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We have reached a decision regarding your submission to International Journal of Integrated Engineering, "The The characteristics, swelling ratio and water content percentage of chitosan-gelatin/limestone-based carbonate hydroxyapatite composite scaffold; chitosan-gelatin/limestone-based carbonate hydroxyapatite composite scaffold".

Our decision is: Revisions Required

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## The characteristics, swelling ratio and water content percentage of chitosan-gelatin/limestone-based carbonate hydroxyapatite composite scaffold

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**Abstract: Background:** The tissue engineering field has developed a scaffold that can be used to increase the bone regeneration process. Carbonate hydroxyapatite (CHA) is a well-known scaffold due to its components resembling human bones. **Methods:** The scaffold was synthesized from K, G, and limestone-based CHA using a freeze-drying method with K-G/CHA ratios (w/w) of 40:60, 30:70, 20:80, and 10:90. A Fourier transform infrared spectroscopy (FTIR), a scanning electron microscope-energy dispersive X-ray (SEM-EDX), and X-ray diffraction (XRD) were used to characterize the scaffold. **Results:** The FTIR test showed some functional groups, such as hydroxyl, amide I, amide II, carbonate, and phosphate. The SEM-EDX test showed micropore (<50  $\mu\text{m}$ ) and macropores (>50  $\mu\text{m}$ ) structures as well as elements of C, N, O, Mg, Al, Si, P, and Ca. The XRD analysis obtained crystalline and amorphous particles. The WCP values obtained were 61.29%, 64.30%, 67.71%, and 67.78%. **Conclusion:** K-G/CHA composite scaffold with a ratio of 30:70 has ideal characteristics, a swelling ratio and a WCP.

**Keywords:** Chitosan, gelatin, limestone-based carbonate hydroxyapatite, composite scaffold, Medicine, swelling ratio.

### 1. INTRODUCTION

Bone damage is one of the health problems that often occurs in the field of dentistry. Bone destruction can be caused by periodontal disease, neoplastic disease, trauma, necrosis, or infection.<sup>[1,2]</sup> Bones can be remodeled; however, severe fractures require surgical intervention to aid the healing process. In this case, a technique was employed to develop a 3D scaffold. The scaffold is combined with stem cells and growth factors to replace damaged tissue by supporting the differentiation of stem cells to new bone.<sup>[3,4]</sup>

The scaffold acts as microenvironment that facilitate stem cells to attach. In addition, it may support cell proliferation that can induces bone formation. There are some requirement for ideal scaffold such as biocompatible, biodegradable, osteoconductive, good porosity and it should have good mechanical properties.<sup>[1,5]</sup> The scaffold's property is determined by the intrinsic properties and the combination of the chemical and physical properties of the material that constructs the scaffold.<sup>[4]</sup>

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The scaffold from chitosan, gelatin, and limestone-based Carbonate hydroxyapatite (CHA) was developed for application in the field of tissue engineering. Chitosan (K) is a polymer derived from chitin, which has a similar structure to glycosaminoglycan, which supports proliferation, the differentiation of osteoprogenitor cells, and bone formation.<sup>[3,6]</sup> Chitosan can be applied clinically because of its good biocompatibility and biodegradation properties.<sup>[7]</sup> Gelatin (G) is a biopolymer resulting from the partial hydrolysis of collagen. Gelatin consists of arginine-glycine-aspartate (RGD) amino acid sequences, which support adhesion and cell migration, and it has good biocompatible and biodegradable properties.<sup>[6]</sup> Carbonate apatite is utilized in the field of bone tissue engineering because its content most closely resembles bone apatite compared to calcium phosphate.<sup>[8]</sup> Carbonate apatite has osteoconductive and osteoinductive properties, and it can also stimulate osteogenesis with minimal immunological reactions.<sup>[9,10,11]</sup>

Chitosan and gelatin combined with carbonate apatite are expected to produce organic and inorganic compositions that resemble bone structures and produce good mechanical properties for implantation in bone defects.<sup>[12]</sup> The organic composition of bones consists of collagen and non-collagen components. The collagen component is represented by gelatin, while the non-collagen component is represented by chitosan.<sup>[13]</sup>

In this study, we used CHA synthesized from Indonesian limestone, which is produced by Balai Besar Keramik Indonesia (CHA). Limestone contains calcium carbonate ( $\text{CaCO}_3$ ), which can be used as a scaffold material in the form of apatite carbonate.<sup>[14]</sup> CHA is often used for scaffold materials because it has physicochemical properties, biocompatibility, and osteoconductivity is similar to inorganic components of bone.<sup>[15]</sup> The K-G/CHA composite scaffold was synthesized in four ratios (w/w), namely 40:60, 30:70, 20:80, and 10:90. These ratios are considered to be in accordance with the composition of the organic (22%) and inorganic (69%) component of bone with variations in the arithmetic series.<sup>[12]</sup>

The K-G/CHA scaffold was synthesized by a freeze-drying method to obtain a porous scaffold.<sup>[16]</sup> The characteristics were analyzed using Fourier transform infrared spectroscopy (FTIR), a scanning electron microscope-energy dispersive X-ray (SEM-EDX), and X-ray diffraction (XRD). In addition, the swelling ratio and the water content percentage (WCP) analysis were also performed. The composition of the scaffold is important to produce the ideal scaffold.<sup>[3]</sup> The difference in the ratios of the scaffold component composition will affect the scaffold's characteristics. Thus, the aim of this study is to analyze the characteristics of various ratios of K-G/CHA scaffold and find the optimal ratios for the scaffold manufacture.

