Controlled release of vascular endothelial growth factor from spray-dried alginate microparticles in collagen–hydroxyapatite scaffolds for promoting vascularization and bone repair

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Abstract

A major limitation with current tissue-engineering approaches is creating functionally vascularized constructs that can successfully integrate with the host; this often leads to implant failure, due to avascular necrosis. In order to overcome this, the objective of the present work was to develop a method to incorporate growth factor-eluting alginate microparticles (MPs) into freeze-dried, collagen-based scaffolds. A collagen–hydroxyapatite (CHA) scaffold, previously optimized for bone regeneration, was functionalized for the sustained delivery of an angiogenic growth factor, vascular endothelial growth factor (VEGF), with the aim of facilitating angiogenesis and enhancing bone regeneration. VEGF was initially encapsulated in alginate MPs by spray-drying, producing particles of < 10 μm in diameter. This process was found to effectively encapsulate and control VEGF release while maintaining its stability and bioactivity post-processing. These VEGF-MPs were then incorporated into CHA scaffolds, leading to homogeneous distribution throughout the interconnected scaffold pore structure. The scaffolds were capable of sustained release of bioactive VEGF for up to 35 days, which was proficient at increasing tubule formation by endothelial cells in vitro. When implanted in vivo in a rat calvarial defect model, this scaffold enhanced vessel formation, resulting in increased bone regeneration compared to empty-defect and VEGF-free scaffolds. This biologically functionalized scaffold, composed entirely of natural-based materials, may offer an ideal platform to promote angiogenesis and tissue regeneration. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords polymeric alginate microparticles; vascular endothelial growth factor; angiogenesis; osteogenesis; collagen scaffolds; tissue engineering; spray-drying

1. Introduction

More than 2.2 million orthopaedic procedures requiring bone grafts are conducted worldwide annually, making bone the second most commonly transplanted tissue, next to blood (Marino and Ziran, 2010). Due to limitations of current therapeutic approaches, the engineering and regeneration of bone via tissue-engineered (TE) scaffolds is being explored extensively. A wide range of collagen-based scaffolds exist for tissue engineering (Wahl and Czernuszka, 2006; Swetha et al., 2010), a number of which have been developed and optimized, in our research laboratory for bone repair, to include synthetic ceramics and natural polymers (Cunniffe et al., 2010; Haugh et al., 2010; Keogh et al., 2010). These scaffolds provide a suitable porous structure and substrate for tissue growth by mimicking the natural extracellular matrix (ECM).
One of the biggest challenges faced in the TE field is promoting the growth of vasculature within engineered tissues, to enable sufficient engraftment and integration within the host (Novosel et al., 2011). The diffusion distance for nutrients and oxygen implicit to cell survival is limited (150–200 μm) and this can lead to necrosis in the centre of the supportive scaffolds designed, for example, for bone repair. Traditional methods of initiating angiogenesis and osteogenesis include delivering recombinant human (Rh) growth factors (GFs), such as vascular endothelial growth factor (VEGF) and bone morphogenetic proteins (BMPs) (Lieberman et al., 2002), as well as direct delivery of genes encoding for these GFs (Lu et al., 2013). Specifically, delivery of VEGF represents a particularly promising means of enhancing angiogenesis and osteogenesis, due to the fact that this protein has the ability to increase vascular network formation and vascular permeability (Stacker and Achen, 1999), resulting in increased blood flow and access to the defect site by progenitor cells. Additionally, VEGF is also an inducer of osteoblast differentiation, serving to promote cell recruitment, acting as a chemo-attractant for mesenchymal stem cells (MSCs), osteoblasts and osteogenic cells and suggesting a role for VEGF in coupling angiogenesis and osteogenesis (Mayr-Wohlfart et al., 2002; Patel et al., 2008). However, current GF delivery approaches for VEGF and other GFs are often associated with limited success, due to the uncontrolled manner in which proteins are released, short half-life, high doses required to reach the target tissue, potential safety concerns within a clinical setting and high costs (Epstein, 2011; Lu et al., 2013).

In an attempt to overcome these issues, several scaffold-based systems, either naturally or synthetically derived, have been developed to deliver GFs to the target sites. The most common approach consists of GFs directly interspersed within a scaffold through soak loading. One example is Medtronic’s INFUSE®, a bone graft substitute consisting of an absorbable collagen sponge soaked with recombinant BMP-2. This product, although approved by the US Food and Drug Administration (FDA), has been associated with side-effects and complications such as heterotopic bone growth or bone overgrowth, as well as haematomas in soft tissue, para-implant bone resorption and osteolysis. This occurs as a result of BMP-2 leakage into other areas of the body, due to the uncontrolled manner in which the GF is released (Epstein, 2011). These shortcomings demonstrate the need for alternative drug delivery strategies capable of controlling growth factor delivery to particular sites. This has led to the emergence of scaffolds integrated with GF delivery devices in the form of polymer microparticles, which serve to protect the GF whilst controlling its release (Richardson et al., 2001; Chen and Mooney, 2003). The scientific literature describes an array of microparticle-engineering methodologies for encapsulating drugs in a variety of matrix materials, including emulsion dispersion (Borselli et al., 2010), ionotropic pregelation (Reis et al., 2006) and spray drying (Schoubben et al., 2010). However, emulsion dispersion and ionotropic gelation possess limitations, in that they are difficult to scale up and typically involve the use of toxic organic solvents. Spray-drying is a rapid method to produce calcium alginate microparticles with few processing parameters compared to other techniques, making it suitable for industrial scale-up (Sivadas et al., 2008; Schoubben et al., 2010). In the current study, the encapsulation of VEGF in spray-dried microparticles produced from the natural polymer alginate was investigated. Alginate is biodegradable, biocompatible and categorized in the ‘generally regarded as safe’ (GRAS) classification by the FDA (George and Abraham, 2006). Furthermore, it has many possible applications in the area of drug delivery and controlled release (Lee and Mooney, 2012).

