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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 less than 10 and 2 μ m, 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.

Graphical abstract

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Keywords

Chitosan; nano-hydroxyapatite; spherical scaffold; compressive strength; osteoblast attachment; mechanical testing

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 noninvasive 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 nano-particles 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 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 cytocompatibility 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 μ m 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 x) and high magnification (3500 x). 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 $4000\text{--}700\text{ cm}^{-1}$. 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-K α radiation generated at 40 mA and 45 kV. The samples were scanned from $20\text{--}70^{\circ}$ 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 Figure 1. L is the force recorded by MTESTQuattro machine. At initial crosshead contact with scaffolds, $x = 0 \text{ mm}$. 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.

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

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

$$\text{Stress} = L/A_e$$

$$\text{Strain} = \frac{x}{2r}$$

In order to compare UCS of our spherical scaffolds with cylindrical scaffolds according to ASTM-C39-05 criteria, we assumed that force applied on cross section area of a cylindrical scaffold is approximately equals to force applied on a one layer of spherical scaffolds arranged in circular area similar to the cross section cylindrical scaffold. Then we calculated number of scaffolds that can be arranged in the specified cross sectional area and multiply by mean UCS of one scaffold since we have observed that number of scaffolds compressed is proportional to the load.

2.6. Cell viability, attachment and proliferation

OB-6 line cell vial was plated on a petri dish (100 mm) with α -MEM supplemented with 9% FBS and 1% Pen Strep. The petri dish was incubated at 37°C in a humidified 5% CO₂/95% air atmosphere in an osteogenic medium. Cells were monitored and the medium was changed every 2–3 days. When the dishes reached 80% confluence, the attached osteoblasts were harvested as follows: Cells were washed with PBS solution, added 1 ml of 0.05% trypsin-EDTA and incubated the petri dish for 10 min, pipetted with α -MEM to extract many cells as possible. After 10 μ l of pipetted sample was added on the hemocytometer and number of cells were counted. Sample volume to obtain 20000 cells was calculated.

Chitosan/nHA scaffolds were sterilized under UV light inside a bio hood for overnight. Scaffolds were added into a 10 ml of alpha-MEM medium in 50 ml beaker and stirred for 12 h. Six settled scaffolds from each beaker were added into a well in 24 well plate. Scaffolds in each well in 24 well-plate were seed with 20000 cells. Medium was changed every 3 days and scaffolds in the seeded well were transferred to new well on the 5th day after washing them with PBS. Live/dead cell assay was conducted on day 3, 6, 10 and 14. Before the live/dead cell assay, scaffolds were washed with PBS and transferred to a new well in 24-well plate and wash the scaffolds again using PBS. Then live/dead cell assay was performed and observed using Total Internal Reflection Fluorescence (TIRF) microscope (Olympus).

2.7. Statistical Analysis

For mechanical testing, five batches were prepared for each composition and minimum of 5 compression tests were carried for each batch to make minimum total of 25 compression tests for each composition of scaffolds. Each compression test was conducted on 5 scaffolds and mean compressive strength per scaffold was calculated. Data were compared using one-way analysis of variance (ANOVA) with 95% confidence interval. IBM SPSS Statistics version 21 software was used.

3. Results

3.1. Morphology of scaffolds

After lyophilization, scaffolds were slightly attached to each other in glass vials and could be separated by stirring slowly using a metal rod without physically harming the scaffolds. Visually, scaffolds were stiff, white, spherical and rough structures on the surface could be observed. Scaffolds can be picked and hold with forceps without altering scaffolds shape but higher force could break the scaffolds or alter their shape. However, soaked scaffolds were spongy at higher force but did not break. SEM images shows that scaffolds' shape has distorted. According to SEM images and caliper measurements of lyophilized and LSD scaffolds had mean diameter of 1.33 (n=25, SD = 0.058) and 0.93 (n=25, SD = 0.083) mm respectively. The porous surface structures can be clearly seen on the SEM images, Figure 2. Further (Figure 2.E, F) show rough texture compared to Figure 2.D because of the presence of nHA on the particle surface. Pore sizes of (Figure 2.D, E) images are range from 2-10 μm while Figure 2.F have pores < 2 μm . Number of SEM images on scaffolds implied that nHA composition of the scaffolds has not influenced in the shape or the pore sizes of the scaffolds but rough surface.