## **2. MATERIALS AND METHODS**

### **A. Material**

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 Balai Besar Keramik Indonesia (BBK Indonesia), natrium hydroxide (Biomedicine), acetic acid (Merck), aquadestilata (Duta Farma), and simulated body fluid (SBF merck).

### **B. Preparation of K-G/CHA scaffold**

The scaffold with a ratio of 40:60 (w/w) consists of 0.5 grams of chitosan, 0.5 grams of gelatin, and 1.5 grams of CHA. The scaffold with a ratio of 30:70 (w/w) consists of 0.375 grams of chitosan, 0.375 grams of gelatin, and 1.75 grams of CHA. The scaffold with a ratio of 20:80 (w/w) consists of 0.25 grams of chitosan, 0.25 grams of gelatin, and 2 grams of CHA. The scaffold with a ratio of 10:90 (w/w) consists of 0.125 grams of chitosan, 0.125 grams of gelatin, and 2.25 grams of CHA.

Gelatin was dissolved in 2 ml of 2% acetic acid by stirring at 50°C. The CHA was mixed with 0.94 ml of distilled water and stirred until homogeneous. The diluted CHA was mixed with gelatin gel, and then chitosan powder was added to form a chitosan-gelatin gel and CHA. 0.5 ml of 0.1 M NaOH were added to neutralize the acid. The pH was checked to get the neutral pH (pH = 7), which was then added into the scaffold mold and frozen at -40°C for 2 x 24 hours.

### **C. Functional group analysis**

The functional groups of chitosan, gelatin, CHA, and K-G/CHA scaffold were analyzed using an FTIR (Thermo Scientific) with a wavelength of 400–4000  $\text{cm}^{-1}$ . The resulting graph was then matched with the peak table.

### **D. X-ray diffraction (XRD) analysis**

The XRD analysis was carried out using the X'Pert PRO PAN analytical tool. The monitor was rotated around the sample and set at an angle of  $2\theta$  to the incident flow. The results of this X-ray diffraction was imprinted on paper with a copper (Cu) radiation source with a nickel filter.

### **E. Scanning electron microscopy-energy dispersive X-ray (SEM-EDX) analysis**

An SEM-EDX analysis was obtained using EDAX-AMATEK, which was conducted at 100x and 500x magnification. In the SEM test, 10 pores at 100x magnification were randomly selected. The pore diameter was then measured using ImageJ software.

### **F. Swelling analysis**



The swelling analysis was carried out by weighing the initial weight of the scaffold ( $W_i$ ), immersing the scaffold in distilled water for 24, 72, and 168 hours and then weighing it to get the final weight ( $W_f$ ). The swelling value is calculated by the formula:

$$\text{Swelling} = \frac{W_f - W_i}{W_i}$$

**G. Water content percentage (WCP) analysis**

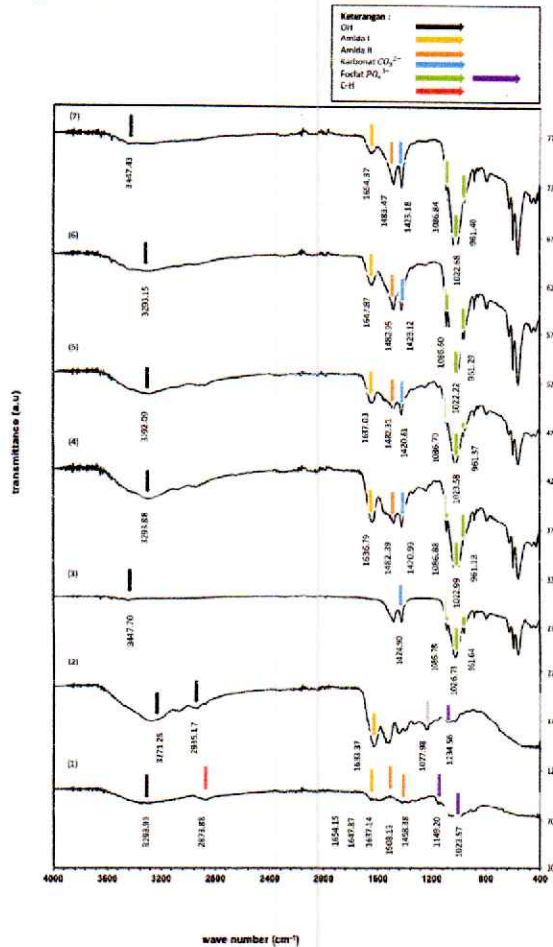
The WCP analysis was carried out by weighing the initial weight of the scaffold ( $W_i$ ), immersing the scaffold in distilled water for 24, 72, and 168 hours and then weighing it to get the final weight ( $W_f$ ). The WCP value is calculated by the formula:

$$\text{WCP} = \frac{W_f - W_i}{W_f} \times 100\%$$

The data were analyzed using Saphiro-Wilk testing to see the normality of the data distribution and a Levene test to see the data homogeneity. If the data were normally distributed and homogeneous, it was continued with the one-way Anova test and Tukey's HSD. If the data were not normally distributed or homogeneous, they were analyzed using Kruskal Wallis and Mann Whitney. The values of  $p < 0.05$  were considered statistically significant.

