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Fabrication and characterization of chitosan–gelatin–chondroitin sulfate-diclofenac scaffold with cross-linked glutaraldehyde for potential application in osteoarthritis

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ABSTRACT

The inadequate regenerative capacity of cartilage renders osteoarthritis (OA) and cartilage injuries difficult to manage. In tissue engineering, a scaffold facilitates establishing an environment favorable to cell proliferation, migration, and adhesion. Moreover, diclofenac sodium can be administered locally due to the scaffold's porous architecture, which possesses anti-inflammatory characteristics. This study investigated the development and characterization of an innovative scaffold formulation intended for potential application in cartilage repair associated with OA and cartilage injuries. The scaffold was cross-linked with varying concentrations of GA (0.00%–2.50%) and comprised chitosan, gelatin, chondroitin sulfate, and PEG 400. The scaffold also contained the anti-inflammatory agent, diclofenac sodium, which was dissolved in PEG 400 for targeted drug delivery. The pore diameter, porosity, compressive strength, and degradation of the scaffolds were assessed following their dried form. The results indicated that GA significantly influenced these attributes, with porosity, mechanical stability, and degradation control improved at an optimal concentration of 0.50 percent. GA cross-linking between polymer chains enhanced the scaffold's integrity and augmented its mechanical properties through the establishment of more rigid structures. The cross-linking of the amino group in chitosan with the sulfonate group in chondroitin sulfate enhanced the scaffold's stability. The study's findings indicated that GA-optimized chitosan–gelatin–chondroitin sulfate-PEG 400-diclofenac scaffolds exhibited suitable physicochemical and mechanical properties, supporting their potential use in localized drug delivery systems for OA management.

INTRODUCTION

The key characteristics of osteoarthritis (OA) include the degeneration of articular cartilage, remodeling of subchondral bone, and inflammation of the synovial membrane. OA is a persistent, degenerative joint disorder [1]. It is the predominant form of arthritis and a significant factor in pain, disability, and diminished quality of life among the aging worldwide population [2]. In 2019, around 528 million individuals globally were affected by OA [3,4]. The prevalence in Asia is 31% for women and 23% for men over 24 years, 61% for women, and 53% for men aged 40–75. In Europe, the prevalence is 14% for women and 12% for men over 22 years, and 29% for women and 16% for men over 55 years [5,6]. In Indonesia, OA prevalence is 5% among individuals under 40, 30% among those aged 40 to 60, and 65% among those over

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60. Meanwhile, according to gender, OA affects 5% of men and 12.7% of women [7,8].

An imbalance in the anabolic and catabolic factors produced by chondrocytes leads to OA, a degenerative joint disease [9]. This imbalance triggers the production of cytokines and inflammatory mediators, resulting in the release of nitric oxide (NO), which induces chondrocyte apoptosis and degradation of the extracellular matrix (ECM) [10]. The progressive degeneration of articular cartilage is a distinctive feature [11,12]. Primary OA is defined as joint degeneration without an identifiable cause. Concurrently, secondary OA arises from abnormal articular cartilage, as observed in rheumatoid arthritis (RA), or from abnormal force concentration inside the joint, as seen in post-traumatic instances [13].

Joint pain, stiffness, and functional limitations are the defining characteristics of OA. Pharmacological management of OA generally includes diclofenac sodium, a member of the Nonsteroidal Anti-Inflammatory Drug (NSAID) class. Diclofenac sodium suppresses prostaglandin synthesis by obstructing the cyclooxygenase (COX) enzyme, which catalyzes the conversion of 2-arachidonic acid (AA) to prostaglandin H2 in prostanoid biosynthesis [14]. Reports indicate that 30% of patients who administered diclofenac sodium orally for an extended duration encountered adverse effects, including renal impairment, increased liver enzymes, and gastric ulcers [15]. The topical dosage form of diclofenac sodium possesses a disadvantage [16,17]. Notwithstanding a lipophilic partition coefficient of 13.4 (log p = 1.13), diclofenac sodium could not efficiently extract its constituent from the vehicle. Moreover, the topical administration of diclofenac sodium solely mitigates symptomatic knee pain; it does not reverse cartilage degeneration [18,19]. Patients with OA will experience disability if cartilage degradation is not fully rectified.

