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The compressive strength and static biodegradation rate of chitosan-gelatin limestone-based carbonate hydroxyapatite composite scaffold

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ABSTRACT

Background: *One of the main components in tissue engineering is the scaffold, which may serve as a medium to support cell and tissue growth. Scaffolds must have good compressive strength and controlled biodegradability to show biological activities while treating bone defects. This study uses Chitosan-gelatin (C–G) with good flexibility and elasticity and high-strength carbonate hydroxyapatite (CHA), which may be the ideal scaffold for tissue engineering.* ***Purpose:*** *To analyze the compressive strength and static biodegradation rate within various ratios of C–G and CHA (C–G:CHA) scaffold as a requirement for bone tissue engineering.* ***Methods:*** *The scaffold is synthesized from C–G:CHA with three ratio variations, which are 40:60, 30:70, and 20:80 (weight for weight [w/w]), made with a freeze-drying method. The compressive strengths are then tested. The biodegradation rate is tested by soaking the scaffold in simulated body fluid for 1, 3, 7, 14, and 21 days. Data are analyzed with a one-way ANOVA parametric test.* ***Results:*** *The compressive strength of each ratio of C–G:CHA scaffold 40:60 (w/w), 30:70 (w/w), and 20:80 (w/w), consecutively, are 4.2 Megapascals (MPa), 3.3 MPa, 2.2 MPa, and there are no significant differences with the p= 0.069 (p>0.05). The static biodegradation percentage after 21 days on each ratio variation of C–G:CHA scaffold 40:60 (w/w), 30:70 (w/w), and 20:80 (w/w) is 25.98%, 24.67%, and 20.64%. One-way ANOVA Welch test shows the result of the p-value as p<0.05.* ***Conclusion:*** *The compressive strength and static biodegradation of the C–G:CHA scaffold with ratio variations of 40:60 (w/w), 30:70 (w/w), and 20:80(w/w) fulfilled the requirements as a scaffold for bone tissue engineering.*

Keywords: *biodegradation; compressive strength; medicine; scaffold; tissue engineering*
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INTRODUCTION

Bone defects may occur in the maxillary and mandibular alveolar bone because of congenital anomaly, trauma, bone deficiency after tumor resection, periodontal diseases, and tooth loss.[1–3] The most common treatment is the application of bone grafts using the concept of tissue engineering, which comprises three fundamental components: cells, scaffolds, and growth factors.[4] Tissue engineering aims to develop new biofunctional tissue to regenerate and repair damaged or diseased tissues.[5,6] In tissue engineering, scaffolds are crucial as they provide support for cell and tissue growth and can imitate natural bone.[7] The primary characteristics required in a scaffold for tissue engineering include biocompatibility, good mechanical properties, controlled biodegradability, osteoinductivity, osteoconductivity, and non-toxicity.[8]

The scaffold's synthesization involves biomaterials consisting of a natural polymer from chitosan-gelatin (C–G) and a bio-ceramic from carbonate hydroxyapatite (CHA), which display ideal scaffold characteristics.[9] Providing flexibility and elasticity, C–G is an organic material, while CHA is high in crystals, which might contribute to the scaffold's structural strength.[10] Chitosan is

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a natural biopolymer derived from chitin with the desirable characteristics of biocompatibility and biodegradability, as well as being antibacterial and non-toxic.[11] Gelatin is a biocompatible, biodegradable, low-toxicity material derived from hydrolyzed and denaturalized collagen, one of the leading organic components in the natural bone.[12,13] The combination of gelatin and chitosan may help improve the bone repair process.[14] With greater homogeneity and fixation ability than hydroxyapatite, CHA is an inorganic compound and is commonly used as a scaffold material for bone repair and replacement due to its bioactive, osteoconductive, and biocompatible characteristics.[15] It is capable of activating cell adhesion differentiation and proliferation with good absorptivity for bone defects, which is essential for tissue engineering.[16,17] CHA may also help increase the ion calcium and phosphate required for new bone formation.[13] In this study, limestone-based CHA from Cirebon, West Java, extracted by the Indonesian Center for Ceramics (BBK Indonesia), has potential application as a bio-ceramic material in the medical field for bone replacement in treating bone defects.