These spray-dried microparticles were incorporated into scaffolds with properties optimized specifically to promote cell infiltration and tissue formation. Recent work in our laboratory has led to the development of a highly porous collagen–hydroxyapatite (CHA) scaffold that combines the biodegradability and biocompatibility characteristics of collagen with the naturally occurring and osteoconductive bone mineral hydroxyapatite (Gleeson et al., 2010). Previously, this has been shown to facilitate bone regeneration in two preclinical models: rat calvaria (Gleeson et al., 2010) and rabbit radii (Lyons et al., 2014). The aim of the current study was thus to enhance the regenerative capacity of this scaffold through the incorporation of therapeutic GFs. Specifically, the scaffold was functionalized with VEGF-releasing alginate microparticles, in an attempt to engineer a material capable of localizing and sustaining the release of VEGF over extended periods, in order to promote vascular ingrowth and enhance bone formation. Herein, we describe the in vitro development and characterization of this functionalized CHA scaffold through the combination of the above materials, with the ultimate aim of assessing the functionality and bioactivity of the scaffold in vivo.

2. Materials and methods

2.1. Preparation of VEGF-loaded alginate microparticles

Alginate microparticles (MPs) were prepared using low-viscosity alginic acid sodium salt from Macrocystis pyrifera (Sigma, Ireland) by a spray-drying method. Fluorescently labelled bovine serum albumin (BSA) with fluorescein isothiocyanate (FITC; Sigma, Ireland) tag was initially incorporated as a model protein cargo, as previously described (Sivadas et al., 2008), to determine the protein-MP distribution within the scaffolds. RhVEGF165 (R&D Systems, UK) was similarly incorporated into alginate MPs. A 0.5% w/v alginate feed solution was mixed with VEGF (1 μg/mg) and spray-dried (Buchi Mini Spray-Dryer B290) according to the following drying parameters: compressed air 5–8 bar, air flow rate 400–600 l/h, inlet temperature 140 °C, aspirator at 80% of maximum capacity and pump flow rate at 15%. These were based on
previously established parameters for fabricating particles of an appropriate size range for the intended application (Sivadas et al., 2008). MPs were obtained in powder form from the spray-drier and subsequently crosslinked. With this purpose, MPs were suspended in acetonitrile to disperse them under sonication (Schoubben et al., 2010) and the suspension was poured into 1.2% w/v calcium chloride solution under magnetic stirring (10 min). The particles were filtered on 0.45 μm nylon filter paper, washed twice with distilled water to remove the solvent and dried at room temperature overnight. The recovered MPs were stored at 4 °C in a dessicator until further use.

2.1.1. Microparticle yield and size

The mass yield of product after spray-drying and crosslinking was determined according to the following equation:

\[
\text{Yield (\%)} = \frac{\text{mass of MPs post-spray-drying (g)}}{\text{(polymer + protein in spray dryer (g)) \times 100}}
\]

The mean particle size and size distribution of the MPs were determined by dynamic light scattering (DLS) with a Malvern MasterSizer Sirocco 2000, by suspending the particles in distilled water prior to analysis. Size and surface morphology of the VEGF-MPs was characterized using a scanning electron microscope (SEM; SMU, Japan) and an optical microscope (Olympus CX-41). Prior to SEM observation, the particles were mounted on metallic studs coated with thermoplastic carbon adhesive and later sputtered with gold.