Bone tissue engineering has significantly advanced as a therapeutic strategy for addressing bone and cartilage defects by restoring and maintaining native tissue functionality. Among the various approaches, scaffolds composed of ceramics or polymers have been widely explored for their ability to support tissue regeneration [20,21]. Diclofenac sodium has been recognized for its potential to be locally and sustainably delivered through scaffolds composed of chitosan, gelatin, and chondroitin sulfate, which are also applicable for cartilage repair. However, systemic oral administration of diclofenac sodium has been associated with severe gastrointestinal and cardiovascular risks, highlighting the need for safer, localized delivery systems [22]. Scaffolds for cartilage regeneration must replicate the morphology, structure, and function of native cartilage. Within these porous structures, neighboring cells migrate, adhere, proliferate, and differentiate to form new cartilage tissue [23]. The release of diclofenac sodium from such scaffolds may reduce acute inflammatory responses by decreasing neutrophil and macrophage infiltration at the injury site, thereby alleviating pain and swelling [24]. This study presents a novel scaffold formulation and systematic physicochemical characterization of diclofenac-loaded chitosan–gelatin–chondroitin sulfate, designed to improve mechanical performance and support controlled local release of anti-inflammatory agents, potentially relevant for cartilage repair applications.

In a previous study, a scaffold composed of chitosan, gelatin, and chondroitin sulfate in a 50:25:25 ratio was employed, resulting in a compressive strength of 10.58 MPa and a high cell viability rate of 102.75%. These findings indicate that the scaffold possesses favorable mechanical properties and excellent biocompatibility, demonstrating its non-toxic nature to cells [25]. Nonetheless, this composition ratio presents a disadvantage, as the scaffold will disintegrate within about two days. A cross-linking agent is required to improve the scaffold's properties and facilitate the progressive release of diclofenac sodium. Upon the addition of a cross-linking agent to the scaffold, it can attach to the polymer's amino groups, resulting in the formation of α-helical connections, which causes the initially linear polymer threads to become thicker and intertwined [23,26]. The pore diameter may consequently diminish. Consequently, the scaffold degradation rate would decrease while the volume of liquid influx would increase somewhat [27,28].

A scaffold composed of chitosan, gelatin, and chondroitin sulfate, incorporating polyethylene glycol (PEG) 400 as a plasticizer and diclofenac sodium as the active pharmaceutical ingredient, was formulated using glutaraldehyde (GA) as a cross-linking agent. GA maintains bone-bonding strength by forming stable cross-links between BHA and gelatin. Previous studies have demonstrated that GA, among various cross-linking agents tested, results in superior mechanical strength. The concentration range of GA used in this study was carefully selected based on prior research [29], which indicated that concentrations up to 2.5% are non-toxic. The most effective concentrations for cross-linking activity were reported to be 0.5% and 1.0% [30]. Accordingly, the scaffold was formulated with varying concentrations of GA: 0%, 0.25%, 0.50%, 1.00%, and 2.50%. This formulation was designed to assess the effect of different GA concentrations on key scaffold properties, including mechanical strength, pore diameter, porosity percentage, and degradation behavior under physiological conditions. These investigations provide a scientific basis for evaluating the efficiency of cross-linking while ensuring the biocompatibility and functional performance of the scaffold.

MATERIALS AND METHODS

Materials

This study utilized shrimp chitosan (CV. Multiguna, Indonesia), gelatin (Cartino, Thailand), chondroitin sulfate, GA (Sigma-Aldrich, USA), pro-analysis NaOH solution (Merck, Germany), diclofenac sodium (Kalbe, Indonesia), pro-analysis acetic acid (Mallinckrodt, UK), PEG-400, phosphate buffer saline pH 7.4, ethanol 96%, and distilled water (Interlab, Indonesia).