This study uses freeze-drying to synthesize the C–G:CHA scaffold. This method can produce porous 3-dimensional scaffolds with more than 90% porosity and a 20–400 micrometer (μm) pore diameter.[18] Combining several natural materials can improve each material's properties to achieve the scaffold's ideal characteristics, particularly good mechanical properties.[19] Chemical cross-linking between the polymer components of C–G and the addition of CHA can affect the mechanical properties of a scaffold.[20]

Scaffolds used as a bone replacement need a 60% to 90% porosity with an average pore size of 150 μm and compressive strength comparable to the cortical bone of 100MPa to 230 MPa or trabecular bone of 1MPa to 12MPa.[21–23] The compressive strength of a scaffold material is mainly studied to determine its maximum load-bearing capacity.[24] The ideal scaffold must have good mechanical properties, including compressive strength to withstand pressure from tissue and maintain space for cell and new bone growth.[25] When a scaffold is implanted into the body, it must maintain its mechanical properties with enough structural integrity, determined by the biodegradability of the biomaterial that it can create space for new bone tissue to grow. Thus, the research aims to determine the mechanical property requirements for a tissue engineering scaffold by testing the compressive strength of the C–G:CHA scaffold composite with ratio variations of 40:60 (w/w), 30:70 (w/w), and 20:80 (w/w) and biodegradation testing to determine how long it takes for the scaffold to degrade into the body completely.[26] A scaffold's controlled and stable degradation process may help regenerate new bone tissue.[27] This study aims to analyze the compressive strength and static degradation rate of the C–G:CHA scaffold composite with specific ratios as requirements for bone regeneration.

MATERIALS AND METHODS

The materials used in this study were chitosan with a medium molecular weight (Sigma Aldrich 448877, USA), bovine gelatin (Sigma Aldrich G9391, USA), CHA powder made from limestone produced by Indonesian Center for Ceramics (BBK Indonesia), natrium hydroxide (Biomedicine), acetic acid (Merck), distilled water (Duta Farma), and simulated body fluid (SBF Merck). The C–G:CHA 40:60 (w/w) scaffold is prepared by weighing 0.5 grams of chitosan powder, 0.5 grams of gelatin powder, and 1.5 grams of hydroxyapatite carbonate powder. Up to 2% acetic acid is added to the weighed gelatin up to 2 milliliters (ml) and stirred with a magnetic agitator at 50°C until the gelatin powder is homogeneous. The weighed CHA is mixed with 0.94 ml of distilled water and then incorporated with a metal spatula until homogeneous. Then, the dilute CHA is incorporated in the gelatin gel and then stirred until homogeneous, while chitosan powder is added gradually to form a C–G:CHA gel. The C–G:CHA gel was mixed with 0.5 ml of 0.1 molar NaOH to neutralize the acid. The C–G:CHA gel was measured using litmus paper until a pH of 7 was obtained. If a pH >7 (alkaline) was obtained, 0.1 ml of acetic acid solution is added, while if a pH <7 (acidic) is obtained, 0.1 ml of NaOH solution is added. The pH measuring must be done simultaneously to ensure the scaffold's pH is exactly 7. The pH 7 C–G:CHA gel is placed into the 48-well plate using a glass spatula and then compacted using a cement stopper until no hollow spaces are left. The mixture is frozen at −40°C for 2x24 hours and freeze-dried for 2x24 hours.[28] Scaffolds with the ratios of 30:70 (w/w) and 20:80 (w/w) are processed the same way. On the 30:70 (w/w) ratio, 0.375 grams of chitosan powder, 0.375 grams of gelatin powder, and 1.75 grams of CHA powder are used. For the 20:80 (w/w) ratio, 0.25 grams of chitosan powder, 0.25 grams of gelatin powder, and 2 grams of CHA powder are applied.

Compressive strengths are tested using the Mini Autograph Universal Testing Machine's sensor load cell L IP3 Class 0.02 with microcontroller software Phyton 2.7 on the cylindrical-shaped scaffolds. The diameter and height of the scaffolds are measured using vernier calipers to measure their surface area. Scaffold samples are placed in the middle of the pressing machine with their vertical axis perpendicular to the flat plane. Activating the Mini Autograph tool, the suppressor crushes the sample slowly with a pressure load of 400 newtons and 2 mm/minute speed until the samples are distorted and break. The tool will be stopped when the graph in the monitor shows that there is an increase after a decrease. When this occurs, the load no longer pressures the scaffold but is distributed only onto the upper and lower suppressor. Calculations will be made from the graph results, which show displacement and force accepted by the scaffold as the maximum amount of load divided by the surface area of the scaffold sample. The data are then put into the compressive strength formula to calculate the compressive strength value with units of MPa.[29]

