IJSCIA
International Journal of Scientific Advances
ISSN: 2708-7972
Volume: 7 | Issue: 6 | Nov – Dec 2026 Available Online: www.ijscia.com
DOI: 10.51542/ijscia.v7i6.11

Differences in Swelling and Water Content Percentage Between Non-Crosslinked and 0.25% Glutaraldehyde-Crosslinked Chitosan–Gelatin–Carbonate Hydroxyapatite Composite Scaffolds

Togi Ozora Sihaloho[1], Devi Rianti[2]*, Titien Hary Agustantina[2]

[1]Undergraduate Student of Dental Medicine, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia

[2]Department of Dental Materials, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia

E-mail: togi.ozora.sihaloho-2023@fkg.unair.ac.id; devi-r@fkg.unair.ac.id; titien-h-a@fkg.unair.ac.id

*Corresponding author: Devi Rianti; devi-r@fkg.unair.ac.id

ABSTRACT

Background: Scaffolds are three-dimensional structures that serve as a site for cell attachment, proliferation, and differentiation in bone tissue engineering. The combination of chitosan, gelatin, and carbonate hydroxyapatite (K-G:CHA) is widely used because of its biocompatibility, bioactivity, and similarity to the natural extracellular matrix of bone. However, its mechanical strength still needs to be improved through a crosslinking process. Crosslinking using 0.25% glutaraldehyde is known to improve the mechanical stability of scaffolds, but it can also affect their physical characteristics, particularly their ability to absorb and retain fluids, which can be evaluated through swelling and water content percentage (WCP). These two parameters are important for supporting nutrient diffusion, metabolite exchange, and providing a suitable physiological environment for cell growth and differentiation. ***Objective:*** This study aimed to analyze the differences in swelling and water content percentage of K-G:CHA composite scaffolds with ratios of 30:70 (w/w) and 40:60 (w/w), both non-crosslinked and crosslinked with 0.25% glutaraldehyde, after immersion in simulated body fluid (SBF). ***Methods:*** K-G:CHA scaffolds with ratios of 30:70 (w/w) and 40:60 (w/w) were synthesized using deep- freezing and freeze-drying methods. The scaffolds were then divided into non-crosslinked and 0.25% glutaraldehyde-crosslinked groups. The samples were immersed in SBF for 1, 3, and 7 days (24, 72, and 168 hours). Swelling and water content percentage were calculated based on changes in scaffold weight before and after immersion. Statistical analysis was performed using the Shapiro–Wilk test, Levene's test, one-way ANOVA, and the post hoc Tukey test. ***Results:*** Both swelling and water content percentage increased with longer immersion time. The scaffolds crosslinked with 0.25% glutaraldehyde showed higher swelling and water content percentage values compared with the non-crosslinked groups. Statistical analysis showed significant differences between the treatment groups ($p < 0.05$). ***Conclusion:*** Crosslinking K-G:CHA composite scaffolds with 0.25% glutaraldehyde increased their swelling and water content percentage values by improving fluid retention capacity and structural stability. Therefore, these scaffolds have the potential to support their function as biomaterials for bone tissue engineering.

Keywords: Scaffold; chitosan–gelatin–carbonate hydroxyapatite; 0.25% glutaraldehyde; swelling; water content percentage

INTRODUCTION

One of the main components used in bone tissue engineering is a scaffold. A scaffold is a three-dimensional structure that supports the formation of new tissue while providing an environment that promotes cell attachment, proliferation, differentiation, and communication between cells. In addition, scaffolds can act as carriers that facilitate the delivery of cells and bioactive materials to the defect area, allowing bone regeneration to occur more effectively (Dasari et al., 2022; Marantson et al., 2023; Milojević et al., 2021; Darus et al., 2020; Jazayeri et al., 2020). An ideal scaffold should have characteristics such as biocompatibility, biodegradability, osteoconductivity, osteoinductivity, non-toxicity, and adequate mechanical properties to maintain its structural integrity during the healing process (Darus et al., 2020).

