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(54) **Title:** BIORESORBABLE-MAGNESIUM COMPOSITE

(57) **Abstract:** The invention relates to biocomposites comprising a polymeric matrix and a magnesium filler such as a water soluble magnesium salt. The use of elemental magnesium or magnesium alloy in the biocomposite is minimized and preferably avoided. The magnesium biocomposites can be used as bone implants.

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BIORESORBABLE-MAGNESIUM COMPOSITE

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of priority of Singapore Patent Application No.

5   10201407605R, filed November 14, 2014, the contents of which being hereby incorporated

by reference in its entirety for all purposes.

TECHNICAL FIELD

The invention relates generally to biocomposites, and in particular, to magnesium

biocomposites. More specifically, the use of elemental magnesium or magnesium alloy in the

10   biocomposite is minimized and preferably avoided. The magnesium biocomposites can be

used in the field of orthopaedic.

BACKGROUND

Magnesium (Mg) is an essential trace element of the human body, and has been

shown to play an important role in regulating biological functions, including that of bone

15   homeostasis. Currently, Mg has been administered as a dietary supplement, to be taken orally

to regulate bone mass and maintain bone health. Many patients who suffer from poor bone

mass and those who are pre-disposed to arthritis have been put on Mg-rich diet due to the fact

that Mg is important for bone mineralization. In addition, medical practitioners employ the

use of Mg for improving calcium (Ca) uptake, particularly in cases where an already-present,

20   exogenous supply of calcium is ineffective. In the case of orthopaedic implants, recent

developments have seen the use of Mg-coated implants for better host-implant integration.

Current systems employ the use of magnesium as an alloy in orthopaedic implants.

These implants are faced with challenges in controlling its degradation in vivo, due to the

potential side-effects of locally produced gas near its surface.

25   Accordingly, there remains a need to provide for an alternative magnesium

composition that overcomes, or at least alleviates, the above problem.

SUMMARY

The present invention makes use of low temperature pulverization of materials for forming biomaterials or biocomposites suitable for delivering magnesium to a subject to facilitate bone growth and repair, regeneration, and/or proliferation of host tissues. In particular, the biocomposite of present invention includes a polymeric matrix and a magnesium filler. The polymeric matrix may be provided for by any suitable biocompatible and/or biodegradable polymer (including copolymer). The magnesium filler may be provided for by any suitable soluble magnesium salt.

The present invention also provides for a method for forming the present biocomposite. The method includes low temperature processing of the biocompatible and/or biodegradable polymer (including copolymer) and the magnesium filler to form powders. The method further includes processing of the powders to form a thin film or a three-dimensional scaffold of the biocomposite.

The present invention further provides for a method for promoting bone growth and repair, regeneration, and/or proliferation of host tissues. The method includes implanting into a subject the present biocomposite at a site in need of bone growth and repair, regeneration, and/or proliferation of host tissues.

BRIEF DESCRIPTION OF THE DRAWINGS

In the drawings, like reference characters generally refer to the same parts throughout the different views. The drawings are not necessarily drawn to scale, emphasis instead generally being placed upon illustrating the principles of various embodiments. In the following description, various embodiments of the invention are described with reference to the following drawings.

FIG. 1 shows scanning electron microscopy images of cryomilled polycaprolactone (PCL)/tricalcium phosphate powders according to Example 1, at 600x and 2500x

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magnification, demonstrating that homogenous distribution of tricalcium phosphate (granular, approximately 2 μm) was achieved.

FIG. 2 shows representative scanning electron microscopy (SEM) images of various PCL/Mg films after immersing in phosphate buffer solution (PBS) at 37 °C for 4 hours according to Example 2. 100/0 represents PCL (100 wt%) without incorporated Mg (0 wt%). As the amount of Mg increases (i.e. 95/5, 85/15, 80/20 PCL/Mg), the size and the number of pores increased. Images were taken at 300x magnification, and scale bar represents 50 microns.

FIG. 3 shows the release profiles of various PCL/Mg films over 4 hours. 100/0 (i.e. pure PCL) did not exhibit any release while increasing amounts of Mg led to increased release, and higher rates of release.

FIG. 4 shows alkaline phosphatase (ALP) activity and Ca deposition of mesenchymal stem cells (MSCs) cultured in the absence of Mg (Mg free), normal serum (0.8 mM), and elevated Mg (8 mM) according to Example 4. Results indicated a peak in ALP activity on Day 3 in the 8 mM group, while showing 9 times higher activity as compared to Mg free and 0.8 mM groups. Ca deposition was markedly higher on Day 7 in the 8 mM group.

FIG. 5 shows osteocalcin expression of MSCs cultured on Day 11 according to Example 4, demonstrating the ability of maintaining osteogenic behaviour in the presence of long, prolonged exposure to elevated levels of Mg, as compared to MSCs cultured in initially high levels of Mg and slowly decreased to normal serum levels (0.8 mM). Images were taken at 4x magnification, and the scale bar represents 600 μm.

FIG. 6 shows mass loss profiles of various PCL/Mg biocomposite films according to Example 5. 100/0 and 95/5 PCL/Mg films behaved similarly, showing minimal mass loss (approximately 5 %) over the first 72 hours. On the other hand, 90/10 and 80/20 PCL/Mg films showed increased mass losses, and attained at least 25 % mass loss within the same time frame.

FIG. 7 shows hematoxylin and eosin (H&E) stains of PCL and PCL/Mg films implanted into the fatty pockets of pigs over a period of 3 months according to Example 6. Darkly stained cell nuclei, indicative of inflammatory events, were seen in the tissue structures surrounding the PCL films, while minimal indications of inflammation were observed in the PCL/Mg films.

FIG. 8 shows an illustration and prototype of a 3D scaffold with gradually increasing porosity, and a bioactive thin film that may be used as an envelope to guide bone tissue regeneration according to Example 7.

FIG. 9 shows the differentiation of human fetal mesenchymal stem cells (hfMSCs) into the following three lineages: adipogenic, chondrogenic, and osteogenic according to Example 8.

FIG. 10 shows the results of proliferation and differentiation of hfMSCs enabled by both magnesium chloride ($MgCl_2$) and magnesium sulphate ($MgSO_4$) according to Example 8. NaCl was used as a control to demonstrate that Cl^- did not influence proliferation and differentiation events.

FIG. 11 shows the effect of various Mg levels on hfMSC proliferation. hfMSC proliferation in the Mg free and 8 mM groups were compared and normalized against the basal level (0.8 mM), in both (A) proliferative and (B) osteogenic media. In both proliferative and osteogenic media, Mg starvation suppressed cell growth to a particularly large extent ($p < 0.001$). On the other hand, 8 mM of Mg supported cell proliferation ($p < 0.001$). Corresponding visualization with live/dead (FDA/PI) imaging led to corroborating results, with higher Mg indicating higher hfMSC proliferation.

FIG. 12 shows the effect of Mg on osteogenic differentiation according to Example 8. hfMSCs cultured under prolonged exposure to high levels of Mg (8 mM) exhibited lower levels of osteonectin (ON), collagen type I (coll-I), and transforming growth factor-*beta*

(TGF-β) expressions (FIG. 12). Upon switching to Mg-free conditions after 4 days, hfMSCs demonstrated higher potential for osteogenic differentiation as compared to 0.8 mM.

FIG. 13 shows osteocalcin (OC) protein expression as determined using immunocytochemical staining. From the results, OC expression was clearly demonstrated in the group exposed to decreasing concentrations of Mg, while prolonged exposure to Mg resulted in suppressed expression of OC from the hfMSCs.

DESCRIPTION

The following detailed description refers to the accompanying drawings that show, by way of illustration, specific details and embodiments in which the invention may be practised. These embodiments are described in sufficient detail to enable those skilled in the art to practise the invention. Other embodiments may be utilized and changes may be made without departing from the scope of the invention. The various embodiments are not necessarily mutually exclusive, as some embodiments can be combined with one or more other embodiments to form new embodiments.

The present invention discloses a fabrication method of a biocomposite comprising a polymeric matrix and a magnesium filler via a solvent-free and a heat-free technique. In other words, the formation technique does not involve a solvent. The formation technique further does not involve a heating step.

The polymeric matrix is preferably a well-studied biomaterial that is approved for use in clinics by the Food and Drug Administration (FDA) of the United States. In one example, polycaprolactone (PCL) has been used as a long-term drug delivery device, and has been employed as scaffolds for tissue engineered bone and cartilage, and more recently, for bone repair. The biomaterial preferably has a long degradation time.

Other suitable biomaterials include, but not limited to, poly(lactic-co-glycolic acid) (PLGA), poly(lactic acid) (PLA), poly(glycolic acid) (PGA), the family of polyhydroxyalkanoates (PHA), polyethylene glycol (PEG), polypropylene glycol (PPG),

polyesteramide (PEA), poly(lactic acid-co-caprolactone), poly(lactide-co-trimethylene carbonate), poly(sebacic acid-co-ricinoleic acid) and a combination thereof. The polymeric matrix may include one or more of the biomaterials.

Magnesium salts are chosen as the filler material due to their role in maintaining normal cellular function, and more specifically, for their role in regulating bone homeostasis. Recent studies have shown that osteogenic activities are regulated by Mg.

In various embodiments, a soluble magnesium salt as the filler is incorporated into the polymeric matrix. Suitable magnesium salts are those that dissolve in an aqueous environment or medium, and include, but not limited to, magnesium chloride ($MgCl_2$), magnesium sulphate ($MgSO_4$), or magnesium phosphate ($Mg_3(PO_4)_2$).

Importantly, the use of elemental magnesium or magnesium alloy in the biocomposite is minimized and preferably avoided.

Advantageously, the biocomposite can be rendered porous if the biocomposite is initially non-porous, or rendered more porous if the biocomposite is initially porous, by leaching or dissolving the magnesium salt upon contact with an aqueous environment or medium. This finds particular use as implants or scaffolds where the biocomposite affords the ability to create a gradually porous scaffold over time, matched by the simultaneous dissolution of Mg into the surrounding microenvironment after implantation into the body of a subject. In this sense, the gradual increasing porosity of a biocomposite scaffold is symbolic of a 'smart' scaffold.

Another advantage of the biocomposite lies in the release of Mg, which has been demonstrated and established to be an important trace element for potentiating osteogenic differentiation.

As illustrated in the examples described in later paragraphs, the amount of magnesium filler initially present in the biocomposite may affect the degradation time of the polymeric matrix and cellular response to the magnesium. For example, based on the

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examples results it is hypothesized that early supplementation of Mg directs osteogenic differentiation of mesenchymal stem cells (MSCs). By exposing MSCs to elevated levels of Mg (8 mM) for four days and subsequently switching back to basal (0.8 mM) and Mg-free conditions, it was demonstrated that osteogenic factors such as ALP, osteonectin (ON),

5   collagen-type 1 (coll-1) were upregulated, as compared to prolonged exposure of elevated Mg levels. Taken together, these results suggest that extracellular Mg may play important roles in bone tissue engineering.