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Static biodegradation testing is achieved by soaking the samples in 1.5 ml SBF in an Eppendorf container at a temperature of 37°C. Before soaking, the pH measurements of the SBF media are taken by weighing the SBF to determine the initial weight of the scaffold in its dry state (Wo). The percentage of biodegradation is obtained after calculating the final weight (Wt) as the scaffolds are dried after soaking for 1, 3, 7, 14, and 21 days. The chosen formula is used to calculate the biodegradation percentage from the scaffolds' W and Wt data.[30]

$$Biodegradation = \frac{Wo-Wt}{Wo} x\ 100\%$$

Research data are then statistically analyzed using the Kolmogorov–Smirnov test to determine if the data distribution is normal, followed by homogeneity testing using the Levene test. If the $p > 0.05$, one-way ANOVA parametric tests are performed to identify the significance of every sample's data results.

RESULTS

The compressive strength value of the C–G:CHA scaffold is obtained after entering the strength test results from the Mini Autograph Universal Testing Machine's sensor load cell L IP3 Class 0.02 with the Python 2.7 microcontroller software. Results and the standard deviation of the C–G:CHA scaffolds' compressive strength value are shown in Table 1 and Figure 1. The average compressive strength value of the C–G:CHA scaffold appears to increase

Table 1. The compressive strengths of various C–G:CHA scaffold ratios (MPa)

Sample	n	Average of compressive strength value	Standard deviation
C–G:CHA scaffold 40:60	6	4.19	0.79
C–G:CHA scaffold 30:70	6	3.29	0.22
C–G:CHA scaffold 20:80	6	2.19	1.19

Table 2. Static biodegradation test of C–G:CHA scaffold ratios (%)

C–G:CHA scaffold ratios	n	Day 1	Day 3	Day 7	Day 14	Day 21
		x + SD	*x + SD*	*x + SD*	*x + SD*	*x + SD*
40:60	6	0.41±0.06	1.69±0.69	6.67±1.64	16.13±5.43	25.98±2.74
30:70	6	0.50±0.13	3.18±1.35	6.07±0.52	12.55±2.03	24.67±3.77
20:80	6	0.42±0.09	1.86±0.80	5.51±0.79	8.27±1.51	20.64±6.40

Notes: *x*: average biodegradation percentage; SD: standard deviation

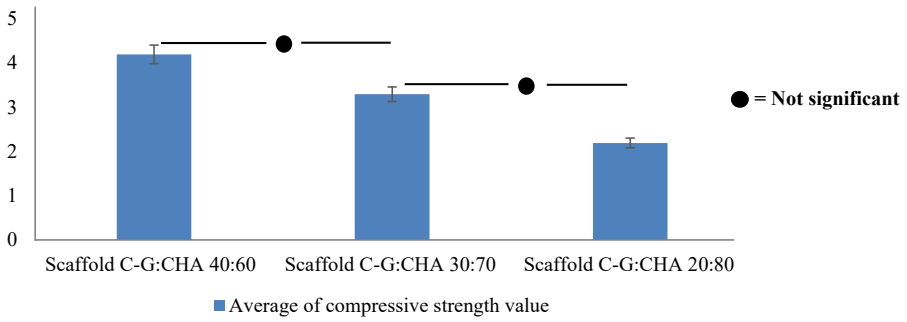

Figure 1. Graph of the average compressive strength of C–G:CHA scaffolds.

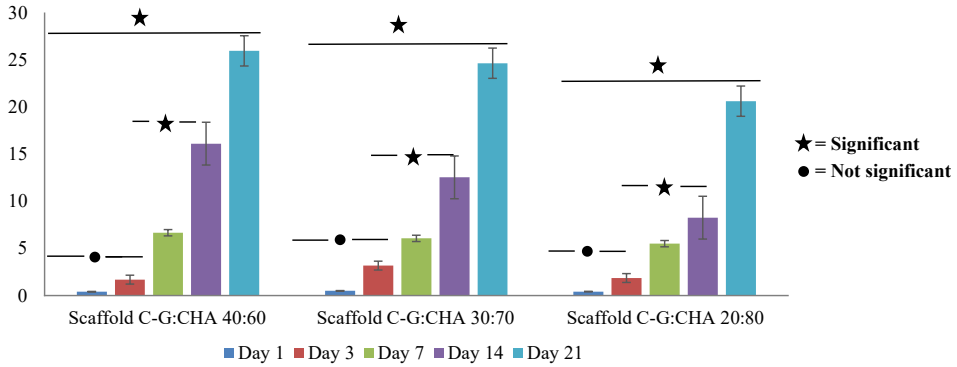

Figure 2. The graph shows the average static biodegradation rate of the C–G:CHA scaffolds on days 1, 3, 7, 14, and 21.

