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Characteristics of Chitosan Gelatine Limestone-Based Carbonate Hydroxyapatite Composite Scaffold After Crosslinking

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Abstract

Bone damage is one of the most common cases in dentistry. Tissue engineering is advancing in biotechnology to aid bone regeneration using scaffolds. Scaffolds need to be biocompatible, bioactive, and bioresorbable. Purpose: Analyzing the characteristics of scaffold C-G:CHA after crosslinking with 0.25% glutaraldehyde. Methods: The scaffold is synthesized from C–G:CHA in ratios of 40:60, 30:70, and 20:80 (w/w) using a freeze-drying technique and crosslinked with 0.25% glutaraldehyde. Compressive strengths are tested with a Universal Testing Machine Mini Autograph. FTIR, XRD, and SEM EDX were used to identify the most optimal base of each ratio. Data are analyzed with a one-way ANOVA parametric test. Results: The FTIR test showed that adding 0.25% glutaraldehyde formed a new chemical group. The XRD test indicated the use of 0.25% glutaraldehyde as a crosslinking agent contributed to the scaffold having an amorphous form. The SEM test results of the porosity of the C-G:CHA were 88.41% to 91.14%. After crosslinking the porosities slightly decreased. The EDX analysis showed that the Ca/P ratio in the C-G:CHA scaffold is 1.79 to 2.07. The average compressive strength of the C-G:CHA scaffold increases after being crosslinked with glutaraldehyde. Conclusion: Scaffold C-G:CHA crosslinked with 0.25% glutaraldehyde effective to increase compressive strength. The 30:70 ratio is ideal because it has a Ca/P ratio and average pore size closest to bone.

Keywords: Scaffold; Carbonate hydroxyapatite; Chitosan; Gelatine; Glutaraldehyde; Characteristic.

1. Introduction

Bone damage in the oral cavity is a common issue in the field of dentistry [1]. It can be caused by periodontal disease, reconstructive surgery, and trauma [2]. One approach to repairing bone damage is through the use of bone grafts or tissue engineering concepts [3]. There are three basic components of tissue engineering, often referred to as the "tissue engineering triad" : cells, scaffolds, and growth factors [4]. Scaffold is a three-dimensional biomaterial that provides a suitable medium for cells to regenerate tissues and organs [5]. The scaffold structure must have high porosity and interconnectivity to allow for cell attachment, facilitating tissue regeneration, proliferation, and differentiation [6].

Previous research on scaffolds with porous structures involved the combination of three materials: chitosan-gelatine (K-G) and carbonate hydroxyapatite (KHA) [7]. Chitosan is a biopolymer with cationic properties and other important characteristics for scaffold applications, such as being non-toxic, biodegradable, and biocompatible [8]. The combination of gelatine and chitosan is often used as a scaffold material [9]. Gelatine is used as one of the scaffold materials due to its good biocompatibility; the addition of gelatine can also enhance osteoblast attachment, cell migration, and tissue mineralization [10]. Carbonate hydroxyapatite exhibits better biological properties due to its low crystallinity and increased surface area, making it a suitable material for biomedical applications [11].

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Scaffolds must also meet the requirements of being biocompatible and biodegradable. Biocompatibility refers to the scaffold's ability to support cell attachment and proliferation without causing excessive inflammatory reactions [1]. Scaffolds for bone tissue engineering have several requirements, including adequate mechanical strength that corresponds to human trabecular bone, ranging from 1-12 MPa, and an appropriate degradation rate of around 2-3 months [12]. In studies conducted by Alqomariyah (2021) and Rifayinqa (2021) on the compressive strength and biodegradation of K-G scaffolds, the highest average compressive strength was found in K-G scaffolds with a ratio of 40:60 (w/w), which measured 4.195 MPa. The biodegradation rate for K-G scaffolds with the same 40:60 (w/w) ratio was 20-25.98% on the 21st day. These values meet the lower end of the standard criteria for compressive strength and biodegradation, but further improvement is needed to bring these values closer to the upper standard limits. These values meet the lower end of the standard criteria for compressive strength and biodegradation of the scaffold, so they need to be improved to bring the compressive strength and biodegradation values closer to the upper standard limits.

