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# Development of Chitosan Based Scaffolds for Bone Regeneration: A Preliminary Report

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## Abstract

An ideal scaffold for bone regeneration will provide adequate mechanical support and eventually degrade after bone healing without causing harm to the body. Chitosan, mixed with carboxymethyl chitosan, creates a scaffold with high porosity, but low mechanical strength. Therefore, the objective of this research is to develop biofunctional chitosan based scaffolds for possible application in bone regeneration, and characterize the mechanical properties, degradation rates, and porosity of these scaffolds. It was hypothesized that chitosan combined with hydroxyapatite will result scaffolds which will provide a stable biodegradation rate and suitable mechanical properties while bone regenerates. Hydroxyapatite was added to the solution of carboxymethyl chitosan to enhance the mechanical properties of the scaffolds created from the mixture. This solution was mixed with a chitosan solution, creating the scaffolds for analysis. Morphology of the scaffolds was determined using a Scanning Electron Microscope to evaluate the porosity of the scaffolds. Compression testing was done to determine the mechanical strength of the scaffolds. Qualitative assessment revealed a porous structure throughout each scaffold. It was noted that the addition of hydroxyapatite increased the mechanical stability of each scaffold tested. It was concluded, chitosan based composite can provide the needed mechanical support for regenerating bone, which was inclusive of a 3D environment.

***Keywords:*** *Chitosan; Scaffold; Bone; Polymer*

## Abbreviations

ASTM: American Society of Testing and Materials; CMC: Carboxymethyl Chitosan; CS: Chitosan; DI: Deionized; ECM: Extra Cellular Matrix; h: Hours; HA: Hydroxyapatite; KPa: Kilopascals; kV: Kilovolts; MPa: Megapascals; μA: Microampere; μm: Micrometer; ml: Milliliter; mm: Millimeter; min: Minutes; N: Newtons; PBS: Phosphate Buffer Saline; RPM: Revolutions Per Minute; SEM: Scanning Electron Microscopy; NaOH: Sodium Hydroxide; 3D: Three Dimensional; 2D: Two Dimensional

## Introduction

Bone remodeling in humans is restricted when tissues are completely destroyed by traumatic injuries or degenerated by age-related or inflammatory diseases. Biodegradable scaffolds may be used as a support structures for regenerative bone or tissues because of good biocompatibility and three-dimensional porous structures. An interconnected porous network facilitates the cell growth and transport the nutrients and metabolic waste [1]. Bone defects are frequently caused by trauma, diseases, developmental deformity, and tumor removal [2]. Patient-derived bone cells can be used in addition to temporary scaffolds, initiating the generation of natural bone tissues. A porous chitosan and carboxymethyl chitosan scaffold using a hydroxyapatite composite is a prime candidate for scaffolding of osteoblast-like cells and a barrier in growth of fibrous connective tissue [3].

Two-dimensional (2-D) scaffolds have significantly contributed to the advancement of the cell culturing, cell growth, and testing. Three-dimensional modifications have shown to be improvements over existing structures [4]. Three-dimensional scaffolding aids growth factor exchange and viability. As the first generation of cell culture medium, 2-D dishes have various limitations. Dishes are rigid and inert structures that restrict cell movement [5]. This restricts cellular growth because it eliminates the possibility of growth factor exchange that would happen in a 3-D environment. In addition, 2-D growth structures are unstable and impossible to use for mechanical testing. A key difference between 2-D and 3-D scaffolds is media diffusion; while in dishes diffusion and exchange will not occur. 3-D scaffolds mimic the exchange more closely [5-7].

The merging of micro and nanotechnology is a promising approach to design 3-D biodegradable scaffolds [8,9]. When creating 3-D scaffolds, there are various requirements that need to be met. First of all the scaffolds should have higher levels of biocompatibility, secondly scaffolds should exhibit mechanical properties similar to the surrounding environment, to prevent stress shielding. In addition, scaffolds need suitable morphology that allows expansion and/or biodegradation as necessary [10]. When 3-D scaffolds act as the ECM, they provide more faithful replication data of *in-vivo* data [6,11]. Most of all, the implementation of the ECM is to deliver the biochemical signals for sustainable differentiation and homeostasis [12]. Since 3-D structure scaffolds act as ECM, it promotes cell to cell interaction, which supports the structure and function of physiologically occurring factors, acting as proliferation surface and promotes the shape and its environment [6].

There are currently several types of biomaterials in development and use. Among the most popular are stainless steel, chromium-cobalt based alloys, titanium and titanium based alloys, as well as various ceramic materials, all of which are not biodegradable. The lacking biodegradability often requires additional surgeries for removal or revision. Additional discomfort is experienced by patients with permanent materials.

