Royal College of Surgeons in Ireland

repository@rcsi.com

Long-term controlled delivery of rhBMP-2 from collagen-hydroxyapatite scaffolds for superior bone tissue regeneration.

AUTHOR(S)

Elaine Quinlan, Emmet M. Thompson, Amos Matsiko, Fergal O'Brien, Adolfo López-Noriega

CITATION

Quinlan, Elaine; Thompson, Emmet M.; Matsiko, Amos; O'Brien, Fergal; López-Noriega, Adolfo (2015): Long-term controlled delivery of rhBMP-2 from collagen-hydroxyapatite scaffolds for superior bone tissue regeneration.. Royal College of Surgeons in Ireland. Journal contribution. https://hdl.handle.net/10779/rcsi.10766594.v1

HANDLE

10779/rcsi.10766594.v1

LICENCE

CC BY-NC-SA 4.0

This work is made available under the above open licence by RCSI and has been printed from https://repository.rcsi.com. For more information please contact repository@rcsi.com

URL

https://repository.rcsi.com/articles/journal_contribution/Long-term_controlled_delivery_of_rhBMP-2_from_collagen-hydroxyapatite_scaffolds_for_superior_bone_tissue_regeneration_/10766594/1

Long-term controlled delivery of rhBMP-2 from collagen–hydroxyapatite scaffolds for superior bone tissue regeneration

Elaine Quinlan[2,3,4], Emmet M. Thompson[2,3,4], Amos Matsiko[2,3,4], Fergal J. O'Brien[2,3,4] and Adolfo López-Noriega*[1,2,3,4]

[1] School of Pharmacy, Royal College of Surgeons in Ireland, Dublin, Ireland

[2] Tissue Engineering Research Group, Dept. of Anatomy, Royal College of Surgeons in Ireland, Dublin, Ireland

[3] Trinity Centre for Bioengineering, Trinity College Dublin, Ireland

[4] Advanced Materials Bio-Engineering Research Centre (AMBER), RCSI and Trinity College Dublin, Dublin, Ireland

* Corresponding author: adolfolnoriega@rcsi.ie

1. Abstract

The clinical utilization of recombinant human bone morphogenetic protein 2 (rhBMP-2) delivery systems for bone regeneration has been associated with very severe side effects, which are due to the non-controlled and non-targeted delivery of the growth factor from its collagen sponge carrier post-implantation which necessitates supraphysiological doses. However, rhBMP-2 presents outstanding regenerative properties and thus there is an unmet need for a biocompatible, fully resorbable delivery system for the controlled, targeted release of this protein. With this in mind, the purpose of this work was to design and develop a delivery system to release low rhBMP-2 doses from a collagen-hydroxyapatite (CHA) scaffold which had previously been optimized for bone regeneration and recently demonstrated significant healing *in vivo*. In order to enhance the potential for clinical translation by minimizing the design complexity and thus upscaling and regulatory hurdles of the device, a microparticle and chemical functionalization-free approach was chosen to fulfil this aim. RhBMP-2 was combined with a CHA scaffold using a lyophilization fabrication process to produce a highly porous CHA scaffold supporting the controlled release of the protein over the course of 21 days while maintaining *in vitro* bioactivity as demonstrated by enhanced alkaline phosphatase activity and calcium production by preosteoblasts cultured on the scaffold. When implanted *in vivo*, these materials demonstrated increased levels of healing of critical-sized rat calvarial defects 8 weeks post-implantation compared to an empty defect and unloaded CHA scaffold, without eliciting bone anomalies or adjacent bone resorption. These results demonstrate that it is possible to achieve bone regeneration using 30 times less rhBMP-2 than INFUSE®, the current clinical gold standard; thus, this work represents the first step of the development of a rhBMP-2 eluting material with immense clinical potential.

2. Keywords

RhBMP-2; osteogenesis; collagen-hydroxyapatite; controlled release; bone tissue regeneration; rat calvarial defect model.

3. Introduction

The FDA approval for the utilization of recombinant human bone morphogenetic protein 2 (rhBMP-2) in 2002, represented a major milestone in the field of bone tissue regeneration[1]. Based on this approval, Medtronic commercialized INFUSE®, a collagen based sponge that is soak-loaded in a solution of several milligrams of rhBMP-2 prior to implantation, for spinal fusion procedures in skeletally mature patients with degenerative disc disease. Driven by this product, in 2010, Medtronic became the leader in the field with 44 % of the $1.9 billion bone graft market. However, an increasing body of evidence has demonstrated INFUSE® to be associated with numerous complications related to off-label use such as heterotopic ossification, osteolysis, increased neurological deficits, or cancer; all these serious side effects have been linked to the uncontrolled and offsite release of rhBMP-2[2-4].

RhBMP-2 is a primary growth factor (GF) for bone tissue regeneration, and it is involved in the commitment of multipotent stem cells towards the osteogenic lineage, eliciting an outstanding pro-osteogenic effect[5]. In this regard, there has been significant effort in the development of a biocompatible substrate that can support the spatiotemporally controlled release of rhBMP-2, in order to overcome the current therapeutic limitations associated with INFUSE®. Such materials while providing a solution to current publicized BMP risks may also facilitate better bone regeneration for those in need. Indeed, a wide array of substrates have been proposed, from inorganic materials, to hydrogels and porous organic composites to name a few, showing some encouraging results[6-11]. However, despite these promising outcomes, it is likely that for most of these materials the path to clinical utilization will take several years,

as they imply complex designs or the usage of novel polymers or their combinations, which will probably incur time consuming upscaling and regulatory hurdles[12].

The tissue engineering research laboratory at the Royal College of Surgeons in Ireland (RCSI) has recently patented and licensed HydroxyColl®, a biomimetic crosslinked collagen-hydroxyapatite (CHA) porous scaffold which is biocompatible, bioresorbable, osteoconductive and osteoinductive[13-16]. This biomaterial, which has shown evidence of healing critical-size defects on a rabbit radius and rat calvaria, is undergoing regulatory examinations for FDA and CE mark approval and is expected to be utilized in clinical trials by the end of 2015. Due to the regenerative properties of this scaffold, the present work focused on investigating the potential of utilizing this CHA scaffold for the sustained delivery of rhBMP-2, further improving its regenerative potential using a method which could be applied to other scaffolds.

In order to facilitate delivery of rhBMP-2 from these CHA scaffolds, we aimed to see if it was possible to adopt a carrier-free approach, without the utilization of pre- or post-fabrication scaffold functionalization processes previously adopted[17-19]. As such, we evaluated the possibility of controlling the dosage and the release of the bioactive GF by incorporating it within the structure of the scaffold, during its lyophilization fabrication process. This approach was investigated based on two particular features of rhBMP-2: i) its high stability in acidic environments[20, 21], which are used in the fabrication of the scaffolds and ii) its documented non covalent bonding with hydroxyapatite crystals, which are a component of the CHA scaffolds, via the -OH, $-NH_2$, and $-COO^-$ functional groups[22, 23]. These unique characteristics were hypothesized to facilitate sustained, long term delivery of low doses of the protein post implantation thereby overcoming the risks associated with INFUSE®.

Initially, this study explored whether it was possible to directly incorporate rhBMP-2 into the scaffold using the proposed lyophilization method and subsequently how the incorporation of

the GF into CHA scaffolds affected their architecture, the release profile of the GF from scaffolds as well as the bioactivity of the protein were studied. The regenerative capacity of this rhBMP-2-loaded scaffold to heal bony defects was subsequently investigated *in vivo* using a critically sized rat calvarial defect model over an 8 week period and the results were compared to unloaded CHA scaffolds and empty defect controls.

