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Magnesium incorporated chitosan based scaffolds for tissue engineering applications

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ABSTRACT

Chitosan based porous scaffolds are of great interest in biomedical applications especially in tissue engineering because of their excellent biocompatibility *in vivo*, controllable degradation rate and tailorable mechanical properties. This paper presents a study of the fabrication and characterization of bioactive scaffolds made of chitosan (CS), carboxymethyl chitosan (CMC) and magnesium gluconate (MgG). Scaffolds were fabricated by subsequent freezing-induced phase separation and lyophilization of polyelectrolyte complexes of CS, CMC and MgG. The scaffolds possess uniform porosity with highly interconnected pores of 50–250 μm size range. Compressive strengths up to 400 kPa, and elastic moduli up to 5 MPa were obtained. The scaffolds were found to remain intact, retaining their original three-dimensional frameworks while testing in in-vitro conditions. These scaffolds exhibited no cytotoxicity to 3T3 fibroblast and osteoblast cells. These observations demonstrate the efficacy of this new approach to preparing scaffold materials suitable for tissue engineering applications.

1. Introduction

Tissue defects and diseases due to trauma, injuries, infections, degeneration, and congenital deformity are a major human health concern. These problems underscore the need for improved tissue regeneration treatment technologies. There has been some recent progress in organ transplantation and surgical reconstruction. Smaller sized defects are best treated by surgical reconstruction, using the ability of tissue to regenerate and spontaneously heal over time. Defects larger than a critical size require a scaffold, or substrate, to support the cell growth and guide the repair process. The current clinical approach mostly involves the use of autografts (from the patient's own tissue) and allografts (tissue other than the patient's own). Several considerations limit the use of these techniques: significant morbidity-related complications at the tissue donation site in the body, the unavailability of matching donor tissue, risk of disease transmission and immune rejection [1–3]. Tissue engineering is evolving as a third approach to overcome these limitations and to develop viable grafts. With this approach, new tissue can be regenerated using a synthetic scaffold as a substrate to promote cell adhesion and proliferation. The scaffold material is designed to biodegrade in a controlled fashion, leaving the space for newly formed tissues [3–6]. The repair process can further be aided by loading drugs and growth factors into such scaffolds [3].

The material properties ideally required for tissue regeneration scaffold drive the choice of material. A partial listing of these properties is: uniform porosity with macro as well as micro-sized pores, non-toxicity to the host tissue, biodegradation and bioresorption, and sufficient mechanical properties [7]. Macropores are required for cell and blood vessels to grow and migrate [8], whereas micropores play a vital role in cell-cell communication, and nutrient transport and removal of waste products [9,10]. Most current candidate materials fail to satisfy all the requirements, due either to insufficient strength during implantation, or the inability to degrade at same rate as that of new tissue growth. If the tissues

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are not mechanically stressed sufficiently during the growth stage, they will not be able to bear the physiological stresses of post-treatment use. The composites of biodegradable natural polymers and ceramics come closest to fulfilling most of these property requirements. The organic polymer phase enhances the biodegradation needed to provide the space for tissue growth. The dispersed phase provides the required mechanical integrity to the scaffold [11,12].

Chitosan, made of glucosamine and N-acetylglucosamine units linked by one to four glycosidic bonds, has been proven to be biologically renewable, biodegradable, biocompatible, nonantigenic, nontoxic, biofunctional. Also, it also bears the proxy structure of glycosaminoglycan (GAG), a major component that constitutes the tissue extracellular matrix (ECM) [13–15]. Chitosan and some of its complexes have also been studied for use in a number of other biomedical applications, including wound dressings, drug delivery systems, and space-filling implants [16,17]. However, the major drawback of chitosan is its lack of proper mechanical strength for hard tissue engineering applications. Several studies have been conducted to improve its strength by reinforcing it with various ceramic phases like wollastonite, hydroxyapatite and beta tricalcium phosphate (β-TCP) and also by polyblending with other synthetic and natural polymers [18]. In this study, chitosan was combined with its oppositely-charged derivative, carboxymethyl chitosan (CMC) to form a stable matrix phase and magnesium gluconate (MgG) as the dispersed phase. MgG is an organic salt of magnesium that readily dissolves to release Mg^{++} ions. A number of studies have demonstrated that divalent cations such as Mg^{++}, Ca^{++}, and Mn^{++} play a critical role in tissue remodeling and development [19–21]. The extracellular matrix (ECM) of tissue contains certain domains that bind divalent cations such as Ca^{++}, Mg^{++} and Mn^{++}. These ECM-bound cations modify the integrin affinity to their respective ligands [20,22–24]. In a study performed by *Zreiqat* et al., human bone-derived cells grown on bioceramic substrate modified with divalent cations showed higher expression levels of β1-, α5, α5α1-, and α3β1-integrin receptors, compared to Mg^{++} free substrates [20]. The choice of Mg for use in implants is further motivated by magnesium's excellent biocompatibility, degradation into non-toxic products and its proven use as an essential nutrient for human metabolism [25].