Methods

Preparation of chitosan–gelatin–chondroitin sulfate-diclofenac scaffold composite

The scaffold was prepared using diclofenac sodium, chondroitin sulfate, gelatin, and chitosan. The composition ratio

of the three biopolymers followed a 50:25:25 proportion of chitosan:gelatin:chondroitin sulfate, which corresponds to 2.5 g of chitosan, 1.25 g of gelatin, and 1.25 g of chondroitin sulfate. This ratio was determined based on previous optimization studies showing favorable properties regarding pore size distribution and interconnectivity. Moreover, incorporating chondroitin sulfate contributed to an increase in compressive strength, reaching 10.58 MPa and a high cell viability of 102.75%. These results indicate that the scaffold exhibits excellent mechanical integrity while remaining non-toxic to cells [24]. Specifically, 2.5 g of chitosan was solubilized in 100 ml of a 2% acetic acid solution and agitated until homogeneous with a magnetic stirrer; 50 ml of warm distilled water (40°C–50°C) was utilized to dissolve 1.25 g of gelatin. The chitosan solution was subsequently included, and the mixture was stirred. The chitosan–gelatin solution was thereafter stirred while 1.25 g of chondroitin sulfate powder was incrementally introduced. A 1% NaOH solution neutralized the composition upon achieving a homogeneous mixture. A 1% sodium diclofenac solution in PEG 400 was incorporated post-neutralization and stirred until fully dissolved and homogeneous. GA was then introduced as a cross-linking agent at varying concentrations of 0.25%, 0.5%, 1%, and 2.5% (v/v) by mixing it directly into the homogeneous solution. Cross-linking was conducted at room temperature (25 °C) for 24 hours. After cross-linking, the scaffolds were thoroughly washed with distilled water three times to remove unreacted GA, minimizing potential cytotoxicity. Finally, the scaffolds were subjected to freeze-drying (lyophilization) to obtain the final porous structure in dry form. Table 1 illustrates the formulation of the chitosan–gelatin–chondroitin sulfate-sodium diclofenac scaffold implant with the inclusion of GA.

Characterization evaluation

Organoleptic tests, pore diameter evaluations utilizing a scanning electron microscope (SEM) (Inspect S-50, FEI, Japan), compressive strength evaluations with Autograph 2.1 (Autograph, Indonesia), and degradation analyses employing PBS solution were conducted to characterize the chitosan–gelatin–chondroitin sulfate scaffold composite incorporating the cross-linking agent GA.

Organoleptic evaluation

The organoleptic evaluation of chitosan–gelatin–chondroitin sulfate-diclofenac sodium scaffolds was conducted to assess their color and physical properties at varying GA concentrations (0%, 0.25%, 0.50%, 1.00%, and 2.50%). Three unbiased evaluators performed the assessments in a regulated laboratory environment, and the results were recorded as qualitative descriptions for subsequent comparison.

Pore diameter

The width of the pore, measured in micrometers (μm), is referred to as the pore diameter. A scaffold must possess an appropriate pore diameter size. Pore size influences gas exchange, nutrient transport, chondrocyte migration, and nutrient infiltration into the scaffold. A scaffold specimen measuring 5 mm in width and 3 mm in height was prepared for testing. A sputter was employed to deposit a 3-minute gold coating onto the scaffold. Upon placement of the coated sample in the sample chamber, it was subjected to an electron beam at a magnification of 1000 and a voltage of 5 kV. The detector would identify the beam post-reflection. A picture may result from an order of micron expansion or the dimensions of the scaffold sample's pores. This test was conducted using SEM [31].

Porosity test

Porosity is a percentage ranging from 0% to 100% that indicates the volume of voids inside the scaffold relative to its total free space volume. The fluid transfer technique is employed to conduct the porosity test. Ethanol was selected due to its efficient absorption in the scaffold without inducing contraction or swelling. The scaffold's dry weight (m1) was measured in its desiccated state. The mass of the ethanol and the container was subsequently recorded as m^2. The scaffold was thereafter placed in a container containing ethanol (m^3). Subsequently, it is immersed for 48 hours in 96% v/v ethanol. The scaffold was dismantled after 48 hours, and the weights of the ethanol and container were recorded (m4) [31]. The following formula can be employed to ascertain porosity:

$$\text{Porosity (\%)} = \frac{(m3\text{-}m4\text{-}m1)}{(m2\text{-}m4)} \times 100\%$$

Compressive test

Compressive strength testing aimed to evaluate whether the scaffold could withstand mechanical forces exerted by surrounding tissues. Scaffold samples, 5 mm in diameter and 3 mm in height, were tested in a dry state at room temperature using an Autograph universal testing machine. The compression test was conducted at a constant speed of 5 mm/minutes. A compressive load was applied to the scaffold during the test

Table 1. Formulation of chitosan–gelatin–chondroitin sulfate-diclofenac sodium scaffolds preparations with the addition of GA.

