Characterization of elasticity and hydration of composite hydrogel based on collagen-iota carrageenan as a corneal tissue engineering

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Abstract. The cornea is a refractive element of the eye that serves to continue the stimulation of light into the eye it has a clear, transparent, elastic and relatively thick tissue. Factors caused corneal blindness, are dystrophy, keratoconus, corneal scaring. Hydrogels can be made from polysaccharide derivatives that have gelation properties such as iota carrageenan. Therefore, it is a need to develop composite hydrogel based collagen-iota carrageenan as an engineered corneal tissue with high elasticity and hydration properties. Collagen hydrogel has a maximum water content an has equilibrium up to 40 %, less than the human cornea, 81 % and under normal hydration conditions, the human cornea can transmit 87 % of visible light. In addition, the refractive index on the surface of the cornea with air is 1.375-1.380. Based on this study, it is necessary to conduct research on the development and composition of hydrogel composite collagen-iota carrageen hydrogen based on.The best result was K5 (5:5) treatment, which has the equilibrium water content of 87.07 % and viscosity of 10.7346 Pa.s.

1. Introduction

The cornea is a refractive element of the eye that serves to continue the stimulation of light into the eye; it has a clear, transparent, elastic and relatively thick tissue [1]. It is estimated that around 10 million people worldwide suffer from vision loss due to corneal damage [2]. Some factors causing corneal blindness are infection (45 %), trauma (25 %), heredity (5 %), and other factors such as nutrinal (13 %) of the total number of people suffering from blindness in the world. Factors that cause corneal blindness can lead to certain conditions on the cornea, including Fuch's dystrophy, keratoconus, keratophaty, dystrophies, and corneal scaring. This indicates that a corneal donor through transplantation (keratoplasty) is able to overcome the problem of blindness due to obstacles to refractive ability, clarity and corneal wholeness [3].

The donor cornea (graft) provides the cornea of a natural donor (allografts) and an artificial cornea (keratoprosthesis). Allografts have good biocompatibility, which can accelerate the proliferation of endothelial cells in the cornea. However, rejection by the recipient often occurs.

Based on medical approach, the ideal replacement cornea must be biodegradable and can be a tissue regeneration promoter. The application of synthesis materials such as glycopolymers produces corneal
membranes that cannot be degraded by the eye stromal tissue. Therefore, they are rarely used. The membrane is relatively unreactive, which will be rejected (immunologically rejected), <10%. The development of replacement corneas in recent years has focused on such materials as soft polymeric hydrogels [4].

The natural ingredients of collagen and its composites have shown good biocompatibility and tissue regeneration in animals [2]. Collagen fibrillar, the composition of corneal stromal layer, is a type I collagen that can be extracted from tilapia species. Due to the increase of zoonotic infection cases in mammals, its use as a source of collagen is decreasing. Alternatively, fish are used as a potential source of low-potency collagen. In addition, fish collagen is easily digested and absorbed by the body with cow/pig collagen. This fibrillar structure can act as a good scaffold on corneal tissue techniques. The application of the collagen as a material for artificial corneas results in membranes with high mechanical strength (elasticity), which is low. So, it needs other materials. Hydrogels can be made from polysaccharide derivatives that have gelatin properties such as carotene iota [5]. The caroteneic ion will undergo a transition into a helix-shaped coil (helix) that carries it on gelation, resulting in a smooth elastic gel.

Patel et al. [6] showed that collagen-polymer hydrogel has a maximum water content equilibrium up to 40%, while the human cornea has 81%. This characteristic is required in corneal tissue engineering for high mechanical and hydration strength. Based on this background, it is necessary to conduct research on the manufacture and composition of composite hydrogel based on collagen-iota karaginan (5:0; 5:1; 5:2; 5:3; 5:4; 5:5) so that the optimum composition characteristics approaching corneal characteristics can be identified.

