Generation of composites for bone tissue engineering applications consisting of gellan gum hydrogels mineralized with calcium and magnesium phosphate phases by enzymatic means

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Abstract

Mineralization of hydrogels, desirable for bone regeneration applications, may be achieved enzymatically by incorporation of alkaline phosphatase (ALP). ALP-loaded gellan gum (GG) hydrogels were mineralized by incubation in mineralization media containing calcium and/or magnesium glycerophosphate (CaGP, MgGP). Mineralization media with CaGP:MgGP concentrations 0.1:0, 0.075:0.025, 0.05:0.05, 0.025:0.075 and 0:0.1 (all values mol dm^{-3}, denoted as A, B, C, D and E, respectively) were compared. Mineral formation was confirmed by IR and Raman, SEM, ICP-OES, XRD, TEM, SAED, TGA and increases in the the mass fraction of the hydrogel not consisting of water.

Ca was incorporated into mineral to a greater extent than Mg in samples mineralized in media A-D. Mg content and amorphicity of mineral formed increased in the order A < B < C < D. Mineral formed in media A and B was calcium-deficient hydroxyapatite (CDHA). Mineral formed in medium C was a combination of CDHA and an amorphous phase. Mineral formed in medium D was an amorphous phase. Mineral formed in medium E was a combination of crystalline and amorphous MgP. Young's modulus and storage modulus decreased in dependence of mineralization medium in the order A > B > C > D, but were significantly higher for samples mineralized in medium E. Attachment and vitality of osteoblastic MC3T3-E1 cells were higher on samples mineralized in media B-E, containing Mg, than in those mineralized in medium A, not containing Mg. Hence, mineral composition, mechanical and biological properties of enzymatically mineralized hydrogels can be adjusted by varying the mineralization medium.

1. Introduction

Gellan gum (GG) is an anionic calcium-binding polysaccharide produced by bacteria (Fialho et al., 2008) (Giavasis et al., 2000) (Okamoto and Kubota, 1996). which has been applied in hydrogel form as a tissue engineering (TE) scaffold (Fan et al., 2010, Oliveira et al., 2010). Further advantages of gellan gum are its low cost and the fact that it is not animal-derived, avoiding regulatory concerns. Gelation is induced by cooling a GG-$CaCl_2$ solution to body temperature, while cells and bioactive substances such as enzymes can be incorporated before gelation at temperatures above 37 ˚C (Oliveira et al., 2010, Douglas et al., 2012a).

Mineralization of hydrogels is desirable for bone TE applications in order to promote bioactivity, i.e. the formation of a chemical bond with surrounding bone tissue after implantation (LeGeros, 1991). Potential further advantages of mineralization are promotion of osteoblastic differentiation through increased stiffness (Engler et al., 2006, Rowlands et al., 2008, Evans et al., 2009) and enhanced binding of growth factors which stimulate bone healing (Ruhe et al., 2005). Furthermore, in contrast to traditional ceramics, hydrogels can serve as delivery vehicles for bioactive substances such as growth factors and enzymes. As mineralized hydrogels consist mainly of water, and contain a polymer component, they are expected to undergo more rapid degradation than “traditional” calcium phosphates such as beta-tricalcium phosphate (beta-TCP) and hydroxyapatite (HA), whose resorption remains incomplete after several months or even years (Draenert et al., 2013, Gunther et al., 1998, Hwang et al., 2012, Moore et al., 2001).

Hydrogel mineralization can be achieved by addition of enzymes such as alkaline phosphatase (ALP) (Gkioni et al., 2010), which causes mineralization of bone by cleavage of phosphate from organic phosphate, thereby increasing the local phosphate concentration and enabling precipitation of insoluble phosphate salts. ALP has been added to hydrogel materials to induce their mineralization with calcium phosphate (CaP) during incubation in solutions

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containing calcium and glycerophosphate (GP), which serves as a substrate for ALP (Beertsen and van den Bos, 1992, Douglas et al., 2012a, Douglas et al., 2012b, Douglas et al., 2012c, Filmon et al., 2000, Spoerke et al., 2009, Yamauchi et al., 2004). ALP has been implanted in vivo as a coating on titanium-based implants (Schouten et al., 2009) and as a component of collagen sheets (Beertsen and van den Bos, 1992, Doi et al., 1996) and synthetic oligo(poly(ethylene glycol)fumarate) (OPF) hydrogels (Bongio et al., 2013). No severe inflammatory reactions were observed in any of these studies, suggesting low immunogenicity of ALP.

However, ALP-induced enzymatic mineralization with CaP enriched with Mg or magnesium phosphate (MgP) remains unexplored. MgP is gaining interest as an alternative to CaP due to its comparable cytocompatibility and ability to support osteoblast adhesion and expression of osteoblastic markers at the mRNA level (Tamimi et al., 2012). Thanks to the cytocompatibility of MgP, bone cements based on MgP have been developed as alternatives to CaP cements (Ewald et al., 2011, Klammert et al., 2010, Mestres and Ginebra, 2011, Moseke et al., 2011, Tay et al., 2007). An addtional justification for the use of magnesium phosphate-based ceramics is their faster resorption in vivo compared to calcium phosphate-based ceramics (Klammert et al., 2011). Mg-substituted hydroxyapatite coatings on titanium implants have promoted proliferation and differentiation of osteoblast-like cells and improved early osseointegration in vivo (Zhao et al., 2012).

In a previous study, enzymatic mineralization of GG with CaP by incubation in a mineralization solution containing calcium glycerophosphate (CaGP), which served as a source of calcium and organic phosphate, led to enhanced mechanical properties and adhesion and proliferation of osteoblastic cells (Douglas et al., 2012a). In this study, this strategy was extended to induce enzymatic mineralization of GG with mineral consisting of CaP and MgP phases by incubation in solutions of CaGP and magnesium glycerophosphate (MgGP). The

CaGP:MgGP concentration ratio in the mineralization was varied in order to investigate its influence on the nature of mineral formed. Resulting mineralized hydrogels were characterized physicochemically using Infrared (IR) and Raman spectroscopy, X-ray diffraction (XRD), selected area electron diffraction (SAED), transmission electron microscopy (TEM), scanning electron microscopy (SEM), Ion-coupled plasma optical emission spectroscopy (ICP-OES), thermogravitational analysis (TGA) and increases in the dry mass percentage, i.e. the mass fraction of the hydrogel not consisting of water. Mechanical properties were evaluated by compressive testing and rheometry. Cytocompatibility of mineralized hydrogels and their ability to support adhesion and proliferation of osteoblasts was also evaluated with a view to their suitability as scaffolds for applications in bone TE.

