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Localised controlled release of simvastatin from porous chitosan–gelatin scaffolds engrafted with simvastatin loaded PLGA-microparticles for bone tissue engineering application

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ABSTRACT

Localised controlled release of simvastatin from porous freeze-dried chitosan–gelatin (CH–G) scaffolds was investigated by incorporating simvastatin loaded poly-(DL-lactide-co-glycolide) acid (PLGA) microparticles (MSIMs) into the scaffolds. MSIMs at 10% w/w simvastatin loading were prepared using a single emulsion-solvent evaporation method. The MSIM optimal amount to be incorporated into the scaffolds was selected by analysing the effect of embedding increasing amounts of blank PLGA microparticles (BL-MPs) on the scaffold physical properties and on the in vitro cell viability using a clonal human osteoblastic cell line (hFOB). Increasing the BL-MP content from 0% to 33.3% w/w showed a significant decrease in swelling degree (from 1245 ± 56% to 570 ± 35%). Scaffold pore size and distribution changed significantly as a function of BL-MP loading. Compressive modulus of scaffolds increased with increasing BL-MP amount up to 16.6% w/w (23.0 ± 1.0 kPa). No significant difference in cell viability was observed with increasing BL-MP loading. Based on these results, a content of 16.6% w/w MSIM particles was incorporated successfully in CH–G scaffolds, showing a controlled localised release of simvastatin able to influence the hFOB cell proliferation and the osteoblastic differentiation after 11 days.

1. Introduction

Among the targets of tissue engineering (TE), the repair of bone defects is an important clinical need as impaired fracture healing or non-union bone defects often result in a functional disability for which there is no effective therapy [1]. Recent TE strategies are based on three primary components: a scaffold, cells and biomolecules [2]. Several researchers have attempted to stimulate bone regeneration by the release of biomolecules, such as growth factors and/or hormones. Application of growth factors, including bone morphogenetic protein-2 (BMP-2), has been reported to stimulate bone formation. However, such growth factors are expensive and when exposed to a physiological environment, they can degrade rapidly. Furthermore, supra-physiological amounts may cause immune responses [3,4]. Alternatively, statins, which are synthetic cholesterol lowering agents, have been used to improve bone health, since they influence bone metabolism by various mechanisms [5]. Simvastatin is reported to inhibit mevalonate, farnesyl pyrophosphate, and geranyl-pyrophosphate pathways and to be responsible for increased bone mineral density by reducing osteoclast activity [6]. Sakoda et al. have reported that simvastatin enhances alkaline phosphatase activity (ALP) and mineralisation, as well as it increases the expression of bone sialoprotein, osteocalcin and type I collagen [7].

Furthermore, Yamashita et al. have demonstrated that simvastatin inhibits BMP-induced osteoclast differentiation by regulating the RANKL/BMP-to-MAPK/AKT/Src pathway, suggesting a potential role of simvastatin in improving bone damage related to excessive bone resorption [8]. However, systemic administration of simvastatin was reported to exhibit negligible results on bone formation [9,10]. The bone regeneration promoting function of statins administered systemically are minimized due to clearance by the liver and administration of higher systemic doses is associated with significant side effects [6].

Local delivery of simvastatin at the desired site of action has been shown to have positive effects on bone formation not only in vitro but also in vivo. For example, Wong et al. developed statin/collagen matrix grafts implanted into rabbit calvaria and showed that the delivery of statins caused expression of BMP-2 and resulted in an increased new

Abbreviations: BL-MP, blank PLGA microparticle; CH–G, chitosan–gelatin blend; GP, genipin; MSIM, simvastatin loaded PLGA microparticle; PLGA, poly-DL-lactide-co-glycolide.

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bone formation with respect to using collagen matrix control grafts [11–13]. The drug release rate and, consequently, the rate of tissue ingrowth in such systems have been shown to be influenced by properties of the scaffold, such as pore size or crosslinking density, polymer composition, hydrophobicity, crystallinity, and degradability [14,15]. Yazawa et al. reported an optimum dose of simvastatin at 10^{-7} to 10^{-8} M for maximum cell proliferation using periodontal ligament cells [16].

Chitosan, a partially deacetylated derivative of chitin, is a fibre-like substance consisting of glucosamine and N-acetylglucosamine and is soluble in dilute acids. It is structurally similar to glycosaminoglycans (GAG) which are important structural elements of the extracellular matrix of many tissues. Chitosan has free amine groups and therefore possesses positive ionic charges, which facilitate its chemical binding with negatively charged lipids, cholesterol, metal ions, proteins and macromolecules such as glycosaminoglycans and proteoglycans. This ability of chitosan for modification and combination with other polymers has allowed its use for the development of a wide range of scaffolds for the regeneration of many tissues such as the bone, liver, neural and vascular tissues, cartilage and skin [17].

Gelatin (G) is a hydrolyzed form of collagen obtained by denaturing the triple-helix structure of collagen into single-strand molecules. Gelatin is non-immunogenic compared to collagen; it retains its RGD sequence and is completely resorbable in vivo [18,19]. Gelatin has been widely utilized for the fabrication of scaffolds generally in combination with other scaffold forming materials, as on its own it possesses low mechanical properties. It possesses both acidic and basic functional groups, which allow its chemical modification with other scaffold forming materials such as chitosan. In this study we prepared scaffolds using CH–G blends crosslinked with genipin (GP), an aglycone derived from geniposide, to increase its mechanical properties and water stability [20].

