Effect of Carbodiimide Crosslinking on Gelatin-Carboxymethylcellulose-Polycaprolactone Scaffold Properties for Wound Dressing Applications

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ABSTRACT

This research investigated the feasibility of fabricating 3D porous scaffolds from gelatin, carboxymethylcellulose (CMC) and polycaprolactone (PCL) using the freeze-drying technique for wound dressing applications. The scaffolds were crosslinked using Dehydrothermal Treatment (DHT) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) combination with N-hydroxysuccinimide (NHS). Their morphological and mechanical properties were analyzed to determine the optimal condition. For DHT crosslinking, the results demonstrated that the average pore size ranged from 127.25 to 150.77 µm, which was smaller than in non-crosslinked scaffolds. The porosity ranged from 64.84% to72.08%, decreasing as CMC content increased. The gelatin scaffold with 35% (w/w) CMC and 30% (w/w) PCL exhibited the best overall properties. It provided the highest average pore size and porosity, and a compressive strength of 47.39 MPa, which was higher than non-crosslinked scaffold. Under EDC/NHS conditions, the average pore size ranged from 145.40 µm to 184.80 µm and porosity from 70.24% to 74.48%. These characteristics indicate a larger pore size and porosity compared to the DHT crosslinked scaffold Although its compressive strength was lower than that of the DHT crosslinked scaffold, it remained higher than that of the non-crosslinked scaffold. Therefore, it can be implied that the gelatin scaffold with 35% CMC and 30% PCL is suitable for use as a skin substitute in wound dressing applications

Keywords-scaffold; carboxymethylcellulose; polycaprolactone; carbodiimide; biodegradation

I. INTRODUCTION

Skin tissue engineering has advanced significantly in recent decades, particularly in the development of biomaterials with properties that aid in the repair and regeneration of damaged tissues or organs that do not naturally heal on their own [1]. Biomaterials play a crucial role in wound dressing applications by serving as scaffolds for tissue regeneration [2]. Ideally, these materials should be biodegradable and non-toxic to fibroblast cells. Three-dimensional (3D) porous scaffolds have gained considerable attention as potential skin substitutes for wound dressing applications. An ideal scaffold should allow cell penetration without triggering inflammation, be biodegradable through biological processes, and possess surface characteristics and porosity that support cell adhesion, differentiation, and the maintenance of a 3D structure during tissue formation. Additionally, it should have mechanical properties comparable to natural tissue and be strong enough to withstand internal and external forces [3].

Gelatin is widely used for fabricating porous scaffolds in skin tissue engineering due to its similarity to collagen, its biocompatibility with fibroblast cells, and its reactive amine groups. Collagen, a protein composed of amino acids, attracts fibroblast cells, facilitating cell adhesion and biocompatibility. Moreover, gelatin can degrade naturally in the body during tissue regeneration without causing damage [4, 5]. Various biodegradable polymers are often incorporated into gelatin scaffolds to enhance mechanical strength, including polycaprolactone (PCL) [6, 7], carboxymethylcellulose (CMC) [7] and polylactic acid (PLA) [8]. These polymers are biocompatible, non-toxic, and biodegradable, making them

ideal for reinforcing scaffold structures. Additionally, they can undergo surface modifications or crosslinking to further improve scaffold stability and strength. Several crosslinking techniques are employed to enhance scaffold structural integrity, including dehydrothermal treatment (DHT), ultraviolet (UV) irradiation, and chemical crosslinking using agents such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) combined with N-hydroxysuccinimide (NHS), glutaraldehyde (GTA), and bio crosslinkers like microbial transglutaminase and genipin. These crosslinking methods have been shown to improve the mechanical properties of scaffolds [9].

This study focuses on using DHT and EDC/NHS for crosslinking. EDC is a zero-length crosslinking agent that facilitates the coupling of carboxyl groups with primary amines by initially reacting with carboxyl groups to form O-acylisourea. Since O-acylisourea is unstable in aqueous environments and prone to hydrolysis, NHS is used to stabilize the reaction by forming a semi-stable, amine-reactive NHS ester [10].