The present study aimed to fill the following gaps in the scientific literature: i) development of composites of hydrogels and mineral containing Mg by enzymatic means; ii) influence of mineralization medium, i.e. different CaGP:MgGP concentration ratios, on the incorporation of Mg and Ca into mineral formed; iii) effect of varying Mg and Ca content on composite mechanical properties and osteoblast behavior.

2. Materials and Methods

2.1 Materials

Unless stated otherwise, all materials, including GG (Gelzan™ CM, G1910), ALP (P7640), CaGP (50043) and MgGP (17766) where obtained from Sigma-Aldrich. The GG preparation used was classified as “Low-Acyl” by the manufacturer.

2.2 Production and characterization of GG hydrogels containing ALP

ALP was incorporated into GG hydrogels by a modification of the method of Oliveira et al (Oliveira et al., 2010). Briefly, an aqueous GG solution was heated to 90 ºC and mixed with an aqueous $CaCl_2$ solution to achieve final GG and $CaCl_2$ concentrations of 0.7% (w/v) and 0.03% (w/v), respectively. The resulting solution was mixed and allowed to cool to 50 ºC. At 50 ºC ALP solution was added to achieve a final ALP concentration of 2.5 mg/ml. This temperature was chosen in order to avoid excessive deactivation of ALP associated with higher temperatures while permitting good mixing and preventing premature gelation during casting due to cooling. ALP in milk has been reported to retain more than 80% of its activity after heating for 10 min at 50 ºC (Fadilogwlu et al., 2004). In another study, ALP in milk showed no loss of activity after 30 min at 50 ºC (Lombardi et al., 2000), while ALP in serum heated for 30 min was not inactivated at all at 45 ºC and only by 28% at 50 ºC (Neale et al., 1965). Hydrogel cylinders of volume 250 µl, diameter 8 mm and thickness 5 mm were prepared by casting 40 ml GG-$CaCl_2$ solution at 50 ºC in glass petri dishes of diameter 10 cm at room temperature and cutting out cylinders with a hole punch. Casting took place immediately after ALP addition to minimize ALP activity loss. Finally, GG-$CaCl_2$ solution was allowed to cool at room temperature for 20 min to gelify.

2.3 Mineralization of gels

Gel mineralization was induced at room temperature by incubation in mineralization medium containing different concentrations of CaGP and MgGP. Five different CaGP:MgGP concentration ratios were compared, namely 0.1:0, 0.075:0.025, 0.05:0.05, 0.025:0.075 and 0:0.1 (all values mol dm^{-3}). These mineralization media were denoted as A, B, C, D and E, respectively (see Table 1). Mineralization medium was changed every day. After conclusion of mineralization after 7 days, gels were rinsed three times in Milli-Q water and subsequently incubated in Milli-Q for 1 day with the aim of removing residual CaGP.

2.4 Calculation of mass change due to mineralization by measurement of dry mass percentage

The dry mass percentage, i.e. the hydrogel weight percentage not consisting of water, was calculated as: (weight after incubation and subsequent freeze-drying/weight after incubation but before freeze-drying)*100. This served as a measure of the extent of mineral formation. Freeze-drying was performed for 24 h. Experiments were performed in triplicate.

2.5 Thermogravimetric analysis (TGA)

Thermogravimetric Analysis (TGA) was performed using a Hi-Res TGA 2950 Thermogravimetric Analyzer (TA Instruments). Lyophilized samples (n=3) were heated from 30 ºC to 800 ºC in a helium atmosphere at a rate of 10 ºC/min under constant monitoring of remaining weight percentage, to determine the mass percentage of lyophilized samples attributable to mineral.

2.6 Physicochemical and morphological characterization: IR and Raman spectroscopy, XRD, TEM, SAED, EDS, ICP-OES, SEM

After mineralization experiments and subsequent lyophilization, the molecular structure of the hydrogels was examined using IR and Raman spectroscopy, XRD, TEM, SAED, EDS, ICP-OES and SEM. Samples were lyophilized for 48 h prior to analysis.

IR spectra of the powdered samples dispersed in KBr tablets were recorded using a Galaxy 6030 Fourier transform IR spectrophotometer (Mattson, Madison, WI, USA).

Raman Spectra were recorded using a Kaiser Optical Systems Rxn1-532 device equipped with a 532 nm (Nd:YAG) laser source. Spectra were recorded after 10 accumulations of 5 s each.

XRD analysis was performed using an X'Pert Pro diffractometer (Panalytical) with the X'Celerator strip detector, $Cu_{K\alpha 1}$radiation (50 kV, 30 mA) and the standard Bragg-Brentano geometry. The diffraction patterns were collected in the 2θ angle range of 10-90º with formal step of 0.001º, the total time of each measurement was 8 hours. The diffractograms were analyzed with the help of ICDD card 01-084-1147 and 01-089-6440 for bobierrite and hydroxyapatite, respectively.

Transmission electron microscopy analysis was performed using a JEM-2200FS FEG (Jeol) instrument operated at 200 kV. Conventional TEM bright field (TEM BF), selected area electron diffraction (SAED) and scanning transmission (STEM) modes were used in this work. An in-column omega filter was used to diminish chromatic aberration caused by inelastic scattering of primary electrons in thick areas of the sample. EDS spectrometry was used in combination with STEM mode to measure chemical compositions and produce elemental mappings of the material agglomerates. Lyophilized hydrogel samples were prepared by chopping them up with a razor blade to obtain powders. A lacey carbon support film on a Cu grid was then repeatedly dipped into this powder.

SEM analysis was performed on a JEOL JSM-5600 instrument. The instrument was used in the secondary electron mode (SEI). The SEM instrument is equipped with an electron microprobe JED 2300. Prior to analysis, lyophilized samples were coated with a thin gold layer (ca 20 nm) using a plasma magnetron sputter coater.

The concentrations of Ca, Mg and P were determined by inductively coupled plasma optical emission spectroscopy (ICP-OES) using a Spectro Arcos Optical Emission Spectrometer (Spectro, Germany). Before analysis, lyophilized hydrogel samples were dissolved in 1 ml 14 M HNO_3, and further diluted (200x) with 0.3 M HNO_3 (HNO_3 of analytical grade, ChemLab, Belgium). The instrument was calibrated by means of 6 standard solutions with Ca, Mg and P concentrations ranging from 0 to 15 mg l^{-1}. Yttrium was added to all solutions as an internal

standard in order to correct for possible instrument instabilities and matrix effects. Measurements were performed in triplicate.

