Acta Biomaterialia xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

A collagen–hydroxyapatite scaffold allows for binding and co-delivery of recombinant bone morphogenetic proteins and bisphosphonates

Ciara M. Murphy [a,b,*], Aaron Schindeler [a,b], John P. Gleeson [c], Nicole Y.C. Yu [a,b], Laurence C. Cantrill [a,b], Kathy Mikulec [a], Lauren Peacock [a], Fergal J. O'Brien [c,d,e], David G. Little [a,b]

[a] Orthopaedic Research & Biotechnology Unit, The Children's Hospital at Westmead, Sydney, NSW, Australia
[b] Discipline of Paediatrics and Child Health, Faculty of Medicine, University of Sydney, Sydney, NSW, Australia
[c] Tissue Engineering Research Group, Department of Anatomy, Royal College of Surgeons in Ireland, Dublin, Ireland
[d] Trinity Centre for Bioengineering, Trinity College, Dublin, Ireland
[e] Advanced Materials and BioEngineering Research Centre (AMBER), RCSI & TCD, Dublin, Ireland

ARTICLE INFO

Article history:
Received 3 October 2013
Received in revised form 16 December 2013
Accepted 14 January 2014
Available online xxxx

Keywords:
Zoledronic acid
Bisphosphonate
rhBMP-2
Collagen
Hydroxyapatite

ABSTRACT

An emerging paradigm in orthopedics is that a bone-healing outcome is the product of the anabolic (bone-forming) and catabolic (bone-resorbing) outcomes. Recently, surgical and tissue engineering strategies have emerged that combine recombinant human bone morphogenetic proteins (rhBMPs) and bisphosphonates (BPs) in order to maximize anabolism and minimize catabolism. Collagen-based scaffolds that are the current surgical standard can bind rhBMPs, but not BPs. We hypothesized that a biomimetic collagen–hydroxyapatite (CHA) scaffold would bind both agents and produce superior in vivo outcomes. Consistent with this concept, in vitro elution studies utilizing rhBMP-2 ELISA assays and scintillation counting of ^{14}C-radiolabeled zoledronic acid (ZA) confirmed delayed release of both agents from the CHA scaffold. Next, scaffolds were tested for their capacity to form ectopic bone after surgical implantation into the rat hind limb. Using CHA, a significant 6-fold increase in bone volume was seen in rhBMP-2/ZA groups compared to rhBMP-2 alone, confirming the ability of ZA to enhance rhBMP-2 bone formation. CHA scaffolds were found to be capable of generating mineralized tissue in the absence of rhBMP-2. This study has implications for future clinical treatments of critical bone defects. It demonstrates the relative advantages of co-delivering anabolic and anti-catabolic agents using a multicomponent scaffold system.

1. Introduction

Bone and fracture repair occurs naturally through a series of anabolic and catabolic processes [1]. However, in the cases of severe trauma or disease, surgical intervention is required. Bone grafts and bone graft substitutes are used in the surgical repair and reconstruction of critical defects. Currently the clinical "gold standard" approach is the use of autografts. This approach is not without complications, such as donor site morbidity, quantity of available tissue and the need for a second surgery. The use of allografts can overcome these obstacles. However, there is a worldwide shortage of donors which, together with an associated risk of disease transmission, hinders allografts as an ideal treatment option [2].

Bone tissue engineering aims to develop bone graft replacements that can repair bone defects without a need for allografts or autografts. Currently, collagen matrices loaded with anabolic agents are clinically utilized as bone graft substitutes. Recombinant human bone morphogenetic proteins (rhBMPs) are potent anabolic agents that are in clinical use for the treatment of non-union and critical-sized bone defects. Treatment with rhBMPs facilitates the differentiation of mesenchymal progenitors into bone-forming osteoblasts [3]. This leads to robust induction of new bone formation. However, in non-load-bearing defects or when used at high local doses, rhBMPs have been shown to both directly and indirectly induce osteoclast differentiation [4,5]. This is problematic as it can lead to the premature resorption of bone [5–7].

An emerging paradigm for orthopedics is that bone formed from an anabolic stimulus can be maximized by use of an anti-catabolic agent [8–11]. Bisphosphonates (BPs) are anti-catabolics clinically used in the treatment of osteoporosis and metabolic bone diseases, and have also been shown to minimize bone loss due to unloading or stress shielding [12]. BPs have a high affinity for the hydroxyapatite

* Corresponding author at: Orthopaedic Research & Biotechnology Unit, The Children's Hospital at Westmead, Sydney, NSW, Australia. Tel.: +61 2 98451451; fax: +61 2 98453078.
E-mail address: ciara.murphy@sydney.edu.au (C.M. Murphy).

http://dx.doi.org/10.1016/j.actbio.2014.01.016
1742-7061/

(HA) within bone mineral. Once bound, they potently inhibit osteoclast-mediated resorption [13,14]. Only the osteoclasts are able to mobilize and internalize BPs bound with high affinity to the bone mineral. As the osteoclasts resorb the bone, they internalize the BPs, leading to inhibition of osteoclast activity and/or osteoclast apoptosis [15–18].

To date, studies utilizing combined anabolic and anti-catabolic treatments have typically relied on systemic BP delivery [12,19]. However, there are a number of adverse affects associated with systemic dosing of PBs, including stomach ulceration (oral BPs) and flu-like symptoms (intravenous dosing) [20,21]. In rare cases major complications have been reported, such as renal failure and osteonecrosis of the jaw, although these are typically linked to underlying kidney diseases or dental problems [22]. Local delivery has emerged as an appealing alternative to systemic delivery as it avoids these complications and can lower the absolute BP dose required. Furthermore, it simplifies treatment for the patient as both drug delivery and surgery are combined into one procedure rather than requiring post-surgical systemic/oral administration. Multiple studies have utilized polymeric-based (poly-L-lactide, PLLA; poly(lactic-*co*-glycolic) acid, PLGA) carriers to demonstrate synergistic benefits for co-delivering rhBMPs and BPs locally [23,24]. However, these materials can be associated with problems, including the release of acidic degradation by-products that can alter the pH in surrounding tissue. In turn, this can cause adverse tissue and inflammatory reactions [25,26].

Collagen, as a naturally derived biomaterial, has many advantages over most synthetically derived polymers. It shows superior biocompatibility, biodegradability, interconnected porous architecture [16] and a capacity to bind rhBMPs. As such, it has been adopted as an rhBMP delivery system now in clinical use (rhBMP-2: Medtronic INFUSE®; and rhBMP-7: Olympus OP-1® bone graft). However, while collagen provides structural stability to a number of endogenous tissues, including bone, scaffolds comprising collagen possess poor load-bearing capabilities.

This study trialed a recently developed composite collagen–hydroxyapatite (CHA) scaffold that incorporates both collagen and HA in a porous scaffold matrix [27]. These materials are the two major constituents of bone and a logical choice as the basis of a biomimetic scaffold capable of supporting and promoting bone regeneration [28]. Furthermore, the CHA scaffold combines the advantages conferred by the mechanical strength of ceramics with the biological advantages of collagen. The CHA scaffold has been developed using a patented fabrication process [20] and incorporates features used in the development of a range of collagen-based scaffolds optimized in terms of composition [26,29], cross-linking density [30,31] and pore architecture [32–35], for use in bone tissue engineering applications. The CHA scaffold has 99% porosity with higher pore interconnectivity than standard collagen, measured in terms of scaffold permeability (0.4×10^{-9} m^4 N^{-1} s^{-1} vs. 4.5×10^{-9} m^4 N^{-1} s^{-1}). The addition of HA confers improved mechanical strength over collagen alone (1.3 kPa vs. 0.5 kPa). This CHA scaffold has previously facilitated healing of a critical-sized rat calvarial defect without the addition of exogenous pro-osteogenic factors [36].