One way to improve the compressive strength and biodegradation of the scaffold is by crosslinking the C-G:CHA scaffold to enhance its mechanical properties and biodegradation using natural or synthetic reagents. One of the most common reagents is glutaraldehyde, due to its high efficiency in stabilizing gelatine-chitosan materials [13]. The differences in the composition ratios of the scaffold components and the use of crosslinking methods will result in different scaffold characteristics. Therefore, it is necessary to test the characteristics of the scaffold using Fourier Transform Infrared (FTIR), Scanning Electron Microscope-Energy Dispersive X-ray (SEM-EDX), and X-Ray Diffraction (XRD) analysis, and compressive strength test.

2. Material and methods

2.1 Scaffold C-G:CHA 30:70 Synthesis

The materials used in this study included chitosan with medium molecular weight (Sigma Aldrich 448877, USA), bovine gelatine (Sigma Aldrich G9391, USA), CHA powder generated from limestone by Balai Besar Keramik Indonesia (BBK Indonesia), sodium hydroxide (Biomedicine), acetic acid (Merck), distilled water (Duta Farma), 7F2 osteoblast cells (ATCC CRL 12557), culture medium containing DMEM (Sigma Alderich, D6429), Penicillin Streptomycin 1% (Sigma Aldrich), dimethyl sulfoxide (Vivantic, PC0906), Foetal Bovine Serum 10% (Sigma Aldrich), MTT (Sigma Aldrich), Amphotericin B Solution (Sigma Aldrich, A2942), Phosphate Buffer Saline (Sigma Aldrich 806552, USA), Kanamycin, and glutaraldehyde (Merck 354400). The scaffold with a ratio of 30:70 (w/w) consisted of 0.375 grams of chitosan, 0.375 grams of gelatine, and 1.75 grams of CHA [14].

The gelatine was dissolved in 2 ml of 2% acetic acid by stirring at a temperature of 50°C. Then, CHA was mixed with 0.94 ml of distilled water and stirred until homogeneous. The diluted CHA was combined with the gelatine gel, and chitosan powder was added to form a chitosan-gelatine gel and CHA mixture. 0.5 ml of 0.1 M NaOH was added to neutralize the acid. The pH was checked to ensure a neutral pH level of 7, then put into a scaffold mold and frozen at -80°C for 24 hours followed by a freeze-dry process for 24 hours [14]. The crosslink scaffold in this study used the immersion method. The scaffold was rehydrated in 0.05 M acetic acid solution for the first 15 minutes, then the crosslink method was carried out by immersing it in a glutaraldehyde solution dissolved in double distilled water with a concentration of 0.25% at a temperature of 4°C for 24 hours. The scaffold was then washed (10 minutes x 5 times), then freeze-dried for 24 hours [15].

2.2 FTIR, XRD, SEM-EDX Analysis

The functional groups of chitosan, gelatine, CHA, and C-G/CHA scaffolds were analyzed using FTIR (Nicolet iS10) by clamping the sample in a sample holder. The resulting graph was then read by matching it to the peak table [16]. XRD analysis was performed using a Rigaku Benchtop Miniflex 600 analyzer. The monitor was rotated around the sample and set at an angle of 2θ to the incident path. The results of this X-ray diffraction will be printed on paper with a copper (Cu) radiation source with a nickel filter [17]. SEM-EDX analysis was obtained using Thermo Fisher Scientific, which was performed at 100x and 500x magnification. The pore diameter was then measured using ImageJ software [18].

2.3 Compressive Strength Test

The compressive strength of the scaffold was analyzed using a Universal Testing Machine Mini Autograph sensor load cell L IP3 Class 0.02 with a Phyton 2.7 microcontroller software. Measure the diameter and height of the KG:CHA scaffold using a vernier caliper, then calculate its surface area. The KG:CHA scaffold sample is placed in the middle of the press tool with the vertical axis perpendicular to the flat plane. The Universal Testing Machine Mini Autograph sensor load cell L IP3 Class 0.02 is activated then the press will slowly press the KG:CHA scaffold sample with a compressive load of 400 N and a speed of 2 mm/min until the KG:CHA scaffold sample experiences distortion. The tool will be stopped after

the graph on the monitor shows an increase after a decrease, this indicates that the load given is no longer pressing the KG:CHA scaffold but is only distributed to the upper and lower presses. Calculations will be made from graph results showing the displacement and force received by the scaffold as the maximum load divided by the surface area of the scaffold sample. The data is then entered into the compressive strength formula to calculate the compressive strength value with MPa units [19].