4. Materials and Methods

4.1. Scaffold fabrication

Collagen-hydroxyapatite (CHA) scaffolds were fabricated using a patented process developed within our laboratory[16] with the omission of the chemical crosslinking step so as to avoid loss of protein in solution prior to use *in vitro* and *in vivo*. Briefly, 1.8 g of micro-fibrillar bovine tendon collagen was added to 320 mL of 0.5 M acetic acid solution. This suspension was blended at 15,000 rpm for 90 min using an overhead blender in a reaction vessel which was kept at 4 °C using a circulation system to prevent heat denaturation of the collagen. Aliquots of 10 mL of a solution of 3.6 g of HA in 40 mL of 0.5 M acetic acid solution were added every 60 min followed by additional blending. The slurry was degassed under vacuum to remove air bubbles which may lead to uncontrolled porosity. In order to incorporate rhBMP-2 into the scaffold, rhBMP-2 (R&D Systems UK) was gently mixed with the CHA slurry after the degassing step at a final dose of 12 µg rhBMP–2/mL slurry and 9 mL of slurry was used to produce a scaffold in a 4.5 × 4.5 cm stainless steel tray (50 ng/cm^2 of scaffold or 1.5 µg per scaffold). The *in vitro* BMP-2 release of a series of CHA scaffolds incorporating different amounts of rhBMP-2 was analyzed prior to this study. Among them, the scaffold fabricated with a slurry containing 12 µg/mL rhBMP-2 was selected with a release rate of 50–100 ng/mL of rhBMP-2 per timepoint since this concentration of rhBMP-2 elicits an optimal response on the osteoblast cell line used for *in vitro* characterization.

The mixtures were lyophilized at -10 °C using a constant cooling freeze-drying protocol. Subsequently, the scaffolds were sterilized under UV light for 4 h (λ=254 nm).

4.2. Assessment of rhBMP-2 release from scaffolds

4.2.1 Scaffold characterization

The morphological characterization of the scaffolds was carried out by Scanning Electron Microscopy (SEM) using a Zeiss Supra Variable Pressure Field Emission Scanning Electron Microscope. Scaffolds were cut (6 mm diameter and 4 mm height) and mounted onto metallic studs with the help of carbon-based glue and sputtered with gold and SEM images were captured at a voltage of 5 kV using secondary electron mode, taken at a working distance between 12 and 18mm.

4.2.2 *In vitro* bioactivity assays

To examine rhBMP-2 release profile, 6 mm diameter scaffolds were placed in 2 mL of PBS in a shaking water bath at 37 ºC. At given time-points up to 21 days, release media were removed, replaced by fresh PBS and the protein concentration was determined by ELISA using a rhBMP-2 ELISA kit (R&D Systems UK) according to the manufacturer's instructions. At later timepoints the scaffolds begins to degrade and lose structural integrity. Prior to this point the concentration of protein delivered from the scaffold was between 50 and 100 ng/mL per time point considered optimal for eliciting a pro-osteogenic effect on osteoblast cells[24].

The bioactivity of the scaffolds loaded with rhBMP-2 was studied using MC3T3-E1 cells, a murine pre-osteoblast cell line, in order to study the effects of the scaffolds on osteoblast proliferation and differentiation *in vitro* since they exhibit similar properties as osteoprogenitor cell lines [25] and form calcified bone tissue[26]. Cells were cultured in α-MEM supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 2% penicillin/streptomycin, the

medium was changed every 3 days. Cells were trypsinized (trypsin–EDTA 0.25%), seeded on 6 well plates at a density of 30,000 cells per well and cultured for 24 h in standard growth media followed by growth media supplemented with ascorbic acid 2-P 50 µM, dexamethasone 100 nM, β-glycerophosphate 10 mM, FBS 10%, 100 U/mL penicillin and 100 µg/mL streptomycin under standard conditions (37 °C, 5% CO2).

To analyze the influence of rhBMP-2 eluting scaffolds on cell proliferation, DNA was quantified after 3 and 7 days of culture. At those timepoints, the cell seeded scaffolds were homogenized in lysis buffer using a rotor–stator homogenizer (Omni International, Germany), the cell/scaffold suspension was centrifuged (2500 ×g, 10 min, 4 ºC) and the supernatant was collected and used for dsDNA analysis using the PicoGreen assay (Quanti-iT ™ PicoGreen dsDNA Molecular Probes, OR, USA) according to the manufacturer's instructions.

The osteogenic potential of MC3T3-E1 cells on the rhBMP-2 eluting scaffolds was assessed and compared to unloaded (rhBMP-2-free) CHA scaffolds by quantifying the activity of ALP, a widely exploited early biochemical marker for osteogenic activity 3 and 7 days post-seeding[27]. At the endpoint of the study constructs containing MC3T3-E1 cells were washed in PBS, lysed according to the manufacturer's protocol in lysis buffer (SensoLyte pNPP Alkaline Phosphatase Assay Kit) and incubated at 4 ºC. The supernatant was collected for quantification of ALP. This method utilizes p-nitrophenylphosphate (pNPP) that is hydrolyzed by ALP to produce a yellow product. The amount of colored product is proportional to the amount of enzyme in the reaction mixture.

Quantification of calcium deposition within osteogenesis experiments was performed using the Calcium Liquicolor kit (Stanbio Laboratories). The scaffolds were removed from culture at day 14 and solubilized in 1 mL of 0.2 M HCl overnight at 4ºC on a shaker and the amount of calcium was determined according to the manufacturer's protocol.

4.3. Assessment of the effects of rhBMP-2 release on bone repair

4.3.1 *In vivo* procedure

Once the bioactivity of the eluting scaffolds was confirmed *in vitro*, scaffolds with a diameter of 7 mm (2 µg rhBMP-2 per scaffold) were implanted in 7 mm critical-sized calvarial defects [28] generated on adult male Wistar rats (n=8, mean weight 375 g, range 360–395 g) (Harlan, UK) in order to assess the ability of BMP-2 CHA scaffolds to promote bone regeneration. For comparative purposes, a second group of animals (n=8) received unloaded CHA scaffolds, while a third group (n=8) received no scaffold and these empty defects served as a negative control.

In vivo analysis was conducted in accordance with protocols approved by the Research Ethics Committee of the Royal College of Surgeons in Ireland and an animal license was granted by the Irish Government Department of Health (Ref. B100/4416). General anesthesia, the creation of the 7-mm-diameter critical-size cranial defect, the placement of the composite materials in the defect site, and postoperative animal care were performed using established methods[28]. The generation of a 7-mm-diameter critical-size calvarial defect, the implantation of the scaffolds in the defect site and the procedures for postoperative animal care were performed using methods established in the Tissue Engineering Research Group (TERG) of the Royal College of Surgeons in Ireland (RCSI)[28, 29]. Anesthesia was induced with intraperitoneal medetomidine hydrochloride 0.3 mg/kg and ketamine hydrochloride 70 mg/kg and maintained with inhalational isoflurane (0.5–2%) and oxygen. Pre-operative analgesia and antibiotic prophylaxis were administered before the skin over the head was shaved and the area painted with 7.5% povidone-iodine antiseptic surgical scrub prior to draping. An ~3mm longitudinal incision was made over the cranial vault, with dissection down to the left parietal bone before a trephine bur drill was used to create a 7 mm circular transosseous defect. Drilling was

performed under constant irrigation with 0.9% NaCl saline solution and the dura was identified to confirm that a transosseous defect had been created. Scaffolds were implanted into the defect, after which the periosteum and superficial connective tissue were closed in layers with 3-0 absorbable monofilament sutures (Monocryl™) and finally a topical skin adhesive (n-butyl cyanoacrylate). Post-operatively, rats were given a subcutaneous injection of the reversal agent atipamezole 1.2 mg/kg and placed in an incubator at 27º C until fully recovered. Animals were housed (2 per cage) in the Biomedical Research Facility at the RCSI with *ad libitum* access to water, food and environmental enrichment.