Preferably, the biocomposite includes the magnesium filler of between 5 and 40 wt% based on the total weight of the biocomposite. For example, the magnesium filler may be

10   present in 5 wt%, 6 wt%, 7 wt%, 8 wt%, 9 wt%, 10 wt%, 11 wt%, 12 wt%, 13 wt%, 14 wt%, 15 wt%, 16 wt%, 17 wt%, 18 wt%, 19 wt%, 20 wt%, 21 wt%, 22 wt%, 23 wt%, 24 wt%, 25 wt%, 26 wt%, 27 wt%, 28 wt%, 29 wt%, 30 wt%, 31 wt%, 32 wt%, 33 wt%, 34 wt%, 35 wt%, 36 wt%, 37 wt%, 38 wt%, 39 wt%, or 40 wt%.

As mentioned in earlier paragraphs, the biocomposite fabrication technique does not

15   involve a solvent or a heating step. In various embodiments, the method for forming the present biocomposite includes first mixing of a polymeric matrix and a magnesium filler. After mixing, the mixture is processed in a cryomill to obtain fine powder. In one embodiment, the composites were pre-weighed using a microbalance and loaded into a cryogenic vial with a ball-to-mass ratio of 30:1. The cryomilling protocol was set to be 6 to 8

20   min of pre-cooling in liquid nitrogen and 20 min of continuous milling for one cycle. One advantage of employing a cryomilling technique is that particle size reduction efficiency is improved and homogenous distribution may be achieved in a single processing step.

The fine powder may be further processed to form a thin film or a three-dimensional (3D) scaffold. In certain embodiments, the 3D scaffold may be fabricated using an additive

25   manufacturing technique or using a die set along with the incorporation of 50 vol% of sodium chloride, followed by leaching in water.

In one embodiment, a thin film, such as 60 microns or less in thickness, of the biocomposite can be formed by pressing the fine powder between two stainless steel sheets. For example, the PCL composite films may be thermally pressed into films of thickness approximately 30 to 60 μm. Briefly, a known mass of composite is placed between two

5   stainless steel sheets on a heat press system with temperature control. Temperature is elevated to 100 °C and pressure is applied for 30 min. The pressed film is then allowed to cool to room temperature via normal convection cooling.

The continuity of the biocomposite thin film structure has been shown to play a considerable role in bone and vascular regeneration, both of which are important in tissue

10 regeneration. The thin film fabricated by the present method is preferably a continuous film and is non-porous. While a porous thin film may be desired for directing ingrowth, a significant trade-off is present in the mechanical properties of the porous biocomposite, as a substantially porous material may have compromised mechanical properties. Present biocomposite provides an important advantage in that the biocomposite can be made

15 substantially non-porous at the beginning to provide better mechanical integrity. Subsequently, upon interaction *in vivo* with body fluid, soluble magnesium may then be leached out over time, gradually creating a porous structure that may bear resemblance to other existing films and/or scaffolds.

Accordingly, the present invention further provides a method for promoting bone

20 growth and repair, regeneration, and/or proliferation of host tissues. The method includes implanting into a subject the present biocomposite at a site in need of bone growth and repair, regeneration, and/or proliferation of host tissues.

In order that the invention may be readily understood and put into practical effect, particular embodiments will now be described by way of the following non-limiting

25 examples.

EXAMPLES

EXAMPLE 1

Cryomilling was employed as a method to achieve efficient and homogenous distribution of presently disclosed biocomposite. Polycaprolactone (PCL) particles were pulverized into fine powder after a cryomilling process of 20 min. It is shown in the high magnification images of FIG. 1 that the filler (in this illustration, tricalcium phosphate) was well distributed in the PCL matrix.

EXAMPLE 2

To demonstrate an increased surface porosity of presently disclosed biocomposites, a biocomposite including PCL and a soluble magnesium salt (i.e. a PCL/Mg biocomposite) was processed by cryomilling, and subsequently fabricated into a continuous film structure (FIG. 2). These biocomposite films were then soaked in a phosphate buffer solution (PBS) at 37 °C. PBS is a buffer solution commonly used to simulate bodily fluids. After immersing for various times up to 4 hours, the films were then retrieved and imaged, revealing a highly porous surface due to the selective leaching of Mg. The porosity of the biocomposite films increased with increasing amounts of Mg salt.

EXAMPLE 3

To demonstrate the release profile of Mg from presently disclosed biocomposites, samples were taken from the PBS in which the PCL/Mg films were immersed according to Example 2. The results are presented in FIG. 3. As expected, 100/0 (i.e. pure PCL) films did not exhibit any release of Mg into PBS over the release period. Mg release correlated directly with the amount of Mg incorporated, increasing with 80/20 PCL/Mg demonstrating the highest release at the end of 4 hours. In addition, the rate of release also increased with increasing amount of Mg.

EXAMPLE 4

To demonstrate that Mg is able to promote osteogenic differentiation, *in vitro*, cellular studies were conducted using mesenchymal stem cells (MSCs) in the presence of elevated levels of Mg. MSCs are known to be present within the bone marrow niche and play a role in regulating bone by differentiating into osteoblastic or osteoclastic phenotypes. From the results, alkaline phosphatase (ALP) activity peaked at Day 3 in the presence of 8 mM of Mg, showing 9 times higher expression levels as compared to MSCs cultured in the absence of Mg (Mg free) and under normal serum conditions (0.8 mM). Calcium deposition was found to be markedly higher on Day 7 as compared to both Mg free and 0.8 mM groups (FIG. 4). Immunocytochemical (ICC) staining of MSCs with osteocalcin demonstrated expression at Day 11 (FIG. 5), regardless of the exposure time to elevated levels of Mg.

EXAMPLE 5

To demonstrate that presently disclosed biocomposites exhibit a reduced degradation time, PCL/Mg biocomposite films were placed in 1 M sodium hydroxide (NaOH) solution at 37 °C. Results were plotted out in terms of mass loss (%) against time (hours) (FIG. 6), and indicated that with increase in Mg incorporation into the PCL biocomposites, degradation was significantly accelerated particularly in the case of 90/10 and 80/20 biocomposite films, where mass loss reached at least 25 %. On the other hand, 95/5 films behaved in a similar fashion to pure PCL films, showing approximately 5% mass loss over the first 72 hours.

EXAMPLE 6

Presently disclosed 80/20 PCL/Mg films were implanted into pigs over a period of three months. While both PCL and PCL/Mg biocomposite films were well-accepted by the host without events of rejection, the inflammatory response was markedly different (FIG. 7). Darkly-stained cell nuclei indicative of inflammatory events persisted at 3 months in the PCL group, while minimal inflammation was observed in the PCL/Mg group, suggesting that PCL/Mg biocomposite films have the advantage in regulating the inflammatory environment upon implantation.

EXAMPLE 7

The presently disclosed PCL/Mg biocomposite may be fabricated as a 3-dimensional (3D) scaffold body for use as a supporting architecture for directing bone in-growth while providing mechanical stability during the regenerative process. Its gradually increasing porosity promotes bone tissue in-growth (FIG. 8) while the release of Mg stimulates osteogenic differentiation of MSCs. In this instance, the 3D scaffold can be used within the craniomaxillofacial area, under slight to moderate mechanical loading.

The PCL/Mg biocomposite may also be fabricated as a thin film to serve as a bioactive sheet that has good flexural properties and high strength for use as an envelope to prevent fibrous tissue invasion while promoting osteogenic growth to bridge the defect area (FIG. 8). For instance, the thin film may be of not more than 50 μm thick.

EXAMPLE 8

In this example, the influence of exogenous magnesium on mesenchymal stem cell proliferation and early osteogenic activity is investigated. Highly osteogenic human fetal mesenchymal stem cells (hfMSCs) were cultured in varying concentrations of Mg and in varying exposure times to Mg in an attempt to study the effects of prolonged and transient exposures to elevated concentrations of Mg on hfMSC proliferation and osteogenesis. From the results, exposure to elevated levels of Mg (8 mM) led to improved proliferation of hfMSCs as compared to basal levels (0.8 mM), while prolonged exposure to Mg-free conditions resulted in significant cell death. When hfMSCs were cultured in 8 mM Mg for 4 days and subsequently maintained in lower Mg concentrations, osteonectin (ON), collagen-1, bone morphogenetic protein-1, -4, -6 (BMP-1, -4, -6) were significantly upregulated as compared to cultures maintained in prolonged, elevated levels of Mg over 8 days. Taken together, these results suggest that an initial elevated level of Mg is necessary to kick-start the osteogenic differentiation of MSCs.

MATERIALS AND METHODS

Human fetal MSCs isolation and culture

Human fetal MSCs (hfMSCs) were obtained as previously described (*Zhang Z-Y,*
Teoh S-H, Chong MSK, Lee ESM, Tan L-G, Mattar CN, et al. Neo-vascularization and bone
formation mediated by fetal mesenchymal stem cell tissue-engineered bone grafts in critical-
5 *size femoral defects. Biomaterials. 2010;31:608-20).* Cells were seeded in a flask (T175,
Nunc, Rochester) at a density of 10^6/ ml in Dulbecco's Modified Eagle's medium (DMEM)
supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin
(pen/strep) (D10). Non-adherent cells were removed with media change on day three. The
remaining adherent cells were subsequently used for this work (Passage 3 – 6).

10   **Multilineage mesenchymal differentiation of hfMSCs**

The multilineage differentiation potential of hfMSCs was evaluated for the following:
adipogenic, chondrogenic, and osteogenic differentiation. To induce adipogenic
differentiation, cells were plated and cultured in adipogenic media (basal D10 supplemented
with 5 µg/ml insulin, 10^{-6} M dexamethasone and 0.6 x 10^{-4} indomethacin (Sigma Aldrich,
15 USA) for 21 days with media change every three days. Oil-Red O staining was then
conducted for the presence of lipid vacuoles. For the induction of chondogenic
differentiation, hfMSCs were pelleted and cultured in chondrogenic media DMEM
supplemented with 0.1 µM dexamethasone, 0.17 mM ascorbic acid, 1.0 mM sodium
pyruvate, 0.35 mM L-Proline, 1 % ITS (BD Pharmingen, USA), 1.25 mg/ml BSA, 5.33
20 µg/ml linoleic acid, 0.01 µg/ml TGF-β) for 28 days with media change every three days. The
pellets were fixed with formalin, embedded in paraffin wax and cut before staining with
Safranin O. To induce osteogenic differentiation, hfMSCs were cultured in osteogenic media
(D10 supplement with 10 mM β-glycerophosphate, 10^{-8} dexamethasone, 0.2 mM ascorbic
acid) for 21 days, with media change every three days. The cells were then fixed in 4 %
25 paraformaldehyde and stained with von Kossa (2 w/v% silver nitrate), and exposed to
ultraviolet light for 30 mins.