**3. RESULTS**

**A. Functional group analysis using FTIR**



**Figure 1.** The FTIR pattern of chitosan (1), gelatin (2), and CHA BBK (3) K-G/CHA scaffold with ratios of 40:60 (4), 30:70 (5), 20:80 (6), and 10:90 (7).

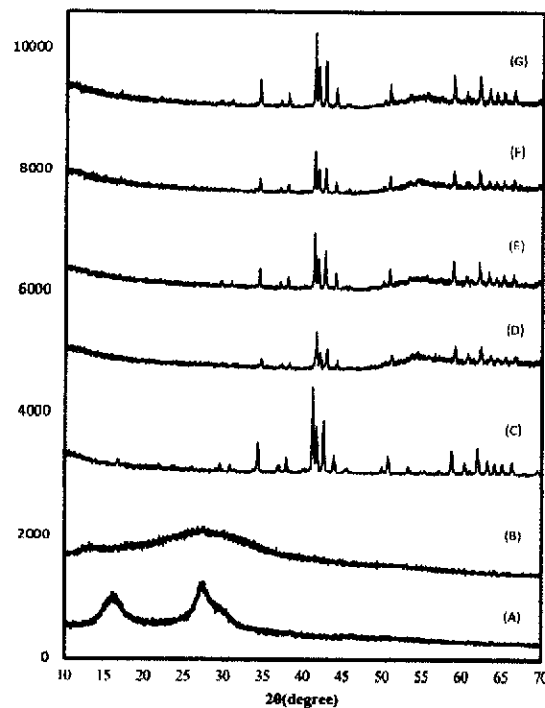
FTIR analysis was performed to analyze the presence of different functional groups for CHA BBK, chitosan, gelatin, and K-G/CHA scaffold, as shown in Figure 1. It was found that CHA BBK has hydroxyl (-OH), carbonate ( $\text{CO}_3^{2-}$ ), and phosphate ( $\text{PO}_4^{3-}$ ) groups. The peak for the hydroxyl groups (-OH) bands at  $3447.70 \text{ cm}^{-1}$ , the carbonate groups ( $\text{CO}_3^{2-}$ ) at  $1424.90 \text{ cm}^{-1}$ , and the phosphate groups ( $\text{PO}_4^{3-}$ ) at  $1086.78 \text{ cm}^{-1}$ ,  $1026.73 \text{ cm}^{-1}$ , and  $961.64 \text{ cm}^{-1}$ .<sup>[17,18]</sup>

Chitosan with a medium molecular weight has hydroxyl (-OH), C-H, amide I, amide II, and amine (C-N) functional groups. The peak for the hydroxyl groups (-OH) bands at  $3293.93\text{ cm}^{-1}$ ; the CH groups at  $2873.88\text{ cm}^{-1}$ ; the amide I groups at  $1654.15\text{ cm}^{-1}$ ,  $1647.87\text{ cm}^{-1}$ , and  $1637.14\text{ cm}^{-1}$ ; the amide II groups at  $1508.13\text{ cm}^{-1}$  and  $1458.38\text{ cm}^{-1}$ ; and the amine groups at  $1149.20\text{ cm}^{-1}$  and  $1023.57\text{ cm}^{-1}$ .<sup>[17,18]</sup>

In gelatin, there are hydroxyl groups (-OH), amide I, carboxylate (-COO), and amine functional groups. The peak for the hydroxyl groups (-OH) bands at  $3271.25\text{ cm}^{-1}$  and  $2935.17\text{ cm}^{-1}$ , the amide I groups at  $1633.37\text{ cm}^{-1}$ , the carboxylate (-COO) groups at  $1234.4\text{ cm}^{-1}$ , and the amine groups at  $1077.98\text{ cm}^{-1}$ .<sup>[17,18]</sup>

The K-G/CHA scaffold with four different ratios showed the presence of the same functional groups, namely the hydroxyl (-OH) groups with peaks at around  $3292.09\text{--}3447.43\text{ cm}^{-1}$ , the amide I groups at around  $1654.37\text{--}1636.79\text{ cm}^{-1}$ , the amide II groups at around  $1483.47\text{--}1482.31\text{ cm}^{-1}$ , the carbonate groups at around  $1423.18\text{--}1420.81\text{ cm}^{-1}$ , and the phosphate groups at around  $1086.88\text{--}961.29\text{ cm}^{-1}$ .