It was hypothesized that this biphasic collagen/HA scaffold would show superior binding of an rhBMP-2/BP combination. Collagen facilitates rhBMP-2 binding [37], and BPs have a high affinity for HA [38]. It was further speculated that this combination of collagen, HA, rhBMP-2 and the BP zoledronic acid (ZA) would achieve superior bone formation. The first aim of this study was to investigate the binding and retention of rhBMP-2 and ZA to the biphasic components of the scaffold in comparison to control collagen scaffolds. The second aim was to then test the efficacy of this system in an ectopic bone formation model in a rodent hind limb.

2. Materials and methods

2.1. Pharmaceuticals

rhBMP-2 from the INFUSE® Bone Graft Small Kit was purchased from Medtronic (Memphis, TN, USA). ZA was purchased from AXXORA, LLC (San Diego, CA, USA). For in vitro studies, a stock solution of ^{14}C-radiolabeled ZA (^{14}C-ZA) with a specific activity of 7.027 MBq mg^{-1} was supplied by Novartis Pharma AG (Switzerland). This was diluted with unlabeled ZA to generate a 1 mg ml^{-1} solution with a specific activity of 703 kBq mg^{-1}. AlexaFluor 647 was purchased from Life Technologies (Victoria, Australia).

2.2. Scaffold fabrication

The CHA scaffold was fabricated as previously described [36]. In brief, collagen slurries were produced by the homogenization of fibrillar collagen (Collagen Matrix, Franklin Lakes, NJ, USA) within a 0.5 M acetic acid solution. Slurries were homogenized in a reaction vessel and cooled to 4 °C by a WK1250 cooling system (Lauda, Westbury, NY, USA) using an overhead blender (IKA Works Inc., Wilmington, NC, USA). In parallel, HA particles with a mean diameter of 5 μm (Plasma Biotal Limited, North Derbyshire, UK) were suspended in a 0.5 M acetic acid solution. Collagen concentration was 0.1 g ml^{-1} and HA concentration was 0.2 g ml^{-1}. The final composite slurry was produced by lyophilization after being pipetted into a stainless steel pan (125 × 125 mm, grade 304 SS) and cooled to −40 °C at a constant cooling rate of 0.9 °C min^{-1}. After freezing, ice crystals were removed by sublimation for 17 h at 0 °C and 200 mtorr [32,39]. This produced scaffolds with a pore size of ~100 μm. Dehydrothermal (DHT) cross-linking treatment was carried out as previously described [40] under a vacuum of 0.05 bar at a temperature of 120 °C for 24 h. CHA scaffolds were sliced into 6 mm × 3 mm sections for both in vitro and in vivo analysis.

A porous collagen sponge was used as a control for in vitro investigations. Rectangular sponges (6 mm × 3 mm) were sliced from 4 mm thick collagen sheets supplied from INFUSE® Bone Graft Small Kit (Medtronic, USA). Each scaffold was loaded with 10 μg rof hBMP-2. Following the manufacturer's instructions, rhBMP-2 solution (20 μl × 1 mg ml^{-1}) was dripped onto the collagen scaffold 20 min prior to surgical insertion.

2.3. BP binding to CHA and collagen scaffolds

Fluorescently labeled BP (Pamidronate, PAM) was used to assess the binding of the BP within the CHA and collagen. PAM was fluorescently labeled with a commercially available AlexaFluor 647 (Life Technologies, Victoria, Australia) according to the manufacturer's protocol. The resultant fluorescently labeled BP was termed AlexaPam 647. PAM has a free amine group and can be readily labeled using protein labeling kits, as opposed to the nitrogen-containing ring structure of the ZA side-chain. The addition of a die moiety does not compromise the "bone hook" involved with mineral avidity, allowing it to be used to investigate BP binding interactions in vitro.

CHA scaffolds and collagen sponges were loaded with 2 μg of AlexaPam 647 and washed for 24 h in phosphate-buffered saline (PBS) at 37 °C. The scaffolds were imaged using a Leica TCS SP5 confocal microscope (Leica Microsystems, NSW, Australia), both pre- and post-washing.

2.4. BP elution from CHA and collagen scaffolds

Release of BP from both CHA and collagen scaffolds was measured using radiolabeled ZA (^{14}C-ZA). Specimens loaded with

Please cite this article in press as: Murphy CM et al. A collagen–hydroxyapatite scaffold allows for binding and co-delivery of recombinant bone morphogenetic proteins and bisphosphonates. Acta Biomater (2014), http://dx.doi.org/10.1016/j.actbio.2014.01.016

either 1 or 2 μg of ^{14}C-ZA were prepared by the addition of 100 μl of ^{14}C-ZA solution via pipette. Both CHA and collagen scaffolds were compared. The scaffolds were washed in PBS for 24 h at 37 °C. ^{14}C-ZA elution was measured with a Tri-Carb scintillation counter (Packard, MN, USA) using a scintillation cocktail (5 ml of Ultima Gold DIPN, PerkinElmer, Inc., MA, USA) and counted in 20 ml Pico Prias polyethylene vials (Perkin-Elmer, Australia).

2.5. RhBMP elution from CHA and collagen scaffolds

The release of rhBMP-2 from both CHA and collagen scaffolds was measured over 6 weeks. $N = 3$ scaffolds were measured per group. Samples were loaded with 10 μg of rhBMP-2 and were incubated at 37 °C in Protein LoBind tubes (Eppendorf, UK) containing 2 ml of elution buffer (1% bovine serum albumin (BSA)/PBS) formulated to prevent adsorption to the vessel. At time points of 1 min (0.01 h), 1 h, 1 day (24 h), 1 week (168 h), 3 weeks (504 h) and 6 weeks (1008 h) scaffolds were transferred to new tubes containing fresh elution buffer. Collected samples were stored at −20 °C. The amount of rhBMP-2 drug released was quantified using a Qunatikine® BMP-2 Immunoassay (PDBP200, R&D Systems, Wiesbaden, Germany) as per the manufacturer's instructions.

2.6. Cell culture and cell viability

MC3T3-E1 murine pre-osteoblasts were cultured in α-minimum essential medium supplemented with 10% fetal bovine serum, 1% L-glutamine and 2% penicillin/streptomycin (Invitrogen) and used to determine the ability of HA within CHA scaffolds to protect cells from cytotoxic ZA. MC3T3-E1 cells were seeded in 24-well plates at a density of 2×10^4 cells per well in 500 μl of standard media containing 50 μM ZA (kindly supplied by Novartis Pharmaceuticals, NSW, Australia). In order to determine the ability of CHA to protect cell viability by binding and retaining BP in comparison to collagen scaffolds, 6 mm × 3 mm sections of both CHA and collagen scaffolds were placed within the wells.

Viability was measured at 4 and 7 days post-seeding. Cellular viability was assessed using the CellTitre 96 Aqueous One Solution Cell Proliferation Assay kit (Promega) according to the manufacturer's instructions. Briefly, cells were incubated with the viability solution for 30 min at 37 °C and read using a spectrophotometer at 490 nm. All samples were assayed in triplicate and the results are representative of $n = 6$.

2.7. Animal care

Male Wistar rats (10–12 weeks old, average weight 400–450 g) were housed in autoclavable polypropylene solid boxes with stainless steel lids and polycarbonate water bottles with food and mouse chow supplied ad libitum. Ethics approval was obtained for all procedures from the Westmead Hospital Animal Ethics Committee.

Table 1
Experimental design for the ectopic bone formation study.