At 8 weeks post-implantation, the animals were euthanized by CO_2 asphyxiation and cervical dislocation. A 20 mm × 20 mm segment of calvarium containing the defect was resected using a dental saw and the explants retrieved were stored in 10% neutral buffered formalin for 4 days and then transferred to PBS prior to decalcification

4.3.2. Microcomputed tomography

The three dimensional (3D) structure of new bone produced within the cranial defect was analyzed using micro computed tomography (microCT). Scans were performed on a Scanco Medical 40 MicroCT system (Scanco Medical, Bassersdorf, Switzerland) with a 70 kVP Xray source and 112 µA (resolution of ~12µm). 3D reconstruction was performed using the standard Scanco software package and a threshold of 140 in a scale from 0 to 1000. Volume of interest (VOI) was defined to analyze a 6 mm defect region in order to assess healing in the center of the defect and to exclude any old bone at the defect edges. Repair was expressed as percentage bone within defect area.

4.3.3 Histological analysis

In addition to microCT, both qualitative and quantitative histological examinations were performed on explants in order to further assess the levels of bone formation. Explants were

first decalcified for a period of 5 weeks by immersing them in a solution of ethylenediaminetetraacetic acid (EDTA) 14% (w/v) at pH 7.4 with renewal of the solution 3–4 times per week. Following decalcification, specimens were dehydrated in a graded series of ethanol solutions using an automated tissue processor, bisected and embedded in single paraffin wax blocks. Sections (7 µm thick) were cut from the mid-portion of each sample using a rotary microtome and mounted on poly-l-lysine slides. Qualitative and quantitative histological examinations were performed on explants in order to assess the levels of bone formation in the defect site. Each section was stained with Hematoxylin and Eosin (H&E). Hematoxylin stains cell nuclei purple, eosin stains extracellular matrix pink and bone appears dark pink/red. Images of each specimen were acquired and digitized using transmitted light and epifluorescence microscopic visualization (Nikon Microscope Eclipse 90i with NIS Elements software v3.06, Nikon Instruments Europe, The Netherlands). Quantitative analysis was performed on n=4 sections from each specimen. Histomorphometrical analysis (blind) was carried out in order to quantify the healing response from the H&E-stained samples. The defect margin was identified and any new bone was quantified by measuring the area of bone nucleation sites (BNS) in each section and calculating the mean total area of these sites per group. The area of new bone formation was calculated using NIS Elements software v3.06 (Nikon Instruments Europe B.V, The Netherlands).

4.4. Statistical analysis

These data are represented as means ± standard error of the mean (SEM). Statistics were carried out using GraphPad Prism software using a general linear model ANOVA with Bonferroni post-test analysis performed for multiple comparisons. All experiments were performed with a sample size of 3 per treatment group unless otherwise stated. Statistical significance was taken at $p<0.05$ unless otherwise stated.

5. Results

SEM analysis of the CHA scaffolds with and without rhBMP-2, shown in Figure 1A, demonstrated that the introduction of the growth factor did not lead to significant changes in the architectural properties of the scaffolds, which possess pore sizes of ca. 100 µm. Encouragingly, after a minor burst delivery in the first 4 h of the study, the growth factor is released from the scaffolds in a sustained manner, as observed in Figure 1B, suggesting a viable method of growth factor incorporation. After 21 days, approximately 25% (ca. 400ng) of the incorporated rhBMP-2 was released.

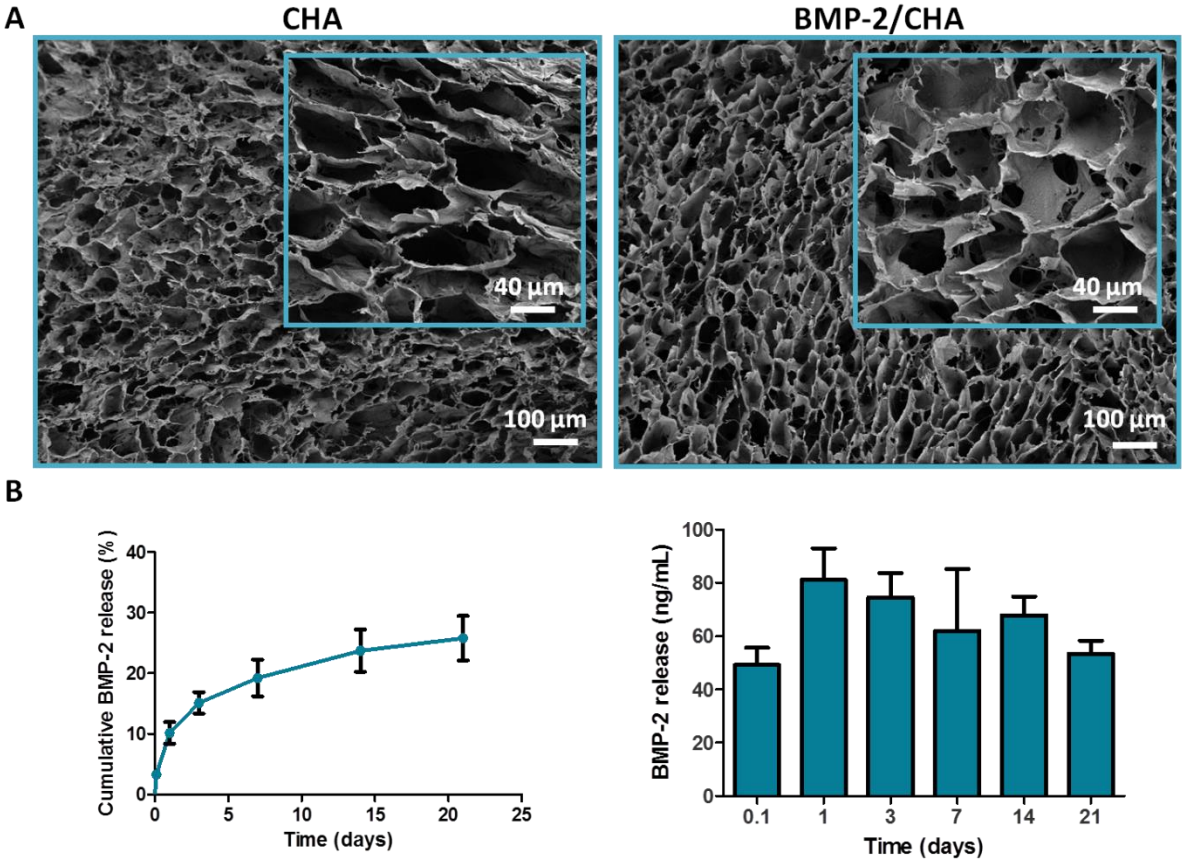


Figure 1. (A) SEM micrographs of collagen–hydroxyapatite scaffolds and collagen–hydroxyapatite scaffolds incorporating rhBMP-2 (BMP-2/CHA). Images were taken at 200 × magnification and inset at 50 × magnification. **(B)** % cumulative and instant dose of RhBMP-2 released from collagen–hydroxyapatite scaffolds.

Once it was established that the rhBMP-2 was delivered in a controlled fashion from the CHA scaffolds, we then investigated whether the protein was stable and bioactive specifically by testing whether it was capable of eliciting an osteoblastic response in terms of ALP activity and subsequent calcium deposition by preosteoblastic cells within non-loaded and rhBMP-2 eluting scaffolds. Figure 2 summarizes the main findings of this study. As shown in Figure 2A, the cell number did not change after 3 days of culture on both scaffold types. However, although non-significant, there was a clear decrease in cell number when osteoblasts were cultured on GF eluting scaffolds for 7 days, compared to those grown on unloaded scaffolds indicative of reduced proliferation due to increased differentiation. The expression of ALP per DNA, depicted in Figure 2B, was enhanced on cells cultured on rhBMP-2 eluting scaffolds during the course of the study, with a significant increase observed after 7 days of culture. Most importantly from a therapeutic perspective, the amount of deposited calcium after 14 days of culture was significantly higher from cells grown on rhBMP-2 eluting scaffolds, as shown in Figure 2C; a cell-free CHA scaffold was utilized as a negative control.