A porous and bioactive scaffolds was fabricated by using a blend mixture of CS, CMC and MgG, and subsequent freezing-induced phase separation and lyophilization. Magnesium gluconate was first introduced into aqueous solution of CMC and mixed the resulted solution with CS solution in acidic pH prior to freezing and lyophilization to obtain the composite the scaffolds. Scaffold morphology was analyzed by SEM, water uptake and retention ability by weighing the amount of water absorbed and retained after centrifugation and cell toxicity using 3T3 fibroblast and osteoblast cells. Mechanical properties of scaffolds were evaluated under compression loading. Additionally, a release study was carried out at different time points using UV-VIS spectrophotometry to quantify the amount of Mg^{++} released from the chitosan-CMC-based composite scaffolds.

2. Materials and methods

2.1. Materials

Chitosan powder (Medium Mw, DD 75–85%) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and carboxymethyl chitosan (CMC) powder (DD 90%) was purchased from Santa Cruz Biotechnology (Dallas, TX, U.S.A.). Magnesium gluconate dihydrate (MgG) was purchased from Pfaltz & Bauer (Waterbury, CT, USA). Glacial acetic acid and sodium hydroxide (NaOH) were purchased from Acros Organics (Morris Plains, NJ, USA). Phosphate buffered saline (PBS) was purchased from Thermo Fisher Scientific (Fair Lawn, NJ, USA). Alamar blue and Xylidyl blue assay kits were obtained from Stanbio Laboratory (Boerne, TX, USA) and Life Technologies (Grand Island, NY, USA) respectively. (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay kit was obtained from Sigma-Aldrich (Oakville, ON, Canada).

2.2. Fabrication of scaffolds

Chitosan solution and CMC solutions were prepared at concentrations of 2, 4 and 5 wt%. Chitosan was dissolved in 2% acetic acid. CMC was dissolved in deionized (DI) water. The two solutions were thoroughly mixed in a 1:1 wt ratio in a container rotating inside a Thinky planetary centrifugal mixer (Planetary Centrifugal Mixer, ARM-310) for 30 min at 2000 rpm (Fig. 1). After thorough mixing, the material was injected via syringe in 48-well cell culture dishes. The cast material was kept at 4 °C for about 30 min, transferred to −20 °C for 4 h, and finally to −80 °C for 12 h. The scaffolds were allowed to lyophilize for about 36 h in the freeze dryer (LabConCo, Kansas City, MO). Excess acetic acid in the dried scaffolds was neutralized by immersing the scaffolds in 0.1 M sodium hydroxide (NaOH) solution for 15 min and then washing thrice with DI water.

As in Table 1, two sets of scaffolds were prepared. For the first set of scaffolds, three concentrations of polymer solutions were used, with no added MgG. For the second set of scaffolds, polymer concentration of CS and CMC was held constant at 5% and the relative amount of MgG was varied. The quantity of MgG added to the scaffolds was 5%, 10%–20% of the total weight of the chitosan and CMC dry powder present in the solution. This was done by dissolving the MgG in 2 ml of DI water and then mixing it with the CMC solution. CMC and MgG were allowed to mix in the Thinky mixer for 15 min at 2000 rpm. CS solution was then added to the CMC − MgG system and allowed to crosslink in the Thinky for 30 min at 2000 rpm. The process of casting, freezing, lyophilization and neutralization was repeated similarly. To measure the required weight (W) of MgG in grams, the following equation was used.

$$W_{MgG} = \frac{\%MgG}{100} * [C_1 * V_1 + C_2 * V_2] \quad (1)$$

where, C_1 and C_2 are the concentrations in wt% while V_1 and V_2 are the volumes (in ml) of CS and CMC solutions, respectively.

2.3. Study of morphology and pore size distribution

The surface morphology of the scaffolds was studied by scanning electron microscopy (SEM) (Hitachi SU8000, Japan). Thin discs of ~1 mm thickness were cut from the scaffolds using surgical scalpel. The samples were mounted on the holder with double-sided carbon tape and sputter-coated with gold using a Polaron SEM coating system (Quorum Technologies, East Sussex, UK) for 2 min at 15 mA. The SEM images were taken at an accelerating voltage of 2 kV and current of 5 μA. Scaffold pore size distributions were evaluated using Image J software (NIH, Gaithersburg, MD) according to previous method on the SEM images [26]. The scale bar length is measured in pixels. Three different images were analyzed for each scaffold composition.

2.4. Water uptake and retention abilities

Water absorption efficiency was determined as follows: initial