^{-1} for 6 h conditioned medium), and at day two in culture, Si levels in the supernatant were significantly reduced upon cell treatment with OssiMend Bioactive and 45S5 Bioglass conditioned media, suggesting Si incorporation/deposition within human osteoblast cultures. Si has been described to play a physiological role in osteoblast proliferation [73], osteogenesis [74, 75], and bone calcification [76-78] although little is known about its mechanism of action [79].

Wang *et al.* showed that the ionic extract of β-calcium silicate/poly-D,L-lactide-co-glycolide, namely Si and Ca ions, induced osteogenic differentiation of rat MSCs, possibly via activation of AMPK-ERK1/2 pathway, also in the absence of osteogenic supplements [80]. Karpov *et al.* showed that bone marrow MSCs cultured on Bioglass coatings of high silica composition differentiated into osteoblasts [81]. Botelho *et al.* showed that human osteoblasts response, when seeded on sHA substrates modified with Si, was dependent on the presence and level of substitution of Si in the sHA substrate [82]. Soluble silica, supposedly released from the Bioglass as silicic acid ($Si(OH)_4$) has been shown to induce collagen Type I production by human osteoblasts at a concentration of 10 mmol [83]. Indeed, media containing 15 – 20 μg ml^{-1} of Si (in the form of soluble silica) induced mature osteoblasts to form mineralized bone nodules [84].

It is clear that Bioglass stimulates bone regeneration due to the release of ionic dissolution products and these studies highlight the ability of bioactive ions to drive osteogenesis; the results also imply additional phosphate can further accelerate osteogenesis. The results provide the basis for the design of synthetic bone grafts and bioactive medical devices (*e.g.* silicate glasses, glass-ceramics and calcium phosphates) for bone regeneration [8, 85]. Although the biological responses to bioactive ions released from inorganic materials have been widely studied, the underlying mechanisms of interaction between cells and the ionic extracts or each individual ionic species, and the signalling pathways involved, are still not fully understood [8, 9, 85].

Human osteoblasts differentiate *in vitro* in response to the dissolution products of OssiMend Bioactive with increasing deposition of Ca and hydropxyapatite in bone-like nodules. However, this did not occur when the cells were exposed to OssiMend and 45S5 Bioglass conditioned media, which only induced osteoblast maturation within the same period in culture. Indeed, OssiMend Bioactive conditioned medium rapidly induced the terminal mineralization stage of osteogenic differentiation without the addition of exogenous osteoinductive factors. Our results highlight the advantage of combining the bioactive Si from 45S5 Bioglass with P dissolution and Ca deposition in OssiMend Bioactive.

5. Conclusions

OssiMend Bioactive conditioned media stimulated human osteoblasts to form bone-like nodules fully surrounded by mineralized matrix, *in vitro*, as confirmed by gene expression analysis and positive specific staining for hydroxyapatite. This did not occur when the cells were exposed to media conditioned with OssiMend, which did not contain 45S5 Bioglass, or even media conditioned with 45S5 Bioglass alone. OssiMend and 45S5 Bioglass conditioned media were only able to induce osteoblast differentiation to mature osteoblasts within the same period in culture. OssiMend Bioactive benefits from the combination of bioactive Si from 45S5 Bioglass with the P dissolution

and Ca deposition of OssiMend. Our observations show that including 45S5 Bioglass in OssiMend Bioactive formulation improves the osteostimulation mediated by the bioactive ions without the addition of exogenous osteogenic factors. Our observations confirm that OssiMend Bioactive releases bioactive ions that modulate osteoblast differentiation and matrix mineralization. These promising observations encourage further studies in animal models to assess OssiMend Bioactive *in vivo* bone bioactivity.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The project was funded by Collagen Matrix Oakland (NJ), who produce and market the medical devices OssiMend and OssiMend Bioactive. They supplied the Bioglass, OssiMend and OssiMend Bioactive samples for the study. Collagen Matrix Inc. approved publication but did not supply input. The authors do not work for Collagen Matrix Inc. All data presented in the manuscript was collected by the authors and the authors take complete responsibility for the integrity of the data and the accuracy of the data analysis.

Acknowledgements

Authors acknowledge funding provided by Collagen Matrix Inc., Oakland (NJ).

