The Effect of Sodium Hyaluronate to the Properties of Collagen-Chitosan Composite as Artificial Cornea – an In Vitro Study

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Abstract

The cornea is in the front of the eye that is prone to damage to the point of blindness. As many as 36 million people in the world suffer from blindness and it is estimated that this number will increase in double by 2020. Allogeneic grafts produce a rejection reaction in the patient's body up to 65% within 4 months of transplantation. An alternative to corneas with appropriate transmittance, morphological, and degradation characteristics needs to be sought. It is known that the addition of 0.5% w / v sodium hyaluronate (NaHA) to collagen 1% w / v + chitosan 2% w / v is capable of producing up to 95% transmittance percentages. This study aims to determine the effect of sodium hyaluronate on artificial corneal biocomposite transmittance and determine the morphological characteristics and artificial corneal biocomposite degradation.Artificial cornea was made from collagen, chitosan, and NaHA with a ratio of 20% w /v collagen, 10% w / v chitosan, and NaHA varied with a ratio of 0%; 0.3%; and 0.6% w /v. The artificial cornea was made by linking collagen with Hydroxypropyl methylcellulose (HPMC) which was mixed with chitosan and NaHA by using a magnetic stirrer. Then, heating was performed by using an incubator.Transmittance test results show that increasing the concentration of NaHA provides a transmittance value that is increasingly in accordance with the human cornea. The degradation test gives an artificially faster corneal degradation rate than the rate of human corneal degradation. Surface morphology test gives a picture of three samples that have a structure that is less strong and fragile.Based on this study, it was concluded that NaHA can increase artificial corneal transmittance, whereas the degradability and morphological nature of artificial corneal biocomposites need to be improved in further studies.

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Introduction

Cornea is in the front of the eye that is prone to damage until it leads to blindness. Cornea damage caused by infection, lack of nutrition, inflammation, heredity, degenerative, traumatic, and iatrogenic.[1] This blindness case is not only a concern of Indonesia, but also the world, where as many as 36 million people suffer from blindness. It is estimated that this number will increase in double by 2020.[2] Handling corneal damage by using an allogeneic graft still produces a rejection reaction in the patient's body up to 65% in 4 months of transplantation, morphology, and degradation.

Chen et. al.[3] have engineered corneal tissue by using type I collagen which is the largest component of the cornea. However, the compressive strength of type I Collagen is still lower than the human cornea, thus it is necessary to add materials that have a higher compressive strength[4]. Collagen combined with chitosan is known to be able to produce biomaterials with better mechanical properties. This is due to chitosan has a high compressive strength value. Besides that, chitosan adds to collagen can stabilize the rate of degradation of collagen[3].

Study of Chen et. al.[3] found that the

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addition of sodium hyaluronate (NaHA) affected the transmittance of the sample. In 1% collagen + 2% chitosan, the addition of 0.5% NaHA can transmit light 95% which means that transparency is 5%. This showed a better value than 1% collagen + 2% chitosan without the addition of NaHA which only has 78% transmittance with 22% transparency. According to Ozcelik et. al.,[4] human corneas allow the transmission of visible light 75% to 95% at wavelengths of 400 - 700 nm and UV transmittance of 30% to 75% at wavelengths of 310 - 400 nm. However, in Chen's study with three sample variations, they were: 10% chitosan + 0.5% NaHa, 20% collagen + 10% chitosan + 0.5% NaHa, and 30% collagen + 10% chitosan + 0.5% NaHa, with concentrated NaHA similarly, it turned out to give different transparency results in the in vivo test using rabbits. The transparency was seen after 5 months implantation in rabbits by using slit-lamp photography which showed several condition, sample A with 10% chitosan + 0.5% NaHa seems dark and not clear, sample B with 20% collagen + 10% chitosan + 0.5% NaHa seems bright and translucent, while sample C with 30% collagen + 10% chitosan + 0.5% NaHa seems clear transparent. Chen's study examined that there were no transmittance and transparency data of three samples in vitro test. Regarding the nature of transmittance that is very influential on the function of the cornea as a successor of light to the parts of the eye, thus the effect of NaHA on the transmittance and transparency of the cornea needs to be further investigated.

Based on this background, an artificial corneal biocomposite based on collagen was made by the addition of chitosan and combined with NaHA. This study aims to determine the effect of sodium hyaluronate on artificial corneal biocomposite transmittance and determine the morphological characteristics and degradation of the artificial cornea.