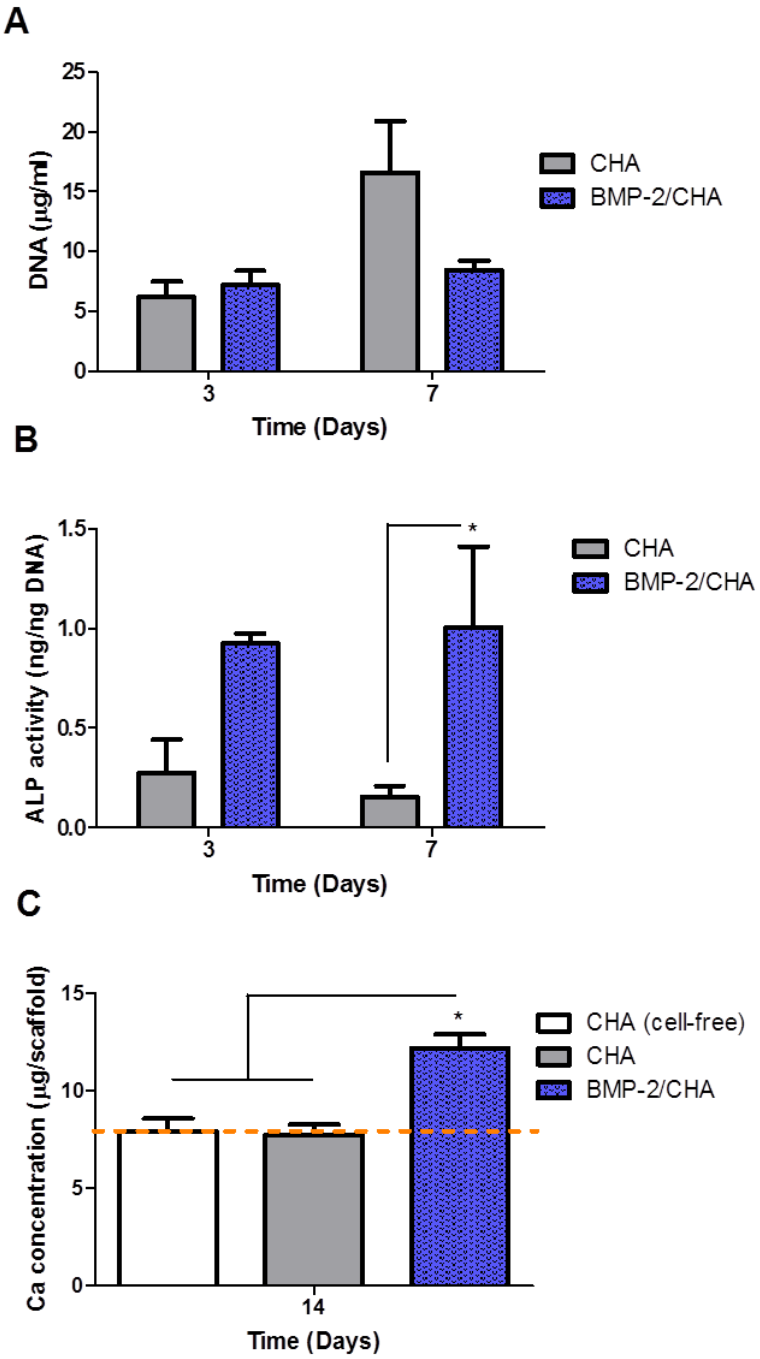


Figure 2. (A) MC3T3 DNA content following 3 and 7 days of culture on collagen–hydroxyapatite and rhBMP-2 eluting collagen–hydroxyapatite scaffolds. **(B)** MC3T3 ALP activity following 3 and 7 days of culture on the scaffolds (*p<0.05). **(C)** Cell mediated calcium deposition on collagen–hydroxyapatite and rhBMP-2 eluting collagen–hydroxyapatite scaffolds following 14 days of culture (*p<0.05).

Having confirmed the pro-osteogenic effect of the rhBMP-2 released from the CHA scaffolds and therefore the *in vitro* viability of the system developed to incorporate the protein, we then tested its ability to enhance bone regeneration *in vivo* using critically sized calvarial defects in male Wistar rats. Unloaded CHA scaffolds were also implanted as well as an untreated empty defect group. The results from the microCT analysis of the explanted defects 8 weeks post-implantation are shown in Figure 3. The microCT images show that there was no *de novo* bone tissue within the defects that were untreated. Unloaded CHA scaffolds were able to elicit a certain level of tissue healing as demonstrated by new regions of bone within the defect. Importantly, the defect was completely filled with new bone in the animals where a BMP-2 eluting scaffold was implanted; additionally, there were no signs of either bone anomalies or bone resorption on defect adjacent areas. Quantification of the newly formed bone within the defect confirmed these observations. It was evident that the new bone volume within the defects treated with GF-eluting CHA scaffolds was approximately 10 and 20 times higher than that of the unloaded CHA scaffold and empty defect respectively.

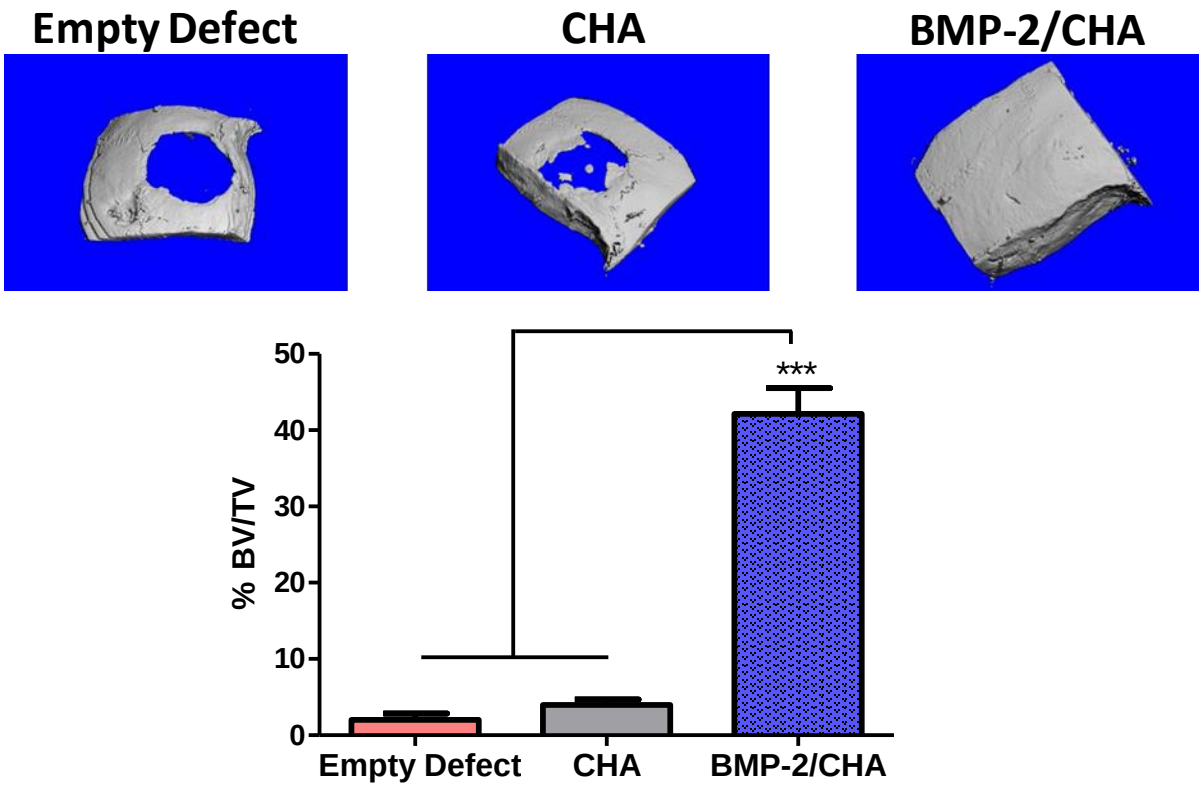


Figure 3. Representative microCT images of empty defect, collagen–hydroxyapatite and rhBMP-2 eluting collagen–hydroxyapatite scaffolds demonstrating the level of bone repair at 8 weeks post-implantation. Quantitative microCT analysis demonstrating that the BMP-2/CHA group supported the highest level of bone formation compared to the empty defect and collagen–hydroxyapatite scaffold (***p<0.001).