2.1.2. VEGF loading and encapsulation efficiency

To determine the content of VEGF encapsulated in the polymer, 5 mg dried and crosslinked VEGF-MPs were dissolved in 10 ml 0.1 M sodium citrate under magnetic stirring for 30 min and the VEGF content was analysed by ELISA, using a rhVEGF DuoSet® ELISA Development Kit (R&D Systems), according to the manufacturer’s instructions. Drug loading and encapsulation efficiency were defined as:

\[
\text{Loading efficiency (LE; \%)} = \frac{\text{encapsulated drug/microparticle weight}}{\text{protein in spray dryer (g)}}
\]

\[
\text{Theoretical loading efficiency (TLE; \%)} = \frac{\text{drug used for encapsulation/(protein + polymer used for encapsulation)}}{\text{protein in spray dryer (g)}}
\]

\[
\text{Encapsulation efficiency (EE; \%)} = \frac{\text{LE}}{\text{TLE}}
\]

2.1.3. In vitro growth factor release kinetics from microparticles

In order to examine VEGF release kinetics from the alginate MPs, 20 mg VEGF-MPs were dispersed in 2 ml Dulbecco’s phosphate-buffered saline (PBS; 140 mM NaCl, 1.5 mM KH2PO4, 8.1 mM NaHPO4, 2.7 mM KCl; Sigma-Aldrich, Germany), placed in a waterbath and shaken at 37 °C. The release medium was removed following centrifugation and replaced by fresh PBS at the following time points for analysis; 4 h, and 1, 3, 7, 14, 21 and 28 days. VEGF in the release medium was quantified using the rhVEGF ELISA above, according to the manufacturer’s instructions.

2.2. In vitro bioactivity of VEGF released from microparticles

2.2.1. Cell culture

To assess the bioactivity of VEGF released from alginate MPs, human umbilical vein endothelial cells (HUVECs) were cultured in complete endothelial medium (EGM-2 BulletKit, Lonza, UK) in T175 flasks under standard culture conditions (37 °C, 5% CO2) until they reached 90% confluence. The medium was replaced every 3–4 days and the cells were passaged every 5–6 days. A confluent flask was rinsed with 10 ml prewarmed PBS to remove any trace of medium and trypsinized (0.25% EDTA/trypsin; Sigma-Aldrich, Germany). The flasks were returned to the flow hood, where 4–5 ml medium was added to deactivate the trypsin and the cells were counted using a haemocytometer. The collected suspension was then centrifuged (5000 rpm for 5 min), the supernatant was discarded and the pellet was resuspended in growth medium without VEGF and seeded according to the parameters given below.

2.2.2. Tubule formation in response to released VEGF

To examine the ability of VEGF released from VEGF-MPs to enhance angiogenesis, Growth Factor Reduced Matrigel™ (BD Biosciences, UK), a basement membrane matrix commonly used to observe in vitro angiogenesis, was placed in a 48-well plate (120 μl/well). The release medium (containing VEGF) was diluted to achieve a VEGF concentration previously shown to enhance tubule formation (50 ng/ml) in vitro in a similar assay (Suehiro et al., 2010). HUVECs were plated at 3 × 10^4/well and placed in an incubator for 20 min, after which time different cell culture media were added, including: 1 ml endothelial medium without the VEGF supplement in all cases; endothelial medium containing VEGF previously released from VEGF-MPs (for 4 h) and diluted to
50 ng/ml; or non-encapsulated VEGF aliquoted from fresh VEGF stock solution at the same concentration used as a bioactivity benchmark control. Phase-contrast light-microscopy images of the cells at x 10 magnification were captured at 6, 12 and 24 h post-seeding with an in situ camera (Leica DFC420C), using a Leica DML microscope (Leica Microsystems, Switzerland). ImageJ software (Abramoff et al., 2004) (National Institutes of Health) was used to determine the total tubule length of each tube in each image (five images/well) and the average cumulative length/well was calculated in response to the various culture conditions and was used as a surrogate marker of angiogenesis.

2.3. Fabrication and characterization of CHA and functionalized CHA scaffolds

Having established the method of VEGF-MP preparation and maintenance of VEGF bioactivity, a collagen–hydroxyapatite (CHA) scaffold was fabricated using a patented freeze-drying process developed by the RCSI Tissue Engineering Research Group (O’Brien et al., 2008; Gleeson et al., 2010; Lyons et al., 2014). Briefly, collagen (Collagen Matrix Inc., NJ, USA) slurries were produced by the homogenization of 1.8 g fibrillar collagen within 320 ml of a 0.5 M acetic acid solution. Slurries were homogenized in a reaction vessel at 4 °C, using an overhead blender. In parallel, 3.6 g hydroxyapatite (HA) particles with a size of 3–8 μm (Plasmabiotal Ltd) were suspended in 40 ml 0.5 M acetic acid solution. The final CHA slurry (200% HA:collagen weight ratio) was produced by the addition, in aliquots, of the HA-acetic acid suspension to the initial collagen slurry during the homogenization process. The slurry was degassed under a vacuum to remove air bubbles and subsequently 9 ml was pipetted into a stainless steel pan (45 × 45 mm, grade 304 SS). The tray was degassed under a vacuum to remove air bubbles and subsequently 9 ml was pipetted into a stainless steel pan (45 × 45 mm, grade 304 SS). The tray was placed into a freeze-dryer and cooled to –10 °C at a constant rate of 0.9 °C/min. Once freezing was complete, the ice crystals were removed by sublimation for 17 h at 200 mTorr. The resulting materials were cut with a punch into 6.5 mm diameter discs with a depth of 3–4 mm.