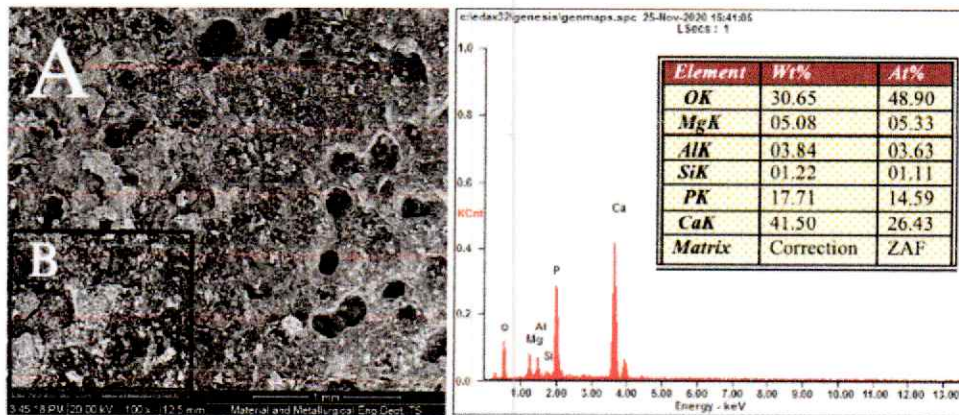
### B. X-ray diffraction (XRD) analysis



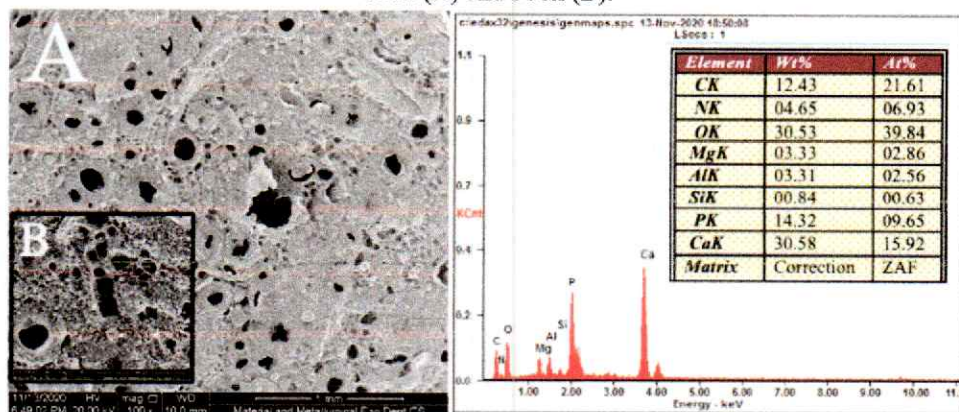
**Figure 2.** X-ray diffraction pattern of chitosan (A), gelatin (B), CHA BBK (C), and K-G/CHA scaffold with ratios of 40:60 (D), 30:70 (E), 20:80 (F), and 10:90 (G).

XRD was performed to analyze the structural properties, as shown in Figure 2. CHA BBK has clear peak patterns, indicating that it has a crystalline form. It showed a similar pattern with the synthetic hydroxyapatite. The XRD patterns of chitosan showed that there are two high peaks with a wide and sloping distance, which indicates that chitosan has a semi-crystalline form. The gelatin sample showed a sloping peak, indicating that gelatin has an amorphous form. The pattern of the K-G/CHA scaffold sample with 40:60 (w/w), 30:70 (w/w), 20:80 (w/w), and 10:90 (w/w) ratios were firm and sloping peakthes. This pattern indicated that the scaffold form is a combination of being crystalline and amorphous.

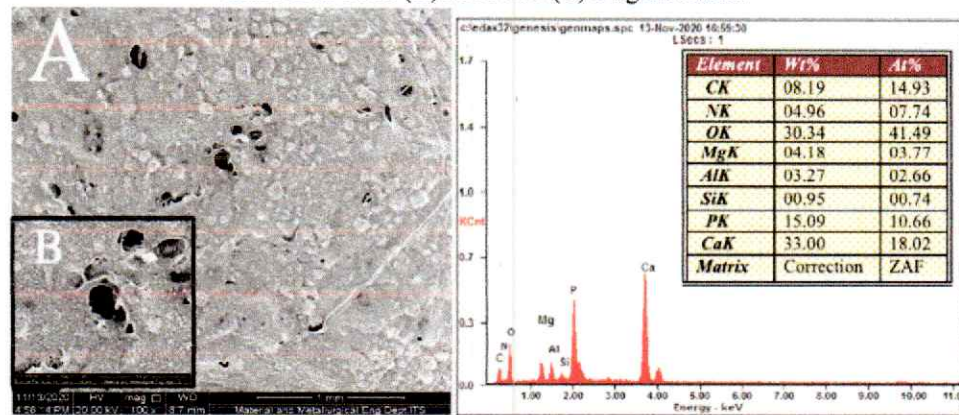
**C. Scanning electron microscopy-energy dispersive X-ray (SEM-EDX) analysis**



**Figure 3.** The SEM-EDX analysis shows the surface morphology and composition of CHA BBK at a magnification of 100x (A) and 500x (B).

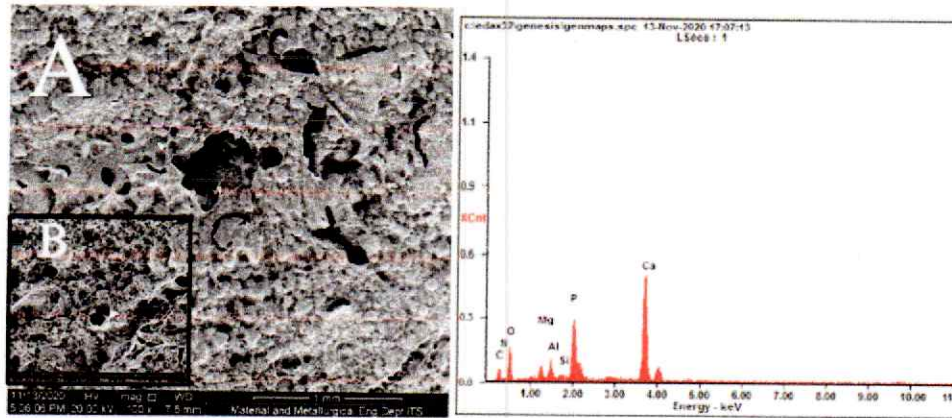


**Figure 4.** The SEM-EDX analysis shows the surface morphology and composition of the K-G/CHA40:60 (w/w) scaffold at 100x (A) and 500x (B) magnification.

