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Nano-fibrin stabilized $CaSO_4$ crystals incorporated injectable chitin composite hydrogel for enhanced angiogenesis & osteogenesis

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ABSTRACT

Calcium sulfate ($CaSO_4$), an excellent biodegradable bone forming agent that is an ideal choice as additive in gels, however, its disadvantage being poor gel rheology and angiogenesis. Here, we have synthesized chitin–$CaSO_4$–nano-fibrin based injectable gel system which shows improved rheology and angiogenic potential. Rheological studies showed that the composite gel was a shear thinning gel with elastic modulus of 15.4 ± 0.275 kPa; a 1.67 fold increase over chitin control. SEM and XRD analyses revealed the effect of nano-fibrin (nFibrin) in transforming $CaSO_4$ crystal shape from needle to hexagonal. It also masked the retarding effect of $CaSO_4$ towards *in vitro* early cell attachment and angiogenesis using rabbit adipose derived mesenchymal stem cells (rASCs) and HUVECs, respectively. rASCs osteogenesis was confirmed by spectrophotometric endpoint assay, which showed 6-fold early increase in alkaline phosphatase levels and immuno-cytochemistry analysis. These *in vitro* results highlight the potential of injectable chitin–$CaSO_4$–nFibrin gel for osteo-regeneration *via* enhanced angiogenesis.

1. Introduction

Chitin, a bio-degradable biopolymer of *N*-acetyl glucosamine and *N*-glucosamine, has been widely used for the tissue engineering applications, as its monomeric units mimic the ECM (Anitha et al., 2014; Kim et al., 2008). Many studies have showed that, the use of chitin will enhance bio-adhesion, cell migration and thereby lead to improved tissue regeneration (Jayakumar, Menon, Manzoor, Nair, & Tamura, 2010; Jayakumar et al., 2011; Wan & Tai, 2013). Chitin can be easily processed into various forms such as scaffolds, fibers, sponges, injectable hydrogels and microgels (Anitha et al., 2014). This gives us a great versatility in handling and tuning the properties of chitin. However, when chitin is used as a gel, its poor mechanical strength and faster degradation rate than the tissue regeneration rate seems to be a disadvantage for its use in bone tissue engineering (Tomihata & Ikada, 1997).

To improve mechanical strength and to slow down degradation rates, chitin gels can be combined with fillers such as ceramics, minerals or composite materials. Calcium sulfate hemihydrate ($CaSO_4 \cdot 1/2H_2O$) is a commercial bone graft substitute material with a long history of use in various medical applications such as ridge augmentation, bone defect filler, sinus augmentation and guided tissue regeneration (Thomas & Puleo, 2009). Advantages of calcium sulfate as a bone graft material are that it is biocompatible, eliciting minimal inflammatory responses, is osteoconductive, completely degradable and Ca^{2+} ions released during dissolution may promote osteogenic differentiation (Thomas & Puleo, 2009). Degradation properties also make calcium sulfate attractive for local drug delivery (Reddy et al., 2014). The advantages of using $CaSO_4$ in an injectable form are reduction of surgical invasiveness, good moldability, ability to fill deep spaces and ability to adapt to a defect area and thereby promoting better cell migration from the healthy surrounding tissues (Sivashanmugam, Arun Kumar, Vishnu Priya, Nair, & Jayakumar, 2015). However, most of the $CaSO_4$ formulations to date are hard setting types and are brittle in nature after setting (Moore, Graves, & Bain, 2001). Therefore, by combining chitin and calcium sulfate in an injectable gel system, will reduce the brittle nature of pure $CaSO_4$ and would be advantageous for the purpose of regeneration of small and non-load bearing bone defects.

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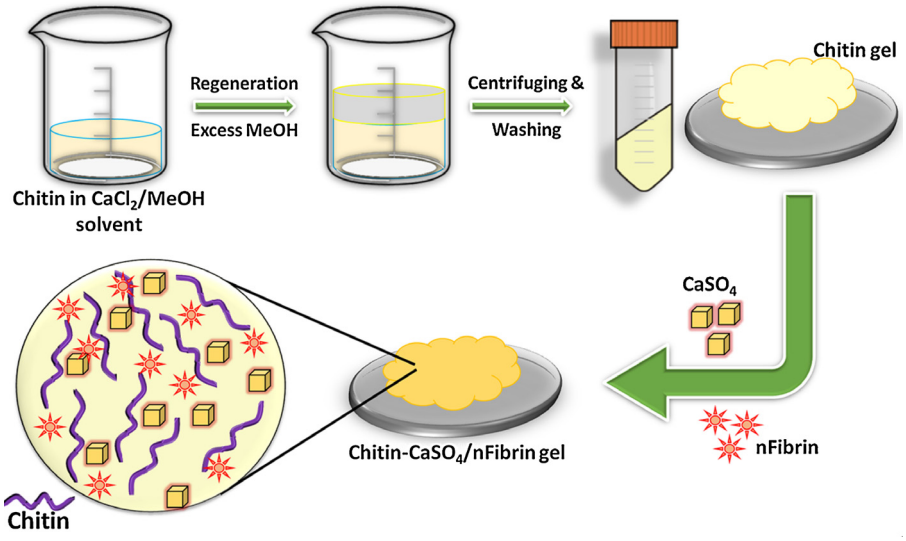

Fig. 1. Schematic illustration showing the preparation of injectable chitin–$CaSO_4$/nano fibrin composite hydrogel.