The data was then analyzed using the Shapiro-Wilk test to determine the normality of the data, then a homogeneity test was performed using the Levene test. If the data is normally distributed (p-value > 0.05), it is continued with the parametric one-way ANOVA and Post Hoc Tukey HSD tests. If the data is not normally distributed (p-value < 0.05), it is continued with non-parametric statistical tests with Kruskal Wallis and Mann-Whitney-Wilcoxon U.

3. Results and discussion

3.1 Functional Group Analysis using FTIR

Based on the results of FTIR tests on the C-G:CHA scaffold without 0.25% glutaraldehyde crosslink and with 0.25% glutaraldehyde crosslink, the presence of hydroxyl functional groups (-OH) can be identified in the spectrum range of 3700-3400, 3550-3500, 3300-2500 cm-1, amide I at a spectrum distance of 1685-1630 cm-1, amine II at a spectrum distance of 1550-1450 cm-1, carbonate (CO32-) at a spectrum distance of 1450-1410 cm-1, phosphate (PO43-) at a spectrum distance of 1300-1050 and C=N at a spectrum distance of 1690-1640 cm-1. The amide I bond is a C=O bond while amide II represents a flexible N-H bond. Carbonate groups and phosphate groups are indicators of the involvement of hydroxyapatite carbonate in the scaffold. The results of the FTIR test showed that in each scaffold ratio C-G:CHA there were groups that were markers of the involvement of hydroxyapatite carbonate, chitosan, and gelatine. The reaction of adding 0.25% glutaraldehyde to chitosan material is indicated by the formation of a new group, namely the C=N group located in the absorption region of 1690-1640 cm-1 which overlaps with amide I which is at the wavelength of 1680-1630 cm-1.

The crosslinking process involves the reaction of two carbonyl groups from glutaraldehyde with amine groups in lysine from gelatine and glucosamine from chitosan, forming imine bonds (-C=N-) through Schiff base reactions. Additionally, the carbonyl group (C=O) bonds with hydroxyl groups in chitosan via acetalization, resulting in a C–O–C–O–C structure [20].

3.2 X-ray Diffraction (XRD) Analysis

From the XRD test results, almost similar results were obtained, namely the presence of a graphic pattern with a firm and sloping peak which indicates that the shape of the C-G:CHA scaffold is a combination of amorphous and crystalline, due to the combination of the composition of hydroxyapatite carbonate, chitosan, and gelatine, the use of 0.25% glutaraldehyde as a crosslink agent is one of the factors that the scaffold has an amorphous form.

Based on the results of XRD testing that has been carried out on scaffold C-G:CHA 40:60 (w/w), 30:70 (w/w) and 20:80 (w/w) and scaffold C-G:CHA 40:60 (w/w), 30:70 (w/w) and 20:80 (w/w) crosslink glutaraldehyde 0.25% showed the presence of a sharp peak shape with high intensity indicating a crystal form and a wide and gentle peak indicating an amorphous form. This is based on the form of hydroxyapatite carbonate and chitosan which are basically crystalline, gelatine whose structure is amorphous according to research conducted by Maji et al. (2016) [21] and according to research conducted by Acharyulu et al. (2014) [22] chitosan crosslinked with glutaraldehyde showed two short and wide peaks 2θ=16 and 2θ=30 which is evidence that chitosan crosslinked with glutaraldehyde has an amorphous form which is also supported by research conducted by Li et al (2013) [23] who crosslinked chitosan with glutaraldehyde showed changes in the structure of chitosan which initially had a high degree of crystallinity, but after crosslinking with glutaraldehyde, the characteristic peaks of chitosan which were initially in two sharp peaks in the diffraction 2θ = 10° and 20° disappeared, then a wide peak appeared at 2θ = 15° which means a decreased crystallinity reaction, this is caused by the deformation of strong hydrogen bonds in chitosan due to the substitution of hydroxyl and amino groups, which are efficiently able to destroy the regularity of the original chitosan chain and cause the crosslinked chitosan to be predominantly amorphous.