Group	Scaffold	Local anabolic agent	Local anti-catabolic agent	Specimens
1	CHA	–	–	8
2	CHA	5 μg rhBMP-2	–	8
3	CHA	5 μg rhBMP-2	10 μg ZA	8
4	CHA	10 μg rhBMP-2	–	8
5	CHA	10 μg rhBMP-2	2 μg ZA	8
6	CHA	10 μg rhBMP-2	10 μg ZA	8

2.8. Surgical model

Ectopic bone formation was assessed in a bilateral quadriceps muscle pouch via surgical implantation of the drug-loaded CHA scaffolds (Table 1). The surgical model and dosing regimens were based on previously published methods where rhBMP-2 and ZA were delivered in a carboxymethylcellulose and polymer carrier [41,42]. Agents were applied to scaffolds 20 min prior to implantation using a pipette.

In brief, anesthesia was induced and maintained using inhaled isofluorane. The operative sites were trimmed and prepared with povidone–iodine. Muscle pouches were created in the hind limb (in line with the femur) using a scalpel. The CHA scaffolds were inserted and the muscle and skin closed using a 5–0 Vicryl suture (Ethicon, Somerville, NJ, USA). Following surgery, animals were recovered on a heated pad and placed in recovery cages. Post-operative pain was managed with subcutaneously injected buprenorphine (0.05 mg kg^{-1}) and dehydration was managed by saline injection, as required. Animals were monitored daily and weighed weekly for the duration of the study. At 4 weeks post-surgery, animals were euthanized via CO_2 asphyxiation, and the femurs and surrounding muscle were collected for subsequent analyses.

2.9. Radiographic analysis

The placement of the ectopic bone nodules relative to the femur was determined by radiography using a digital X-ray (XR) machine (Faxitron X-ray Corp, Wheeling, IL, USA) of whole hind limbs (27 kV, ×2 magnification). Ectopic bone samples and the surrounding soft tissue were further isolated and individual XR images captured (27 kV, 3× magnification). Samples were examined by micro-computed tomography (μCT) scanning using a SkyScan 1174 compact μCT scanner (SkyScan, Kontich, Belgium). The primary outcome measure for the study was total bone volume (BV) of the entire ectopic bone nodule. Samples were scanned in 70% ethanol at 14.7 μm pixel resolution, 0.5 mm aluminum filter, 50 kV XR tube voltage and 800 μA tube electric current. The images were reconstructed using NRecon, version 1.5.1.5 (SkyScan) and 3-D assessments of the entire ectopic bone nodule were conducted with CTAnalyser software, version 1.9.2.3 (SkyScan). The bone tissue was defined as calcified tissue detected by μCT using a predetermined global threshold set at 0.4 g cm^{-3} of CHA. Secondary outcome measurements included bone tissue alone mineral density (BTMD, g cm^{-3} of CHA) of the entire ectopic bone nodule. In addition, the trabecular-like region of the ectopic bone nodule was delineated from the denser cortical sheath. Additional outcome measurements of the trabecular measurements included trabecular thickness (TbTh, mm) and trabecular number (TbN, mm^{-1}). A global threshold representing bone (44–255) was defined following analysis of consecutive 2-D slices of bone samples on CTAnalyser software. Representative 3-D bone pellets were reconstructed with transaxial slices (50 slices) from the pellet's mid-section, using CTVol Realistic Visualization software version 2.1.0.0 (SkyScan). Representative samples from each group were selected as having a BV closest to the group's mean.

2.10. Tissue histology

For paraffin histology, samples were decalcified and embedded in paraffin blocks. 0.5 mm thick sections were cut using a Leica RM 2155 Microtome (Leica Microsystems, Wetzlar, Germany). Sections were stained with tartrate-resistant acid phosphatase (TRAP) for osteoclasts. Stained sections were scanned with a ScanScope digital slide scanner (Aperio Technologies, Vista, CA, USA), and images were captured with ImageScope (Aperio Technologies). Represen-

Please cite this article in press as: Murphy CM et al. A collagen–hydroxyapatite scaffold allows for binding and co-delivery of recombinant bone morphogenetic proteins and bisphosphonates. Acta Biomater (2014), http://dx.doi.org/10.1016/j.actbio.2014.01.016

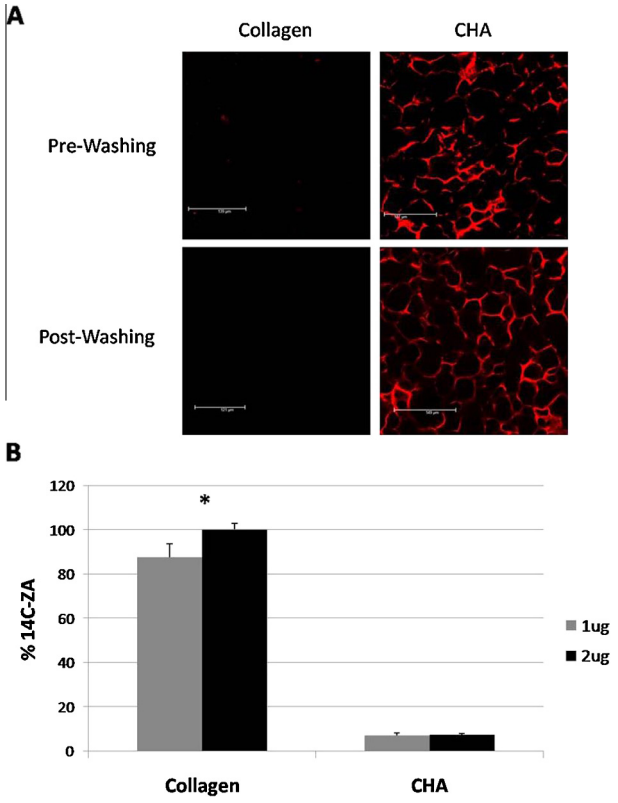

Fig. 1. (A) Representative images of the fluorescently labeled BP (AlexaPam 647 – red in color) within both scaffold types. No AlexaPam 647 is visible in the collagen scaffolds pre- or post-washing. Within CHA scaffolds the struts are labeled red both pre- and post-washing due to the retention of AlexaPam 647 by the HA. (B) Quantification of ^{14}C-ZA elution from porous collagen and CHA scaffolds post-washing. $^{*}p < 0.01$ in comparison to CHA elution of both 1 and 2 μg ^{14}C-ZA.

tative images were selected and cropped using ScanScope and Photoshop (Adobe Technologies) software.

2.11. Statistical analysis

For the statistical analysis of in vitro elution assays, significance was determined by Student's *t*-test. For the in vivo studies we were not able to assume that the formation of ectopic bone followed a normalized distribution. Thus non-parametric Kruskal–Wallis and Mann–Whitney *U*-tests were performed. All analyses were performed using SPSS Statistics version 17 (SPSS, Chicago, IL, USA). Statistical significance was set at $\alpha < 0.05$.

3. Results

3.1. BP binding potential of CHA Scaffolds

To examine the binding affinity of BP to the HA particles within CHA scaffolds, the incorporation of AlexaPam 647 within the scaffold was assessed via fluorescent confocal microscopy. Control collagen sponge was used as a comparative. Both scaffold types were assessed immediately after loading with AlexaPam 647 and thorough washing. No florescent BP was observed to be bound to the collagen scaffold. In contrast, AlexaPam 647 bound to the HA particles within the struts of the CHA scaffold (Fig. 1A).