Experimental culture of hfMSCs

hfMSCs that were isolated were exposed to the following conditions: For this purpose, Mg-free DMEM (BioRev, Singapore) was supplemented with 10 % FBS/1% pen/strept, and supplemented with variable amounts of magnesium chloride (Sigma Aldrich, Singapore) to achieve the following concentrations: 0.8 mM, and 8 mM. This shall henceforth be denoted as "proliferative media". "Osteogenic media" was prepared by supplementing various proliferative media with 10 mM β-glycerophosphate, 10^{-8} dexamethasone, and 0.2 mM ascorbic acid.

Cell proliferation and viability

hfMSCs were seeded at a density of 7.5 k/cm^2 in 6-well plates in the various proliferative and osteogenic medium. At days 3 and 7, AlamarBlue® reagent (Invitrogen, Singapore) was added according to the manufacturer's instruction, and incubated in the dark for 1.5 hours before fluorescence reading at 590 nm with a microplate reader (Spectramax). In addition, cells were stained with fluorescein diacetate (FDA) and propidium iodide (PI) to visualize live and dead cells.

Alizarin red and von Kossa

hfMSCs were seeded at confluence (20 k/cm^2), and cultured in both proliferative and osteogenic media for 14 days. Alizarin red stains were prepared according to the manufacturer's instruction, and maintained at pH 4.2. hfMSCs were fixed with 4 % paraformaldehyde for 5 mins, washed and stained with Alizarin red for 10 mins under gentle shaking. Subsequently, they were thoroughly washed and air-dried before visualization with a microscope. von Kossa staining was done as mentioned earlier.

ALP and calcium

hfMSCs cultured in both proliferative and osteogenic medium were rinsed with phosphate buffer saline (PBS) solution and incubated in a mixture of collagenase and trypsin for 4 hours at 37 °C. Subsequently, they underwent three freeze-thaw cycles to lyse the cells,

and the lysates were evaluated for ALP activity according to the manufacturer's instructions. The pellet obtained was stored separately for evaluating calcium deposition. The pellet obtained previously was dissolved overnight in 0.5 N acetic acid. A calcium assay was then used to quantify the amount of Ca deposited by measuring its absorbance at 612 nm, in

5   accordance with the manufacturer's instructions.

Real-time polymerase chain reaction

Real-time polymerase chain reaction (RT-PCR) was performed to study the expression of early osteogenic genes by hfMSCs cultured in 6-well plates under proliferative and osteogenic conditions. Total ribonucleic acid (RNA) was harvested by using a Reverse

10   Transcription System (Promega, USA) on days 4 and 8. Next, 1 mg total RNA was reverse-transcribed to complementary deoxyribonucleic acid (cDNA). Finally, the CFX Connect system (BioRad, Singapore) was used to conduct quantitative real-time PCR with TaqMan Universal PCR Master Mix and gene-specific PCR primers including osteocalcin, coll-1, transforming growth factor-beta (TGF-β) and glyceraldehyde-3-phosphate dehydrogenase

15   (GAPDH). Gene expression was normalized to GAPDH by using the comparative $2^{-\Delta\Delta Ct}$ method. The primers used in this experiment are shown in Table 1. All PCRs were carried out in triplicate.

Table 1. Details of Primers

Gene	Primer sequence (both 5' to 3')	Annealing Temperature (°C)	Product size (basepairs)	Accession Number
GAPDH	F: CCACCCATGGCAAATTCC	58	67	NM_00128974 6.1
	R: GGATTTCCATTGATGACAAGCTT			
ON	F: CGCGGTCCTTCAGACTGCCC	58	85	NM_003118.3
	R: AGGCCCTCATGGTGCTGGGA			
COL1A1	F: AGGACAAGAGGCATGTCTGGTT	58	70	XM_006721170 3.1
	R: CCCTGGCCGCCATACTC			
TGFβ1	F: GGCAGTGGTTGAGCCGTGGA	58	531	NM_000660.5
	R: TGTTGGACAGCTGCTCCACCT			

Fabrication of Mg-releasing PCL films

PCL was placed together with $MgCl_2$ in a cryomill (Retsch®, Germany). Using similar settings as described elsewhere (*Lim J, Chong MS, Chan JK, Teoh SH. Polymer Powder Processing of Cryomilled Polycaprolactone for Solvent-free Generation of Homogeneous Bioactive Tissue Engineering Scaffolds. Small. 2014;17:201302389*), fine powders of PCL/Mg were generated, and subsequently pressed between two stainless steel sheets to obtain PCL/Mg films with a thickness of 30 to 40 μm. In this example, composites were fabricated using cryomilling. Composites were pre-weighed using a microbalance and loaded into the cryogenic vial with a ball-to-mass ratio of 30:1. The cryomilling protocol was: 6 to 8 min of pre-cooling in liquid nitrogen and 20 min of continuous milling for one cycle. The PCL composite films may be thermally pressed into films of thickness approximately 30 to 60 μm. Briefly, a known mass of composite is placed between two stainless steel sheets on a heat press system with temperature control. Temperature is elevated to 100 °C and pressure added for 30 min. The pressed film is then allowed to cool to room temperature via normal convection cooling. 4 compositions were fabricated: (PCL/Mg) 100/0, 95/5, 90/10, 80/20.

Statistics

Student's t-test (two-tailed) was conducted on all data to determine statistical significance. A confidence level of 95 % was taken to be statistically significant, as represented by $p < 0.05$.

RESULTS

Multilineage differentiation potential of hfMSCs

hfMSCs were shown here to be able to differentiate into the following three lineages: adipogenic, chondrogenic, and osteogenic (FIG. 9), a result in agreement with previous reports (*Zhang ZY, Teoh SH, Chong MSK, Schantz JT, Fisk NM, Choolani MA, et al. Superior Osteogenic Capacity for Bone Tissue Engineering of Fetal Compared with*

Perinatal and Adult Mesenchymal Stem Cells. Stem Cells. 2009;27:126-37). Accordingly, hfMSCs that were differentiated down the adipogenic pathway presented lipid vacuoles; chondrogenic hfMSCs displayed positive staining for Safranin O; osteogenic hfMSCs displayed positive staining for von Kossa.

5   **Validating the source of Mg**

The sources of Mg were evaluated to determine their suitability for this study. From the results, both $MgCl_2$ and $MgSO_4$ allowed proliferation and differentiation of hfMSCs (FIG. 10).

Effect of various Mg levels on hfMSC proliferation

10   hfMSC proliferation in the Mg free and 8 mM groups were compared and normalized against the basal level (0.8 mM), in both proliferative and osteogenic media. In both proliferative and osteogenic media, Mg starvation suppressed cell growth to a particularly large extent (FIG. 11; $p < 0.001$). On the other hand, 8 mM of Mg supported cell proliferation ($p < 0.001$). Corresponding visualization with live/dead (FDA/PI) imaging led to

15   corroborating results, with higher Mg indicating higher hfMSC proliferation (FIG. 11).

Effect of Mg on osteogenic differentiation

hfMSCs cultured under prolonged exposure to high levels of Mg (8 mM) exhibited lower levels of ON, coll-1, and TGF-β expressions (FIG. 12). Upon switching to Mg-free conditions after 4 days, hfMSCs demonstrated higher potential for osteogenic differentiation

20   as compared to 0.8 mM.

Osteocalcin protein expression

Expression of osteocalcin (OC) protein was determined using immunocytochemical staining. From the results (FIG. 13), OC expression was clearly demonstrated in the group exposed to decreasing concentrations of Mg, while prolonged exposure to Mg resulted in

25   suppressed expression of OC from the hfMSCs.

Discussion

The intriguing role of Mg in directing osteogenesis has been of recent interest, due to the seemingly phenomenological observations of enhanced osseointegration in coated implants. Given that Mg is complexed to adenosine triphosphate (ATP), which is ternary complex of the catalytic subunit of cAMP-dependent protein kinase, it is understandable that Mg plays an important role in regulating many cellular processes, including that of cell adhesion to substrates. However, its purported role in osteogenesis remains profound knowledge, and an attempt was made in this study to understand its importance by hypothesizing the temporal effect of Mg on osteogensis.

First, it is attempted to understand the effect of various sources of Mg on MSC proliferation. It was established and demonstrated that soluble magnesium salts including $MgCl_2$ and $MgSO_4$ were suitable. On this note, the use of $MgCl_2$ and $MgSO_4$ supplementation for the study of osteogenesis has previously been validated. Thereafter, it was shown here that higher levels of Mg (8 mM) resulted in higher MSC proliferation over time in both culture and osteogenic medium while the lack of Mg (Mg-free) resulted in inhibited cell proliferation. This was similarly reported in human osteoblast-like cells (MG-63, SaOS, and U2-OS), clearly cementing the role of Mg in DNA and protein synthesis through melastatin-like transient receptor potential 6 and 7 (TRPM6 and 7). More accurately, studies have shown that the mammalian target of rapamycin (mTOR), a protein kinase in the PI3-K pathway, is regulated by MgATP. These studies, together with the present results, verified and confirmed that Mg has a positive influence on MSC proliferation.

In the presence of soluble osteogenic factors such as dexamethasone and β-glycerophosphate, MSCs already have a strong predisposition towards the osteogenic lineage. When further supplemented with higher Mg (8 mM), characteristic hallmarks of osteogenesis such as ALP activity and calcium deposition were further upregulated. Over 7 days, it was demonstrated temporal expression of ALP in response to varying Mg concentrations, with ALP expression peaking on day 3 in 8 mM of Mg as compared to basal levels (0.8 mM),

which possibly occurred either on day 7 or beyond. In tandem with ALP expression on day 3 in the 8 mM Mg group, calcium deposition was significantly expressed on day 7. From the literature, Leem et al. (*Leem Y-H, Lee K-S, Kim J-H, Seok H-K, Chang J-S, Lee D-H. Magnesium ions facilitate integrin alpha 2- and alpha 3-mediated proliferation and enhance alkaline phosphatase expression and activity in hBMSCs. Journal of Tissue Engineering and Regenerative Medicine. 2014; doi:10.1002/term.1861*) also reported enhanced ALP activity within the first 72 hours (3 days) in the presence of 2.5 mM of Mg. While the expression of ALP is understandably transient, it is an important, early indication of osteogenesis. ALP may traditionally be known as pyrophosphatase, which is an enzyme that is responsible for the production of inorganic phosphate, which is then transported through the cell membrane via vesicles for interaction with available, unbound calcium ions to form calcium phosphate (CaP) crystals.

According to a report by Li et al. (*Li RW, Kirkland NT, Truong J, Wang J, Smith PN, Birbilis N, et al. The influence of biodegradable magnesium alloys on the osteogenic differentiation of human mesenchymal stem cells. Journal of Biomedical Materials Research Part A. 2014*), hfMSCs proliferated well in the presence of 0.5-0.8 mM of Mg, while at high levels of Mg (ca. 5-8 mM), they showed poorer proliferation. On the other hand, when hfMSCs were exposed to higher levels of Mg, their differentiation towards the osteogenic phenotype was increased (14 day culture), which is in agreement with the present results. However, the medium extracts used in the previous study were diluted with other alloying metals, possibly resulting in the delayed onset of ALP activity. In the present study, supplementation of Mg to culture medium was a more direct way of understanding its effects on MSC differentiation, to which it was demonstrated an earlier peak in ALP expression on day 3.

