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Hypoxia-mimicking bioactive glass/collagen glycosaminoglycan composite scaffolds to enhance angiogenesis and bone repair

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ABSTRACT

One of the biggest challenges in regenerative medicine is promoting sufficient vascularisation of tissue-engineered constructs. One approach to overcome this challenge is to target the cellular hypoxia inducible factor (HIF-1α) pathway, which responds to low oxygen concentration (hypoxia) and results in the activation of numerous pro-angiogenic genes including vascular endothelial growth factor (VEGF). Cobalt ions are known to mimic hypoxia by artificially stabilising the HIF-1α transcription factor. Here, resorbable bioactive glass particles (38 μm and 100 μm) with cobalt ions incorporated into the glass network were used to create bioactive glass/collagen–glycosaminoglycan scaffolds optimised for bone tissue engineering. Inclusion of the bioactive glass improved the compressive modulus of the resulting composite scaffolds while maintaining high degrees of porosity (>97%). Moreover, *in vitro* analysis demonstrated that the incorporation of cobalt bioactive glass with a mean particle size of 100 μm significantly enhanced the production and expression of VEGF in endothelial cells, and cobalt bioactive glass/collagen–glycosaminoglycan scaffold conditioned media also promoted enhanced tubule formation. Furthermore, our results prove the ability of these scaffolds to support osteoblast cell proliferation and osteogenesis in all bioactive glass/collagen–glycosaminoglycan scaffolds irrespective of the particle size. In summary, we have developed a hypoxia-mimicking tissue-engineered scaffold with pro-angiogenic and pro-osteogenic capabilities that may encourage bone tissue regeneration and overcome the problem of inadequate vascularisation of grafts commonly seen in the field of tissue engineering.

1. Introduction

For bone repair, the surgeons preferred choice of a bone substitute remains the bone graft, specifically the autograft, creating an elevated demand for these materials worldwide. The main disadvantage associated with this approach is the requirement of surgery to harvest the autologous bone, and the pain associated with the harvest site is often said to be more painful than the recipient site [1]. Hence, the field of regenerative medicine aims to address this issue by developing new substitutes that can activate the body's own natural repair process omitting the need for donor tissue [2]. Scaffolds provide sites for cell attachment, mechanical stability within the defect site, and a porous and interconnected pore network for interaction with the host (for cell migration, and nutrient and waste removal) [3]. We have developed a series of scaffolds from type I collagen and the polysaccharide, glycosaminoglycan, to produce highly porous collagen–glycosaminoglycan (CG) scaffolds by using a controlled freeze-drying process [4–6]. These scaffolds have an optimised composition to facilitate osteogenesis [7] and have been shown to enhance bone repair *in vivo* in minimally loaded calvarial defects [8–10].

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The traditional role of the scaffold as simply a template for tissue formation has evolved and the new generation of scaffolds are increasingly being used as delivery vehicles for therapeutic molecules such as drugs, proteins and genes that initiate biological events leading to the regeneration of tissue [5]. Ions can also be classified as therapeutics; for instance, it has been shown that silicon (Si) and calcium (Ca) ions initiate osteogenesis when released in biologically relevant ranges (15–30 ppm for Si and 60–90 ppm for Ca) [11,12]. One method of delivery is the release of Si and Ca ions from bioactive glasses, which are defined as inorganic surface-active bioceramics. When exposed to biological fluids, bioactive glasses form a hydroxyl carbonate apatite layer; this layer then forms a bond between the bioactive glass and bone, imparting pro-regenerative ability to the bioactive glass allowing for bone ingrowth [11]. An approach to further enhance the therapeutic potential of the bioactive glass is to introduce ions such as strontium [13], magnesium or zinc [14], which are known to have anabolic responses in bone metabolism. Controlled rates of dissolution of the bioactive glass provide the physiologically relevant concentrations of the biologically active ions to the cells when exposed to body fluids [11]. 45S5 Bioglass particles, NovaBone and PerioGlas are examples of commercially available bioactive glass products that are used in the treatment of a wide range of dental and orthopaedic diseases [15].

One of the biggest challenges faced in the field of regenerative medicine is promoting the growth of vasculature within engineered tissues to enable sufficient engraftment and integration within the host [16]. Lack of vascularisation can lead to graft failure due to avascular necrosis. Methods of initiating angiogenesis include using expensive recombinant pro-angiogenic vascular endothelial growth factor (VEGF) proteins and genes encoding for VEGF. However, these approaches have limited success due to the uncontrolled manner in which proteins are released, high doses of protein required, short protein half-life, low transfection efficiencies associated with gene-based approaches and potential safety concerns within a clinical setting [17,18]. Furthermore, single growth factor release has previously been shown to lead to the formation of immature vasculature [19]. An alternative strategy is to target the cellular Hypoxia Inducible Factor (HIF-1α) pathway, which responds to low oxygen concentration (hypoxia) and results in the activation of a cascade of pro-vasculogenic genes critical for angiogenesis, including VEGF, thus mimicking the normal regenerative response [20]. Cobalt ions (Co^{2+}) have the potential to mimic hypoxia, they artificially stabilise the transcription factor HIF-1α [20,21], which then translocates into the nucleus to stimulate the upregulation of pro-vasculogenic genes such as VEGF [22]; this approach has been adopted as a potential neovascularisation strategy in a number of studies [23,24]. Recently, cobalt-releasing bioactive glasses have been developed [25], and their ability to activate the HIF pathway under normoxic conditions was demonstrated [26].

