International Journal of Chemical and Biochemical Sciences
(ISSN 2226-9614)

Journal Home page: www.iscientific.org/Journal.html

Optimization of gel content and swelling properties of hyaluronic acid/*Gracilaria changgi* hydrogel

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Abstract

Hyaluronic acid (HA) is a biocompatible and biodegradable that is naturally found within the extracellular matrix of many human tissues. HA-based hydrogels have been widely utilized in tissue engineering, drug delivery, regenerative medicine and hyperthermia treatment, and other biomedical applications. Various types of seaweeds are extensively used in the life science as source of compounds with diverse structural forms and biological activities, therefore, potential source of natural nutrients for body. *Gracilaria changgi* (Gc) is edible seaweed can be found at many places in Malaysia. Due its chemical composition and structure, it can be utilized in hydrogel formulation that could help to improve the performance of hydrogel in medical applications. In this study, the preparation of HA/Gc hydrogel was optimized by using three parameters; concentration of 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDC), ratio of HA to Gc, and pH values. The optimum ratio of gel content was found at 70:30 ratio of HA/Gc with 30 mM of EDC in pH 6.0. As HA was blended with Gc, the gel content and swelling studies of the HA/Gc hydrogel film improved when compared to HA alone.

Keywords: Hyaluronic acid, *Gracilaria changgi*, hydrogel, seaweed, 1-Ethyl-3-[3-(dimethylamino)- propyl]carbodiimide

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1. Introduction

Hydrogel are polymer networks having hydrophilic properties, capable to retain high amount of water content as high as 90% to 99% [1, 2]. Hydrogel water absorption capacity is due to the presence of hydrophilic groups such as -CONH, -OH, -SO_3H and -$CONH_2$ [1]. The hydrogel porous structure may be readily controlled by manipulating crosslink density between polymer chains that join by covalent bonds, hydrogen bonds, or physical entanglements using chemical or physical crosslinks [3]. When the environment changes, such as temperature, pH, in presence of glucose and enzyme, hydrogel is extremely sensitive [3]. Their porosity of hydrogels allows loading of drugs into the gel network which promote the interest of hydrogels in drug delivery applications [1, 3, 4]. Hyaluronic acid (HA) is a natural linear anionic polysaccharide made up of alternating units of N-acetyl-D-glucosamine and β-D-glucuronic acid positioned at 1,4 and 1,3 respectively which usually found in vitreous body, umbilical cords, skin, in synovial fluid and skin [5, 6]. The physico-chemical properties of HA are unique, such as biodegradability, biocompatibility, hygroscopicity, viscoelasticity and mucoadhesivity which involved in number of essential processes, including wound reparation, cell signaling, matrix organization, pathobiology, morphogenesis, and tissue regeneration. The outstanding properties of HA made it useful in many applications including treatment in cancer and drug delivery system, rhinology, ophthalmology, urology, aesthetic medicine and cosmetic [7]. However, the mechanical properties of HA based hydrogel are commonly very poor in vitro environment and rapidly degrade in vivo system which limit its use in biomedical application [8, 9]. The physico-chemical properties and stability of HA can be improved by pre-modified with crosslinkable functional groups or by adding a crosslinker directly and three-dimensional (3D) network is formed [7-9]. Seaweed is a general name for many species of macroscopic, multicellular, marine algae that grow in the ocean as well as in lakes, rivers, and others. Various seaweeds are extensively used in the life science as source of compounds with diverse structural forms and biological activities, therefore, potential source of novel antioxidants.

