ADDITION OF HYDROXYAPATITE IMPROVES STIFFNESS, INTERCONNECTIVITY AND OSTEOGENIC POTENTIAL OF A HIGHLY POROUS COLLAGEN-BASED SCAFFOLD FOR BONE TISSUE REGENERATION

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Abstract

There is an enduring and unmet need for a bioactive, load-bearing tissue-engineering scaffold, which is biocompatible, biodegradable and capable of facilitating and promoting osteogenesis when implanted *in vivo*. This study set out to develop a biomimetic scaffold by incorporating osteoinductive hydroxyapatite (HA) particles into a highly porous and extremely biocompatible collagen-based scaffold developed within our laboratory over the last number of years to improve osteogenic performance. Specifically we investigated how the addition of discrete quantities of HA affected scaffold porosity, interconnectivity, mechanical properties, *in vitro* mineralisation and *in vivo* bone healing potential. The results show that the addition of HA up to a 200 weight percentage (wt%) relative to collagen content led to significantly increased scaffold stiffness and pore interconnectivity (approximately 10 fold) while achieving a scaffold porosity of 99%. In addition, this biomimetic collagen-HA scaffold exhibited significantly improved bioactivity, *in vitro* mineralisation after 28 days in culture, and *in vivo* healing of a critical-sized bone defect. These findings demonstrate the regenerative potential of these biodegradable scaffolds as viable bone graft substitute materials, comprised only of bone's natural constituent materials, and capable of promoting osteogenesis *in vitro* and *in vivo* repair of critical-sized bone defects.

Keywords: Collagen, hydroxyapatite, scaffold, bone tissue engineering, bone regeneration.

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Introduction

Bone grafts and bone graft substitutes are used in the repair and reconstruction of bone tissue defects throughout the body that can arise as a result of any number injuries to the tissue. Currently, the "gold standard" clinical approach involves the surgical harvesting of autograft tissue, taken from the patient's own body and subsequently re-implanted into the patient's defect site. However, there are significant practical and surgical complications associated with this approach, specifically donor site morbidity, quantity of harvest tissue available (Laurencin *et al.*, 2006; Toolan, 2006; Desai, 2007), quality of geriatric/pathological source tissue (Bridwell *et al.*, 2004) and need for a second surgical procedure (Arrington *et al.*, 1996). Tissue-derived substitutes such as allografts and xenografts offer significant practical advantages over autograft material (e.g. no need for additional surgery, "off the shelf" availability, size of graft material). However, significant drawbacks such as worldwide donor shortage (Greenwald *et al.*, 2001) and associated risk of disease transmission (Mroz *et al.*, 2008) ensure that allografting is insufficient as a viable long-term approach to bone autografting.

Focus has recently switched towards the use of alternative approaches to attempt to promote and facilitate the body's own bone tissue healing ability. These approaches have included stem cell technology, tissue engineering and the development of cell-free scaffolds to act as bone graft substitutes. Synthetically-derived bone graft substitutes, such as ceramic (hydroxyapatite, β-TCP) or polymeric-based (poly-L-lactide, PLLA; poly(lactic-*co*-glycolic) acid, PLGA) scaffolds have a number of advantages such as high mechanical strength, osteoinductivity and biodegradability. Unfortunately these current solutions have a number of associated disadvantages (such as low porosity, toxic degradation by-products and long term mechanical integrity issues (Athanasiou *et al.*, 1998; Revell *et al.*, 1998; Spain *et al.*, 1998; Bohner, 2000; Bohner *et al.*, 2000; Hunziker *et al.*, 2002; Woodfield *et al.*, 2002) and have enjoyed limited clinical success (Ratcliffe, 2008). These strategies prioritise mechanically-competent scaffolds at the expense of biocompatibility and biological performance. This has resulted in an enduring and unmet need for a bioactive, load-bearing scaffold, capable of promoting osteogenesis *in vivo* (Barrere *et al.*, 2008).

Recent advances in composite biomaterials have led to a paradigm shift towards biomimetic tissue engineering scaffolds for use in the regeneration of bone tissue defects. Biomimetics, both in terms of composition and fabrication,

process may provide a compromise between the competing mechanical and the biological prerequisites needed to rapidly promote healing of bone tissue defects. Given bone's native composition of predominantly type I collagen and hydroxyapatite, these materials are an obvious choice as the basis for a composite biomaterial capable of supporting and promoting the bone regenerative process (Wahl and Czernuszka, 2006). Recent studies have shown that improvements in the interaction between osteoblasts and PLLA scaffolds can be improved by the application of a collagen-HA coating (Li *et al*., 2010) clearly demonstrating the potential of a composite material composed of only collagen and hydroxyapatite for use as a bioactive bone graft.

One of the barriers to the successful development of a collagen-HA scaffold is the difficulty in achieving a homogenous distribution of the HA throughout polymer-based matrices (Supová, 2009), an issue that can have a significant effect on a collagen-HA biomaterial's *in vivo* vascularisation and production of newly formed bone tissue (Lyons *et al*., 2010; Zhang *et al*., 2010a). As a result, many recent studies have utilised biocompatible or bioactive dispersants, such as chitosan (Zhang *et al*., 2010b) or biomimetic fabrication methods for the *in situ* mineralisation of collagen-HA scaffolds during the fabrication process (Kikuchi *et al*., 2004; Xu *et al*., 2010; Yoshida *et al*., 2010; Zhang *et al*., 2010a). However, control and regulation of this process and the resulting nature of the fabricated HA can be difficult with implications for the purity and crystallinity of the resulting mineral phase. Given that HA crystallinity and purity plays a significant role in promoting bone tissue formation *in vivo* (ter Brugge *et al*., 2002; Zhang *et al*., 2010a), the ability to produce pure collagen-HA scaffolds of high purity and crystallinity is desirable from a tissue engineering perspective.

Our laboratory's approach has involved the development a number of highly porous and biocompatible collagen-based scaffolds optimised in terms of composition (Tierney *et al*., 2009a; Tierney *et al*., 2009b), cross linking density (Haugh *et al*., 2009) and pore architecture (O'Brien *et al*., 2005; O'Brien *et al*., 2007a; Murphy *et al*., 2010a,b) for use in bone tissue engineering applications. Collagen is an ideal material when used as a scaffold as it fulfils many of the biological determinants required for successful implantation such as biocompatibility, cell adhesion and proliferation (Doillon *et al.,* 1986; Berry *et al*., 2004; O'Brien *et al*., 2005; Byrne *et al.,* 2008; Murphy *et al*., 2010a,b). Unfortunately, these scaffolds do not possess the load-bearing capability required when used in orthopaedic tissue engineering applications.