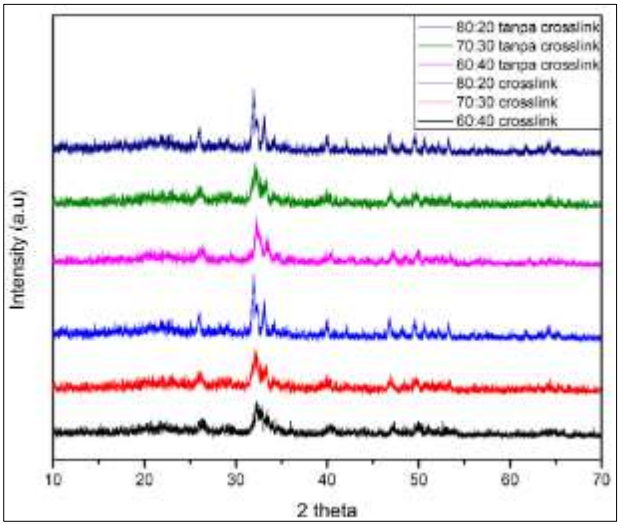

Figure 1 Graph of X-Ray Diffraction (XRD) results

3.3 Scanning Electron Microscopy-energy Dispersive X-ray (SEM-EDX) Analysis

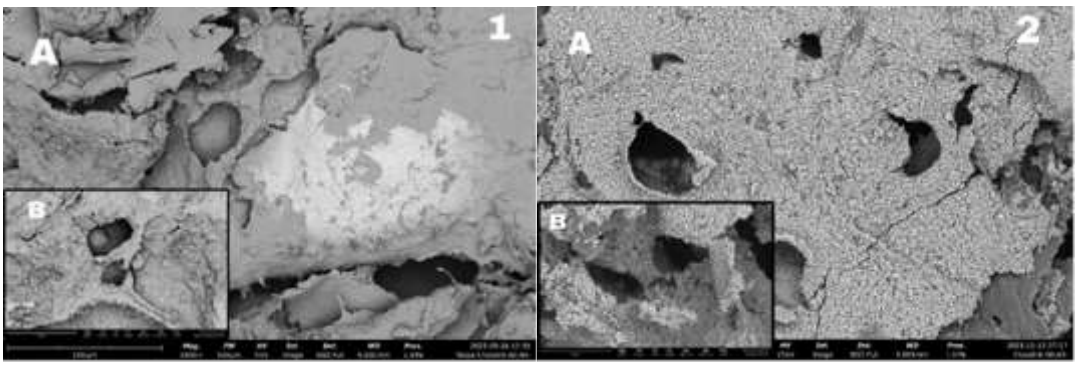

Figure 2 SEM results of C-G:CHA 40:60 scaffold without crosslink (1) and with 0.25% glutaraldehyde crosslink (2) at 100x (A) and 500x (B) magnification.

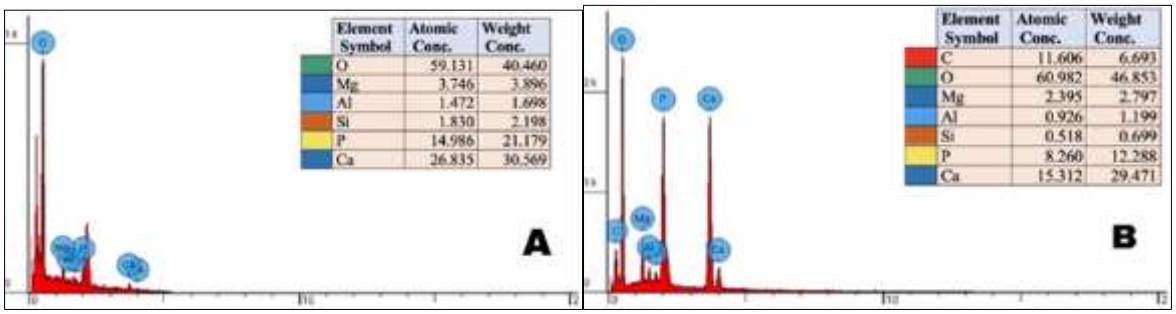

Figure 3 EDX results of K-G:KHA 40:60 scaffold without crosslinking (A) and with 0.25% glutaraldehyde crosslinking (B)