II. MATERIALS AND METHODS

A. Preparation of Gelatin-CMC-PCL Scaffold

In this research, gelatin type A (Bio Basic Inc.) was mixed with CMC (Sigma-Aldrich) to fabricate 3D scaffolds utilizing the freeze-drying process. The scaffolds were first frozen at -80°C for 48 hours and then lyophilized at -105°C to control their porosity. The freeze-drying technique was chosen due to its efficiency, convenience, and ability to produce highly porous 3D scaffolds while preserving material properties [11]. After initial freeze-drying, the scaffold was immersed in a polycaprolactone (PCL) solution (Sigma-Aldrich) and subsequently freeze-dried again. A non-crosslinked scaffold was used as a control for comparison with the modified scaffolds.

To prepare gelatin-CMC-PCL porous scaffolds, the following steps were performed:

- A gelatin solution with a concentration of 0.8 wt.% was prepared by dissolving in deionized (DI) water.
- The solution was kept at room temperature for 45 minutes, then stirred at 50°C for 30 minutes until fully dissolved
- A 0.8 wt.% CMC solution was prepared by dissolving CMC with DI water and stirring it at 50 °C until homogeneous.
- The gelatin solution was mixed with the CMC solution, stirred until uniform, and pipetted into a 24-well tissue culture plate.
- The solution was frozen at -80 °C (Biologix model CKF-UL858) for 48 hours and lyophilized at a temperature of -105 °C (BUCHI model Lyovapor™ L-300 P) for 24 hours to obtain 3D porous structure.
- After gelatin-CMC scaffolds were obtained, it was soaked in a PCL solution prepared by dissolving it in chloroform (RCI Labscan) and stirring it at 70 °C for 30 minutes with a concentration of 20 and 30 wt.%.
- The gelatin-CMC scaffolds were soaked in the PCL solution for 5 minutes, frozen at -80 °C for 48 hours, and then freeze-dried again.

The mixing of the gelatin-CMC-PCL scaffolds in different mixing ratios is presented in Table I.

TABLE I. TABLE TYPE STYLES

Code	Concentration (wt%, w/w)		Code	Concentration (wt%, w/w)
	Gelatin	CMC		PCL
GC00	100	0	GC00P20	20
			GC00P30	30
GC20	80	20	GC20P20	20
			GC20P30	30
GC25	75	25	GC25P20	20
			GC25P30	30
GC30	70	30	GC30P20	20
			GC30P30	30
GC35	65	35	GC35P20	20
			GC35P30	30
GC40	60	40	GC40P20	20
			GC40P30	30

B. Crosslinking Methods

Crosslinking involves the formation of covalent bonds within or between molecules in the scaffold structure. This process enhances scaffold stability, improves mechanical properties, and slows down degradation rates [12]. It is widely used to optimize scaffold performance in tissue engineering applications. In this study, two crosslinking methods were used: DHT and EDC/NHS [13]. The EDC/NHS crosslinking mechanism for the gelatin-CMC-PCL scaffold is illustrated in Figure 1 [14]. A non-crosslinked scaffold was used as a control.

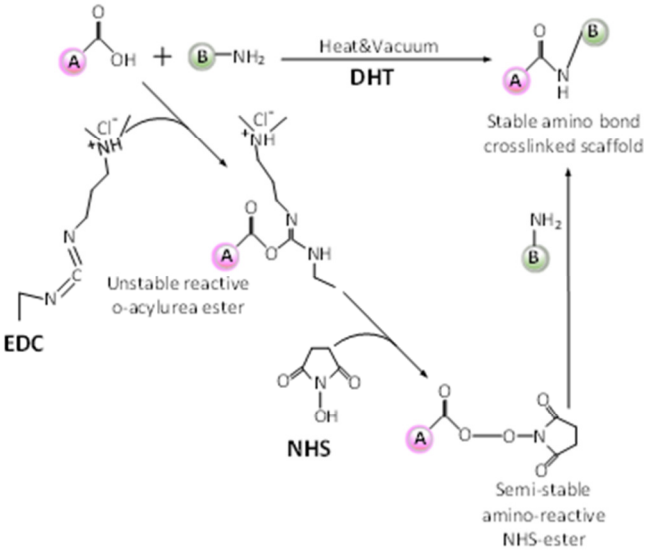

Fig. 1. Mechanism of EDC/NHS on gelatin-CMC-PCL scaffold.