Materials and methods

The materials used in this study were collagen (Col), chitosan (Chi), NaHA, hydrochloric acid (HCl), acetic acid, hydroxypropyl methylcellulose (HPMC), Phosphate Buffered Saline (PBS), and aquadestt.

The study began with the preparation of materials and tools. The material was prepared by dissolving 20% w/v collagen (Col) in 0.1 M acetic acid and 10% w / v chitosan (Chi) in 0.6 M acetic acid. Collagen solution that has been crosslinked with HPMC was mixed and sterilized with chitosan solution for 60 minutes. Then, NaHA with 0% concentration variation; 0.3%; and 0.6% was added to the Col + Chi solution and stirred for 30 minutes. Concentration ratios of collagen, chitosan, and NaHA can be looked at Table 1. Homogeneous Col + Chi + NaHA solution was casted on a Perspex plate and heated by using an incubator for 24 hours at 35℃ until the membrane dries. After that, the membrane was soaked in PBS until the pH becomes neutral.

Three samples from each treatment were tested by using UV-Vis spectrophotometer. The spectrometer fires light at the wavelength of visible light (400 - 700 nm). Then, the photometer would record the light transmittance of each wavelength, hence the transmittance graph (% T) of the wavelength (nm) was obtained.

Degradation was performed by immersing the sample in a solution of Phosphate Buffered Saline (PBS). Sample reliability was obtained by measuring the actual sample mass before degradation and the mass of sample after degradation at each interval of 3 days, 6 days, and 9 days. Next, the calculation was conducted to know the percentage of mass loss using Equation 1 in order to obtain the value of sample degradability.

The sample preparation procedure for the SEM test began with cutting the sample to a size of 10mm. The coating process was conducted by using a Quorum Q150RS engine. After that, the morphology of the samples was observed with a Hitachi TM3000 electron microscope (SEM) with a magnification of 1800-3000x. SEM test results in the form of surface photographs of the sample.

Results

Synthesis Results

An artificial corneal membrane has been made from collagen (Col), chitosan (Chi), and sodium hyaluronic acid (NaHA) material in this study. Type I collagen 20% w / v completely dissolved in 0.1 M acetic acid. Hydrochloric acid was not used due to collagen can dissolve completely only in organic acids, while hydrochloric acid was a type of inorganic acid in this study. One organic solvent that was able to

extract collagen better than other solvents was acetic acid.[5]

Chitosan 10% w / v also dissolved completely in acetic acid. This was due to chitosan can only dissolve completely in organic solvents and only partially dissolve in inorganic solvents.[6] The concentration of acetic acid used was 75% v / v with a molarity of 13.4 M. It was found that with high concentrations of acetic acid and chitosan 10% w / v was completely dissolved compared to use 0.1 M acetic acid. Chitosan solubility was also affected by the degree of deacetylation. In this study, the degree of deacetylation (DD) of chitosan used was 95%. The degree of deacetylation (DD) was one of the chitosan quality parameters which showed the percentage of the number of free amino groups in the chitosan polysaccharide[5].

Collagen and chitosan, each completely dissolved, were mixed using a magnetic stirrer for 1 hour as a sample without the addition of NaHA or control samples. The control sample was successfully mixed perfectly.

The next sample was a variation of the control sample by adding NaHA solution. 0.1 M acetic acid was used as a solvent for NaHA and can dissolve NaHA perfectly using a magnetic stirrer for 30 minutes. The second sample added 0.3% w/v NaHA, while the third sample added 0.6% w / v NaHA. The mixing process was conducted at high speed and the pouring of NaHA solution into the Col + Chi compound was completed slowly. This was conducted due to NaHA and chitosan were able to form polyelectrolyte complexes (PECs) which can cause them not to mix properly. PECs were supramolecular structures formed from ionic bonds between two polyelectrolytes that have opposite charges. This structure can be formed when the mixing pH was above pKa NaHA and below pKa chitosan, thus the opposite charge appears.[7]

The next process was cross linking with hydroxypropyl methylcellulose (HPMC). The addition of HPMC to collagen was also able to increase light transmittance, increase cell adhesion, and proliferation, as well as have good cytocompatibility in artificial corneal applications.[8] Thus, HPMC was added to the collagen solution as a cross-linker in this study. The ratio of collagen and HMPC used was 1: 1 by weight. Hence, the overall concentration of collagen and chitosan did not change, the HPMC was added without adding solvent volume. Collagen that had been added by HPMC was stirred by using a magnetic stirrer for 12 hours and the temperature was maintained at 4℃. After the collagen solution has been crosslinked, the next procedure was conducted, namely the addition of chitosan as a control sample and the addition of chitosan and NaHA solutions respectively 0.3% w/v and 0.6% w/ v.