The aim of this study was to develop a biomimetic and highly porous (>95%) composite scaffold by incorporating an osteoinductive ceramic phase into our optimised collagen-based scaffolds and to assess its regenerative potential as a bone graft substitute. Our approach seeks to optimise a compliant scaffold to promote mineralisation upon implantation (Hutmacher *et al*., 2000), rapidly facilitating a load bearing capacity within the newly mineralised bone tissue graft. By combining the two primary constituents of human bone tissue, namely type 1 collagen and hydroxyapatite using a patented mixing process (O'Brien *et al*., 2007b, WO200896334A2), a highly porous composite tissue engineering scaffold with a high degree of pore interconnectivity, improved mechanical strength, permeability and cellular bioactivity was developed. The combination of the extremely biocompatible and biodegradable collagen scaffold with an osteoinductive mineral component (Gosain *et al*., 2002; Yuan *et al*., 2002; Barrere *et al*., 2003; Le Nihouannen *et al*., 2005; Habibovic *et al*., 2006) provides an ideal mechanical and biological environment to facilitate cell recruitment and maintain pore structure in order to promote healing. The objective of this study was to investigate the effect of the addition of HA to our highly porous collagen scaffolds on (i) mechanical stiffness, (ii) scaffold porosity, (iii) pore interconnectivity (measured in terms of permeability), (iv) *in vitro* osteogenic potential and (v) *in vivo* healing potential of these biomimetic scaffolds.

Materials and Methods

Scaffold fabrication

Collagen slurries were produced by the homogenisation of fibrillar collagen (Collagen Matrix, Franklin Lakes, NJ, USA) within a 0.5 M acetic acid solution. Slurries were homogenised in a reaction vessel, 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, hydroxyapatite (HA) particles with a mean particle diameter of 5 μm (Plasma Biotal Limited, North Derbyshire, UK) were suspended in a 0.5 M acetic acid solution. The final collagen-hydroxyapatite (CHA) composite slurry was produced by the addition, in aliquots, of the HA/acetic acid suspension to the initial collagen slurry during the homogenisation process. Collagen concentration in all scaffolds was 0.1g per ml acetic acid solution. HA concentration within the CHA scaffolds was varied as a weight percentage of the collagen concentration, resulting in four distinct scaffolds, namely control collagen-only (0 wt% HA), 50 wt% HA, 100 wt% HA and 200 wt% HA scaffolds (0g HA/ml, 0.05g HA/ml, 0.1g HA/ml and 0.2g HA/ml respectively). The resulting solution was degassed to remove any air bubbles and subsequently stored at 4°C prior to lyophilisation.

Collagen and CHA scaffolds were fabricated using a previously described lyophilisation technique by O'Brien *et al*. (2004; 2005). Briefly, 67.25 ml of the CHA slurry was pipetted into a stainless steel pan (125 x 125 mm, grade 304 SS). The tray was placed onto the freeze-dryer shelf (Advantage EL, Vir-Tis Co., Gardiner, NY, USA) and cooled to -40°C at a constant cooling rate of 0.9°C/min. Once freezing was complete, the ice crystals were removed by sublimation for 17 h at 0°C and 200 mTorr. This process produces a highly porous sheet of scaffold of dimensions 125 mm (W) x 125 mm (L) x 4 mm (D). Dehydrothermal (DHT) cross linking treatment was carried out as previously described (Haugh *et al*., 2009) by placing the scaffolds in an aluminium foil packet inside a vacuum

oven (Vacucell 22, MMM, Brno, Czech Republic) under a vacuum of 0.05 bar at a temperature of 120°C for 24 hours. This process improves the mechanical properties and also sterilises the scaffolds. Scaffold samples were further cross linked by immersion in an EDC/NHS solution (14 mM N-(3-Dimethylaminopropyl) -N'-ethylcarbodiimide hydrochloride/5.5 mM N-hydroxysuccinimide; Sigma-Aldrich, St. Louis, MO, USA) for two hours (Haugh *et al.*, 2009) to additionally improve the mechanical characteristics of the scaffolds.

The microstructure of the different scaffolds was examined after their production. No significant difference was found between the average pore size of the scaffold groups, with the average pore size seen to be 120 μm. Average pore size was not altered by the addition of HA particles. This allowed the exclusion of pore size as a variable. Hydroxyapatite particle distribution was assessed using Energy Dispersive X-Ray analysis and microCT and particles were found to be homogenously distributed in all three CHA scaffold groups. Particle size was assessed qualitatively using scanning electron microscopy (SEM) and was found to be unaffected by the fabrication process.

Mechanical testing

Unconfined compression testing was carried out using a mechanical testing machine (Z050, Zwick/Roell, Ulm, Germany) fitted with a 5-N load cell. Samples (n=20) were prehydrated in phosphate buffered saline (PBS) for 1 hour prior to testing and all testing was carried out with samples submerged in a bath of PBS. Samples of 9.5 mm diameter were cut from the scaffolds using a punch and were subsequently placed between two impermeable, unlubricated platens. Compressive tests were conducted up to a maximum compressive strain of 10%, at a strain rate of 10% per minute. The compressive modulus was defined as the slope of a linear fit to the stress-strain curve over 2-5% strain (Haugh *et al.*, 2009).

Scaffold porosity

The dry weight of 9.5 mm diameter scaffold samples was determined using a mass balance, with height and diameter measured using digital Vernier callipers (Krunstoffwerke, Radionics, Dublin, Ireland) to determine scaffold sample volume. The relative density of the scaffolds was calculated from the dry weight and volume of each scaffold disc using the density of bulk collagen (1.3 mg/mm^3) and hydroxyapatite (3.153 mg/mm^3). The percentage porosity was calculated using eqn (1) below;

$$Porosity\ \ (\%) = (1 - \rho_{scaffold} / \rho_{Solid}) \times 100 \quad (1)$$

Results of eight measurements (n=8) were averaged to determine mean scaffold porosity for each scaffold variant (Tierney *et al.*, 2009a).

Scaffold permeability

Scaffold pore interconnectivity was assessed by quantifying fluid mobility (permeability) of the scaffolds. Scaffold samples were inserted into a custom permeability rig under a column of water. Validation experiments were carried out to validate fluid flow through the compliant scaffolds. The flow rate of water through the constructs (n=5) was measured over a flow period of 300 seconds and used to calculate the steady state permeability from eqn (2);

$$k = Qh / AP \quad (2)$$

where k is the hydraulic permeability in m^4/Ns, Q is the volume flow rate in m^3/s, h is the height of the scaffold, A is the cross sectional area of the flow path and P is the pressure of the column of water, given by eqn (3):

$$P = \rho g h \quad (3)$$

where ρ is the density of water, g is the acceleration due to gravity and h is the height of the water column used.

Cell culture

Scaffold samples were seeded with 2 million MC3T3-E1 pre-osteoblast cells (ATCC-LGC, Teddington, Middlesex, UK). Cell-seeded scaffolds were cultured in non-osteogenic media (alpha-minimum essential medium (α-MEM), BioSera, East Sussex, UK) supplemented with 2% penicillin/streptomycin, 1% L-glutamine, 10% foetal bovine serum and 0.1% amphotericin (Sigma-Aldrich Ireland, Dublin, Ireland) for 3 days to allow proliferation before the medium was supplemented with osteogenic factors (10 mM β-glycerophosphate and 50 μg/mL ascorbic acid (Sigma-Aldrich). Cell-seeded scaffolds were cultured for 7, 14, 21 and 28 days at a temperature of 37°C and a carbon dioxide concentration of 5% CO_2. The osteogenic medium was changed every 2 to 3 days during the culture period.