Highly biocompatibility, which enhances cell adhesion and proliferation [13-15], often make use of natural polymer scaffolds desirable. Alginate, collagen, chitosan, hyaluronan, gelatin and silk are the most commonly used polymers for tissue engineering [16,17]. Chitosan(CS) is natural polysaccharide formed by the partial deacetylation (derivative) of chitin, which formed from the exoskeletons of crustaceans, shells, insects, and spiders [18]. Chitosan is a cationic polymer, it has been considered as a multi-factor natural material with several biomaterial and bone healing applications, because of its biodegradability and biocompatibility [19] and has high biocompatibility. Carboxymethyl chitosan (CMC) is derivative of chitosan that can be engineered in multiple ways. Carboxymethyl chitosan has a very similar trait as chitosan, such as natural biodegradability and biocompatibility. After chemically modifying CS to CMC, it becomes an anionic polymer, turning the mixture between CMC and CS into a strong bond [19,20]. Chitosan has many biomedical applications, but does not dissolve in water. Moreover, carboxymethyl chitosan easily dissolves in water without any acetic acid. In addition, CMC has unique physiochemical and biological properties including high viscosity, low toxicity, high biocompatibility, and mechanical versatility in film, fiber and hydrogel process [18]. Hydroxyapatite (HA) is a calcium phosphate known to be chemically bonded to bone tissues [18]. Hydroxyapatite enhances the mechanical properties of the scaffolds also to support the bone growth [21-24]. Hydroxyapatite [$Ca_{10}(PO_4)_6(OH_2)$], a bioactivated biomaterial is minimally degraded in the body, and on implantation into bone defects will induce formation of new bone by osteoblast proliferation [25].

The objective of this research is to develop novel biofunctional chitosan based scaffolds for cancellous bone regeneration. The development of the scaffolds includes the characterization of porosity and mechanical properties of the scaffolds. It was hypothesized that chitosan based scaffold combined with hydroxyapatite would provide a stable biodegradation after performing the bone integrality and mechanical properties for bone regeneration.

## Methodology

Chitosan (≈200 kDa, 90% DD), and acetic acid were purchased from Fisher Scientific (Waltham, Massachusetts, USA). Carboxymethyl chitosan (≈200 kDa, 90% DD) was purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). Hydroxyapatite (99.995%) was purchased from Sigma Aldridge (St. Louis, Missouri, USA). Phosphate buffered saline (PBS) and sodium hydroxide (NaOH) were purchased from Fisher Scientific (Waltham, Massachusetts, USA). For mixing CMC solution with chitosan solution Thinky was used, it is a Planetary Centrifugal Mixture (ARM-310) purchased from (Iwamoto-cho, Chiyoda-Ku, Tokyo, Japan). Cell Culture Dishes (48 well Immunoassay Cell Culture Dish) was used to fabricate the scaffolds, was purchased from Fisher Scientific. The Freeze-dryer (FreeZone Plus 2.5 Liter Cascade Benchtop Freeze Dry System) was purchased from LabConCo (Kansas City, MO, USA), and was used for lyophilization. The Dubnoff Metabolic Shaking Incubator (water bath) at 37.5˚C, 65 RPM was used to simulate *in vivo* condition.

All solutions were prepared using weight percentages. Chitosan solution was prepared with a diluted mixture of acetic acid. Solution was mixed at room temperature using magnetic stir bar for 6 - 8 hours then stored in a 4°C refrigerator. The solution was taken out the freezer after 6 - 8 hours to hand stir because of its high viscosity which prevented magnetic stir bar usage. This process was repeated till the solution fully dissolved, took about 4 - 5 days. The final product of chitosan solution was dark brown color and highly viscous. The solution was stored in a 4°C refrigerator unit use.

Separately the CMC solution was prepared with deionized (DI) water. Solution mixed at room temperature using the magnetic stir bar for 5 - 6 hours, till the solution dissolved fully, then stored in a 4°C refrigerator unit use. The results of fully dissolved CMC were clear and less viscous compared to chitosan solution.