Reported by Jensen [10], some edible seaweed contains high amounts of vitamins, protein and mineral that important for human nutrition. *Gracilaria Changgi* (Gc) is an edible seaweed that can be found in various places in Malaysia. Due its chemical composition and structure, it can also be utilized into the hydrogel matrix. There are many studies have been carried out on the modification of HA based hydrogel, however, to the best of our knowledge, no study has been reported on HA blend *Gracilaria changgi* seaweed hydrogel. Cross linking in polymer chemistry is a stabilization process which the formation of network structure resulting from the multidimensional extension of polymeric chain. The physical properties of polymer are affected by addition of cross linker between polymer chains which depend on crosslinking degree and crystallinity (presence or absence). Various cross-linking agents including glutaraldehyde (GTA), epoxy compounds, divinyl sulfone and carbodiimides have been studied for chemically modified of HA [10, 11]. Among them, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) is extensively used due to its low cytotoxicity [11, 12]. It was found that, EDC crosslinked HA had a faster degradation rate, smoother surface structure and reduced in cytotoxicity as compared to the GTA cross-linker [11]. It can be fabricated mechanically reinforced, structurally stable, transparent, cytocompatible and readily deformable HA hydrogel discs with the potentiality to be used as delivery carrier for corneal endothelial cell therapy. In this study, HA and Gc were chosen as the main materials for hydrogel formulation due to their excellent properties in drug delivery applications, such as biocompatibility and non-toxic. EDC was chosen to be used as crosslinker.

2. Materials and methods

2.1. Materials

The raw *Gracilaria Changgi* (Gc) seaweed was obtained from coastal area of Sabah, Malaysia. The hyaluronic acid (HA, sodium salt, M_w=1.5×10^6) and the 1-Ethyl-3-[3-(di-methylamino)-propyl]carbodiimide(EDC) were purchased from the Sigma-Aldrich.

2.2. Synthesis of HA/Gc hydrogel

A dried Gc seaweed was washed properly under running water and was soaked in water for few days (daily exchange of water). The seaweed then was rinsed and dried for few days. A dried seaweed was cut and chopped to small size and ground into powder form. After sieved, it was kept in plastic containers and placed in desiccators. Three parameters were varied to prepare HA/Gc 0.5% (w/v) which were concentration of EDC, ratio of HA to Gc, and pH value. Solution mixture of HA and Gc was prepared with various ratio of HA to Gc; 90:10, 80:20, 70:30, 60:40 and 50:50 by dissolving them in distilled water and stirred at 350 rpm until homogeneous. Then, the EDC with concentration of 10 mM, 30 mM and 50 mM was added into homogeneous HA/Gc solution and continue stirred. The samples were left for 3 days at 37℃ for drying purpose.

2.3. Measurement of gel content of the HA/Gc hydrogel

Before being immersed in distilled water for 24 hours, the dry HA/Gc hydrogel film was weighed (W_i). After the hydrogel was immersed in distilled water 24 hours, hydrogel was dried again at 37℃ for drying in oven until constant weight. After that, the hydrogel was weighted again (W_d). The percentage of gel content and soluble gel was calculated according to the following equations.

$$\textbf{Soluble gel (\%)} = (W_i - W_d)/W_i \times 100 \ \textbf{Gel content (\%)}$$

$$\textbf{Gel content (\%)} = 100 - \textbf{Soluble gel (\%)}$$

Where W_d is the weight of dried hydrogel after immersion in water 24 hours and W_i is the initial weight of dried hydrogel.

2.4. Measurement of swelling properties of the HA/Gc hydrogel

The dried HA/Gc hydrogel film was weighed to determine the hydrogel's swelling properties. The dried HA/Gc was immersed in 10 mL of distilled water for 24 hours at room temperature. Then, swollen HA/Gc hydrogels were filtered, excess water was wiped and weighed. The experiments were repeated three times, the average weight of hydrogel was determined. Same methods were used for the other pH values which were 2.0, 4.0 and 6.0. The swelling percentage of the hydrogel was calculated using the following equation.

$$\textbf{Weight of swollen hydrogel} = W_i / W_d \times 100\%$$

2.5. FT-IR analysis

Fourier transform infrared-attenuated total reflection (FTIR-ATR) analysis of the samples was performed by using Bruker Alpha II. The seaweed was analyzed in powder form, while hydrogels were analyzed in the form of thin film. The hydrogel was cut into small pieces and scanned within range of 4000-400 cm^{-1}.