2. Methodology
The research was conducted using fish collagen (pure), semi refined iota karaginan from Eucheuma spinosum, Ca(OH)₂, ethanol 96%, HCl 0.1N, glutaraldehyde, Phospate Buffer Saline, glycerine and distilled water. This research employed experimental methods with completely randomized design, which consists of six treatments. Are K0 (5:0) as control, K1 (5:1), K2 (5:2), K3 (5:3), K4 (5:4) and K5 (5:5) with three repetitions for each treatment.

K0: 50 mg collagen: 0 mg iota carrageenan
K1: 50 mg collagen: 10 mg iota carrageenan
K2: 50 mg collagen: 20 mg iota carrageenan
K3: 50 mg collagen: 30 mg iota carrageenan
K4: 50 mg collagen: 40 mg iota carrageenan
K5: 50 mg collagen: 50 mg iota carrageenan

Hydrogel synthesis was performed with blinding technique. Ten percents (w/v) of collagen solution was prepared by dissolving 50 mg of granular collagen using 5 ml distilled water with a temperature of 50°C. It was then stirred at 600 rpm for 20 minutes. Furthermore, using the same step, 10% (w/v) iota carrageenan solution was prepared by dissolving iota carrageen into distilled water with a temperature of 50°C, which was then stirred at 500 rpm for 35 minutes. Iota carrageenan solution was then poured into the collagen solution with 200 rpm stirring for 10 min at 50°C. After that, a cross linker of 0.5 ml gluteraldehyde and 0.5 ml ethanol and 0.05ml 0.1 N HCl sequentially and 3% of glycerin were added at each treatment to form viscous. Furthermore, it was printed in a 15x2 cm mica mold and left in room temperature for 15-30 minutes.

The main parameters of this study were equilibrium water content and viscosity of the hydrogel. Observation of equilibrium water content was conducted by cutting the gel into square with the size of 1x1 cm² as much as five pieces taken from each mold sampling point (top, bottom, middle) and weighed (W₀), then dipped into phosphate buffer saline (pH 7.4) solution. After being charged for 3 hours, the hydrogel was then removed and weighed wet weight (W). Then, the hydrogel was dried to a constant weight, and
the dry weight ($W_0$) was weighed. Equilibrium water content ($W_t$) was calculated using the following equation:

$$W_t = \frac{W_w - W_0}{W_0} \times 100\% \quad (1)$$

Viscosity test was aimed at measuring the elasticity value of hydrogel, which indicates the mechanical strength. The viscosity test was carried out at a temperature of 30°C. Hydrogel samples were cut in a circle form with a diameter of 2 cm and thickness of ± 1mm. The samples were thawed by heating and diluted using distilled water 1:4. The samples were then inserted into Oswald's viscometer and the time when hydrogel dropped from the first line to the second line was calculated. Viscosity number was calculated using the following Poiseville equation.

$$\eta = \frac{\pi PR^4T}{8LV} \quad (2)$$

Supported parameters are refractive index and light transmission. Refractive Index measurements were performed using a VEE GEE refractometer at a temperature of 30°C by dripping the gel on the prismic surface of the refractometer reading the refractive index scale. Transparency testing was performed by measuring the transmittance of the gel using a UV-Vis spectrophotometer at 600 nm wavelength. The hydrogel solution was taken ± 3 ml, put into the cuvette. Cuvette was inserted into UV-Vis Spectrophotometer. Then, the percentage of transmittance was calculated.

Data of sperm motility and motion duration that have been obtained were analyzed using ANOVA. The data were then analyzed using Duncan's Multiple Range Test at the resulting p-value lower than 0.05 ($p < 0.05$).