To understand the effect of decreasing local Mg concentration over time (as per *in vivo* orthopaedic implants), hfMSCs were cultured in 8 mM of Mg over 4 days, before

switching to 0.8 mM and Mg-free conditions. Present results demonstrated that the osteogenic potential of MSCs was significantly upregulated with decreasing concentrations of Mg due to the upregulation of osteogenic genes such as ON, coll-I, and TGF-β. On the other hand, prolonged exposure of hfMSCs to elevated Mg did not result in increased

5   osteogenic activity, which is in agreement with a previous study by Leidi et al. (*Leidi M, Dellera F, Mariotti M, Maier JA. High magnesium inhibits human osteoblast differentiation in vitro. Magnes Res. 2011;24:1-6*) and Yang et al. (*Yang C, Yuan G, Zhang J, Tang Z, Zhang X, Dai K. Effects of magnesium alloys extracts on adult human bone marrow-derived stromal cell viability and osteogenic differentiation. Biomedical Materials. 2010;5:045005*).

10   In the latter study by Yang et al., human bone marrow MSCs (bMSCs) maintained in culture extracts taken from Mg alloys (AZ91D, NZ30K) and Mg metals demonstrated upregulation of osteopontin (OPN) at day 6 at the transcription level but not at the protein level. ALP levels were also similar to control (no added Mg) throughout the study period. These evidences, taken in consideration with the present observation here, suggest that transient

15   exposure to elevated levels of Mg may potentiate early differentiation of hfMSCs.

Conclusion

In summary of this example, the response of hfMSCs to different levels of Mg was studied, with the aim of understanding the positive effect of Mg-coated orthopaedic implants. It is hypothesized and demonstrated that transient exposure to elevated levels of Mg led to

20   significant upregulation of osteogenic genes and proteins, leading to substantial calcium deposition. These results are likely to facilitate understanding of the observations related to the osteogenic effects of Mg-coated implants *in vivo*.

By "comprising" it is meant including, but not limited to, whatever follows the word "comprising". Thus, use of the term "comprising" indicates that the listed elements are

25   required or mandatory, but that other elements are optional and may or may not be present.

By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of". Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present.

The inventions illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

By "about" in relation to a given numerical value, such as for temperature and period of time, it is meant to include numerical values within 10% of the specified value.

The invention has been described broadly and generically herein. Each of the narrower species and sub-generic groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

Other embodiments are within the following claims and non- limiting examples. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

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CLAIMS

1.   A biocomposite comprising a polymeric matrix and a magnesium filler, wherein the magnesium filler comprises a soluble magnesium salt.

2.   The biocomposite of claim 1, wherein the magnesium filler does not comprise a magnesium alloy or elemental magnesium.

3.   The biocomposite of claim 1 or 2, wherein the magnesium salt comprises magnesium chloride ($MgCl_2$), magnesium sulphate ($MgSO_4$), or magnesium phosphate ($Mg_3(PO_4)_2$).

4.   The biocomposite of any one of claims 1 to 3, wherein the magnesium filler comprises 5 to 40 wt% based on the total weight of the biocomposite.

5.   The biocomposite of claim 4, wherein the polymeric matrix comprises a polymer selected from the group consisting of polycaprolactone (PCL), poly(lactic-co-glycolic acid) (PLGA), poly(lactic acid) (PLA), poly(glycolic acid) (PGA), the family of polyhydroxyalkanoates (PHA), polyethylene glycol (PEG), polypropylene glycol (PPG), polyesteramide (PEA), poly(lactic acid-co-caprolactone), poly(lactide-co-trimethylene carbonate), poly(sebacic acid-co-ricinoleic acid) and a combination thereof.

6.   A method for forming a biocomposite comprising a polymeric matrix and a magnesium filler, wherein the magnesium filler comprises a soluble magnesium salt, the method comprising:

mixing the polymeric matrix and magnesium filler;

processing the mixture of the polymeric matrix and magnesium filler in a cryomill to obtain fine powder and processing the fine powder to form a thin film or a three-dimensional (3D) scaffold.

7.   The method of claim 6, wherein processing the mixture of the polymeric matrix and magnesium filler in a cryomill to obtain fine powder comprises loading pre-weighed mixture into a cryogenic vial with a ball-to-mass ratio of 30:1, pre-cooling the

cryogenic vial in liquid nitrogen for 6 to 8 minutes, and continuous milling for one cycle for 20 minutes.

8.　　The method of claim 6 or 7, wherein the biocomposite thin film is formed by thermally pressing the fine powder between two stainless steel sheets in a heat press system.

9.　　The method of claim 8, wherein the fine powder are thermally pressed at 100 °C with pressure applied for a period of time, followed by cooling the pressed film to room temperature.

10.　　The method of any one of claims 6 to 9, wherein the 3D biocomposite scaffold is formed by an additive manufacturing technique, or using a die set along with the incorporation of 50 vol% of sodium chloride, followed by leaching in water.

11.　　The method of any one of claims 6 to 10, wherein the magnesium filler does not comprise a magnesium alloy or elemental magnesium.

12.　　The method of any one of claims 6 to 11, wherein the magnesium salt comprises magnesium chloride ($MgCl_2$), magnesium sulphate ($MgSO_4$), or magnesium phosphate ($Mg_3(PO_4)_2$).

13.　　The method of any one of claims 6 to 12, wherein the magnesium filler comprises 5 to 40 wt% based on the total weight of the biocomposite.

14.　　The method of any one of claims 6 to 13, wherein the polymeric matrix comprises a polymer selected from the group consisting of polycaprolactone (PCL), poly(lactic-co-glycolic acid) (PLGA), poly(lactic acid) (PLA), poly(glycolic acid) (PGA), the family of polyhydroxyalkanoates (PHA), polyethylene glycol (PEG), polypropylene glycol (PPG), polyesteramide (PEA), poly(lactic acid-co-caprolactone), poly(lactide-co-trimethylene carbonate), poly(sebacic acid-co-ricinoleic acid) and a combination thereof.
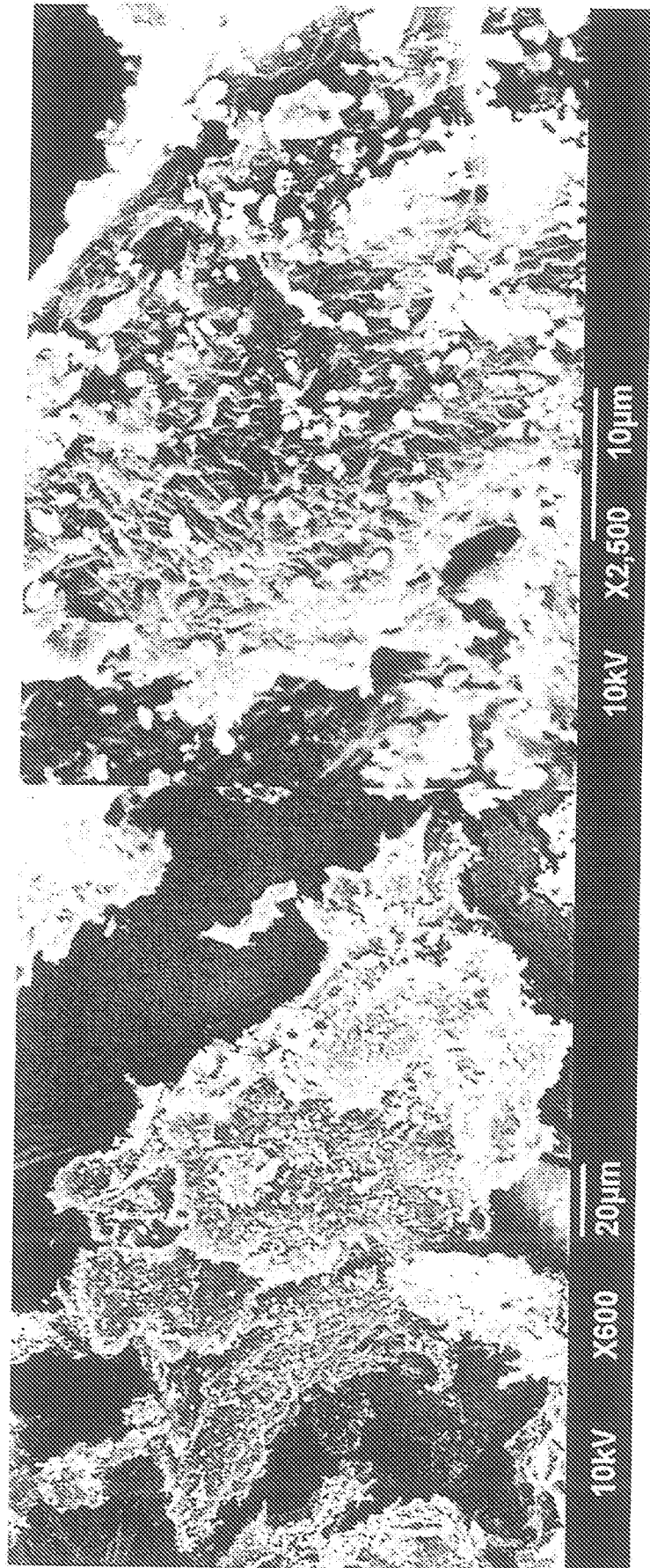
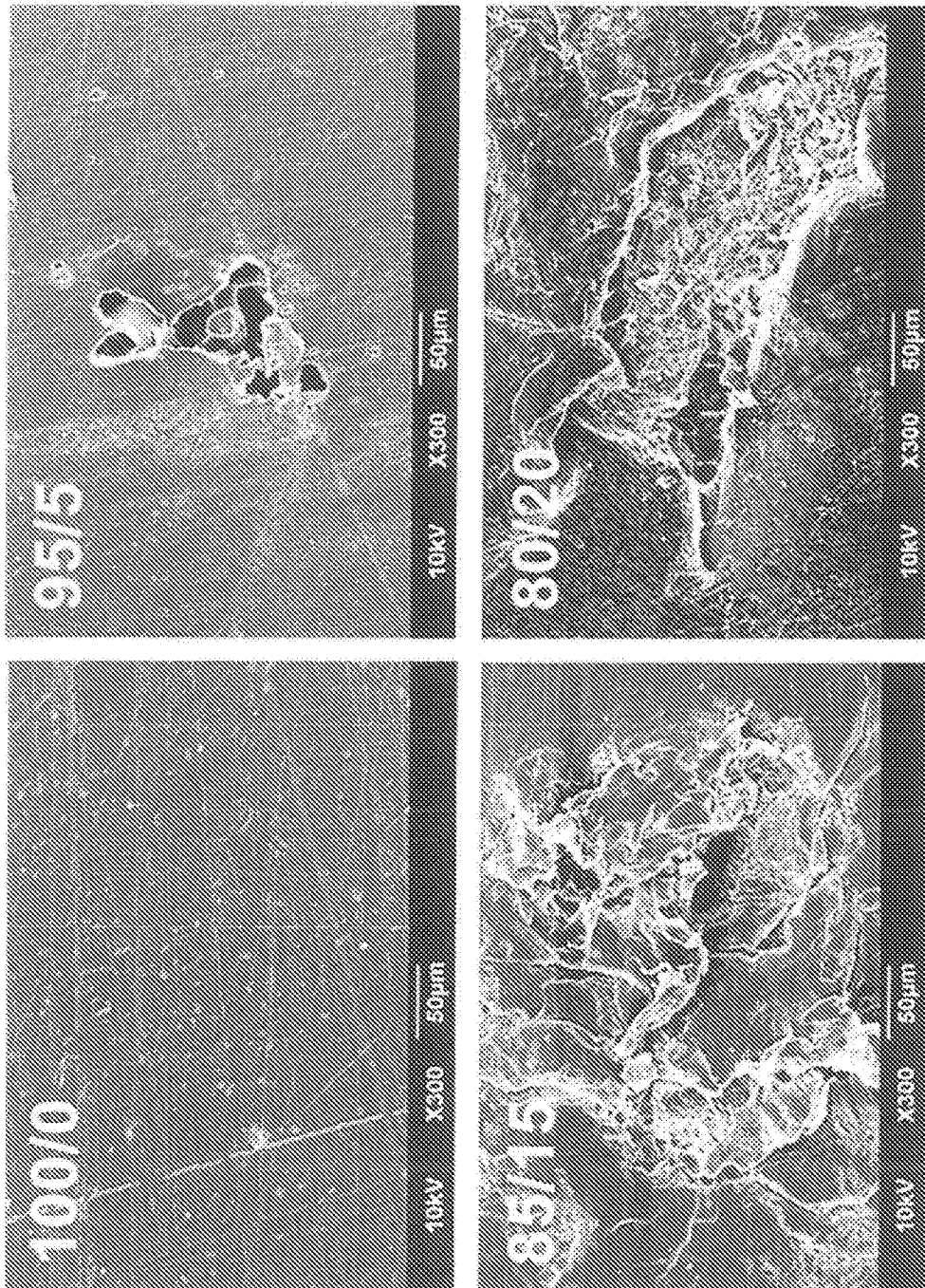15.　　A method for promoting bone growth and repair, regeneration, and/or proliferation of host tissues, the method comprising implanting into a subject a biocomposite at a site in need of bone growth and repair, regeneration, and/or proliferation of host tissues,