DNA quantification

Four scaffolds (n=4) per group (collagen-only, 50 wt% HA, 100 wt% HA, 200 wt% HA) at each of the four time points (64 samples in total) were homogenised in 1 mL of Qiazol (Qiagen, Valencia, CA, USA) using a high speed, hand-held homogeniser (Finemech, Portola Valley, CA, USA) equipped with a T6 homogenising shaft attachment (Finemech). After the addition of chloroform and centrifugation to separate RNA and DNA, the RNA layer was pipetted off carefully and stored. Cell number on the constructs was quantified using a Hoechst 33258 assay (Sigma-Aldrich). The fluorescence of the samples was measured at 460 nm after excitation at 355 nm in a Wallac Victor2™ 1420 multilabel counter (Perkin Elmer Life Sciences, Waltham, MA, USA) and compared to a standard curve to determine cell number.

Histological analysis

At each time point, scaffold samples were placed into a solution of 10% formalin for 30 min and then processed with an automatic tissue processor (ASP300, Leica, Wetzlar, Germany). All constructs were embedded in paraffin wax and sectioned at a thickness of 10 μm using a rotary microtome (RM2255, Leica microtome, Leica). Sections were placed in an oven at 70°C overnight and

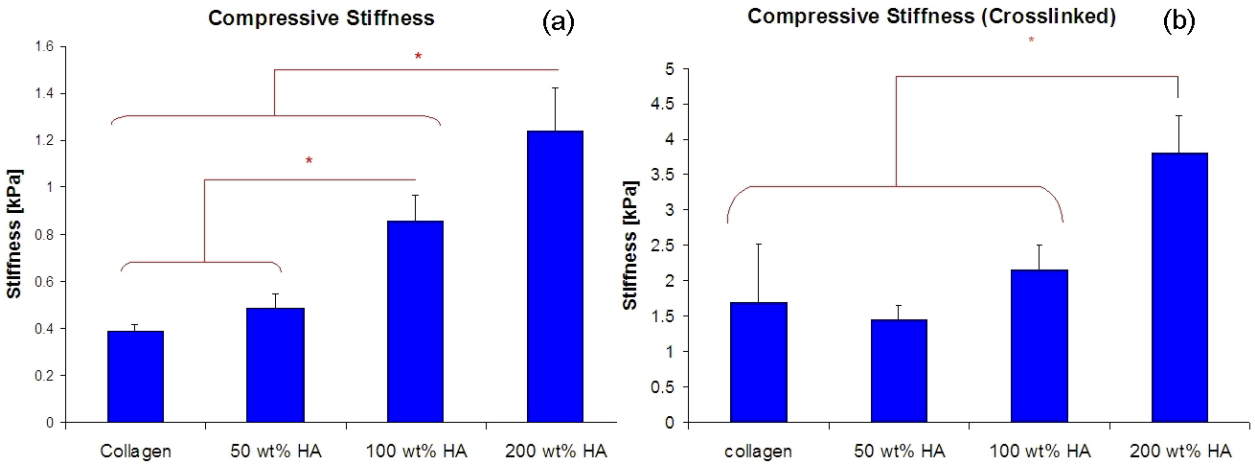

Fig. 1. (a) Effect of hydroxyapatite addition on the compressive stiffness of collagen-based scaffolds (*p<0.05); **(b)** Effect of hydroxyapatite addition on the compressive stiffness of DHT and chemically cross linked collagen-based scaffolds (*p<0.05). The addition of hydroxyapatite results in a linear increase in wet unconfined compressive stiffness in both non-cross-linked (R^2=0.99) and cross linked (R^2=0.95) CHA scaffolds. Cross-linked 200 wt% HA scaffold is ten times stiffer than non-cross linked collagen-only scaffolds (0.4kPa vs. 4 kPa).

residual wax was removed from the sections in a xylene bath. Sections were stained in 2% alizarin red for 5 min after wax removal and hydration. Quantification of mineralisation was carried out using 10% cetylpyridinium chloride to absorb the alizarin red stain from sections that had been exposed to this stain (Venugopal *et al.*, 2008). Four scaffold sections were attached per slide: two per slide were quantified, leaving two other sections per slide for examination under the microscope. 400µl of cetylpyridinium chloride solution was pipetted onto the slides and the stain was desorbed for 15 minutes. 100µl was pipetted in triplicate into the wells of a 96 well plate. Absorbance readings at 540 nm were obtained on a Titerek Multiskan MCC/340 spectrometer (Titertek, Pforzheim, Germany) after subtraction of cetylpyridinium solution baseline readings. Digital images of all stained sections were captured at 200X magnification using an imaging system (AnalySIS, Olympus, Tokyo, Japan or NIS Elements Basic Research Version 3.0, Nikon, Tokyo, Japan) in conjunction with a microscope (Olympus IX51 or Nikon Eclipse 90i).

Pre-clinical trial

A small preliminary pre-clinical trial was carried out to investigate the regenerative potential of the collagen hydroxyapatite (CHA) scaffolds. Pre-clinical investigation was carried out under approval by the RCSI Research Ethics Committee and following acquisition of an animal license from the Irish Government Department of Health. 5 mm diameter transosseous critical sized defects were created in calvariae of 3 adult Wistar rats. One animal was left with an empty defect as a control. The remaining two calvarial defects were filled with the optimised 200 wt% HA scaffolds. Animals were anaesthetised prior to surgical intervention. Calvarial bone was exposed and a critically-sized defect was introduced into the bone (5 mm diameter) using a trephine bur. Scaffolds were located within these cylindrical defect sites. The periosteum was subsequently sutured over the scaffold-filled defect, followed by suturing of the skin. Animals were closely monitored postoperatively with regular administration of suitable antibiotics and analgesias. After 28 days implantation within the rat calvariae, the animals were sacrificed and the calvarial bones were removed. These were analysed using microCT to investigate the capacity of the 200 wt% HA scaffold to promote healing. Scans were performed on a Scanco Medical 40 Micro CT system (Bassersdorf, Switzerland) with 70 kVP X-ray source and 112 µA using a high-resolution of 8 µm. Due to the high porosity of the CHA scaffolds, a threshold level greater than 35 renders the scaffold invisible (Al-Munajjed *et al.*, 2009) and a threshold of 140 (grayscale value between 0 and 1000) was required to image mineralised tissue (Kennedy *et al.*, 2009). Consequently a threshold value of 140 was used to assess new host tissue mineralisation within the defects without any influence of the original porous CHA scaffold.

Statistical analysis

All error bars represent standard deviations. Statistical analysis was carried out using Minitab 15 (Minitab Inc., State College, PA, USA) by applying a general linear model ANOVA with the Tukey test as the post-hoc test. Non-normal data was normalised using logarithmic or square root transforms so that the conditions of the statistical test were met. Statistical significance was taken at $p < 0.05$.