Next, this phenomenon was quantified by assaying the release of radiolabeled ^{14}C-ZA from both scaffold types (Fig. 1B). Background controls were assayed using both no eluent and eluent from CHA scaffolds without ^{14}C-ZA loading and neither showed a

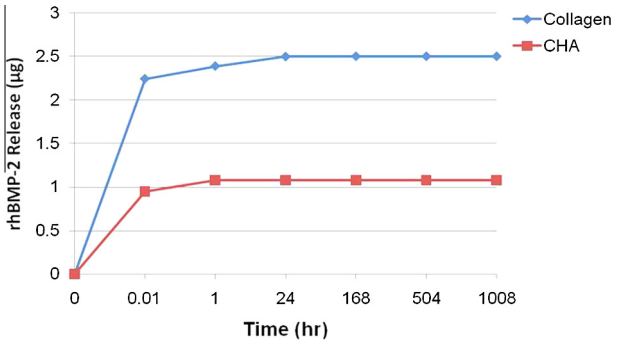

Fig. 2. In vitro rhBMP-2 elution characteristics over 6 weeks from porous collagen and CHA scaffolds.

significant reading. There was a significant difference between the two scaffold types in terms of total ^{14}C-ZA eluted ($p < 0.001$). The collagen scaffolds released >90% ^{14}C-ZA after 24 h. Only 7% of the ^{14}C-ZA was eluted from CHA scaffolds. Increasing the amount of ^{14}C-ZA from 1 to 2 μg within the scaffolds did not change the proportion of BP eluted from either scaffold type.

These results demonstrate the potential of the CHA scaffold to retain and slowly release BP in comparison to collagen alone.

3.2. Elution of rhBMP-2 from porous CHA and collagen scaffolds

In order to confirm the binding of rhBMP-2 to CHA scaffolds, an in vitro elution study was performed over 6 weeks with eluents quantified by ELISA. The release profile of control porous collagen scaffold was used as a comparison.

In both scaffold types the most rapid release of rhBMP-2 was observed within the first minute, showing an initial burst. This was followed by a sustained release of rhBMP-2 (Fig. 2). The amount of rhBMP-2 released from CHA scaffolds was 45–50% of the amount of rhBMP-2 released from collagen, indicating a greater retention of rhBMP-2 within the CHA scaffolds. Although the total release of rhBMP-2 from both scaffold types represented less than 30% of the total rhBMP-2 incorporated into the scaffold, it is important to note that the scaffolds did not undergo the substantive biodegradation seen after surgical implantation.

3.3. HA within CHA scaffolds yields protection against the cytotoxic effects of ZA in vitro

A cell culture study was performed to determine whether the presence of CHA scaffolds containing HA could protect cells from

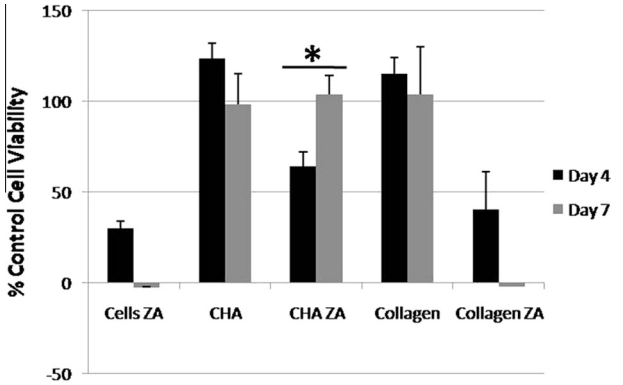

Fig. 3. MC3T3 cell viability in the presence of collagen and CHA with and without ZA. CHA has a protective effect against the cytotoxic effects of ZA on cell viability in comparison to collagen. $^{*}p < 0.05$ in relation to Collagen ZA at days 4 and 7.

Please cite this article in press as: Murphy CM et al. A collagen–hydroxyapatite scaffold allows for binding and co-delivery of recombinant bone morphogenetic proteins and bisphosphonates. Acta Biomater (2014), http://dx.doi.org/10.1016/j.actbio.2014.01.016

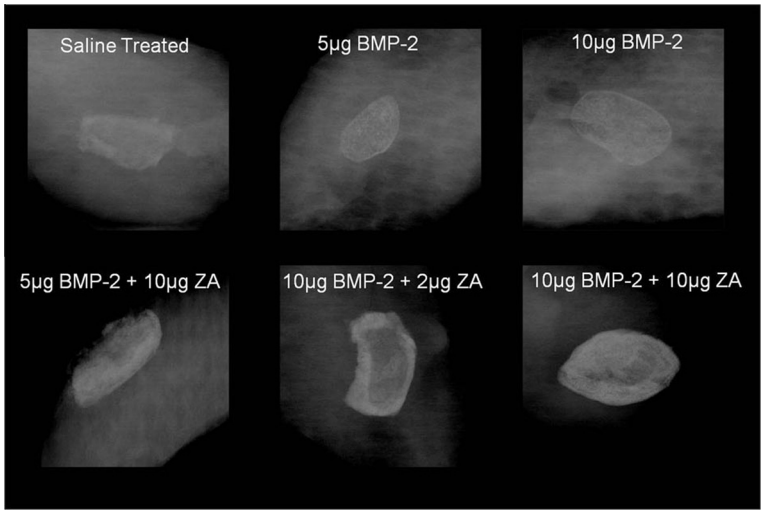

Fig. 4. Representative XR images of pellets within all treatment groups at 4 weeks post-implantation, demonstrating bone formation.

the cytotoxic effects of high-dose BP. MC3T3 pre-osteoblasts were cultured in 50 μM ZA, a dose that has been previously shown to be cytotoxic to cells [43]. Consistent with prior reports, ZA treatment significantly decreased viable cell numbers in 4 days and no viable cells were detectable by day 7. However, the presence of HA in the CHA scaffolds afforded substantive protection against these effects (Fig. 3), with cells surviving at 7 days, while in contrast, the Collagen ZA group failed to offer a cytotoxic-protective benefit against