2.7 Mechanical testing

Hydrogel samples were subjected to compressive testing using a Hounsfield Universal Testing Machine H10KM. Samples were placed between piston heads at a predefined distance. Displacement was applied at a rate of 4 mm/min until samples were compressed to 50% of their original height up to a maximum load of 80 N. During displacement, force was recorded with a 100 N load cell every 0.5 s using Qmat software. Compressive stress was calculated as force recorded divided by cross-sectional area. Compressive strain was calculated as distance moved during compression divided by initial distance between piston heads. Finally, Young's modulus was calculated as the gradient of the stress-strain curve.

In addition, viscoelastic properties of all types of gels were determined using a rheometer (Anton Paar Physica, MCR 301) equipped with a PP25 rotating head of diameter 25 mm. Storage modulus (G') and loss modulus (G'') values were recorded at a strain of 0.01% and an angular frequency of 10 rad s^{-1}. For all hydrogels evaluated, n=5.

2.8 Degradation testing

Degradation of hydrogel samples was evaluated using an extreme solution test and a simulation solution test according to ISO 10993-14 norm "Identification and quantification of degradation products from ceramics". Prior to testing, hydrogel samples were sterilized by autoclaving for 20 min in 50 ml Milli-Q water at 115 ºC and 1.2 bar.

For the extreme solution test, a citric acid buffer solution at pH 3 at 37 ºC was prepared by dissolution of 21 g of citric acid monohydrate in 500 ml Milli-Q water in a 1000 ml volumetric flask, addition of 200 ml of 1 M sodium hydroxide solution and dilution to the

mark with Milli-Q water. 40.4 ml of this solution was mixed with 59.6 ml of 0.1 M hydrochloric acid to yield the desired buffered citric acid solution.

Hydrogel samples were weighed, placed in glass vessels and 2 ml citric acid solution was added per 0.1 g sample. After 120 h incubaton at 37 °C, samples were removed from solution and reweighed. Experiments were repeated 5 times per sample group. Samples containing 2.5 mg ALP/ml hydrogel mineralized in media A-E were compared. Samples containing 0 mg ALP/ml hydrogel mineralized in media A served as controls.

For the simulation solution test, a Tris-HCl buffer at pH 7.4 was prepared by dissolving 13.25 g tris(hydroxymethyl)aminomethane in 500 ml of Milli-Q water and adjusting the pH with an appropriate amount of 1 M hydrochloric acid to pH 7.4 at a temperature of 37 °C. Milli-Q water was added until a final volume of 1000 ml was reached.

Hydrogel samples were weighed, placed in containers and 15.6 ml Tris-HCl buffer was added. After 120 h incubaton at 37 °C, samples were removed from buffer and reweighed. Buffer was retained and 1 ml was mixed with 1 ml 14 M HNO_3 for ICP-OES analysis as described in section 2.6 Experiments were repeated 5 times per sample group. Samples containing 2.5 mg ALP/ml hydrogel mineralized in media A-E were compared.

2.9 Cell biological characterization

2.9.1 Sterilization

Prior to cell experiments, samples were sterilized as described in section 2.8.

2.9.2 Cytocompatibility testing

Cytocompatibility was evaluated by determining the viability of human fibroblastic cells HFF-1 (human foreskin fibroblasts, ATCC) after culture in eluate from hydrogel samples. HFF cells were cultured in DMEM Glutamax™ supplemented with 15% fetal bovine serum, 0.1% sodium pyruvate and 0.1% penicillin/streptomycin (all Gibco, Invitrogen).

The colorimetric MTT assay, using a 3-(4, 5-dimethyldiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Merck Promega) was performed to quantify cell viability. The tetrazolium component is reduced in living cells by mitochondrial dehydrogenase enzymes to a water-insoluble purple formazan product, which can be solubilized by addition of lysis buffer and measured using spectrophotometry.

Eluate was produced by incubating four hydrogel samples in 2 ml cell culture medium (corresponding to a surface to volume ratio of 5.52 cm^2/ml) for 48 h. Eluate was diluted by factors of 1 (undiluted), 2, 4, 6, 8, 16, 32 and 64. HFF cells (10000 per well of a 96-well plate) were subsequently incubated in eluate at the aforementioned dilutions for 72 h. Afterwards, the eluate was replaced by 0.2 ml (0.5 mg/ml) MTT reagent and cells were incubated for 4 h at 37 °C. The MTT reagent was removed and replaced by 0.2 ml lysis buffer (1% Triton X-100 in isopropanol/0.04 N HCl) for 30 min. The dissolved formazan solution was measured spectrophotometrically at 580 nm (Universal microplate reader EL 800, Biotek Instruments). Triplicate measurements were performed. The viability was calculated as a percentage of control cultures.

2.9.3 Osteoblast seeding on GG hydrogels

Cells of the osteoblastic cell line MC3T3-E1 (ATCC) were seeded onto hydrogel samples. Each hydrogel sample was placed in a well of a 24-well plate and a suspension of 100000 cells in 1 ml cell culture medium (alpha-MEM supplemented with 10% fetal bovine serum and 0.5% Penicilin-Streptomycin, (all Gibco, Invitrogen)) was added. After incubation for 4 hours to allow attachment, 1 ml supplementary cell culture medium was added.

2.9.4 Osteoblast adhesion

To visualize cell attachment and distribution on the hydrogels, the cell cultures were evaluated using fluorescence microscopy, which was performed as follows. A live/dead staining (Calcein AM/propidium iodide) was performed to evaluate cell viability. Cells were

rinsed and the supernatant was replaced by 1 ml PBS solution supplemented with 2 µl (1 mg/ml) calcein AM (Anaspec, USA) and 2 µl (1 mg/ml) propidium iodide. Cultures were incubated for 10 min at room temperature, washed twice with PBS solution and evaluated by fluorescence microscopy (Type U-RFL-T, Olympus, Aartselaar, Belgium). Evaluations were performed 1 day and 6 days post-seeding.