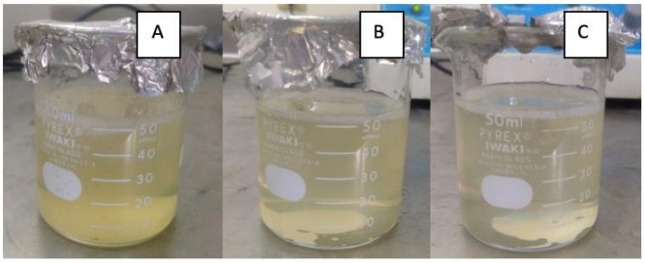

Figure 1. Compound of Solution Col 20% w/v cross linking on HPMC + Chi 10% w/v DD 95% + (A) NaHA 0% w/v, (B) 0,3% w/v, and (C) 0,6% w/v.

Three compound solutions were prepared as shown in Figure 1, then poured in a petri dish and heated for 24 hours by using an incubator at 35℃. Furthermore, an artificial corneal membrane was successfully formed and neutralized by using aquadestt.

Transmittance Test

Transmittance test using UV-Vis Spectrophotometer produced a wavelength graph of the percentage of transmittance in Figure 1 and a graph of the average transmittance value can be seen in Figure 2.

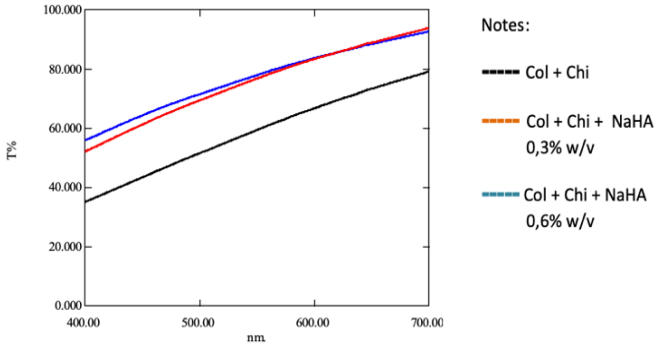

Figure 2. Graphic of Sample Transmission used UV-Vis Spectrophotometer on wavelength of 400-700 nm.

Transmittance test results showed that compared to samples without additional NaHA,

samples with additional NaHA have a greater percentage of transmittance. This showed that the addition of NaHA can increase the value of sample transmittance.

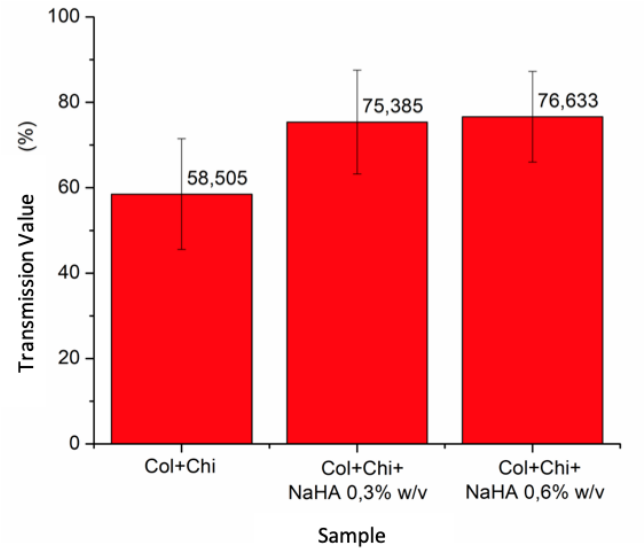

Figure 3. Diagram of Transmission Mean Value of Samples.

The transmittance of visible light in the human cornea was 75% - 95% (400-700 nm).[9] Without the addition of NaHA, the average percentage of transmittance obtained was 58.505% which did not fall within the normal transmittance range of the human cornea. Samples with additional NaHA 0.3% w / v and NaHA 0.6% w / v have an average percentage of transmittance of 75.835% and 76.633%, thus they were in accordance with the normal range of human corneal transmittance and fulfil the optical properties of human corneal light transmittance.

