Comparison on mechanical properties of single layered and bilayered chitosan-gelatin coated porous hydroxyapatite scaffold prepared through freeze drying method

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Abstract. Biopolymer coated porous hydroxyapatite (HA) scaffolds were prepared for tissue engineering through freeze drying method and impregnation. In this study, to mimic the mineral and organic component of natural bone, synthetic hydroxyapatite (HA) scaffolds coated by polymer were prepared. Highly porous Hap scaffolds, fabricated by synthetic HA impregnation method on polyurethane foam, were coated with polymer coating solution, consisting of chitosan, Gelatin, and bilayered chitosan-gelatin prepared by aging and impregnating technique. For the purpose of comparison, the bare scaffolds without polymer coating layer were investigated. The Bare scaffolds were highly porous and interconnected with a pore size of around 150 µm - 714 µm, has porosity at around 67.7% - 85.7%, and has mechanical strength at around 0.06 Mpa - 0.071 Mpa, which is suitable for osteoblast cell Proliferation. Chitosan coated porous HA scaffold and gelatin coated porous HA scaffold had mechanical strength at around 0.81 - 0.85 Mpa and 1.32 - 1.34 Mpa, respectively, with weight ratio of biopolymer and Hap was around 18% - 22%. To compare these results, the coating on the bare scaffold with gelatin and chitosan had been conducted. Based on the result of FTIR, it could be concluded that coating procedure applied on porous hydroxyapatite (HA) coated by Gelatin, chitosan coated HA scaffold, and bilayered Gelatin-chitosan coated porous HA scaffold, confirming that all samples had no significant chemical effect on the coating structure. The compressive strength of bilayered Gelatin-chitosan coated HA scaffold had middle values between the rest, at around 1.06 - 1.2 Mpa for the samples at the same weight ratio of biopolymer: HA (around 18% - 22%). These results also confirming that coating by gelatin on porous hydroxyapatite was highest compressive strength and can be applied to improve mechanical properties of porous hydroxyapatite bare scaffold.

1. Introduction
Hydroxyapatite (Ca_{10}(PO_{4})_{6}(OH)_{2}, HA) has been widely used in medicine and dentistry because it is biocompatible, osteoconductive, and has excellent chemical and biological affinity with bony tissue [1]. As a result, HAp is accepted as a bioactive scaffold material for guided bone regeneration. Hydroxyapatite (HA) ceramics also have been used mainly as non-load-bearing parts in the form of powders and granules [2]. Compared to dense bodies or granules, the porous scaffolds are highly attractive for their biological benefits, such as osteoconductivity and fast bone ingrowth, due to high surface area and sufficient blood circulation [1][3]. However, the intrinsically poor mechanical properties of HA, such as low compressive strength and fracture toughness restrict its application only...
to small sizes of granules and powders or non-load bearing implant [2]. In order to expand its applicability in hard tissue applications, the brittleness of HA needs to be overcome. In addition to the requirements for the chemical composition of the scaffold material, an interconnected porous structure is necessary to allow cell attachment, proliferation, and differentiation, and to provide pathways for biofluids. With this approach, the porous scaffold serves an important role in the manipulation of the functions of osteoblasts and a central role in the guidance of new bone formation into desired shapes. Therefore, the scaffold materials must be biocompatible, osteoconductive, and osteointegrative, and have enough mechanical strength to provide structural support.

Chitosan is a natural cationic polymer that is biologically renewable, biodegradable, biocompatible, non-antigenic non-toxic, and biofunctional [4]. Gelatin is a natural protein derived from the organic constituent of bone (collagenteype I). Therefore its combination with the natural mineral constituent of bone (HA) is supposed to provide closer properties to the natural bone. Gelatin is readily assimilated by the body [5]. A composite scaffold of HA and Gelatin is therefore expected to show increased osteoconductivity and biodegradation together with sufficient mechanical strength [6]. beside that, gelatin, a natural protein derived from the organic phase of bone is much cheaper and more easily obtainable in solutions than collagen. In this study, a coating design of the HA porous scaffold was proposed to optimize these requirements. The polymer layer is expected to improve the brittleness of the porous scaffold, and the HA powder to enhance biocompatibility and hydrophilicity of the polymer coating layer, it assumed close to the mineral and organic component of natural bone.

2. Experimental details

2.1. Fabrication of Porous Scaffolds

Porous scaffolds were prepared by mixing hydroxyapatite powder (BPPT, synthesized from limestone with characteristic are: > Purity 99%, crystallinity: > 99%, Density 1.865 (gram/cc), Particle Size (nm): 50 – 100) were mixed with distilled water (DW). and suspension agent (Duramax D3005, Aldrich) was added to make a viscous HA slurry (HA/DW = 0.5 mg/mL). A polyurethane foam template (45 ppi, 3M) was replicated by immersing into the slurry, drying, and heat-treating at 600 °C for 3 h. Porosity was calculated by measuring the dimension and weight of specimen as well as by the Archimedes method ($n$ = 3).