Author contributions

SAF, GY developed experimental protocols, designed the experiments, conducted experiments and analysed the data. SMR and JRJ supervised the project. All authors wrote, revised and approved the manuscript.

Data availability statement

The authors declare that all data supporting the findings of this study are available within the article and its supplementary information file. Raw data is available on request from rdm-enquiries@imperial.ac.uk.

References

[1] J.R. Jones, D.S. Brauer, L. Hupa, D.C. Greenspan. Bioglass and bioactive glasses and their impact on healthcare. Int. J. Appl. Glass 7 (2016) 423-434.
[2] H. Oonishi, S. Kushitani, E. Yasukawa, H. Iwaki, L.L. Hench, J. Wilson, E.I. Tsuji, T. Sugihara. Particulate bioglass compared with hydroxyapatite as a bone graft substitute. Clin. Orthop. Rel. Res. (1997) 316-325.
[3] H. Oonishi, L.L. Hench, J. Wilson, F. Sugihara, E. Tsuji, S. Kushitani, H. Iwaki. Comparative bone growth behavior in granules of bioceramic materials of various sizes. J. Biomed. Mater. Res. 44 (1999) 31-43.
[4] H. Oonishi, L.L. Hench, J. Wilson, F. Sugihara, E. Tsuji, M. Matsuura, S. Kin, T. Yamamoto, S. Mizokawa. Quantitative comparison of bone growth behavior in granules of Bioglass (R), A-W glass-ceramic, and hydroxyapatite. J. Biomed. Mater. Res. 51 (2000) 37-46.
[5] L.L. Hench, J.M. Polak. Third-generation biomedical materials. Science 295 (2002) 1014-1017.
[6] I. Xynos, A. Edgar, L. Buttery, L. Hench, J. Polak. Ionic products of bioactive glass dissolution increase proliferation of human osteoblasts and induce insulin-like growth factor II mRNA expression and protein Synthesis. Biochem. Biophys. Res. Commun. 276 (2000) 461-465.
[7] I. Xynos, A. Edgar, L. Buttery, J. Polak. Gene-expression profiling of human osteoblasts following treatment with the ionic products of Bioglass 45S5 dissolution. J. Biomed. Mater. Res. 55 (2001) 151-157.
[8] A. Hoppe, N.S. Guldal, A.R. Boccaccini. A review of the biological response to ionic dissolution products from bioactive glasses and glass-ceramics. Biomaterials 32 (2011) 2757-2774.
[9] S. Kargozar, F. Baino, S. Hamzehlou, R.G. Hill, M. Mozafari. Bioactive glasses entering the mainstream. Drug Discov. Today 23 (2018) 1700-1704.