2.9.5 Osteoblast proliferation

Cell vitality was investigated using an MTT Assay. 2 ml MTT reagent was added directly to the hydrogel samples in 24-well plates and incubated for 4 hours at 37°C. Subsequently, samples were transferred to 48-well plates. 0.5 ml lysis buffer was added and incubated for 30 minutes at 37°C on a gyratory shaker (70 rpm). 0.2 ml of the resulting solution was used for the spectrophotometric measurement. Evaluations were performed 1 day and 11 days post-seeding. The viability was expressed as a percentage of control cultures after 11 days.

2.10 Statistical analysis

Statistical analysis was performed using SPSS statistics software (IBM corporation, USA). Results of dry mass percentage, TGA, compressive testing and rheometrical measurements were analysed using a one-way Analysis of Variance (ANOVA) combined with Tukey's post-hoc test. Values of $p < 0.05$ were considered significant.

3. Results

3.1 Physicochemical characterization of mineralized hydrogels

Formation of mineral was investigated by IR and Raman spectroscopy, XRD, SAED, TEM, EDS, ICP-OES and SEM.

The IR spectrum of GG without the presence of ALP and mineralized for 7 days in mineralization medium A was representative for the IR spectrum of GG without ALP and mineralized for 7 days in mineralization medium E (Figure 1). The absorption bands in these IR spectra were comparable to the absorptions present in the IR spectrum of pure GG. Typical absorption bands of CaP or MgP were not found. The IR spectrum of GG with ALP and mineralized in medium A was representative for the IR spectrum of GG with ALP and mineralized in medium B. In these IR spectra absorption bands typical for apatite could be found at 568 and 605 cm^{-1} due to the ν_4 vibration components of PO_4^{3-}. The peaks around 970, 1045 and 1100 cm^{-1} arose from the ν_1 and ν_3 stretching of PO_4^{3-} groups. The typical absorptions due to the vibration and stretching of OH^- groups in hydroxyapatite appeared as weak shoulders around 640 and 3570 cm^{-1}. The small absorption band at 870 cm^{-1} arose from the ν_5 P-O(H) deformation of HPO_4^{2-} groups. The absorbance at 1138 cm^{-1}, arising from the ν_3 vibration component of HPO_4^{2-} groups, confirmed the presence of HPO_4^{2-} in the apatite lattice (Van Den Vreken et al., 2006). The precipitated mineral in these samples is not pure hydroxyapatite, but a calcium deficient apatite (CDHA). In the IR spectra of samples mineralized in media C, D and E, the band profiles of the PO_4^{3-} absorptions in the regions 530 – 640 cm^{-1} and 950 – 1120 cm^{-1} were clearly different, indicating another mineral phase than CDHA was precipitated in the GG. The absorption bands of the PO_4^{3-} absorptions in the IR spectrum of sample D were not resolved which is typical for the presence of an amorphous mineral phase. In the IR spectrum of the GG with ALP and mineralized in medium E, only the

absorption band in the region 530 – 640 cm^{-1} was not resolved. This might indicate that both an amorphous and a crystalline magnesium phosphate were present in this sample.

In Raman spectra (Figure 2) bands typical for PO_4^{3-} and HPO_4^{2-} groups were detected in samples containing ALP, while no bands were seen in the ALP-free control sample. In samples mineralized in media A-D, Raman bands characteristic for the ν_1 stretching of PO_4^{3-} groups were detected at 960 cm^{-1} (Koutsopoulos, 2002, Ryu et al., 2010). Other bands observed at 1010 cm^{-1} may be characteristic for the ν_1 stretching of HPO_4^{2-} groups in CaP phases containing HPO_4^{2-}, such as CDHA (Koutsopoulos, 2002). The spectrum of the sample mineralized in medium E was markedly different from the others. A band corresponding to ν_1 stretching of PO_4^{3-} groups was detected at 960 cm^{-1}, while the band at 1010 cm^{-1} corresponding to ν_1 stretching of HPO_4^{2-} groups was much less intense. Further bands were observed at 583 cm^{-1} (ν_4 bending of PO_4^{3-} groups) and 281 cm^{-1}, 216 cm^{-1} and 184 cm^{-1}.

The X-ray diffraction patterns (Figure 3) of the samples mineralized in media A and B show the presence of peaks at about 26° and 32° (Koutsopoulos, 2002, Ryu et al., 2010) corresponding to the (002) and (211) planes of apatite, respectively. The low intensity and low resolution of these peaks implies that the crystalline phase is not highly crystalline or present in high amounts. Peaks could not be identified in the samples mineralized in media C and D, suggesting the presence of an amorphous mineral phase only. The X-ray diffraction pattern of the sample incubated in medium E is markedly different, with peaks observed at approximately 11° and 13° which are consistent with the presence of a hydrated magnesium phosphate phase (bobierrite) (Brown et al., 1986).

TEM BF micrographs and corresponding SAED patterns, presented as negatives, are shown in Figure 4. The SAED patterns of samples mineralized in media A, B and C (Figure 4 Aii, Bii, and Cii) exhibit ring electron diffraction pattern, typical for polycrystalline specimens with random orientations of individual crystals, although there is also a contribution from the

amorphous part of the samples. Samples A and B display diffraction rings corresponding to the (002) plane and the (211) planes that are characteristic of a crystalline apatite phase (Mahamid et al., 2008, Ryu et al., 2010). These diffraction rings are less well defined in the pattern of the samples mineralized in medium C, suggesting a higher degree of amorphicity. SAED patterns of samples mineralized in media D and E exhibit broad, blurred rings typical for an amorphous mineral phase (Figure 8 Dii & Eii). No diffraction spots from a crystalline state were detected.

TEM images of samples A and B (Figure 4 Ai & Bi) show needle-like crystals of length of approximately 50-100 nm characteristic of CDHA (Juhasz et al., 2010), while deposits in sample C (Figure 4 Ci & Di) were more sphere-shaped with an approximate diameter of 10 nm. Deposits in samples D and E were irregularly shaped (Figure 4 Di & Ei).

TEM images and EDS spectrometry-based elemental mapping (Figure 5) demonstrate that the strength of the signal for Ca relative to Mg was dependent on mineralization medium and decreased in the order A > B > C > D > E. The Ca signal was stronger than the Mg signal in samples A, B and C and D and weaker in sample E. No Mg signal was seen in sample A. Ca and Mg present were homogeneously distributed on the surface of specimens.