3.2. Physiochemical properties

The FTIR spectrums of chitosan bulk, nHA bulk and, nHA/chitosan composite scaffolds are presented in Figure 3. The nHA FTIR spectrum shows characteristic bands corresponding to triply degenerated asymmetric stretching mode of the P-O bonds of ν_{3a} and ν_{3b} at 1086 cm^{-1} and 1020 cm^{-1} respectively. The nondegenerated symmetric stretching mode of the P-O bonds are present at 962 cm^{-1} . The chitosan FTIR spectrum exhibits: N-H symmetric stretching vibration peak at 3360 cm^{-1} and 3287 cm^{-1} ; amide I (-C=O-) stretching vibration peaks at 1653 cm^{-1} ; amine bending vibration at 1589 cm^{-1} ; CH_3 bending deformation peaks at 1420 cm^{-1} , 1375 cm^{-1} and 1315 cm^{-1} ; and 1150 cm^{-1} , 1060 cm^{-1} and

1028 cm^{-1} peaks corresponds to stretching vibration of C-O-C bond [7]. The spectrum of nHA/chitosan composite scaffold exhibits characteristic peaks typically present in chitosan. Effect of nHA characteristic bands on the composites cannot be distinguished clearly on nHA/chitosan composite spectra. However, with the increment of nHA percentage in composite particle; the C-O stretching vibration absorption band which appeared at 1060 cm^{-1} and 1067 cm^{-1} on bulk chitosan and 0% nHA/chitosan spectra has disappeared, and chitosan characteristic stretching vibration of N-H has broadened. Although one peak corresponding to C-O band has disappeared, 1028 cm^{-1} peak remains strongly on spectra as it overlaps with peak at 1020 cm^{-1} corresponding to P-O bond in nHA bulk spectrum. This also indicates the presence of nHA in the nHA/chitosan composite particle. The stretching vibrations of amide I and amine bond of the scaffolds have switched from 1653 cm^{-1} to 1645 cm^{-1} and 1589 cm^{-1} and 1558 cm^{-1} respectively [7].

XRD pattern of nHA/chitosan composites shows the nHA crystalline nature remains in the scaffolds as observed by the all peaks in nHA bulk (Figure 4-A) are present in the all composite spectra (Figure 4-B,C,D). Major peaks present at 25.93°, 31.84°, 32.24°, 32.95°, 46.75° and 49.52° correspond to reflections of 002, 211, 112, 300, 222 and 213 Miller indices respectively [39]. XRD patterns also show increasing amount of nHA percentage on composites also increases the intensity of peaks present in the spectra of nHA/chitosan composites. Chitosan is characterized by the peak present at 20° and this peak is present in nHA/chitosan composites as presented in Figure 4.B, C and D. The increase in the nHA resulted in the decrease in the characterized peak of chitosan.

3.3. Mechanical Properties

Mechanical testing of the lyophilized scaffolds showed increase of nHA percentage could increase the UCS (Figure 5). 2% nHA/chitosan scaffolds exhibited highest UCS of 3.93 (n = 25, SD = 0.7) MPa while 0% nHA/chitosan scaffolds had only 2.8 (n = 25, SD = 0.28) MPa. 1% and 2% nHA/chitosan scaffolds have significantly improved ($p < 0.001$) their UCS compared to 0% nHA/chitosan scaffolds. 0.5% nHA/chitosan scaffolds haven't increased their mechanical strength significantly. 1% and 2% nHA/chitosan scaffolds increased ($p < 0.001$) their UCS by 23% and 40% respectively. Approximate standardized UCS was calculated to be 79.98 MPa for 2% nHA/chitosan scaffolds.

2% nHA/chitosan scaffolds showed loss of mean UCS by 95% ($p < 0.001$) in wet state condition (0.61 MPa) compared to dry state (3.93 MPa) after immersing in PBS for 24 h. Loss of mean UCS even greater for scaffolds immersed for more than 24 h. Mean UCS of LSD scaffolds in dry state were 220% higher ($p < 0.001$) compared to lyophilized scaffolds in dry state. Mean UCS for 2% nHA/chitosan LSD scaffold was measured to be 8.59 (n = 25, SD = 0.77) MPa and standardized UCS was calculated to be 357.54 MPa.

3.4. Cell cytotoxicity

Ethidium homodimer-1 (EthD-1) staining and fluorescence microscopy were used to observe the cell viability on scaffolds. EthD-1 is a high affinity fluorescent nucleic acid stain that binds to DNA and RNA of cells with disintegrated membranes. Few dead cells can be observed on 2% nHA/Chitosan scaffolds on the 14th day (Figure 7.D). This few number

of dead cells are comparatively very low to the large number of proliferated live cells on the same particle (Figure 8.D).