Formulation	Chitosan (gram)	Gelatin (gram)	Chondroitin sulfate (gram)	Diclofenac sodium (gram)	GA (%v/v)
Control	2.5	1.25	1.25	0.05	0
I	2.5	1.25	1.25	0.05	0.25
II	2.5	1.25	1.25	0.05	0.50
III	2.5	1.25	1.25	0.05	1.00
IV	2.5	1.25	1.25	0.05	2.50

until structural deformation was observed. The diameter and height of the scaffold were recorded prior to testing to ensure accuracy. The compressive strength value was calculated based on the maximum force (in Newtons) applied before deformation occurred, and the result was automatically displayed upon test completion [31].

Degradation test

The degradation test was carried out by immersing the scaffold in a pH 7.4 phosphate-buffered saline (PBS) solution at 37 °C in an incubator. A cubic device measuring 1 × 1 × 1 cm with four interconnected holes was used. The scaffold was prepared and dried using a freeze-dryer prior to immersion. The scaffold was immersed for a total of 14 days, and the mass change was measured at specific time intervals: 1 day, 2 days, 3 days, 5 days, 7 days, 10 days, 12 days, and 14 days [31].

RESULTS AND DISCUSSION

This study focused on a scaffold utilizing sodium diclofenac as the active component, composed of chitosan, gelatin, chondroitin sulfate, and PEG-400. The scaffold exhibits non-toxic, biodegradable characteristics and material compatibility, which are essential prerequisites for further development in cartilage repair applications for OA. GA was employed as a cross-linking agent to enhance structural stability. The maximum GA concentration was limited to 2.5%, which has been reported to cause no cytotoxic effects—such as cell damage or death—in human fibroblast cell lines (WI-38) [29]. This finding is consistent with the study by Budiatin *et al.* [23], which demonstrated that scaffolds containing up to 2.5% GA maintained ≥60% cell viability in MTT assays, thereby classifying them as non-toxic.

The cytotoxicity of GA is also known to be time-dependent. Sun *et al.* [29] reported that prolonged exposure to GA (24 hours) significantly increased its toxicity, lowering the 50% toxic concentration (TC_{50}) from 4.83 mM to 2.09 mM. However, short-term exposure with appropriate post-treatment—such as extensive washing—can maintain GA levels within biologically acceptable limits. Accordingly, in this study, the cross-linking process was conducted with limited GA exposure (24 hours), followed by three cycles of washing with distilled water to remove unreacted GA, in line with procedures recommended by previous studies [29,30]. This strategy was implemented to minimize residual GA content and reduce the risk of cytotoxicity. Furthermore, PEG-400 was incorporated as a plasticizer to improve the scaffold's elasticity and flexibility by increasing intermolecular spacing, thereby enhancing its resemblance to the mechanical properties of native cartilage tissue [32].

Organoleptic evaluation

Dry samples (after freeze-drying) underwent organoleptic evaluations through visual inspection. An organoleptic study of the color of each chitosan–gelatin–chondroitin sulfate-PEG 400-diclofenac sodium scaffold implant sample indicated a color variation, as illustrated in Figure 1, where the scaffold transitions from white to brown with increasing GA concentration.

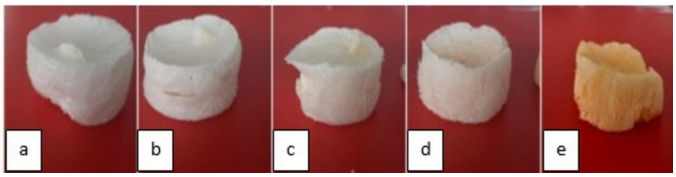

Figure 1. Differences in the color of implant scaffold preparations with the addition of different GA concentrations. (a) Control, (b) GA 0.25, (c) 0.50, (d) 1.00, (e) 2.50%.