The histological examination of the explants after H&E staining, shown in Figure 4, confirms the results obtained by microCT. Only few, thin areas of new bone were observed in untreated defects or those treated with unloaded CHA scaffolds and scaffold resorption was evident (supplementary 1). In comparison the rhBMP-2 eluting scaffolds supported the formation of thick, mature, new bone within the defect site. Encouragingly, the appearance of the *de novo* bone tissue within these defects treated with rhBMP-2-loaded scaffolds was very similar to that of native bone. In all cases, the scaffolds had been completely resorbed 8 weeks after implantation and replaced with new bone, which completely bridged the defect sites. Similar

to microCT, there were no signs of bone anomalies or bone resorption around the defect site and the scaffolds were almost completely degraded and replaced with new bone.

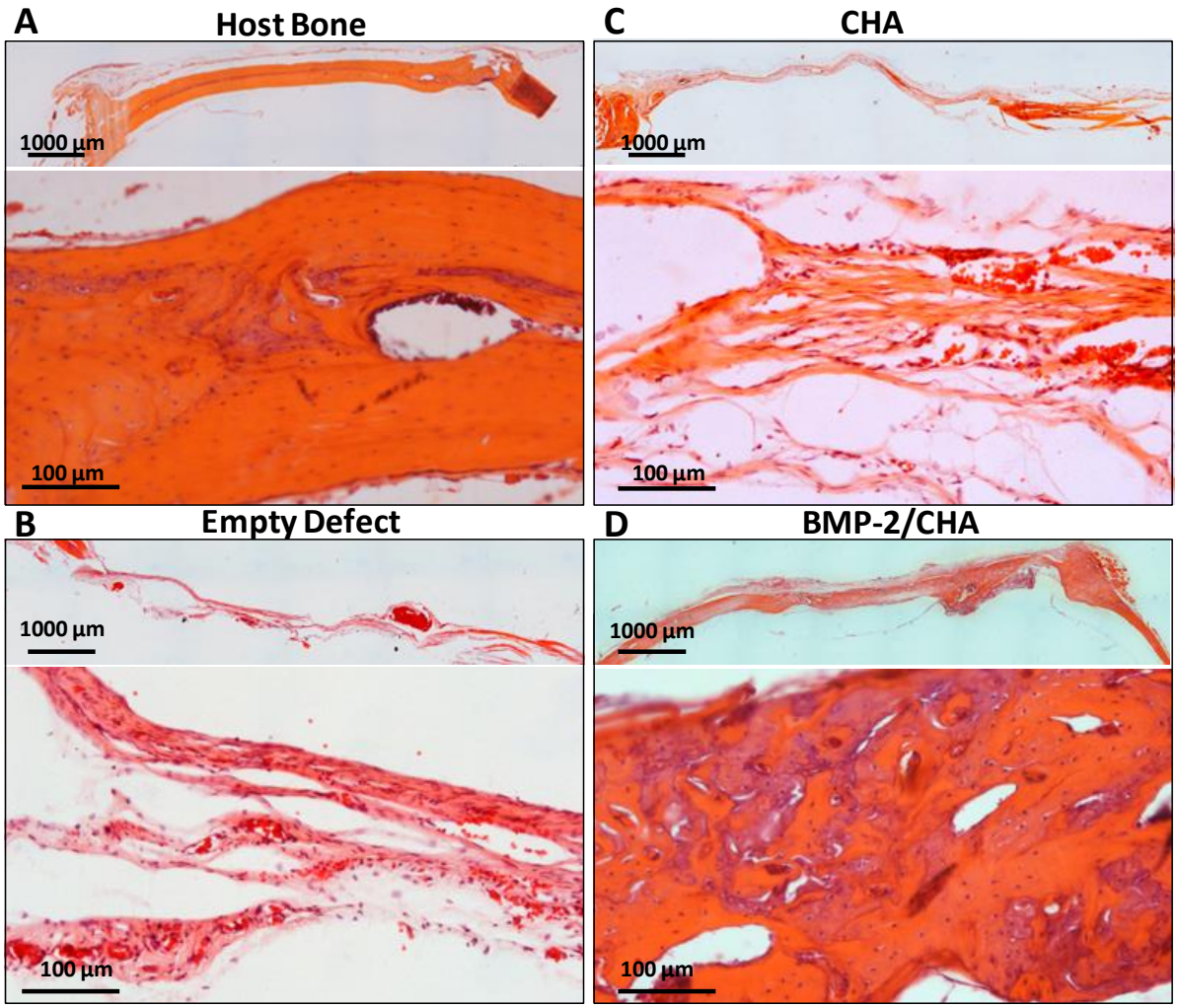


Figure 4. (A) Representative histological sections of the calvarium of an unoperated rat demonstrating the appearance of host bone. Representative histological section of explants from 7 mm calvarial defects in rats left empty **(B)**, treated with collagen–hydroxyapatite scaffolds **(C)** or rhBMP-2 eluting collagen–hydroxyapatite scaffolds **(D)** following 8 weeks of implantation. Bone healing was considerably enhanced in defects where the rhBMP-2 eluting scaffold was implanted and defects were fully bridged following 8 weeks of implantation with an appearance similar to that of the unoperated control.

The quantitative analysis of the histomorphometrical analysis (Figure 5) corroborates the superior healing properties of BMP-2 eluting CHA scaffolds seen on micro CT analysis. At 8 weeks post-implantation, the area of new bone in the defects treated with the GF eluting scaffolds was found to be *circa* 3 and 8 times higher than unloaded CHA and untreated empty defect controls respectively.

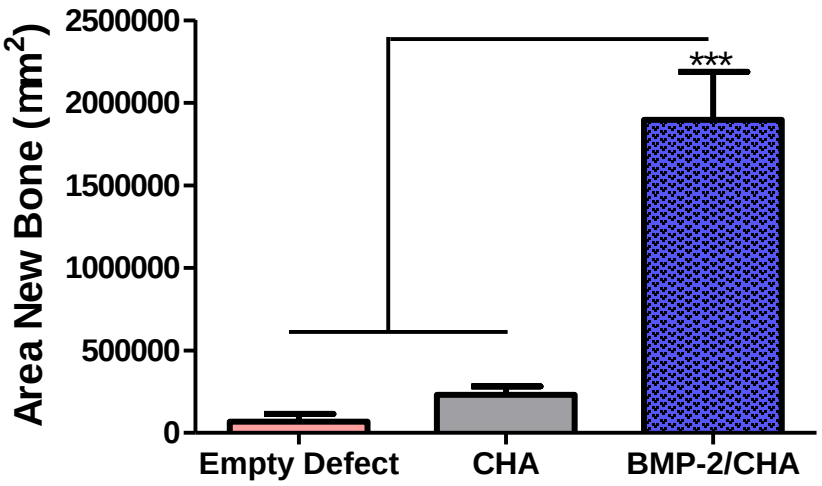


Figure 5. Quantitative histomorphometric analysis indicating the mean total area of bone nucleation sites and accelerated bone healing in the rhBMP-2 eluting collagen–hydroxyapatite scaffold (BMP-2/CHA) compared to the empty defect and non-eluting collagen–hydroxyapatite scaffold (***p<0.001).