As previously mentioned two different mixing methods were used for optimization. Method 1: previously 5% chitosan and 5% CMC solution was prepared and kept in the freezer at 4°C. One percent HA powder was added into dissolved chitosan and mixed then mixing with CMC. Method 2: 1% Hydroxyapatite powder was dissolved in 2% acetic acid (2.5 ml); which took approximately 15 - 20 minutes in a conical tube. After the solution was dissolved into 2% acetic acid, HA solution was then place in a container and 5% chitosan solution was mixed in a same container using the magnetic stir for 30 minute in a stir. Then CMC was mixed into the HA and chitosan solution. After the Thinky container was weighted, a necessary step to set the counter balance in a Thinky to physically mixing the chitosan and CMC together in a centrifugal mixture was used for 40 minutes. After the solutions were mixed, they were placed in 48 cell culture dish using syringe. Method 3 followed the same procedure as method 2, except HA powder was dissolved into DI water then mixed in CMC solution then mixing with chitosan using Thinky. Three different percentage of HA were added into the CMC and chitosan solution to find the proper balance of porous structure and the increased mechanical strength of the scaffolds. Starting with 1% HA then 3 and 5% HA scaffolds were created using method 3.

A freeze dryer was used in the lyophilization process. After mixing the CMC and Chitosan with HA and put into the 48 well cell culture dish. The scaffolds were placed in 4°C refrigerator then transferred into -20°C for 4 hours then into -80°C and kept for overnight (approximately 10 - 12 hours). The frozen scaffolds were placed in a glass LabConCo containment jar and kept in that state for 36 - 40 hours. After lyophilization, scaffolds were removed from the freeze dryer then kept in the room temperature for 24 hours to reconstruct themselves in ambient air. This allowed the scaffolds to expand to their normal diameter, keeping the porous structure in inside the cell culture dish.

This process was to remove the acetic acid that was used to dissolve chitosan. A small concentration of sodium hydroxide (NaOH) (0.1 molar) solution was used. Each scaffold was submerged completely into the NaOH solution then rinsed it out with DI water. The DI water was used to eliminate excess NaOH after neutralization. For scanning electron microscope image and other experiments, stabilizing process was followed.

### Scanning Electron Microscope Assessment

The surface morphology of HA/CS/CMC composite scaffolds was examined by scanning electron microscope (SEM). Tracor Northern TN5400 was used to assess the structure of the scaffolds. Polaron E5400 High Resolution Coater (United Kingdom), was used to gold sputter the samples for SEM usage. Six samples were tested, using two per mixing method. The 5% CMC: CS 1:1 ratio was compared using 1%, 3%, and 5% HA, respectively. The scaffolds were cut into four pieces, the center parts were used because the outer layer is not smooth surface and uneven. Place in the proper holder for the samples and adjust the height. Double sided copper tape was used for attaching the specimens to its holder. Due to its non-metallic properties, a liquid strip of graphite glue was used on an edge of the scaffold to act as a conductor in order to generate the 3-D image. Samples were then gold sputtered using a Polaron. The samples were imaged using 5 kV low voltages and 5 μA conduction for visual readings. A low magnification setting was used to capture the image because of its size and finding porous in the scaffolds. Images were taken at 500 μm and 1.00 mm in magnifications. All SEM images have the same voltage and conduction as well as same sample size.

### Mechanical Testing

Compression testing provides information regarding the behavior of cellular material under compressive load. Living tissues are continuously interacting with high and low mechanical loads of internal or external stresses including vascular, cardiac, ligament, and cartilage tissues, which are very responsive to the compression forces [26]. Therefore, compression testing was done to obtain a complete load-deformation curve. The test was able to compute the compressive stress and the effective modulus of elasticity in accordance with ASTM Standard D1621-10 [27]. An Instron 5525 was setup with the scaffold within the cross-head unit before testing had begun, and the testing was completed under displacement control. A 500 N load cell was connected to the compression chamber, interfacing to a computer directing mechanical test conditions (extension, load, rate, and the data collection) using Merlin software. Testing machine was set to ASTM D 695-10 specifications. The samples were compressed at a rate of 1.3 ± 0.3 mm/min [28].

Sixty samples were tested, using five per conditions and per mixing method. The 5% CMC: CS 1:1 ratio was compared using 0%, 1%, 3%, and 5% HA, respectively. A set of samples were tested before stabilization while it was still at dry conditions. Another set of samples were stabilized and compression tested using the Instron 5525. The other set of samples were stabilized and placed inside of a conical tube containing approximately 25 ml of 1X PBS solution. The conical tube was then placed into the incubator at 37.5°C for six hours before doing any testing of those five samples. Test specimens need to be prepared carefully in the control environment, smooth surface, and clean edges. The standard test specimen shall be the form of right cylinder or prism, whose length is one and half of its principle of width or diameter. When testing syntactic foam, the standard test specimen shall be in the form of a right cylinder approximately 10 mm in diameter by 15 mm in length.