3.5. Cell attachment

OB-6 cells attachment and proliferation on both 0% and 2% nHA/chitosan scaffolds were analyzed using Calcein AM staining and fluorescence microscope. Calcein AM permeates into intact live cells and intracellular esterase hydrolyze Calcein AM to Calcein, strongly fluorescent compound. Scaffolds were washed carefully with PBS and moved to a different well on 24 well plate before staining with Calcein AM to make sure only OB-6 cells attached to the scaffolds are stained and analyzed. Results showed that qualitatively higher cell proliferation can be observed on 2% nHA/chitosan on 3rd day. By the end of 14th day 2% nHA/chitosan scaffolds are full confluent with osteoblasts thus images are blurred due to high cell density and expulsions from the scaffold. Osteoblast proliferation on 0% nHA/chitosan scaffold has increased on 14th day compared to 3rd day of the study.

4. Discussion

In this study we were able to fabricate porous spherical scaffolds using chitosan biopolymer and nHA using a simple fabrication technique. We have adopted the fabrication procedure used by our group in previous studies [6, 10, 37, 40, 41]. The objective of this study was to obtain porous spherical scaffolds using chitosan, increase the mechanical strength of the scaffolds using nHA and characterized the scaffolds for its morphological, mechanical, physiochemical, cell attachment and cell cytotoxicity properties.

We fabricated scaffolds using solutions having 0-2% (w/v) nHA in 2% (w/v) chitosan. The viscosity of the chitosan solution was increased as we increase the nHA percentage. This lead to high friction in the syringe causing difficult to drip the solution. We also noticed that when nHA concentration increases, frequency of clogging the needle is also increased. Therefore 2% nHA was chosen as the highest concentration. We used simple coacervation method to crosslink nHA/chitosan drops with TPP solution which was proven to have minimal toxicity towards cells. Instead of using the traditional freeze drying method which first freeze the sample and dry using a lyophilizer, we have lyophilized the scaffolds by directly exposing them to an instant temperature and pressure drop. By instantly dropping the pressure and temperature, nHA/chitosan beads are exposed to rapid freezing front. This method derived mechanically sound scaffolds compared to fragile scaffolds obtained from traditional freeze drying method.

We have used the coacervation method to ionically crosslink nHA/chitosan droplets. The droplets are crosslinked between positively charged amino groups of chitosan present in nHA/chitosan mixture and negatively charged ion of TPP. This ionic crosslink formation can be confirmed by the translation in antisymmetric deformation N-H vibrations in NH^{3+} in chitosan at 1589 cm^{-1} to a lower wave number of present at 1558 cm^{-1} on all composite spectrums [42-44].

We can observe slight change in characteristics bands in all nHA/chitosan crosslinked scaffolds' spectrums because coacervation procedure is based on diffusion of anions in TPP

into the nHA/chitosan droplet and crosslink at the droplet. Since we have powdered nHA/chitosan scaffolds before micro-ATR/FTIR procedure, spectrums might have varied due to crosslinked chitosan density present at the Ge crystal of the micro-ATR. The antisymmetric stretching vibrations of PO_4^{3-} group which is another confirmation to crosslink of chitosan with TPP could not be distinguish in spectrums [18]. This indicates low crosslinking density which was mainly due to low crosslinking time of 30 min. This analysis shows higher density of the crosslinked nHA/chitosan composite scaffolds can be obtained by longer crosslink time thus resulting scaffolds with higher mechanical strength.

The UCS of scaffolds were calculated using a novel method as illustrated in the materials and methods section. We selected UCS as analyzing parameter as it can be easily distinguish on the stress-strain graphs and we were unable to set sound assumptions for compression modulus calculations due to the absence of linear region on stress-strain curve. We noticed that increasing the number of nHA/chitosan scaffolds in the compression test increases compressive force proportionally which determined the method of using 5 scaffolds per a one compression test and calculate the mean UCS for a scaffold. The 5 scaffolds compression test records a large load thus minimize the sensitivity errors of load cell and also reduce the noise on the load-displacement curve. The maximum compressive strength of 3.93 (79.98 standardized UCS, $\text{SD}=0.74$) MPa was shown by 2% nHA/chitosan lyophilized scaffolds. This method is suitable mainly for comparative assessment of UCS between several groups of scaffolds. We have noticed that increase in the nHA percentage from 1% to 2% increases the UCS at ($p < 0.01$) significant level. However, there was no significant difference of UCS between 0% and 0.5% nHA scaffolds. This increase in UCS with the increase in nHA percentage can be interpret as an indication of the interfacial bonding strength between chitosan and nHA as illustrated in XRD and FTIR analysis. In addition proper stress transfer between nHA phase and chitosan phase might have occurred to provide higher mechanical strength to the scaffolds [45, 46].