wherein the biocomposite comprises a polymeric matrix and a magnesium filler, and wherein the magnesium filler comprises a soluble magnesium salt.

16.   The method of claim 15, wherein the magnesium filler does not comprise a magnesium alloy or elemental magnesium.

17.   The method of claim 15 or 16, wherein the magnesium salt comprises magnesium chloride ($MgCl_2$), magnesium sulphate ($MgSO_4$), or magnesium phosphate ($Mg_3(PO_4)_2$).

18.   The method of any one of claims 15 to 17, wherein the magnesium filler comprises 5 to 40 wt% based on the total weight of the biocomposite.

19.   The method of any one of claims 15 to 18, wherein the polymeric matrix comprises a polymer selected from the group consisting of polycaprolactone (PCL), poly(lactic-co-glycolic acid) (PLGA), poly(lactic acid) (PLA), poly(glycolic acid) (PGA), the family of polyhydroxyalkanoates (PHA), polyethylene glycol (PEG), polypropylene glycol (PPG), polyesteramide (PEA), poly(lactic acid-co-caprolactone), poly(lactide-co-trimethylene carbonate), poly(sebacic acid-co-ricinoleic acid) and a combination thereof.
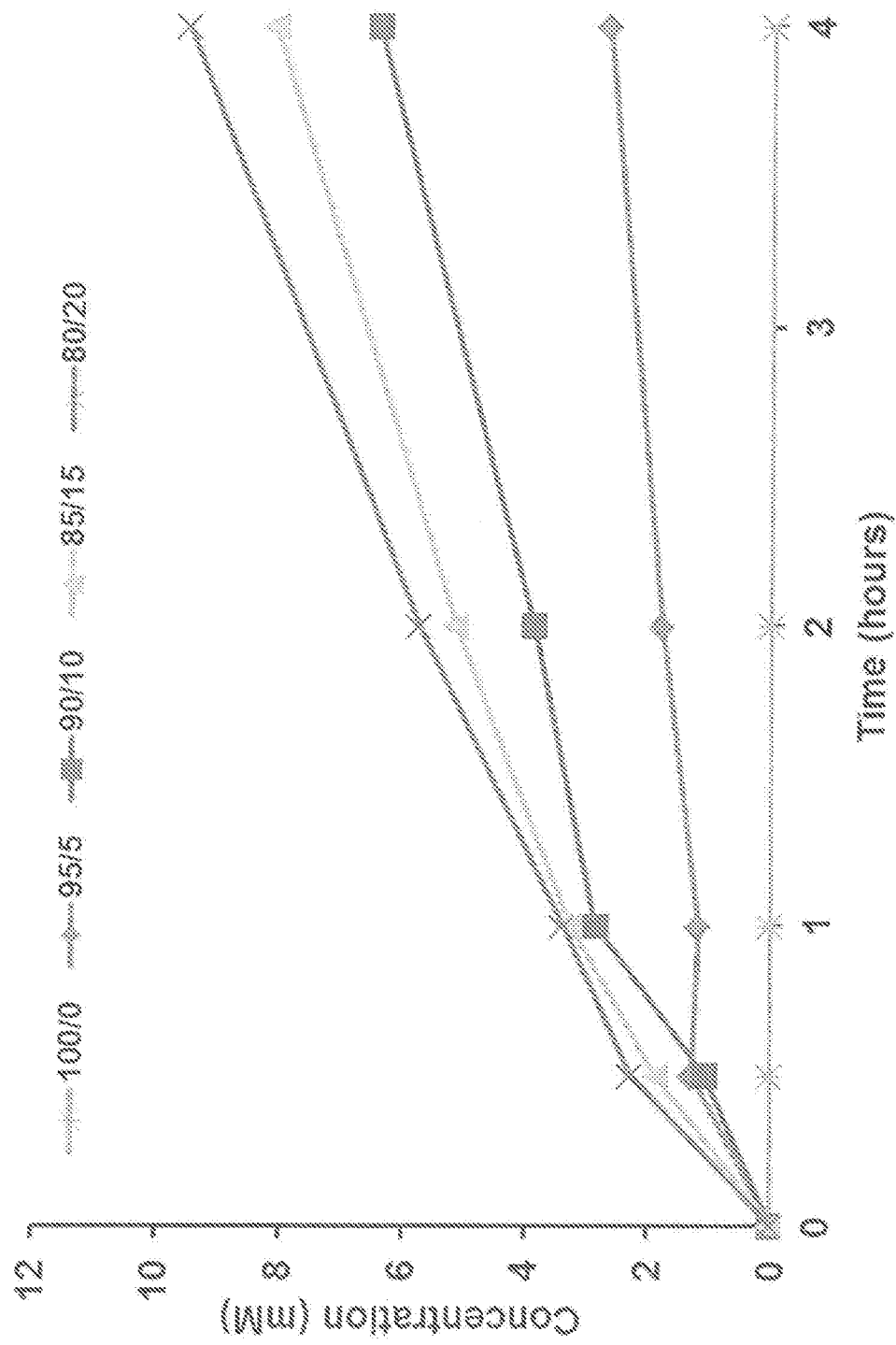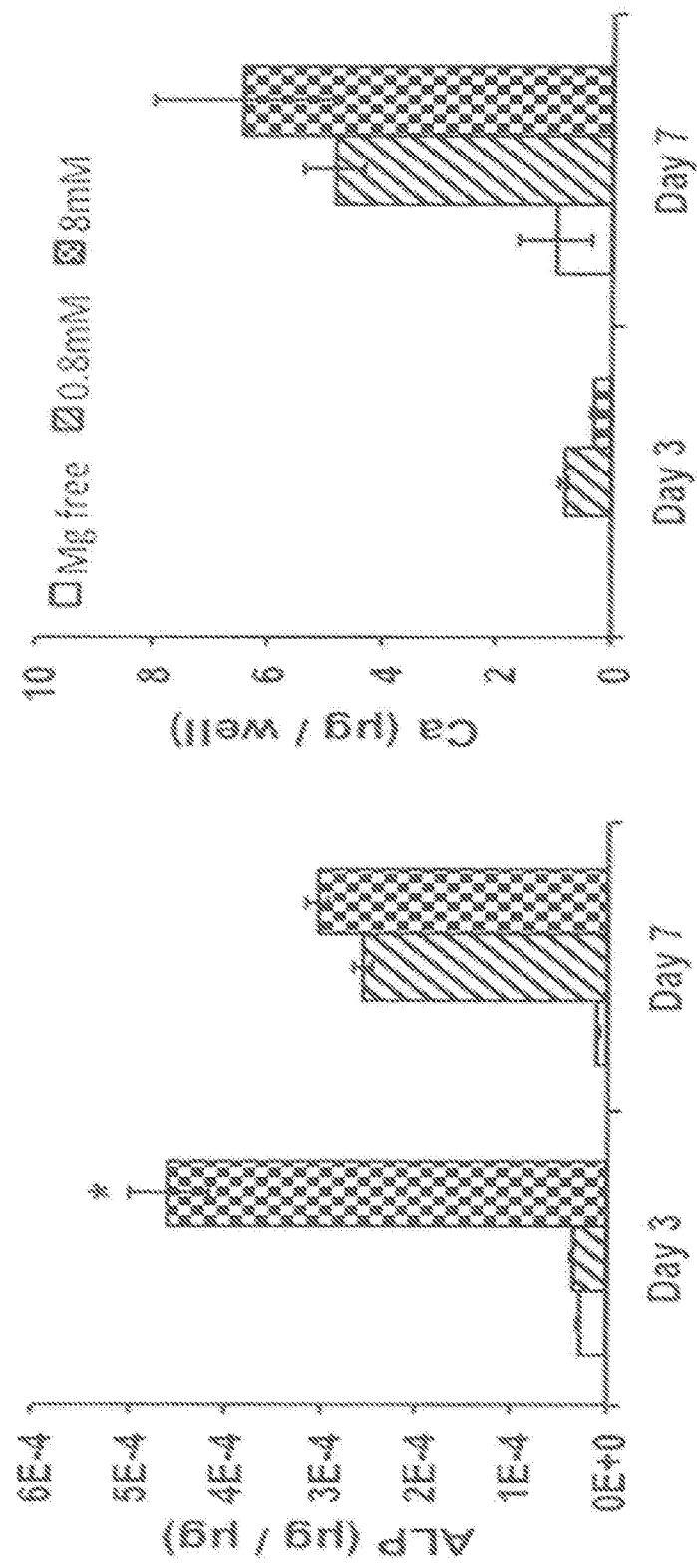
FIG. 1