[10] E. Fiume, J. Barberi, E. Verné, F. Baino. Bioactive Glasses: From Parent 45S5 Composition to Scaffold-Assisted Tissue-Healing Therapies. J. Funct. Biomater. 9 (2018) 24.
[11] S. Midha, T.B. Kim, W. van den Bergh, P.D. Lee, J.R. Jones, C.A. Mitchell. Preconditioned 70S30C bioactive glass foams promote osteogenesis in vivo. Acta Biomater. 9 (2013) 9169-9182.
[12] I. Xynos, M. Hukkanen, J. Batten, L. Buttery, L. Hench, J. Polak. Bioglass 45S5 stimulates osteoblast turnover and enhances bone formation In vitro: implications and applications for bone tissue engineering. Calcif Tissue Int. 67 (2000) 321-329.
[13] M. Lynch, C. Capparelli, J. Stein, G. Stein, J. Lian. Apoptosis during bone-like tissue development in vitro. J. Cell Biochem. 68 (1998) 31-49.
[14] N. Fratzl-Zelman, H. Horandner, E. Luegmayr, F. Varga, A. Ellinger, M. Erlee, K. Klaushofer. Effects of triiodothyronine on the morphology of cells and matrix, the localization of alkaline phosphatase, and the frequency of apoptosis in long-term cultures of MC3T3-E1 cells. Bone 20 (1997) 225-236.
[15] G. Camci-Unal, A. Laromaine, E. Hong, R. Derda, G. Whitesides. Biomineralization Guided by Paper Templates. Sci. Rep. 6 (2016) 27693-27693.
[16] Y. Liu, Q. Lu, R. Pei, H. Ji, G. Zhou, X. Zhao, R. Tang, M. Zhang. The effect of extracellular calcium and inorganic phosphate on the growth and osteogenic differentiation of mesenchymal stem cells in vitro: Implication for bone tissue engineering. Biomed. Mater. 4 (2009) 025004.
[17] G.R. Beck Jr. Inorganic phosphate as a signaling molecule in osteoblast differentiation. J. Cell. Biochem. 90 (2003) 234-243.
[18] J. Aubin. Regulation of osteoblast formation and function. Rev. Endocr. Metab. Disord. 2 (2001) 81-94.
[19] G.R. Beck Jr, E. Sullivan, E. Moran, B. Zerler. Relationship between alkaline phosphatase levels, osteopontin expression, and mineralization in differentiating MC3T3-E1 osteoblasts. J. Cell. Biochem. 68 (1998) 269-280.
[20] G.R. Beck Jr, E. Moran, N. Knecht. Inorganic phosphate regulates multiple genes during osteoblast differentiation, including Nrf2. Exp. Cell Res. 288 (2003) 288-300.
[21] K. Conrads, M. Yi, K. Simpson, D. Lucas, C. Camalier, L. Yu, T. Veenstra, R. Stephens, T. Conrads, G. Beck. A combined proteome and microarray investigation of inorganic phosphate-induced pre-osteoblast cells. Mol. Cell Proteomics 4 (2005) 1284-1296.
[22] E. Jensen, R. Gopalakrishnan, J. Westendorf. Regulation of gene expression in osteoblasts. BioFactors 36 (2010) 25-32.
[23] K. Hu, B. Olsen. The roles of vascular endothelial growth factor in bone repair and regeneration. Bone 91 (2016) 30-38.
[24] K. Hu, B. Olsen. Osteoblast-derived VEGF regulates osteoblast differentiation and bone formation during bone repair. J. Clin. Invest. 126 (2016) 509-526.
[25] C. Conover, C. Rosen, J. Bilezikian, L. Raisz, G. Rodan. Chapter 45 - The role of insulin-like growth factors and binding proteins in bone cell biology. Principles of Bone Biology (Second Edition). San Diego: Academic Press; 2002. p. 801-815.
[26] J. Sandy, M. Davies, S. Prime, R. Farndale. Signal pathways that transduce growth factor-stimulated mitogenesis in bone cells. Bone 23 (1998) 17-26.
[27] B. Langdahl, M. Kassem, M. Moller, E. Eriksen. The effects of IGF-I and IGF-II on proliferation and differentiation of human osteoblasts and interactions with growth hormone. Eur. J. Clin. Invest. 28 (1998) 176-183.
[28] R. Marsell, T. Einhorn. The biology of fracture healing. Injury 42 (2011) 551-555.
[29] H. Tenenbaum. Levamisole and inorganic pyrophosphate inhibit beta-glycerophosphate induced mineralization of bone formed in vitro. J. Bone Miner. Res. 3 (1987) 13-26.
[30] K. Nakashima, X. Zhou, G. Kunkel, Z. Zhang, J. Deng, R. Behringer, B. de Crombrugghe. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 108 (2002) 17-29.
[31] K. Nakashima, B. de Crombrugghe. Transcriptional mechanisms in osteoblast differentiation and bone formation. Trends Genet. 19 (2003) 458-466.
[32] X. Zhou, Z. Zhang, J. Feng, V. Dusevich, K. Sinha, H. Zhang, B. Darnay, B. de Crombrugghe. Multiple functions of Osterix are required for bone growth and homeostasis in postnatal mice. Proc. Natl. Acad. Sci. U.S.A. 107 (2010) 12919-12924.