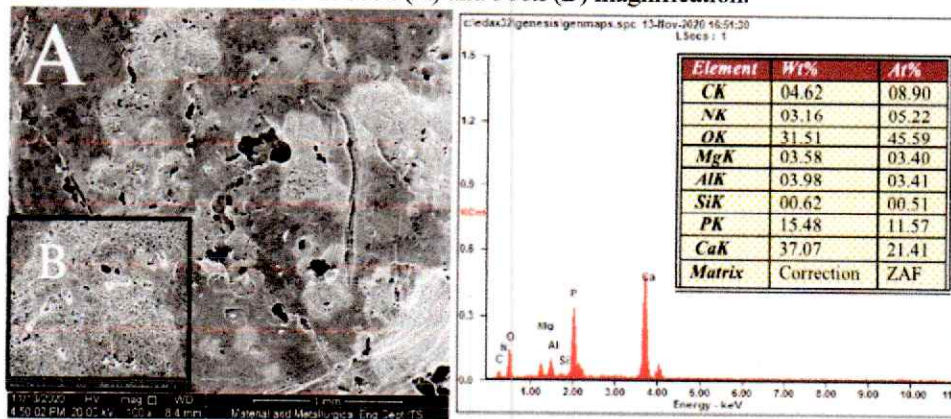


**Figure 5.** The SEM-EDX analysis shows the surface morphology and composition of the K-G/CHA30:70 (w/w) scaffold at 100x (A) and 500x (B) magnification.





**Figure 6.** The SEM-EDX analysis shows the surface morphology and composition of the K-G/CHA20:80 (w/w) scaffold at 100x (A) and 500x (B) magnification.



**Figure 7.** The SEM-EDX analysis shows the surface morphology and composition of the K-G/CHA10:90 (w/w) scaffold at 100x (A) and 500x (B) magnification.

**Table 2.** Pore size of various K-G/CHA scaffold ratios

No	K-G/CHA scaffold ratios	Pore size range	Mean	SD
1	40:60	30,42-282,7 μm	108,72 μm	75.27
2	30:70	40,59-153,8 μm	69,405 μm	35.32
3	20:80	43,71-441 μm	127,69 μm	118.67
4	10:90	44,10-145,2 μm	109,75 μm	37.77

Based on the SEM analysis, the K-G/CHA scaffold with ratios of 40:60 (w/w), 30:70 (w / w), 20:80 (w/ w), and 10:90 (w/w) has a pore size range of 30.42-282.7 μm, 40.59-153.8 μm, 43.71-441.0 μm, and 44.10-145.2 μm , respectively (Table 2).

Based on the results of the EDX analysis shown in Figures 3–7, CHA BBK contained six elements, namely O, Mg, Al, Si, P, and Ca. Moreover, there were eight elements contained in the K-G/CHA scaffold, namely C, N, O, Mg, Al, Si, P, and Ca. The Ca/P ratio was calculated using the atomic percentages of Ca and P, which were obtained from the EDX analysis. It was found that CHA BBK has a Ca/P ratio of 1.81. After the CHA BKK was combined with chitosan and gelatin to form the K-G/CHA scaffold, it was found that the ratio of 40:60 (w / w) had the smallest Ca/P ratio (1.65) and the ratio of 20:80 (w/ w) had the largest Ca/P ratio (1.99) (Table 3).

**Table 3.** Energy dispersive X-ray (EDX) analysis at 100x magnification

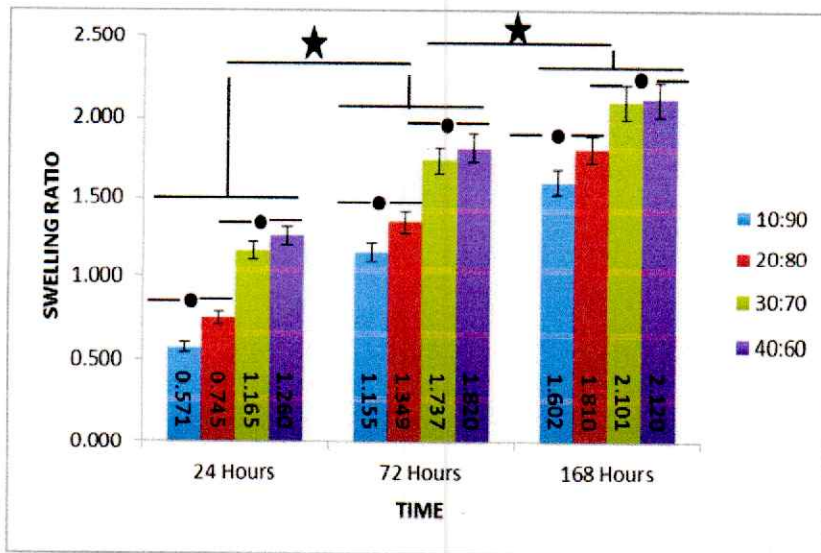
Sample	Ca (At %)	P (At %)	Ca/P ratio
CHA BBK	26.43	14.59	1.81
K-G/CHA 40:60	15.92	09.65	1.65
K-G/CHA 30:70	18.02	10.66	1.69
K-G/CHA 20:80	20.44	10.27	1.99
K-G/CHA 10:90	21.41	11.57	1.85