FIG. 2

FIG. 3

FIG. 4

Osteocalcin   Hoechst 33342   Combined

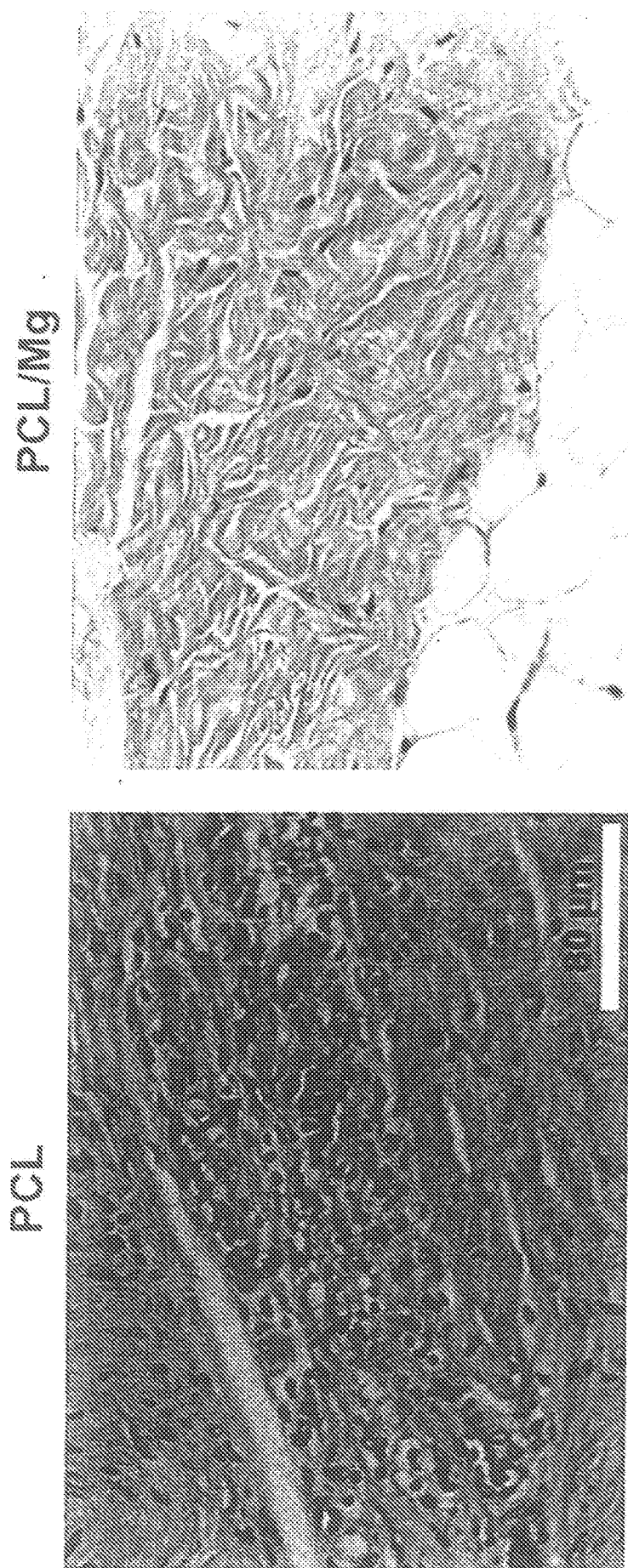Day 11 (decreasing)   Day 11 (8mM)