[33] Y. Mochida, W. Duarte, H. Tanzawa, E. Paschalis, M. Yamauchi. Decorin modulates matrix mineralization in vitro. Biochem. Biophys. Res. Commun. 305 (2003) 6-9.
[34] K. Hoshi, S. Kemmotsu, Y. Takeuchi, N. Amizuka, H. Ozawa. The Primary Calcification in Bones Follows Removal of Decorin and Fusion of Collagen Fibrils. J. Bone Miner. Res. 14 (1999) 273-280.
[35] P. Hauschka, J. Lian, D. Cole, C. Gundberg. Osteocalcin and matrix Gla protein: vitamin K-dependent proteins in bone. Physiol. Rev. 69 (1989) 990-1047.
[36] M. Weinreb, D. Shinar, G. Rodan. Different patterns of alkaline phosphatase, osteopontin, and osteocalcin expression in developing rat bone visualized by in situ hybridization. J. Bone Miner. Res. 5 (1990) 831-842.
[37] C. Chenu, S. Colucci, M. Grano, P. Zigrino, R. Barattolo, G. Zambonin, N. Baldini, P. Vergnaud, P.D. Delmas, A.Z. Zallone. Osteocalcin induces chemotaxis, secretion of matrix proteins, and calcium-mediated intracellular signaling in human osteoclast-like cells. J. Cell Biol. 127 (1994) 1149-1158.
[38] G. Hunter. Role of osteopontin in modulation of hydroxyapatite formation. Calcif. Tissue Int. 93 (2013) 348-354.
[39] W. Neuman, M. Neuman. The nature of the mineral phase of bone. Chem. Rev. 53 (1953) 1-45.
[40] H. Anderson. Matrix vesicles and calcification. Curr. Rheumatol. Rep. 5 (2003) 222–226.
[41] C. Tye, G. Hunter, H. Goldberg. Identification of the type I collagen-binding domain of bone sialoprotein and characterization of the mechanism of interaction. J. Biol. Chem. 280 (2005) 13487-13492.
[42] T.P. Scott, K.H. Phan, H. Tian, A. Suzuki, S.R. Montgomery, J.S. Johnson, E. Atti, S. Tetratis, R.C. Pereira, J.C. Wang, M.D. Daubs, F. Stappenbeck, F. Parhami. Comparison of a novel oxysterol molecule and rhBMP2 fusion rates in a rabbit posterolateral lumbar spine model. Spine J 15 (2015) 733-742.
[43] R. Florencio-Silva, G. Sasso, E. Sasso-Cerri, M. Simoes, P. Cerri. Biology of bone tissue: structure, function, and factors that influence bone cells. BioMed Res. Int. 2015 (2015) 17.
[44] P. Sepulveda, J. Jones, L. Hench. In vitro dissolution of melt-derived 45S5 and sol-gel derived 58S bioactive glasses. J. Biomed. Mater. Res. 61 (2002) 301-311.
[45] P. Ducy, C. Desbois, B. Boyce, G. Pinero, B. Story, C. Dunstan, E. Smith, J. Bonadio, S. Goldstein, C. Gundberg, A. Bradley, G. Karsenty. Increased bone formation in osteocalcin-deficient mice. Nature 382 (1996) 448-452.
[46] L. Malaval, D. Modrowski, A. Gupta, J. Aubin. Cellular expression of bone-related proteins during in vitro osteogenesis in rat bone marrow stromal cell cultures. J. Cell Physiol. 158 (1994) 555-572.
[47] E. Burger, J. Klein-Nulend. Mechanotransduction in bone-role of the lacuno-canalicular network. FASEB J. 13 (1999) S101-S112.
[48] R. Jilka, R. Weinstein, T. Bellido, A. Parfitt, S. Manolagas. Osteoblast programmed cell death (apoptosis): modulation by growth factors and cytokines. J. Bone Miner. Res. 13 (1998) 793-802.
[49] K. Paiva, J. Granjeiro. Matrix metalloproteinases in bone resorption, remodeling, and repair. Progress in Molecular Biology and Translational Science: Academic Press; 2017. p. 203-303.
[50] T. Franz-Odendaal, B. Hall, P. Witten. Buried alive: how osteoblasts become osteocytes. Dev. Dyn. 235 (2006) 176-190.
[51] L. Bonewald. The amazing osteocyte. J. Bone Miner. Res. 26 (2011) 229-238.
[52] Y. Mikuni-Takagaki, Y. Kakai, M. Satoyoshi, E. Kawano, Y. Suzuki, T. Kawase, S. Saito. Matrix mineralization and the differentiation of osteocyte-like cells in culture. J. Bone Miner. Res . 10 (1995) 231-242.
[53] K. Poole, R. van Bezooijen, N. Loveridge, H. Hamersma, S. Papapoulos, C. Lowik, J. Reeve. Sclerostin is a delayed secreted product of osteocytes that inhibits bone formation. FASEB J. 19 (2005) 1842-1844.
[54] S. Ubaidus, M. Li, S. Sultana, P. de Freitas, K. Oda, T. Maeda, R. Takagi, N. Amizuka. FGF23 is mainly synthesized by osteocytes in the regularly distributed osteocytic lacunar canalicular system established after physiological bone remodeling. Microscopy 58 (2009) 381-392.

