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Development of collagen–hydroxyapatite scaffolds incorporating PLGA and alginate microparticles for the controlled delivery of rhBMP-2 for bone tissue engineering

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ABSTRACT

The spatiotemporally controlled delivery of the pro-osteogenic factor rhBMP-2 would overcome most of the severe secondary effects linked to the products delivering this protein for bone regeneration. With this in mind, the aim of the present work was to develop a controlled rhBMP-2 release system using collagen–hydroxyapatite (CHA) scaffolds, which had been previously optimized for bone regeneration, as delivery platforms to produce a device with enhanced capacity for bone repair. Spray-drying and emulsion techniques were used to encapsulate bioactive rhBMP-2 in alginate and PLGA microparticles, with a high encapsulation efficiency. After incorporation of these microparticles into the scaffolds, rhBMP-2 was delivered in a sustained fashion for up to 28 days. When tested in vitro with osteoblasts, these eluting materials showed an enhanced pro-osteogenic effect. From these results, an optimal rhBMP-2 eluting scaffold composition was selected and implanted in critical-sized calvarial defects in a rat model, where it demonstrated an excellent healing capacity in vivo. These platforms have an immense potential in the field of tissue regeneration; by tuning the specific therapeutic molecule to the tissue of interest and by utilizing different collagen-based scaffolds, similar systems can be developed for enhancing the healing of a diverse range of tissues and organs.

1. Introduction

Bone morphogenetic proteins (BMPs) are the most important growth factors (GFs) in bone formation and healing. It is well known that they enable skeletal tissue formation during embryogenesis and throughout adulthood as well as bone growth and repair [1]. To date, more than 30 B.P. have been reported and among them BMP-2, -4, and -7 are each known to stimulate new bone formation at ectopic sites in vivo in critical sized defects [2,3]. In particular, BMP-2 is known as a primary GF in bone formation involved in the commitment of multipotent stem cells to the osteogenic lineage. Additionally, recombinant human BMPs (rhBMPs) have been isolated by purification and cloning and are widely available for regenerative applications [4].

BMPs were first described in 1965 but it was not until 1998 that Johnson and Urist found the possibility of their clinical application by purifying endogenous bone and utilizing it in human patients to heal long bone non-unions and segmental defects [5,6]. rhBMP-2 was approved by the FDA in 2002 to be used in spinal fusion. Based on this, Medtronic's INFUSE®, a collagen sponge soaked with rhBMP-2, was commercialized the same year. This product is the market leader in the bone graft field with 44% of the $1.9 billion market; however, it has been associated with numerous complications such as heterotopic ossification, osteolysis, increased neurological deficits, retrograde ejaculation, or cancer. These serious side effects have been linked to the uncontrolled and offsite release of rhBMP-2 [7,8]. Thus while problems exist, due to the outstanding pro-osteogenic effect of this growth factor, a major focus of research is on developing biomaterials for the spatiotemporally controlled delivery of rhBMP-2 to the defect site. In this sense, a myriad of polymeric matrices, ceramic scaffolds or hydrogel based materials have been tested in the last decade, showing very promising results [9–13].

We have recently developed a porous collagen–hydroxyapatite (CHA) scaffold with immense potential for bone repair as it is biodegradable, biocompatible, osteoconductive and osteoinductive [14–16]. The localized and sustained delivery of rhBMP-2 to the defect site once implanted would further enhance the regenerative capacity of this material which may be applied to an expanded range of bone regeneration applications. One way to incorporate rhBMP-2 into these CHA scaffolds would be to pre-load the protein into polymeric carriers

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that could be subsequently integrated into the CHA network during fabrication, an approach that has been successfully used in other collagen-based materials [17–20]. Ideally, these carriers would ensure the stability of the rhBMP-2 during the fabrication process and allow its controlled delivery after implantation while maintaining the existing pore architecture, mechanical properties and proven biological activity of the underlying scaffold. In addition, the scaffold should act as a reservoir, protecting the therapeutics from degradation in vivo.

With this in mind, the present work evaluated the potential of alginate and poly(lactic-co-glycolic acid) (PLGA) microparticles as rhBMP-2 carriers and assessed the effect of the incorporation of different amounts of these polymers into the CHA scaffold. The rhBMP-2 release kinetics from these constructs was determined and the bioactivity of the growth factor following both the polymeric encapsulation and inclusion into the CHA scaffold was tested in vitro on pre-osteoblastic cells. An optimal rhBMP-2 eluting microparticles/CHA scaffold composition was selected and its capability for enhancing bone tissue regeneration in vivo was determined by implanting it into a critical size calvarial defect of young male rats.

2. Materials and methods

2.1. Materials

Sodium alginate, calcium chloride, sodium chloride, polyvinyl alcohol (PVA), sodium hydroxide, sodium dodecyl sulfate (SDS) and PLGA (50:50, Mw 24,000–38,000) were purchased from Sigma Aldrich (Arklow, Ireland). Acetonitrile and dichloromethane (DCM) were purchased from Fisher Scientific (Ballycoolin, Ireland). Physiogel® was obtained as a kind gift from Braun Medical (Emmenbrucke, Switzerland). RhBMP-2 was purchased from R&D Systems Europe Ltd. (UK). All chemicals were used as obtained, without further purification.