Lyophilized scaffolds have shown hydrogel like behavior when immersed in PBS. Because of its hydrogel like properties, scaffolds have shown very low compressive strength values such that scaffolds have lost 95% of UCS compared to its dry state. However, we have noticed that after the wet state compression, scaffolds remains intact. These compressed scaffolds have become very rigid that after a one day of drying, scaffold's mean UCS has increased to 14.92 MPa ($n=10$). This value is higher compared to UCS of LSD particles which shown 8.59 MPa in dry state. The boost in UCS on LSD and compressed scaffolds could be due to shrinkage of scaffold's inherent pore structure which is also highlighted by the reduction in the diameter of the scaffolds and surface pore sizes as shown in SEM analysis. The compression of wet state scaffolds doesn't break the particle but squeeze out the liquid by reducing pore sizes.

Scaffolds have shown no toxicity towards cells as shown in Figure 7. This is an indication that particle constituents and preparation methods are benign to osteoblasts. Previous studies have shown that chitosan polymerization with TPP is conducive towards the cell adhesion [6]. Cell attachment study shows every good osteoblast attachment to both 0% and 2% nHA/chitosan scaffolds. Moreover, 2% nHA/chitosan scaffolds are completely confluent with osteoblasts at the end of 14th day (Figure 8.D). This implies the presence of hydroxyapatite

on scaffolds expedite the osteoblast cell adhesion and support proliferation over the particle. This osteoblast adhesion and proliferation is also supported by highly porous surface structure of the all scaffolds.

5. Conclusion

This study has modified the microparticles preparation method used by our group in previous studies by introducing porosity and incorporating nHA to spherical chitosan scaffolds. Our new fabrication process is free of toxic substances to provided benign environment for osteoblasts. Depending on the scaffold's treatment method, scaffolds had diameters in the range of 0.9-1.3 mm and pore sizes from 2-10 μm which supports vascularization and transport of nutrients. Incorporation of nHA to chitosan has increased the ultimate compressive strength of scaffolds. And further we found that mechanical strength of scaffolds can be further improved by reducing pore sizes of scaffolds using a simple treatment. We also found that increasing amount of nHA supports the osteoblasts adhesion and proliferation on scaffolds. Thus novel injectable porous nHA/chitosan scaffolds can be used to in vivo studies for further assessment of its bone regeneration capacity.

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Highlights

- Injectable porous scaffolds prepared from chitosan and nano-hydroxyapatite.
- Scaffolds are fabricated using non-toxic coacervation and lyophilization techniques.
- 2% nHA/chitosan scaffolds exhibited highest ultimate compressive strength.
- Scaffolds' compressive strength can be increased by simple soaking and drying.
- Scaffolds showed very good osteoblast adhesion and proliferation.

Figure 1.

Experimental and calculation methods used to obtain compressive stress-strain graphs. (A) Side view of a particle before the compression test; (r) initial radius and (O) center of the particle. (B) Side view of a partially compressed particle; (R_e) effective radius, (r) initial radius and (x) movement of the crosshead. R_e is the radius of the surface in touch with the crosshead. R_e changes as the crosshead moves.

Figure 2.
SEM images of lyophilized and LSD scaffolds at low (A, B, C) and high (D, E, F) magnification: (A, D) 0% nHA/Chitosan lyophilized particle; (B, E) 2% nHA/Chitosan lyophilized particle; (C, F) 2% nHA/Chitosan LSD particle.

Figure 3. Fourier Transform Infrared (FTIR) Spectrums: (A) 2% nHA/chitosan scaffolds; (B) 1% nHA/chitosan scaffolds; (C) 0.5% nHA/chitosan scaffolds; (D) 0% nHA/chitosan scaffolds; (E) Chitosan bulk and (F) nHA bulk.

Figure 4.
X-Ray Diffraction spectrums: (A) nHA bulk; (B) 2% nHA/chitosan scaffolds; (C) 1% nHA/
chitosan scaffolds; (D) 0.5% nHA/chitosan scaffolds; (E) Chitosan bulk

Figure 5. Mean ultimate compressive strength of dry state lyophilized scaffolds vs nHA percentage graph

Figure 6. Mean ultimate compressive strength of scaffolds vs state of the scaffolds: wet state-lyophilized; dry state lyophilized; dry state LSD.

Figure 7.

Live and Dead cell assay result showing scaffolds' cytotoxicity towards OB-6 cells on day 3 and 14: (A) 0% nHA/chitosan particle on 3rd day; (B) 0% nHA/chitosan particle on 14th day; (C) 2% nHA/chitosan particle on 3rd day; (D) 2% nHA/chitosan on 14th day

Figure 8.

Live and Dead cell assay results showing OB-6 murine cells attachment on day 3 and 14: (A) 0% nHA/chitosan particle on 3rd day; (B) 0% nHA/chitosan particle on 14th day; (C) 2% nHA/chitosan particle on 3rd day; (D) 2% nHA/chitosan particle on 14th day.