6. Discussion

The present study aimed to develop a novel rhBMP-2 delivery system for the sustained release of low doses of rhBMP-2 incorporated directly within collagen–hydroxyapatite scaffolds without the use of carriers or pre/post fabrication functionalization processes. In order to accomplish this, rhBMP-2 was incorporated within the CHA scaffold during an intermediate fabrication step using a lyophilization process. The release profile of the growth factor from

these scaffolds and its bioactivity were evaluated *in vitro*, demonstrating the controlled delivery of bioactive growth factor for three weeks. Importantly, the superior healing properties of the rhBMP-2 eluting CHA scaffolds were also confirmed *in vivo* as shown by the outstanding regeneration of bone tissue within a rat calvarial defect 8 weeks post implantation.

A biomimetic collagen-hydroxyapatite (CHA) porous scaffold for bone regeneration was chosen as an optimal matrix for the immobilization and controlled release of rhBMP-2. The CHA scaffold is a fully resorbable biomaterial and has been shown to be osteoconductive and osteoinductive in different animal models. Therefore, the sustained delivery of rhBMP-2 might enhance even further the regenerative capacity of the scaffold and thus its clinical application to new indications of use. Our goal was to facilitate the sustained delivery of the protein without the utilization of polymeric carriers or using chemical functionalization after scaffold fabrication processes, strategies approached previously by our group[17, 18]. The fabrication of the CHA scaffolds traditionally involves the blending of collagen and hydroxyapatite in an acidic suspension which is subsequently freeze-dried in order to produce a macroporous material. RhBMP-2 is stable at acidic pH such as that utilized in this suspension and, in addition, it has an affinity for HA crystals, a property that has motivated many researchers to fabricate calcium phosphate-based biomaterials as long-term delivery vehicles for rhBMP-2[30-33]. In this regard, we utilized a similar approach by incorporating rhBMP-2 directly via an intermediate fabrication step into this suspension of collagen and hydroxyapatite, at a concentration that would elicit a pro-osteogenic response by MC3T3-E1 preosteoblastic cells upon release.

One major advantage of the bioactive CHA scaffold developed in this study is that the amount of BMP-2 incorporated was 30 times lower (in $\mu g/ cm^2$ of scaffold) than that present in the only currently approved material utilizing rhBMP-2 and the clinical gold standard for bone tissue regeneration in humans (INFUSE® bone graft) as well as being considerably lower than the

amount of rhBMP-7 used clinically (OP-1® bone graft)[34, 35]. RhBMP-2 was delivered in a sustained fashion for 21 days and, interestingly, there was only a minor burst release within the first 4 h, suggesting a strong interaction between the scaffold and the growth factor. According to previous studies, this interaction is based on water bridged hydrogen bonds between the -OH, -NH_2, and -COOH groups of the protein and the HA. Based on the different orientations of the protein, each of these groups might interact with the HA crystallites individually or multiple groups cooperatively[22, 23]. The strong bond between the growth factor and the HA component of the scaffold was further confirmed by the fact that only 25% of the incorporated rhBMP-2 had been released at the end of the study. Theoretically, this scaffold also offers the potential for increased rhBMP-2 release due to substantive biodegradation typically seen following surgical implantation. The rhBMP-2 release profile, without a significant initial burst and showing sustained delivery of the protein, represents a significant advantage compared to the current commercial alternative, which has been linked to very severe secondary effects due to the untargeted and uncontrolled release of the growth factor.

Having demonstrated that the rhBMP-2 was released in a controlled profile from the CHA scaffolds, we investigated whether these eluting bioactive scaffolds were capable of eliciting a pro-osteogenic response *in vitro*. With this purpose in mind, osteoblasts were seeded on rhBMP-2 eluting CHA scaffolds and their bioactivity was compared to that of cells grown on non-loaded scaffolds, used as control. The decrease in osteoblast cell number, together with the increase in ALP activity after 7 days of culture on rhBMP-2 eluting scaffolds compared to controls demonstrates that the delivered growth factor is promoting the differentiation of the cells towards an osteogenic lineage. This fact was further confirmed by the significant increase in calcium production from the osteoblasts cultured on rhBMP-2-loaded scaffolds compared to unloaded CHA scaffolds after 14 days of culture. These outcomes have been already observed by a number of studies utilizing rhBMP-2 on preosteoblasts[24, 36]. However, the fact that the

protein is capable of eliciting such a positive response following immobilization within the scaffolds without the use of carriers or functionalization is a significant finding.

Based on these promising findings, the next step was to confirm the regenerative capacity of the delivery system *in vivo*. To this aim, rhBMP-2-loaded scaffolds were implanted within critical-sized defects in the calvariae of adult male Wistar rats. The characterization of the explanted defects 8 weeks post-implantation demonstrates the healing capacity of these scaffolds. The microCT images demonstrated that the bone defect was completely bridged with new bone when treated with rhBMP-2-loaded CHA scaffolds to a greater extent than previously seen with rhBMP-2 releasing materials in the same animal model[37, 38]. The histomorphometry analyses demonstrated evidence of scaffold resorption and a histological appearance similar to that of native bone when defects were treated with rhBMP-2-loaded CHA scaffolds. Importantly, there were no signs of bone anomalies or adjacent bone resorption on the analyzed explants; features that are indicative of an over dosage of rhBMP-2[39, 40]. These positive findings contradict some previous studies which suggested that rhBMP-2 should ideally be released within the defect area following a very specific release profile *in vitro*: a high burst followed by a sustained release of a much lower dose for achieving optimal bone healing[41].

The results shown in this work demonstrate that, following a relatively simple intermediate step introduced during the scaffold fabrication process, it is possible to obtain rhBMP-2 eluting CHA scaffolds with superior bone healing properties. These materials overcome the offsite release and non-controlled release of rhBMP-2, which are the main drawbacks of INFUSE®, the clinical gold standard for similar applications. In addition, the novelty of this work lies in the fact that the CHA matrix used as delivery platform for the long-term sustained delivery of BMP-2 is biomimetic, osteoinductive, osteoconductive and fully resorbable without the need for microparticle carriers or chemical functionalization. Furthermore, from a cost perspective,

the amount of rhBMP-2 utilized for achieving an optimal regenerative response is lower than that of the current commercial alternative. Thus, this study represents the first step on the development of a material with an immense clinical and commercial potential. In this sense, further studies are being carried out at the moment by our group, such as testing clinically acceptable sterilization techniques or evaluating the regenerative capacity of these materials on weight-wearing defects in conjunction with the INFUSE® delivery system at the same dose and in larger animal models.

7. Conclusions

Novel collagen–hydroxyapatite scaffolds releasing low doses of rhBMP-2 in a long-term, controlled fashion have been fabricated following a microparticle and chemical functionalization-free approach thus minimizing design complexity while overcoming the risks associated with high growth factor doses. The growth factor was incorporated into the scaffolds structure during their fabrication and, after release, elicited a pro-osteogenic response on preosteoblastic cells *in vitro*. These scaffolds induced the complete regeneration of a non-healing critically sized calvarial defect in rats and were fully resorbed 8 weeks after implantation, demonstrating their superior healing properties *in vivo.*

8. Acknowledgments

This publication has emanated from research conducted with the financial support of Science Foundation Ireland under Grant SFI 12/TIDA/B2383. E.Q. & F.J.O'B acknowledge the European Research Council for the funding (239685-CollRegen-ERC-2009-STG). E.T. acknowledges the Health Research Board of Ireland (HRA_POR/2011/27).

9. References

[1] W.F. McKay, S.M. Peckham, J.M. Badura, A comprehensive clinical review of recombinant human bone morphogenetic protein-2 (INFUSE (R) Bone Graft), International Orthopaedics, 31 (2007) 729-734.