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with an increase in the C–G ratio and a decrease in the CHA ratio.

Initial and final weights are calculated on each sample at every time stamp during the research. Time-stamp variations for the biodegradation tests are 1, 3, 7, 14, and 21 days. Data from the initial and final weights are used to calculate the degradation percentage of the C–G:CHA scaffolds with various ratios. Results and the standard deviation of the C–G:CHA scaffolds' degradation are shown in Table 2 and Figure 2.

DISCUSSION

The scaffold's mechanical properties are essential in the manufacturing process. Developing a porous structured scaffold compatible with bone is a central issue in tissue engineering.[31,32] The porous structure of the scaffold is to facilitate cell attachment and proliferation, but then it must also have the sufficient mechanical strength to enhance biostability.[33]

Based on the results, the average compressive strength value increases when the CHA ratio decreases, and the C–G ratio rises. A one-way ANOVA statistical analysis showed no significant difference in the compressive strength values of the three variations of the C–G:CHA scaffold ratios of 20:80 (w/w), 30:70 (w/w), and 40:60 (w/w). This result means that the average compressive strength value increase was insignificant. In previous studies, the C–G:CHA scaffold had been tested by FTIR, SEM-EDX, and XRD, and the results contained a phosphate group (PO_4^{3-}), a carbonate group (CO_3^{2-}), and a hydroxyl group (OH^-). Chitosan has several hydroxyl groups (-OH) and amine groups (-NH_2) in its chain, while gelatin has active hydroxyl groups (-OH), carboxylic groups (-COOH), and amine groups (-NH_2). The groups in chitosan and gelatin form hydrogen bonds between the amine and carboxylic groups or with phosphate and carbonate groups.[33]

Bonds between carboxyl groups in gelatin and amine groups in chitosan also produce ionic bonds, which cause the formation of a scaffold with denser properties.[34] Bonds in chitosan and gelatin will form intermolecular hydrogen bonds. Hydrogen bonds will be created due to bonds with –NH_2 and carbonates hydroxyapatite or interactions between –COOH and carbonates apatite. Combining these 3 materials will form crystalline particles, which are dominantly formed from CHA material containing the elements O, Ca, and P and has crystalline particles.[33] During the freeze-drying process, the mixture of the three materials will produce crystals and amorphs, which will balance the crystallinity of the scaffold so that the addition of the C–G ratio can increase the bond to the 3 materials.

The above confirms the opinion that chemical cross-linking between polymer components, namely C–G, can affect the mechanical properties of the scaffold.[20] The compressive strength value in this study is still in the range of compressive strength values in trabecular bone of 0.1 to 16 MPa. Engineering scaffold tissue must possess sufficient mechanical properties to support new bone tissue at the implantation site and maintain good integrity for cells in vitro and in vivo.[35-38] Thus, it is vital for a bone scaffold to have identical mechanical properties as trabecular bone.[23]

In the results of previous studies, the trabecular bone mechanical properties have a value of compressive strength of at least 1 MPa.[39] A study by Waletzko-Hellwig showed that the compressive strength of trabecular bone is 2 to 48 MPa. In comparison, a study by Mohaghegh suggested a compressive strength of 1.5 to 45 MPa, and research by Gerhardt and Boccaccini showed a compressive strength of 0.1 to 16 MPa.[37,40,41] This indicates that trabecular bone has a highly anisotropic and heterogeneous structure whose mechanical properties depend highly on anatomical location.[42] In addition, the stiffness level of the bone scaffold must not be too low to provide mechanical stability and not too high to prevent stress shielding, resulting in friction under continuous pressure and damage to the surrounding bones.[43] This can affect bone remodeling.[44] Previous research on Balai Besar Keramik's hydroxyapatite (HABBK) scaffold, combined with C–G, proved the HABBK:C–G scaffold composite with a ratio of 60:40 (w/w) had the highest average compressive strength value, i.e. 0.81 MPa, less than the compressive strength value of this study using CHA.[29,45] This study shows that all ratios meet the requirements as a scaffold in tissue engineering: the compressive strength in the C–G:CHA scaffold with the ratio of 40:60 (w/w) is 4.19 MPa; the C–G:CHA 30:70 (w/w) is 3.29 MPa; and the C–G:CHA scaffold 20:80 (w/w) is 2.19 MPa. All these values are still within the range of compressive strength values of the trabecular bone. Developing chitosan, gelatin, and CHA materials into a three-dimensional structural scaffold with good mechanical strength and biological function will increase the recovery of bone defects. It can be used as a good candidate for designing biomimetic bone scaffolds.