Results

Compressive stiffness

The addition of hydroxyapatite particles added to the collagen scaffolds in 50 wt%, 100 wt% and 200 wt% quantities resulted in an approximately linear increase (R^2=0.99) in unconfined compressive stiffness of the hydrated scaffolds. Average stiffness values for the non-cross linked 50 wt%, 100 wt% and 200 wt% HA scaffolds were approximately 0.5 kPa, 0.9 kPa and 1.3 kPa respectively, with the 200 wt% HA scaffolds being

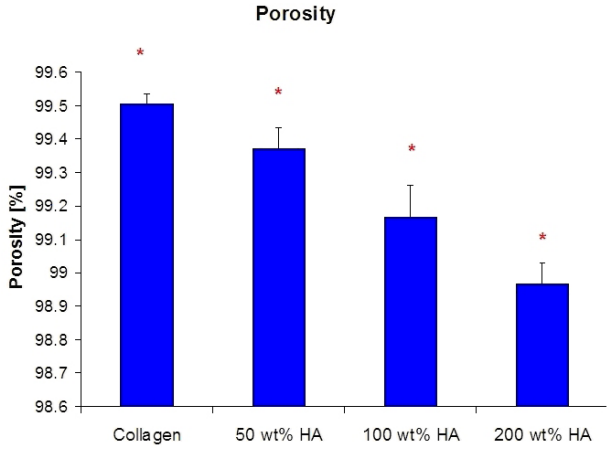

Fig. 2. Effect of hydroxyapatite addition on the porosity of collagen-based scaffolds (*p<0.05). The addition of hydroxyapatite results in statistically significant but negligible linear decrease (R^2=0.99) in overall scaffold porosity for all groups. The 200 wt% HA scaffold porosity is still as high as 99%.

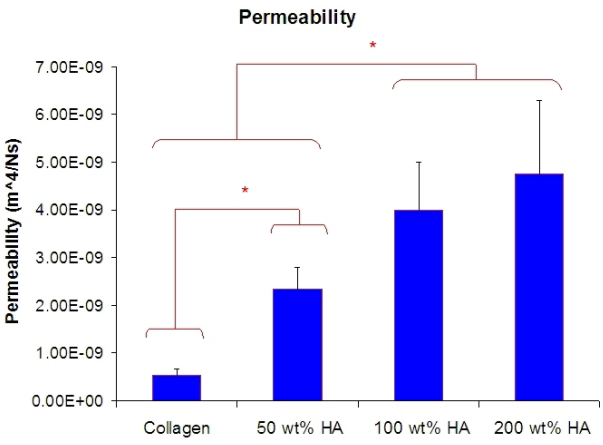

Fig. 3. Effect of hydroxyapatite addition on scaffold permeability (*p<0.05). The addition of hydroxyapatite results in a linear increase (R^2=0.97) in scaffold permeability for all CHA scaffold groups. 200 wt% HA scaffold is approximately ten times more permeable than collagen-only scaffolds (0.4 x 10^{-9} m^4/Ns vs. 4.5 x 10^{-9} m^4/Ns).

significantly stiffer than all other scaffolds (p<0.05) (Fig. 1a). A similar trend (R^2=0.95) was seen in all scaffold variants after the scaffold groups were dehydrothermally and chemically cross linked, with the absolute stiffness values being substantially increased as the quantity of HA added was increased (1.5 kPa, 2.2 kPa and 3.5 kPa respectively) (Fig. 1b), with the 200 wt% HA scaffolds showing a nearly tenfold increase in mechanical stiffness (p<0.05) relative to non-cross linked collagen controls.

Construct porosity

Average scaffold porosity significantly decreased (p<0.05) as the quantity of HA was increased as function of collagen weight (Fig. 2). Collagen controls were found to exhibit an average porosity of approximately 99.5%, with porosity levels decreasing in an approximately linear fashion (R^2=0.99) as the quantity of HA was increased to 50 wt% HA, 100 wt% HA and 200 wt% HA (99.4%, 99.2% and 99% respectively). This decrease in scaffold porosity level was expected due to the addition of HA but was negligible in real terms, even in the 200 wt% HA scaffolds. The largest decrease in porosity was seen in the 200 wt% HA scaffolds (≅ 0.5% decrease).

Construct permeability

Scaffold permeability was seen to increase in an approximately linear fashion (R^2=0.97) as the quantity of HA added to the scaffold increased up to 200 wt% HA. 50 wt% HA scaffolds exhibited a significantly higher permeability relative to collagen control scaffolds (p<0.05) while 100 wt% HA and 200 wt% HA scaffolds were significantly more permeable than 50wt% HA scaffolds and controls (Fig. 3).

DNA quantification

Cells were viable on all scaffolds at every time point up to 28 days based on cell number quantification. Cell number was seen to significantly increase (p<0.05) in the 50 wt%

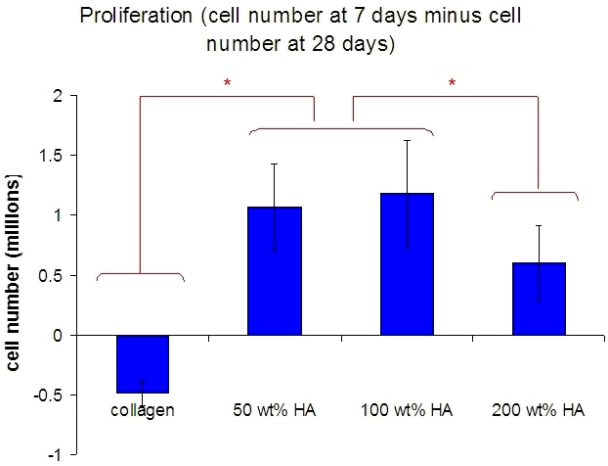

Fig. 4. Effect of hydroxyapatite addition on scaffold bioactivity (*p<0.05). The addition of hydroxyapatite resulted in a significantly increase (p<0.05) in cell number in the 50 wt% and 100 wt% HA scaffolds while 200 wt% HA scaffolds exhibited a non-significant increase in number relative to collagen-only controls over the 28 day culture period.

and 100 wt% HA scaffolds while 200 wt% HA scaffolds exhibited a non significant increase in cell number relative to collagen-only controls over the 28 day culture period (Fig. 4).