[2] N.E. Epstein, Pros, cons, and costs of INFUSE in spinal surgery, Surgical neurology international, 2 (2011) 10-10.

[3] N.E. Epstein, Complications due to the use of BMP/INFUSE in spine surgery: The evidence continues to mount, Surgical neurology international, 4 (2013) S343-352.

[4] C.A. Tannoury, H.S. An, Complications with the use of bone morphogenetic protein 2 (BMP-2) in spine surgery, Spine Journal, 14 (2014) 552-559.

[5] E.A. Wang, V. Rosen, J.S. Dalessandro, M. Bauduy, P. Cordes, T. Harada, D.I. Israel, R.M. Hewick, K.M. Kerns, P. Lapan, D.P. Luxenberg, D. McQuaid, I.K. Moutsatsos, J. Nove, J.M. Wozney, RECOMBINANT HUMAN BONE MORPHOGENETIC PROTEIN INDUCES BONE-FORMATION, Proceedings of the National Academy of Sciences of the United States of America, 87 (1990) 2220-2224.

[6] F.-M. Chen, M. Zhang, Z.-F. Wu, Toward delivery of multiple growth factors in tissue engineering, Biomaterials, 31 (2010) 6279-6308.

[7] T. Crouzier, K. Ren, C. Nicolas, C. Roy, C. Picart, Layer-By-Layer Films as a Biomimetic Reservoir for rhBMP-2 Delivery: Controlled Differentiation of Myoblasts to Osteoblasts, Small, 5 (2009) 598-608.

[8] D.H.R. Kempen, L. Lu, A. Heijink, T.E. Hefferan, L.B. Creemers, A. Maran, M.J. Yaszemski, W.J.A. Dhert, Effect of local sequential VEGF and BMP-2 delivery on ectopic and orthotopic bone regeneration, Biomaterials, 30 (2009) 2816-2825.

[9] Y. Takahashi, M. Yamamoto, Y. Tabata, Enhanced osteoinduction by controlled release of bone morphogenetic protein-2 from biodegradable sponge composed of gelatin and beta-tricalcium phosphate, Biomaterials, 26 (2005) 4856-4865.

[10] M. Alhag, E. Farrell, M. Toner, N. Claffey, T.C. Lee, F. O'Brien, Evaluation of early healing events around mesenchymal stem cell-seeded collagen-glycosaminoglycan scaffold. An experimental study in Wistar rats, Oral Maxillofac Surg, 15 (2011) 31-39.

[11] S. Suliman, Z. Xing, X. Wu, Y. Xue, T.O. Pedersen, Y. Sun, A.P. Døskeland, J. Nickel, T. Waag, H. Lygre, A. Finne-Wistrand, D. Steinmüller-Nethl, A. Krueger, K. Mustafa, Release and bioactivity of bone morphogenetic protein-2 are affected by scaffold binding techniques in vitro and in vivo, Journal of Controlled Release, 197 (2015) 148-157.

[12] B.-B.C. Youan, Chronopharmaceutical drug delivery systems: Hurdles, hype or hope?, Advanced Drug Delivery Reviews, 62 (2010) 898-903.

[13] J.P. Gleeson, N.A. Plunkett, F.J. O'Brien, Addition of hydroxyapatite improves stiffness, interconnectivity and osteogenic potential of a highly porous collagen-based scaffold for bone tissue regeneration, European cells & materials, 20 (2010) 218-230.

[14] F.G. Lyons, J.P. Gleeson, S. Partap, K. Coghlan, F.J. O'Brien, Novel Microhydroxyapatite Particles in a Collagen Scaffold: A Bioactive Bone Void Filler?, Clin Orthop Relat Res, (2014) 1-11.

[15] C.M. Murphy, A. Schindeler, J.P. Gleeson, N.Y.C. Yu, L.C. Cantrill, K. Mikulec, L. Peacock, F.J. O'Brien, D.G. Little, A collagen-hydroxyapatite scaffold allows for binding and co-delivery of recombinant bone morphogenetic proteins and bisphosphonates, Acta Biomaterialia, 10 (2014) 2250-2258.

[16] F.J. O'Brien, Plunkett, N.A, Gleeson, J.P, A collagen/hydroxyapatite composite scaffold, and process for the production thereof, WO/2008/096334, (2008).

[17] E. Quinlan, S. Partap, M.M. Azevedo, G. Jell, M.M. Stevens, F.J. O'Brien, Hypoxia-mimicking bioactive glass/collagen glycosaminoglycan composite scaffolds to enhance angiogenesis and bone repair, Biomaterials, 52 (2015) 358-366.

[18] A. López-Noriega, E. Quinlan, N. Celikkin, F.J. O'Brien, Incorporation of polymeric microparticles into collagen-hydroxyapatite scaffolds for the delivery of a pro-osteogenic peptide for bone tissue engineering, APL Materials, 3 (2015) 014910.

[19] J. Wang, J. Guo, J. Liu, L. Wei, G. Wu, BMP-Functionalised Coatings to Promote Osteogenesis for Orthopaedic Implants, International Journal of Molecular Sciences, 15 (2014) 10150-10168.
[20] M. Kisiel, A.S. Klar, M. Ventura, J. Buijs, M.-K. Mafina, S.M. Cool, J. Hilborn, Complexation and Sequestration of BMP-2 from an ECM Mimetic Hyaluronan Gel for Improved Bone Formation, Plos One, 8 (2013).
[21] M. Kisiel, M. Ventura, O.P. Oommen, A. George, X.F. Walboomers, J. Hilborn, O.P. Varghese, Critical assessment of rhBMP-2 mediated bone induction: An in vitro and in vivo evaluation, Journal of Controlled Release, 162 (2012) 646-653.
[22] X. Dong, Q. Wang, T. Wu, H. Pan, Understanding adsorption-desorption dynamics of BMP-2 on hydroxyapatite (001) surface, Biophysical Journal, 93 (2007) 750-759.
[23] J.W. Shen, T. Wu, Q. Wang, H.H. Pan, Molecular simulation of protein adsorption and desorption on hydroxyapatite surfaces, Biomaterials, 29 (2008) 513-532.
[24] Y. Takuwa, C. Ohse, E.A. Wang, J.M. Wozney, K. Yamashita, BONE MORPHOGENETIC PROTEIN-2 STIMULATES ALKALINE-PHOSPHATASE ACTIVITY AND COLLAGEN-SYNTHESIS IN CULTURED OSTEOBLASTIC CELLS, MC3T3-E1, Biochemical and Biophysical Research Communications, 174 (1991) 96-101.
[25] V. Kartsogiannis, K.W. Ng, Cell lines and primary cell cultures in the study of bone cell biology, Mol. Cell. Endocrinol., 228 (2004) 79-102.
[26] H. Sudo, H.A. Kodama, Y. Amagai, S. Yamamoto, S. Kasai, INVITRO DIFFERENTIATION AND CALCIFICATION IN A NEW CLONAL OSTEOGENIC CELL-LINE DERIVED FROM NEWBORN MOUSE CALVARIA, J. Cell Biol., 96 (1983) 191-198.
[27] A. Yamaguchi, T. Komori, T. Suda, Regulation of osteoblast differentiation mediated by bone morphogenetic proteins, hedgehogs, and Cbfa1, Endocr. Rev., 21 (2000) 393-411.
[28] F.G. Lyons, A.A. Al-Munajjed, S.M. Kieran, M.E. Toner, C.M. Murphy, G.P. Duffy, F.J. O'Brien, The healing of bony defects by cell-free collagen-based scaffolds compared to stem cell-seeded tissue engineered constructs, Biomaterials, 31 (2010) 9232-9243.
[29] M. Alhag, E. Farrell, M. Toner, T.C. Lee, F.J. O'Brien, N. Claffey, Evaluation of the ability of collagen-glycosaminoglycan scaffolds with or without mesenchymal stem cells to heal bone defects in Wistar rats, Oral and maxillofacial surgery, 16 (2012) 47-55.
[30] H. Autefage, F. Briand-Mesange, S. Cazalbou, C. Drouet, D. Fourmy, S. Goncalves, J.-P. Salles, C. Combes, P. Swider, C. Rey, Adsorption and Release of BMP-2 on Nanocrystalline Apatite-Coated and Uncoated Hydroxyapatite/beta-Tricalcium Phosphate Porous Ceramics, Journal of Biomedical Materials Research Part B-Applied Biomaterials, 91B (2009) 706-715.
[31] T. Crouzier, F. Sailhan, P. Becquart, R. Guillot, D. Logeart-Avramoglou, C. Picart, The performance of BMP-2 loaded TCP/HAP porous ceramics with a polyelectrolyte multilayer film coating, Biomaterials, 32 (2011) 7543-7554.
[32] S. Piskounova, J. Forsgren, U. Brohede, H. Engqvist, M. Stromme, In Vitro Characterization of Bioactive Titanium Dioxide/Hydroxyapatite Surfaces Functionalized with BMP-2, Journal of Biomedical Materials Research Part B-Applied Biomaterials, 91B (2009) 780-787.
[33] W.J. King, P.H. Krebsbach, Growth factor delivery: How surface interactions modulate release in vitro and in vivo, Advanced Drug Delivery Reviews, 64 (2012) 1239-1256.
[34] M. Pecina, L.R. Giltaij, S. Vukicevic, Orthopaedic applications of osteogenic protein-1 (BMP-7), Int Orthop, 25 (2001) 203-208.
[35] L.B.E. Shields, G.H. Raque, S.D. Glassman, M. Campbell, T. Vitaz, J. Harpring, C.B. Shields, Adverse effects associated with high-dose recombinant human bone morphogenetic protein-2 use in anterior cervical spine fusion, Spine, 31 (2006) 542-547.
[36] E.-C. Kim, T.-H. Kim, J.-H. Jung, S.O. Hong, D.-W. Lee, Enhanced osteogenic differentiation of MC3T3-E1 on rhBMP-2-immobilized titanium via click reaction, Carbohydrate Polymers, 103 (2014) 170-178.