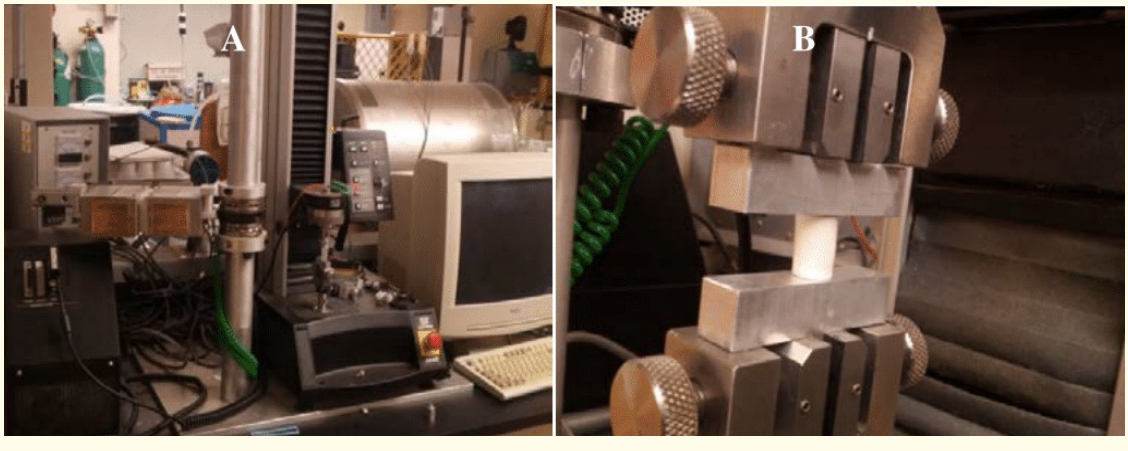


***Figure 1:*** *Instron 5525. (A) Full set-up of the machine and (B) Cross-head unit with scaffolds.*

A vertical line was drawn to intersect the linear elastic line to fine the yield point of the curve. The modulus of elasticity was calculated according to ASTM Standard D1621-10 by extending a line from the initial linear portion of the load deformation curve. The slope of the curve was calculated using Origin software, which gave the modulus of elasticity. The results were then expressed in Megapascals (MPa) to three significant figures.

## Results

### Chitosan Based Composite Scaffolds

After mixing Carboxymethyl chitosan with the chitosan at 5% ratio of 1:1 showed the best results. The 5% ratio was the best by comparing with its 4 and 6% solution counterparts. The results of 6% CS and CMC mixture was not homogeneous, also making it too viscous for CS and CMC mixing. Results of the 5% 1:1 CMC: CS was centrifuged in the Thinky are shown. A 5% solution was homogeneous (perfectly mixed).

### Comparison of Different Types of Mixing Methods

Three different methods were used to see which methods was the best to mix HA into CS and CMC scaffolds. All solutions used 5% 1:1 CMC: CS, but the method of hydroxyapatite dissolution into mixture was changed.

| Method | Hydroxyapatite and 5% 1:1 CMC: CS Scaffolds |
|---|---|
| 1 | Non-homogeneous to the eye, method not used |
| 2 | Homogeneous to the eye, method used |
| 3 | Homogeneous to the eye, method used |

***Table 1:*** *Summary of the results of mixing methods for HA and %5 1:1 CMC:CS.*

### Assessment of Scanning Electron Microscope

**Hydroxyapatite and 5% 1:1 CMC: CS Scaffolds Method 2:** SEM images (Figure 2) show the morphology that HA did not dissolve fully as the white crystals are still visible in figure 2A. Also Figure 2B shows that the structure of the scaffolds is not uniform and has very minimum porosity.