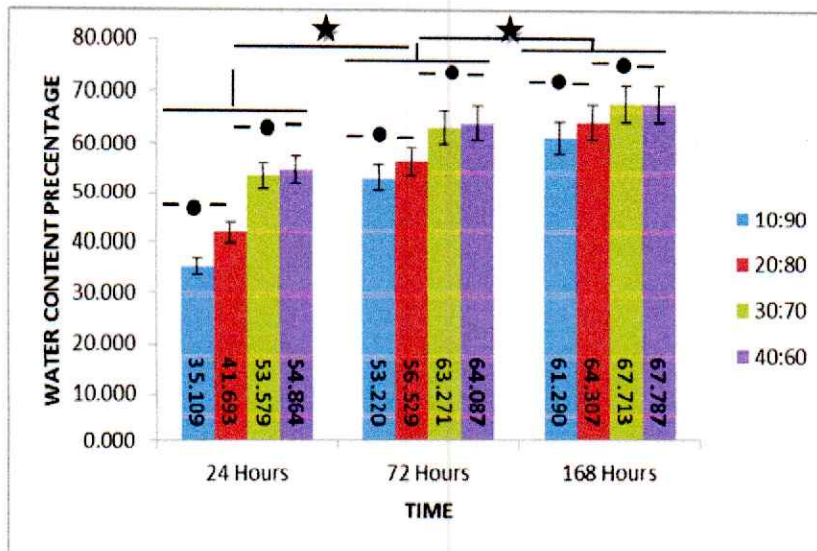
**D. Swelling ratio and water content percentage (WCP) analysis**

Figures 8 and 9 shows that the less CHA composition, the greater the mean value of the WCP. On the other hand, the duration of immersion affects the swelling ratio, the longer the immersion duration, the higher the swelling ratio. The results of the normality test using the Saphiro-Wilk test and the homogeneity using the Levene test showed that the swelling data were normally distributed and homogeneous ( $p > 0.05$ ).

The statistical tests using the one-way ANOVA showed that there is a significant difference ( $p = 0.000$ ) between the K-G/CHA scaffold ratio and the swelling ratio value, indicating that the higher the CHA composition, the lower the swelling ratio value. Using Tukey's HSD statistical test, a significant difference was found between the K-G/CHA scaffold ratios of 10:90, 30:70, and 40:60 also between the ratios of 20:80 and 40:60 ( $p < 0.05$ ). There was a significant difference between 24, 72, and 168 hours of immersion ( $p < 0.05$ ).



**Figure 8.** The swelling ratio for each K-G/CHA scaffold ratio at 24, 72, and 168 hours of immersion.



**Figure 9.** The water content percentage for each K-G/CHA scaffold ratio at 24, 72, and 168 hours of immersion.

Figures 8 and 9 show that the less the CHA composition, the greater the swelling and WCP mean values. When immersed for 24, 72, and 168 hours, it showed that the longer the immersion duration, the more the swelling ratio and WCP were increased. The Saphiro-Wilk and Levene tests showed that the swelling data were normally distributed and homogeneous ( $p > 0.05$ ), while the WCP data were not normally distributed ( $p < 0.05$ ) but were homogeneous ( $p > 0.05$ ).

The one-way Anova and Kruskal Wallis tests showed that there were significant differences between each K-G/CHA scaffold ratio to swelling ratio ( $p = 0.000$ ), and the WCP ( $p = 0.001$ ) indicated that the higher the CHA composition, the lower the swelling ratio and WCP values.

Furthermore, to determine which ratio had a significant difference, the Tukey HSD test for swelling and the Mann Whitney test for the WCP were obtained. There were significant differences in the swelling ratio and the WCP on the



K-G/CHA scaffold ratios of 10:90, 30:70, and 40:60 and between the ratios of 20:80, 30:70, and 40:80. There was a significant difference between 24, 72, and 168 hours of immersion ( $p < 0.05$ ).

#### 4. DISCUSSION

The spectra and FTIR peak values of the K-G/CHA scaffolds can be seen in Figure 2, which shows the presence of hydroxyl groups (OH-) and indicates the involvement of the three biomaterials in the scaffold formation. The hydroxyl group (-OH) comes from the presence of hydrated inorganic compounds.

The presence of the carbonate and phosphate groups were found in the CHA. The carbonate group is a marker of carbonate substitution in the apatite structure, while the phosphate group is a marker for the presence of minerals that support bone growth. A previous study showed the presence of hydroxyl, carbonate, and phosphate functional groups in carbonate apatite synthesized by the precipitation method.<sup>[19]</sup> These three groups were identified in the K-G/CHA scaffold, which indicates carbonate apatite involvement in the scaffold.

The presence of amide I, carboxylate, and amine groups could be identified in type B gelatin from bovine. Gelatin contains Arg-Gly-Asp (RGD) sequences, which are composed of several amino acids. Amino acids are organic compounds that contain amine and carboxylic functional groups, which can be identified from a FTIR band. RGD sequences support bone cell adhesion and migration.<sup>[6,20]</sup> The involvement of gelatin in the K-G/CHA scaffold is characterized by the presence of an amide group I.

Chitosan with a medium molecular weight has C-H, amide I, amide II, and amine groups. The C-H group is a marker of the polysaccharide structure of chitosan, while the hydroxyl and amine groups are reactive functional groups that increase the bonds between the biomaterials.<sup>[20,21]</sup> Hydroxyl groups, C-H, amide I, amide II, and amine groups were found in commercial chitosan (Sigma-Aldrich).<sup>[22,23]</sup> The involvement of chitosan in K-G/CHA is characterized by the presence of amide groups I and amide II.