Carbonate hydroxyapatite is one of the inorganic biomaterials commonly used in scaffold fabrication because its mineral composition is similar to that of

human bone tissue. It is also bioactive and biocompatible and can support new bone formation. The addition of carbonate groups to hydroxyapatite produces a material that is more soluble and has characteristics that are closer to those of natural bone minerals (Triyono et al., 2021; Sari et al., 2024). To obtain scaffolds with better biological and mechanical characteristics, carbonate hydroxyapatite can be combined with natural polymers such as chitosan and gelatin, which represent the organic components of the bone extracellular matrix. This combination can improve the bioactivity of the scaffold while providing an environment that is more suitable for supporting cellular activity (Trisnawati et al., 2019; Jazayeri et al., 2020; Agnes et al., 2023; Hu et al., 2020; Moshiri et al., 2020).

Previous studies showed that a chitosan-gelatin-carbonate hydroxyapatite (K-G:CHA) composite scaffold with a ratio of 30:70 (w/w) had a composition that was closest to the bone extracellular matrix and had a Ca/P ratio of 1.69. Meanwhile, the scaffold with a ratio of 40:60 (w/w) showed the highest compressive strength, reaching 4.19 MPa. However, the mechanical strength of these scaffolds still needs to be improved so that they can better withstand pressure during the bone regeneration process (Putri et al., 2021; Rianti et al., 2022; Rianti et al., 2023).

One method commonly used to improve the mechanical properties of scaffolds is crosslinking with glutaraldehyde. Glutaraldehyde is used because it can form crosslinks between the amino groups of chitosan and gelatin, which can improve the structural stability of the scaffold. In addition, 0.25% glutaraldehyde has been reported to be non-toxic and able to improve the attachment, proliferation, and differentiation of osteoprogenitor cells and osteoblasts (Gerhardt et al., 2010; Rahmitasari et al., 2019; Rianti et al., 2023; Pinto et al., 2020; Budiatin et al., 2022).

Besides having good mechanical strength, a scaffold also needs to interact well with the physiological environment through its ability to absorb, retain, and distribute fluids. Two parameters commonly used to evaluate these characteristics are swelling and water content percentage (WCP). Swelling describes the ability of a scaffold to absorb fluid and expand as the fluid enters its structure, while water content percentage describes the ability of the scaffold to retain the absorbed fluid. Both parameters are important because they support nutrient diffusion, oxygen transport, removal of metabolic waste, and provide a suitable environment for cell attachment and growth (Yilmaz et al., 2020; Wang et al., 2022; Thi et al., 2019; Zhang et al., 2019).

Swelling and water content percentage tests are commonly performed using simulated body fluid (SBF) because its ionic composition is similar to human blood plasma. Immersing scaffolds in SBF for 1, 3, and 7 days allows interactions between the scaffold and the physiological environment to occur, making changes in fluid absorption and retention capacity possible to observe over time (Yilmaz et al., 2020; Kokubo et al., 2006).

Based on the description above, this study investigated differences in swelling and water content percentage of chitosan–gelatin–carbonate hydroxyapatite composite scaffolds with ratios of 30:70 (w/w) and 40:60 (w/w), both without crosslinking and with 0.25% glutaraldehyde crosslinking. The evaluation of these two parameters provides insight into the scaffold's ability to maintain structural integrity while supporting bone tissue regeneration.

MATERIALS AND METHODS

Synthesis of Chitosan–Gelatin–Carbonate Hydroxyapatite (K-G:CHA) Composite Scaffolds

K-G:CHA composite scaffolds were prepared using two different ratios, namely 30:70 (w/w) and 40:60 (w/w). The composition of each material is presented in Table 1 (Rianti et al., 2022).

TABLE 1: K-G:CHA material ratios.