FIG. 5

FIG. 6
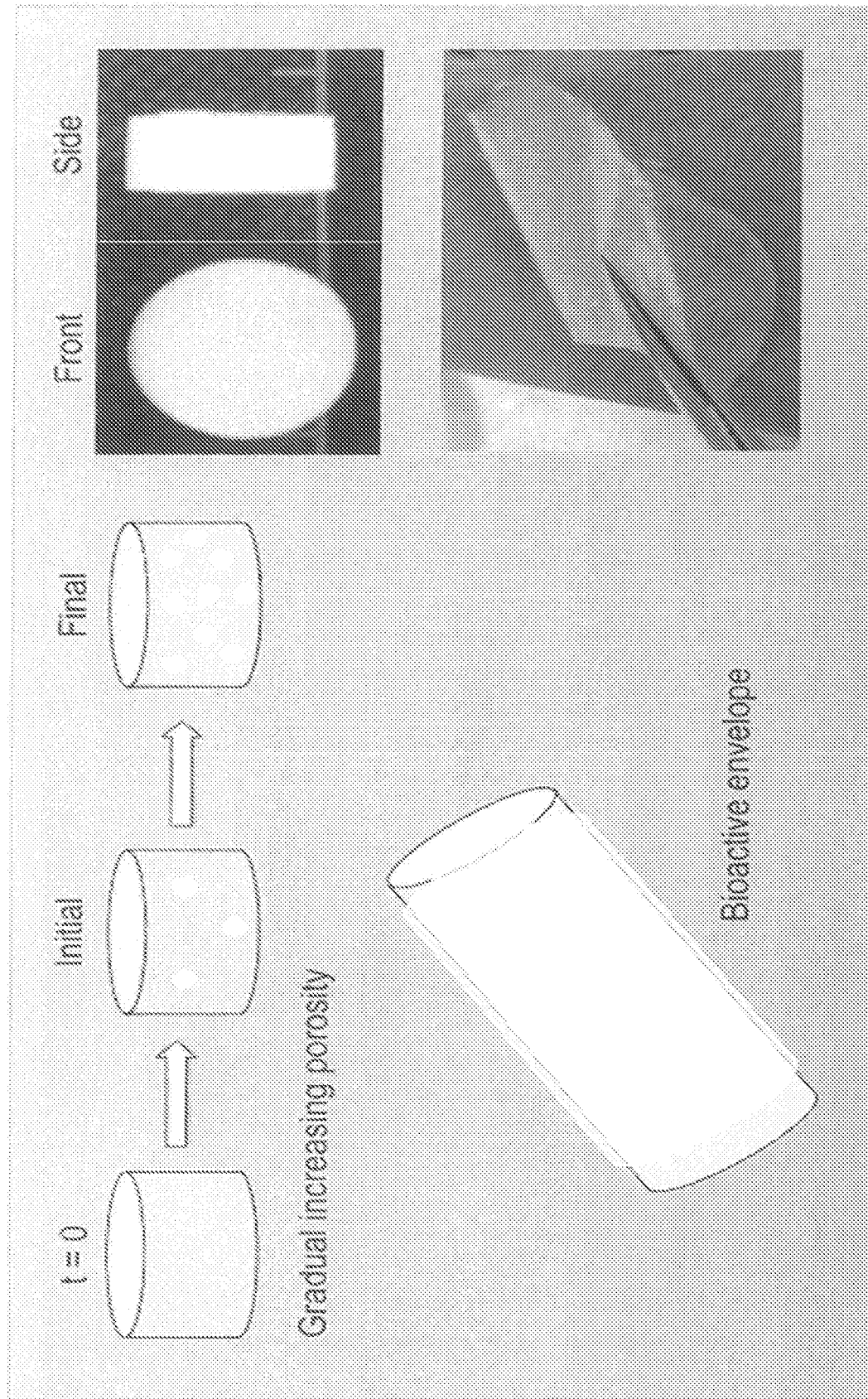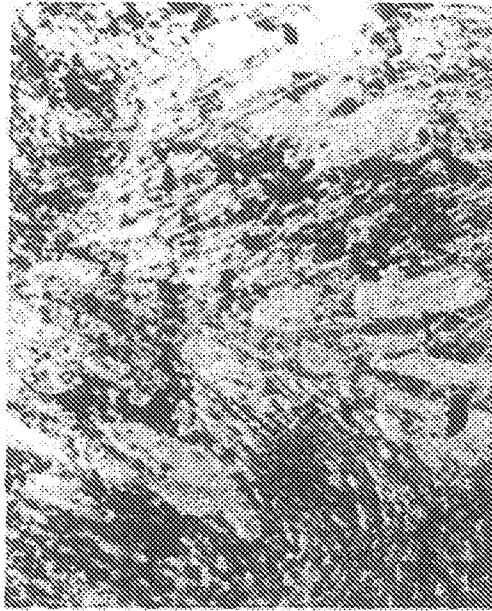
PCL/Mg
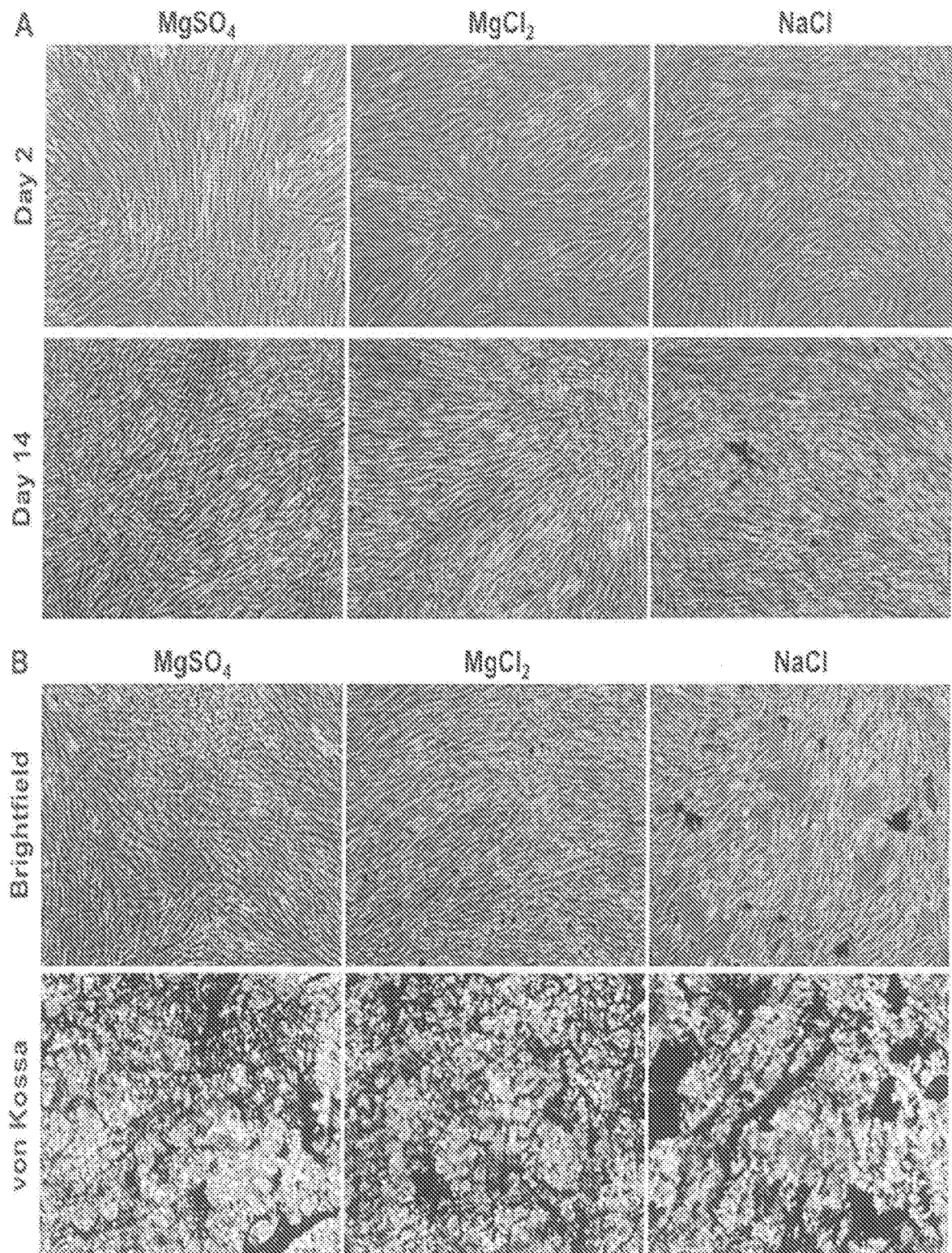
PCL
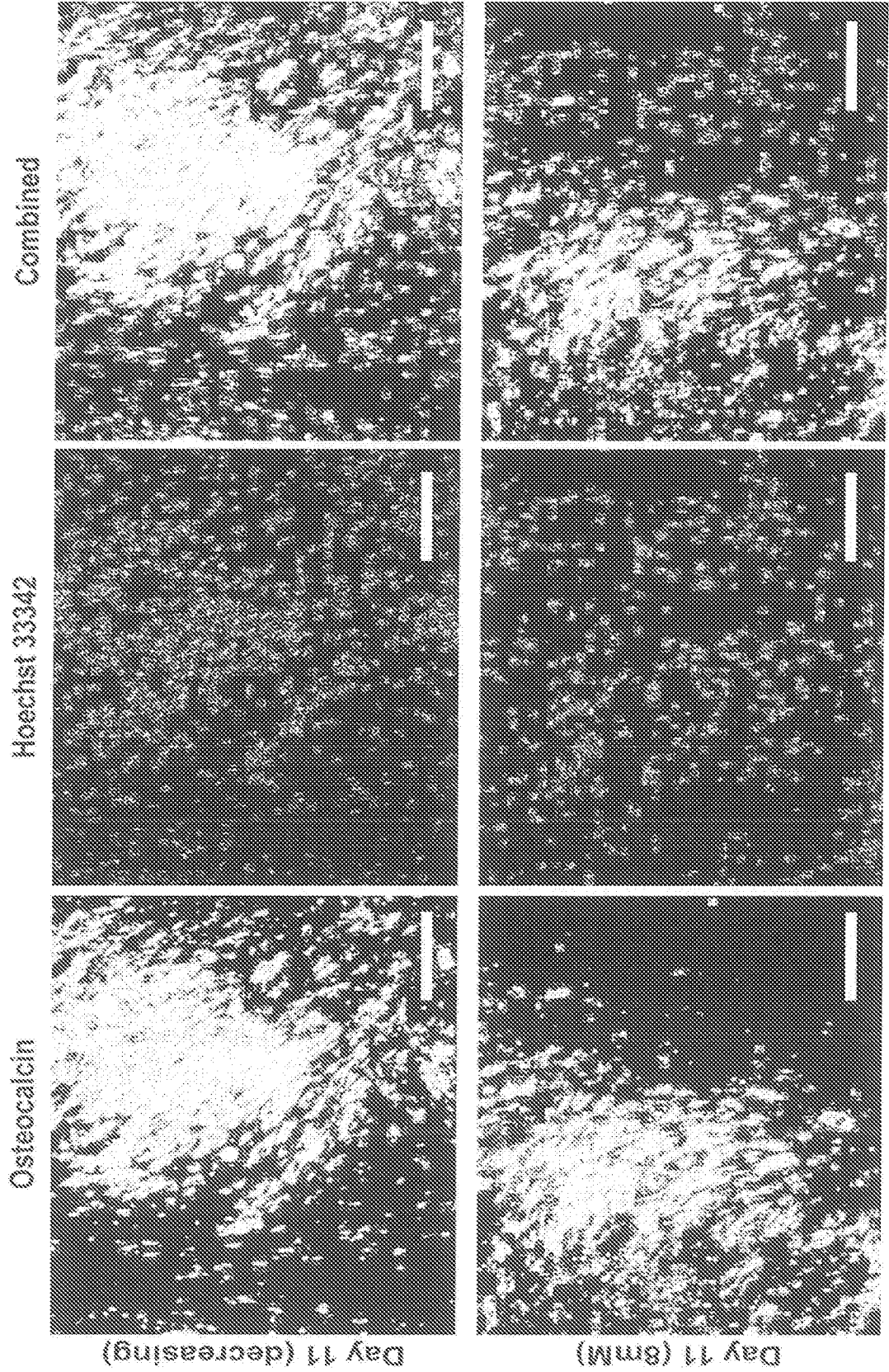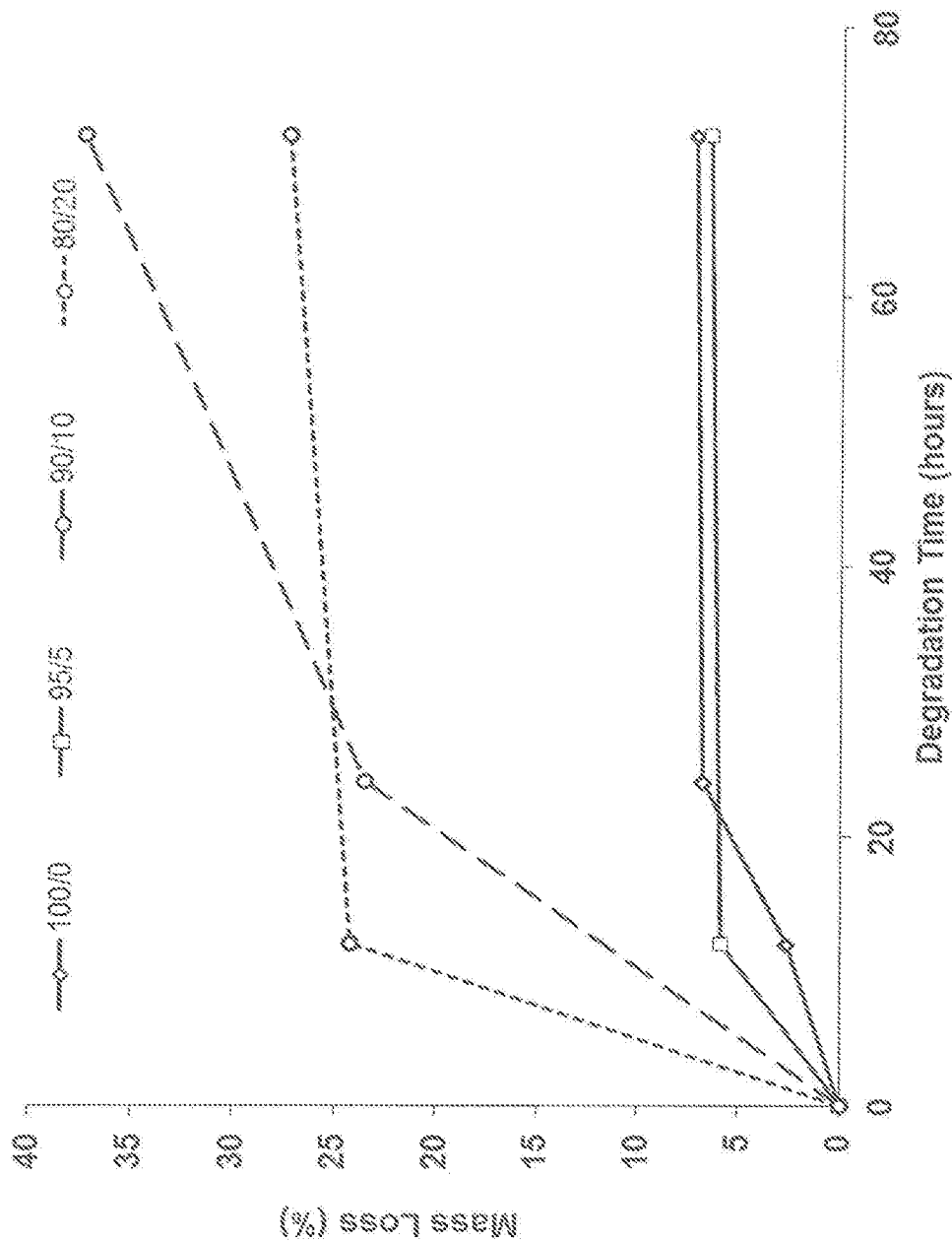
FIG. 7