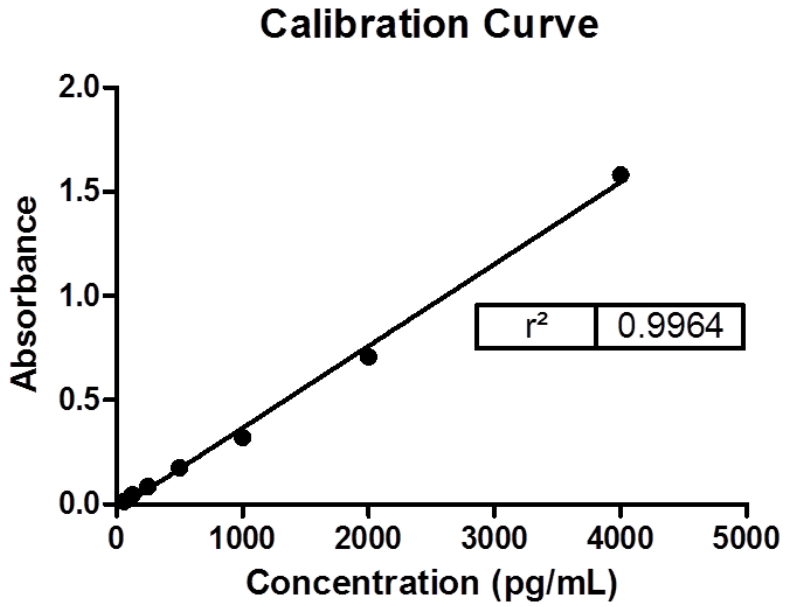
[37] E.W.H. Bodde, O.C. Boerman, F.G.M. Russel, A.G. Mikos, P.H.M. Spauwen, J.A. Jansen, The kinetic and biological activity of different loaded rhBMP-2 calcium phosphate cement implants in rats, Journal of Biomedical Materials Research Part A, 87A (2008) 780-791.

[38] C.V. Rahman, D. Ben-David, A. Dhillon, G. Kuhn, T.W.A. Gould, R. Müller, F.R.A.J. Rose, K.M. Shakesheff, E. Livne, Controlled release of BMP-2 from a sintered polymer scaffold enhances bone repair in a mouse calvarial defect model, Journal of Tissue Engineering and Regenerative Medicine, 8 (2014) 59-66.

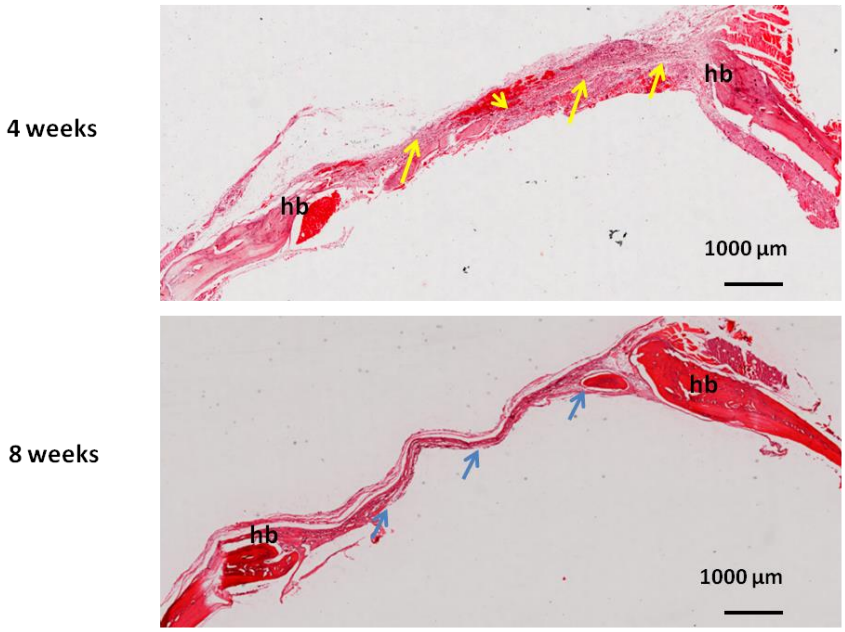
[39] J.N. Zara, R.K. Siu, X. Zhang, J. Shen, R. Ngo, M. Lee, W. Li, M. Chiang, J. Chung, J. Kwak, B.M. Wu, K. Ting, C. Soo, High Doses of Bone Morphogenetic Protein 2 Induce Structurally Abnormal Bone and Inflammation In Vivo, Tissue Engineering Part A, 17 (2011) 1389-1399.

[40] K.-U. Lewandrowski, C. Nanson, R. Calderon, Vertebral osteolysis after posterior interbody lumbar fusion with recombinant human bone morphogenetic protein 2: A report of five cases, Spine Journal, 7 (2007) 609-614.

[41] B. Li, T. Yoshii, A.E. Hafeman, J.S. Nyman, J.C. Wenke, S.A. Guelcher, The effects of rhBMP-2 released from biodegradable polyurethane/microsphere composite scaffolds on new bone formation in rat femora, Biomaterials, 30 (2009) 6768-6779.



Supplementary 1. Standard curve of corrected mean absorbance for each rhBMP-2 standard against concentration of each standard. Optical density was determined at 450 nm with wavelength correction at 540 nm.



Supplementary 2. Histological images of collagen-hydroxyapatite scaffolds at 4 and 8 weeks post implantation in a rat calvarial defect model demonstrating that the scaffold is significantly resorbed following 8 weeks implantation. Yellow arrows represent areas of remaining scaffold, blue arrows represent bone nucleation sites/areas of new bone formation and host bone = hb.