1) Mechanism of EDC/NHS to Gelatin-CMC-PCL Scaffold

Chemical crosslinking utilizing EDC activates carboxylic acid groups, forming O-acylurea ester groups, which are water-soluble. The crosslinked scaffold is formed when free amine groups from lysine or hydroxylysine residues attack nucleophilically. To enhance crosslinking efficiency, NHS is introduced. The addition of NHS converts the O-acylurea ester group into an NHS-ester, which is more stable and less prone to hydrolysis at acidic pH compared to O-acylisourea. However, two side reactions may occur during EDC activation of carboxylic acid groups. Firstly, water can act as a nucleophile, leading to the hydrolysis of the O-acylisourea group, regenerating the carboxylic acid and forming a substituted area byproduct. Secondly, the highly reactive O-acylurea group can rearrange to a stable amino bond crosslinked scaffold. Other chemical crosslinking agents, such as genipin, glutaraldehyde, and formaldehyde, are also commonly employed. However, they may pose cytotoxicity risks upon implantation, making EDC/NHS a safer alternative [15].

2) Gelatin-CMC-PCL scaffold crosslinked by DHT

The freeze dry gelatin-CMC-PCL scaffold was crosslinked by DHT. Firstly, the various conditions of gelatin-CMC-PCL scaffold were immersed in 14/5.5 mM of DHT in 50 mM MES buffer for 2 hours at room temperature. After that, the crosslinked gelatin-CMC-PCL scaffold was thoroughly rinsed with DI water for 30 min for 3 times. Then, the crosslinked scaffold was frozen at -80 ^{o}C for 48 hours and lyophilized at -105 ^{o}C for 48 h. The obtained DHT crosslinked scaffold was kept in humidity control container.

C. Pore Size and Porosity

The pore size of a scaffold significantly influences the movement and distribution of fibroblast cells within the scaffold structure. In general, as the pore size increases, the structural strength of the scaffold decreases [16]. In this study, the entire surface structure of the scaffold was observed using a Scanning Electron Microscope (SEM) (JEOL: JCM6000). The scaffold was cut transversely using a surgical blade, and the exposed surface was gold-coated before imaging. An acceleration voltage of 10 kV was applied. To analyze pore size, the diameter of randomly selected 30 pores from different scaffold conditions were measured, and the average pore size was calculated. The porosity of the scaffold was analyzed utilizing the Mercury Intrusion Porosimetry (MIP) technique, which is based on the Washburn equation, describing the relationship between pressure and pore radius [17]. Scaffolds weighting between 3-5 grams were tested, employing a maximum of 5 milligrams of mercury.

D. Surface morphology

The surface morphology of the scaffolds was examined using SEM. Observations were conducted on both the vertical and horizontal surfaces of the scaffolds. The scaffold was sectioned using a surgical blade, and images were captured using the Backscattered Electron Imaging (BEI) signal in High Vacuum (HV) mode at an electron energy of 10 kV and 50x magnification.

E. Compressive Testing

A compressive test was conducted to evaluate the mechanical strength and resilience of the scaffold. Adequate mechanical strength is crucial for maintaining the 3D porous structure, ensuring the transportation of nutrients and oxygen for fibroblast cell growth. Additionally, the scaffold should resist shrinkage during application. The test was performed according to ASTM D3574-03, a foam compression test standard applicable to porous structures. Scaffolds were prepared with a diameter of 6 ± 1 mm, and a height of 5 ± 1 mm. Compression testing was performed using a universal testing machine (Zwickoell: Z1.0) under dry conditions applying a 20 N pressure, and a crosshead speed of 5 mm/sec.