In order to incorporate the MPs into the scaffold for the formation of a functionalized CHA scaffold, they were suspended in water to achieve three different concentrations within the CHA slurry; 0% (control scaffolds), 0.5%, 1.1% and 2.2% w/v (weight of microparticles/volume of slurry), equating to 0, 1.5, 3.3 and 6.6 μg protein, respectively. They were then dispersed with the CHA slurry and freeze-dried as above. This fabrication process is the subject of a patent filing (O’Brien et al., 2012). Blank and BSA–FITC loaded MPs were initially incorporated to establish the maximum MP loading achievable, so as not to affect the porosity and mechanical properties of the scaffold. VEGF-MPs were subsequently incorporated into the slurry at the maximum MP-loading capacity, determined to be 0.5% w/v CHA slurry, based on scaffold characterization studies (see results shown in the supporting information, Figure 2A (ii), B (i), C). This VEGF-releasing scaffold will be denoted VEGF-MP scaffold in the remainder of the manuscript. All scaffolds were crosslinked under an ultra-violet (subtype C, 365 nm) lamp for 15 min and were turned half-way through to enhance the mechanical and enzymatic resistance properties of the materials (Weadock et al., 1995, 1996). The scaffolds were subsequently sterilized under the UV lamp (254 nm).

Confocal laser-scanning microscopy, using a LSM 510 Axio Plan 2 upright confocal microscope (Carl Zeiss, Germany), was used for assessing the distribution of fluorescently labelled MPs in different layers and regions of the BSA–FITC-loaded MP scaffolds. Three-dimensional (3D) reconstructions were carried out from these images. Scaffolds were cut and mounted onto metallic studs, with the help of carbon-based glue, and sputtered with gold, and SEM images were captured at 5 kV, using secondary electron mode, taken at a working distance of 12–18 mm. Both the surfaces and the cross-sections of the MP scaffolds were examined.

2.4. Analysis of VEGF release and bioactivity from VEGF-MP scaffolds

In order to examine VEGF bioactivity following scaffold fabrication, protein was released from VEGF-MP scaffolds by placing 6 mm scaffolds in 2 ml PBS. At set time points of 4 h and 1, 3, 7, 14, 21, 28 and 35 days, buffer was removed and a fresh aliquot of PBS was added to the scaffold. ELISA was used for protein quantification as described above. To examine the ability of VEGF released from scaffolds to enhance angiogenesis, Matrigel was similarly employed as described for VEGF-MPs (section 2.2). Endothelial medium containing VEGF released from VEGF-MP scaffolds (for 4 h) and diluted to 50 ng/ml, instead of protein released from VEGF-MPs, was used.

2.5. Effect of functionalized VEGF-MP scaffolds on in vivo bone formation and vascularization

2.5.1. Surgical procedure

In vivo analysis was conducted in accordance with protocols approved by the Research Ethics Committee of the Royal College of Surgeons in Ireland, and an animal licence was granted by the Irish Government Department of Health (Ref. No. B100/4416). A total of 24 young adult, male Wistar rats, aged ca. 12 weeks, mean weight 375 (range 360–395) g, were divided into three groups: (a) empty defect (n = 8), denoted ‘empty defect’; (b) CHA scaffold alone (no MPs or VEGF; n = 8), denoted ‘empty scaffold’; and (c) CHA scaffold containing alginate MPs encapsulating VEGF (n = 8), denoted ‘VEGF-MP scaffold’. General anaesthesia, the creation of the 7 mm diameter
critical-sized cranial defect, the placement of the composite materials in the defect site and postoperative animal care were performed using established methods in our laboratory (Lyons et al., 2010, Quinlan et al., 2015). In all cases a trephine was used to create a transosseous defect, confirmed by identification of the dura at surgery, which remained intact. The rehydrated scaffold was implanted into the defect, the peristeam kept intact and oversewn with 3/0 absorbable monofilament suture (Ethicon, NJ, USA), and the skin closed with skin glue (3M™ Vetbond™ Tissue Adhesive (n-butyl cyanoacrylate)). All rats were housed in the Biomedical Research Facility at the Royal College of Surgeons in Ireland. At 8 weeks post-implantation, the animals were euthanized by CO₂ asphyxiation, a 20 × 20 mm² segment of calvarium containing the defect was resected using a dental saw (Dentalfarm, Torino, Italy) and the explants retrieved were stored in 10% formalin for 4 days and then transferred to PBS prior to analysis.

