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# Injectable porous nano-hydroxyapatite/chitosan/tripolyphosphate scaffolds with improved compressive strength for bone regeneration

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ABSTRACT

In this study we have fabricated porous injectable spherical scaffolds using chitosan biopolymer, sodium tripolyphosphate (TPP) and nano-hydroxyapatite (nHA). TPP was primarily used as an ionic crosslinker to crosslink nHA/chitosan droplets. We hypothesized that incorporating nHA into chitosan could support osteoconduction by emulating the mineralized cortical bone structure, and improve the Ultimate Compressive Strength (UCS) of the scaffolds. We prepared chitosan solutions with 0.5%, 1% and 2% (w/v) nHA concentration and used simple coacervation and lyophilization techniques to obtain spherical scaffolds. Lyophilized spherical scaffolds had a mean diameter of 1.33 mm ($n = 25$). Further, portion from each group lyophilized scaffolds were soaked and dried to obtain Lyophilized Soaked and Dried (LSD) scaffolds. LSD scaffolds had a mean diameter of 0.93 mm ($n = 25$) which is promising property for the injectability. Scanning Electron Microscopy images showed porous surface morphology and interconnected pore structures inside the scaffolds. Lyophilized and LSD scaffolds had surface pores <10 and 2 μm, respectively. 2% nHA/chitosan LSD scaffolds exhibited UCS of 8.59 MPa compared to UCS of 2% nHA/chitosan lyophilized scaffolds at 3.93 MPa. Standardize UCS values were 79.98 MPa and 357 MPa for 2% nHA/chitosan lyophilized and LSD particles respectively. One-way ANOVA results showed a significant increase ($p < 0.001$) in UCS of 1% and 2% nHA/chitosan lyophilized scaffolds compared to 0% and 0.5% nHA/chitosan lyophilized scaffolds. Moreover, 2% nHA LSD scaffolds had significantly increased ($p < 0.005$) their mean UCS by 120% compared to 2% nHA lyophilized scaffolds. In a drawback, all scaffolds have lost their mechanical properties by 95% on the 2nd day when fully immersed in phosphate buffered saline. Additionally live and dead cell assay showed no cytotoxicity and excellent osteoblast attachment to both lyophilized and LSD scaffolds at the end of 14th day of *in vitro* studies. 2% nHA/chitosan scaffolds showed higher osteoblast attachment than 0% nHA/chitosan scaffolds.



## 1. Introduction

Recent advances in tissue engineering have started to focus on using injectable scaffold as they can be inserted to the defect site with a non-invasive surgery to prevent infection, morbidity, surgical scars and extensive blood loss. Injectable scaffolds must possess mechanical strength similar to surrounding bone to provide structural stability at the defect site and support mechanotransduction [1,2]. Promising injectable scaffolds should be embodied with growth factors and they must be released in a controlled manner to promote cell attachment, proliferation and differentiation [3,4]. Different types of injectable scaffolds such as microparticles, hydrogel, nano-composite films and nanoparticles have been developed using biomaterials such as natural and synthetic polymers, biocomposites and bioceramics [3–5]. Among many biomaterials, chitosan and hydroxyapatite have been widely investigated for injectable scaffolds fabrication [6,7].

Chitosan (CS) is a semi crystalline polysaccharide derived from chitin which is the second most abundant natural biopolymer commonly found in invertebrates [8–10]. Chitosan has been gaining increase importance in tissue engineering, wound healing and drug delivery as degradation products of chitosan are nontoxic, nonimmunogenic, noncarcinogenic, biocompatible, and biodegradable [9,11–15]. Polysaccharide backbone of chitosan has gained increasing attention in bone and cartilage regeneration because of its structural similarity to glycosaminoglycan, which is a major component of bone extracellular matrix [16]. Chitosan has an amine group which can be cross-linked with anionic nontoxic sodium tripolyphosphate (TPP) to obtain rigid chitosan scaffolds with higher mechanical strength and control release of encapsulated growth factors [17–19].

The mechanical strength of scaffolds is a significant property required in bone tissue engineering. To obtain higher mechanically rigid scaffolds, chitosan can be incorporated with synthetic and natural polymers, ceramics and composite materials [20–23]. Bioceramics such as

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hydroxyapatite (HA) and its powdered subgroup β-tricalcium phosphate have proven to improve the biocompatibility, mimic the inorganic component of the bone and improving the mechanical strength of scaffolds [24–26].

Hydroxyapatite is one of the major materials that incorporates with chitosan because it has shown to increase the mechanical properties of chitosan scaffolds and mimic the role of main inorganic component in calcified bone tissue [7,26]. HA-chitosan scaffolds have shown good cytocompatibilty with osteoblasts which is an indication for HA to be used in bone tissue engineering applications [27,28]. Additionally, substantial research has shown that presence of HA in bone scaffolds could improve the differentiation of pre-osteoblasts, human mesenchymal stem cells and vascular smooth muscle cells to osteoblasts in the presence of osteogenic factors [29–31].

Mechanical properties of bone scaffolds are important for bones to support load bearing applications and transmit mechanical force between the parts of body. Bone scaffolds should possess similar compressive strength to the host bone and provide support until complete regeneration and functional restoration of the damaged host bone tissue. Number of studies have incorporated synthetic materials or biopolymers with HA because HA alone is a brittle material with low fracture toughness [32,33]. Researchers have also conducted studies using HA as an additional constituent in scaffolds to strengthen natural polymers [7,34].