III. EXPERIMENTAL RESULTS

The gelatin-CMC-PCL scaffold was analyzed for its morphological, mechanical, and biodegradation properties to determine the most suitable condition for tissue engineering applications. The results are as follows:

A. Surface Morphology

The gelatin-CMC-PCL 3D scaffold, fabricated using the freeze-drying technique, exhibited a cylindrical shape with an average diameter of 7 ± 1 mm and an average height of 6 ± 0.5 mm. The external appearance was uniformly porous, resembling a sponge-like structure with a white to yellowish color. Figure 2 presents the physical characteristics, including diameter and height measurements, of the obtained gelatin-CMC-PCL 3D scaffold.

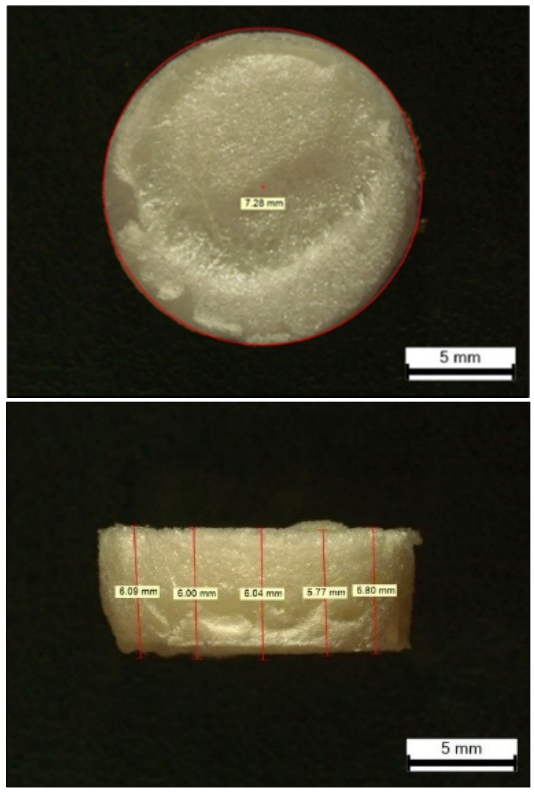

Fig. 2. 3D gelatin-CMC-PCL scaffold: (a) front view and (b) side view.

To examine the surface structure, scaffolds with different mixing ratios were sectioned horizontally and vertically using a surgical blade. The structural properties were then observed using SEM at 50x magnification. It was found that the cross-sectional SEM images of the DHT-crosslinked gelatin-CMC-PCL scaffold revealed continuous circular pores (Figure 3(a)). However, the pore size was smaller compared to the non-crosslinked scaffold. The non-crosslinked gelatin-CMC-PCL scaffold exhibited larger, continuous circular pores (Figure 3(b)). The EDC/NHS-crosslinked gelatin-CMC-PCL scaffold maintained a circular pore structure with lateral expansion. Upon visual inspection, the pore size was larger than that of the DHT-crosslinked scaffold but smaller than the non-crosslinked scaffold (Figure 3(c)).