Elemental composition of samples was determined using ICP-OES (Tables 2 & 3). Mg was absent in samples mineralized in medium A. A small amount of Ca was detected in samples mineralized in medium E due to the use of $CaCl_2$ as a crosslinking agent during hydrogel formation. In ALP-free samples, Ca, Mg and P were detected, presumably due to incomplete removal of CaGP and/or MgGP by washing, which in turn is probably due to binding of these species to GG. In samples containing ALP, Ca, Mg and P were detected in greater amounts than in ALP-free samples, demonstrating the formation of mineral. ICP-OES measurements also revealed that Ca was incorporated to a greater extent than Mg in mineral formed. For samples mineralized in media B, C and D, elemental mass percentages of Mg were inferior to

those of Ca by factors of over 30, 9 and 2.5, respectively (Table 2), while elemental molar concentrations were inferior by factors of over 20, 5 and 1.5, respectively (Table 3). Elemental mass percentage (Table 2) and molar concentration (Table 3) were higher for Ca in samples mineralized in medium A, which contains only CaGP, than for Mg in samples mineralized in medium E, which contains only MgGP.

SEM showed the formation of mineral-like deposits in the samples containing ALP mineralized in media A-E (Figure 6 a,b,c,d,ei,eiii), while in the absence of ALP, no mineral deposits were observed (Figure 6 ac, ec). In all samples containing ALP, the GG polymer network was visible. In samples mineralized in media A and B (Figure 6a,b), roughly spherical deposits with an approximate diameter of 200 nm were observed, as well as larger aggregates of such deposits. In samples mineralized in media C and D (Figure 6c,d), deposits were of the same dimensions, but with a more spherical morphology and smoother surface. Aggregates of such deposits were not seen. In samples mineralized in medium E, irregularly shaped deposits were seen (Figure 6ei) but at certain points, plate-shaped crystals were observed (Figure 6eii) (Tamimi et al., 2012).

3.2 Calculation of dry mass percentage and TGA analysis

The dry mass percentages of GG samples mineralized in different mineralization media are shown in Figure 7a. Dry mass percentages were significantly higher in samples containing ALP. Regarding the influence of mineralization medium, dry mass percentage was similar for the samples mineralized in media A, B and C, ranging from approximately 6 to 7% with no significant differences. In contrast, dry mass percentages of samples mineralized in media D and E (4-5%) were similar to each other with no significant difference, but were also significantly lower than values for samples mineralized in media A, B and C.

TGA analysis of lyophilized hydrogels (Figure 7b) revealed that the mass percentage remaining at 800 ºC, which is attributable to mineral, was significantly higher for samples containing ALP than for ALP-free controls. The mass percentage remaining at 800 ºC was similar for samples mineralized in media A-D (approximately 65%), and was lower in samples mineralized in medium E (approximately 50%). However, no significant differences were observed between samples containing ALP mineralized in media A-E, or between ALP-free samples minealized in media A or E.

3.3 Influence of mineralization on hydrogel mechanical properties

Compressive testing of hydrogels (Figure 8a) after 7 days incubation in mineralization solution revealed an increase in Young's modulus due to ALP incorporation. For samples containing ALP, Young's modulus was dependent on the mineralization medium. Samples mineralized in media A were significantly stiffer than those mineralized in media B, C and D. The samples mineralized in medium E displayed a Young's modulus which was at least three times higher than that of all other groups.

Rheometric testing results (Figure 8b) were consistent with the results of compressive testing. Storage modulus of samples mineralized in media A and B were significantly higher than those of samples mineralized in media C and D. The storage modulus of samples mineralized in medium E was four or more times higher than that of all other groups.

3.4 Degradation of mineralized hydrogels

Degradation tests in extreme solution (Figure 9a) revealed no apparent differences in percentage mass remaining after incubation. Samples mineralized in media A-E lost opacity and became transparent, but maintained their shape, suggesting that the ceramic phase was washed out but that hydrogel integrity was maintained.

Degradation tests in extreme solution (Figure 9b) also revealed no apparent differences in percentage mass remaining after incubation. Samples mineralized in media E became less opaque without becoming fully transparent. ICP-OES of degradation products (Table 4) showed that Ca, P and Mg were released from all samples. Release of Mg and P increased in the order A < B < C < D < E. Release of Ca and P was similar for groups A and B but increased in the order A, B < C < D < E. In groups C and D, amounts of of Ca and Mg released were comparable.

3.5 Cell biological characterization

Cytocompatibility testing revealed comparable cell viability for all sample groups at all eluate dilutions (Figure 9). The most pronounced differences were seen for cells cultured in undiluted eluate (1/1), where samples mineralized in medium A displayed markedly inferior viability (approx. 40%) compared to all other groups (approx. 60%) except samples mineralized in medium E (approx 50%). This suggests that toxic substances are released by sample groups, and that the presence of magnesium in samples reduces cytotoxicity.

Live/dead staining (Figure 10) of cells revealed that few cells were observed on GG without ALP after 1 day (Figure 10m) and 6 days (Figure 10n), and that these cells had a poorly-spread morphology, typical for non-attached cells. In contrast, larger numbers of cells adhered to GG containing ALP after 1 day, independently of the mineralization medium (Figure 10c,e,g,i,k). The cells had a well-spread morphology typical for attached cells and similar to that seen on the positive control, tissue culture polystyrene (Figure 10a). After 6 days, layers of viable cells were observed the positive control (Figure 10b) and samples mineralized in media C (Figure 10h), D (Figure 10j) and E (Figure 10l) with no dead cells. On samples mineralized in media A (Figure 12d) and B (Figure 10f), only isolated groups of cells were observed. On samples mineralized in medium A (Figure 10d), non-viable, red cells were seen.

Cell viability assays (Figure 11) showed, as expected, that cell number after 11 days was higher than after 1 day. Cell viability on samples containing ALP was superior to that on ALP-free controls, independently of mineralization medium used. After 1 day, viability was highest on samples mineralized in medium E and lowest for those mineralized in medium A. After 11 days, viability was similar on all samples except for samples mineralized in medium A, which displayed markedly lower viability (approximately 50-60% of viability for other mineralized groups).

4. Discussion

4.1 Influence of mineralization medium on nature and amount of mineral formed

The formation of mineral in hydrogels in the presence of ALP was demonstrated directly by IR (Figure 1) and Raman spectroscopy (Figure 2), XRD (Figure 3), TEM and SAED (Figure 4), EDS-based elemental mapping (Figure 5), ICP-OES (Tables 2 & 3) and SEM (Figure 6). Indirect evidence was provided by the increases in dry mass percentages in samples containing ALP (Figure 7a), increases in the mass percentage of lyophilized samples attributable to mineral measured by TGA (Figure 7b), and an increased Young's Modulus (Figure 8a) and rheometric storage modulus (Figure 8b).