Ratio (w/w)	Chitosan (g)	Gelatin (g)	Carbonate hydroxyapatite (g)
30:70	0.375	0.375	1.75
40:60	0.5	0.5	1.5

Gelatin was dissolved in a 2% acetic acid solution (2 mL) in a glass beaker and stirred using a magnetic stirrer at 50°C until homogeneous and a gelatin gel was formed. Carbonate hydroxyapatite was then dissolved in 0.94 mL of distilled water and stirred until homogeneous. The carbonate hydroxyapatite suspension was added to the gelatin gel and stirred again until homogeneous, while chitosan powder was gradually added until a K-G:CHA composite gel was formed.

The K-G:CHA gel was then mixed with 0.5 mL of 0.1 M NaOH solution to neutralize the solution until it reached pH 7. The pH was measured using litmus paper. If the gel pH was below 7, 0.1 mL of 0.1 M NaOH was added, while if the pH was above 7, 0.1 mL of acetic acid was added until the desired pH was obtained.

The neutralized K-G:CHA gel was placed into a sample mold using a cement spatula on a vibrator and then compacted using a cement stopper to remove air cavities inside the scaffold. The gel was then frozen using the deep-freezing method at −40°C for 1 × 24 hours (Yuliati et al., 2019; Rianti et al., 2022; Habiburrohman et al., 2025).

Freeze-Drying Method

The scaffold that had undergone the deep-freezing process was placed in a freeze dryer. The equipment was activated by pressing the COND button and waiting until the condenser temperature reached −55°C for approximately 20–30 minutes. After the condenser reached the required temperature, the vacuum process was started by pressing the VAC

button until the indicator light turned on. The freeze-drying process was then carried out for 2 × 24 hours. The scaffold was observed periodically until it appeared dry, indicating that the freeze-drying process had been completed. After the process was finished, the equipment was turned off, and the scaffold was removed. The DEFR button was then pressed to melt the ice on the condenser, and the condenser was cleaned using a baking soda solution or detergent (Lou et al., 2015; Habiburrohman et al., 2025).

Glutaraldehyde Crosslinking Method

The K-G:CHA scaffold was rehydrated using 2 mL of 0.05 M acetic acid solution for 15 minutes. The solution was then removed, and the scaffold was rinsed with distilled water. The crosslinking process was performed by immersing the scaffold in 2 mL of 0.25% glutaraldehyde solution at 4°C for 24 hours. After the immersion process was completed, the glutaraldehyde solution was removed, and the scaffold was washed five times using distilled water at 10-minute intervals. The scaffold was then dried again using the freeze-drying method at −55°C for 1 × 24 hours, resulting in a K-G:CHA scaffold that had undergone the crosslinking process (Lou et al., 2015; Rianti et al., 2024).

Swelling Test

Before testing, the scaffold was weighed using an analytical balance to obtain its initial weight (Wi). Each scaffold was then placed into an Eppendorf tube and immersed in 2 mL of simulated body fluid (SBF) at 37°C for 24, 72, and 168 hours (days 1, 3, and 7). After each immersion period, the scaffold was removed and gently dried using tissue to remove excess liquid from its surface. The scaffold was then weighed again to obtain its final weight (Wf). The swelling value was calculated using the following formula: Swelling (%) = (Wf − Wi) / Wi × 100% (Kokubo et al., 2006).

Water Content Percentage Test

The scaffold was weighed using an analytical balance to obtain its initial weight (W1). The scaffold was then placed in an Eppendorf tube with gauze placed at the bottom of the tube. Each sample was immersed in 2 mL of simulated body fluid (SBF) at 37°C for 1, 3, and 7 days. After the immersion period was completed, the SBF solution was removed through the gauze until only the scaffold remained. The scaffold was then gently pressed using tissue paper to remove excess fluid from its surface and weighed again to obtain its final weight (W2). The water content percentage (WCP) was calculated using the following formula: Water Content Percentage (%) = (W2 − W1) / W2 × 100% (Kokubo et al., 2006; Rianti et al., 2022).