Amide I and amide II contain C-N groups that show the cross-linking between gelatin and chitosan, which forms the interconnectivity of the scaffold. The chitosan-gelatin complex shows structural similarities with glycosaminoglycans and collagen of an extracellular matrix. In addition, cross-linking between chitosan and gelatin improves the mechanical properties and pore interconnectivity of the scaffold.<sup>[24]</sup>

An XRD analysis was carried out to determine the shape of the sample's particles (Figure 3). The carbonate apatite obtained a clear pattern at each peak and high intensity, indicating that it has a crystalline form. The peak pattern of carbonate apatite is similar to synthetic hydroxyapatite (Sigma-Aldrich) because the carbonate apatite used in this study (CHA BBK) is carbonate ions (calcium carbonate and magnesium carbonate to form CHA) added to hydroxyapatite. The addition of carbonate ions causes changes in the crystal morphology and decreases the crystallinity, which thereby increases the solubility of the materials.<sup>[25]</sup>

The pattern of the XRD analysis on the chitosan sample shows a wide diffraction peak at  $2\theta = 10.01^\circ$  and  $20.05^\circ$  then the intensity decreased (Figure 2). This pattern indicates a semi-crystalline characteristic, and it has a dominant amorphous structure. The XRD pattern on the gelatin sample showed a sloping diffraction between the peaks of  $19.95^\circ$ – $41.66^\circ$ , which indicated the presence of an amorphous form (Figure 3). The results of the study were due to the sloping diffraction because of signals from the amorphous polysaccharides.<sup>[26]</sup> Former study also argued that there was a dominant amorphous structure in chitosan and the gelatin samples.<sup>[6]</sup>

The XRD analysis carried out on the K-G/CHA scaffold with ratios of 40:60 (w/w), 30:70 (w/w), 20:80 (w/w), and 10:90 (w/w) shows a firm peak pattern with high intensity and gentle peaks (Figure 2). This shows that the form of the particle structure of the K-G/CHA scaffold is a crystalline and amorphous combination due to the presence of carbonate apatite, chitosan, and gelatin on the scaffold. The crystalline form is obtained from apatite carbonate, while the amorphous form is obtained from chitosan and gelatin.

The results of the SEM-EDX analysis can be seen in Figures 3–7. A freeze-drying technique produces a scaffold morphology with a porosity of up to 90%.<sup>[16]</sup> The data from our unpublished data showed that the K-G/CHA scaffold with four ratios has porosity of around 57.40%. The lower the polymer concentration, the higher the porosity percentage. This is because at low polymer concentrations, the viscosity of the gel will be lower and contain more water. During the freeze-drying process, the water contained will sublime and produce a larger pore size and porosity. The results of this study are in accordance with other studies that stated an increase in the percentage of porosity due to the reduction in the concentration of alginate as a polymer.<sup>[27]</sup> The porosity, which is suitable for application in the tissue engineering field, is around 50-90%. The scaffold with this porosity percentage shows the ideal pore interconnectivity for cell tissue growth, and it supports the osteoinduction properties.<sup>[6,28]</sup> Based on these results, it can be concluded that it is feasible to apply the K-G/CHA scaffold with four different ratios in the field of tissue engineering due to the supports of cell growth.

A scaffold as bone substitute material must have a micropore and macropore structure. Micropores (<50  $\mu\text{m}$  in diameter) support osteoblast adhesion and growth factors, while macropores (>50  $\mu\text{m}$  in diameter) support bone growth and bone tissue vascularization.<sup>[29,30]</sup> The pores required for bone mineralization is at least 100  $\mu\text{m}$  to provide a conducive environment for cell survival and bone remodeling. Moreover, micropores are required for bone maturation and formation.<sup>[31]</sup>

Based on Table 2, it shows that there are micropore and macropore structures in the K-G/CHA scaffold with a ratio of 40:60 (w/w), 30:70 (w/w), 20:80 (w/w), and 10:90 (w/w). According to previous research data, the pore size of around 50–300  $\mu\text{m}$  allows for adhesion, growth, and migration of vascular tissue.<sup>[15]</sup>

An EDX analysis is used to identify and measure the percentage of elemental composition contained in the scaffold.<sup>[32]</sup> CHA BKK consists of oxygen (O), magnesium (Mg), aluminum (Al), silicon (Si), phosphorus (P), and calcium (Ca). The K-G/CHA scaffold with four different ratios consists of the same elements as CHA BKK, namely carbon (C), nitrogen (N), oxygen (O), magnesium (Mg), aluminum (Al), silicon (Si), phosphorus (P), and calcium (Ca). These results indicated the involvement of apatite carbonate in the K-G/CHA scaffold (Figures 4–7).

CHA on the scaffold plays a role in attracting calcium and phosphorus ions to induce osteogenesis.<sup>[32]</sup> The Ca/P ratio of adult bone tissue is 1.71, and hydroxyapatite is 1.67. It is considered the most effective ratio for bone regeneration.<sup>[33,34]</sup>

The atomic ratio of Ca/P in table 3 were supported by other studies, which stated that carbonate apatite synthesized from calcium sulfate hemihydrate and calcium hydroxide obtained a Ca/P ratio of around 1.53–2.01.<sup>[35]</sup> Based on the results of the study, the four different ratios meet the criteria for use in tissue engineering. A K-G/CHA scaffold with a ratio of 30:70 (w/w) is considered the most optimal because it has a Ca/P ratio that is almost the same as the Ca/P ratio of hydroxyapatite by stoichiometry and bone Ca/P.