Although different shapes of scaffolds have been fabricated for bone tissue engineering applications, standards for testing mechanical properties have not been established for most of the shapes of scaffolds. Commonly used standard for mechanical testing of scaffold is compression testing of standard cylindrical shape scaffold under the American Society for Testing Materials (ASTM) [35,36]. It has been a problem to test mechanical properties of non-cylindrical scaffolds due to the absence of standard procedure. As a solution, researchers have prepared cylindrical scaffolds or films by aggregating primary scaffold materials or using the same compositions of the scaffolds [35,37,38]. However, these methods do not present the exact mechanical properties of original scaffold because of the binding methods of scaffolds and the area exposed to the force on scaffolds. In this study, we have developed a new comparative method to assess scaffolds' Ultimate Compressive Strength (UCS) using an unconfined uniaxial compression test.

In this study we have fabricated injectable scaffolds using chitosan biopolymer and nano-hydroxyapatite using coacervation and lyophilization method. We hypothesized that incorporation of nHA could improve the UCS of the composite scaffolds. Mechanical properties are evaluated using a new comparative assessment method. Additionally, scaffolds were characterized for its morphology, physiochemical properties, cell cytotoxicity and cell attachment.

## 2. Experimental procedures

### 2.1. Materials

Medium molecular weight chitosan (85% deacetylation), hydroxyapatite nanopowder (nHA, <200 nm particle size), sodium tripolyphosphate (TPP, technical grade, 85%) and acetic acid (A.C.S reagent, 99.7%) were purchased from Sigma-Aldrich (USA). Murine Osteoblast (OB-6 cell line) was used for this study. Alpha minimum essential medium (α-MEM), fetal bovine serum (FBS), penicillin–streptomycin (Pen Strep) and 0.25% trypsin-EDTA phenol red were purchased from Gibco.

### 2.2. Fabrication of scaffolds

Spherical scaffolds were fabricated by coacervation followed by lyophilization. 2% (w/v) chitosan solution was prepared by dissolving chitosan into 1% (v/v) acetic acid. Then 0.5%, 1% and 2% (w/v) nHA/chitosan solutions were prepared by mixing nano-hydroxyapatite with 2% (w/v) chitosan solution and vigorously stirring using a magnetic stirrer. Further, nHA nano powder dispersed in the chitosan solution by sonication. Sonicated nHA/chitosan suspensions were filtered by 50 μm nylon mesh to remove nHA agglomerates thus to avoid clogging in the needle. Then suspensions were dripped into 27.18 mM TPP/deionized water solutions using 30 gauge needles and stirred at 600 rpm. After 30 min, TPP crosslinked nHA/chitosan beads were filtered out from the TPP/deionized water solutions and lyophilized at −52 °C temperature and 0.02 mbar pressure for 24 h. Portion of lyophilized scaffolds from each sample were soaked in 300 ml distilled water and stirred at 300 rpm for an hour. Then scaffolds were separated and dried inside the chemical hood for 24 h. Particles were named as lyophilized soaked and dried (LSD) scaffolds.

### 2.3. Surface morphology of scaffolds

The surface morphology was examined Scanning Electron Microscopy (SEM) images taken by FEI Quanta 3D Field Emission Gun (FEG) Dual Beam Electron Microscope. Scaffolds were mounted on the aluminum stubs using double coated, carbon conductive tapes and were sputter coated for 30 s using a gold target. SEM images of each particle were taken at low magnification (80×) and high magnification (3500×). Three scaffolds from each group were examined.

### 2.4. Physiochemical properties of scaffolds

Scaffolds were grinded using mortar and pestle. Fourier Transform infrared (FTIR) spectrums of each finely grinded sample was obtained by micro Attenuated Total Reflectance (micro-ATR) method. Varian UMA 600 microscope was used with Ge crystal and scanning were performed in the wave number range 4000–700 $cm^{-1}$. Spectrums were analyzed using Bio-Rad spectral library.

X-ray diffraction was carried out to determine crystal phases of the grinded particle samples. XPERT PRO diffractometer system was used with Cu-Kα radiation generated at 40 mA and 45 kV. The samples were scanned from 20 to 70° 2θ angle with 2θ step size of 0.033° and 40 s step scan time.

### 2.5. Mechanical properties of scaffolds

Spherical scaffolds were compressed using ADMET eXpert 2600 series Universal testing machine. Five spherical scaffolds from same sample were placed on the flat smooth steel bottom fixture. Then the flat stainless steel crosshead with Interface SM-250 load cell was moved at a rate of 0.05 mm/s to compress scaffolds. Force on the load transducer and crosshead position data were generated by the ADMET's MTESTQuattro software. The acquired data were exported to Microsoft Excel to generate Compressive Stress-Strain graph using following calculation method. Compression testing was done for scaffolds in dry state and wet state. For wet state compression test, scaffolds were immersed in phosphate buffered saline (PBS) for 24 h before the compression test.

Variables r, $R_e$ and x are defined in the Fig. 1. $L$ is the force recorded by MTESTQuattro machine. At initial crosshead contact with scaffolds, $x = 0$ $mm$. Effective surface area ($A_e$) is defined as the area of the particle in touch with the crosshead. We assumed that the horizontal deformation of the scaffolds due to crosshead movement is minimal thus the radius of the particle remains constant.

$$R_e = \sqrt{\left[r^2 - \left(r - \frac{x}{2}\right)^2\right]}$$

$$A_e = \pi \times (R_e)^2$$

$$Stress = L/A_e$$