Preparation of Simulated Body Fluid (SBF)

One liter of simulated body fluid (SBF) was prepared using the same composition as the two previous studies, as shown in Table 2 (Kokubo et al., 2006).

TABLE 2: Composition of simulated body fluid (SBF).

Reagent	Amount per 1 liter
NaCl	8.035 g
$NaHCO_3$	0.355 g
KCl	0.225 g
$K_2HPO_4 \cdot 3H_2O$	0.231 g
$MgCl_2 \cdot 6H_2O$	0.311 g
HCl 1.0 M	39 mL
$CaCl_2$	0.292 g
Na_2SO_4	0.072 g
Tris	6.118 g
HCl 1.0 M	0–5 mL

Statistical Analysis

The data obtained from the swelling and water content percentage tests were analyzed using statistical software. The normality test was performed using the Shapiro–Wilk test, while the homogeneity of variance was analyzed using Levene's test. When the data were normally distributed and homogeneous, the analysis was continued using one-way ANOVA. Differences between groups were then analyzed using the post hoc Tukey test with a significance level of $p < 0.05$.

RESULTS

Scaffold Synthesis Results

Chitosan–gelatin–carbonate hydroxyapatite (K-G:CHA) composite scaffolds with ratios of 30:70 (w/w) and 40:60 (w/w) were successfully synthesized using the deep-freezing method for 1 × 24 hours, followed by freeze-drying for 2 × 24 hours. The resulting scaffolds had a cylindrical shape with a homogeneous surface and maintained their mold shape after the drying process.

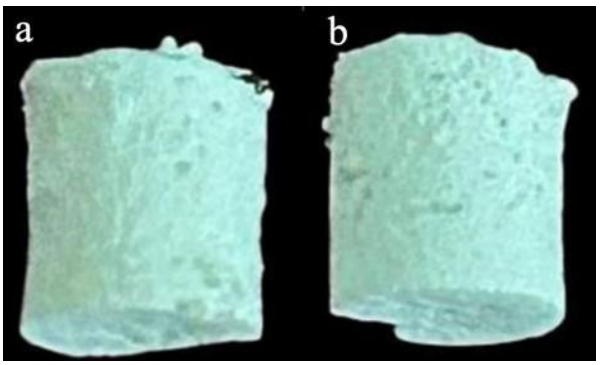


FIGURE 1: K-G:CHA scaffold synthesis with ratios of 30:70 (w/w) and 40:60 (w/w).

The scaffolds were then crosslinked using 0.25% glutaraldehyde for 24 hours at 4°C. After the crosslinking and second freeze-drying processes were completed, the scaffolds maintained their macroscopic shape and were ready for swelling and water content percentage testing.

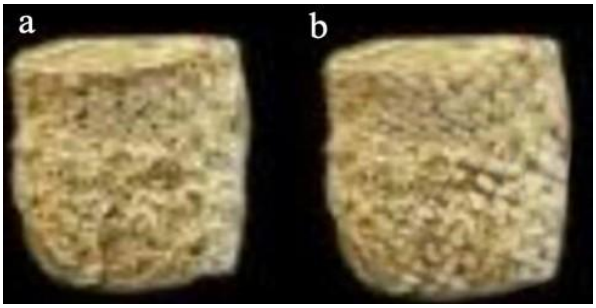


FIGURE 2: K-G:CHA scaffolds after crosslinking with 0.25% glutaraldehyde.

Swelling Test Results

The results showed that all scaffold groups experienced an increase in swelling values as the immersion time in simulated body fluid (SBF) increased over 24, 72, and 168 hours. The highest mean swelling percentage was found in the K-G:CHA scaffold with a ratio of 40:60 (w/w), crosslinked with 0.25% glutaraldehyde, after 168 hours of immersion.