Samples mineralized in media A and B contained CDHA, as evidenced by characteristic bands detected by IR (Figure 1) and Raman spectroscopy (Figure 2) as well as characteristic needle-shaped morphology and diffraction patterns observed using TEM and SAED, respectively (Figure 4). The formation of CDHA as a result of mineralization in medium A and the diameters of the individual mineral deposits observed using SEM (Figure 6) are in agreement with previous work, which revealed apatite formation in catechol-poly(ethylene glycol) and oligo[poly(ethylene glycol) fumarate] hydrogels after 6 days and GG hydrogels after 14 days (Douglas et al., 2012a, Douglas et al., 2012c). Samples mineralized in medium B contained considerably less Mg than Ca (Tables 2 & 3). This may be due to hydroxyapatite's higher affinity for Ca^{2+} than Mg^{2+}, which was reported by Aoba et al. (Aoba et al., 1992). Neuman and Mulryan showed that most Mg associated with hydroxyapatite is located at the surface of crystals and thus excluded from the crystal bulk (Neuman and Mulryan, 1971). An explanation proposed by Martin and Brown (Martin and Brown, 1997), who investigated the effect of Mg^{2+} on hydroxyapatite formation at 37 ºC, was based on the higher degree of hydration of Mg^{2+} ions and the slower rate of dehydration compared to Ca^{2+}

ions. Mg^{2+} ions adsorb to CaP surfaces but do not dehydrate rapidly enough to become incorporated into CaP crystal structures and instead remain complexed in aqueous species on the surfaces. As a result, the solution does not reach the critical supersaturation required for the precipitation of magnesium-containing solids and therefore Mg is incorporated to a much lesser extent than Ca. Hydroxyapatite is less soluble than MgP phases. For example, solubility products of 3.37×10^{-59} and 1×10^{-36} at 25°C have been reported for hydroxyapatite (McDowell et al., 1977) and the MgP phase bobierrite $Mg_3(PO_4)_2 \cdot 8H_2O$ (Taylor et al., 1963), respectively.

Samples mineralized in media C and D contained more Mg than those mineralized in media A and B (Tables 2 & 3), They were also less crystalline, as shown by changes in the bands detected by IR (Figure 1) as well as the disappearance of diffraction peaks characteristic for apatite, blurring of diffraction rings and changes in deposit morphology to more rounded shapes on the nanometer and millimeter scale, as observed using XRD, SAED, TEM and SEM, respectively (Figures 3, 4 & 6). Samples mineralized in medium C appeared to contain a mixture of CDHA and an amorphous mineral phase, while those mineralized in medium D contained an amorphous mineral phase. Samples mineralized in medium D contained approximately twice as much Mg and two-thirds as much Ca compared to samples mineralized in medium C (Tables 2 & 3).

This increase in amorphicity with increasing Mg is consistent with the findings of other authors. Bachra et al found that increasing Mg concentrations in solutions of Ca^{2+} and PO_4^{3-} led to a decrease in the crystallinity of the CaP phase formed, from apatite to amorphous calcium phosphate (ACP) (Bachra et al., 1965). This may be due to the stabilization of ACP by Mg^{2+}, which decreases its solubility, leading to an increase in the rate of ACP precipitation and a decrease in transformation of ACP to hydroxyapatite, as reported by Boskey et al and Blumethal et al (Boskey and Posner, 1974, Blumenthal et al., 1977).

Abbona et al. caused precipitation of mineral phases by mixing solutions of phosphate ions with solutions containing an equal concentration of Ca and Mg ions (i.e. $[Ca^{2+} + Mg^{2+}] = [PO_4^{2-}]$) at Mg/(Ca+Mg) ratios between 0 and 1 (Abbona et al., 1986). At all $[Ca^{2+} + Mg^{2+}]$ and $[PO_4^{2-}]$ concentrations ranging from 0.01 M to 0.5 M, predominantly amorphous calcium phosphate was formed initially at all Mg/(Ca+Mg) ratios except 1, i.e. in the absence of Ca^{2+} ions. Hence, the differences in mineral phases in dependence of mineralization medium can be explained as follows. In medium A, the ACP formed initially transforms to CDHA over 7 days. In medium B, the concentration of Mg^{2+} is too low to inhibit transformation of ACP to CDHA. In media C & D, the concentrations of Mg^{2+} are sufficiently high to partially and totally inhibit transformation, respectively.

Pure MgP mineral phases were only detected in the calcium-free mineralization medium E, which is in agreement with the results of Abbona et al (Abbona et al., 1986). Regarding the nature of the MgP formed, SAED results (Figure 4) suggest the presence of amorphous MgP while XRD results (Figure 3) suggest the presence of crystalline MgP and IR (Figure 1) and SEM (Figure 6) results suggest the presence of both crystalline and amorphous MgP. Hence, it would appear that both crystalline and amorphous phases are present. Regarding the type of crystalline MgP formed, the peaks in the X-ray diffraction pattern (Figure 3) and Raman spectrum bands (Figure 6) are consistent with the presence of bobierrite $Mg_3(PO_4)_2 \cdot 8H_2O$ (Frost et al., 2002).

It is conceivable that GG possesses a higher affinity for Ca^{2+} than Mg^{2+} ions, which would favour Ca incorporation in mineral over Mg incorporation. However due to the lack of published data, discussion must remain speculative.

Regarding amount of mineral formed, dry mass percentage measurements (Figure 7a) showed a clear decrease for samples mineralized in media D and E. However, TGA measurements revealed no clear differences in mineral content in dependence of mineralization medium

(Figure 7b). A possible explanation might be increased GG degradation in mineralization media of higher Mg concentration (D, E), however this remains speculative due to lack of proof.

4.2 Influence of mineralization media on mechanical properties and degradation

Compressive and rheometric testing (Figure 8a,b) and determination of dry mass percentage and mass percentage of lyophilized samples attributable to mineral (Figure 7a,b) showed that amount of mineral formed does not correlate with mechanical properties. Samples mineralized in medium E were markedly stiffer. Samples mineralized in media A and B were stiffer than those mineralized in media C and D. On the basis of the results presented in this paper it is not possible to determine the underlying reasons for these differences. Assuming that stiffness increases with increasing contact between mineral deposits, it can be speculated that the deposits in samples of higher stiffness are more tightly packed, leading to increased contact area with surrounding deposits. For example, the deposits in samples mineralized in media C and D were in the form of individual spheres, while the deposits in samples mineralized in media A and B were more irregularly shaped and formed larger aggregates, suggesting superior packing (Figure 6). Further research is needed to determine the influence of mineral deposit morphology on compressive modulus.