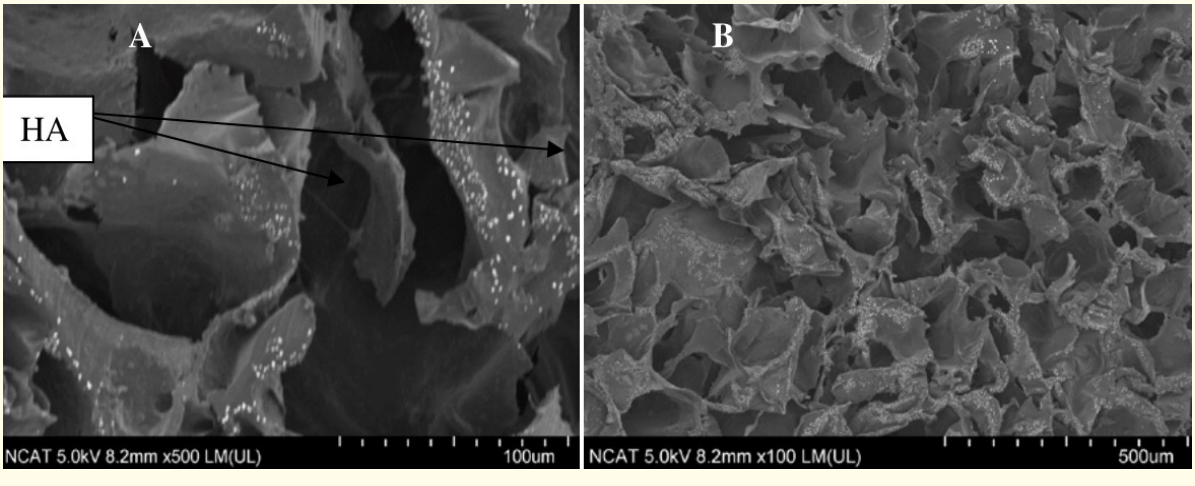


***Figure 2:*** *SEM illustrating 1% HA added into CS then mixing with CMC.*

**Hydroxyapatite and 5% 1:1 CMC: CS Scaffolds Method 3:** Figure 3 shows the morphology of the scaffolds using this method, the HA did fully dissolve and nor mix well. SEM image shows no white crystal (HA) present even at 400X magnifications. Figure 3 also shows that the structure of the scaffolds is uniform and contains porosity.

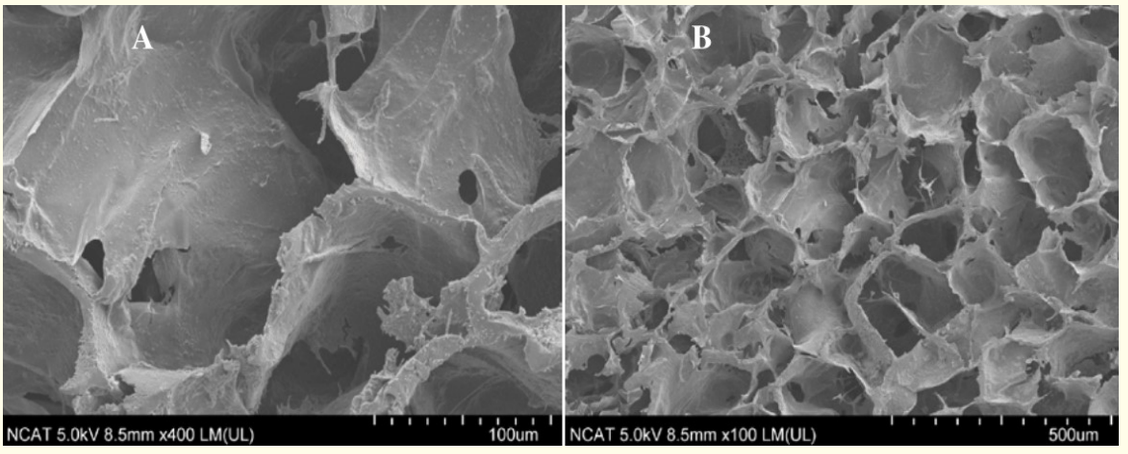


***Figure 3:*** *SSEM illustrating 1% HA added into CMC then mixing with CS.*

**Comparison of 1%, 3% and 5% Hydroxyapatite into 5% 1:1 CMC: CS:** After determining that method 3 was the best for adding hydroxyapatite, the next step was to find out the proper ratio of HA contains best porous structure. Figure 4 shows the results of the different ratios of HA. Figure 4A and B are 1% HA illustrates that the porous structures are being observed at 100X magnification. There are also small inner pores throughout the inner wall structure. Fifty times magnification clearly shows the porosity is not uniform throughout the surface of the scaffolds. This porosity helps to maintain the cell to cell interaction when seeded, as it would *in-vivo*. Figure 4C and D are 3% HA added to the solution illustrating the presence of a porous structure and a small, porous, inner wall at 100X magnifications. At 50X the structure is fairly uniform and has a better porosity than 1% HA. Figure 4E and F are 5% HA added to the solution. While a porous structure is present from 100X magnification, no inner wall porosity was visible; only the top surface of the scaffold was porous. At 50X magnification, it clearly shows that 5% HA is not uniform nor does it contain acceptable porosity.