In contrast, the non-crosslinked groups showed lower swelling values at all observation times compared with the crosslinked groups. Overall, the crosslinked scaffolds had a higher swelling capacity than the non-crosslinked scaffolds at both K-G:CHA ratios. This increase in swelling values indicates that the crosslinked scaffolds had a better fluid retention capacity during the immersion process.

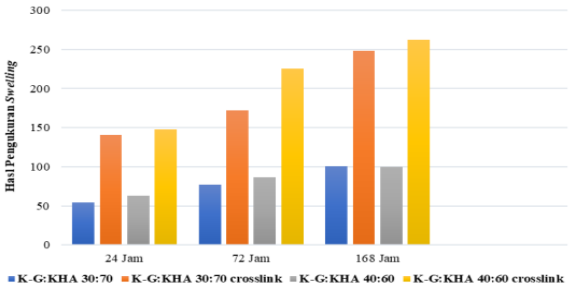


FIGURE 3: Mean swelling percentage of K-G:CHA scaffolds at different immersion times.

Water Content Percentage Test Results

The results showed that all scaffold groups experienced an increase in water content percentage (WCP) during immersion in simulated body fluid (SBF) on days 1, 3, and 7. The scaffolds crosslinked with 0.25% glutaraldehyde showed higher WCP values than the non-crosslinked groups at both K-G:CHA ratios.

In addition, the scaffold with a ratio of 40:60 (w/w) tended to have a higher WCP value than the 30:70 (w/w) ratio. The increase in WCP occurred as the immersion time increased, indicating that the scaffold's ability to retain fluid increased during contact with the physiological environment.

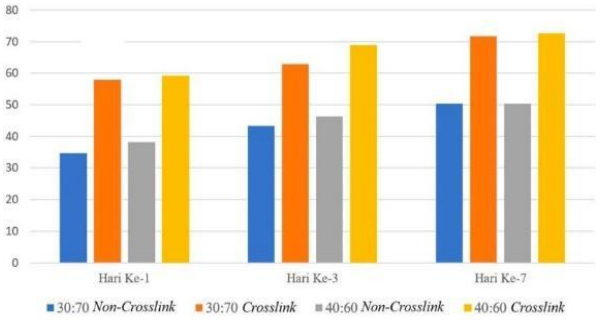


FIGURE 4: Mean water content percentage of K-G:CHA scaffolds.

Statistical Analysis of the Swelling Test

The normality test using the Shapiro–Wilk test showed that all groups had p-values > 0.05, indicating that the data were normally distributed. The homogeneity test using Levene's test also showed p-values > 0.05, indicating that the data variances among the groups were homogeneous. Based on the one-way ANOVA results, a p-value < 0.001 was obtained, indicating a significant difference in swelling values among the treatment groups (Rianti et al.).

TABLE 3: One-way ANOVA results for the swelling test.

Source of variation	Sum of squares	df	Mean square	F	Sig.
Between groups	174906.666	11	15900.606	632.915	<0.001
Within groups	602.948	24	25.123		
Total	175509.614	35			

TABLE 4: Post hoc Tukey analysis results for the swelling test.

8						*	*	*	*	*	*
P1.24							*	*	0,7 77	*	*
P1.72								*	*	*	*
P1.168									*	*	0,0 96
P2.24										*	*
P2.72											*
P2.168											

*Note: * indicates a significant difference (p < 0.05).*

The post hoc Tukey test was then performed to determine the differences between treatment groups. The analysis showed that most treatment groups had significant differences ($p < 0.05$), while several groups did not show statistically significant differences.

Statistical Analysis of Water Content Percentage

The Shapiro–Wilk normality test showed that all groups had p-values > 0.05, indicating that the data were normally distributed. The Levene homogeneity test also showed p-values > 0.05, indicating that the variances among the groups were homogeneous. The one-way ANOVA analysis resulted in a p-value of < 0.001, indicating a significant difference in water content percentage among the treatment groups.