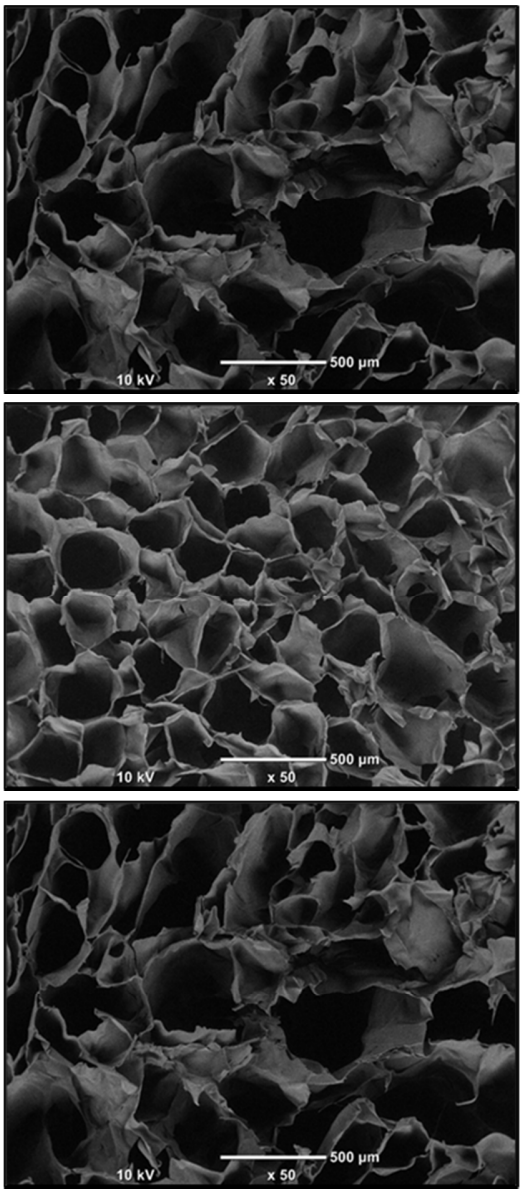

Fig. 3. SEM images of gelatin-CMC-PCL scaffolds: (a) GC40P30, (b) GC40P30 (crosslinked by DHT), (c) GC40P30 (crosslinked by EDC/NHS).

B. *Pore Size and Porosity*

1) *Pore Size Test*

To determine the average pore size, SEM images at 50x magnification were analyzed using ZEN Core software (ZEISS). The pore shape was assumed to be circular for measurement comparisons. The results demonstrated that the gelatin-only scaffold had a relatively large average pore size of 164 µm. However, when CMC was added to gelatin, the average pore size decreased at each mixing ratio. Interestingly, the pore size tended to increase with higher CMC concentrations. When PCL was incorporated at 20 wt% and 30 wt%, the average pore size increased compared to the gelatin-CMC scaffolds without PCL. This increase was observed for gelatin-CMC scaffolds containing 30 wt%, 35 wt%, and 40 wt% CMC when combined with 20% and 30% PCL. The gelatin-CMC-PCL scaffold crosslinked with DHT exhibited a smaller pore size at the same mixing ratio compared to non-crosslinked scaffolds. At the same time, the gelatin-CMC-PCL scaffold crosslinked with EDC/NHS showed a larger pore size than DHT-crosslinked scaffolds but remained smaller than non-crosslinked scaffolds. The GC35P30 scaffold exhibited the largest average pore size among the DHT-crosslinked scaffolds, measuring 147.77 µm. In the case of EDC/NHS-crosslinked scaffolds, the GC35P30 scaffold exhibited a pore size of 184.40 µm, which was 12.55% larger than the gelatin-only scaffold and greater than the pore size of the DHT-crosslinked gelatin scaffold. The largest average pore size was observed in non-crosslinked scaffold measuring 192 µm. The highest pore size was recorded in the GC35P30 scaffold, measuring 22.57% which facilitated greater fibroblast cell attachment, migration, and growth, as depicted in Figure 4. A previous study on PCL-CMC scaffolds reported an average porosity range of 81.44% to 98.88%. The highest porosity was observed in the P5 scaffold (PCL 80% (w/w) - CMC 20% (w/w)), which had a porosity of 98.88%, whereas the pure PCL scaffold (P0 scaffold) had the lowest porosity at 81.44% [18].