3. Results and discussion

3.1. Effect of concentration of EDC on gel content of HA/Gc hydrogel film

Figure 1 shows the gel content of several ratio of HA/Gc hydrogel composition at different concentration of cross linker, EDC from 10 mM to 50 mM. Hydrogel HA is a hydrogel consist of HA only was prepared for comparison purpose. Similar trend was observed as a high concentration of cross linker was resulted high percentage of gel content of hydrogels. A maximum percentage of gel content was obtained at 30 mM of EDC for all ratio of HA to Gc. In comparison with previous study conducted by Yacob et al. [13], the gel content of the PVP/carrageenan hydrogel slightly increased as irradiation dose increased, which may be explained by the formation of denser and tighter hydrogels network structure in polymer chain. However, as shown in Figure 1, further increased the concentration of EDC to 50 mM, the gel content slightly reduced compared to 30 mM. The amide formation in the hydrogel polymer is affected by the concentration of EDC. The higher concentration of EDC produced more amide linkage. From the Figure 1, 50 mM of EDC was excessive in HA/Gc hydrogel to crosslink with carboxyl groups, leading to more side reaction of N-acylurea formation [14]. From figure 1, the gel content increased as the ratio of HA to Gc decreased from 100:0 (HA hydrogel) to 70:30. The hydrogel of HA/Gc with ratio of HA to Gc of 60:40 and 50:50, had a lower percentage of gel content because they have lower percent of HA which give less side chain for crosslinking reaction. Similar studies by Zhang et al, high percent, 7 % of HA derivative carrying aldehyde (HAALD) had lowest gel content due to the high viscosity of HAALD. HAALD is a well-known property of HA derivatives with applied biological functions in tissue engineering. The high viscosity caused blocking in the conjugation between the active groups of HA derivative and other polymers, and resulting a low gel content of hydrogel [13]. Figure 2 shows chemical structure of HA and Gc. Carboxylate groups from the chemical structure of HA, can be reacted with EDC to form amide linkage. However, the structure of Gc also consists of carboxyl, hydroxyl and ester stretching bonds. The crosslinking reaction forming an insoluble gel from HA/Gc solution were mostly due to reaction of EDC and HA. Hence, increasing in the amount of HA (from 50% to 70%) leads to more functional groups available for crosslinking which resulted in high percentage of gel content. Thus, high concentration of EDC is probably needed in HA/Gc mixture for more covalent crosslinking formation [14]. 70:30 ratio of HA/Gc hydrogel was chosen to proceed to the next optimization parameter because of their high gel content which is suitable for drug-loading.

3.2. Effect of pH value on gel content of HA/Gc hydrogel film

Figure 3 shows gel content of HA/Gc hydrogel with HA to Gc ratio 70:30 prepared at different pH values. From Figure 3, 70:30 HA/Gc hydrogel showed a maximum percentage of gel content (62.03%) at pH 6.0, followed by pH 4.0, pH 2.0, and distilled water. HA/Gc hydrogel exhibited excellent gel content in acidic condition because of its hydrophilic characteristic of HA. At the same time, these HA/Gc hydrogels are sensitive to pH. Previous study showed HA is highly sensitive to pH and degrade at $4 < pH > 11$ [5].

3.3. Effects of concentration of EDC on swelling properties of HA/Gc hydrogel film

The swelling percentage of hydrogel is one of the most important parameters in swelling studies. Figure 4 shows the images of dried and swelled HA/Gc hydrogel film after 4 hours of immersion in water. The HA/Gc hydrogel film swelled immediately and the shape is maintained without dissolving. This indicates that the crosslinking of polymer chains was accomplished.