GA serves as a cross-linking agent in the construction of this scaffold. GA is essential for enhancing the stability and porosity of the scaffold by facilitating cross-linking among the amine groups of chitosan and gelatin, as well as between chitosan and chondroitin sulfate [26]. This study illustrates that the scaffold's color alters with increasing GA content. The observed color changes indicated that the chemical reaction between GA and chitosan facilitated the formation of chromophores [33,34]. A study by Budiatin *et al.* [23] showed that the chitosan–gelatin–diclofenac scaffold underwent a color change upon adding GA. The color change is a consequence of a diazotization reaction between GA and the scaffold material. An intensified brownish hue is produced with an increase in GA concentration due to the formation of more cross-linking bonds between the carbonyl group (-C=O) in GA and the amine group (-NH2) from gelatin and chitosan, along with the S-group from chondroitin sulfate [35].

Pore diameter

Figure 2 illustrates that SEM analysis was employed to conduct morphological observations and ascertain the scaffold's pore width. Based on the results of the obtained SEM observations, data were subsequently created to analyze the observed pore diameters. Table 2 illustrates that increased GA content reduces the scaffold's pore diameter. The study's results revealed that all scaffold samples, save for the highest concentration of 2.5%, possess pore diameters above 100 µm.

The presence of GA influences the dimensions of the pores that develop in the scaffold. The resultant cross-linking will form an α-helix bond, causing the initially linear polymer strands to tighten and coil around each other, enhancing the scaffold's density [36,37]. The scaffold necessitates pore dimensions ranging from 100 to 200 µm [38,39]. Narrow pore widths may impede nutrition transfer and metabolic waste removal and restrict cellular mobility. This may lead to scaffold necrosis. Conversely, if the hole size is enormous, cells would detach more easily from the scaffold, leading to poor differentiation and proliferation processes [40–42]. The results of this work align with the prior research conducted by Zadeh and Zamanian [43], which showed that elevating the concentration of cross-linking GA may reduce the scaffold's average pore size. Similarly, Samirah *et al.* [30] reported that higher concentrations of GA result in smaller pore diameters, indicating that GA significantly influences the structural characteristics of bioscrew pores.

Porosity presentation

Table 3 presents the average porosity of the chitosan–gelatin–chondroitin sulfate-diclofenac scaffold at varying

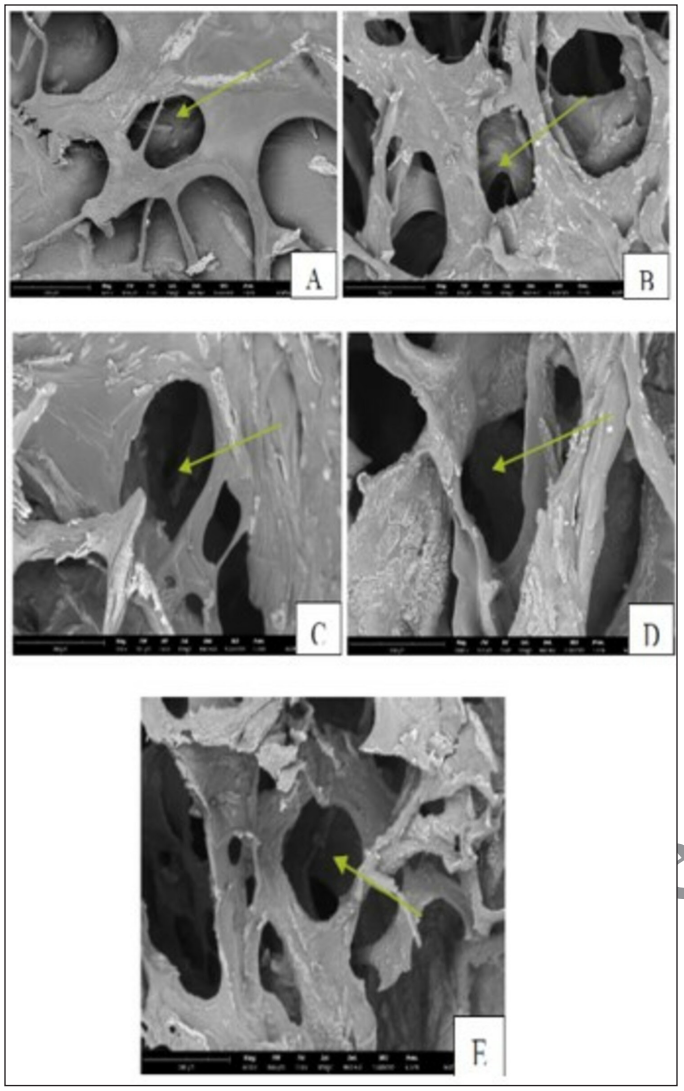

Figure 2. The appearance of the pore diameter of scaffolds with the addition of different GA concentrations using SEM with a magnification of 600–1000x. (A) Control; (B) GA 0.25%; (C) 0.50%; (D) 1.00%; and (E) 2.50%.

