



Cross-linked chitosan improves the mechanical properties of calcium phosphate–chitosan cement



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ABSTRACT

Calcium phosphate (CaP) cements are highly applicable and valuable materials for filling bone defects by minimally invasive procedures. The chitosan (CS) biopolymer is also considered as one of the promising biomaterial candidates in bone tissue engineering. In the present study, some key features of CaP–CS were significantly improved by developing a novel CaP–CS composite. For this purpose, CS was the first cross-linked with tripolyphosphate (TPP) and then mixed with CaP matrix. A group of CaP–CS samples without cross-linking was also prepared. Samples were fabricated and tested based on the known standards. Additionally, the effect of different powder (P) to liquid (L) ratios was also investigated. Both cross-linked and uncross-linked CaP–CS samples showed excellent washout resistance. The most significant effects were observed on Young's modulus and compressive strength in wet condition as well as surface hardness. In dry conditions, the Young's modulus of cross-linked samples was slightly improved. Based on the presented results, cross-linking does not have a significant effect on porosity. As expected, by increasing the P/L ratio of a sample, ductility and injectability were decreased. However, in the most cases, mechanical properties were enhanced. The results have shown that cross-linking can improve the mechanical properties of CaP–CS and hence it can be used for bone tissue engineering applications.

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1. Introduction

In tissue engineering, chitosan (CS), derived from chitin has been explored on several fronts as an organic substance to complex with calcium phosphate (CaP). Part of what makes chitosan a desirable to use substance is its biodegradability, which allows the body to break it down after healing [1,2], and biocompatibility, which includes minimal foreign body reaction while allowing human cells to grow onto it [3–6].

With such biocompatibility, CS is an ideal substance to complex into a scaffold with other additives [7]. One of the promising candidates for bone paste biomaterials is CaP, the main component of the inorganic portions of bone. CaP cements have gained clinical acceptance as valuable bone replacing biomaterials for almost three decades [8]. These scaffolds would form the basis of a moldable cement intended for bone regeneration applications while also allowing cells such as osteoblasts to grow normally onto them. Porosity, set time and mechanical properties such as, flexural strength, tensile strength, and hardness have been explored *in vitro* and *in vivo*. For the sake of clinical

applications, traits such as washout resistance and injectability have also been heavily explored [9–13]. These properties have a significant effect on the feasibility of injectable cements in bone tissue engineering. CaP cements have drawbacks in terms of graft migration, brittleness, fatigue fracture, and handling difficulties *etc.* These properties need to be improved for clinical applications. For instance, washout resistance holds significance due to the intended environment of the CaP–CS complexes, which generally involves being inside the human body. The other important property is injectability, which involves the force required to move the CaP–CS cement through a syringe. Injectable CaP–CS cement can be administered to bone defects using a minimally invasive technique.

CaP–CS scaffolds have been created with a variety of different methods to further explore possible methods for enhancements of their mechanical properties. The more recent methods have involved using nanoparticles or nanofibers [14–17]. Other methods involved precipitating beads of CaP–CS complexes out of a solution and lyophilization freeze drying [18]. Also the fabrication process has been heavily varied by the addition of different additives including organic acids, epoxides, and celluloses [19–21]. The addition of the additives has enhanced mechanical properties, injectability, and washout. On a chemical level, some of these additives have even induced cross-linking between chitosan.

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Nonetheless, cross-linked CS containing CaP–CS scaffolds have not been fully explored. CS contains abundant amino and hydroxyl groups which enable it to form particles via both physical and chemical cross-linking. Ionic cross-linking of CS is a typical non-covalent interaction, which can be realized by association with negatively charged multivalent ions such as tripolyphosphate (TPP) [22]. This paper seeks to focus on the effects of cross-linking of CS in the CaP–CS scaffold and explore its effects on Young's modulus in both dry and wet conditions, porosity, and hardness. Moreover, different powder to liquid ratios (P/L) were examined. Also taken into account were washout resistance and injectability.

2. Materials and methods

2.1. Materials

All components of CaP cement were purchased from Fisher Scientific. CS (85%, deacetylated), TPP and other chemical materials were purchased from Sigma-Aldrich. Statistical analysis was done using one-way ANOVA and $p < 0.05$ was considered as statistically significant. For each group, three samples were tested.