As shown in Figure 5, as the concentration of EDC cross linker increased, the percentage of swelling was decreased. According to Flory's theory, higher concentration of cross linker will induce the generation of more crosslink points, thus, the density of crosslinking increase [15]. Highest percentage of swelling of 70:30 HA/Gc hydrogel was obtained at 10 mM of EDC. This may be due to the insufficient concentration of cross linker which cause failure in the hydrogel network formation and resulting in increasing amount of soluble portion [15]. The percentage of swelling of HA/Gc hydrogel was decreased as the concentration of EDC increased from 10 mM to 30 mM.

The decreased swelling percentage of 30 mM would be expected due to the more compact and tight structure of crosslinked hydrogel formation. Similar outcomes were found by Kanafi et al [3], the swelling percentage decreased beyond 10% (w/w) of citric acid which may be due to a formation of strong and tight bonding in crosslinked hydrogel structure, thus, repels penetration of large volume of water into hydrogels. Moreover, as the higher concentration of EDC was used, more carboxyl and hydroxyl groups in HA and Gc were consumed during reaction of crosslinking, thus, leaving less hydroxyl groups that available for hydrogen bonding with water molecules. Thus, the swelling percentage is decreased. When comparing the percentage of swelling between 10 mM and 50 mM of EDC, the percentage of swelling of 50 mM much lower than 10 mM of EDC which due to the rigid network structure.

The pH of the medium also affected the swelling behavior of hydrogel. The 70:30 HA/Gc at difference concentration of EDC were swelled in several pH 2.0, 4.0, 6.0 and distilled water (pH 6.3) at room temperature. For 10 mM of EDC, the highest swelling percentage of HA/Gc hydrogel was in distilled water followed by in pH 2.0, pH 4.0 and pH 6.0. The high swelling percentage of hydrogel is attributed to hydrogel's ability to absorb water. The penetration of water in the HA/Gc hydrogel (70:30) with in distilled water shows the maximum swell within the crosslinked HA/Gc network structure. However, the nature properties of HA hydrogel that behaves as a hydrophilic gel while swelling at neutral pH as most of the carboxyl groups were ionized at higher pH (near 7) and become deprotonated at lower pH [16]. In this study, it is found that pH 6.0 was the optimum for percentage of swelling of the hydrogel.

3.4. Fourier Transform Infrared Spectroscopy Studies

Figure 6 shows the FTIR spectra of HA/Gc hydrogel prepared using 30 mM EDC with different ratio of HA to Gc. As shown in Figure 6, HA structure has spectral characteristics for carboxylate anions, which absorption bands at 1401 cm^{-1} assigned symmetric stretching vibrations of carboxylate anion. There are also amide bonds present at

1622 cm^{-1}. The crosslinking EDC with HA/Gc hydrogels was confirmed through formation of HA/Gc hydrogels from its solution. Two new absorption peaks showed at 1400 cm^{-1} and 1134 cm^{-1} which were assigned to symmetric stretching vibration of COO- and C-C(O)-C stretch of ester bonds of HA. As the amount of HA increased as different ratio, the C=N peak shift to high wavelength. As the amount of Gc increased, the broad hydroxyl group was recorded at around 3290-3306 cm^{-1}. The hydroxyl group from Gc structure and new crosslinking were overlapped. In Figure 6, it shows the increased in intensity as the amount of Gc is higher in ratio (10% to 50%) while constant concentration of EDC for all ratios of HA/Gc. Furthermore, at 1210-1233 cm^{-1} peak which belong to C-O stretching of GC, the intensity weakened as lesser amount of Gc in ratio were used. This proven that, crosslinking may also occur at Gc structure. However, as the ratio of Gc increased (10% to 50%), the peak shifted from 1601 cm^{-1} to 1636 cm^{-1} due to the high number of carboxylate bond formation. This may due to reaction of the ester band from unreacted EDC.

