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Mineralization regulation and biological influence of bioactive glass-collagen-phosphatidylserine composite scaffolds

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Biomimetic scaffolds are appealing products for the repair of bone defects using tissue engineering strategies. In the present study, novel biomimetic composite scaffolds, with similar properties to natural bone, were prepared, blended and cross-linked with bioactive glass, type I collagen and phosphatidylserine. When exposed to cell culture solution in the absence of a cellular source, the composite scaffolds form crystals with octahedral structure. These crystals are similar to the products derived from MC3T3-E1 cell mineralization within the composite scaffolds, with respect to both composition and morphology. Furthermore, crystals with octahedral structure were observed to develop into plate-like hydroxyapatite. The bio-mineralization behavior of the composite scaffolds is likely influenced by inorganic components. Finally, a rabbit tibia defect model shows that the highly bioactive properties of the investigated composites result in excellent bone repair.

composite, scaffold, cell, bio-mineralization, regulation

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Bone-like tissue engineering composite materials, with properties optimized for specific repair functions, are under development and promising results have been achieved [1–3]. Although the majority of materials are biocompatible, they often require addition of cells, growth factors and other agents to improve cell response and subsequent mineralization, which makes this approach complicated and expensive [4–6].

Importantly, the regulatory influence of biomaterials, at the biological level, is a fundamental issue to consider during their evaluation. The physicochemical properties of biomaterials influence initial cellular responses on the cell-material interface, including cellular adhesion, proliferation and overall cell performance, in particular their ability to promote bio-mineralization [7–9].

Biomimetic applications have permitted development of biomimetic matrices, based on the process of natural bone formation and structure, in order to provide a suitably comparable microenvironment. The extracellular matrices of hard tissue are composed of organic and inorganic phases. The inorganic phase consists primarily of hydroxyapatite (HA), whilst the organic phase consists mainly of type I collagen (COL1) and small amounts of ground substance, including glycosaminoglycans, proteoglycans, glycoproteins and velum lipid, all with unique distributions reflecting their biological roles. Select compositions of silicate glass, phosphate glass and glass ceramic have been proven to react with physiological fluid, forming strong bonds between hard and soft tissues following cellular activity [10]. Naturally-derived collagen has the advantage of permitting specific cellular interactions, owing to its hydrophilic property [11]. Phosphatidylserine (PS) is an important compo-

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nent of cell velum lipid and plays a key role in tissue development, repair and function [12].

This study aimed to develop a novel composite scaffold, using a biomimetic approach, with optimized biodegradability and bioactive properties for bone tissue engineering applications. In particular, the influence of biomolecules, including COL1 and PS, on bio-mineralization was investigated. The major advantages of this approach are its simplicity and low cost. Strategies for studying the regulatory influence of biomolecules on cellular activity and bio-mineralization, may be useful in bone tissue engineering when using bioactive glass (BG)-COL1-PS biomimetic composite scaffolds.

1 Materials and methods

1.1 Preparation of composite scaffolds

58s-BG was synthesized using a sol-gel method and consisted of 60 wt% SiO_2, 35 wt% CaO and 5 wt% P_2O_5. BG-COL1-PS composite scaffolds consisted of 65% part by weight of inorganic components (BG) and 35% part by weight of organic components (80 wt% COL1 and 20 wt% PS). Composite scaffolds were prepared using a freeze-drying technique. The BG particles were gradually added to a solution of COL1-PS and intensively mixed. 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS, 4:1 ratio) were then incorporated in a 2.5 mg mL^{-1} solution at pH 5.5, adjusted with 2-(4-morpholino)ethanesulfonic acid (MES). The solution was kept at 4°C for 24 h to allow cross-linking. The mixture was then transferred to a freezer (VXE380, Jouran, Nantes, France) at −60°C for 12 h to solidify the solvent and induce solid-liquid-phase separation. The solidified mixture was freeze-dried in a freeze dryer (ALPHA2-4, Christ, Osterode, Germany) at −50°C for 12 h to form porous scaffolds.