Table 2 and Figure 2 show that the average value of the static biodegradation rate of the C–G:CHA scaffold in each ratio variation increased during the study from day 1 to day 21. The test results for the highest average degradation rate value of the C–G:CHA scaffold sample for each ratio variation were obtained on the 21st day. In the C–G:CHA scaffold with a variation of the ratio of 40:60 (w/w), the static biodegradation rate is faster compared to the other ratio variations. One of the ideal properties of biomaterials in tissue engineering is biodegradability. Biomaterials used for scaffolds have an essential role in the success of tissue engineering. Tissue engineering in a scaffold must support the tissue growth process that acts as a temporary extracellular matrix during the cell attachment and adhesion processes. This matrix must be made of biodegradable materials capable of being metabolized by the body and eventually gradually degraded when cells begin to undergo a process of proliferation and differentiation.[46,47]

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The static biodegradation rate of the 40:60, 30:70, and 20:80 scaffolds throughout 1, 3, 7, 14, and 21 days increased due to bonds between the scaffold components and calcium and phosphate ions from the SBF solution. This bond gradually damages the scaffold components and causes a decrease in the weight of the scaffold, increasing the degradation rate due to the interaction between the scaffold and the SBF media. In the degradation process of the C–G:CHA scaffold in the SBF, the increasing weight loss was affected by the bond between the scaffold components with Ca^{2+} ions and PO_4^{3+} ions originating from the SBF solution. The interaction of the scaffold and the SBF also caused the degradation of chitosan in the form of an amide complex such as NH_2 in the SBF. This process will cause the SBF to gradually damage the scaffold components and increase the percentage of scaffold weight loss as the static immersion time rises to 21 days.[33,48]

The static biodegradation test, the gold standard, is used in this study.[49] According to previous research, the reduction in scaffold weight should not be too fast or slow, following the bone remodeling process of around 3–6 months.[50,51] In another opinion, the minimum degradation process is between 1–2 weeks because this period is the bone repair stage, starting with the elimination of damaged cells and replacing the weak fibrin clot with a more mechanically stable structure called a callus.[52]

The biodegradation rate of the C–G:CHA scaffold is 20% to 25.98% on day 21, the largest ratio variation in the study. Based on previous research, the trabecular bone regeneration process takes place for about 2–3 months.[53]According to other studies, bone regeneration in the trabecular bone takes about 200 days (6 months).[54] In this study, the degradation rate of C–G:CHA scaffold of 20% to 25.98% for 21 days is expected to lead to complete degradation within 3–6 months, showing that the C–G:CHA scaffold has a biodegradation rate suitable for the bone regeneration process. All of the C–G:CHA scaffold ratios demonstrate biodegradable properties. Over 1, 3, 7, 14, and 21 days, the scaffold underwent in vitro bone regeneration, or it could be said that the scaffold was degraded. Therefore, the C–G:CHA scaffold with the ratios of 40:60 (w/w), 30:70 (w/w), and 20:80 (w/w) all have reasonable degradation rates for a scaffold in tissue engineering. This study has limitations because it has not been able to observe complete biodegradation, which will occur in the future. It is also necessary to undertake further research using dynamic degradation techniques by simulating the movement of bone marrow in the trabecular bone.

In conclusion, the compressive strength value of the C–G:CHA scaffold composite with ratio variations of 40:60 (w/w), 30:70 (w/w), and 20:80 (w/w) met the requirements of a scaffold in tissue engineering. The variation of the ratio of the C–G:CHA scaffold composite 40:60 (w/w), 30:70 (w/w), and 20:80 (w/w) increased throughout 1, 3, 7, and 14 days, with the highest percentage of biodegradation on day 21 of 25.98% on the C–G:CHA scaffold with a ratio variation of 40:60 (w/w). With good flexibility and elasticity, C–G combined with high-strength CHA, is an ideal scaffold for tissue engineering.

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