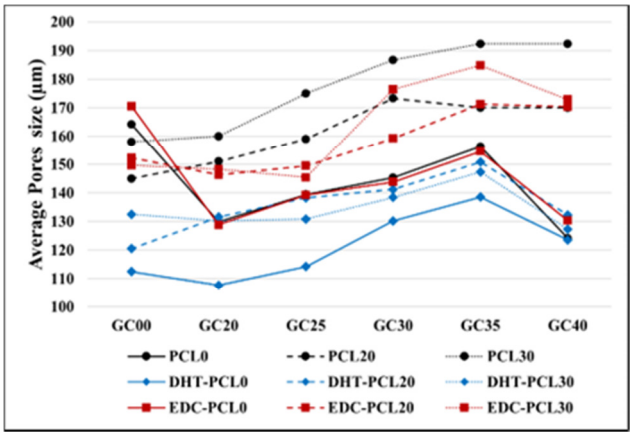

Fig. 4. Average pores size at various mixing proportions of gelatin-CMC-PCL scaffolds with non-crosslink, crosslinked by DHT and EDC/NHS.

2) *Porosity Test*

The porosity test results indicates that the pure gelatin scaffold had a porosity of 85.40%. When CMC was

incorporated into the gelatin scaffold, the porosity gradually decreased as the CMC content increased. Similarly, when PCL was added to the gelatin scaffold at 20% and 30% concentrations, the porosity slightly decreased to 84.95% and 84.88%, respectively. The addition of PCL to gelatin-CMC scaffolds resulted in higher porosity compared to gelatin-CMC scaffolds without PCL, at the same mixing ratios. Higher PCL concentrations (30%) produced slightly higher porosity than lower PCL concentrations (20%). The addition of PCL to gelatin-CMC scaffolds and crosslinking with DHT resulted in a slight reduction in porosity, with an average porosity reduction of approximately 2.29% compared to non-PCL scaffolds. The highest porosity among DHT-crosslinked scaffolds was found in the GC20P20 scaffold, with a porosity of 72.08%. The GC20P20 scaffold crosslinked with DHT followed by EDC/NHS exhibited the highest porosity at 74.48%. The porosity values for different gelatin-CMC-PCL scaffolds with varying mixing ratios, crosslinked by DHT and EDC/NHS, are summarized in Figure 5. A previous study on PCL-CMC scaffolds reported an average porosity range of 81.44% to 98.88%. The highest porosity was found in the P5 scaffold (PCL 80% (w/w) - CMC 20% (w/w)), measuring 98.88%, while the pure PCL scaffold (P0 scaffold) had the lowest porosity at 81.44% [18].

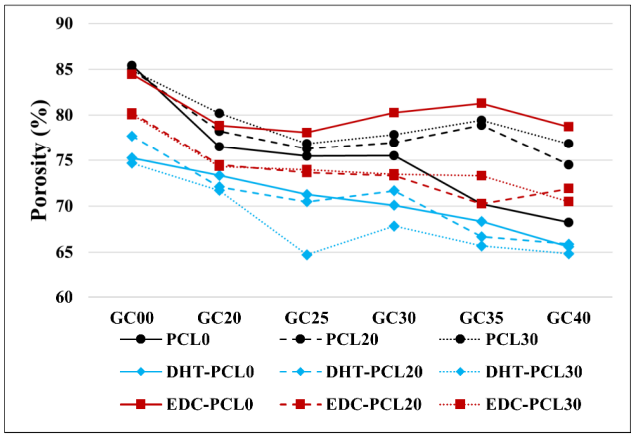

Fig. 5. Average porosity at various mixing proportions of gelatin-CMC-PCL scaffolds with non-crosslink, crosslinked by DHT and EDC/NHS.

C. Compressive Strength

The compressive strength of the gelatin-CMC-PCL scaffold was evaluated under different crosslinking conditions. It was found that gelatin-CMC-PCL scaffold when crosslinked by DHT and EDC/ NHS expressed increased compressive strength compared to non-crosslinked scaffolds. Additionally, compressive strength increased as the CMC-PCL proportion increased in the scaffold. The mixed scaffold (GC40P30(DHT) that crosslinked by DHT showed the highest compressive strength. The GC40P30(DHT) scaffold exhibited the highest compressive strength of 59.34 MPa, which represented a 32.37% increase compared to the non-crosslinked gelatin-CMC-PCL scaffold. The GC40P30(EDC/NHS) scaffold had a compressive strength of 48.67 MPa, which was 17.98% higher than the non-crosslinked scaffold.