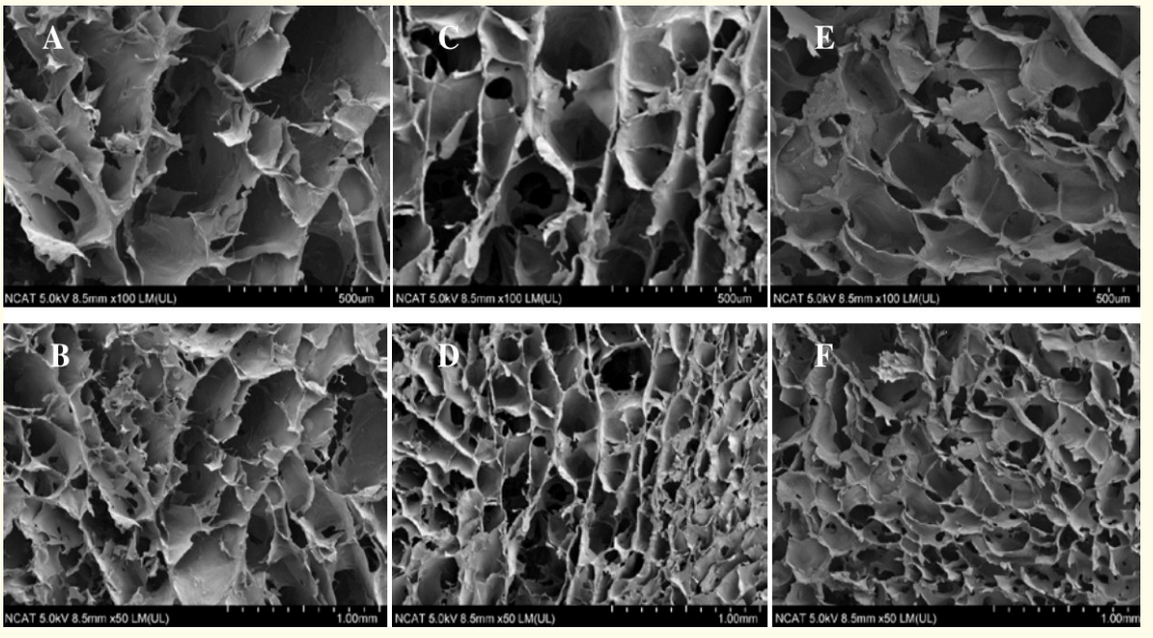


***Figure 4:*** *SEM illustration comparing 1%, 3% and 5% HA added into CMC then mixing with CS.*

### Mechanical Testing

During the compression testing of 5% HA scaffolds, it is believed that scaffolds broke from inside, and it occurred for all samples that were stabilized also after keeping the solution in the incubator for six hours. All scaffolds failed inside, As a result, data was not collected to further evaluation. 1% and 3% scaffold structures did not change besides compressing to a minimal (approximately 70%).

### Compression Testing Comparison

To compare how the scaffold reacts to the three different conditions (dry – unstabilized, wet – stabilized, and wet – incubated), scaffolds of the same ratio were used. For 1 and 3 percent HA, 5 samples for each test were prepared. In comparison, the 1 percent HA dry sample had a higher stress and the yield point than the 3 percent. Figure 5 below shows the stress-strain curve and a yield of approximately 0.8 MPa for the 1% HA and approximately 0.65 for that of the 3 percent.

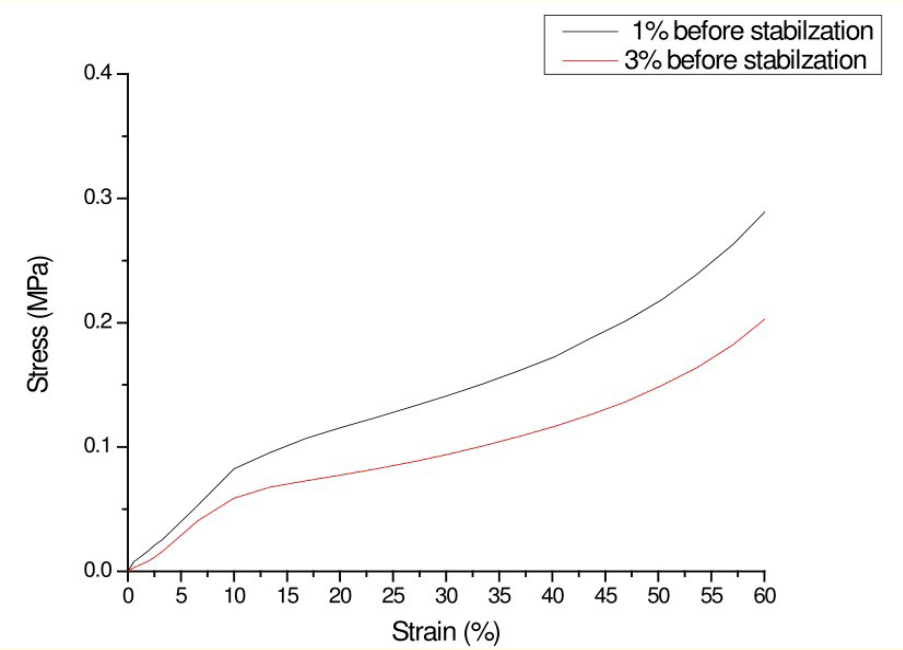


***Figure 5:*** *5% CMC: CS scaffolds comparison with 1% HA vs. 3% HA before stabilization.*

One and three percent HA scaffolds was tested as wet samples. Figure 6 on the right has the yield point for one percent is approximately 0.018 MPa and for three percent yield point is approximately 0.014 MPa. Figure 6 on the right which showed the graph of 6 hours after submerged in body bath, after compressing the scaffolds to 45%, linear elastic region did not have peak for one nor three percent HA scaffolds.