2.5.2. Immunohistochemical staining

The endothelial cell marker platelet endothelial cell adhesion marker (PECAM/CD31) was analysed to observe the ability of VEGF released from the scaffolds to promote vessel formation, as it is highly expressed in endothelium (Zocchi et al., 1996; Norrby and Ridell, 2003). CD31 is a commonly used endothelial marker for quantifying angiogenesis by calculation of microvessel density (MVD) (Norrby and Ridell, 2003). For quantification, cross-sections (7 μm) of decalci
cified, paraffin-embedded tissue (described below), representing the peripheral and central regions of the scaffold (eight scaffolds/group, with n = 6 sections/scaffold analysed) were deparaffinized, rinsed in PBS, surrounded by a Pap pen (Abcam, Cambridge, USA) and incubated in blocking buffer, consisting of horse serum and 1% BSA in PBS ( Vectastain Elite Kit, Vector Laboratories, CA, USA) for 1 h. The specimens were washed in PBS/Tween 20 and incubated in mouse anti-ram primary CD31 antibody (BD Biosciences, UK) at a dilution of 1:650 in 1% BSA in PBS for 1 h at room temperature, and then mounted with Vectashield (Vectastain Elite Kit). Images were taken with a fluorescence microscope (Olympus IX51-AnalySIS imaging system), where FITC-labelled ECs in sections were visualized. Nuclei were stained with DAPI to identify the presence of vessel-forming ECs. The vessel area was then measured, using the DAPI images to select for vessel-forming ECs, subtracting any staining due to the presence of scaffold. Vessel area (average of the total area covered by red-stained vessels in the sections) and density (total number of vessels found in the biomaterial) were quantified using ImageJ software.

2.5.3. Microcomputed tomography analysis

The 3D structure of the new bone produced within the cranial defect was analysed using micro-computed tomography (microCT). Scans were performed on a Scanco Medical 40 MicroCT system (Scanco Medical, Bassersdorf, Switzerland) with 70 kVP X-ray source and 112 μA (resolution of −12 μm). 3D reconstruction was performed using the standard Scanco software package at a threshold of 140 in a scale of 0–1000. Volume of interest (VOI) was defined to analyse a 6 mm defect region, to assess healing in the centre of the defect, excluding any old bone at the defect edges. Repair was expressed as percentage bone within the defect area.

2.5.4. Histological analysis

Along with microCT, both qualitative and quantitative histological examinations were performed on explants, in order to assess the levels of bone and vascular formation throughout the scaffold following implantation for 8 weeks. After microCT analysis, the implants were decalcified for 3–4 weeks depending on the level of mineralization, using ethylene diamine tetra-cetic acid (EDTA) 14% w/v, pH 7.4. The specimens were bisected, wax-embedded, sectioned (7 μm) and stained with haematoxylin and eosin (H&E). Images were taken using transmitted light and epifluorescence microscope visualization (Nikon Microscope Eclipse 90i, Nikon Instruments Europe, The Netherlands). The defect margin was identified and quantitative histomorphometric analysis was carried out, using a blind scoring method, to quantify the healing response from the H&E-stained samples. The average area of bone nucleation sites (stained pink) formed in cross-sections representing the peripheral and central regions of the scaffold were calculated using ImageJ (eight scaffolds/group, with n = 6 sections/scaffold analysed).

2.6. Statistical analysis

Results are expressed as mean ± standard error (SE) of the mean. Statistics were carried out using GraphPad Prism software, using a general linear model ANOVA followed by Tukey and Bonferroni post hoc analysis, unless otherwise stated. The sample size was n = 3 for in vitro and n = 8 for in vivo studies, where p ≤ 0.05 values were considered statistically significant.

3. Results

3.1. VEGF microparticle characterization

A process yield after spray-drying of 54 ± 4% was determined for blank alginate and BSA–FITC-loaded MPs and 65 ± 4% for VEGF-loaded MPs. Scanning electron microscopy (SEM) images of the VEGF-MPs showed particles which were irregularly shaped, although generally smooth and spherical, features which have previously been described for alginate microparticles fabricated by spray-drying (Bowey et al., 2013), with diameters < 10 μm (Figure 1A). DLS measurements confirmed these
observations, as diameters of the VEGF-MPs were found to be distributed in the range 1–20 (average 3.9) μm. Protein encapsulation efficiency of the VEGF-loaded alginate MPs was next determined by relating the loading efficiency (0.045%) to the total theoretical loading efficiency (0.1% protein:polymer). The content of encapsulated protein, as determined by ELISA, was found to be 49% of the original amount of VEGF incorporated during the fabrication process.

### 3.2. Analysis of VEGF release and bioactivity from VEGF microparticles

The release profile of VEGF from the MPs, displayed in Figure 1C, was continuous and sustained at high levels for up to 4 days, after which time protein release dropped at 7 days. Following this, a decrease in VEGF release was seen thereafter from day 10, and minimum concentrations were recorded until the end of the study. At this point, 28% of the protein was cumulatively released. Surprisingly, when the particles were digested at the end-point of the study, they were shown to still contain 20% of the initially incorporated protein.

The ability of the encapsulated VEGF to stimulate the tubule formation potential of HUVECs was determined by culturing ECs with VEGF released from VEGF-MPs and subsequently quantified and diluted to 50 ng/ml. There HUVECs displayed enhanced tubule network formation (6 and 12 h) compared to the rhVEGF control (non-encapsulated) added to the medium at the same concentration. In addition, the ability of encapsulated VEGF to stimulate EC proliferation was also enhanced to a similar extent as the rhVEGF control when compared to cells in the absence of VEGF (see supporting information, Figure S1B).