Table 2. The average pore diameter values chitosan–gelatin–chondroitin sulfate-diclofenac sodium scaffolds preparations with the addition of different GA concentration.

GA concentration	Pore diameter		*p*-value
	Min-Max (*μm*)	Mean ± SD (*μm*)	
Control	200–241	216.80 ± 21.27	
GA 0.25%	180–215	195.03 ±18.60	0.767
GA 0.50%	128–171	153.93 ± 22.23*	0.043
GA 1.00%	110–112	111.62 ± 0.97*	0.002
GA 2.50%	29–102	65.57 ± 36.05*	0.000

Data are expressed as the mean ± SD of 3 replicates and analyzed by one-way ANOVA. *p-value<0.05 compared to the control group.

doses of the GA cross-linking agent. As the concentration of GA rose, the porosity percentages of the scaffold markedly increased. Nonetheless, according to these findings, two

Table 3. The porosity average of chitosan–gelatin–chondroitin sulfate-diclofenac sodium scaffolds containing different concentrations of GA.

GA Concentration	Porosity (%)	*p*-value
Control	64.53 ± 1.09	
GA 0.25%	74.65 ± 0.29*	0.002
GA 0.50%	77.79 ± 1.49*	0.002
GA 1.00%	82.53 ± 0.96*	0.000
GA 2.50%	94.33 ± 2.08*	0.000

Data are expressed as the mean ± SD of 3 replicates and analyzed by one-way ANOVA. *p-value<0.05 compared to the control group.

Table 4. The compressive strength average of chitosan–gelatin–chondroitin sulfate-diclofenac sodium scaffolds containing different concentrations of GA.

GA Concentration	Average ± SD (MPa)	*p*-value
Control	0.069 ± 0.006	
GA 0.25%	0.097 ± 0.009	0.995
GA 0.50%	0.156 ± 0.007	0.769
GA 1.00%	0.368 ± 0.115*	0.017
GA 2.50%	1.463 ± 0.168*	0.000

Data are expressed as the mean ± SD of 3 replicates and analyzed by one-way ANOVA. *p-value<0.05 compared to the control group.

scaffold samples—one with a GA concentration of 0.5% and another with 1%—exhibit porosity values within the requisite 75%–90% range for scaffolds.

Incorporating GA facilitates the proliferation and differentiation of chondrocyte cells, enhances cell migration and vascularization, and increases the scaffold's porosity to the requisite range of 75%–90% porosity percentage values [44]. The configuration of this hole is essential for enhancing bioapplicability, accelerating bone repair, and providing an increased surface area for gas and nutrient exchange [38,39]. Similar findings were seen in the work by Azami *et al.* [45], who reported analogous results, indicating that the porosity of the GEL-HA scaffold with GA was 85.1% more than that of the GEL-HA scaffold devoid of GA, which measured 84.6%. This study further illustrates that GA cross-linking enhances the mechanical strength and compressive resistance of the scaffold [23]. The cross-linking between the scaffold's polymers is the reason for this. The study's results met the criteria for a potential scaffold compressive strength (0.01–3 MPa) based on the mechanical properties of the target cartilage tissue [46,47].

Compressive strength

Table 4 presents the outcomes of experiments assessing the compressive strength of scaffolds composed of chitosan, gelatin, chondroitin sulfate, and diclofenac sodium, which incorporate differing quantities of GA. The results indicated that increasing the GA concentration significantly enhances the compressive strength of the scaffold. Scaffolds containing 0.5% GA exhibited superior compressive strength compared

Table 5. Degradation percentage average of chitosan–gelatin–chondroitin sulfate-diclofenac scaffolds containing different concentrations of GA after 14 days.