3. Results

Hydrogel characterization was conducted physically. The result of the composite hydrogel characterization is presented in table 1. It shows the average result of the composite hydrogel characterization from the first treatment (K0), K2, K3, K4, until K5. As the table indicates, the equilibrium water content is more varied than the literature. The viscosity and transparency were similar to those indicated by results in the literature. Meanwhile, the refractive index was smaller than the literature.

| Parameter          | Research result       |
|--------------------|-----------------------|
| Equilibrium water content | 0.87.07%            |
| Viscosity         | 1.4847-10.7346 Pa.s   |
| Refractive index   | 1.3473-1.3513         |
| Transparency      | 17.67-97.80%          |
The hydration of hydrogel was observed by the percentage of equilibrium water content, which was displayed in Table 1. ANOVA test results indicated that the composition of hydrogel has a significant effect (p < 0.05) on the average equilibrium water content. Duncan's multiple range test indicates that the lowest average of equilibrium water content was present in K0 (5:0) treatment that significantly differs (p < 0.05) from K1 (5:1), K2 (5:2), K3 (5:3), K4 (5:4) and K5 (5:5) treatments. Meanwhile, the K1 (5:1), K2 (5:2), K3 (5:3), K4 (5:4) and K5 (5:5) are not significantly different.

**Table 2.** Equilibrium water content of hydrogel.

| Treatment  | Eq. Water Content Average (%) ± SD |
|------------|-----------------------------------|
| K0 (5:0)  | 0±0.00                            |
| K1 (5:1)  | 76.18±12.08                       |
| K2 (5:2)  | 76.78±10.11                       |
| K3 (5:3)  | 74.58±14.35                       |
| K4 (5:4)  | 80.40±8.51                        |
| K5 (5:5)  | 87.07±7.28                        |

Note: Different superscript in one column indicates significant difference (p<0.05)

K0 treatment is significantly different from the K1 (5:1), K2 (5:2), K3 (5:3), K4 (5:4) and K5 (5:5) treatments. K1 (5:1), K2 (5:2), K3 (5:3), K4 (5:4) and K5 (5:5) were not significantly different.

The mechanical strength of hydrogel was then observed by the viscosity of hydrogel using Oswald viscosimeter to measure the time of hydrogel flow. Then, the viscosity was calculated using Poiseuville equation. ANOVA test results indicate that the composition of hydrogel has a significant effect (p < 0.05) on the viscosity. Duncan's Multiple Range Test results show that the lowest average of viscosity is in K0 (5:0), and the highest viscosity is in K5 treatment that is significantly different (p < 0.05) from K1 (5:1), K2 (5:2), K3 (5:3), and K4 (5:4) treatments. However, both K2 and K3 treatments were not significantly different. Table 3 below illustrates the viscosity of hydrogel.

**Table 3.** Viscosity of hydrogel.

| Treatment  | Average of viscosity of hydrogel (cP) |
|------------|--------------------------------------|
| K0 (5:0)  | 1.4847a                              |
| K1 (5:1)  | 3.6433b                              |
| K2 (5:2)  | 5.0931c                              |
| K3 (5:3)  | 6.1111c                              |
| K4 (5:4)  | 8.1274d                              |
| K5 (5:5)  | 10.7346e                             |

Note: Different superscript in one column indicates significant difference (p<0.05)

The refractive index of hydrogel was observed using refractometer at a temperature of 29°C. The refractive index of hydrogel is shown in Table 4. ANOVA test results indicate that the composition of hydrogel has no significant effect (p < 0.05) on the refractive index of hydrogel.
Table 4. Refractive Index of hydrogel.

| Treatment  | Average of Refractive Index |
|------------|-----------------------------|
| K0 (5:0)   | 1.3513<sup>a</sup>         |
| K1 (5:1)   | 1.3570<sup>a</sup>         |
| K2 (5:2)   | 1.3490<sup>a</sup>         |
| K3 (5:3)   | 1.3480<sup>a</sup>         |
| K4 (5:4)   | 1.3477<sup>a</sup>         |
| K5 (5:5)   | 1.3473<sup>a</sup>         |

Note: Different superscript in one column indicates significant difference (p<0.05)

The transparency of hydrogel was observed in terms of its light transmission using UV-Vis spectrophotometer at 600 nm wavelength as shown in table 5.

