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Bio-artificial dura mater in head trauma patients based on a kombucha-chitosan-collagen combination

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Abstract

Introduction: Kombucha scoby shows potential as a biomaterial applicable to patients with head trauma that results in tearing of the dura mater layer. This research aims to characterize kombucha scoby with chitosan and collagen as in vitro modification materials. The artificial dura mater is derived from kombucha scoby synthesized in green tea media and added with glycerol as a plasticizer.

Objective: This study used four composition variations: Scoby-Kombucha (Control), Kombucha-Glycerol, Kombucha-Chitosan-Glycerol, and Kombucha-Chitosan-Collagen-Glycerol. Characterization was performed based on functional group analysis through Fourier Transform Infrared (FTIR) testing, tensile strength, swelling, degradation, Scanning Electron Microscope (SEM), and (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (MTT Assay). The characterization of functional groups confirmed the presence of functional groups from kombucha Scoby, chitosan, collagen, and glycerol. The data were analyzed using descriptive analysis.

Results: The results of tensile strength characterization showed that the three sample composition variations have UTS values (4-20 MPa) and Elongation (7-20%) in accordance with artificial dura mater standards. The swelling characterization of the four samples showed a liquid absorption value that does not exceed the swelling ratio limit (<70%). The highest degradation was obtained in the Kombucha-Chitosan-Collagen-Glycerol sample composition with a degradation rate of 20%. The SEM test found a pore diameter that meets the standards for artificial dura mater in the sample compositions of Kombucha-Chitosan-Glycerol and Kombucha-Chitosan-Collagen-Glycerol. Cytotoxicity characterization through the MTT Assay showed that the material does not contain substances toxic to the body.

Conclusion: The Kombucha-Chitosan-Collagen-Glycerol membrane was an optimal composition as a candidate for artificial dura mater.

Keywords: Artificial Dura mater; Scoby Kombucha; Chitosan; Collagen; Glycerol

1. Introduction

Head injuries can occur due to various factors originating from daily activities such as traffic accidents, work accidents, sports, accidents due to falling blunt objects, or due to being struck by hard objects [1]. According to WHO data, the prevalence of traffic accidents occurs mostly in developing countries, including Indonesia, accounting for 90%. Data recorded by the Indonesian Police in 2011 also mentioned that 55.1% of accident victims suffered severe concussions.

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Accidents or trauma affecting the head can cause the brain's dura mater layer to tear, leading to death. The dura mater is the outermost layer of the meninges that prevents cerebrospinal fluid from leaking and is the largest within the central nervous system (CNS). This layer acts as a protective membrane for the brain and spinal cord [2]. Therefore, artificial dura mater is required in cases of head trauma.

Applications of artificial dura mater have developed commercially using various materials. Artificial dura mater using xenogeneic collagen material currently on the market has the disadvantage of requiring a long operation time, is not easy to apply to hard-to-reach locations, and is fragile. Dura mater made of amnion tends to be too thin and cannot be adjusted for thickness. Meanwhile, synthetic dura mater such as silicone tends not to be biodegradable for the body, causing chronic stimulation to the surrounding area [3]. There have even been reports of synthetic materials used in dura mater reconstruction in the field of nerves such as expanded polytetrafluoroethylene (PTFE) sheets with several reports of artificial dura mater infections [4]. Synthetic materials also have a very high cost in their application.

One natural material candidate that can be used in the field of medical biomaterials is kombucha. Kombucha is a symbiosis between bacteria and fungi in making green tea. The result of the symbiosis of bacteria and fungi can release a gel-like membrane consisting of cellulose fibers [5]. The soft kombucha gel is the result of a water layer surrounding the polyglucose chains from cellulose bacterial molecules and has a sponge structure with varying pore sizes. In addition, the presence of cellulose bacteria in kombucha is another advantage. Therefore, cellulose bacteria (microbial cellulose) have the potential with several characteristics, including being biocompatible, non-pyrogenic, non-toxic, easy to sterilize, absorbable, and more cost-effective in production [6]. Kombucha membrane has biocompatibility properties and does not cause toxic hematological and histological effects on nerve tissue [7]. The kombucha membrane also has a dense structure, and its cellulose bacteria are easy and effective to synthesize in kombucha. In addition, the cost of making kombucha itself is relatively cheap compared to synthetic or other materials.

The effectiveness of the kombucha membrane can be enhanced by modification. Modification is done by adding active polysaccharides, such as chitosan [2]. Based on previous research, biocomposite products from cellulose-chitosan bacteria tested in vitro have bacteriostatic or antibacterial activity. Fibers and scaffold membranes made from chitosan are also suitable for sutures. This is one condition that is an additional ideal material substitute for dura mater. In addition, other modification materials such as collagen and plasticizers. The plasticizer to be used is glycerol, as it is amorphous so it does not degrade quickly and can meet the minimum degradation standard for artificial dura mater, which is about one to three months [4].