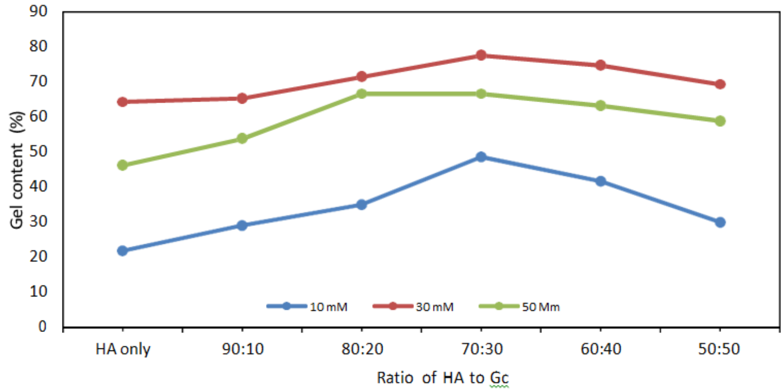

Fig. 1. Effect of different concentration of EDC on gel content of HA/Gc hydrogels at different HA to Gc ratio

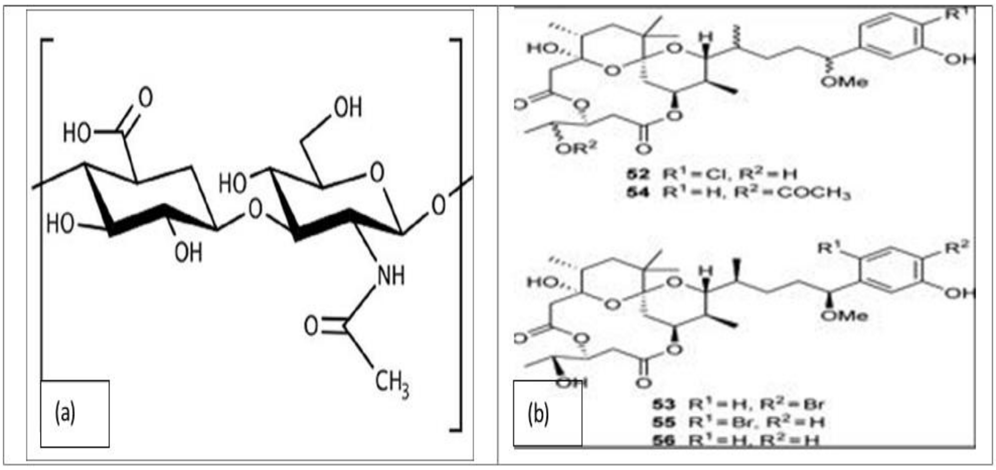

Fig. 2. General chemical structure of (a) hyaluronic acid and (b) *Gracilaria changgi*

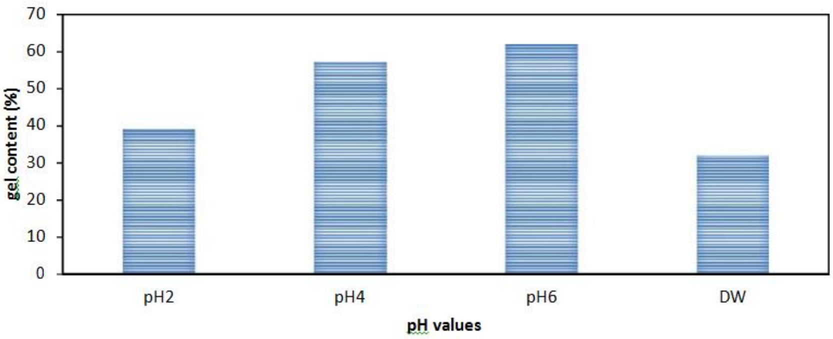

Fig. 3. Effect of gel content of 70:30 HA/Gc on pH values, EDC was kept constant at 30 mM