In vitro mineralisation

200 wt% HA scaffolds seeded with cells and cultured *in vitro* were the only group at days 14 and 21 that exhibited evidence of mineralisation. After the 28 day culture period, collagen-only scaffolds showed deeper mineralisation staining than the blank scaffolds while 50 wt%, 100 wt% and particularly 200 wt% HA constructs stained positive for calcium deposition (Fig. 5). Alizarin red stain quantification showed significantly increased staining

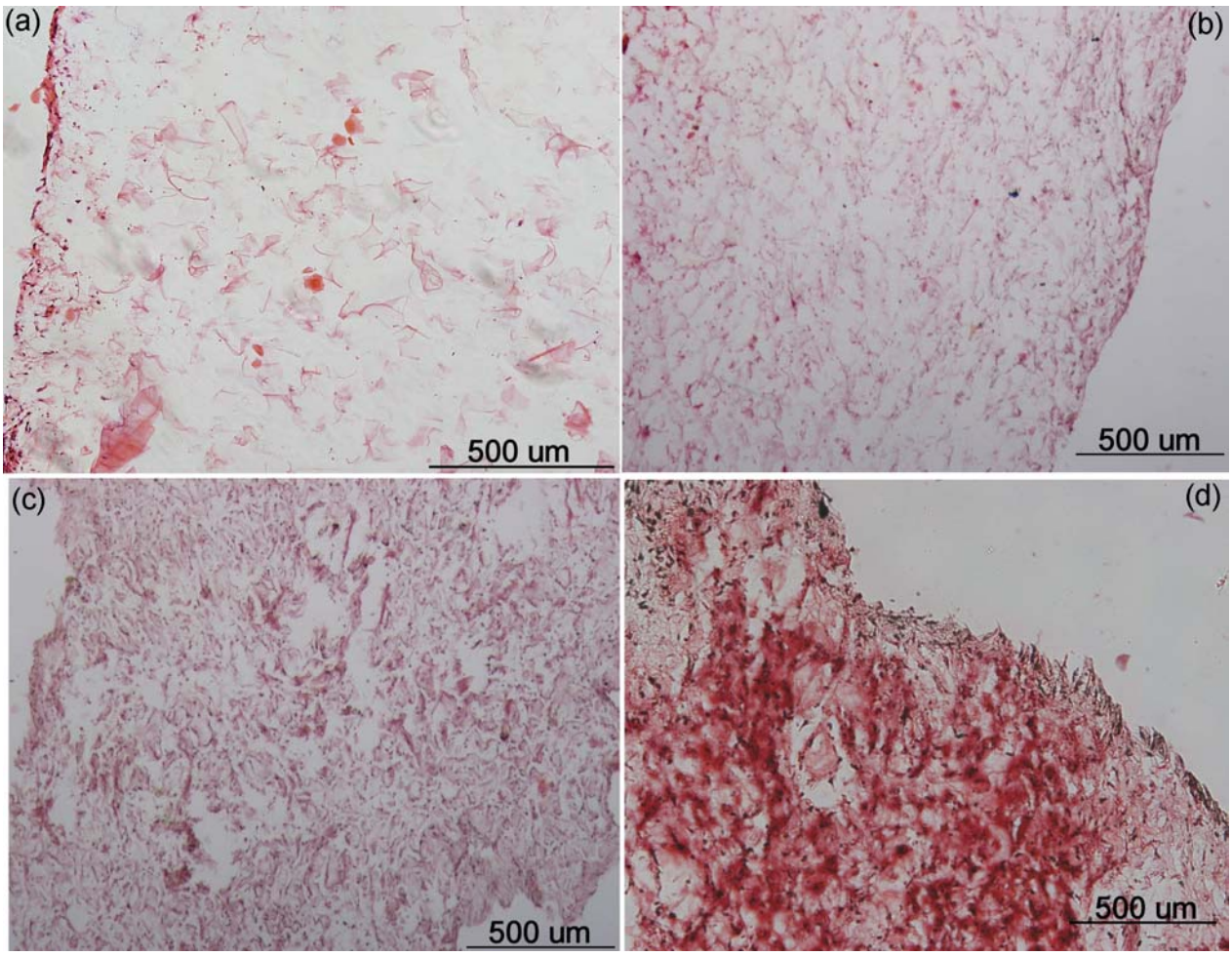

Fig. 5. Alizarin red staining of all four scaffold groups after 28 days in culture (**A**: Collagen-only, **B**: 50 wt% HA, **C**: 100 wt% HA, **D**: 200 wt% HA). Collagen-only scaffolds show no negligible Alizarin red staining. 50 wt% and 100 wt% HA groups show increased staining while 200 wt% HA group shows the highest levels of Alizarin red staining.

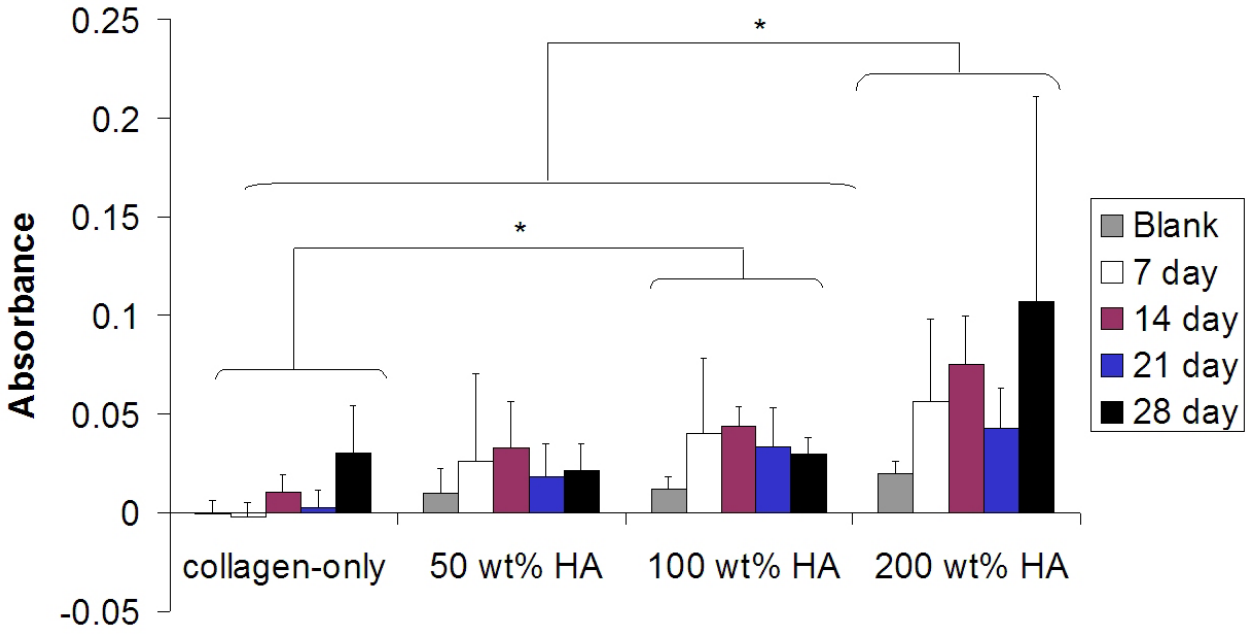

Fig. 6. Quantified alizarin red readings for the four groups over the 28 day culture period (*p<0.05). These results confirm histological results. Collagen-only scaffolds show no significant Alizarin red staining, 50 wt% and 100 wt% HA groups show staining which is significantly higher than collagen-only staining in the 100 wt% HA group while the 200 wt% HA group shows the highest levels of staining which is significantly increased relative to all other groups.