The focus of this study was to incorporate cobalt bioactive glass [25] into CG scaffolds that have been developed and optimised for bone tissue regeneration [7,27–29] with a view to improving the mechanical and structural properties of the CG scaffold and, most importantly, enhancing the initial angiogenic step vital for bone regeneration [30–32]. Specifically, the aims were to assess the effect of the bioactive glass on the pore structure, porosity, compressive moduli and biological activity of the resultant composites by examining their ability to induce an angiogenic and osteogenic response from cells.

2. Materials and methods

2.1. Synthesis of bioactive glass

A series of bioactive glasses containing either no cobalt or 4 mol% cobalt were prepared by the melt-quench route as previously described [25]. The addition of cobalt to bioactive glass has been shown to delay bioactive glass dissolution, ion release and HCA (hydroxyl carbonate apatite) formation in a concentration dependant manner. However, this non-cytotoxic cobalt concentration [33,34] allowed for controlled release of cobalt from the bioactive glass whilst controlling bioactive glass bioactivity or apatite forming ability. The resulting powder was sieved to obtain particle sizes with a mean diameter of 38 μm and 100 μm.

2.2. Scaffold fabrication

A CG slurry was produced by mixing type I collagen (1.8 g) isolated from bovine tendon (Integra, New Jersey, USA) in 300 mL aqueous 0.5 M glacial acetic acid solution, followed by the dropwise addition of 0.32 g of the glycosaminoglycan chondroitin-6-sulphate sodium salt (Sigma–Aldrich, Ireland) dissolved in 60 mL of 0.5 M aqueous acetic acid solution [4]. The slurry was then degassed for a few hours. For the composites, three different types of bioactive glass were investigated: (1) cobalt-free with an average particle diameter of 38 μm; (2) cobalt bioactive glass with an average particle diameter of 38 μm; and (3) cobalt bioactive glass with an average particle diameter of 100 μm. The cobalt bioactive glasses had a concentration of 4 mol% cobalt. The bioactive glass was suspended in distilled water at a concentration of 0.14 g/mL. Various volumes (0.5, 0.2 and 0.1 mL) of the bioactive glass suspension were added dropwise to 20 mL of the CG slurry. The slurries were homogenised at a low speed to ensure a homogeneous distribution of the bioactive glass, however, even at this low speed, excess air could be incorporated during the addition. At this stage, either the slurry was degassed a second time to remove excess air or the slurry was immediately frozen in a controlled manner.

For the latter process, 2 mL of the slurry was pipetted immediately into each well of a 24-well plate and lyophilised (Advantage EL, VirTis Co., Gardiner, NY) for 24 h using a final freezing temperature of −40 °C. Initial freezing rates of either 1 °C/min or 4 °C/min were investigated. A range of scaffolds were fabricated by this method with final concentrations of 0, 1.4, 2.8 and 7 mg of bioactive glass per scaffold.

All scaffold variants were sterilised after fabrication using a dehydrothermal treatment for 24 h at 105 °C and then further chemically crosslinked using 14 mM *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride and 5.5 mM *N*-hydroxysuccinimide (Sigma–Aldrich, Ireland) in distilled water for 2 h followed by 2 × 30 min rinses in phosphate buffered saline (PBS) [28].

2.3. Physical characterisation of bioactive glass/CG scaffolds

2.3.1. Release of cobalt from scaffolds

Ion chromatography plasma mass spectrometry (ICP-MS) was employed. Scaffolds were incubated in 5 mL TRIS buffer that was completely replaced at 24 h and then again at 7 days. TRIS buffer collected at the 24 h and 7 day timepoints was frozen at −80 °C until analysis. ICP-MS was performed on a Varian 8200 machine. All samples were run in triplicate.

2.3.2. Effect of bioactive glass incorporation on scaffold mechanical properties

Compressive modulus of the scaffolds was determined using a Z050 mechanical testing machine (Z050, Zwick/Reoll) fitted with a 5-N load cell. Unconfined, wet compression testing was performed on 9-mm-diameter scaffolds with a thickness of 6–7 mm that were immersed in PBS and tested at a rate of 10% strain per min. The modulus was calculated from the slope of the stress–strain curve over the range 2–5% strain ($n = 4$).

2.3.3. Effect of bioactive glass incorporation on scaffold porosity

Scaffolds were embedded in JB4 glycomethacrylate resin according to manufacturer's instructions (Polysciences, Germany). The embedded scaffolds were sectioned at 10 μm thicknesses (Leica RM 2255, Leica, Germany microtome). The sections were mounted on slides and then stained with an aqueous 1 wt% Toludine Blue solution for 5 min. The slides were rinsed in distilled water and left to dry, then were mounted with coverslips using DPX mountant. The sections were imaged using a Nikon microscope (Optimphot2, Nikon, Japan). The pore diameters were determined from the images using MatLab pore topology analyser software as previously described [4].

The porosity of the scaffolds with and without bioactive glass was calculated using the following equation:

$$\%\ \text{porosity} = 100 \times [1 - (\rho_{\text{actual}}/\rho_{\text{theoretical}})]$$

The actual density (ρ_{actual}) of the scaffolds was calculated by dividing the actual mass of the scaffolds by the volume of the scaffolds, which was then divided by the theoretical density ($\rho_{\text{theoretical}}$) of the materials.

2.4. Biological response of bioactive glass/CG scaffolds

2.4.1. Cell culture and seeding

To assess the angiogenic and osteogenic response of the scaffolds, two commonly used cell lines were employed: (1) Human umbilical vein endothelial cells (HUVECs) were cultured to confluence in complete endothelial media (EGM-2, Lonza, UK) in T175 flasks (Sarstedt, Dublin, Ireland) under standard conditions