[55] Y. Mochida, D. Parisuthiman, S. Pornprasertsuk-Damrongsri, P. Atsawasuwan, M. Sricholpech, A. Boskey, M. Yamauchi. Decorin modulates collagen matrix assembly and mineralization. Matrix Biol. 28 (2009) 44-52.
[56] M. Schaffler, W. Cheung, R. Majeska, O. Kennedy. Osteocytes: master orchestrators of bone. Calcif. Tissue Int. 94 (2014) 5-24.
[57] A.M.C. Barradas, V. Monticone, M. Hulsman, C. Danoux, H. Fernandes, Z. Tahmasebi Birgani, F. Barrère-de Groot, H. Yuan, M. Reinders, P. Habibovic, C. van Blitterswijk, J. de Boer. Molecular mechanisms of biomaterial-driven osteogenic differentiation in human mesenchymal stromal cells. Integr. Biol. 5 (2013) 920-931.
[58] L. Wen, Y. Wang, H. Wang, L. Kong, L. Zhang, X. Chen, Y. Ding. L-type calcium channels play a crucial role in the proliferation and osteogenic differentiation of bone marrow mesenchymal stem cells. Biochem. Biophys. Res. Commun. 424 (2012) 439-445.
[59] A.M.C. Barradas, H.A.M. Fernandes, N. Groen, Y.C. Chai, J. Schrooten, J. van de Peppel, J.P.T.M. van Leeuwen, C.A. van Blitterswijk, J. de Boer. A calcium-induced signaling cascade leading to osteogenic differentiation of human bone marrow-derived mesenchymal stromal cells. Biomaterials 33 (2012) 3205-3215.
[60] C.E. Camalier, M. Yi, L.-R. Yu, B.L. Hood, K.A. Conrads, Y.J. Lee, Y. Lin, L.M. Garneys, G.F. Bouloux, M.R. Young, T.D. Veenstra, R.M. Stephens, N.H. Colburn, T.P. Conrads, G.R. Beck Jr. An integrated understanding of the physiological response to elevated extracellular phosphate. J. Cell. Physiol. 228 (2013) 1536-1550.
[61] M. Yadav, A. Simão, S. Narisawa, C. Huesa, M. McKee, C. Farquharson, J. Millán. Loss of skeletal mineralization by the simultaneous ablation of PHOSPHO1 and alkaline phosphatase function: A unified model of the mechanisms of initiation of skeletal calcification. J. Bone Miner. Res. 26 (2011) 286-297.
[62] S. Chande, C. Bergwitz. Role of phosphate sensing in bone and mineral metabolism. Nat. Rev. Endocrinol. 14 (2018) 637-655.
[63] C.G. Bellows, J.N.M. Heersche, J.E. Aubin. Inorganic phosphate added exogenously or released from β-glycerophosphate initiates mineralization of osteoid nodules in vitro. Bone Miner. 17 (1992) 15-29.
[64] G. Boivin, P. Meunier. The degree of mineralization of bone tissue measured by computerized quantitative contact microradiography. Calcif. Tissue Int. 70 (2002) 503-511.
[65] C. Wennberg, L. Hessle, P. Lundberg, S. Mauro, S. Narisawa, U. Lerner, J. Millan. Functional characterization of osteoblasts and osteoclasts from alkaline phosphatase knockout mice. J. Bone Miner. Res. 15 (2000) 1879-1888.
[66] G.R. Beck Jr, B. Zerler, E. Moran. Phosphate is a specific signal for induction of osteopontin gene expression. Proc. Natl. Acad. Sci. U S A. 97 (2000) 8352-8357.
[67] Y. Yoshiko, G.A. Candeliere, N. Maeda, J.E. Aubin. Osteoblast autonomous Pi regulation via Pit1 plays a role in bone mineralization. Mol. Cell Biol. 27 (2007) 4465-4474.
[68] K. Miyagawa, M. Yamazaki, M. Kawai, J. Nishino, T. Koshimizu, Y. Ohata, K. Tachikawa, Y. Mikuni-Takagaki, M. Kogo, K. Ozono, T. Michigami. Dysregulated gene expression in the primary osteoblasts and osteocytes isolated from hypophosphatemic Hyp mice. Plos One 9 (2014) e93840.
[69] B. Wang, Y. Yang, L. Liu, H.C. Blair, P.A. Friedman. NHERF1 regulation of PTH-dependent bimodal Pi transport in osteoblasts. Bone 52 (2013) 268-277.
[70] N.W. Chavkin, J.J. Chia, M.H. Crouthamel, C.M. Giachelli. Phosphate uptake-independent signaling functions of the type III sodium-dependent phosphate transporter, PiT-1, in vascular smooth muscle cells. Exp. Cell Res. 333 (2015) 39-48.
[71] Y.-R.V. Shih, Y. Hwang, A. Phadke, H. Kang, N.S. Hwang, E.J. Caro, S. Nguyen, M. Siu, E.A. Theodorakis, N.C. Gianneschi, K.S. Vecchio, S. Chien, O.K. Lee, S. Varghese. Calcium phosphate-bearing matrices induce osteogenic differentiation of stem cells through adenosine signaling. Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 990-995.
[72] M. Julien, S. Khoshniat, A. Lacreusette, M. Gatius, A. Bozec, E.F. Wagner, Y. Wittrant, M. Masson, P. Weiss, L. Beck, D. Magne, J. Guicheux. Phosphate-dependent regulation of MGP in osteoblasts: Role of ERK1/2 and Fra-1. J. Bone Miner. Res. 24 (2009) 1856-1868.