GA Concentration	Day (%)								*p*-value
	1	2	3	5	7	10	12	14	
Control	23.81 ± 0.43	32.64 ± 0.87	38.82 ± 0.28	45.85 ± 0.33	51.73 ± 0.72	59.74 ± 0.78	67.13 ± 0.63	89.02 ± 7.15	<0.001*
GA 0.25%	22.98 ± 0.14	30.98 ± 0.32	37.74 ± 0.75	45.07 ± 0.69	50.29 ± 0.52	58.78 ± 0.13	65.44 ± 0.72	80.54 ± 3.31	<0.001*
GA 0.50%	22.06 ± 0.44	28.78 ± 1.15	36.09 ± 0.16	41.50 ± 0.82	49.51 ± 0.17	58.38 ± 0.21	64.49 ± 0.06	73.16 ± 2.06	<0.001*
GA 1.00%	20.89 ± 0.60	26.25 ± 0.59	35.13 ± 0.56	40.54 ± 0.32	48.54 ± 0.32	56.23 ± 0.35	62.89 ± 1.29	70.38 ± 0.30	<0.001*
GA 2.50%	19.50 ± 0.58	25.00 ± 0.68	33.75 ± 0.20	39.46 ± 0.55	46.77 ± 0.51	54.33 ± 1.23	60.89 ± 0.07	68.05 ± 0.30	<0.001*

Data are expressed as the mean ± SD of 3 replicates and analyzed by repeated measure ANOVA.

*p-value<0.05 compared to the control group.

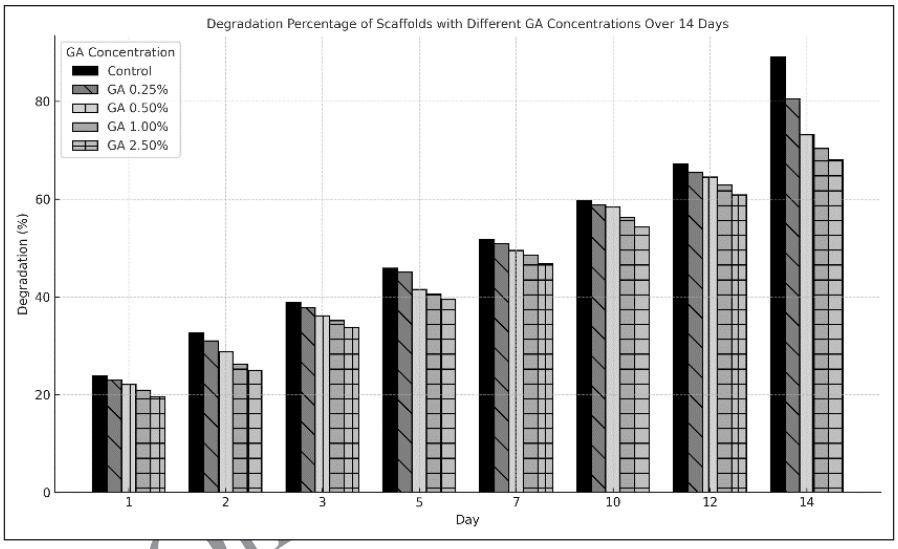

Figure 3. Degradation percentage of scaffolds with different GA concentrations over 14 days.

to scaffolds with different GA concentrations. Conversely, scaffolds lacking GA had reduced compressive strength.

Degradation test

The degradation test in this study was conducted by immersing the scaffold in phosphate-buffered saline (PBS) solution at pH 7.4. While enzymatic degradation more closely replicates the physiological breakdown of cartilage tissue, PBS-only models are routinely used for preliminary scaffold assessment, as supported by recent literature. Yue *et al.* [48] evaluated the degradation behavior of gelatin methacrylate and PLA-silk-based scaffolds in PBS at pH 7.4 over a 30-day period. Despite the absence of enzymatic agents, the scaffolds showed progressive weight loss over time. These findings reinforced the reliability of PBS as a suitable medium for simulating early-stage scaffold degradation and drug release kinetics in physiological-like conditions.

Table 5 presents the results of the degradation test conducted in this study. The results show that increasing the GA concentration might reduce the weight loss % of the chitosan–gelatin–chondroitin sulfate-diclofenac scaffold. Figure 3 presents a bar chart illustrating the scaffold's weight loss tendency over 14 days across different GA concentrations. The proportion of weight decrease was greater for scaffolds without GA than for those containing GA. All groups had a consistent increase in degradation over time.