### 3.3. Characterization of MP distribution and pore size of functionalized scaffolds

Confocal microscopy characterization of scaffolds incorporating BSA–FITC-loaded MPs was used to demonstrate the uniform spreading of the MPs throughout different layers within the scaffolds at all MP concentrations incorporated (Figure 2A; see also supporting information, Figure S2B). SEM images, displayed in Figure 2, corroborated these results, as they demonstrate the homogeneous distribution of the polymer particles through the collagen matrix. The pore diameters of the scaffolds decreased as a function of the increasing concentrations of MPs, with pore size significantly highest (100 μm) in scaffolds containing the lowest MP concentration (see supporting information, Figure S2A, C). Since the optimal range of scaffold pore sizes for bone tissue regeneration is 100–135 μm (Murphy and O’Brien, 2010), scaffolds with a microparticle concentration of 0.5% were selected for further studies.

![Figure 1. Microparticle characterization. (A) SEM image of VEGF-MPs prepared by the spray-drying method, showing particle sizes < 10 μm; scale bars = 10 μm. (B) Microparticle size distribution, indicating particle diameters in the range < 10 μm. (C) Cumulative VEGF release from alginate MPs. [Colour figure can be viewed at wileyonlinelibrary.com]](image)
3.4. Analysis of VEGF release and bioactivity from VEGF-MP scaffolds

The kinetics of VEGF eluted from the MPs that were incorporated within the scaffold was next assessed. Similarly to the delivery from the microparticles alone, the total release achieved at the end-point of the study from the scaffolds was 26% (427 ng) of the initially incorporated VEGF (Figure 2D). A high burst release of >160 ng VEGF (10% of the total loaded protein) was recorded from the VEGF-MP scaffolds, in contrast to the release kinetics observed from the MPs alone (i.e. not embedded in a scaffold), where no burst release was detected. This was followed by a lower VEGF release at 24 h and a burst again of 7% of loaded protein at day 4. After 4 days, a slow release of VEGF was sustained until the end of the assay.

Having determined the release kinetics of VEGF from the scaffolds the next step was to examine whether the process of VEGF-MP scaffold preparation had any detrimental effect on VEGF bioactivity. This was shown not to be the case, and VEGF bioactivity was retained following the various process parameters (Figure 3A, B). It was observed that ECs cultured with VEGF released from VEGF-MP scaffolds displayed enhanced tubule network formation, to a similar extent to rhVEGF added directly to the medium at the same concentration compared to cells cultured in the absence of VEGF for 6 h. Regression of the tubules observed between 12 and 24 h is a typical behaviour of these capillary-like structures, as previously reported (Kubota et al., 1988; McCoy et al., 2013).

3.5. Effect of functionalized scaffolds on in vivo vascularization and bone formation

3.5.1. Assessment of the angiogenic effect of VEGF-MP scaffolds

The ability of the scaffolds to promote in vivo angiogenesis was evaluated by quantifying immunohistochemically stained endothelial cells (Figure 4A). An observably higher angiogenic response was evident in the VEGF-MP group, correlating to a high level of neovascularization compared to all other groups. Empty scaffolds had no effect on angiogenesis when compared to empty defects. Importantly, in the VEGF-MP group, quantitative analysis revealed a three-fold increase in the number of de novo blood vessels compared with that of the empty defect (Figure 4C). Furthermore, the VEGF-MP scaffolds led to a two-fold increase in vessel area compared with the empty defect, and a five-fold increase compared to the empty scaffold (Figure 4D). In summary, these results demonstrate the ability of VEGF eluted from the functionalized scaffolds to enhance angiogenesis.

3.5.2. Assessment of the osteogenic effect of VEGF-MP scaffolds

Having determined the angiogenic effects of the VEGF-releasing functionalized scaffold, the effects of the scaffold on bone formation were studied by microCT imaging (Figure 5A), revealing the higher extent of new bone
formation in the functionalized scaffolds when compared to the empty defect control (significant) and the empty scaffold (not significant) (Figure 5B). While the empty scaffold group also displayed higher bone volume compared to the empty defect, the volume was reduced compared to the VEGF-MP group. To further characterize the levels of healing, H&E staining was carried out on explants after 8 weeks, the results of which are shown in Figure 5C, D. Overall, less healing was found in the empty defect compared to all other groups. In the empty defect group, the defect space was primarily occupied by thin, fibrotic tissue (high-magnification image, Figure 5C) and very little bone formation had occurred. In the empty scaffold group, bone formation occurred predominantly at the defect edges. Cellular infiltration was seen in all scaffolds, with some level of bridging occurring, as confirmed in the microCT images of empty defect versus scaffold groups (Figure 5A). In the defects treated with VEGF-MP scaffolds, enhanced bone regeneration was evident when compared to the CHA scaffold alone and empty defect (high-magnification images, Figure 5C). A much thicker region of bone formation was obvious, with good cell infiltration throughout the construct (Figure 5C) and high levels of bone bridging. These observations were further confirmed by quantitative histomorphometrical analysis, demonstrating the enhanced area of new bone formed resulting from VEGF-MP treatment. This was significantly higher than in the empty defect group, as well as the defects treated with VEGF-free empty CHA scaffolds (Figure 5D). Taken together, these data demonstrate the ability of the VEGF-MP scaffold to promote bone formation in vivo.