Based on the SEM test results, the pore size of the C-G:CHA 40:60 (w/w), 30:70 (w/w) and 20:80 (w/w) scaffolds at 100x magnification had a pore diameter range of 37.15-144.28 µm, 40.34-103.44 µm and 50.18-126.15 µm, while the C-G:CHA 40:60 (w/w), 30:70 (w/w) and 20:80 (w/w) crosslinked glutaraldehyde 0.25% scaffolds had a pore diameter range of 23.78-116.30 µm, 21.18-103.23 µm and 43.68-106.50 µm and the results of the porosity calculation (Table 2) showed the results of the porosity of the C-G:CHA 40:60 (w/w), 30:70 (w/w) and 20:80 (w/w) scaffolds were 88.41%, 90.15% and 91.14% respectively, while the C-G:CHA 40:60 (w/w), 30:70 (w/w) and 20:80 (w/w) scaffolds with 0.25% glutaraldehyde crosslink yielded 86.66%, 89.37% and 89.60%.

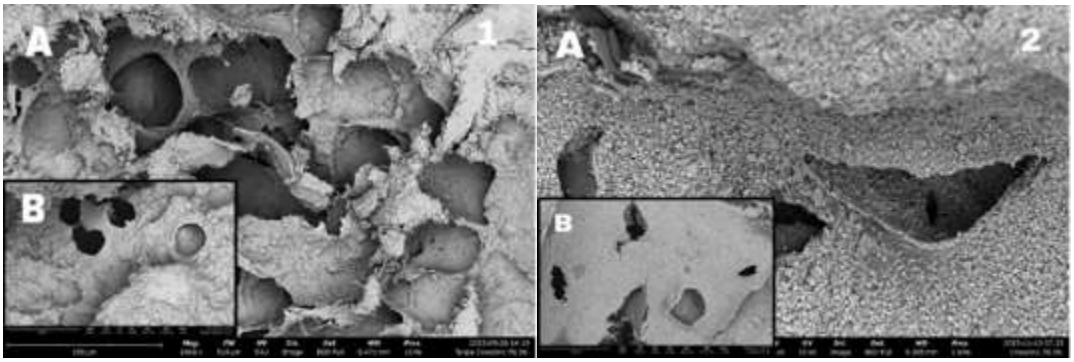

Figure 4 SEM results of C-G:CHA 30:70 scaffold without crosslink (1) and with 0.25% glutaraldehyde crosslink (2) at 100x (A) and 500x (B) magnification.

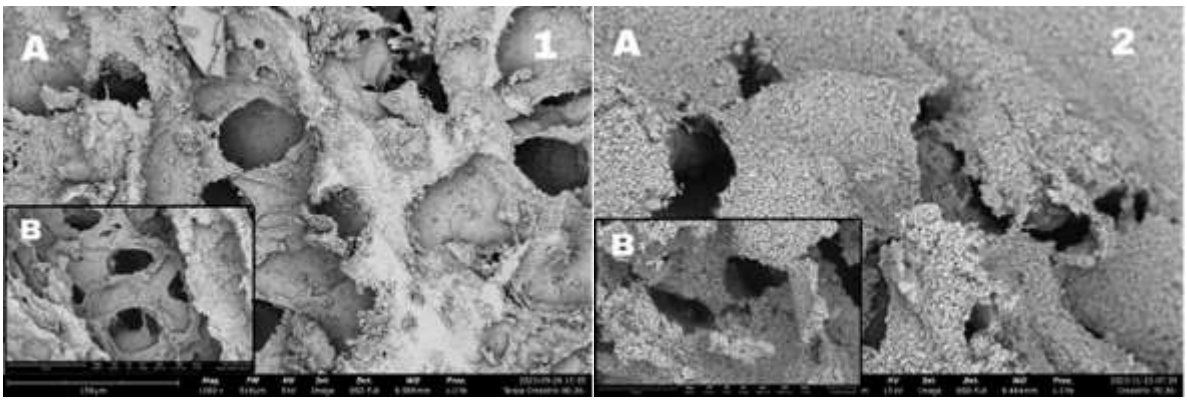

Figure 5 EDX results of K-G:KHA 30:70 scaffold without crosslinking (A) and with 0.25% glutaraldehyde crosslinking (B)