2.2. Scaffold fabrication

The CaP blend was created with the intention of mimicking the inorganic component of bone. The blend comprised of 55% alpha tricalcium phosphate (α -TCP), 45% dicalcium phosphate anhydrous (DCPA), and 15% calcium carbonate (CaCO_3) similar to that which was previously reported [23]. The CS solution was mixed at 2 wt.% with acetic acid or a ratio of 200 mg per 10 mL of acetic acid. The solution was stirred with a magnetic stirrer for 20 min and then set for 20 min allowing the air bubbles to disappear. A CaP powder blend was mixed with the CS solution at powder to liquid mass ratios (P/L) of 1.5, 1.7, and 2.0. The mixing was done manually until a consistent paste-like substance was formed. The paste-like substance was then put into cylindrical molds with dimensions of approximately $d \approx 6$ mm and $l \approx 12$ mm based on the ASTM-C39-05 standard. The molds were placed between two glass slides at 100% relative humidity and stored at 37 °C for 4 h. After 4 h, the samples were removed from the molds and placed into a saliva-like solution (SLS) for 20 h at 37 °C. SLS comprised 1.2 mM CaCl_2 , 0.72 mM KH_2PO_4 , 30 mM KCl, and 50 mM HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). This buffer was previously used to mimic plasma in teeth environment [9]. However, phosphate buffered saline (PBS) is another candidate to replace SLS to accurately mimic bone environment. After 20 h, the samples were removed from the SLS and they were ready for testing. The purpose of fabricating CaP–CS scaffolds using the molds is to measure the mechanical properties of CaP–CS cement.

2.3. Cross-linked scaffolds

To create cross-linked samples, instead of placing the samples in the molding between glass slides at 100% humidity at 37 °C for 4 h, they were only placed in SLS for 2 h. After 2 h, the samples were moved into 80% (wt) TPP solution for 2 h at 37 °C to induce the chitosan within the scaffolds to cross-link at a molecular level. Samples had a high contact area to the TPP solution and based on the calculated porosity, it is assumed that the TPP solution penetrates into the entire sample. This time is fairly enough to form cross-linking between CS molecules [24]. Similar to uncross-linked scaffolds, the CaP–CS cross-linked scaffolds were wetted by immersing in the SLS solution for 20 h.

2.4. Washout resistance

Ideally, a washout resistance test should be done after mixing and preparing paste-like CaP–CS but the test was done after 4 h which was

specified for cross-linking CS using TPP. Washout resistance was indicated by the sample maintaining integrity and not being dissolved by the SLS. Samples were carefully moved to a beaker with 20 mL of SLS and kept at room temperature for about 1 h. Then the washout properties were investigated.

2.5. Compressive strength and injectability testing

Using the ADMET 2611 mechanical testing machine with an MTestQuatro controller, compressive strength and injectability tests were performed. For the compression test, the crosshead speed was set at 1 mm/min. This speed is suitable for CaP cements reported before [5]. Compressive moduli were tested for both wet and dry samples. Wet samples were the samples that were immediately taken out of the SLS. The dry samples were taken out of the SLS and incubated in a dry environment for 24 h to allow the fluid (mainly SLS) inside to evaporate out. This step was done for both cross-linked and uncross-linked groups.

By attaching an appropriate custom made grip, an injectability test was also performed. A syringe was fitted to the machine and filled to about 5 mL of the CaP–CS paste. The force required for expulsion from the syringe was then tested. Since this test was done immediately after making CaP–CS paste, it was only done with uncross-linked samples. Injection speed was set at 6 mm/min. CaP cement pastes are usually considered as non-Newtonian fluid. However, the Hagen–Poiseuille relationship can be used to determine the viscosity [25].

2.6. Porosity

For porosity, samples were initially fully dry. They were then massed and had their exact dimensions measured. Each sample was then immersed into 25 mL of deionized H_2O . After 2 h of immersion, the samples were removed and massed again. The mass difference was used to calculate the volume of H_2O absorbed by each sample at 24 °C. The amount of H_2O absorbed was placed in a ratio with the volume of the CaP–CS scaffold itself ($\frac{V_{\text{water absorbed}}}{V_{\text{CaP scaffold}}}$) to derive a dimensionless value that indicated degree of porosity [13].

2.7. Hardness

For hardness test, dried samples were brought before a CM400AT Clark Microhardness tester. To properly prepare the samples, each was sandpapered at the round ends to create a flatter surface. The round ends were also marked with a marker to add contrast so as to better view with a microscope. The machine would generate small indentations at various forces. The applied force was 200 g force (gf). The indentations' dimensions, both horizontal and vertical diameter, would then be measured to generate a hardness value. Final hardness value can be calculated as follows:

$$\text{HV} = \frac{1.854F}{d^2}.$$

In this equation, HV is the value of Vicker's hardness, F is the adjusted load and d is the average of square's diagonals.

3. Results

3.1. Washout resistance

After 4 h, almost all samples were easily removed from the mold and upon placement into the SLS solution, none of the CaP–CS scaffolds washed out. They maintained structural integrity and did not dissolve into the solution throughout the incubation time as shown in Fig. 1. Particularly, this figure shows the integrity of the samples with P/L = 1.7. For other samples with different P/L ratios, the same behavior was

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