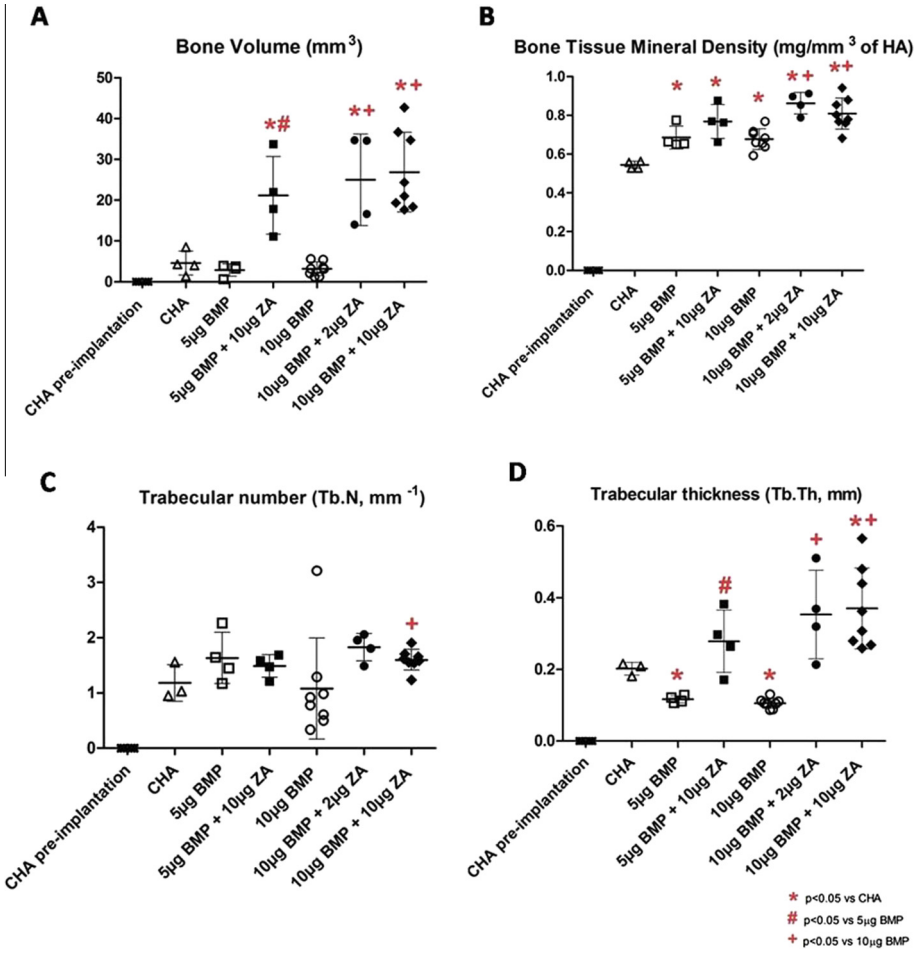

Fig. 5. μCT analysis of ectopic bone nodules at 4 weeks post-scaffold implantation. BV (mm^3) significantly increased in the combined rhBMP-2/ZA treatment groups compared to their respective rhBMP-2 controls (A). BTMD (mg mm^{-3}) significantly increased with the addition of rhBMP-2. This was further increased in the 10 μg rhBMP-2/ZA groups compared to respective 10 μg rhBMP-2 control group (B). (TbN (mm^{-1}) increased with the highest doses of rhBMP-2/ZA. There was no difference seen between the other groups (C). The addition of ZA increased TbTh (mm) in comparison to rhBMP-2 alone treatment groups (D). Error bars represent standard deviation.

Please cite this article in press as: Murphy CM et al. A collagen–hydroxyapatite scaffold allows for binding and co-delivery of recombinant bone morphogenetic proteins and bisphosphonates. Acta Biomater (2014), http://dx.doi.org/10.1016/j.actbio.2014.01.016

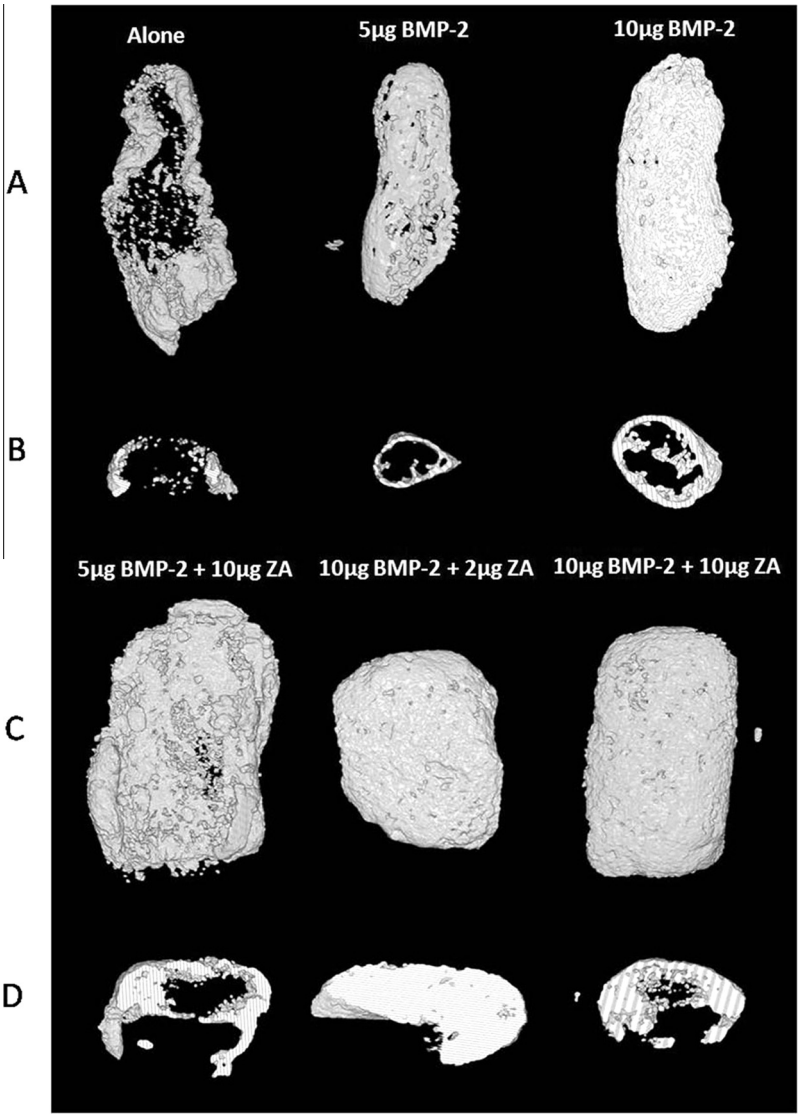

Fig. 6. (A, B) Representative μCT reconstructions, (C, D) corresponding transaxial slices (stack of 50 slices) of μCT images of bone nodules resulting from CHA scaffold loaded with saline/rhBMP-2/rhBMP-2 + ZA treatments.

the effects of BP treatment on cell viability at either time point, such that no cell viability was detected by day 7.

These data indicate that the presence of the CHA scaffolds is able to ameliorate the cytotoxic effects of unbound BP on culture osteoprogenitors.

3.4. Local co-delivery of rhBMP-2 and BP via porous CHA scaffolds

The capacity of rhBMP-2 and BPs to act synergistically when delivered by CHA scaffolds was assessed in an in vivo ectopic bone formation model (Table 1). No complications occurred with surgical implantation. At 4 weeks post-op, specimens were harvested and analyzed by XR, μCT and tissue histology. XR revealed ectopic bone formation in the hind limb in all the treatment groups, including some radiodense tissue in the saline-soaked CHA scaffold controls (Fig. 4). Via radiographs, greater bone formation was apparent in the rhBMP-2 + ZA combination groups compared to rhBMP-2 alone.

The volume of bone was subsequently measured by μCT (Fig. 5). Net BV (mm^3) significantly increased with local co-delivery of rhBMP-2 and ZA in comparison to the rhBMP-2 and saline control groups (Fig. 5A). ZA increased BV by 6-fold rhBMP-2/ZA treatment groups in comparison to rhBMP-2 alone controls ($p < 0.01$). In treatment groups containing ZA, no difference in BV was observed between 5 and 10 μg doses of rhBMP-2. Prior to implantation, the HA within the CHA scaffold was not detected via μCT scanning or XR. Therefore the BV noted within the CHA control group was an indication of the osteogenic properties of this scaffold. Furthermore, no significant additional benefit was seen with the addition of either a low or high dose of rhBMP-2 (5 or 10 μg), highlighting the capability of the CHA scaffold to produce de novo mineralized tissue in an ectopic site without the need for growth factors.

BTMD and both TbTh and TbN were investigated to determine the degree of bone formation as a result of the different treatment groups. In the combined rhBMP-2/ZA dosing regimens, 10 μg doses of rhBMP-2 led to significantly higher BTMD in comparison to the 10 μg rhBMP-2 alone groups. The BTMD of CHA control scaffold was significantly lower in comparison to both the rhBMP-2 alone groups and the combined rhBMP-2/ZA groups. There was no difference within the 5 μg rhBMP-2 groups, either with or without ZA (Fig. 5B). Furthermore, TbTh increased significantly in the combined rhBMP-2/ZA groups in comparison to the rhBMP-2 alone

Please cite this article in press as: Murphy CM et al. A collagen–hydroxyapatite scaffold allows for binding and co-delivery of recombinant bone morphogenetic proteins and bisphosphonates. Acta Biomater (2014), http://dx.doi.org/10.1016/j.actbio.2014.01.016