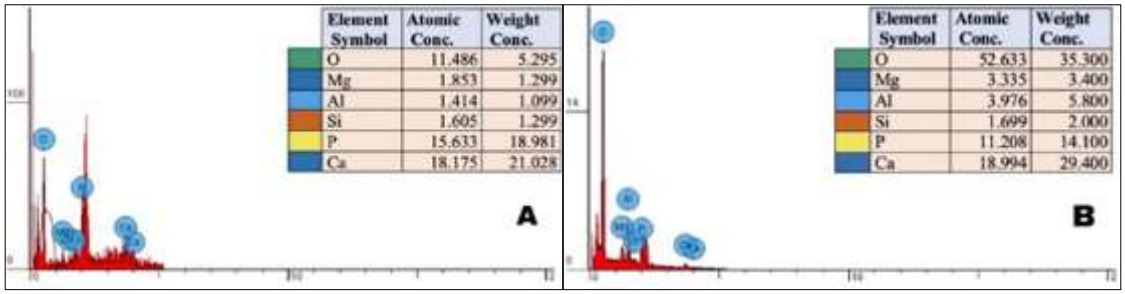

Figure 6 SEM results of C-G:CHA 20:80 scaffold without crosslink (1) and with 0.25% glutaraldehyde crosslink (2) at 100x (A) and 500x (B) magnification.

Scaffolds as bone substitutes must have the same morphology as real bones, namely having pores with varying pore diameters, namely having a micropore structure with a diameter of <50 μm and macropores with a diameter of >50 μm to support the acceleration of the bone regeneration process and bone tissue vascularization [24]. The scaffold pores needed for bone mineralization to occur at least 100 μm in size to provide a conductive environment and cell survival and bone remodeling, but micropores (<50 μm) are also needed for bone maturation and formation [25]

The bonds formed during crosslinking increase the viscosity of the scaffold mixture, leading to a denser and more robust structure. These bonds make the solid structure resistant to damage during the separation of solid and liquid phases in freeze drying, resulting in smaller pore sizes and lower porosity after the process [26]. Smaller pore sizes increase the density of scaffold particles, so when subjected to compressive forces, more particles bear the load, thereby increasing the compressive strength, which is inversely related to porosity.

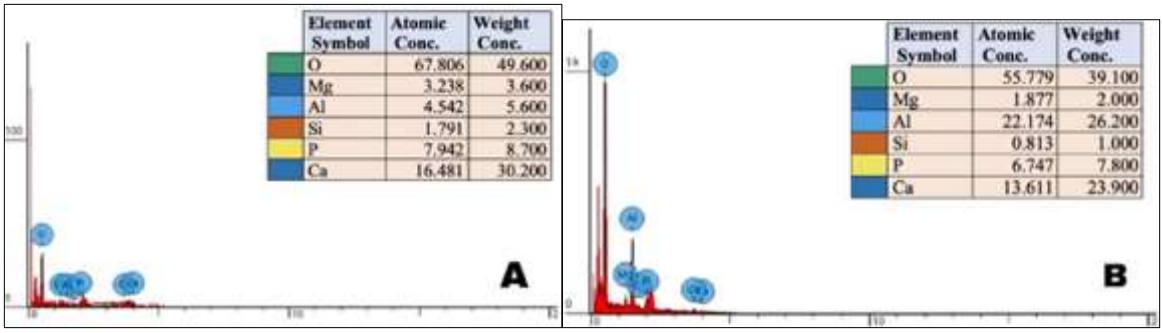

Figure 7 EDX results of K-G:KHA 20:80 scaffold without crosslinking (A) and with 0.25% glutaraldehyde crosslinking (B)