However, crosslinking by DHT process removed water molecules, leading to stronger bonding between polymer chains. This creates a more stable mechanically reinforced structure. A for the EDC/NHS crosslinking, it facilitates chemical bonding between polymer chains via covalent linkages. This resulted in a lattice-like structure, increasing overall mechanical strength. A previous study on CMC-PCL scaffolds reported a compressive modulus range of 0.519-0.790 MPa. The highest compressive modulus was observed in the P2 scaffold (93.5/6.5% of PCL/CMC) which was 0.79 MPa and the lowest compressive modulus showed in P3 scaffold (89/11% of PCL/CMC) which was 0.519 MPa [19].

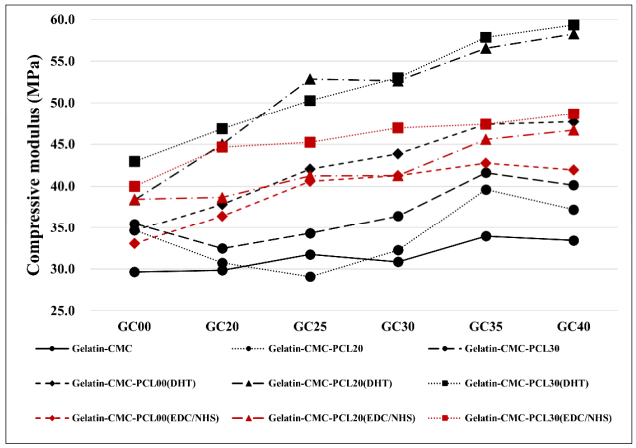

Fig. 6. Average compressive modulus (MPa) at various mixing proportions of gelatin-CMC-PCL scaffolds of non-crosslink, crosslinked by DHT and EDC/NHS.

IV. CONCLUSION

This study examined the morphological and mechanical properties of gelatin-CMC-PCL scaffolds, including pore size, porosity, and compressive strength, to determine the optimal composition and crosslinking method for potential wound dressing applications.

The experimental results demonstrated that the gelatin-CMC-PCL scaffold with 35% (w/w) CMC and 30% (w/w) PCL exhibited the best overall properties. Scanning Electron Microscopy (SEM) images confirmed an interconnected porous structure for both DHT and EDC/NHS crosslinked scaffolds, with circular and laterally expanded pores. The largest average pore size (192 µm) was observed in the non-crosslinked scaffold, which shrank by 23.44% to 147.28 µm after DHT crosslinking, while EDC/NHS crosslinking resulted in a 20.45% larger pore size than the DHT-crosslinked scaffold (184.80 µm). The porosity results aligned with the pore size measurements, with the highest porosity (79.40%) in the non-crosslinked scaffold, which decreased to 65.68% after DHT crosslinking, but increased to 73.28% with EDC/NHS crosslinking. The highest compressive strength was observed in the DHT-crosslinked scaffold (57.87 MPa) due to its denser structure and lower porosity, followed by EDC/NHS crosslinked scaffold (47.39 MPa), while the non-crosslinked scaffold exhibited the lowest strength (41.61 MPa). These findings confirm that PCL enhances the mechanical and

structural properties of gelatin-CMC scaffolds, increasing porosity by 11.55% and compressive strength by 18.40%, with DHT crosslinking further improving strength by 28.11% but reducing porosity by 17.28%, while EDC/NHS crosslinking increased compressive modulus by 12.21% and slightly reduced density by 7.71%.

Future research should focus on biocompatibility and biodegradation properties to ensure the scaffold supports cell growth, is non-toxic, and has an appropriate degradation rate for wound dressing applications.

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