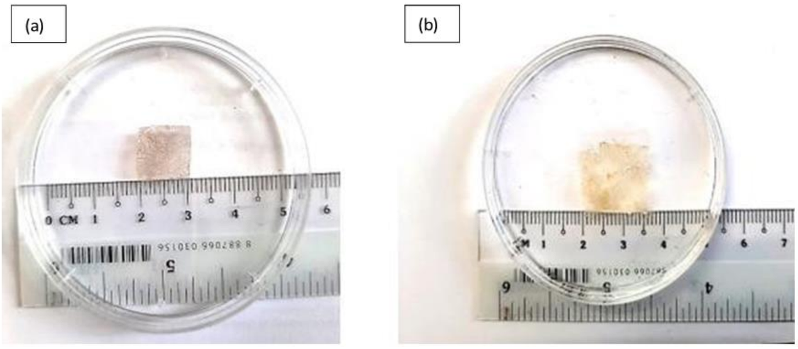

Fig. 4. a) Dry crosslinked HA/Gc and b) swelled HA/Gc hydrogel film

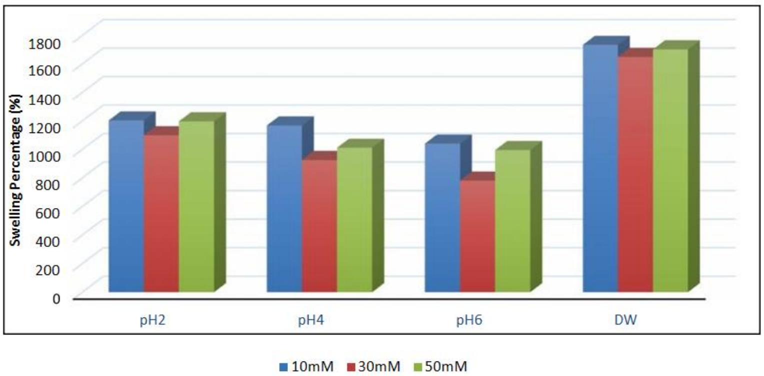

Fig. 5. Effect of different concentration EDC and pH value on swelling percentage of 70:30 HA/Gc

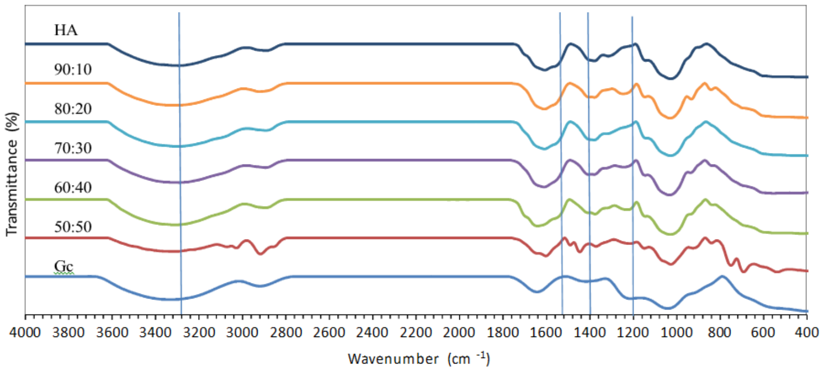

Fig. 6. FTIR spectra of HA/Gc hydrogel with different ratio of HA to Gc. (Parameters of hydrogel preparation: EDC 30 mM)

4. Conclusions

A HA/Gc hydrogel film was successfully prepared and optimized. The results showed that the crosslinking is very important in preparation of HA/Gc hydrogel because it give great effect on the properties of the hydrogel produced. The effect of concentration of EDC on the properties of HA/Gc was analyzed by measuring its gel content, swelling percentage and characterized using Fourier Transformed Infrared (FTIR) spectroscopy. The optimum formation of hydrogel was obtained at ratio 70:30 HA/Gc with 30 mM concentration of EDC at pH 6.0. This preliminary work has illustrated the potential of crosslinked HA/Gc as a drug carrier.

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