TABLE 5: One-way ANOVA results for the water content percentage test.

Source of variation	Sum of squares	df	Mean square	F	Sig.
Between groups	174906.666	11	15900.606	632.915	<0.001
Within groups	602.948	24	25.123		
Total	175509.614	35			

TABLE 6: Post hoc Tukey analysis results for the water content percentage test.

	K1.1	K1.3	K1.7	K2.1	K2.3	K2.7	P1.1	P1.3	P1.7	P2.1	P2.3	P2.7
K1.1	<0,001*	<0,001*	0,002*	<0,001*	<0,001*	<0,001*	<0,001*	<0,001*	<0,001*	<0,001*	<0,001*	
K1.3		<0,001*	0,011*	<0,001*	<0,001*	<0,001*	<0,001*	<0,001*	<0,001*	<0,001*	<0,001*	
K1.7			1,000	<0,001*	<0,001*	<0,001*	<0,001*	<0,001*	<0,001*	<0,001*	<0,001*	
K2.1				<0,001*	<0,001*	<0,001*	<0,001*	<0,001*	<0,001*	<0,001*	<0,001*	
K2.3					<0,001*	<0,001*	<0,001*	<0,001*	<0,001*	<0,001*	<0,001*	
K2.7						<0,001*	<0,001*	<0,001*	<0,001*	<0,001*	<0,001*	
P1.1							*	<0,001*	1,000	<0,001*	<0,001*	
P1.3								<0,001*	<0,001*	<0,001*	<0,001*	
P1.7									0,026*	<0,001*	<0,001*	
P2.1										<0,001*	<0,001*	
P2.3											<0,001*	
P2.7												

*Note: * indicates a significant difference ($p < 0.05$).*

The post hoc Tukey test showed that several scaffold groups had significant differences in water content percentage ($p < 0.05$), while some other groups did not show statistically significant differences.

SUMMARY OF RESEARCH RESULTS

Overall, the K-G:CHA composite scaffolds crosslinked with 0.25% glutaraldehyde showed higher swelling and water content percentage values compared with the non-crosslinked scaffolds at both K-G:CHA ratios. The highest values were generally observed in the 40:60 (w/w) scaffold group after 168 hours of immersion. These findings indicate that crosslinking affects the fluid absorption and retention characteristics of K-G:CHA scaffolds.

DISCUSSION

This study was conducted to compare the swelling and water content percentage (WCP) values of chitosan–gelatin–carbonate hydroxyapatite (K-G:CHA) composite scaffolds with ratios of 30:70 (w/w) and 40:60 (w/w), with and without crosslinking using 0.25% glutaraldehyde.

The results showed that the crosslinked scaffolds had higher swelling values than the non-crosslinked groups at both K-G:CHA ratios. The increase in swelling values occurred because glutaraldehyde forms crosslinks between its aldehyde groups and the amino groups of chitosan and gelatin, resulting in the formation of imine bonds (Schiff bases). These crosslinks increase the number of bonds between polymer chains, making the scaffold structure more stable and improving its structural integrity (Rahmitasari et al., 2019).

The increased number of crosslinks allows the scaffold to retain absorbed fluid more effectively. Although the crosslinking process reduces the pore size and porosity of the scaffold, the more stable structure can retain fluid within the scaffold network, resulting in higher swelling values compared with the non-crosslinked groups (Kumari et al., 2023; Zulkifli et al., 2025; Doustdar et al., 2022; Erinda et al., 2022). Erinda (2021) also reported that crosslinked K-G:CHA scaffolds showed a decrease in pore diameter and porosity, making the scaffold structurally stronger (Erinda et al., 2022).