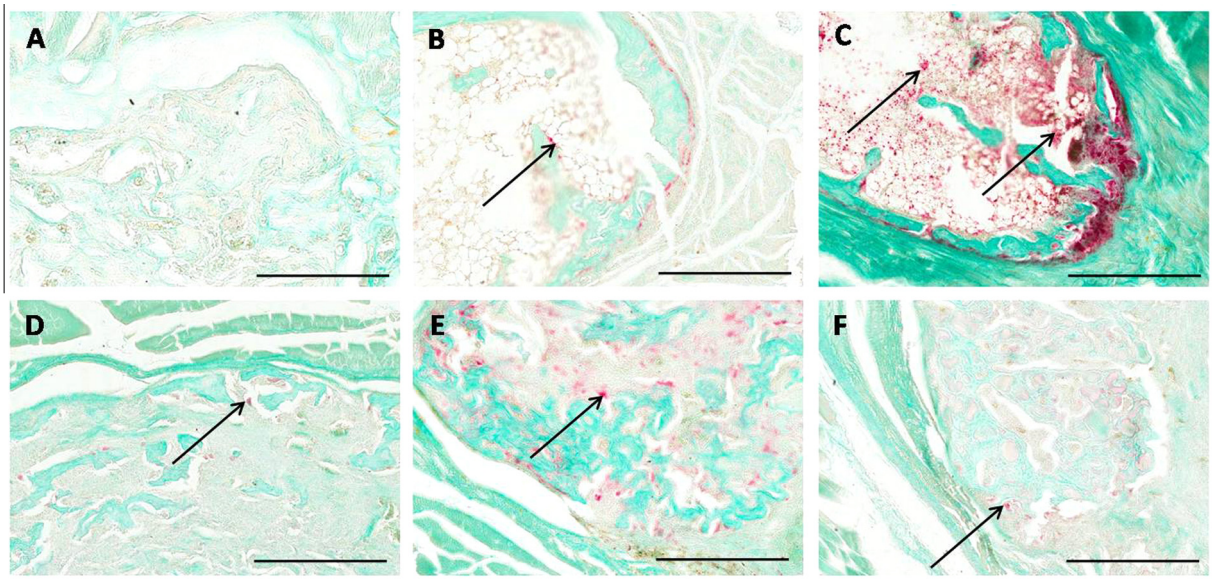

Fig. 7. Representative TRAP-stained histological sections of osteoclasts (stained in red) in trabecular-like structure of ectopic bone formed following 4 weeks intramuscular implantation. Arrows indicate stained osteoclasts. (A) CHA alone, (B) 5 μg rhBMP-2, (C) 10 μg rhBMP-2, (D) 5 μg BMP + 10 μg ZA, (E) 10 μg BMP + 2 μg ZA, (F) 10 μg BMP + 10 μg ZA. Scale bars = 500 μm.

controls. A significant increase in TbN was observed only in the 10 μg rhBMP-2 + 10 μg ZA group compared to the 10 μg rhBMP-2 control. There was no significant difference between the other groups (Fig. 5C and D).

As a secondary outcome measure, the microarchitecture of the ectopic bone nodules was examined by 3-D μCT reconstructions of transaxial mid-sections of bone nodule (Fig. 6). Larger pellets with greater internal trabecular-like bone were observed to form as a result of combined rhBMP-2/ZA treatment compared to rhBMP-2 alone groups and CHA control scaffolds, which formed smaller, hollow nodules.

Histological analysis comparing TRAP positive staining in all the treatment groups demonstrated a reduction in osteoclasts in the ZA co-treatment groups compared to the corresponding rhBMP-2 controls (Fig. 7B–F). This is consistent with reduced BV rhBMP-2 control groups compared to the rhBMP-2/ZA groups, suggestive of reduced catabolism. No TRAP positive cells were observed the in the saline-soaked CHA group (Fig. 7A).

4. Discussion

An emerging paradigm in orthopedics is the concept of synergistically harnessing anabolic and anti-catabolic drug treatments for improved bone healing [44–47]. BPs alone inhibit bone resorption and do not promote bone formation. However, when combined with an anabolic agent such as rhBMPs, bone formation is increased significantly in comparison to BMPs alone [12]. Historically, a majority of studies have used local rhBMPs delivered with systemic BPs, in accordance with current clinical practice. However, local delivery has increasing appeal as it can minimize the total BP dose and avoid adverse effects associated with systemic BP treatment. This study describes a combination rhBMP and BP delivered via a CHA scaffold. Critically, fluorescent AlexaPam 647 and ^{14}C-ZA were found to bind to the CHA scaffold but not control collagen sponge (Fig. 1) and both showed a similar sustained release profile for rhBMP-2 (Fig. 2).

Currently, porous collagen sponge remains the standard for orthopedic delivery of rhBMP-2, with rhBMP binding being cited as a major advantage. However, we hypothesized that collagen would show poor BP binding, and this was confirmed experimentally. The addition of 0.2 g ml^{-1} HA in the CHA scaffolds was sufficient to bind BP and, with no difference in the proportion bound between 1 and 2 μg of ZA, this indicates that the scaffolds were not saturated. The binding and sequestration of BP within the CHA scaffold could have multiple advantages. High local doses of BPs are potentially toxic and can impede healing or bone-graft fixation [48]. The presence of a ceramic phase that can prevent the rapid uptake of BP by cells other than osteoclasts is hypothesized to ameliorate these negative effects. This concept is further supported by our data showing that the cytotoxicity of high dose ZA to cultured pre-osteoblasts can be counteracted by the presence of CHA scaffolds but cannot control collagen (Fig. 3). This concept of preventing local BP toxicity using scaffolds containing ceramic microparticles is likely to be broadly translatable to a range of polymeric and natural scaffolds but the CHA scaffold also has the advantage of binding rhBMP-2.

The capacity of collagen scaffolds to bind rhBMP-2 has been previously reported [49,50]. It was hypothesized that the inclusion of HA in the CHA scaffold would not significantly impair rhBMP-2 binding, as collagen remained a major component. ELISA assays of serial eluent samples demonstrate that the release rate of rhBMP-2 from CHA is slower than that of control collagen, although following a similar sustained release profile. While burst release is an important initiator of new bone formation, sustained delivery may reduce the initial inflammatory response [51], and has been associated with increased bone formation in prior reports [52]. The combination of collagen and HA has been utilized in a number of previous studies [53,54], but many compositions demonstrate only limited porosity and osteoconductivity. The CHA scaffold is highly porous and exhibits significantly improved bioactivity and in vitro mineralization over collagen alone [36]. CHA has the benefit of the biocompatibility and rapid degradation associated with collagen combined with the more robust mechanical properties conferred by the HA component. Both the composition (HA for ZA binding and collagen for rhBMP-2 adsorption) and hydrophilic qualities (for absorption of rhBMP-2/ZA solution) of CHA ensure easy application of the drugs onto the scaffold.

To investigate the relative advantages of an rhBMP-2 vs. rhBMP-2/ZA combined treatment, a dosed CHA scaffold was implanted

into the hind limbs of rats to induce ectopic bone formation. Doses of rhBMP-2 were chosen at a lower range (5 and 10 μg) than other synthetic scaffold systems [10,11] due to the favorable release profile of rhBMP-2 from collagen. Moreover, rhBMPs are costly to produce and one translational benefit of combined therapies is the potential to reduce the amount of rhBMP required to produce a comparable bone formation outcome. A significant increase in both BV and TbTh (Fig. 5) was observed in the rhBMP-2/ZA treatment groups in comparison to their rhBMP-2 alone controls. This highlights the considerable increase in net bone that can be achieved by co-delivery of an anti-resorptive agent, which has been borne out by the prior art [11,23,24,48,55–57]. 3-D μCT reconstructions confirmed larger bone nodules with greater internal structure in the samples containing ZA (Fig. 6). Mechanistically, the anti-resorptive effects of ZA were confirmed by histology, and revealed decreased osteoclasts in the presence of ZA (Fig. 7). This decrease in remodeling is desirable within an unloaded model, but future work will be required to determine dosing that produces bone with good material properties and eventual remodeling.