2. Material and methods

This research was conducted in several laboratories at Universitas Airlangga, Surabaya, Indonesia. The synthesis process of the kombucha membrane was carried out at the Veterinary Medicine Faculty's Pharmacy Laboratory, sample drying at the Institute of Tropical Disease (ITD), and FTIR testing at the Faculty of Pharmacy. Tensile strength tests were performed at the Material Physics Laboratory, Faculty of Science and Technology, cytotoxicity at the Research Center of the Faculty of Dentistry, swelling and degradation at the Microbiology Laboratory of the Faculty of Veterinary Medicine, and SEM at the Institute of Life Sciences, Engineering, and Technology (LIHTR).

2.1. Materials

The equipment used in the production of the kombucha-chitosan-collagen biocomposite membrane as an artificial candidate includes synthesis tools and test tools. The synthesis equipment includes a 250 ml glass beaker, measuring glass, pipette drops, magnetic stirrer, magnetic bar, digital scale, stove, pot, pH meter, micropipette, thermometer, flack reactor, fermentation container, and tweezers. Test tools used during FTIR testing, swelling test, MTT Assay. The materials used for artificial dura mater candidates are bacteria, green tea, kombucha scoby, glycerol, sucrose, collagen, chitosan, and aquades.

2.2. Method

2.2.1. Synthesis of Kombucha-Glycerol Membrane Using Kombucha scoby

The synthesis starts with the preparation of tea fermentation materials by mixing 1.25% (w/v) green tea, 0.75% (v/v) glycerol, and 10% (w/v) sucrose in 600 ml and boiling for 5 minutes. The resulting tea solution is added with 150 ml of kombucha starter. The tea solution with the starter is placed in a glass jar and added with kombucha scoby seed. Fermentation is carried out for 14 days at room temperature of 30º C. The scoby membrane formed is washed with distilled water and dried using a freeze-dry technique for 6 hours.

2.2.2. Synthesis of Kombucha-Glycerol-Chitosan Membrane

Chitosan solution is prepared with 2% (v/v) acetic acid added into 100 ml aquades for 20 minutes, then added with 0.5% (w/v) chitosan and stirred until homogeneous using a magnetic stirrer at a speed of 100-200 rpm with a temperature of 24-25º C. The chitosan solution is soaked for 6 hours and dried again using the freeze-dry technique for three hours.

2.2.3. Synthesis of Kombucha-Glycerol-Chitosan-Collagen Membrane at 0.1% (m/v) Concentration

The preparation of kombucha-collagen membrane begins with making a collagen solution in gram form at a concentration of 0.1% (m/v). 2 grams of citric acid is mixed into 100 ml aquades and collagen 0.1 gram is gradually added with a magnetic stirrer. After forming a homogeneous collagen solution, the kombucha membrane already formed is immersed in the collagen solution for 6 hours. Then, the membrane is sterilized using alcohol and stored in normal saline in a refrigerator [8].

2.2.4. Membrane Characterization Tests

Fourier Transform Infrared (FTIR) Test

FTIR is a test aimed at analyzing functional groups by observing the absorption of infrared radiation at different wavenumber ranges. The sample is characterized with infrared laser spectroscopy reflected by a prism [9]. The test sample is cut to a size of 0.5-1 mm, then the sample is ground together with KBr. The sample is ground into a pellet form, then placed on a stainless steel plate, then inserted into the FTIR equipment and its infrared spectrum is tested at the wavenumber range 4000-200 cm^{-1}.

Tensile Strength Test

The tensile strength test is conducted to complete information on the strength of a material and as a complement to the material specification [10]. The tensile strength test aims to determine the endurance of a material when subjected to loading following the standards set by the American Society for Testing Material (ASTM) D638 type V. Tensile testing is conducted with three repetitions for each sample. Before testing, the sample is first formed into a dog bone. The thickness of the sample is first measured with a Coatting Thickness Gauge type TT210 with the precision of the tool reaching 0.05 μm. Thickness measurements are made at 3 points, namely top, middle, and bottom then the average value is calculated. The thickness of artificial dura mater ranges from 100-300 μm.

Swelling Test

The swelling test is conducted to determine the ability of the biocomposite membrane to absorb fluid. Sample preparation is done by cutting the sample to a size of 1 × 1 cm. The swelling test is conducted by weighing the prepared sample, then all four sample variations are immersed in a Phosphate Buffer Saline (PBS) solution with a pH of 7 for 2 hours at a temperature of 37°C. After that, reweighing is done, and calculations are made to determine the swelling ratio using the equation:

$$(\%) = \frac{Wwet - Wdry}{Wdry} \times 100\%$$

2.2.5. Degradation Test

The degradation test is conducted to observe the ability of the material to degrade due to interaction with the environment or static fluid. The in vitro degradation test is conducted by cutting all samples to a size of 1 × 1 cm then the samples are weighed and recorded as initial weight (g). In this test, 10 ml of Phosphate Buffer Saline (PBS) with a pH of 7 is used where the sample is immersed for 14 days in an incubator at a temperature of 37°C. After that, calculations are made to determine the percentage of degradation using the equation:

$$(\%) = \frac{W0 - Wt}{W0} \times 100\%$$

2.2.6. Scanning Electron Microscope (SEM)

The Scanning Electron Microscope (SEM) test is conducted to determine the surface morphology and cross-section or cross section of artificial dura mater membrane using SEM (Phenom P-Series, Netherlands). SEM characterization will produce images of the sample surface, making it easy to identify whether the variation in each sample composition affects the surface structure and membrane pores [9]. For observation using a Scanning Electron Microscope (SEM), the

membrane first undergoes a drying process using a freeze dryer for 6 hours. The membrane is coated with gold to prevent surface charging and create a homogeneous surface for imaging.