In addition to improving structural stability, crosslinking also affects the degradation process of the scaffold. Scaffolds with smaller pore sizes have a smaller surface area in contact with the degradation medium, resulting in a slower hydrolysis process of the polymer chains compared with non-crosslinked scaffolds. The reduced breakdown of polymer chains allows the scaffold to retain a greater amount of fluid during immersion, resulting in higher swelling values (Kumari et al., 2023; Zulkifli et al., 2025; Mihai et al., 2016; Hynes et al., 2023).

Zadeh and Zamanian (2020) also reported that crosslinked scaffolds had a lower degradation rate than non-crosslinked scaffolds due to limited fluid penetration into the scaffold structure (Zadeh et al., 2022).

The results also showed that the crosslinked scaffolds had higher water content percentage values than the non-crosslinked scaffolds at both K-G:CHA ratios. This increase occurred because crosslinks were formed between the aldehyde groups of glutaraldehyde and the amino groups of chitosan and gelatin, resulting in a denser and more stable scaffold structure with better fluid retention capacity. These crosslinks form Schiff base bonds (-C=N-) that improve the stability of the scaffold polymer network during immersion (Qian et al., 2011). The results of this study differ from those reported by Doustdar et al. (2022), who found that glutaraldehyde crosslinking decreased the water content percentage because it produced a denser scaffold structure and reduced its ability to absorb fluid. A more rigid scaffold structure can reduce microporosity, causing fluid penetration to occur more slowly compared with non-crosslinked scaffolds. The differences between these results may be influenced by variations in scaffold composition, glutaraldehyde concentration, synthesis methods, and the characteristics of the biomaterials used, which may result in different responses to the crosslinking process.

In this study, the scaffold with a ratio of 40:60 (w/w) showed higher swelling and water content percentage values than the scaffold with a ratio of 30:70 (w/w). The increase in both parameters was assumed to be related to the higher content of chitosan and gelatin, which resulted in a greater number of hydrophilic groups, such as hydroxyl and amino groups. These hydrophilic groups can interact with water molecules through hydrogen bonding, increasing the ability of the scaffold to bind and retain fluid (Ryglova et al., 2017; Ari et al., 2018; Rianti et al., 2022). Besides being affected by scaffold composition, swelling and water content percentage also increased with longer immersion times. On day 1, fluid began to diffuse into the scaffold pores, so the increase in both parameters was still relatively low. From day 3 to day 7, fluid penetration became more effective, resulting in an increase in the amount of fluid absorbed and retained by the scaffold. This condition indicates that the scaffold was able to adapt to the physiological environment during immersion in SBF (Yilmaz et al., 2020; Marantson et al., 2023).

The simulated body fluid (SBF) used in this study had an ionic composition similar to human blood plasma, allowing it to represent physiological conditions during the tissue regeneration process. Calcium and phosphate ions contained in SBF can interact with carbonate hydroxyapatite in the scaffold and support the structural stability of the scaffold during immersion (Yilmaz et al., 2020).

The results of this study show that swelling and water content percentage are two related parameters for evaluating scaffold characteristics. Swelling describes the ability of a scaffold to absorb fluid and expand as fluid enters its structure, while water content percentage describes the ability of the scaffold to retain the absorbed fluid. Scaffolds with good fluid retention capacity can maintain moisture around the cells, supporting nutrient diffusion, oxygen transport, removal of metabolic waste, as well as cell attachment, proliferation, and differentiation during bone tissue regeneration (Wang et al., 2022; Zhang et al., 2019).

Based on the overall results, crosslinking with 0.25% glutaraldehyde not only improves the mechanical stability of the scaffold but also increases its ability to retain fluid during immersion. The combination of improved mechanical properties and high fluid retention capacity is expected to create a more favorable microenvironment for bone tissue regeneration. Therefore, crosslinked K-G:CHA composite scaffolds have greater potential to be further developed as biomaterials for bone tissue engineering applications (Rahmitasari et al., 2019; Rianti et al., 2023; Budiatin et al., 2022).

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