One noteworthy observation was the formation of mineralized tissue in all treatment groups, including saline-soaked CHA scaffolds. This was further confirmed using μCT. While the CHA scaffolds contain HA that would produce a basal level of radiopacity, saline-soaked CHA was not visible by XR. Further, saline-soaked CHA analyzed by μCT and a comparison to implanted specimens showed a net and significant increase in BV. The potential of CHA scaffolds to promote mineralization and heal bone voids was observed previously within a calvarial defect [1]. However this study demonstrates for the first time the capacity of this material to promote matrix mineralization in an ectopic and non-osseous site. The significantly greater BMTD levels compared to the rhBMP-2 groups as well as lack of TRAP positive cells suggest that this may be the result of dense mineral depositions initially forming along the struts of the scaffold. This capacity for mineralization independent of rhBMP-2 may be suggestive of increased osteoconduction and/or osteoinduction in the context of an osseous defect, although this will need to be confirmed experimentally.

5. Conclusion

This study demonstrates the advantages of co-delivery of anabolic and anti-catabolic agents using CHA, a scaffold system comprising collagen and HA. CHA demonstrates a capacity for both rhBMP-2 and BP binding and delivery, which may make it superior for combination treatments compared to commercially available pure collagen scaffolds. Notably, the CHA scaffolds demonstrate a capacity for mineralization in the absence of additional osteogenic factors. This approach can be seen as a template for developing multicomponent scaffolds to allow for improved delivery of multiple pharmaceutical or biological agents.

Disclosures

C.M.M., A.S., N.Y.C.Y., L.C.C., K.M., L.P. and D.G.L. have no conflicts of interest. J.P.G. and F.J.O. hold IP with a commercial product of related composition to the collagen–HA scaffolds used in this study.

Acknowledgements

N.Y.C.Y. receives salary support from the Australian National Health Medical Research Council (NHMRC) Project Grant 1020987 and A.S. receives salary support from the Australian NHMRC Project Grant 1003478. The Leica SP5 in the CLEM Suite at KRI was supported by the following Grants: the Cancer Institute New South Wales Research Equipment (10/REG/1-23), the Australian National Health and Medical Research Council (2009-02759), the Ian Potter Foundation (20100508), the Perpetual Foundation (730), the Ramaciotti Foundation (3037/2010) and the Sydney Medical School Research Infrastructure Major Equipment Scheme.

Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figures 1, 2, 5 and 7, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2014.01.016.

References

[1] Little DG, Ramachandran M, Schindeler A. The anabolic and catabolic responses in bone repair. J Bone Joint Surg Br 2007;89:425–33.
[2] Giannoudis PV, Dinopoulos H, Tsiridis E. Bone substitutes: an update. Injury 2005;36:S20–7.
[3] Hughes F, Collyer J, Stanfield M, Goodman S. The effects of bone morphogenetic protein-2,-4, and-6 on differentiation of rat osteoblast cells in vitro. Endocrinology 1995;136:2671–7.
[4] Itoh K, Udagawa N, Katagiri T, Iemura S, Ueno N, Yasuda H, et al. Bone morphogenetic protein 2 stimulates osteoclast differentiation and survival supported by receptor activator of nuclear factor-κB ligand. Endocrinology 2001;142:3656–62.
[5] Kaneko H, Arakawa T, Mano H, Kaneda T, Ogasawara A, Nakagawa M, et al. Direct stimulation of osteoclastic bone resorption by bone morphogenetic protein (BMP)-2 and expression of BMP receptors in mature osteoclasts. Bone 2000;27:479–86.
[6] Ito M. Assessment of bone quality – present and future. Clin Calcium 2004;14:27–32.
[7] Pradhan BB, Bae HW, Dawson EG, Patel VV, Delamarter RB. Graft resorption with the use of bone morphogenetic protein: lessons from anterior lumbar interbody fusion using femoral ring allografts and recombinant human bone morphogenetic protein-2. Spine 2006;31:E277–84.
[8] Little D, McDonald M, Bransford R, Godfrey C, Amanat N. Manipulation of the anabolic and catabolic responses with OP-1 and zoledronic acid in a rat critical defect model. J Bone Miner Res 2005;20:2044–52.
[9] Little DG, Ramachandran M, Schindeler A. The anabolic and catabolic responses in bone repair. J Bone Joint Surg Br 2007;89-B:425–33.
[10] Yu N, Schindeler A, Peacock L, Mikulec K, Fitzpatrick J, Ruys A, et al. Modulation of anabolic and catabolic responses via a porous polymer scaffold manufactured using thermally induced phase separation. Eur Cell Mater 2013;25:190–203.
[11] Yu NYC, Schindeler A, Peacock L, Mikulec K, Baldock PA, Ruys AJ, et al. In vivo local co-delivery of recombinant human bone morphogenetic protein-7 and pamidronate via poly-D, L-lactic acid. Eur Cell Mater 2010;20:431–42.
[12] Little DG, McDonald M, Bransford R, Godfrey CB, Amanat N. Manipulation of the anabolic and catabolic responses with OP-1 and zoledronic acid in a rat critical defect model. J Bone Miner Res 2005;20:2044–52.
[13] Lin JH. Bisphosphonates: a review of their pharmacokinetic properties. Bone 1996;18:75–85.
[14] Rogers MJ, Gordon S, Benford H, Coxon F, Luckman S, Monkkonen J, et al. Cellular and molecular mechanisms of action of bisphosphonates. Cancer 2000;88:2961–78.
[15] Coxon FP, Benford HL, Russell RG, Rogers MJ. Protein synthesis is required for caspase activation and induction of apoptosis by bisphosphonate drugs. Mol Pharmacol 1998;54:631–8.
[16] Coxon FP, Thompson K, Roelofs AJ, Ebetino FH, Rogers MJ. Visualizing mineral binding and uptake of bisphosphonate by osteoclasts and non-resorbing cells. Bone 2008;42:848–60.
[17] Nancollas GH, Tang R, Phipps RJ, Henneman Z, Gulde S, Wu W, et al. Novel insights into actions of bisphosphonates on bone: differences in interactions with hydroxyapatite. Bone 2006;38:617–27.
[18] Russell R, Watts N, Ebetino F, Rogers M. Mechanisms of action of bisphosphonates: similarities and differences and their potential influence on clinical efficacy. Osteoporosis Int 2008;19:733–59.
[19] Gong L, Hoshi K, Ejiri S, Nakajima T, Shingaki S, Ozawa H. Bisphosphonate incadronate inhibits maturation of ectopic bone induced by recombinant human bone morphogenetic protein 2. J Bone Miner Metab 2003;21:5–11.
[20] Monkkonen J, Simila J, Rogers MJ. Effects of tiludronate and ibandronate on the secretion of proinflammatory cytokines and nitric oxide from macrophages in vitro. Life Sci 1998;62:PL95–PL102.
[21] Thiebaud D, Sauty A, Burckhardt P, Leuenberger P, Sitzler L, Green JR, et al. An in vitro and in vivo study of cytokines in the acute-phase response associated with bisphosphonates. Calcif Tissue Int 1997;61:386–92.
[22] Marx RE, Sawatari Y, Fortin M, Broumand V. Bisphosphonate-induced exposed bone (osteonecrosis/osteopetrosis) of the jaws: risk factors, recognition, prevention, and treatment. J Oral Maxillofac Surg 2005;63:1567–75.