Table 1 Pore diameter, average pore diameter and percentage of porosity value

SN.	Sample	Pore diameter range	Average pore diameter	Porosity (%)
1	Scaffold C-G:CHA 40:60	37.15-144.28 µm	84.87 µm	88,41%
2	Scaffold C-G:CHA 40:60 crosslink glutaraldehid 0.25%	23.78-116.30 µm	75.03 µm	85,75%
3	Scaffold C-G:CHA 30:70	40.34 – 103.44 µm	76,79 µm	90,15%
4	Scaffold C-G:CHA 30:70 crosslink glutaraldehid 0.25%	21.81-103.23 µm	65.88 µm	86,65%
5	Scaffold C-G:CHA 20:80	50.18-126.15 µm	81.06 µm	91,14%
6	Scaffold C-G:CHA 20:80 crosslink glutaraldehid 0.25%	43.68-106.50 µm	68.76 µm	87,92%

In the EDX results, the atomic percentage of each element is obtained, the atomic percentage of carbonate (Ca) and Phosphate (P) is calculated and then the Ca/P ratio is obtained as in Table 5.4. In the C-G:CHA scaffold sample, the percentage of Ca/P ratio at a ratio of 40:60 is 1.79, a ratio of 30:70 is 1.99 and a ratio of 20:80 is 2.07. While in the C-G:CHA scaffold sample crosslink glutaraldehyde 0.25% shows the percentage of Ca/P at a ratio of 40:60 is 1.77, a ratio of 70:30 is 1.69 and a ratio of 20:80 is 2.01. So in the results of this EDX test, the C-G:CHA scaffold that has the largest and ideal ratio is the C-G:CHA scaffold crosslink glutaraldehyde 0.25% with a ratio of 30:70 with a ratio of 1.69.

Table 2 Energy Dispersive X-ray (EDX) test results

SN.	Sample	Ca (At%)	P (At%)	Ca/P ratio
1	Scaffold C-G:CHA 40:60	26.83	14.98	1.79
2	Scaffold C-G:CHA 40:60 crosslink glutaraldehid 0.25%	15.31	8.26	1.77
3	Scaffold C-G:CHA 30:70	31.18	15.63	1.99
4	Scaffold C-G:CHA 30:70 crosslink glutaraldehid 0.25%	18.99	11.20	1.69
5	Scaffold C-G:CHA 20:80	16.48	7.94	2.07
6	Scaffold C-G:CHA 20:80 crosslink glutaraldehid 0.25%	13.61	6.74	2.01

The EDX test can determine the atomic ratio of calcium (Ca) and phosphate (P) or the Ca/P ratio. The Ca/P results on the C-G:CHA 40:60 (w/w), 30:70 (w/w) and 20:80 (w/w) scaffolds were 1.79, 1.99, 2.07 respectively, while for the C-G:CHA 40:60 (w/w), 30:70 (w/w) and 20:80 (w/w) crosslinked glutaraldehyde 0.25% scaffolds were 1.77, 1.69, and 2.01 respectively. The Ca/P ratio in adult bone tissue is 1.71 and the Ca/P ratio of hydroxyapatite carbonate has an optimal Ca/P ratio value of generally 1.67. The Ca/P value affects the strength and mechanical properties of

hydroxyapatite carbonate, because the greater the Ca/P ratio, the strength increases and reaches a maximum at a Ca/P value of 1.67 [26]. The Ca/P value ratio will decrease if it has reached a maximum (1.67) [27].

3.4 Compressive Strength of Scaffold C-G:CHA

The compressive strength value of the C-G:CHA scaffold is determined by inputting the test results from the Mini Autograph Universal Testing Machine's L IP3 Class 0.02 load cell sensor into Python 2.7 microcontroller software. The results, along with their standard deviations, are presented in Table 4 and Figure 4. The average compressive strength of the C-G:CHA scaffold across all ratios increases after being crosslinked with glutaraldehyde.

Table 3 The compressive strengths of various C–G:CHA scaffold ratios (MPa)

SN.	Sample	n	Average of compressive strength value	Standard deviation
1	Scaffold C-G:CHA 40:60	5	3.3488	0.27643
2	Scaffold C-G:CHA 40:60 crosslink glutaraldehid 0.25%	5	4.284	0.37137
3	Scaffold C-G:CHA 30:70	5	3.337	0.44665
4	Scaffold C-G:CHA 30:70 crosslink glutaraldehid 0.25%	5	3.4614	0.50437
5	Scaffold C-G:CHA 20:80	5	1.4694	0.35154
6	Scaffold C-G:CHA 20:80 crosslink glutaraldehid 0.25%	5	3.054	0.46913

The bonds formed during crosslinking increase the viscosity of the scaffold mixture, leading to a denser and more robust structure. These bonds make the solid structure resistant to damage during the separation of solid and liquid phases in freeze drying, resulting in smaller pore sizes and lower porosity after the process [28]. Smaller pore sizes increase the density of scaffold particles, so when subjected to compressive forces, more particles bear the load, thereby increasing the compressive strength, which is inversely related to porosity.