[73] P. Valerio, M.M. Pereira, A.M. Goes, M.F. Leite. The effect of ionic products from bioactive glass dissolution on osteoblast proliferation and collagen production. Biomaterials 25 (2004) 2941-2948.
[74] H. Li, K. Xue, N. Kong, K. Liu, J. Chang. Silicate bioceramics enhanced vascularization and osteogenesis through stimulating interactions between endothelia cells and bone marrow stromal cells. Biomaterials 35 (2014) 3803-3818.
[75] G. Jell, I. Notingher, O. Tsigkou, P. Notingher, J.M. Polak, L.L. Hench, M.M. Stevens. Bioactive glass-induced osteoblast differentiation: A noninvasive spectroscopic study. J. Biomed. Mater. Res. A 86A (2008) 31-40.
[76] E.M. Carlisle. Silicon: A possible factor in bone calcification. Science 167 (1970) 279-280.
[77] E.M. Carlisle. Silicon: A requirement in bone formation independent of vitamin D1. Calcif. Tissue Int. 33 (1981) 27-34.
[78] K. Schwarz, D.B. Milne. Growth-promoting effects of silicon in rats. Nature 239 (1972) 333-334.
[79] W. Götz, E. Tobiasch, S. Witzleben, M. Schulze. Effects of silicon compounds on biomineralization, osteogenesis, and hard tissue formation. Pharmaceutics 11 (2019) 117.
[80] C. Wang, K. Lin, J. Chang, J. Sun. Osteogenesis and angiogenesis induced by porous beta-CaSiO3/PDLGA composite scaffold via activation of AMPK/ERK1/2 and PI3K/Akt pathways. Biomaterials 34 (2013) 64-77.
[81] M. Karpov, M. Laczka, P.S. Leboy, A.M. Osyczka. Sol-gel bioactive glasses support both osteoblast and osteoclast formation from human bone marrow cells. J. Biomed. Mater. Res. A 84A (2008) 718-726.
[82] C.M. Botelho, R.A. Brooks, S.M. Best, M.A. Lopes, J.D. Santos, N. Rushton, W. Bonfield. Human osteoblast response to silicon-substituted hydroxyapatite. J. Biomed. Mater. Res. A 79A (2006) 723-730.
[83] D.M. Reffitt, N. Ogston, R. Jugdaohsingh, H.F.J. Cheung, B.A.J. Evans, R.P.H. Thompson, J.J. Powell, G.N. Hampson. Orthosilicic acid stimulates collagen type 1 synthesis and osteoblastic differentiation in human osteoblast-like cells in vitro. Bone 32 (2003) 127-135.
[84] O. Tsigkou, J.R. Jones, J.M. Polak, M.M. Stevens. Differentiation of fetal osteoblasts and formation of mineralized bone nodules by 45S5 Bioglass conditioned medium in the absence of osteogenic supplements. Biomaterials 30 (2009) 3542-3550.
[85] A. Hoppe, V. Mouriño, A.R. Boccaccini. Therapeutic inorganic ions in bioactive glasses to enhance bone formation and beyond. Biomater. Sci. 1 (2013) 254-256.