4. Discussion

The role of scaffolds for bone tissue repair is progressively evolving from simple support materials for cell growth to bioactive matrices, which can also deliver pro-regenerative factors to enhance tissue regeneration. The aim of this study was to deliver VEGF in a controlled and sustained manner from polymer microparticles embedded within a collagen–hydroxyapatite scaffold previously developed for bone regeneration, in order to enhance its therapeutic potential by increasing angiogenesis. The extensive characterization carried out in this study demonstrates that, with the proposed fabrication technique, it is possible to create these functionalized scaffolds, which were shown to be capable of enhancing blood vessel formation. In addition, the VEGF-releasing functionalized scaffold also facilitated increased bone repair in a critical-sized rat calvarial defect model.

To the best of our knowledge, the present work describes for the first time the encapsulation of VEGF in alginate, using a spray-drying technique and a complex
multistep process for the subsequent incorporation of these MPs into a 3D scaffold developed for tissue-engineering applications. The spray-drying process was chosen since it is widely used at an industrial level for the manufacture of particulate systems, as it enables production of small-diameter MPs with high yields and drug-encapsulation efficiencies (Hascicek et al., 2003). In the present work, the VEGF-eluting microparticles were sized in the range 1–20 μm, in accordance with alginate materials fabricated similarly (Bowey et al., 2013). Importantly, VEGF was released at time points relevant to its in vivo expression profile reported in fracture-healing models, suggesting the potential of this delivery system to mimic the innate in vivo response (Tsiridis et al., 2007). Furthermore, the growth factor maintained its bioactivity during the encapsulation process; VEGF released from the MPs was proficient at increasing tubule formation by ECs in vitro. Additionally, when compared with non-encapsulated, freshly aliquoted rhVEGF used as positive control, tubule formation was enhanced, probably due to alginate dissolving from particles into the release medium, which may alter the binding of VEGF to VEGF receptors on ECs, thereby prolonging the effects (Peters et al., 1998). At the end-point of the study, MPs were shown to still retain 20% of the originally loaded protein, indicating the physical integrity of VEGF even after 28 days in an aqueous solution. The remainder of the protein may have degraded to a variable extent in the PBS. Taken together, the results confirmed the feasibility of spray-drying as a method of MP fabrication for encapsulation of VEGF. Thus, these VEGF-loaded MPs were incorporated into scaffolds in order to enhance their angiogenic capacity. Such scaffolds would possess dual functionality; they would enhance bone regeneration, Figure 4. Immunohistochemical analysis of vascular network formation. (A) CD31-labelled endothelial cells, and (B) DAPI-stained nuclei in sections of empty defect, empty scaffold and VEGF-MP scaffolds, indicating enhanced vessel density (white arrow) in VEGF-MP groups compared to the controls at 8 weeks; scale bars = 50 μm. (C) Vessel density in implants, indicating an increase in the VEGF-MP group compared to the empty defect (*p < 0.05) and empty scaffold. (D) Total area of new vessel formation, indicating an increase in the VEGF-MP group compared to the empty defect (**p < 0.01) and empty scaffold (***p < 0.001); one-way ANOVA and post hoc Tukey post-test were performed. [Colour figure can be viewed at wileyonlinelibrary.com]
due to the osteo-inductive nature of the matrix (Gleeson et al., 2010), while providing a physical support for the MPs to release VEGF in the target tissue, thereby promoting angiogenesis.

The final fabrication process resulted in scaffolds with mean pore sizes $>100\,\mu m$, a size range known to facilitate proliferation of osteoblasts and osteogenic differentiation of MSCs (Hulbert et al., 1970; O’Brien et al., 2005; Byrne et al., 2008; Murphy and O’Brien, 2010). Furthermore, the fabrication process resulted in a homogeneous distribution of the MPs throughout the scaffold – a beneficial result that allows controlled spatial delivery of the GF, an effect that can be lost when particles aggregate (Langer, 1998). Cumulative release data suggested a controlled release profile from the scaffolds, with a high initial burst release followed by a slower release for up to 4 days, after which time a steady-state sustained VEGF delivery profile was observed. The high burst release is likely a result of
protein being released from MPs at first contact with the acidic environment of the CHA slurry prior to freeze-drying. Spray-dried MPs typically accumulate their cargo on the outer surfaces (Vehring, 2008) and, since MPs may partially dissolve in contact with the slurry, the aggregated drug on the outer surface is released in an uncontrolled manner. Although there is little conclusive information regarding the specific VEGF concentration required to elicit an in vivo biological response, it has been demonstrated that higher concentrations of VEGF for a period of up to 7 days, followed by lower concentrations as well as a distributed pattern of delivery, as achieved with this scaffold, is more effective at permitting neovascularization (Stacker and Achen, 1999; Street et al., 2002; Silva and Mooney, 2010). Several previous studies using a bolus delivery method have demonstrated that doses of 10–30 ng/g resulted in increased neovascularization in vivo. Other studies have shown that a lower dose of 4 ng/g VEGF released from PLGA microparticles in an alginate gel was sufficient to induce angiogenesis in vivo (Lee and Lee, 2009). The delivery system developed in this study is thus desirable, since the kinetics achieved enabled the release of therapeutically relevant VEGF concentrations which may promote enhanced vasculogenesis (Pufe et al., 2002). Another critically important characteristic of the delivery system is its ability to maintain the bioactivity of the GF pre- and postprocessing. The results demonstrated that VEGF released from the MPs enhanced tubule network formation by endothelial cells, suggesting that alginate encapsulation provided protection to degradation from the acidic environment of the scaffold slurry and during the freeze-drying process. Taken together, these encouraging results demonstrate that a fabrication process for scaffolds showing spatiotemporally controlled GF delivery was developed.