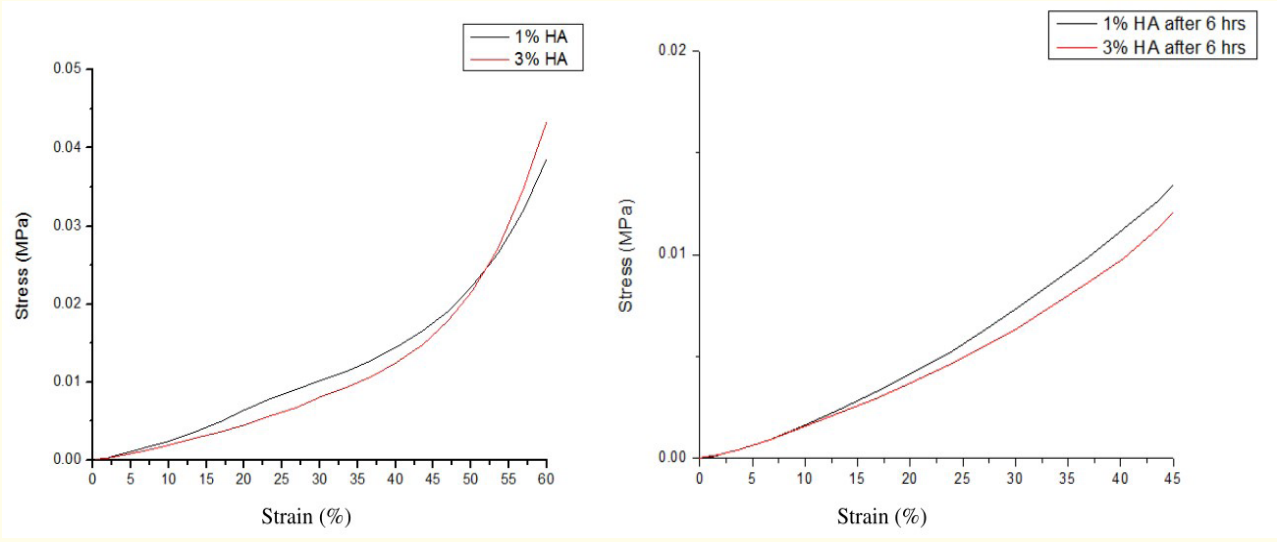


***Figure 6:*** *1 and 3% HA, 5% CMC: CS scaffolds comparison.*

To calculate the modulus of elasticity, average of 5 samples were taken. The linear region of the stress-strain was taken to evaluate the elastic region. Table 2 below shows the different modulus of elasticity also the degradation of the scaffolds.

| | **Modulus of Elasticity (MPa)** | | |
|---|---|---|---|
| Samples (5% CMC: CS) | As synthesized | After Stabilization (Wet samples) | After Incubation (6 h, PBS, 37°C) |
| 0% HA | 0.159 ± 0.013 | 0.0246 ± 0.006 | 0.0212 ± 0.0066 |
| 1% HA | 0.857 ± 0.0165 | 0.0359 ± 0.0693 | 0.0343 ± 0.0123 |
| 3% HA | 0.651 ± 0.0226 | 0.0293 ± 0.0103 | 0.0263 ± 0.0924 |

***Table 2:*** *Modulus of Elasticity of composite scaffolds.*

## Discussion

The objective of this work was to develop novel biofunctional chitosan based scaffolds for cancellous bone regeneration. Based on the results, the 5% CS mixing with the 5% CMC was the best mixture. The 4% CMC: CS was too liquid for mixing and would not create proper scaffolds and the 6% solution was too viscous to mix together, making it difficult to obtain a homogeneous mixture. CMC and CS were chosen due to their excellent biocompatibility and biodegradability, and possibility for medical applications [29].

### Chitosan Based Scaffolds Under SEM

The final products of methods 2 and 3 were assessed through SEM. Method 2 morphology showed that the scaffolds did have porosity, but were not uniform in the inner and outer layers of the scaffolds. Also, the SEM images showed that HA did not fully dissolve into the solution. However, scaffolds from method 3 showed uniform distribution. In addition, no HA crystals were visible, which suggests method 3 as the best choice with which to move forward.