2.2. Biopolymer coated porous hydroxyapatite (HA) scaffold procedure

The scaffold, prepared in a dimension of (diameter x height) 10 mm × 20 mm, was immersed into the biopolymer containing solutions (with concentration was 2% chitosan solution and 20% Gelatin solution) for 24 h at room temperature. For samples containing double immerse of biopolymers, the scaffold was immersed into 20% gelatin solution and was applied to freeze drying method for 24 hours, and then immersed into 2% chitosan solution and was applied to freeze drying for 24 hours to obtain bilayered gelatin-chitosan coated HA scaffold.

2.3. Characterization and compressive strength test

The morphology of the coated scaffolds was evaluated using optical microscope (Olympus BH-2) and polarized light microscope (Olympus CX31). The coating structure was analyzed using Fourier transformed infrared (FT-IR; System 2000, Perkin Elmer, USA) spectroscopy. Compressive mechanical tests were performed on scaffolds of a dimension (diameter x height) 10 mm × 20 mm using a Shimadzu TrapeziumX at a crosshead speed of 0.1 mm/min. The stress-strain curve was obtained, and the compressive strength and elastic modulus were determined from the maximum load recorded and the initial slope of the curve (<2% strain). The capacity of energy absorption ($W_{ab}$) of the coated and the bare scaffolds was defined as the energy necessary to deform a specimen to a strain ($\varepsilon$), and was calculated from the area under the stress-strain curve at a given strain, as follows [7]
Six samples were tested for each condition, and data were analyzed statistically using Student $t$ test and significance was considered at $p < 0.05$.

**Water absorption.** Water absorption of bare scaffold, gelatin coated porous scaffold, chitosan coated porous scaffold and gelatin/chitosan coated porous Hap scaffold were studied to evaluate the effect of HA content on the size and stability of material. The ratio of water absorption ($W_a$) at time $t$ was calculated using the following equation:

$$W_a = \frac{m_t - m_0}{m_0} \times 100\%$$

Where $W_t$ and $W_0$ are the weights of sample at time $t$ and the dry state at room temperature, respectively.

**Measurement of porosity and density.** The density measurements provided information about pore size and distribution, permeability, and the presence of structural faults in sintered ceramic structures [8]. A scaffold of weight $W$ was immersed in a graded cylinder containing a known volume ($V_1$) of ethanol. The cylinder was placed in a vacuum to force the ethanol into the pores of the scaffold until no air bubble emerged from the scaffold. The total volume of the ethanol and scaffold was then recorded as $V_2$. The volume difference ($V_2 - V_1$) was the volume of the skeleton of the scaffold. The scaffold was removed from the ethanol and the residual ethanol volume was measured as $V_3$. The total volume of the scaffold, $V$, was then

$$V = V_2 - V_3$$

The apparent density of the scaffold, $\rho$ was evaluated using,

$$\rho = \frac{W}{(V_2 - V_3)}$$

The porosity of the open pores in the scaffold, $\varepsilon$ was evaluated using [8],

$$\varepsilon = \frac{(V_1 - V_3)}{(V_2 - V_3)}$$

3. Results and discussion

3.1. Bare scaffold morphology

![Figure 1](image)  
**Figure 1.** Optical microscopy morphology with 4x magnification of bare scaffold on the a) X axis cross section, b) middle cross section
The microscopy morphologies of the porous scaffold are represented in figure 1a) and b). The bare HA scaffold, obtained by polyurethane reticulated foam method, exhibited a well-developed open pore structure. The porosity and average pore size were approximately 84% and 200–700 μm, respectively. It occurs caused by replication of morphology of polyurethane foam applied.

3.2. Coated scaffold morphology

When a chitosan and gelatin was coated as single layered and bilayered applied on the scaffold, the pore structure changed slightly. The pore wall became thicker and some pores were partially closed (figure 2a), 2b), 2c) and 2e). The porosity of the coated scaffold decreased slightly (around 78%). At high magnification, the coating layer was somewhat rough but uniformly covered the scaffold surface throughout (figure 2b,2d and 2f). Figure 2e) and 2ef) showed that bilayered coated gelatin (dark side) and chitosan (bright side) had covered scaffold surface.