Supplementary Tables:

Supplementary Table 1. Primers sequences used in quantitative polymerase chain reaction.

Gene	F / R	Sequence	Tm (°C)	Amplicon size (bp)	Primer Conc. (nM)	Accession Number
VEGF	F	AGGGCAGAATCATCACGAAGT	61.2	75	250	284172466c1
	R	AGGGTCTCGATTGGATGGCA	62.9			
MMP14	F	CGAGGTGCCCTATGCCTAC	61.6	178	150	13027797c2
	R	CTCGGCAGAGTCAAAGTGG	60.1			
MAPKAPK2	F	CCAGGAGAAATTCGCCCTCAA	62.1	117	500	341865588c2
	R	TCGTACACATCCACGATCCGT	63.0			
IGF2	F	GTGGCATCGTTGAGGAGTG	60.5	92	100	183603938c1
	R	CACGTCCCTCTCGGACTTG	61.7			
ALPL	F	AACATCAGGGACATTGACGTG	60.3	159	150	294660769c3
	R	GTATCTCGGTTTGAAGCTCTTCC	60.7			
OSX	F	CCTCTGCGGGACTCAACAAC	61.0	128	500	22902135c1
	R	AGCCCATTAGTGCTTGTAAAGG	58.6			
BGLAP	F	ATGAGAGCCCTCACACTCCT	60.0	117	100	NM_199173.3
	R	CTTGGACACAAAGGCTGCAC	60.0			
SPP1	F	CTCCATTGACTCGAACGACTC	60.2	230	250	352962175c1
	R	CAGGTCTGCGAAACTTCTTAGAT	60.0			
IBSP	F	GAACCTCGTGGGGACAATTAC	60.4	79	500	167466186c3
	R	CATCATAGCCATCGTAGCCTTG	60.6			
DCN	F	ATGAAGGCCACTATCATCCTCC	61.0	135	100	47419922c1
	R	GTCGCGGTCATCAGGAACTT	62.2			
GAPDH	F	CTCTGCTCCTCCTGTTCGACA	61.5	204	150	
	R	ACGACCAAATCCGTTGACTC	58.2			
EEF1A1	F	GCTGAGCGTGAACGTGGTAT	62.5	89	250	83367078c2
	R	CCTGGGGCATCAATGATAGTCA	61.6			
RPL13A	F	GCCCTACGACAAGAAAAAGCG	60.1	117	250	14591905c2
	R	TACTTCCAGCCAACCTCGTGA	61.1			

Vascular endothelial growth factor precursor (VEGF), *Matrix metalloproteinase 14 precursor (MMP14), MAP kinase-activated protein kinase 2 (MAPKAPK2)*, *Insulin-like growth factor II (IGF2), Alkaline phosphatase (ALPL), Osterix* (*OSX*), *Osteocalcin (BGALP), Osteopontin (SPP1), Integrin binding sialoprotein (IBSP), Decorin, bone proteoglycan II precursor (DCN)*, *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *Eukaryotic translation elongation factor 1 alpha 1* (*EEF1A1)*, and *Ribosomal protein L13a* (*RPL13A*). Primers were obtained from Sigma or Life Technologies.