With regard to degradation testing, no differences in mass percentage remaining were observed between sample groups, neither in extreme nor in simulation solution (Figure 8). Furthermore, values appeared to be similar after incubation in both solutions. Taking into account the fact that mineral was washed out of samples in extreme solution, and that the hydrogel component appeared impervious to both extreme and simulaton solutions, one possible explanation is that the loss of mass due to dissolution of mineral may be compensated by entry of solution into the hydrogel component. Another reason for the lack of

differences observed may be the low mass percentage attributable to mineral in hydrated mineralized samples (Figure 7a).

Samples were sterilized by autoclaving in Milli-Q water prior to degradation testing in citric acid or Tris-HCl buffer. As the hydrogel component appeared to withstand degradation, the decrease in percentage mass remaining from 100% to average values in the range 70-90% (Figure 8) may possibly be due to osmosis of water out of the samples into the buffer.

Increased release of Ca, P in the order A, B < C < D (Table 4) is consistent with the decrease in crystallinity (Figures 1-4). Considering the ratios of the amounts of Ca and Mg present in sample groups B, C and D (Tables 2 & 3), the amounts of Mg released were disproportonally high. This suggests that Mg was present predominantly on the surface of CaP deposits and/or that any Mg-containg mineral phase present was more soluble than the CaP phases present, as discussed in section 4.1 above. Elemental release was highest from sample group E, suggesting higher solubility of the amorphous and/or crystalline MgP phases compared to the amorphous and/or crystalline CaP phases present in sample groups A-D.

On the basis of these results, it can be concluded that all sample groups undergo degradation.

4.3 Influence of mineralization media on cytocompatibility and cell proliferation

Enzymatically mineralized samples with different Ca and Mg contents showed similar cytocompatibilities (Figure 9). Hence, changes in Ca and/or Mg content do not have a marked influence.

Enzymatic mineralization of GG led to far superior adhesion of viable cells, as demonstrated by live/dead staining (Figure 10) and cell vitality testing (Figure 11). The poor cell adhesion on GG without ALP is consistent with the results of a previous study (Douglas et al., 2012a) and may be due to poor adsorption of adhesive proteins from serum and hence cell adhesion.

The presence of mineral on the surface of mineralized GG may enable adsorption of adhesive serum proteins and thus aid adhesion.

A possible reason for higher proliferation on samples containing Mg might be a stimulatory effect of Mg on proliferation, which has been reported for Mg-doped tricalcium phosphate (TCP) (Sader et al., 2009, Xue et al., 2008) octocalcium phosphate (OCP) (Boanini et al., 2012) and hydroxyapatite (Cai et al., 2010, Bracci et al., 2009, Landi et al., 2006). Pure MgP in the form of newberyite ($MgHPO_4 \cdot 3H_2O$) showed good cytocompatibility and supported colonization by osteoblastic cells (Tamimi et al., 2012). Magnesium phosphate cements based on struvite ($MgNH_4PO_4 \cdot 8H_2O$) have shown superior cytocompatibility to brushite ($CaHPO_4 \cdot 2H_2O$) and CDHA cements (Ewald et al., 2011). In this study, the stimulatory effect was comparable for samples mineralized in media B-E, despite differences in Mg content (Tables 2 & 3). In addition the Mg-containing mineral phase present (B: CDHA, C: CDHA and amorphous mineral phase, D: amorphous mineral phase, E: crystalline and amorphous MgP) and the increase of Mg release in the order B > C > D > E did not influence proliferation. Therefore the influence of Mg is independent of mineral phase and extent of Mg release and manifests itself at a low Mg content equal to or less than the Mg content in samples mineralized in medium B (Tables 2 & 3), but does not increase further with increasing Mg content.

Despite the lower amount of mineral formed in samples only containing MgP, vitality was superior after 1 day, suggesting superior adhesion. Cell adhesion has been improved by addition of Mg to hydroxyapatite (Webster et al., 2002) and carbonated apatite (Yamasaki et al., 2003, Yamasaki et al., 2002), and Mg^{2+} has been reported to promote cell adhesion and migration better than Ca^{2+} (Lange et al., 1994, Lange et al., 1995). One possible explanation might be enhanced integrin receptor expression due to Mg, which has been described before for Mg-doped Al_2O_3 (Zreiqat et al., 2002).

In summary, the results of physicochemical and cell biological characterization suggest that enzymatic mineralization GG with MgP, ACP containing Mg or CDHA containing Mg may be preferable to mineralization with CDHA for bone TE applications.

5. Conclusions

This work showed the feasibility of creating mineralized GG hydrogels and tailoring the Ca and Mg contents by incorporation of ALP during hydrogel formation and varying the concentrations of CaGP and MgGP in mineralization media. Mineral formed in mineralization media A, B, C, D and E appeared to be CDHA, CDHA, CDHA and an amorphous mineral phase, an amorphous mineral phase and a mixture of crystalline and amorphous MgP, respectively. Ca was incorporated into mineral to a greater extent than Mg. Mineralization led to an increase in stiffness, which was not proportional to amount of mineral formed and was dependent on mineralization medium, decreasing in the order E > A,B > C, D. Samples with Mg incorporated into mineral, i.e. those mineralized in media B-E, enhanced osteoblast attachment and proliferation.

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Tables

Mineralization medium	Concentration	
	CaGP	MgGP
	(mol dm^{-3})	(mol dm^{-3})
A	0.1	0
B	0.075	0.025
C	0.05	0.05
D	0.025	0.075
E	0	0.1

Table 1. Mineralization media used in study.

ALP Concentration mg/ml	Mineralization medium	mass percentage of element in samples					
		Ca		P		Mg	
		%	s.d.	%	s.d.	%	s.d.
2.5	A	26.8	0.9	15.0	0.6	0.0	0.0
2.5	B	25.1	0.6	14.8	0.3	0.7	0.0
2.5	C	22.1	2.0	14.9	1.4	2.4	0.2
2.5	D	14.2	0.1	12.8	0.0	5.0	0.0
2.5	E	1.5	0.1	12.2	0.1	12.6	0.2
0	A	8.8	1.3	5.0	0.7	0.0	0.0
0	B	9.8	0.9	6.7	0.6	0.9	0.0
0	C	7.3	1.4	6.3	1.1	2.1	0.3
0	D	3.9	0.1	6.0	0.1	3.6	0.2
0	E	0.2	0.0	5.0	0.2	4.7	0.2

Table 2. ICP-OES determination of elemental Ca, P and Mg mass percentages in GG hydrogels containing 2.5 and 0 mg/ml ALP after mineralization for 7 days in media A-E and subsequent lyophilization. Values are presented as mean ± standard deviation (s.d.) (n=3).