2.2. Microparticle fabrication and characterization

Alginate and PLGA were selected as the polymers for encapsulating rhBMP-2 as they have been widely used for drug delivery purposes; in addition, both are biodegradable, biocompatible and classified as generally regarded as safe (GRAS) by the FDA [21–23]. rhBMP-2 eluting alginate microparticles were manufactured by spray-drying. In this procedure, a polymeric feed solution is atomized and passed through a drying chamber, solvent from the droplets is evaporated and particulate powder is collected. In our study, rhBMP-2 was added to a sodium alginate solution (0.5% w/v) at a protein:polymer ratio of 1 μg/mg. This mixture was spray-dried using a Buchi Mini Spray-Dryer B290 with the following parameters: compressed air 5–8 bar, air flow rate 400–600 L/h, inlet temperature 140 °C, aspirator 80% of maximum capacity and pump flow rate 15%. Alginate microparticles were crosslinked after fabrication; the microparticles were suspended in acetonitrile, poured into 1.2% (w/v) calcium chloride while stirring for 10 min after which they were vacuum filtered through a 0.45 μm nylon membrane, washed twice with 10 mL distilled water and freeze-dried at −56 °C overnight.

PLGA (50:50) microparticles were fabricated by a water–oil–water (W/O/W) emulsion method adapted from a previously described method [24]. Briefly, 0.5 mL of an aqueous solution of 16 μg of growth factor was added to 125 μL of Physiogel® (80 mg/mL of succinylated gelatin) which has been reported to protect proteins from degradation (W_1-Phase) [25]. PLGA (375 mg) was dissolved in 5 mL of dichloromethane (O-Phase). After mixing the W_1-Phase with the O-Phase, the mixture was ultrasonicated to obtain the primary W_1/O emulsion. This W_1/O emulsion was added dropwise while sonicating to 30 mL of an aqueous solution containing 1 g of polyvinyl alcohol (PVA) and 1.13 g of NaCl. The resulting $W_1/O/W_2$ emulsion was dispersed in 70 mL of the same aqueous solution. This emulsion was stirred for 4 h at room temperature and the resulting microparticles were collected by centrifugation (5000 rpm, 10 min). The particles were washed three times with 1.13% (w/v) NaCl and freeze-dried at −56 °C overnight. Blank, non-rhBMP-2 loaded alginate and PLGA microparticles were fabricated by adding distilled water instead of growth factor solutions during the respective procedures.

The encapsulation efficiency of both processes was determined by digesting the microparticles and analyzing the concentration of rhBMP-2 in the medium. With this aim, 5 mg of alginate microparticles was dissolved in 10 mL of 0.1 M sodium citrate under magnetic stirring for 30 min and 20 mg of PLGA microparticles were added to 3 mL of a 0.1 M NaOH, 5% w/v sodium dodecyl sulfate (SDS) solution in water at 37 °C while shaking (50 rpm, 2 h). Digestion media were centrifuged (10,000 rpm, 2 min) and the supernatant was analyzed for protein content using a rhBMP-2 ELISA kit (R&D Systems UK) according to the manufacturer's instructions. The encapsulation efficiency was calculated using the following formula:

$$EE(\%) = (\text{amount of rhBMP}-2 \text{ in the microparticles}/\text{rhBMP}-2 \text{ loaded}) * 100.$$

Microparticles were morphologically characterized with Scanning Electron Microscopy (SEM) using a Zeiss Supra Variable Pressure Field Emission SEM. For SEM observations, samples were mounted on copper studs with carbon glue and then sputtered with gold.

To examine protein release profiles, 20 mg of rhBMP-2 microparticles dispersed in 2 mL of phosphate buffer solution (PBS) was placed in a water bath at 37 °C under agitation. At certain timepoints, samples were centrifuged (10,000 rpm, 5 min) and the release medium was removed and replaced by fresh PBS. The concentration of rhBMP-2 in the media was determined by ELISA as above.

2.3. In vitro bioactivity assessment of rhBMP-2 encapsulated in the microparticles

MC3T3-E1 murine pre-osteoblasts were used to study the effects of the released rhBMP-2 on osteoblast proliferation and differentiation in vitro since they exhibit similar properties as osteoprogenitor cell lines including high alkaline phosphatase (ALP) activity [26] and form calcified bone tissue [27]. Cells were cultured in α-MEM supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 2% penicillin/streptomycin; the media were changed every 3 days. Cells were trypsinized (trypsin–EDTA 0.25%), seeded on 6 well plates at a density of 30,000 cells per well and cultured for 24 h in standard growth media followed by growth media supplemented with ascorbic acid 2-P 50 μM, dexamethasone 100 nM, β-glycerophosphate 10 mM, FBS 10%, 100 U/mL penicillin, and 100 μg/mL streptomycin under standard conditions (37 °C, 5% CO_2).

In order to assess the bioactivity of the rhBMP-2 after the encapsulation process, 20 mg of alginate or PLGA microparticles was dispersed in 2 mL PBS and placed in a water bath at 37 °C under agitation. After 4 h, the samples were centrifuged (10,000 rpm, 5 min), the release media were collected and the concentration of rhBMP-2 was determined by ELISA. Release medium was added to the osteogenic cell culture media at a final concentration of 50 ng/mL of rhBMP-2 for each formulation (Alginate BMP-2 (50 ng/mL), PLGA BMP-2 (50 ng/mL)). To determine the effect of the dissolution product of blank microparticles on MC3T3 bioactivity, the same amount of media from blank microparticles that had been agitating for 4 h at 37 °C was added to another set of cells (blank alginate, blank PLGA). As a positive control, non-encapsulated rhBMP-2 at the same concentration (50 ng/mL) was added to the culture media (BMP-2 (50 ng/mL)); this bioactive concentration is capable of inducing an osteogenic response from these cells [28]. Cells in the absence of rhBMP-2 or any additional media other than osteogenic differentiation media were used as negative control (cells alone).

DNA was quantified from MC3T3-E1 cells cultured after 7 days in the presence of the different media. At the endpoint of the study cells were washed in PBS, lysed using cell lysis buffer according to the