Supplementary Table 2: Statistical analyses to detect differences in the elemental content ((µg mL^{-1}) graphs in Figure 2) in the conditioned media prepared after 6 h incubation at 37 °C with Basal medium, 45S5 Bioglass, OssiMend, and OssiMend Bioactive. A non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test was used. The significant differences are highlighted in blue.

	Calcium	Phosphorus	Silicon
45S5 Bioglass vs. Basal medium	$p > 0.9999$	$p > 0.9999$	$p = 0.0024$
OssiMend vs. Basal medium	$p = 0.0766$	$p = 0.7566$	$p = 0.7594$
OssiMend Bioactive vs. Basal medium	$p = 0.0225$	$p = 0.0196$	$p = 0.0002$
OssiMend vs. 45S5 Bioglass	$p = 0.0173$	$p = 0.0191$	$p = 0.2160$
OssiMend vs. OssiMend Bioactive	$p > 0.9999$	$p = 0.9476$	$p = 0.0366$
45S5 Bioglass vs. OssiMend Bioactive	$p = 0.0042$	$p < 0.0001$	$p > 0.9999$

Supplementary Table 3: Statistical analyses of stability during storage over 14 days at 4 °C of the elemental content ((µg mL^{-1}) graphs in Figure 2) of conditioned media prepared after 6 h incubation at 37 °C with Basal medium, 45S5 Bioglass, OssiMend, and OssiMend Bioactive. A non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test was used.

	Basal medium	45S5 Bioglass	OssiMend	OssiMend Bioactive
Calcium	$p = 0.1228$	$p = 0.0640$	$p = 0.5907$	$p = 0.3858$
Phosphorus	$p = 0.0692$	$p = 0.1126$	$p = 0.6720$	$p = 0.1657$
Silicon	$p = 0.1413$	$p = 0.1535$	$p = 0.4641$	$p = 0.0637$

Supplementary Table 4: Statistical analyses to detect differences in the elemental content ((µg mL^{-1}) graphs in Figure 4) in conditioned media given to the cells versus the cell supernatant for each time point. A Mann-Whitney test (two-tailed) was used. The significant differences are highlighted in blue.

	Calcium	Phosphorus	Silicon
45S5 Bioglass			
day 2	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
day 4	$p = 0.2581$	$p < 0.0001$	$p = 0.9314$
day 6	$p = 0.4363$	$p = 0.0019$	$p = 0.1359$
day 7	$p = 0.3865$	$p < 0.0001$	$p = 0.2313$
day 8	$p = 0.0315$	$p < 0.0001$	$p = 0.0008$
day 10	$p = 0.0111$	$p = 0.0335$	$p = 0.0153$
day 12	$p > 0.9999$	$p < 0.0001$	$p = 0.5043$
day 14	$p < 0.0001$	$p = 0.3521$	$p = 0.0078$
OssiMend			
day 2	$p < 0.0001$	$p = 0.2581$	$p = 0.0503$
day 4	$p < 0.0001$	$p = 0.0487$	$p = 0.0503$
day 6	$p = 0.5457$	$p = 0.3080$	$p = 0.0625$
day 7	$p = 0.1359$	$p = 0.3518$	$p = 0.0503$
day 8	$p = 0.1827$	$p = 0.2485$	$p = 0.9314$
day 10	$p = 0.0473$	$p = 0.7304$	$p = 0.0939$
day 12	$p = 0.0315$	$p = 0.2483$	$p = 0.1615$
day 14	$p = 0.0625$	$p = 0.3401$	$p = 0.1903$
OssiMend Bioactive			
day 2	$p = 0.0503$	$p = 0.6048$	$p = 0.0037$
day 4	$p < 0.0001$	$p = 0.0008$	$p = 0.0503$
day 6	$p = 0.0038$	$p = 0.9314$	$p = 0.3401$
day 7	$p = 0.0038$	$p = 0.9497$	$p = 0.2581$
day 8	$p = 0.0038$	$p = 0.0939$	$p = 0.8633$
day 10	$p < 0.0001$	$p > 0.9999$	$p = 0.0939$
day 12	$p < 0.0001$	$p = 0.7104$	$p = 0.2973$
day 14	$p < 0.0001$	$p = 0.5289$	$p = 0.0503$