Further, recent studies have noted the importance of revascularization of sites for effective bone regeneration and that lack of revascularization or avascular necrosis has been reported to be a major failure of bone graft substitutes (Glowacki, 1998). It has been shown in previous studies that, use of fibrin can promote neovascularization and also improves cell adhesion (Yang, Seol, Kim, Cho, & Lee, 2007). However, large quantities of fibrin are needed to function itself as a scaffold, which is not cost effective. Therefore, we intend to limit the requirement of fibrin by using nano-fibrin (nFibrin), but still maintaining the advantages of it in a graft substitute.

In this work, we have designed and synthesized an injectable gel system comprising of chitin, calcium sulfate and nFibrin (chitin–$CaSO_4$–nFibrin), for regeneration of non-load bearing bone tissues. We hypothesize that combining all three materials will yield a scaffold that has collective advantages of improved bio-mimicking, cell migration, mechanical properties and neovascularization.

2. Materials and methods

2.1. Materials

α-Chitin (Molecular weight—100 kDa) was obtained from Koyo Chemical Co., Japan, with degree of acetylation-85%, in powder form. α-Calcium Sulfate hemihydrate was purchased from Fischer Scientific, USA. Calcium Chloride ($CaCl_2$) and Methanol (MeOH) was purchased from Merck Chemicals, U.S.A. Alamar Blue, Trypsin-EDTA, DAPI, Pen-Strep, growth factor reduced Geltrex®, Fetal Bovine Serum (FBS) and Iscove's modified Dulbecco's medium (IMDM) were obtained from Gibco, Invitrogen Corporation. MilliQ water (18.2 MΩ cm) was used wherever needed. Fibrinogen was obtained from Himedia, India. Dulbecco's modified Eagle medium (DMEM) and endothelial cell growth factor [ECGF] were purchased from Sigma Aldrich. All chemicals were used with no further modifications and purification.

2.2. Methods

2.2.1. Preparation of chitin-$CaSO_4$-nFibrin hydrogel

nFibrin was prepared by the method previously reported from our laboratory with modifications (Praveen, Sreerekha, Menon, Nair, & Chennazhi, 2012). Briefly, fibrinogen dissolved in tris-NaCl buffer and thrombin in $CaCl_2$ was injected to 70 °C preheated vegetable oil to form water in oil emulsion and was stirred for 8 h. The resultant nanoparticles were pelleted, washed and lyophilized prior usage. 0.5% (w/v) Chitin solution was prepared by dissolving chitin in saturated $CaCl_2$/MeOH solvent (Tamura, Nagahama, & Tokura, 2006). The chitin gel was regenerated by adding excess of MeOH to the chitin solution. This was centrifuged and washed with MilliQ water. Centrifuging and washing was repeated several times to completely remove the $CaCl_2$ and MeOH. The gel was mixed with MilliQ water and centrifuged again. The yield of chitin gel thus prepared was ~80% calculated based on the formula dry mass of chitin gel obtained/initial mass of chitin powder taken × 100. The final gel was uniformly mixed with required quantities of $CaSO_4$ or nFibrin or $CaSO_4$ and nFibrin (Fig. 1).The weight ratios of chitin: $CaSO_4$: nFibrin was maintained at 20:5:2. For chitin–$CaSO_4$ gel the weight ratio was 20:5 and for chitin–nFibrin gel the weight ratio was 20:2. Further, the physicochemical characterizations and other studies were carried out using chitin gel, chitin–$CaSO_4$ gel, chitin–nFibrin gel and chitin–$CaSO_4$–nFibrin gel.

2.2.2. Physico-chemical characterization of gels

The prepared gels were subjected to scanning electron microscopy (SEM) analysis using JEOL JSM-6490LA Analytical SEM. Briefly, after the gels were prepared, a volume of 500 μL was placed on to the sample stub, allowed to air dry, gold sputtered and then imaged. From the SEM images obtained the aspect ratio of the $CaSO_4$ crystal structure was analyzed using Image J 1.48V (NIH, USA). X-ray diffraction (XRD) spectra were collected in the 2θ range 5° to 60° using a PAN analytical X'Pert PRO X-ray diffractometer. Fourier transformed infrared spectroscopy (FT-IR) analysis was carried out using Shimadzu IRAffinity-1S in attenuated total reflectance (ATR) mode. The air dried samples were directly pressed against the ATR crystal and IR transmitted spectra were obtained from 4000 to 500 cm^{-1}.

2.2.3. Rheological studies

2.2.3.1. Viscoelastic measurements. The rheological properties of the gels were analyzed in Malvern Kinexus pro rheometer. All rheological measurements were conducted using parallel plates with a 500 μm gap between plates. A constant temperature of 25 °C was used for tests to determine modulus and shear stress values.

The amplitude sweep study was analyzed from 10^{-1}% of strain and the instrument was set to detect the end of linear viscoelastic region (LVER) automatically. Then the strain percentage value within the LVER region was used for further rheological