When compared with previous studies, artificial corneal transmittance obtained from this study was lower than Chen's[3] study, where artificial corneal transmittance of Col 1% w / v + Chi 2% w / v + NaHA 0.5% w/v reached 95%. Probably, this can happen due to several things, including the ratio of concentration of ingredients, types of ingredients, and solvents used in this study were different from previous studies.

Morphological Test

The surface morphology test results using Scanning Electron Microscope (SEM) showed the surface morphology of each sample as in Figure 4. It can be seen that there were irregular black lines in three samples. Lines on the biocomposite surface of the artificial cornea indicated cracks due to the structure of the sample which was not strong or brittle in dry conditions. Chen et al.[3] stated that chitosan was able to improve the mechanical properties of collagen, whereas the use of chitosan in this study showed results that contradict with that statement. This can be caused by several things, such as the comparison of the concentration of collagen-chitosan that was used incorrectly, stirring time, and heating temperature. In the surface morphology test results also cannot be seen the existence of pores in the sample. This can occur due to several factors, such as the mechanical properties of the poor sample or the size of the pore that was too small.

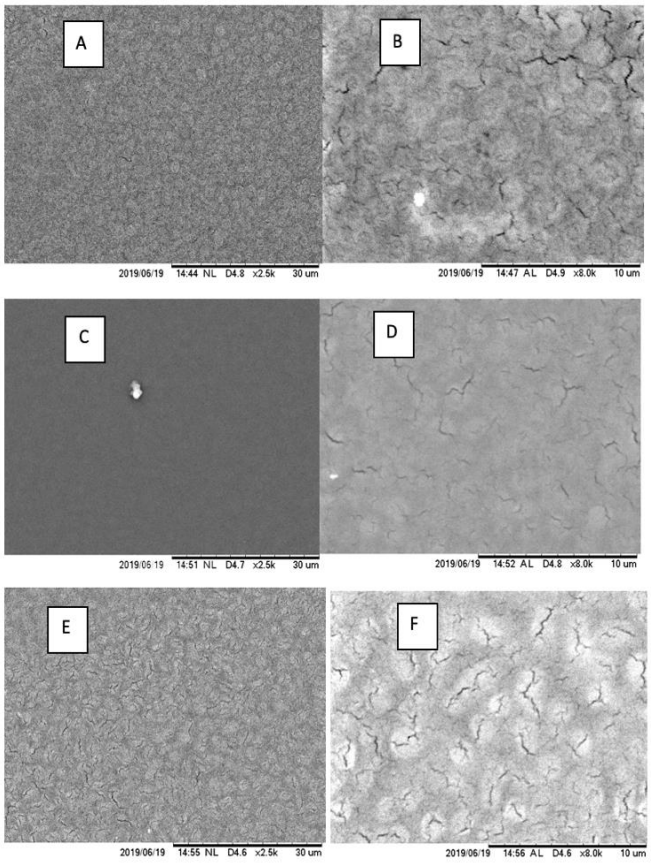

Figure 4. Surface Morphology of Sample (A) Col + Chi at 2500x Magnification and (B) 8000x; (C) Col + Chi + NaHA 0.3% w / v at 2500x Magnification and (D) 8000x; (E) Col + Chi + NaHA 0.6% w / v at 2500x Magnification and (F) 8000x.

Degradation Test

Figure 5 showed the percentage of the sample mass that was degraded on the 3rd, 6th, and 9th days. Based on these figures, it was known that samples with the addition of 0.3% w/v

NaHA have a higher degradation percentage than the other two samples.

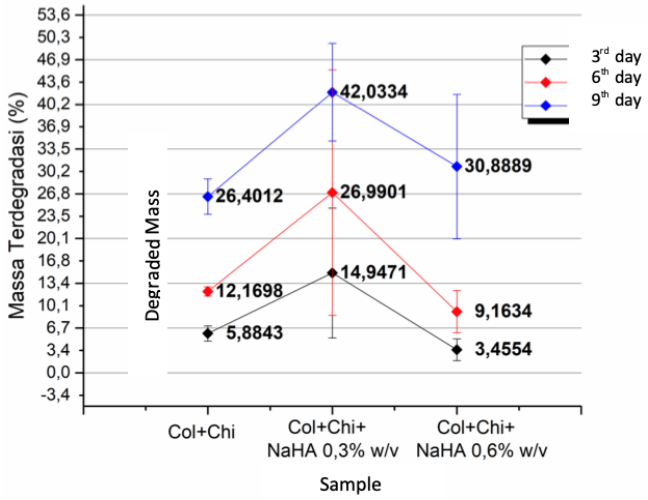

Figure 5. The Percentage of Degraded Sample Mass.