**Figure 2.** Optical microscopy with 4x magnification of Gelatin coated HA scaffold, and b)10x magnification ;c) 4x magnification of chitosan coating layer covered scaffold surface and d) 10x magnification, e) 4x magnification of bilayer gelatin-chitosan coating layer covered scaffold surface and f) 10x magnification.
3.3. Coated scaffold structure

The coating structure was evaluated with FTIR spectroscopy and is shown in figure 3 and figure 4. Data on pure 20% Gelatin, 2% chitosan and HA powders are represented as references as below:

![Figure 3](image1.png)

**Figure 3.** FTIR spectroscopies of a) pure 2% solution chitosan, b) pure 20% gelatin solution, pure hap powder c) were represented as references;

![Figure 4](image2.png)

**Figure 4.** FTIR spectroscopies of d) chitosan coated porous Hap scaffold d) the chitosan/gelatin coated porous Hap scaffold e) and gelatin coated porous Hap scaffold
Figure 2a) represented mixed bands typical of Gelatin. The absorption at 1628 cm$^{-1}$ and 1539 cm$^{-1}$ are attributed to ((C=O)) and ( (C-N)) vibrations of amide I and amide II, which is representative of the collagen secondary structure. According to Yakimet & al [9], the bond at 1628 cm$^{-1}$ gives information on the helicity of the protein. A broad absorption band at 3390 cm$^{-1}$ of OH groups corresponding to absorbed water, secondary alcohols (CMC) and (intramolecular/ intermolecular) hydrogen bonding. [10] [11]. Figure 2b) represented mixed bands typical of HA (P-O at 827,963 – 1026 cm$^{-1}$ and O H at 3370 cm$^{-1}$ (broad)). Figure 2c) represented The spectrum of chitosan depicts characteristic absorption bands at 3280 cm$^{-1}$, 2872 cm$^{-1}$, attributed to the -OH and -CH$_3$ groups. Furthermore, bands were identified at 1539 and 1406 cm$^{-1}$ typical of the N-H group bending vibration and vibrations of -OH group of the primary alcoholic group, respectively. The bands at 1406 cm$^{-1}$ and 1025 cm$^{-1}$ correspond to the stretching of C-O-N and C-O groups. The bands at 1062 and 898 cm$^{-1}$ are attributed to the glycosidic bondings. The shoulder at 1632 cm$^{-1}$ represents the stretching of C=O. According to those IR absorption references, it could be concluded that process applied on porous HA coated by gelatin, chitosan coated HA scaffold and mixed Gelatin/chitosan coated porous HA scaffold, confirming that for all samples had no significant chemical effect on the coating structure as shown in figure 2d), figure 2e), and figure 2f) respectively.

3.4. Mechanical properties

The mechanical properties of a scaffold used for tissue engineering are very important due to the need for the structural stability to oppose the various stresses incurred during culture in vitro or implantation in vivo. The effects of the content of different biopolymer in the coated scaffolds on the mechanical properties are shown in Table 1, which were remarkably higher that those of the bare HA scaffolds (0.06 Mpa - 0.071 Mpa).

| Sample(s) | Bare scaffold (BS) weight, gr | Coated scaffold weight after freeze drying | Biopolymer fraction, (%) | Max. Compressive strenght (Mpa) |
|-----------|-----------------------------|---------------------------------|----------------|---------------------|
| BS        | 1.812- 1.855                | -                              | -              | 0.067-0.071         |
| G20       | 1.825- 2.188                | 2.145 -2.54                    | 18.09% - 17.53% | 2.95-3.27          |
| K2        | 1.33-1.52                   | 1.455- 1.76                    | 15.79% - 16.17% | 0.41-0.45          |
| GK        | 1.779 – 1.915               | 2.16 - 2.275                   | 18,80%-21,42%  | 2.02-2.77          |

The mechanical properties of the different concentrations of these samples were evaluated by compressive strength measurements. The compressive strength obtained in this study were quite low and within the margin of natural trabecular bones, presumably due to the high porosities (at least over 80%) where the ultimate compressive strength of the trabecular bone ranged from 0.22 to 10.44 Mpa with a mean value of 3.9 Mpa [12]. Practically, the trabecular bones had mechanical properties in a wide range depending on the porosity, position, and direction, as well as testing methods. [13-15]. The energy-absorption values in the coated scaffolds had higher values than those reported HA scaffolds (4.5–13 N * cm) with much lower porosities (∞60–70%).[15][17].

The thicker stems and consequently reduced porosity with cyclic coating also played some role in improving the mechanical properties. In this respect, the polymer component is quite beneficial in terms of providing mechanical flexibility and efficient energy absorption, where the gelatin coated porous hydroxyapatite has shown better value of compressive strength than other scaffolds.

4. Conclusions

A hydroxyapatite (HA) porous scaffold was coated with gelatin, chitosan and immersed gelatin and chitosan respectively shown the improvement of compressive strength related to the bare scaffold (without coating applied). The process applied on porous hydroxyapatite confirming that the mix-
polymer had no significant chemical effect on the coating structure. It means that the individual characteristics of components such as biocompatible, osteoconductive, and osteointegrative were no change. Besides, the polymer layer could be improve the brittleness of the porous scaffold. Compared to trabecular bone, The compressive strength of all types of biopolymer coated HA scaffolds were quite low and within the margin of natural trabecular bones

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