After determining scaffold creation method 3 was the best choice, additional scaffolds were prepared using this method and 1%, 3%, and 5% HA with 5% CMC: CS solution. This step was to determine which ratio of HA would be the best in 5% CMC: CS scaffold. The one percent HA scaffolds showed that the structure of the scaffolds contains porosity, and had a rough surface. There were also small inner pores throughout the inner wall structure of the scaffolds. This architectural design is needed for the bone cells to move freely while the scaffold is incorporated into the bone. Three percent HA scaffolds also had the porosity structure and inner porous wall. A five percent HA didn't have any uniform porous structure, and the inner porous wall was very small and only had few inner porous walls.

Aronow M., *et al*. (1990) identified that the porous size of the compressive scaffolds varies from 100-200 μm, this provided the pathway for the cells which are in size of 10 - 30 μm to migrate into the scaffolds. After analyzing scaffolds SEM images, it was determined that the pore size was limited, ranging from 150 to 200 μm. It was concluded that scaffolds can provide a pathway for the cells to interact and migrate. The porous structure of this scaffold created a non-uniform, rough surface.

### Mechanical Testing of Chitosan Based Scaffolds

Chitosan or CMC by itself is not a strong polymer for any application. Combining these together can create a much stronger bond. Thein-Han., *et al*. (2009) compared and contrast the mechanical properties between just CS and CS with HA and results were, 6.0 ± 0.3 KPa and 9.2 ± 0.3 KPa, respectively [30].

Mechanical testing was done using an Instron 5525. All four different types of scaffolds were tested in three different conditions to validate which ratio was the best for creating scaffolds. In all three conditions only CMC: CS scaffolds lacked mechanical strength at the yield point and modulus of elasticity. Five percent HA scaffolds did not provide enough data for analysis the mechanical testing. During the testing, scaffolds broke from the inside for all the stabilized scaffolds. It was confirmed that no HA added to the CMC: CS scaffolds results in a much lower mechanical strength, also by adding too much of HA to the CMC: CS the scaffolds do not show higher mechanical strength.

One and three percent HA scaffolds, after stabilization showed linear behavior from the toe region to the yield point, exhibiting great energy absorption. The results show that adding HA in small percentages will increase the mechanical strength of the scaffolds, even after submerging the scaffolds in the PBS solution, then incubating for 6 hours. The modulus of elasticity did not change significantly.

## Limitations

Limitations of this work included the method for adding HA powder into the CS and CMC solution. Small amounts of HA powder remained in the conical tube, from human error, which may have impacted the studied properties. Future work should consider new methods to mix HA with CMC. During the lyophilization, well plates containing frozen scaffolds were placed at approximately 45º due to the design and shape of the apparatus valve. As observed most of the time scaffolds that were in the center of the well plate came out improper, containing cracks in the middle and the bottom of the scaffolds. Also, if more than two valves were used, scaffolds needed to stay in the freeze-dryer longer. During the mechanical testing, ambient temperature may have a significant effect. It is possible that high temperature may increase the interfacial bonding between CS and HA [31]. Therefore, all the testing that was done in the lab was in controlled room temperature to ensure the consistency of test samples.

Future research to analyze scaffold degradation rates are needed. Scaffold will need to be observed at various time points to identify structural changes along with the previously described compression tests to assess scaffold strength.

## Conclusion

The objective of this research was to develop novel biofunctional chitosan based scaffolds and characterize the porosity and mechanical properties of these scaffolds. It was hypothesized that chitosan combined with hydroxyapatite would result in a porous scaffold with appropriate mechanical properties suitable for bone regeneration. Results show that the composite scaffolds contained porous structure,

even in the inner wall of the structure. Assessment of SEM proves that the structure was stable after adding hydroxyapatite. One percent HA created the best structure for the bone regeneration containing porosity of the surface and a rough and rigid surface for better cell to cell interaction. For all the conditions tested, strain-stress curve did not have a high peak of yield point nor linear elastic region. It is due to scaffolds in wet conditions can have high linear elastic region. No ultimate yield strength was determined due to limits in the physical testing. Because, the porous structure collapsed and the graph is no longer informative. Compression testing concludes that adding hydroxyapatite in current scaffolds CMC: CS does increase the mechanical strength in dry and wet conditions. The results support the research hypothesis. In conclusion, chitosan based composite scaffolds show great promise in providing mechanical support for regenerating bone. Such technology can be used to improve the quality of life of the individuals suffering from bone defects.

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