Dulbecco's minimum essential medium (DMEM) and fetal bovine serum (FBS) were purchased from Sigma (St. Louis, MO, USA). T-75 and T-175 cell-culture flasks and 24-well cell-culture plates were purchased from Corning (Midland, MI, USA). All other reagents were of analytical grade.

1.2 Mineralization examination

MC3T3-E1 cells (Riken Cell Collection, Saitama, Japan) were trypsinized from T-175 culture flasks, washed once in DMEM-10%-FBS medium and collected by centrifugation at 7000 rpm for 10 min. Cell pellets were suspended in medium and the cell density was adjusted to 1.0×10^4 cells mL^{-1}. 2 mL of cell suspension was seeded to the samples, placed on the bottom of a 24-well cell- culture plate. Cells were left to attach to the materials and remained undisturbed in a humidified incubator (37°C and 5% CO_2) for 1, 7 or 15 d. After incubation, specimens were washed twice with phosphate-buffered saline (PBS) and immersed in PBS containing 3% glutaraldehyde (pH 7.4) for 1 h. Specimens were then dehydrated through an ethanol series (from 30%, 50%, 70%, 90%, 95% to 100%), followed by lyophilization. Specimens were then mounted on aluminum stumps, coated with gold in a sputtering device for 3 min at 15 mA and examined using a scanning electron microscope (SEM) (30XLFEG, Philips, Amsterdam, The Netherlands) and energy dispersive X-ray (EDX) (INCA, Oxford, England). An X-ray diffractometer (XRD) (X'PertPRO, PANalytical, Almelo, The Netherlands) was used to verify the formation of HA on the surface of composite scaffolds treated in culture solution, using a flat camera and 40 keV Cu-Ka radiation. The samples were 2θ scanned from 10° to 70° with a scan speed of 15.24° min^{-1}.

1.3 Surgical protocol and histological examination

The reaction of bone-tissue to the composites was examined using a rabbit tibia defect model. Composites with 15 mm×20 mm dimensions were used. Five rabbits (2–2.5 kg, one year old) were sedated by intravenous administration of butorphanol tartrate (0.2 mg kg^{-1}) and midazolam (0.02 mg kg^{-1}). Anesthesia was achieved by administration of propofol (3 mg kg^{-1}) and was maintained with 2% isoflurane in oxygen administered through an endotracheal tube. The composites were implanted into 20 mm bone defects in the tibiae of each rabbit.

The implanted composite and surrounding tissues were removed 12 weeks after operation. Samples were decalcified, embedded in paraffin and sectioned prior to histological staining using hematoxylin and eosin (HE) and observed using light microscopy.

2 Results

2.1 Bio-mineralization of MC3T3-E1 cell-seeded BG-COL1-PS composite scaffolds

After culture of MC3T3-E1 cells within the BG-COL1-PS composite scaffolds for 7 d, mineralization was observed using phase contrast microscopy (Figure 1A). SEM showed the mineralized crystals presented an octahedral structure (Figure 2). The components of the crystals were analyzed and are shown in Table 1. From days 8 to 12, extensive cell mineral formed and macroscopic growth of the coalesced crystals was achieved by assembly of a large number of growth units (Figure 1B–D).

To illustrate further the regulatory influence of BG-COL1-PS composite scaffolds, mineralization in culture solution alone, in absence of cell supplementation, was comparatively studied. Crystals with octahedral structure