[23] Jeppsson C, Astrand J, Tagil M, Aspenberg P. A combination of bisphosphonate and BMP additives in impacted bone allografts. Acta Orthop Scand 2003;74:483–9.
[24] Belfrage O, Flivik G, Sundberg M, Kesteris U, Tagil M. Local treatment of cancellous bone grafts with BMP-7 and zoledronate increases both the bone formation rate and bone density: a bone chamber study in rats. Acta Orthop 2011;82:228–33.
[25] Yang S, Leong KF, Du Z, Chua CK. The design of scaffolds for use in tissue engineering. Part I. Traditional factors. Tissue Eng 2001;7:679–89.
[26] Jiang T, Abdel-Fattah WI, Laurencin CT. In vitro evaluation of chitosan/poly(lactic acid-glycolic acid) sintered microsphere scaffolds for bone tissue engineering. Biomaterials 2006;27:4894–903.
[27] O'Brien FJ, Gleeson J, Plunkett NA, A collagen/hydroxyapatite composite scaffold, and the process for the production thereof. Patent WO 2008/096334 A2, 2008.
[28] Wahl D, Czernuszka J. Collagen–hydroxyapatite composites for hard tissue repair. Eur Cell Mater 2006;11:43–56.
[29] Tierney CT, Jaasma MJ, O'Brien FJ. Osteoblast activity on collagen–GAG scaffolds is affected by collagen and GAG concentrations. J Biomed Mater Res 2009;91:92–101.
[30] Wu B, Zheng Q, Guo X, Wu Y, Wang Y, Cui F. Preparation and ectopic osteogenesis in vivo of scaffold based on mineralized recombinant human-like collagen loaded with synthetic BMP-2-derived peptide. Biomed Mater 2008;3:044111.
[31] Haugh MG, Murphy CM, McKiernan RC, Altenbuchner C, O'Brien FJ. Crosslinking and mechanical properties significantly influence cell attachment, proliferation, and migration within collagen glycosaminoglycan scaffolds. Tissue Eng A 2011;17:1201–8.
[32] O'Brien FJ, Harley BA, Yannas IV, Gibson LJ. The effect of pore size on cell adhesion in collagen–GAG scaffolds. Biomaterials 2005;26:433–41.
[33] O'Brien FJ, Harley BA, Waller MA, Yannas IV, Gibson LJ, Prendergast PJ. The effect of pore size on permeability and cell attachment in collagen scaffolds for tissue engineering. Technol Health Care 2007;15:3–17.
[34] Murphy CM, Haugh MG, O'Brien FJ. The effect of mean pore size on cell attachment, proliferation and migration in collagen–glycosaminoglycan scaffolds for bone tissue engineering. Biomaterials 2010;31:461–6.
[35] Sawyer AA, Song SJ, Susanto E, Chuan P, Lam CXF, Woodruff MA, et al. The stimulation of healing within a rat calvarial defect by mPCL–TCP/collagen scaffolds loaded with rhBMP-2. Biomaterials 2009;30:2479–88.
[36] Gleeson JP, Plunkett NA, O'Brien FJ. Addition of hydroxyapatite improves stiffness, interconnectivity and osteogenic potential of a highly porous collagen-based scaffold for bone tissue regeneration. Eur Cells Mater 2010;20:218–30.
[37] Geiger M, Li RH, Friess W. Collagen sponges for bone regeneration with rhBMP-2. Adv Drug Deliv Rev 2003;55:1613–29.
[38] Lawson MA, Xia Z, Barnett BL, Triffitt JT, Phipps RJ, Dunford JE, et al. Differences between bisphosphonates in binding affinities for hydroxyapatite. J Biomed Mater Res B: Appl Biomater 2010;92B:149–55.
[39] O'Brien FJ, Harley BA, Yannas IV, Gibson L. Influence of freezing rate on pore structure in freeze-dried collagen–GAG scaffolds. Biomaterials 2004;25:1077–86.
[40] Haugh MG, Jaasma MJ, O'Brien FJ. The effect of dehydrothermal treatment on the mechanical and structural properties of collagen–GAG scaffolds. J Biomed Mater Res A 2009;89:363–9.
[41] Cole BJ, Bostrom MP, Pritchard TL, Sumner DR, Tomin E, Lane JM, et al. Use of bone morphogenetic protein 2 on ectopic porous coated implants in the rat. Clin Orthop Relat Res 1997;345:219–28.
[42] Scott MA, Levi B, Askarinam A, Nguyen A, Rackohn T, Ting K, et al. Brief review of models of ectopic bone formation. Stem Cells Dev 2012;21:655–67.
[43] Schindeler A, Little DG. Osteoclasts but not osteoblasts are affected by a calcified surface treated with zoledronic acid in vitro. Biochem Biophys Res Commun 2005;338:710–6.
[44] Seeherman HJ, Li XJ, Bouxsein ML, Wozney JM. RhBMP-2 induces transient bone resorption followed by bone formation in a nonhuman primate core-defect model. J Bone Joint Surg Am 2010;92:411–26.
[45] Tagil M, Aspenberg P, Astrand J. Systemic zoledronate precoating of a bone graft reduces bone resorption during remodeling. Acta Orthop 2006;77:23–6.
[46] Little DG, McDonald M, Bransford R, Godfrey CB, Amanat N. Manipulation of the anabolic and catabolic responses with OP-1 and zoledronic acid in a rat critical defect model. J Bone Miner Res 2005;20:2044–52.
[47] Harding AK, Aspenberg P, Kataoka M, Bylski D, Tagil M. Manipulating the anabolic and catabolic response in bone graft remodeling: synergism by a combination of local BMP-7 and a single systemic dosis of zoledronate. J Orthop Res 2008;26:1245–9.
[48] Jakobsen T, Baas J, Bechtold JE, Elmengaard B, Soballe K. Soaking morselized allograft in bisphosphonate can impair implant fixation. Clin Orthop Relat Res 2007;463:195–201.
[49] Geiger M, Li RH, Friess W. Collagen sponges for bone regeneration with rhBMP-2. Adv Drug Deliv Rev 2003;55:1613–29.
[50] Friess W. Collagen–biomaterial for drug delivery. Eur J Pharm Biopharm 1998;45:113–36.
[51] Lee KB, Taghavi CE, Murray SS, Song KJ, Keorochana G, Wang JC. BMP induced inflammation: a comparison of rhBMP-7 and rhBMP-2. J Orthop Res 2012;30:1985–94.
[52] Brown KV, Li B, Guda T, Perrien DS, Guelcher SA, Wenke JC. Improving bone formation in a rat femur segmental defect by controlling bone morphogenetic protein-2 release. Tissue Eng A 2011;17:1735–46.
[53] Wahl DA, Sachlos E, Liu C, Czernuszka JT. Controlling the processing of collagen–hydroxyapatite scaffolds for bone tissue engineering. J Mater Sci Mater Med 2007;18:201–9.
[54] Dey S, Pal S. Evaluation of collagen–hydroxyapatite scaffold for bone tissue engineering. In: Lim C, Goh JH, editors. 13th International conference on biomedical engineering. Berlin: Springer; 2009. p. 1267–70.
[55] Chen WJ, Jingushi S, Hirata G, Matsumoto Y, Iwamoto Y. Intramuscular bone induction by the simultaneous administration of recombinant human bone morphogenetic protein 2 and bisphosphonate for autobone graft. Tissue Eng 2004;10:1652–61.
[56] Chen WJ, Jingushi S, Jingushi K, Iwamoto Y. In vivo banking for vascularized autograft bone by intramuscular inoculation of recombinant human bone morphogenetic protein-2 and beta-tricalcium phosphate. J Orthop Sci 2006;11:283–8.
[57] Jakobsen T, Baas J, Bechtold JE, Elmengaard B, Soballe K. The effect of soaking allograft in bisphosphonate: a pilot dose–response study. Clin Orthop Relat Res 2010;468:867–74.

Please cite this article in press as: Murphy CM et al. A collagen–hydroxyapatite scaffold allows for binding and co-delivery of recombinant bone morphogenetic proteins and bisphosphonates. Acta Biomater (2014), http://dx.doi.org/10.1016/j.actbio.2014.01.016