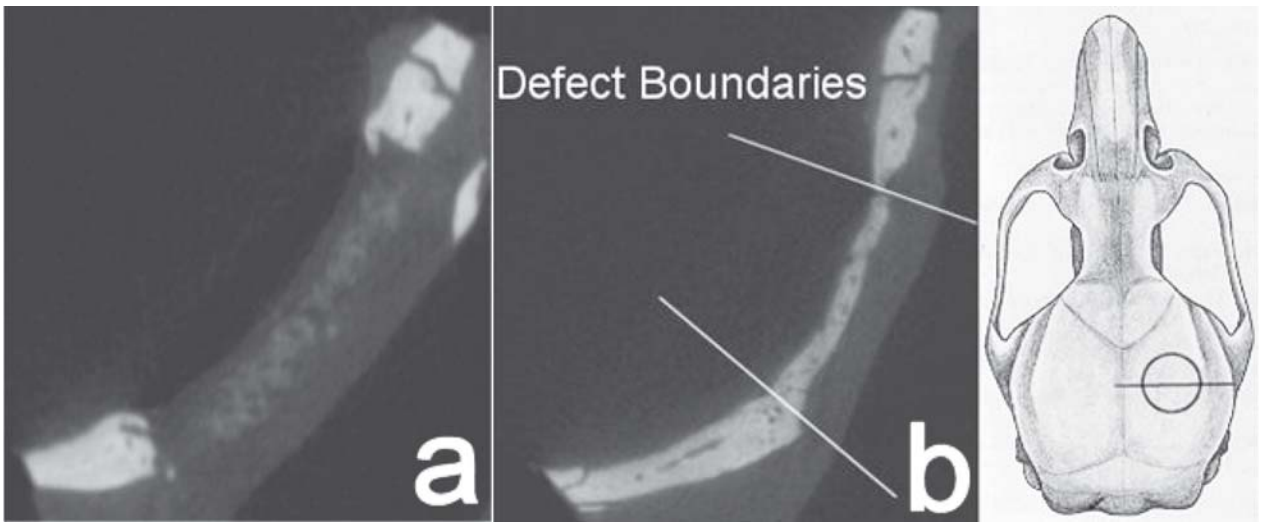

Fig. 7. MicroCT slice of representative level of mineralisation within the defect centre showing defect boundary edges in **(a)** empty defect group, **(b)** 200 wt% HA scaffold group after 28 days implantation with schematic of rat skull highlighting slice anatomical location. Almost complete defect bridging was observed in the 200 wt% HA group, with mineralisation level comparable to surrounding native calvarial bone tissue.

(p<0.05) of the 200 wt% HA scaffolds compared to the 100 wt% HA, 50 wt% HA and collagen-only scaffolds (Fig. 6). 100 wt% HA scaffolds showed significantly elevated staining compared to the collagen-only scaffold (p<0.05). 50 wt% HA scaffold mineralisation was non-significantly increased after 28 days in culture compared to the collagen-only scaffolds. By 28 days, there was significantly increased staining in the 200 wt% HA scaffolds compared to all other cultured scaffold groups and the blank scaffolds (p<0.05).

Pre-clinical trial

Throughout the study period, animals showed no signs of body weight loss or other deterioration in general health following surgery. The animals appeared healthy and alert and showed no signs of pain or discomfort. In the animal with an unfilled defect, the defect was filled in with loose fibrous tissue. Some evidence of localised mineralisation loci were seen within the empty defect after 28 days, in the form of small particles of dense material but these were sparsely distributed and not sufficiently dense to indicate significant healing within the empty defect sample. The 200 wt% HA samples showed significant levels of mineralisation at the periphery and were seen to progress towards the centre of the critical sized defects. This mineralised tissue was continuous in nature and was almost full thickness across the width of the defect (Fig. 7).

Discussion

The aim of this study was to develop a biomimetic scaffold by incorporating osteoinductive hydroxyapatite (HA) particles into a highly porous and extremely biocompatible collagen-based scaffold developed within our laboratory over the last number of years and to investigate the effect on osteogenic capacity of these scaffolds as potential bone graft substitutes. Current clinical standards of autografts and allografts are associated with donor site morbidity, limited volume of donor tissue, disease transmission, infection and chronic pain. Attention has turned to alternative treatments including bone tissue engineering but despite numerous teams worldwide working in the area, progress to date in engineering significant quantities of functional bone tissue *in vitro* for implantation has been disappointing (Meikle, 2007; Partap *et al*., 2010). Alternatively, regeneration of bone tissue *in situ* using tissue engineering scaffolds as potential bone graft substitutes, comprised of collagen and hydroxyapatite, has been attempted by numerous studies in the past (Swetha *et al*., 2010; Dawson *et al*., 2008) but this approach has shown limited clinical success (Ilan and Ladd, 2003; Barrere *et al*., 2008; Carter *et al*., 2009). One reason for this lack of success is the issue of core degradation, arising from lack of nutrient delivery and waste removal from the centre of tissue engineered constructs. This is caused by insufficient blood supply to the implanted tissue. As a result, tissue engineered constructs that appear to demonstrate great potential *in vitro* often fail once implanted *in vivo* due to acellular necrosis. This is of major concern in the field of tissue engineering, and is a major obstacle in the formation of a viable tissue *in vitro*. With this in mind, we hypothesised that the combination of a strong reinforcing and osteoinductive ceramic phase (HA) with a tough but biodegradable polymer phase (type I collagen) would produce a highly porous composite structure which possesses all the prerequisite biological, morphological and mechanical characteristics necessary to facilitate the body's own natural bone regenerative process *in vivo*. The results showed that the high porosity achieved in this scaffold, combined with the increased mechanical properties and improved permeability, seen as a result of the addition of the osteoinductive HA phase, make this scaffold an ideal template for the promotion of cell ingrowth and *in vivo* vascularisation.

Mechanical properties of tissue engineering scaffolds are vital to ensure long-term structural and functional viability *in vivo*. In addition, substrate mechanical