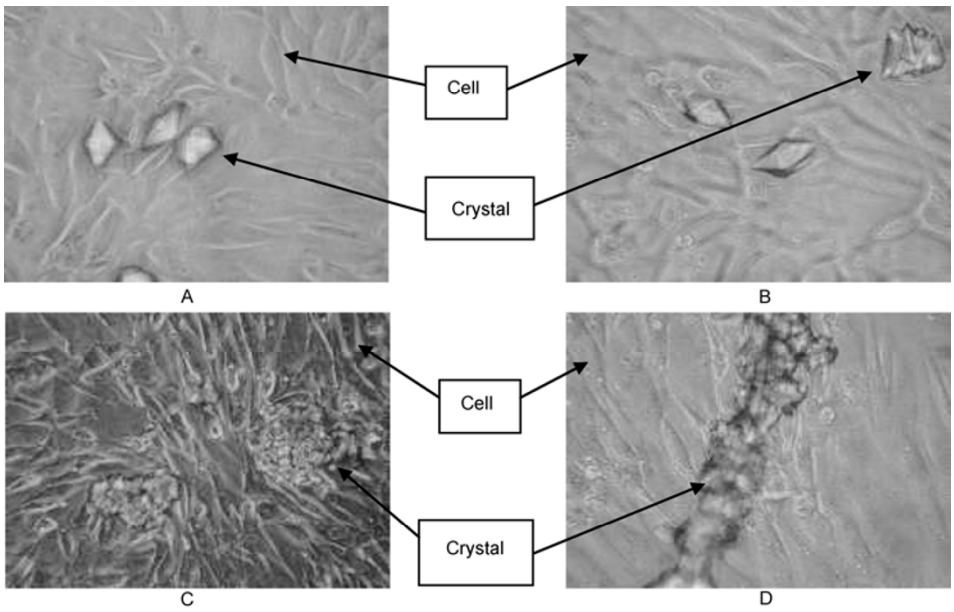

Figure 1 Optical micrographs (×200) of the bio-mineralization process regulated by the BG-COL1-PS composite scaffolds in the presence of MC3T3-E1 cells after 7 (A), 8 (B), 10 (C) and 12 (D) days culture.

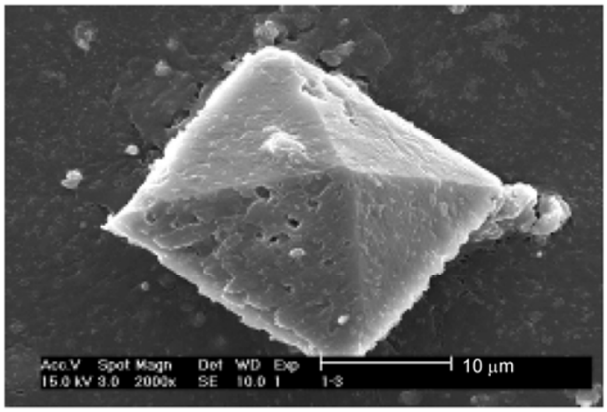

Figure 2 SEM micrograph showing the crystal structure regulated by the BG-COL1-PS composite scaffolds in the presence of MC3T3-E1 cells after 7 days culture.

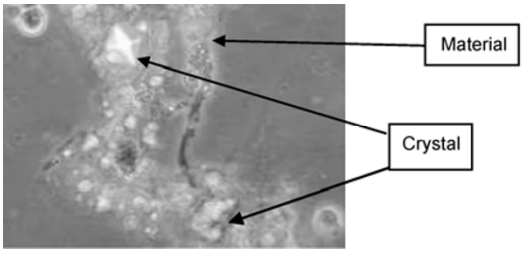

Figure 3 Optical micrograph (×200) of crystals regulated by the BG-COL1-PS composite scaffolds after 7 days culture in the absence of cells.