After the swelling ratio and WCP analysis were conducted, it showed that the higher the CHA composition, the lower the swelling ratio and WCP values. This is probably because it used CHA derived from hydroxyapatite, which is a carbonate group substitution. Hydroxyapatite has the same chemical structure as nano-hydroxyapatite [ $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ ]. The CHA components affect the cross-linking between CHA with chitosan and gelatin. Nano-hydroxyapatite forms cross-links between chains and reduces the hydrophilicity of gelatin by binding calcium and phosphate to hydrophilic groups—COOH or NH<sub>2</sub>. Some NH<sub>2</sub> from chitosan binds to calcium groups, and then the OH groups cannot form hydrogen bonds, which thereby reduces the swelling ratio of the scaffold.<sup>[36,37]</sup>

The swelling and water absorption is also affected by the duration of immersion, where the values of the swelling ratio and WCP increases to the highest peak at 168 hours of immersion. The value of the swelling ratio and WCP is also affected by the hydrophilic scaffold materials, namely chitosan, gelatin, and carbonate apatite.<sup>[38,39,40]</sup> At a high swelling ratio and at high WCP values, the liquid is easier to penetrate the scaffold so that the scaffold degrades faster.<sup>[41]</sup>

The scaffold was developed to be an efficient protein transport, which is not only in large quantities but also gradually.<sup>[42]</sup> The ability of the swelling affects the hydrophilic properties of the scaffold. This ability is important for the scaffold to carry calcium and phosphate ions into the scaffold for new tissue formation.<sup>[43]</sup> The values of the swelling ratio and WCP affects the hydrolysis and the physical properties, which are good for the scaffold in nutrient transport, which is important for the bone regeneration process.<sup>[44]</sup> Based on previous studies, it is stated that the swelling ratio and WCP can affect the hydrophilic properties of a scaffold. The higher the value of the swelling ratio and WCP, the better the hydrophilic properties. In our study, the highest swelling ratio value was obtained from a K-G/CHA scaffold with a ratio of 40:60, namely  $2.120 \pm 0.11$ , and the highest WCP value of  $67.78\% \pm 1.21$  was also obtained from the K-G/CHA scaffold with a ratio of 40:60. The K-G/CHA scaffold ratio of 40:60 and 30:70 has no significant difference; therefore, the two K-G/CHA scaffold ratios have the same properties. The K-G/CHA scaffold with a ratio of 30:70 was considered suitable because it is similar to the components of the bone extracellular matrix.

## 5. CONCLUSION

A K-G/CHA scaffold with four different ratios, 40:60 (w/w), 30:70 (w/w), 20:80 (w/w), and 10:90 (w/w) has the appropriate functional groups, the crystal and amorphous particles form, micropore and macropore structures, and the same elemental content. A K-G/CHA scaffold with a ratio of 30:70 (w/w) has the most ideal ratio (1.69). A K-G/CHA scaffold with a ratio of 30:70 has an ideal value of the swelling ratio ( $2.10 \pm 0.11$ ) and WCP ( $67.71\% \pm 0.56$ ).

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### Comments:

1. This paper is pretty well written.
2. Please label the equations used in this study, include also the descriptions to the parameters used.
3. Many references are not written in complete form, please pay a close look into them (journal name, vol, issue, etc).
4. The conclusion to work is too short, please consider adding few more sentences to conclude more aspects of the findings.



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#### **8792-Article Text-37718-1-4-20210522 – Reviews**

1. Minor grammatical errors need to be corrected, important.
2. In abstract, need to explain what WCP short form is meant.
3. Introduction part – paragraph 5 – superscript '3' in the chemical formula
4. Do space between sub-headings, for example in materials & methods
5. Page 2 – para 1 – line 7 - Carbonate apatite is utilized in the field of bone tissue engineering because of its content that is closely resembling bone apatite as compared to calcium phosphate (This is only a suggestion. Authors may re-phrase accordingly once proofread)
6. Page 2 – para 2 – line 1 - Carbonate Chitosan and gelatin combined with carbonate apatite are – May be changed to – The combination of carbonate chitosan and gelatin with the carbonate apatite are .....
7. Page 4 – last line – what is 'peakthes'?
8. Overall, the paper is good. It needs to be properly proofread. There are minor mistakes in the write up. It will be great if authors can highlight the benefit of the locally produced CHA in combination with chitosan-gelatin as compared to other bone scaffold mix by other researchers.



12:43



pt 14 Sep



to Anita, Devi, Geo, Rania, Alq...

Anita Yuliati, Devi Rianti, Geo Fanny, Rania Vivian Nathania, Alqomariyah Eka Purnamasari, Rifayinqa Ruyani Putri, Helal Soekartono, Soebagio Soebagio, Ardiyansyah Syahrom:

We have reached a decision regarding your submission to International Journal of Integrated Engineering, "The characteristics, swelling ratio and water content percentage of chitosan-gelatin/limestone-based carbonate hydroxyapatite composite scaffold: chitosan-gelatin/limestone-based carbonate hydroxyapatite composite scaffold".

Our decision is to: Accept Submission subject to revision required as in the reviewer report.

Prof. Madya Ir. Dr. Rubita binti Sudirman  
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