properties of these scaffolds have been shown to be a determining factor in directing cellular activity (Engler *et al.*, 2004; Engler *et al.*, 2006). The addition of hydroxyapatite and the application of DHT and chemical cross-linking treatments resulted in a significant increase in scaffold mechanical stiffness. When 200 wt% HA was added in conjunction with DHT and chemical cross linking treatments, an approximate ten-fold increase in substrate stiffness was achieved, specifically up to 4 kPa. Recent unpublished work from our laboratory has demonstrated that collagen-based scaffolds with a stiffness in this range exhibit increased cell attachment, proliferation and migration compared to less stiff scaffolds. From a bone tissue regeneration perspective, these scaffolds are within a bulk stiffness range close to that shown to favour osteogenic differentiation of mesenchymal stem cells (MSCs) (Engler *et al.*, 2006). Interestingly, recent studies have investigated the bulk and localised mechanical properties of collagen-based scaffolds manufactured using an identical fabrication process to the one employed in this study (Harley *et al*., 2007). The nature of high porosity structures means that their bulk mechanical properties are dramatically different to the mechanical properties of the individual struts within the open foam network. As a result, the substrate stiffness that a cell 'feels' while attached to one or multiple struts within the porous scaffold can be significantly higher than that predicted by bulk assessment of the material. Based on their study, it was estimated that the substrate stiffness experienced by a cell attached within a CHA scaffold pore would be of the order of approximately 50 to 100 MPa. This level of localised strut stiffness would appear to be sufficiently high to promote osteogenic differentiation (Khatiwala *et al*., 2007; Rowlands *et al*., 2008) but comparisons are difficult as these substrate stiffness studies were carried within two-dimensional environments as distinct from the three-dimensional environment of the CHA scaffolds. Due to the relatively small pore size in the scaffolds produced using the lyophilisation technique used in this study, the stiffness the cell's actually sense will be governed by a combination of both bulk and tissue modulus because, it is known from research carried out in our laboratory, that up to 75% of cells will bridge pores (Jungreuthmayer *et al*., 2009) and thus they are not seeing a flat planar surface (i.e. a 2D environment). Therefore, the effect of local substrate stiffness in a three-dimensional environment such as that of the CHA scaffold is still an area that requires significant future investigation. However, what is clear is that these scaffolds clearly show potential for bone tissue regeneration when implanted into either an osteoprogenitor-rich osseous defect (such as oral or maxillofacial reconstruction) or alternatively as a bone void filler, used in load-bearing bone tissue defects in combination with mechanical fixation.

All scaffolds investigated as part of this study exhibited an extremely high degree of porosity (≅ 99.5%). While the addition of hydroxyapatite in increasing quantities up to 200 wt% relative to scaffold collagen weight resulted in an expected decrease in scaffold porosity level, this decrease was negligible in real terms, even in the 200 wt% HA scaffolds where a porosity as a high as 99% was maintained. This exceeded our goal of achieving a porosity as high as 95% while improving the mechanical properties compared to the collagen-only scaffold. Porosity is a critical characteristic of tissue engineering scaffolds as high levels of porosity play a critical role in *in vitro* and *in vivo* bone formation (Karageorgiou and Kaplan, 2005). Higher scaffold porosity has been shown to increase cell proliferation levels, due to improved transport of oxygen and nutrients (Takahashi and Tabata, 2004) as a result of increased vascularity which results in an increase in bone ingrowth and new bone formation *in vivo* (Roy *et al.*, 2003).

Flow conductivity or permeability is a measure of the resistance within a porous construct to fluid flow under pressure. High permeability scaffolds are attractive from a tissue engineering point of view as they facilitate increased levels of fluid flow *in vivo* and consequent cellular diffusion. Fluid flow has also been shown in a number of studies to have a stimulatory effect on early-stage bone formation markers in three-dimensional tissue engineering scaffolds (Jaasma and O'Brien, 2008) and stimulating mineral deposition *in vitro* (Bancroft *et al.*, 2002; Sikavitsas *et al.*, 2005). The extremely high level of porosity retained in scaffolds fabricated using twice as much hydroxyapatite per weight than collagen ensures a high degree of pore interconnectivity and presents an extremely large internal surface area for cellular attachment (O'Brien *et al.*, 2005). Permeability values increased significantly in all CHA scaffolds relative to collagen-only scaffolds. It can be hypothesised that this increase in permeability is directly related to the incremental increase in scaffold stiffness as the proportion of hydroxyapatite was increased. Scaffolds possessing increased mechanical stiffness would be better able to withstand the hydrostatic pressure and this resistance to deformation would have a significant benefit in terms of pore shape and size retention, reducing resistance to fluid flow under constant pressure. It could be postulated that this effect would have a positive effect *in vivo* by ensuring high levels of fluid diffusion and cellular material perfusion throughout the scaffolds during low level *in vivo* loading, ensuring homogenous cellular attachment, proliferation and mineral deposition. Consequently, scaffolds with as much HA as 200 wt% can potentially provide an ideal environment capable of supporting long-term cellular attachment and proliferation while significantly reducing the threat of avascular necrosis occurring within the scaffold core during long-term *in vitro* cell culture and *in vivo* implantation (Kelly and Prendergast, 2004). This problem becomes increasingly manifested as cells on the periphery of the construct grow and secrete extracellular matrix. As a result, diffusion of nutrients to the centre of the construct becomes increasingly more difficult due to impeded movement of fluid into the core. The resulting encapsulation effect eventually leads to acellular necrosis occurring in the scaffold centre which acts as a major obstacle in the formation of a viable tissue *in vitro* (Plunkett *et al.*, 2010). Thus, the high porosity achieved in this scaffold, combined with increased mechanical properties and improved

permeability make it an ideal template to promote cell ingrowth and subsequent vascularisation and prevent the problem of core degradation occurring following implantation.

DNA quantification showed increased cell number on all CHA scaffold groups while in contrast, there was a modest decrease in the number of cells on collagen-only constructs over time. While some of these changes were not statistically significant, it is interesting to note that this increased trend was present on all CHA scaffolds. Critically, all CHA scaffold groups are at least as biocompatible as the collagen-only constructs. This is an important finding given collagen's well accepted position as a biocompatibility "gold standard" in tissue engineering and strongly supports the potential use of CHA scaffolds as potential bone graft substitutes.

A significant increase in cell-mediated mineral deposition was observed in scaffolds that exhibited the highest substrate stiffness, highest degree of permeability and contained the largest amount of the osteoinductive HA particles. The significant increase in alizarin red staining on 200 wt% HA scaffolds after 28 days *in vitro* culture is encouraging from an *in vitro* osteogenesis perspective. This calcium deposition was seen throughout the 200 wt% HA scaffolds at earlier time points but increased dramatically at 28 days. This result was promising as it illustrates the potential of the 200 wt% HA scaffolds to support the development of bone tissue from an osteoblast proliferation stage through to extra cellular matrix (ECM) deposition and on to cell-mediated early-stage mineralisation. Interestingly, this effect was not observed in the 50 wt% HA or the 100 wt% HA scaffolds after 28 days in culture. Clearly the addition of small amounts of HA to the scaffolds results in a significantly increased level of calcium deposition and has a mild osteogenic effect. However, there appeared to be a threshold level of 200 wt% HA required to cause a dramatic increase in the osteogenic potential of the scaffolds, seen in the 200 wt% HA 28 day scaffolds. The results of this study suggest that this may be due to two distinct effects as a result of the inclusion of hydroxyapatite particle within the composite, namely (i) the osteoinductive effect of the HA particles when their inclusion does not significantly increase scaffold stiffness (seen in the 100 wt% HA scaffolds, Fig. 1b) and (ii) the combination of the osteoinductive HA particles in combination with an increase in scaffold stiffness (seen in the 200 wt% HA scaffolds) that results in a dramatic increase in mineral deposition within the scaffolds after 28 days. The osteogenic influence of substrate stiffness alone has been seen previously (Engler *et al.*, 2006) but clearly the ability to increase the substrate stiffness within these constructs using a biocompatible and osteoinductive HA phase has significant advantages in bone tissue engineering applications.