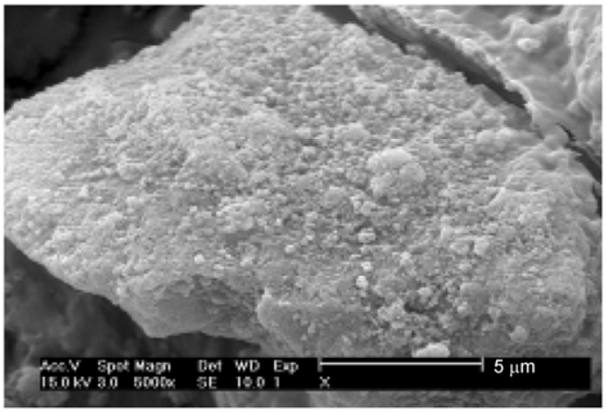

Figure 4 SEM micrograph of crystals regulated by the BG-COL1-PS composite scaffolds after 15 days culture in the absence of cells.

were formed within the material after 7 days culture in cell-free solution (Figure 3) and were similar to the crystals derived following culture of BG-COL1-PS composite scaffolds with cells. EDX analysis confirmed that the octahedral crystal products from the two bio-mineralization processes comprised identical elements and in similar amounts (Tables 1 and 2). After 15 days in culture solution alone, crystals with octahedral structure continued to grow into plate-like minerals and material matrix was not found (Figure 4).

Change in crystal morphology indicates that BG-COL1-PS composite scaffolds have significant control of crystal structure, guiding the mineralization process and promoting crystal growth into specific shapes. Our experiments suggest that deposition of crystals onto the material affects the fusion of COL1 and PS matrices (Figure 3), which may inversely facilitate self-assembly of minerals.

In terms of free energy, COL1 and PS matrices have a driving force to spontaneously create minima during miner-

Table 1 EDX analysis of crystals regulated by the BG-COL1-PS composite scaffolds in the presence of MC3T3-E1 cells after 7 days culture

Element	*K* ratio	Weight %	Atom %
C	0.1404	14.037	33.629
O	0.1929	19.285	34.685
P	0.0084	0.842	0.783
Au	0.2861	28.612	4.180
Ca	0.3722	37.224	26.724
Total		100.000	100.000

Table 2 EDX analysis of crystals regulated by the BG-COL1-PS composite scaffolds after 7 days culture in the absence of cells

Element	*K* ratio	Weight %	Atom %
C	0.1312	13.121	34.556
O	0.1816	18.160	35.904
P	0.0171	1.709	1.746
Au	0.3991	39.916	6.510
Ca	0.2710	27.094	21.384
Total		100.000	100.000

alization. The evolution of the structure requires an overall reduction in the system entropy and it is such energetic ordering influences that cause the minerals to adopt regular shapes with a high level of organization. This in turn may facilitate the macroscopic growth of the coalesced crystals and therefore help promote bone repair [11].

The crystallographic structure of the mineral regulated by the BG-COL1-PS composite scaffolds after 15 days in culture, was studied with X-ray diffraction, which confirmed the formation of HA (Figure 5). When compared with standard HA however, the inorganic phase peaks in the mineralized product were wider with several overlapping peaks. This indicates that the crystallinity of the inorganic phase was very low with small crystallite size, closely resembling natural bone tissue [12].

Mineralized HA was formed in the absence of cells, demonstrating that the BG-COL1-PS bioactive composites possess a remarkable ability to cause and regulate mineralization. This is highly important with regard to development of materials for bone repair or bone tissue engineering/regenera- tion. Although biocompatible, many materials require the addition of growth factors and other agents to improve cell responses and tissue repair. This study shows that even in the absence of additional agents and cells, a similar cell response was observed, including the formation of important HA.

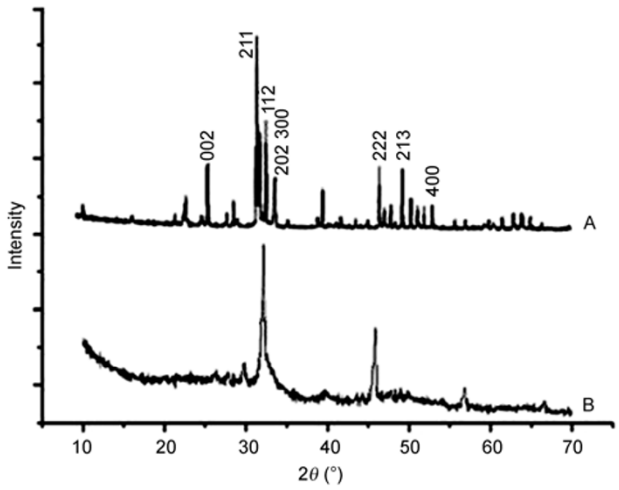

Figure 5 XRD patterns of standard HA (A) and crystals regulated by the BG-COL1-PS composite scaffolds after 15 days culture in the absence of cells (B).