Table 5. Light Transmission Index of hydrogel.

| Treatment  | Light Transmission (%) |
|------------|------------------------|
| K0 (5:0)   | 97.80<sup>a</sup>     |
| K1 (5:1)   | 75.00<sup>b</sup>     |
| K2 (5:2)   | 59.90<sup>bc</sup>    |
| K3 (5:3)   | 50.00<sup>cd</sup>    |
| K4 (5:4)   | 39.47<sup>d</sup>     |
| K5 (5:5)   | 17.67<sup>e</sup>     |

Note: Different superscript in one column indicates significant difference (p<0.05)

ANOVA test results indicate that the composition of hydrogel has a significant effect (p < 0.05) on the light transmission. Duncan's Multiple Range Test results show that the highest light transmission is in K0 (5:0), and the lowest light transmission is in K5 treatment, that is significantly different (p < 0.05) from K1 (5:1), K2 (5:2), K3 (5:3), and K4 (5:4) treatments. However, both K1 and K2, as well as K2 and K3 treatments were not significantly different.
Figure 1. Composite hydrogel Collagen-Iota Carrageenan Based each treatment, A: K0 (5:0), B: K1(5:1), C: K2(5:2), D: K3(5:3), E:K4(5:4), F:K5(5:5).

Figure 2. FTIR result of Composite hydrogel Collagen-Iota Carrageenan Based.

The FTIR test showed a high intensity peak at a wavelength of 687.57 cm\(^{-1}\), an amine group. The others, the peak is at the wavelength of 1243.24 cm\(^{-1}\), which can be interpreted as the vibrational range of the C-N group, 1425.27 cm\(^{-1}\) for the carboxylate group and 2910.36 cm\(^{-1}\) for the C-C-C-H range of medium intensity.
The SEM test result at 5.000x magnification shows that the composite hydrogel morphology is solid and has an uneven surface. The absence of a film layer formed also signifies the linked (collagen) with iota carrageenan because of crosslinking. According to Gong et al. [7], polymer scaffolds should be able to provide space for appropriate cells to grow into new tissue on transplants. High porosity is required for growth and invasion of surrounding tissues. Furthermore, the connected pore tissue is essential for the growth, visualization and diffusion of nutrients in the cell.

4. Discussion
The main parameters observed in this study are equilibrium water content and viscosity, while the supporting parameters consisted of refractive index and transparency of hydrogel. Based on the results of variance analysis, it is known that the composition of collagen- iota carragennan gives a significant effect on the equilibrium water content of the hydrogel. Equilibrium water content indicates the hydration state of the cornea. According to Hayes et al. [8], the addition of iota carrageenan on the hydrogel affects the equilibrium water content of hydrogel. The test results show that the greater number of iota carrageenan added on the composite, the greater the value of equilibrium water content. This is because the iota carrageenan is a hydrophilic substance. Iota carrageenan is anionic. Some of the hydroxyl piranal groups are substituted by sulfate-half esters. These sulphate groups possess highly acidic and hydrophilic properties. So, water will rapidly bond into sulfate groups. K5 treatment has the highest equilibrium water content, up to 87.07%. This value exceeds the water content limit of human cornea (78-80 %). According to Hayes et al. [8], an increasingly decreased hydration rate will reduce Bragg's average intermolecular spacing of collagen. If hydration gets higher, the intermolecular spacing of hydrogel will be higher, causing hydrogel to be more porous. In addition, Dumitriu [9] stated that the higher water content in hydrogel, the faster the degradation, resulting in lower biocompatibility. However, from the physical feature, hydrogel of K5 treatment has better physical properties. This is because, the more water content within hydrogel, the higher the swelling degree that hydrogel has. This means that hydrogels have a high water resistance capability [10].