The regenerative potential of the 200 wt% HA scaffold was clearly observed in the pilot pre-clinical trial carried out as part of this study. After only 28 days implantation within a critical sized calvarial defect, evidence of new bone formation was observed with almost complete bridging of the defect. Most interestingly, mineralisation was seen to progress into the core of the circular osseous defect and was almost full thickness across the width of the defect. Mineralisation levels were comparable to the existing calvarial bone tissue, with an approximate mineralisation level of 75% assessed radiographically compared to normal bone tissue surrounding the defect. Although a larger study would be required to conclusively investigate the extent of bone regeneration possible, the data provide strong evidence for the regenerative potential of these scaffolds. Most importantly, it is clear that these scaffolds have the potential to support long-term healing of osseous defects and can support cellular infiltration and diffusion *in vivo* into their core. It remains to be seen whether these scaffolds could regenerate full thickness healing in load bearing defects greater than the current limits of scaffold diffusion but the mineralisation of the scaffold core to nearly full defect thickness seen in this preliminary study is promising. Additionally, the speed at which mineralisation occurred (approximate mineralisation level of 75% compared to the surrounding calvarial bone tissue) after only 28 days implantation strongly suggests that further investigation of these scaffolds in larger load bearing pre-clinical investigations is warranted.

Conclusions

In conclusion, a highly porous biomimetic tissue engineering scaffold has been developed that exhibits increased stiffness, interconnectivity and *in vitro* bioactivity due to the addition of an osteoinductive hydroxyapatite phase. This scaffold is comprised only of bone's natural constituent materials, ensuring non-toxic degradation by-products, excellent biocompatibility and the potential to degrade in parallel with the process of new bone formation *in vivo*. Coupled with the short-term *in vitro* and *in vivo* experimental results, this CHA scaffold demonstrates real potential as a bone graft substitute material, capable of facilitating and promoting osteogenesis *in vivo*.

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Discussion with Reviewers

Reviewer I: At the end of the first paragraph of the Discussion, the authors state that "the high porosity, combined with increased mechanical properties and improved permeability make it an ideal template to promote cell ingrowth and subsequent vascularisation". The authors do not mention pore size as playing a role? In addition, did the authors see vascularisation in their *in vivo* study?

Authors: We agree with the reviewer's comment that pore size plays a role in cell ingrowth and subsequent vascularisation. (The reviewer may be aware that our group has published extensively on the role of scaffold pore size on cell behaviour e.g.: O'Brien *et al*., 2005, 2007a; Byrne *et al*., 2008; Murphy *et al*., 2010a,b). However, the scaffolds investigated in this study have similar pore sizes so pore size did not affect the results obtained i.e. any variability between groups was not as a result of scaffold pore size (see discussion below). Regarding vascularisation of the scaffolds, the authors did not specifically quantify vascularisation as part of this study. Ongoing work within our laboratory is focussed on a more extensive *in vivo* study (in rabbit long bones) than that shown in this manuscript, quantitative histomorphometry will be used to quantify to both osteogenesis and angiogenesis. Unfortunately, such an analysis was beyond the scope of the current study due to ethical approval being granted only for a preliminary trial.

Reviewer I: Did the authors look at the microstructure of the different scaffolds after production? For example does the mineral particle size change or alter at all after the in-vitro assay? Porosity and permeability give some information about the microstructure, but it is known that pore size also plays a role. What is the pore size of the different scaffolds?

Authors: The authors have examined the microstructure of the different scaffolds after their production. The four scaffold groups investigated as part of the study (namely collagen, 50 wt% HA, 100 wt% HA and 200 wt% HA groups) have similar pore sizes, homogeneity and pore distribution. This allowed the authors to exclude pore size as a variable affecting the results obtained. Hydroxyapatite particle distribution was assessed using Energy Dispersive X-Ray analysis and microCT and particles were found to be homogenously distributed in all three CHA scaffold groups. Particle size was assessed qualitatively using SEM and was found to be unaffected by the fabrication process. Average scaffold pore size was 120 μm.

Reviewer I: It is now well known (from Engler *et al*., 2004; Engler *et al*., 2006, text references) that stiffness controls cell response; however substrate chemistry also plays a role. Changing the composition in terms of the HA/collagen ratio also changes the chemistry, could the authors comment on the relative importance of these two features? Can they be separated?

Authors: The authors agree with the reviewer's comment that changing the composition of the scaffolds in terms of HA/collagen ratio might change the chemistry of the cellular interactions within the CHA scaffolds. The results suggest that increased permeability as a result of improved stiffness increases cell infiltration and thus cell number within the scaffold (Fig. 4), while the increased levels of the osteoinductive HA phase (predominantly chemical effect), leads to the overall increased levels of mineralisation (Fig. 5 and Fig. 6). However, it is difficult to directly uncouple the effects of chemical and mechanical interaction between the scaffold and the cells. It is likely that it is a combined effect that leads to the improved cellular responses seen.

Reviewer I: Macroscopic compression tests were made to calculate stiffness. This measure is of course important to predict the stability of the scaffold, however is not necessarily the value of stiffness the cells will feel. Can the authors comment on this?

Authors: Recent studies have investigated the bulk and localised mechanical properties of collagen-based scaffolds manufactured using an identical fabrication process to the one employed throughout this study (Harley *et al*., 2007). Harley and colleagues used a combination of empirical and theoretical models to investigate differences in bulk and local stiffness values for highly porous collagen-based scaffolds and found that when hydrated, strut stiffness was approximately four orders of magnitude greater than the stiffness values measured using standard mechanical testing of bulk specimens. The nature of high porosity structures means that their bulk mechanical properties are dramatically different to the mechanical properties of the individual struts within the open foam network. As a result, the substrate stiffness that a cell feels while attached to one or multiple struts within the porous scaffold can be significantly higher than that predicted by bulk assessment of the material. Due to the relatively small pore size in the scaffolds produced using the lyophilisation technique used in this study, the stiffness the cell's sense will be governed by a combination of both bulk and tissue modulus because we know that 75% of cells will bridge pores (Jungreuthmayer *et al*., 2009, text reference) and thus they are not seeing a flat planar surface. Using a cellular solids model, such as the Gibson Ashby approach, it was estimated that the substrate stiffness experienced by a cell attached within the pores of the CHA scaffold would be of the order of approximately 50 to 100 MPa.

Reviewer II: Why is there a reduction in the number of cells at 28 days in the collagen scaffolds (especially as collagen scaffold is considered 'gold standard'? Or is this change not significant?

Authors: The decrease in cell number on the collagen control scaffolds was not statistically significant after 28 days. However, the trend of increasing cell number seen in all CHA scaffolds (and statistically significant in the 50 wt% and 100 wt% HA scaffolds) was interesting with respect to previous studies within our group that have looked at cell attachment. There are a number of factors that can affect cell attachment, most notably scaffold pore size for example (Jungreuthmayer *et al.*, 2009, text reference) but the scaffolds investigated throughout this study have similar pore sizes. Interestingly, only the collagen control scaffolds showed a net decrease in cell number after 28 days in culture and we hypothesise that this may be due to the increased permeability seen in the CHA scaffolds.