Biomolecules within the composite scaffolds, such as COL1 and PS, are likely to be the key factors in promoting mineralization and HA formation, because little change in the concentration of calcium and phosphate was recorded in this study.

In natural bone tissue, molecular modulation is the basic factor during the process of bio-mineralization [13]. The carboxyl (–COOH) and phosphate ($-PO_4H_2$) enabling groups, existing within the biomolecules, catalyze and regulate mineralization. They have a strong affinity with the calcium ions. COL1 plays an important role in the initial stage of bio-mineralization, but fails to control completion of the full functionality of mineralization. COL1 is therefore unable to lead and guide the mineralization process and these tasks require completion by phosphoprotein. Phosphoprotein is a class of protein, rich in aspartic acid and PS. Different biological systems may have different mineralization matrices, but in all cases, phosphate groups bind calcium ions first and then further combine with calcium phosphate crystals. Another important feature of phosphoprotein is that it can combine with collagen through the binding of carboxyl and amino groups which exist in the phosphoprotein and collagen. Such coordination creates the conditions required for precipitation and assembly of the calcium phosphate crystals.

2.2 Histological evaluation

Figure 6 shows a HE-stained histological section of the composite scaffold and surrounding tissue after 12 weeks implantation. Newly formed bone, characterized by the presence of a Haversian system and non-calcified soft tissue, had formed around the composite and direct bonding was apparent at the natural bone-composite interface.

Therefore, the composite was incorporated into the *in vivo* bone remodeling process because phagocytosis, by osteoclasts and osteogenesis, by osteoblasts, occurred simultaneously. In general, such behavior *in vivo* appears typically around autografted bone, but has not been clearly observed around artificial implants. Although *in vivo* and *in vitro* resorption by osteoclasts has been reported for some materials [14–16], the resorption and dissolution rates of these materials are too slow for their application in bone remodeling. The BG-COL1-PS composite is likely to be easily resorbed by cells and/or readily dissolved in body fluid because it is made of bioactive glass crystals, COL1 and PS extracellular matrix components and it is these same components that are likely identified by the interacting cells.

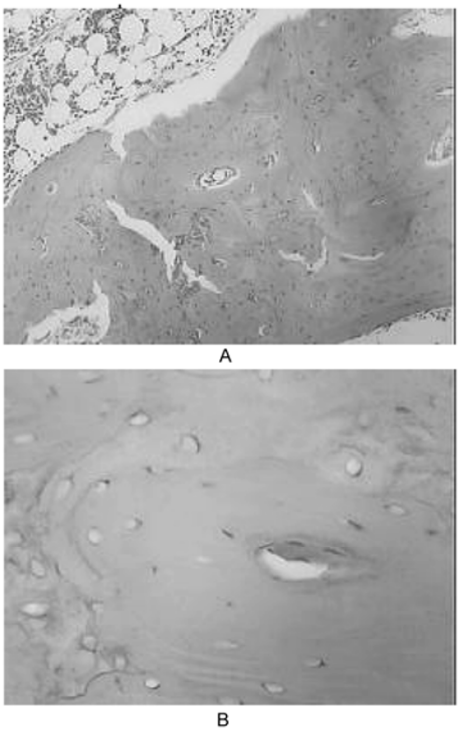

Figure 6 Histological morphology (HE staining) of bone defects filled with BG-COL1-PS composite scaffolds, 12 weeks after implantation. A, ×10. B, ×40.

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