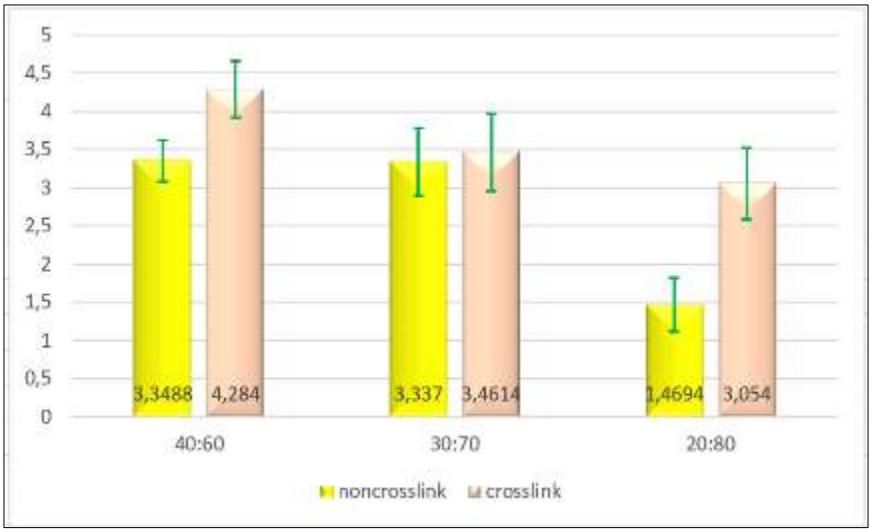

Figure 8 Graph of the average compressive strength of C–G:CHA scaffold

A high carbonate hydroxyapatite ratio in the scaffold KG:KHA 20:80 structure leads to larger pores, which affects the compressive strength of the scaffold. Sefarzadeh (2019) [29] study indicates that larger pore sizes reduce the scaffold's strength under pressure, as higher porosity results in fewer particles bearing the load, leading to the lowest compressive strength for the scaffold KG:KHA 20:80.

The highest increase in compressive strength was observed in the scaffold KG:KHA 40:60. Compressive strength increases when the carbonate hydroxyapatite ratio decreases and the chitosan-gelatine ratio increases. A higher chitosan-gelatine ratio strengthens the bonds between the carboxyl groups in gelatine and the amine groups in chitosan, resulting in a denser scaffold structure [30].

A higher chitosan-gelatine ratio also produces more imine bonds between aldehyde groups and amine groups in chitosan and gelatine, forming a more robust scaffold structure [31]. The increase in compressive strength at higher chitosan and gelatine ratios is also due to the C-N groups in amide I and amide II of gelatine and chitosan, which indicate crosslinking between gelatine and chitosan, forming interconnections that enhance the mechanical properties of the scaffold C-G:CHA

4. Conclusion

The scaffold C-G:CHA with ratios of 20:80, 30:70, and 40:60 (w:w), crosslinked with 0.25% glutaraldehyde, shows enhanced properties, particularly at the 30:70 ratio. The scaffold C-G:CHA at a 30:70 ratio, crosslinked with glutaraldehyde, has a reduced porosity of 89.37%, resulting in more favorable pore interconnectivity for supporting bone regeneration. The Ca/P ratio in the scaffold C-G: CHA 30:70 scaffold crosslinked with 0.25% glutaraldehyde is the most optimal at 1.69, closely resembling natural bone tissue. Furthermore, the scaffold exhibits improved compressive strength after crosslinking with glutaraldehyde. The compressive strength of scaffold C-G: CHA after crosslinking with 0.25% glutaraldehyde meets the minimum standard for scaffold compressive strength, which is 1-12 MPa. Scaffolds that meet this standard can withstand pressure during the formation of natural bone tissue.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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