GA enhances the durability of scaffolds, ensuring their stability even after a fortnight. This study corroborates other research indicating that GA can inhibit degradation by augmenting resistance to the scaffold [30,49,50]. Research conducted by Samirah *et al.* [30] demonstrated that GA stabilizes gelatin, which is susceptible to deterioration, with an optimal concentration ranging from 0.1% to 1%. Pinto *et al.* [51] indicated that GA concentrations beyond 1% may lead to cross-linking saturation, thus diminishing its benefits. The principal cause is the formation of cross-linking bonds between the carbonyl group (-C=O) in GA and the carboxyl group (COOH) from chondroitin sulfate, along with the amine group ($-NH_2$) from gelatin and chitosan [52]. Increased cross-linking bonds are established, necessitating the rupture of additional bonds prior to the polymer's degradation in the liquid at elevated GA concentrations. As the concentration of GA increases, the quantity of degraded particles will diminish due to a reduced fluid passage through the scaffold [53]. Furthermore, incorporating GA as a cross-linking agent in the

scaffold formulation facilitates sustained release of diclofenac sodium, which may minimize potential interference with cartilage repair and provide a platform for further investigation in regenerative applications.

This study successfully characterized scaffolds incorporating various concentrations of GA as a cross-linking agent. However, several limitations should be acknowledged. First, the visual assessment primarily focused on color changes, which limits the generalizability of the findings and should be interpreted cautiously. Second, the degradation testing conditions did not fully replicate the physiological environment and thus may not accurately reflect in vivo performance. Moreover, the study did not include in vivo testing to evaluate the scaffold's functional efficacy. Another limitation lies in the absence of cytotoxicity and in vitro cell compatibility assessments, which are essential for determining the scaffold's biological safety, particularly considering the potential cytotoxic effects of residual GA. Furthermore, the drug release profile of sodium diclofenac, including cumulative release, burst release, and sustained release behavior, was not evaluated, despite its critical importance for understanding the scaffold's therapeutic potential and anti-inflammatory performance. Biological and anti-inflammatory validations were likewise not included.

Further research is required to assess the scaffold's effects on cell proliferation, viability, and differentiation, as well as to conduct quantitative drug release studies, GA cytotoxicity testing, and investigations into chondrocyte behavior and cytokine response assays. These efforts are crucial for comprehensively determining the scaffold's safety and efficacy for biomedical applications. Despite these limitations, the findings suggest that scaffolds composed of chitosan, gelatin, chondroitin sulfate, and PEG-400, cross-linked with GA, exhibit enhanced mechanical strength, structural stability, and physicochemical properties, supporting their potential for further development in cartilage-mimicking scaffolds and localized drug delivery systems.

CONCLUSION

This study highlights the potential of GA-cross-linked chitosan–gelatin–chondroitin sulfate scaffolds as a platform for further development in OA-related cartilage repair, based on their physicochemical properties and drug-release behavior. The findings indicate that GA content significantly influences the optimization of scaffold properties. With additional GA, the resultant scaffold exhibits a more pronounced color, reduced pore diameter, increased porosity percentage, enhanced compressive strength, and diminished degradation percentage. The optimal concentration of GA as a cross-linking agent to improve the characteristics of diclofenac sodium scaffold implants made from chitosan, gelatin, and chondroitin sulfate is 0.5%.

This study did not include biocompatibility testing or in vitro assays, representing key limitations. Future studies should investigate cytotoxicity, drug release profiles, and cellular responses to comprehensively evaluate the scaffold's safety and functionality. While this research provides critical insights into scaffold design and characterization, subsequent investigations should focus on in vivo validation to assess biocompatibility and therapeutic efficacy in relevant animal models. Exploring additional therapeutic agents and their synergistic interactions with scaffold-based delivery systems is recommended to enhance the scaffold's applicability in various tissue engineering contexts. Interdisciplinary strategies combining biomaterials development, controlled drug delivery, and clinical translation represent a promising direction for advancing cartilage repair approaches and improving OA treatment outcomes.

AUTHORS CONTRIBUTION

All authors made substantial contributions to the conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY STATEMENT

All the data is available with the authors and shall be provided upon request.

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The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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