FIG. 8

Adipogenic   Chondrogenic   Osteogenic

FIG. 9

FIG. 10
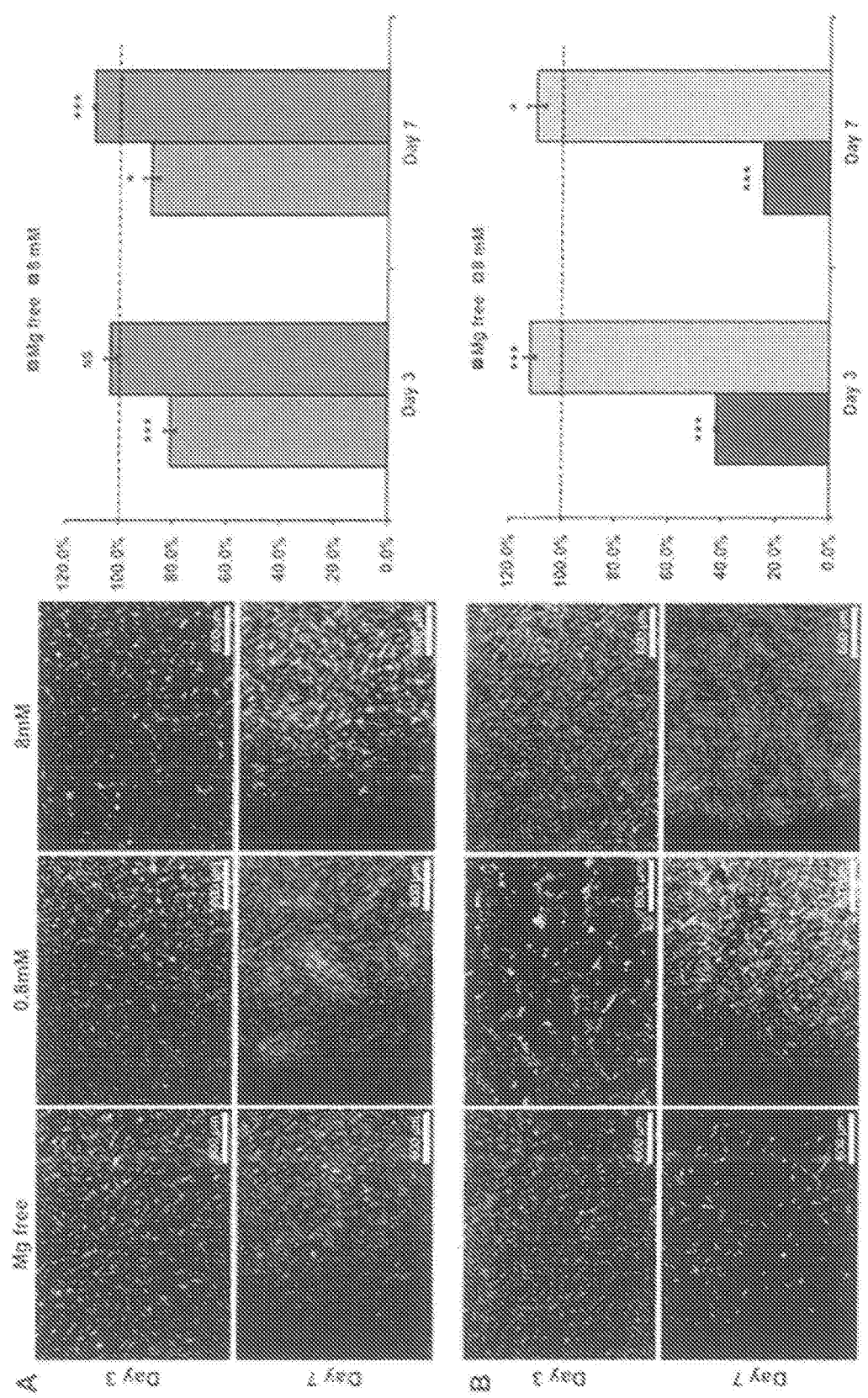
FIG. 11

A: in proliferative media
B: in osteogenic media
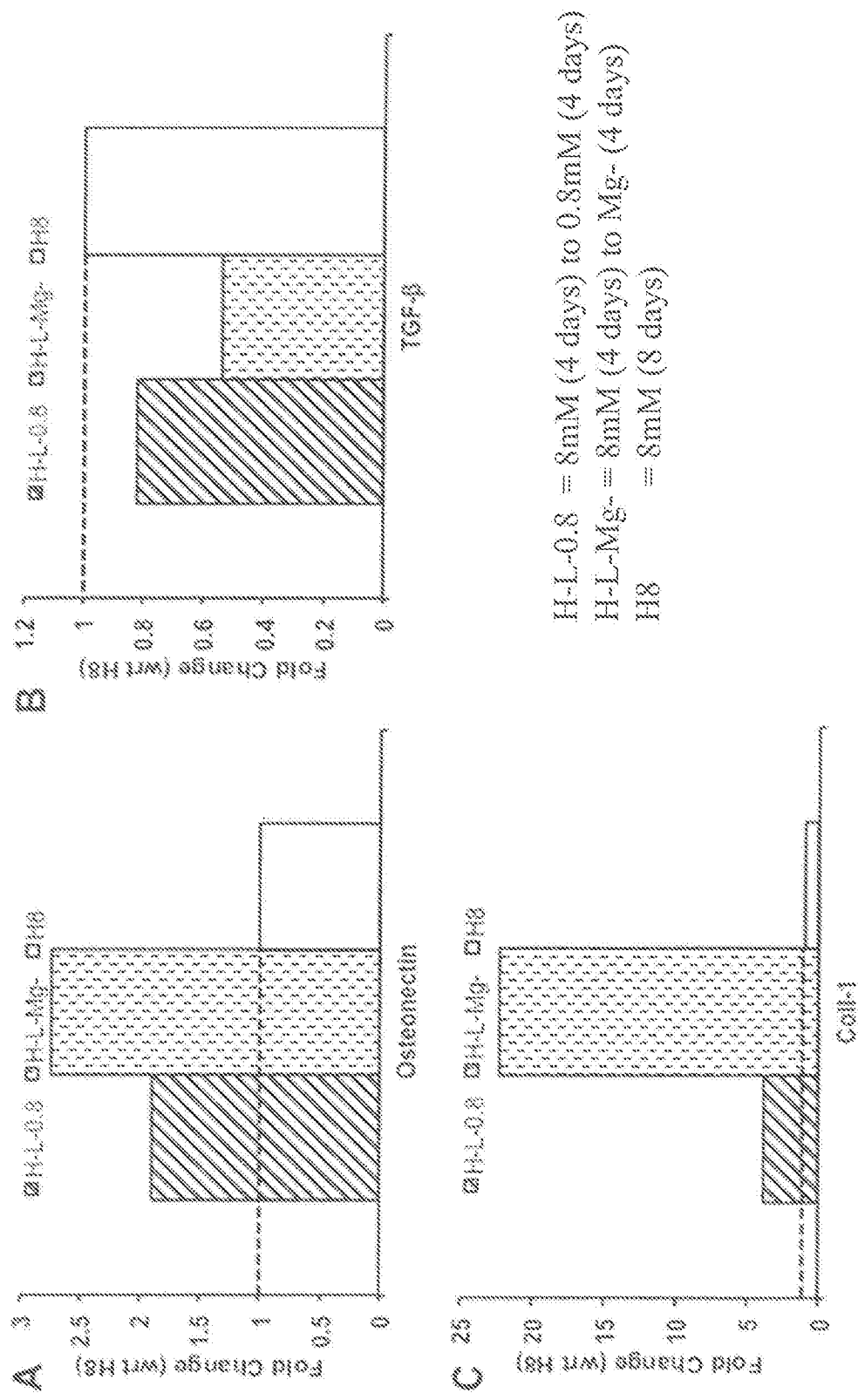
FIG. 12

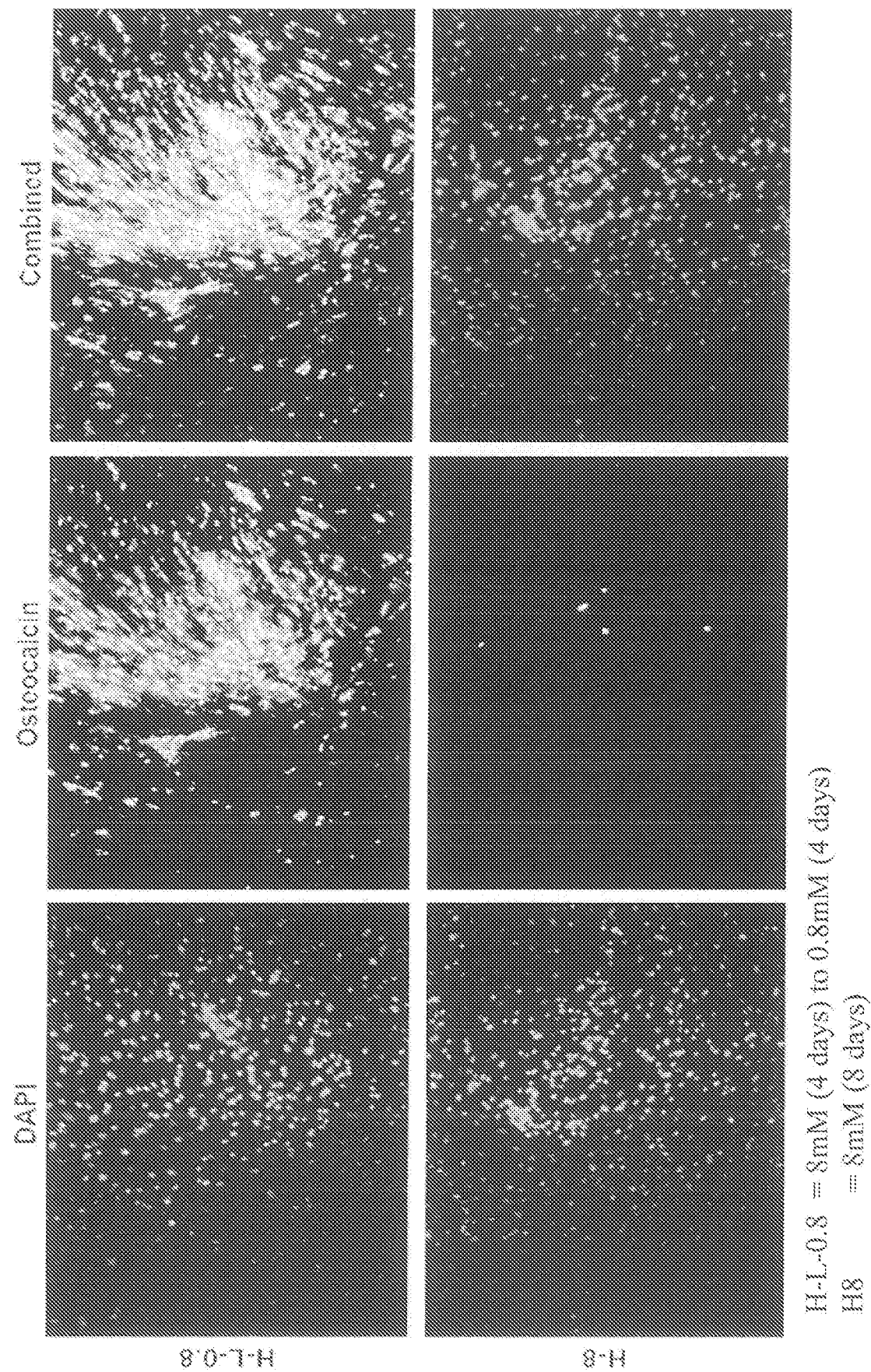H-L-0.8 = 8mM (4 days) to 0.8mM (4 days)
H-L-Mg- = 8mM (4 days) to Mg- (4 days)
H8 = 8mM (8 days)

DAPI   Osteocalcin   Combined

H-L-0.8

H-8

H-L-0.8 = 8mM (4 days) to 0.8mM (4 days)
H8   = 8mM (8 days)

FIG. 13

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

See Supplemental Box

According to International Patent Classification (IPC)

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Database: CAPlus, EPODOC, WPIAP, Scopus
Keywords: magnesium, sulfate, chloride, phosphate, polycaprolactone, polylactic, bone, implant, soluble, cryomill and related terms.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 2749301 A1 (DE PUY SYNTHES PRODUCTS, LLC) 2 July 2014 Paragraphs 0006, 0032, 0052, 0053 Claim 4	1-5 AND 15-19
X	KWON B. K. ET AL., Magnesium Chloride in a Polyethylene Glycol Formulation as a Neuroprotective Therapy for Acute Spinal Cord Injury: Preclinical Refinement and Optimization. *J. Neurotrauma*, 3 August 2009, Vol. 26, No. 8, pages 1379-1393 [Retrieved on 2015-12-28] (DOI: 10.1089/NEU.2009.0884) Page 1380 Methods Page 1381 Dose Optimization	1-5 AND 15-19

☒ Further documents are listed in the continuation of Box C.   ☒ See patent family annex.

*Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29/12/2015 (day/month/year)	12/01/2016 (day/month/year)

Name and mailing address of the ISA/SG	Authorized officer
Intellectual Property Office of Singapore 51 Bras Basah Road #01-01 Manulife Centre Singapore 189554 Email: pct@ipos.gov.sg	Amylia Abdul Ghani IPOS Customer Service Tel. No.: (+65) 6339 8616

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2015/050449

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