Different systems, loaded directly with simvastatin, were reported in literature [21–23], mainly based on synthetic polymers. These systems are characterised by a constant rate of drug release from polymeric substrates which may undergo bulk degradation, modifying dramatically the physico-chemical properties of the device, mainly in terms of mechanical properties. Therefore, the purpose of this research was to propose a localised and controlled delivery system in order to: (1) reduce the released simvastatin amount, preventing systemic side effects, and (2) preserve the physico-chemical properties of the scaffolds without incorporating the drug into the bulk of the material. Previously, using poly-(DL-lactide-co-glycolide) acid (PLGA), an FDA approved biodegradable and biocompatible polymer [24], we formulated simvastatin containing PLGA microparticles, which released simvastatin at a slow and controlled rate and observed that these microparticles were able to induce bio-mineralisation in vitro in clonal human osteoblast (hFOB) cells [25]. In addition, we showed that the incorporation of PLGA nanoparticles within CH–G scaffolds resulted in scaffolds with enhanced mechanical properties, without affecting its cell attachment properties [26].

In consideration of the background knowledge described so far, we describe in this paper the realisation of porous scaffold(s) of chitosan–gelatin, engrafted with simvastatin containing PLGA microparticles, which deliver a localised and controlled release of simvastatin. The amount of PLGA microparticles to be engrafted within the porous scaffolds for the localised and controlled release of simvastatin was first optimised by studying the effect of increasing amounts of blank PLGA microparticles (BL-MPs) on the physical (scaffold micro-architecture, swelling degree, mechanical compressive modulus, in vitro dissolution) and biological (cell viability and cell proliferation) properties of the CH–G scaffolds. Porous CH–G scaffolds were subsequently engrafted with the optimised amount of simvastatin loaded PLGA microparticles and were characterised for simvastatin release properties. The ability of the released simvastatin to enhance cell proliferation and in vitro bio-mineralisation using hFOB cells was examined.

2. Materials

Chitosan, gelatin, poly (vinyl alcohol) (Mw: 30–70 kD, >87–90% hydrolysed; PVA) and trehalose were purchased from Sigma-Aldrich. Poly (DL-lactide-co-glycolide) (LA:GA 50:50) (RG 504 H, Mw 48.3 kD) was obtained from Boehringer Ingelheim Pharma GmbH & Co. KG (Ingelheim, Germany). Genipin was acquired from Challenge Bioproducts Co., Taiwan. All reagents and solvents used were of HPLC grade or analytical grade.

3. Methods

3.1. Preparation and characterisation of simvastatin loaded PLGA microparticles (MSIMs)

PLGA microparticles were prepared by the modified single emulsion-solvent diffusion method as previously described by Nandagiri et al. [25]. Briefly, PLGA solution in ethyl acetate was prepared and Simvastatin at 10% w/w loading of PLGA was added. The resulting organic phase was added slowly to 1% (w/v) aqueous PVA solution and homogenized at 13,500 rpm. The resulting emulsion was then transferred into 25 ml of water and stirred to complete solvent evaporation. Simvastatin-loaded PLGA microparticles (MSIMs) were recovered by centrifugation, washed with water to remove residual PVA, re-suspended in distilled H_2O (dH_2O) and then lyophilized (Freezone 6, Labconco, MO: −57 °C, 0.03 mbar, 24 h). Blank PLGA microparticles (BL-MPs) were prepared following the same procedure but without simvastatin addition to the PLGA solution [25].

Microparticle median size was determined by laser diffraction using a Malvern Master Sizer Sirocco 2000 (Malvern Instrument Ltd., Worcestershire, UK). Lyophilized microparticles (<5 mg) were re-suspended in 1 ml of dH_2O and analysed in triplicate. The surface morphology of microparticles was studied by scanning electron microscopy (SEM) (LEO 1450 VP, Leo Electron microscopy Ltd., Cambridge, UK) as previously described [25,26]. The drug content in 1 mg of simvastatin loaded PLGA microparticles was determined by using a suitably modified HPLC method as described by Krishna et al. [27] and Nandagiri et al. [25]. Microparticles (1 mg) were weighed and dissolved in ethyl acetate (1 ml) by vortexing. After agitation, the suspension was centrifuged at 10,000 rpm for 15 min and the supernatant collected and analysed for simvastatin content by HPLC. HPLC analysis was performed with an Agilent Series 1120 HPLC (Agilent Technologies, Wilmington, DE). The chromatographic separations were performed using Phenomenex® 100 C18, 5 μm, 250 mm × 4.6 mm column, at 45 °C, eluted with mobile phase at a flow rate of 1.0 ml/min. The mobile phase consisted of acetonitrile (Sigma-Aldrich) and 50 mM potassium di-hydrogen phosphate buffer (Sigma-Aldrich) (65:35, v/v) adjusted to a pH of 4.5 ± 0.1 by addition of phosphoric acid (Sigma-Aldrich) solution. Each sample was filtered through a 0.22 μm poly(tetrafluoroethylene) (PTFE) membrane filter and analysed in triplicates and the results were calculated as an average of n = 3 measurements ± standard deviation (SD).

Simvastatin loading and encapsulation efficiency of the microparticles were calculated using Eqs. (1) and (2), respectively:

$$\%\,\text{Drug loading} = (\text{Amount of drug}/\text{Amount of microparticles}) \times 100 \quad (1)$$

$$\text{Encapsulation efficiency} = (\text{Drug loading}/\text{Theoretical drug loading}) \times 100. \quad (2)$$

In vitro simvastatin release from PLGA microparticles was determined, as previously described [25,26]. Lyophilized MSIMs (10 mg) were dispersed in phosphate buffered saline (1 ml, pH 7.4) (PBS, Sigma-Aldrich) containing 0.02% w/v sodium azide (Sigma-Aldrich) as