ALP Concentration mg/ml	Mineralization medium	µmol element/mg sample					
		Ca		P		Mg	
		µmol/mg	s.d.	µmol/mg	s.d.	µmol/mg	s.d.
2.5	A	6.7	0.2	4.8	0.2	0.0	0.0
2.5	B	6.3	0.1	4.8	0.1	0.3	0.0
2.5	C	5.5	0.5	4.8	0.4	1.0	0.1
2.5	D	3.5	0.0	4.1	0.0	2.1	0.0
2.5	E	0.4	0.0	3.9	0.0	5.2	0.1
0	A	2.2	0.3	1.6	0.2	0.0	0.0
0	B	2.5	0.2	2.1	0.2	0.4	0.0
0	C	1.8	0.3	2.0	0.3	0.9	0.1
0	D	1.0	0.0	1.9	0.0	1.5	0.1
0	E	0.0	0.0	1.6	0.1	2.0	0.1

Table 3. ICP-OES determination of elemental molar concentrations of Ca, P and Mg per unit mass sample (µmol/mg) in GG containing 2.5 and 0 mg/ml ALP after mineralization for 7 days in media A-E and subsequent lyophilization. Values are presented as mean ± standard deviation (s.d.) (n=3).

ALP Concentration mg/ml	Mineralization medium	µg element released/mg sample					
		Ca		P		Mg	
		µg/mg	s.d.	µg/mg	s.d.	µg/mg	s.d.
2.5	A	0.21	0.19	0.50	0.19	b.d.l.	b.d.l.
2.5	B	0.18	0.03	0.44	0.05	0.07	0.01
2.5	C	0.29	0.04	0.06	0.06	0.26	0.03
2.5	D	0.68	0.07	1.07	0.13	0.60	0.08
2.5	E	b.d.l.	b.d.l.	1.80	0.09	2.02	0.11

Table 4. ICP-OES determination of mass of elemental Ca, P and Mg released per mg sample after 120 h incubation in Tris-HCl buffer at pH 7.4. Prior to incubation in buffer, samples (GG hydrogels containing 2.5 mg/ml ALP) were mineralized for 7 days in media A-E. Values are presented as mean ± standard deviation (s.d.) (n=3). b.d.l. : below detection limit of apparatus.

Figure captions

Figure 1. Infrared spectra of the Gellan Gum (GG) hydrogels without (A control) and with ALP after mineralization for 7 days in media A-E. The spectrum obtained after mineralization in media A and B were indistinguishable. For comparison the IR spectra of pure GG and CDHA are shown. The dotted lines mark the OH^- and HPO_4^{2-} absorptions. Transmittance is expressed in arbitrary units.

Figure 2. Raman spectra of GG gels containing 2.5 mg/ml ALP mineralized for 7 days in media A-E. Control: GG containing 0 mg/ml ALP mineralized for 7 days in medium A.

Figure 3. XRD analysis of GG gels containing 2.5 mg/ml ALP mineralized for 7 days in media A-E.

Figure 4. TEM images (i, left column) and SAED diffraction patterns (ii, right column) of GG hydrogels containing 2.5 mg/ml ALP mineralized for 7 days in mineralization media A-E.

Figure 5. TEM images (left column) and corresponding EDS elemental mapping images showing distribution of the elements Ca (second column from left), Mg (third column from left) and P (fourth column from left) in GG hydrogels containing 2.5 mg/ml ALP mineralized for 7 days in media A-E.

Figure 6. SEM images of GG hydrogels containing 2.5 mg/ml ALP mineralized for 7 days in media A (A), B (B), C (C), D (D) and E (Ei, Eii). Samples containing 0 mg/ml ALP

mineralized for 7 days in medium A (A c) and medium E (E c) served as controls. Scale bar = 1 μm in all cases except for (Eii), where scale bar = 10 μm.

Figure 7. a: Dry mass percentage (a) of GG gels containing 2.5 mg/ml ALP mineralized for 7 days in media A-E. b: TGA determination of mass percentage attributable to mineral of GG hydrogels containing 2.5 mg/ml ALP after mineralization for 7 days in media A-E and subsequent lyophilization. Error bars show standard deviation.

Figure 8. a: Young's Modulus (Y.M.) of disc-shaped GG hydrogels containing 2.5 mg/ml ALP mineralized for 7 days in media A-E. b: Rheometrical measurement of storage modulus of disc-shaped GG hydrogels containing 2.5 mg/ml ALP mineralized for 7 days in media A-E. ^: $p < 0.001$ relative to all other groups; *: $p < 0.01$. Error bars show standard deviation.

Figure 9. a: Remaining mass percentage of GG gels containing 2.5 mg/ml ALP mineralized for 7 days in media A-E after 120 h incubation in a: extreme solution (citric acid buffer at pH 3) and b: simulation solution (Tris-HCl buffer at pH 7.4). Error bars show standard deviation.

Figure 10. Cytocompatibility testing of GG hydrogels containing 2.5 mg/ml ALP mineralized for 7 days in media A-E. GG containing 0 mg/ml ALP incubated for 7 days in medium A served as a control.

Figure 11. Live/dead staining of MC3T3-E1 cells cultured for 1 day (left column) and 6 days (right column) on GG hydrogels containing 2.5 mg/ml ALP mineralized for 7 days in media A (c,d), B (e,f), C (g,h), D (i,j) and E (k,l). GG containing 0 mg/ml ALP incubated for 7 days

in medium A served as a control (m,n). Tissue culture polystyrene served as a positive control (a,b). Green: viable cells; red: dead cells.

Figure 12. Viability of MC3T3-E1 cells cultured for 1 days and 11 days on GG hydrogels containing 2.5 mg/ml ALP mineralized for 7 days in n media A-E. GG containing 0 mg/ml ALP incubated for 7 days in medium A served as a control. Tissue culture polystyrene served as a positive control. Error bars show standard deviation.

Conflict of Interest statement: The authors have no conflict of interest.

No ethical approval was required for this study