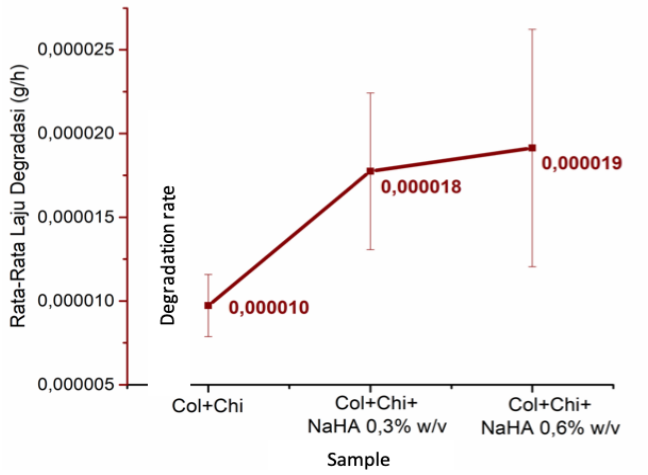

Figure 6. The Mean Value of Samples Degradation Rate.

Based on the degradation mass data obtained, the calculation of the degradation rate per sample was performed. Figure 6 showed the average rate of degradation, where the addition of NaHA can increase the rate of artificial corneal degradation. Based on the rate of degradation obtained, the time needed for the cornea to be fully degraded can be seen in Figure 7.

Huang et. al.[10] stated that corneal donor recipient patients were able to regenerate corneal tissue within 3-6 months. When the corneal tissue has completely regenerated, the artificial cornea must have been degraded from the body. In this study, the results showed that the time of artificial corneal degradation was faster than the regeneration time of the human corneal tissue. Chitosan suffered degradation much slower than the collagen.[11]

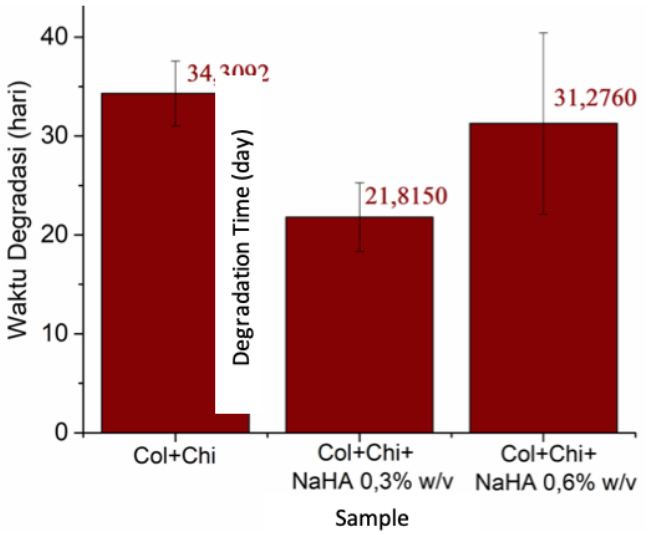

Figure 7. Degradation Time of Samples.

The higher the concentration of chitosan, the lesser the degradation rate is due to crosslinking existency.[12] The more chitosan composition the longer the time of degradation.[13] When chitosan is implanted, it will eventually disappear completely after some time and the degradation speed seems to depend on Deacethylation Degree (DD).[14] The DD has an effect on biological properties, such as in vitro and in vivo biodegradation. It has been well known that, at a greater DD (between 84 and 90%), the degradation process would be slower than the lower DD. Highly deacetylated chitosan (over 85%) shows a low degradation index in the aqueous environment and will degrade after a few months, and a lower DD (between 82 and 65%) would lead to a faster degradation. The commercially available preparations have a DD between 60 and 90%.[15,16] Chitosan as one of polymers type is providing good deal of attention due to their unique features, such as ability to control biodegradation, and mechanical properties. They have several biocompatibility benefits, versatility in surface chemistry, and biological properties that are important in tissue engineering application and in regenerative medicine.[17] In the further study, possibly step which could considered are to increase the DD/ concentration of chitosan or to add material which has the ability to slow the degradation time.

Conclusions

Based on this study, it can be concluded that NaHA can increase artificial corneal transmittance, while the morphological and degradability of artificial corneal biocomposites need to be improved in further studies.

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Declaration of Interest

There is no conflict of interest.

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