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A combinatorial approach towards achieving an injectable, self-contained, phosphate-releasing scaffold for promoting biomineralization in critical size bone defects

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ABSTRACT

An injectable, guanosine 5′-diphosphate (GDP)-crosslinked chitosan sponge was investigated as a drug delivery system (DDS) for accelerating biomineralization in critical size bone defects (CSBDs). Two approaches were examined both individually, and in combination, in order to achieve this goal. The first approach involved the encapsulation and release of Bone Morphogenetic Protein 7 (BMP-7), a powerful mineralization stimulant. Results confirmed that the rapid gelation of the chitosan sponge prompted high encapsulation of BMP-7 and provided a controlled release over a period of 30 days with no burst release. The second approach was aimed at encapsulating pyrophosphatase (PPtase) in the chitosan sponge to cleave pyrophosphate (PPi) – a mineralization inhibitor and a degradation by-product of the chitosan sponge – into phosphate ions (Pi). PPtase was successfully encapsulated in the chitosan sponge and was able to completely eliminate PPi from the media by cleaving them to Pi. Chitosan sponges releasing Pi into the media were shown to increase overall biomineralization fourfold as compared to controls, an amount equivalent to biomineralization caused by direct injection of 1 μg of free BMP-7 to the cells. Even though the combined encapsulation of 1 μg BMP-7 and PPtase in the sponges did not demonstrate an additional increase in biomineralization, encapsulation of low concentrations of BMP-7 can promote mesenchymal stem cell migration into the sponge after application in vivo. The findings suggest that the sponge-PPtase system likely allows excellent bone regeneration with lower concentrations of BMP-7, reducing risks and expense of the treatment.

Statement of significance

There are bone defects, known as critical size defects, which do not heal on their own and require a therapeutic intervention. The current commercially-available therapies use large quantities of growth factors, such as Bone Morphogenetic Proteins (BMPs), which makes them expensive and a source for a myriad of unwanted side effects. In this manuscript we demonstrate, for the first time, the use of an injectable chitosan-based sponge that contains no inorganic components, but can nonetheless act as a source of phosphate ions to improve bone mineralization. We also demonstrate that this sponge can entrap small concentrations of BMP-7 and provide controlled release over time. The ability to release phosphate ions and low concentrations of BMP-7 makes this therapeutic intervention clinically-relevant, affordable, and safe.

1. Introduction

Critical size bone defects (CSBD) are non-healing injuries involving the loss of large segments of bone that cannot be regenerated spontaneously by the body, and therefore require a therapeutic intervention [1]. Grafting autologous bone is the most

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commonly used method to stimulate and accelerate bone growth in such cases. However, invasive surgeries are needed to both harvest and implant these grafts, which increases risk of post-surgical infection and prolongs hospitalization time. Therefore, there remains a need to design viable therapeutic alternatives to enhance bone regeneration [1,2]. Injectable scaffolds are an emerging class of biomaterials that solidify into three-dimensional (3D) substrates after application in vivo, thus eliminating the need for invasive surgery to implant the scaffold [3]. In addition to the favorable hydrated environment they provide for cells, the gelation mechanism of these scaffolds allows easy encapsulation of other mineralization stimulants like stem cells, bioceramics and growth factors [4–6].

In this study we investigate a newly-developed rapidly-gelling injectable chitosan sponge as a candidate for bone regeneration in CSBD applications. The gelation of the chitosan sponge occurs readily upon mixing chitosan and guanosine 5′-diphosphate (GDP) solutions due to ionic interactions between the anionic phosphate groups in GDP and the cationic amine groups in chitosan [7]. The sponge is one of the most rapidly-gelling systems currently available (t_{gel} < 1.6 s). Fast gelation allows for the efficient entrapment of growth factors in vivo, in addition to excellent localization post-injection [7]. The sponge is also desirable because when injected it takes the full size and shape of the CSBD, allowing evenly distributed regeneration over all areas of the defect.

Preliminary experiments exploring the effect of chitosan sponges on mineralization showed that the degradation products of the sponge contained pyrophosphate (PPi), a known inhibitor of mineralization. It has been well established in the literature that increasing the ratio of pyrophosphate to phosphate ions (PPi/Pi) significantly reduces mineralization [8,9]. It is suggested that PPi is produced by enzymatic cleavage of GDP into guanosine and PPi by alkaline phosphatase (ALP) [10].

This study examines an approach to overcoming this inhibitory effect, and another to enhancing biomineralization using the chitosan sponges. The first approach is to encapsulate Bone Morphogenetic Protein 7 (BMP-7), an osteogenic factor that is one of the most powerful in inducing mineralization, in the chitosan sponges [11]. BMP-7 can counteract the effects of PPi and can also act as a chemotactic agent to attract more mesenchymal stem cells into the scaffold during bone regeneration in vivo [12]. The sponge's rapid gelation ensures high encapsulation efficiency of BMP-7 in vivo, and decreases unwanted diffusion of BMP-7 to surrounding tissues. Moreover, BMP-7 is expensive, and therefore a controlled release system can reduce the concentration required to induce mineralization.