The functionalized scaffold was shown to enhance angiogenesis and bone regeneration in a critical-sized rat calvarial defect model by 8 weeks post-implantation. Since angiogenesis involves the movement of ECs towards gradients of VEGF, it is likely that the scaffold sustained sufficient VEGF gradients across the scaffold–tissue interface to elicit an enhanced biological response in vivo. In addition to the number of vessels increasing, the size of the vessels was shown to increase in the VEGF-functionalized scaffold. It is likely that some of these large vessels were derived from newly formed capillaries resulting from increased vessel density in the VEGF-MP group. VEGF release from the material may have increased bone formation indirectly as a consequence of increasing blood flow in the defect site, by laying down a supportive vascular network. Furthermore, the ability of VEGF to promote chemotaxis and differentiation of osteoblasts is known (Patel et al., 2008) and is consistent with previous studies, which showed that the release of VEGF from biomaterials can enhance bone regeneration in critical-sized defects (Geiger et al., 2005; Kaigler et al., 2006; Clarke et al., 2007; Wernike et al., 2010). The potential of CHA scaffolds to promote mineralization within a calvarial defect has been demonstrated previously. Histomorphometry revealed that functionalized scaffolds significantly enhanced bone repair compared to the non-VEGF-eluting empty CHA scaffolds. The discrepancy between this result and the microCT analysis, which demonstrated a non-significant enhancement in bone volume in the functionalized scaffolds compared to the empty scaffold control, may be explained by the higher area of mature bone compared to earlier mineralized tissue in the microCT analysis. Nonetheless, this study has shown that controlled VEGF release led to increased vessel density and vessel size, accompanied by de novo bone formation and early bone healing of a critical-sized bone defect.

The functionalized scaffolds described herein represent a promising approach for overcoming the associated problems of uncontrolled drug delivery for tissue engineering. Both the spray-drying and freeze-drying techniques, used for the fabrication of MPs and scaffolds, respectively, are reproducible processes that can easily be scaled up to industrial production (I Ré, 1998; George and Abraham, 2006). Another potential avenue which could be explored is the adaptability of this pro-angiogenic material to a range of other applications to enhance vascularization in ischemic tissues. Freeze-dried, collagen-based scaffolds are currently being used for the regeneration of a wide range of tissues, such as cartilage (De Franceschi et al., 2005), cornea (Griffith et al., 2009) and blood vessels (Buttafoco et al., 2006). Thus, this system has enormous potential in regenerative medicine, as it could be tuned in terms of the composition of the collagen-based scaffold and released therapeutic to be optimized for the healing of very diverse organs.

### 5. Conclusion

This study demonstrated the successful fabrication of a novel, functionalized, collagen-based scaffold capable of the spatiotemporal controlled release of VEGF, through the incorporation of spray-dried alginate microparticles. The procedure for functionalizing these scaffolds maintained VEGF bioactivity, as it was proficient at increasing tubule formation by endothelial cells in vitro. These functionalized scaffolds were capable of sustaining the release of VEGF for up to 35 days. When implanted in vivo in a rat calvarial bone defect model, this functionalized scaffold led to enhanced vessel formation after 8 weeks. In addition, the release of VEGF also resulted in increased bone regeneration. The process developed to functionalize these scaffolds may offer an ideal platform to promote angiogenesis and tissue regeneration for a wide variety of applications in addition to bone.

### Conflict of interest

The authors have declared that there is no conflict of interest.
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Supporting information
The following supporting information may be found in the online version of this article:

Figure S1. Tubule formation by endothelial cells in a Matrigel assay, and endothelial cell numbers in response to released VEGF

Figure S2. SEM images of surface of empty and microparticle-loaded scaffolds, confocal laser-scanning microscopy images of BSA-FITC MPs incorporated into the scaffold, and graph of scaffold pore diameters, showing a decrease with increasing particle concentration