Viscosity is the degree of consistency at a certain concentration and temperature. According to variance analysis, the addition of iota carrageenan on the composition gives a significant effect to the
viscosity value of the composite hydrogel produced. The greater the addition of iota carrageenan to the composition of hydrogel, the higher its viscosity. This is in accordance with the statement of Nussinovitch [11], that the addition of gelling agents such as carrageenans can increase the viscosity or other physical properties of the gel, such as elasticity. The carrageenan ion may form a gel in low concentration due to the electrostatic interaction between the positive charge of collagen and the negative charge of the sulphate group in the carrageenan, which leads to an increase in the reactivity of collagen [12]. The more iota carrageenan concentration added, the more the negative charge of the sulphate group in the carrageenan induces a positive charge on collagen electrostatically, resulting in an increase in the reactivity of collagen which will lead to an increase of its viscosity. In addition, viscosity is also influenced by crosslinking activities. These activities can easily affect collagen fibers during intrafibrils crosslink process. When the collagen fibrils are connected to iota carrageenan groups, the composite structure becomes more rigid, making it difficult to separate. This makes the hydrogel viscosity higher. Consequently, the hydrogel elasticity becomes higher [13]. The higher the elasticity, the higher the mechanical strength of the hydrogel is.

Refractive index is related to total suspended solid in the sample. Based on the results of the analysis of variance, it is known that the addition of iota carrageenan composition did not give a significant effect on the refractive index value of the resulting composite hydrogel. There is a correlation between refractive index and corneal hydration, but no detailed explanation has been found on this subject. According to Patel et al. [6] theoretically, it is assumed that the natural optics and water distribution in the cornea in mammals are uniform. In the anterior and posterior portions of the stroma, there are differences in the characteristics of swelling, hydration and collagen fibril structure. The anterior part of the cornea is more likely to be low in hydration, and more resistant to water flow than the posterior part. Thus, refractive index measurements on the lower portion of hydration will result in higher refractive index values. Similarly, with regard to the refractive index hydrogel value, if the sampling is not homogeneous, then the refractive index becomes inaccurate. The test results show that the higher iota carrageenan added, the lower the refractive index. This is associated with hydrogel hydration, the more iota carrageenan addition, the higher the resulting hydration. This causes the refractive index measurement to become smaller, in accordance with the theories that have been proposed by Patel et al. [6]. In all treatments, the refractive index value is less than the refractive index value of the human cornea. So, no treatment is eligible for this parameter. However, all treatments have a refractive index value approaching the refractive index of human cornea.

Transparency is measured using the percentage of light transmission. Transmission is the amount of light passed on the speculator. Transmission of light is correlated with refractive index hydrogel. Organic materials do not absorb light. Therefore, more light damping is caused by the spreading of light. Due to differences in the refractive index between the nearest fibrils and the aqueous environment, a glimmer of light will be scattered from each fibril. However, due to the arrangement of random hydrogel fibrils, the result is bias in light interference in all directions [14]. In addition, according to Lambert-Beer's Law, the thickness of the sample will affect the visible light transmission. From the variance analysis, it is known that the addition of iota carrageenan composition gives a real effect to the value of the light transmission of hydrogel. The more iota carrageenan composition, the smaller the light transmission value of the hydrogel. This may be possible because the arrangement of collagen fibrils is randomized by the collagen-iota molecule, which results in uneven distribution of light, so that more light is absorbed in the sample than passed through the sample [14]. The decrease in the value of the light transmission may also be related to the presence of inorganic materials present in the composite hydrogel. Carrageenous ions are known to have sulfate groups [5], wherein this group is an inorganic group. The presence of this inorganic group causes the light to be absorbed into the inorganic material, so that the light that is passed will decrease, resulting in the smaller transmission [15].
5. Conclusion
It can be concluded that the higher composition of collagen: iota carrageenan leads to higher equilibrium water content, viscosity but lower refractive index and transparency. The best equilibrium water content and viscosity were found in K5 (5:5) treatments. The best refractive index and transparency were found in K0 treatment. This study suggests that K5 (5:5) can be performed as a point to create good hydrogel for corneal tissue engineering because of its equilibrium water content and viscosity. Accordingly, the development of hydrogel collagen-iota carrageenan based can use the composition of (5:5) with a blending method.

6. References
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