The second approach involves the encapsulation of the enzyme pyrophosphatase (PPtase) in order to reverse the inhibitory effect of PPi. The schematic diagram below summarizes the expected enzymatic reactions in this method. PPtase delivered gradually from the sponge cleaves each PPi molecule formed from GDP into 2 phosphate ions (Pi), which significantly increases the Pi/PPi ratio and thus improves mineralization.

$$\mathrm{GDP} \xrightarrow{\mathrm{ALP}} \mathrm{PPi} \quad \text{(a)}$$

$$1\,\mathrm{PPi} \xrightarrow{\mathrm{PPtase}} 2\,\mathrm{Pi} \quad \text{(b)}$$

Schematic 1. Rationale behind encapsulating PPtase in the chitosan sponges. (a) GDP released from the chitosan sponges is cleaved by the cellular enzyme ALP into PPi (inhibitor of biomineralization) and guanosine; (b) inorganic PPtase encapsulated in the chitosan sponges will then further cleave the PPi into 2 Pi to eliminate the inhibitory effect of PPi and enhance biomineralization by increasing the supply of Pi.

The enzyme is especially interesting because not only does it eradicate the cause of the inhibition but it converts it to a source of phosphate ions which are known to enhance biomineralization especially when exposed to the cells in small amounts for prolonged times [13–15]. The phosphate ions both act as building blocks for the mineral, hydroxyapatite, and as signaling molecules that upregulate mineralization [15]. Scaffolds that work by gradual supply of phosphate ions to enhance bone regeneration have always been ceramic or had a ceramic component [16–18]. This is the first report to our knowledge of a soft scaffold with no ceramic components, which is able to enhance biomineralization by the gradual delivery of phosphates through the use of protein delivery to enzymatically convert the sponge's biodegradation products to phosphate ions.

2. Materials and methods

2.1. Materials

Recombinant Human BMP-7 and Human BMP-7 Quantikine ELISA Kit were purchased from R&D system; High Molecular Weight Chitosan (Degree of Deacetylation >90%; 3000 cp viscosity) from MP Biomedicals, LLC; guanosine 5′-diphophate (GDP), L-ascorbic acid, Bovine Serum Albumin and β-Glycerophosphate from Sigma Aldrich; Hydrochloric Acid (50% v/v) from LabChem Inc; Protein LoBind tubes (1.5 ml) from Eppendorf; MC-3T3-E1 (sub-clone 14) from ATCC; Minimum Essential Medium (MEM-Alpha), Fetal Bovine Serum, Penicillin–Streptomycin (PenStrep), and a Quant-iT Picogreen dsDNA assay from Invitrogen; Inorganic pyrophosphatase from Baker's Yeast (Saccharomyces *cerevisiae*) from Sigma, PPiLight Inorganic pyrophosphate assay from Lonza, Alizarin Red Staining Solution from EMD Millipore, Millicell cell culture inserts (pore size of 1 μm; PET) from Millipore; alkaline phosphatase assay kit from Abcam; and finally a double-barrel syringe system (M system) from MedMix Systems.

2.2. Methods

2.2.1. Chitosan sponge fabrication, BMP-7 encapsulation and encapsulation efficiency calculation

A chitosan solution was prepared as previously reported [7,19]. Briefly, 60 mg of chitosan was dissolved in 10 ml of 0.06 M HCl solution under magnetic stirring for 30 min. The pH of the solution was adjusted to 6 using a 1 M sodium bicarbonate solution. A GDP solution (100 mg/ml) was prepared by dissolving GDP in distilled water. The chitosan and GDP solutions were sterilized by filtration through 0.22 μm syringe filters under a laminar hood. To prepare sponge containing BMP-7, 1 μg of BMP-7 was dissolved in 100 μl of sterile distilled water and added to a sterile LoBind eppendorf tube containing 1.6 ml of chitosan solution then mixed thoroughly. Immediately after, 0.3 ml of the GDP solution was rapidly injected into the chitosan solution to form the sponge. The eppendorf was closed and inverted repeatedly to ensure complete gelation. The sponge was then removed using tweezers, placed in another LoBind tube and rinsed once with PBS.

The supernatant left from the sponge formation was centrifuged for 1 min to pellet down any sponge debris and the volume of supernatant was measured. ELISA was then used to determine the concentration of the free BMP-7 in the supernatant and the weight of free BMP-7 (W_{free}) was determined. The entrapment efficiency (EE) was calculated using Eq. (1). For comparison, BMP-7 loaded liposomes were fabricated using a previously reported method [20] and were separated from free BMP-7 using size exclusion chromatography. The encapsulation efficiency of the separated liposomes was calculated by their dissolution using 0.1% TritonX and quantifying the encapsulated BMP-7.

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