2.2.7. Cytotoxicity Test

One of the toxicity tests used is the MTT Assay test. The MTT Assay is a test to determine cellular metabolic activity, drug toxicity, and cell viability [11]. The working principle of the MTT Assay is the decomposition of MTT reagent by dehydrogenase enzymes in cell mitochondria into Formazan solution. Then the Formazan solution will react with Dimethyl Sulfoxide (DMSO) and form a crystal that is insoluble in water. Measuring the colorimeter absorbance value (optical density) on the crystal and the number of crystals produced can show the number of cells that survive and the activity in the cells [12].

3. Results and discussion

Modifications of several ingredients such as the addition of glycerol, chitosan and collagen were read in the FTIR test. The main characteristics of bacterial cellulose membranes are characterized by the presence of OH-, CH-, -CH3 Bending, and C-O Glycosidic functional groups which are owned by the four variables. C-O Glycosidic indicates the hydrolysis of sucrose into glucose and fructose by microorganisms in the scoby [13]. The addition of glycerol to variables A, B and C is characterized by a decrease in the OH- wavelength with the average wavelength of variables 2 to 4 being 3437. The addition of chitosan to variables B and C is characterized by the presence of the NH functional group which is read at a wavelength of 1647.21 and 1546.91 (on variable B) and 1539.20 (on variable C). The addition of collagen in variable 4 is indicated by the presence of C=O Stretch which is read at wave 1649.14.

Table 1 Results of Fourier Transform Infrared (FTIR) Functional Group Analysis

Function Group	**Standard Wave Number (cm^{-1})**	**Peak Sample Wave Number (cm^{-1})**			
		Variable Control	Variable A (Kombucha-Glycerol)	Variable B (Kombucha-Glycerol-Chitosan)	Variable C (Kombucha-Glycerol-Chitosan- Collagen)
-OH *Stretching*	3200–3600	3462.22	3433.29	3437.15	3441.01
C-O Glikosidik	1050–1150	1118.71	1062.78	1064.71	1114.86 1062.78
CH_3 *Bending*	1368–1380	1369.46	1373.32	1371.39	1373.32
-CH	2850–3000	2912.51 2862.36	2922.16 2860.43	2922.16 2858.51	2920.23 2858.51
C-O-C	1100–1260	1163.08	1162.15 1062.78	1161.15 1112.93	1161.15 1114.86
C-O *Stretch*	1000–1320	–	1062.78	1064.71	1161.15 1114.86 1062.78
C=C *Stretch*	1620–1680	1645.28	1643.55	1647.21	1649.14
-NH	1535–1680	–	–	1647.21 1546.91	1539.20
C=O *Stretching* (Amida)	1630–1680	–	–	–	1649.14

Table 2 Tensile Strength and Elongation Test Results

Variation of Sample	Thickness (µm)	Ultimate Tensile	Strength (MPa) Elongation Strain (%)
Control (Scoby-Kombucha)	133	5.80	42%
A (Kombucha-Gliserol)	156	6.25	18%
B (Kombucha-Gliserol-Chitosan)	250	5.23	19%
C (Kombucha-Gliserol-Chitosan-Collagen)	267	4.70	16%

Based on Table 2, the Ultimate Tensile Strength (UTS) values were obtained for various samples. The control sample, consisting of a scoby-kombucha membrane, exhibited a UTS of 5.80 MPa with an elongation value of 42%. Sample A, which is a kombucha-glycerol variation, had a UTS of 6.25 MPa with an elongation value of 18%. Sample B, consisting of a kombucha-glycerol-chitosan variation, had a UTS of 5.23 MPa with an elongation value of 19%. Sample C, which is a kombucha-glycerol-chitosan-collagen variation, had a UTS of 4.70 MPa with an elongation value of 16%. According to Yamauchi et al. (2003) [3], artificial dura mater has a UTS value in the range of 4-20 MPa. Samples Control, A, B, and C fall within the standard range for artificial dura mater. Based on research by Zhuvravlova et al. [14], the elongation value for dura mater is 7-20%. Samples A, B, and C meet the standard elongation values for artificial dura mater.

Based on the tensile strength test results analysis, the addition of glycerol increases the flexibility and tensile strength of the membrane. The addition of chitosan and collagen can decrease the tensile strength of the membrane. Chitosan, with its rigid structure, reduces the membrane's flexibility [15]. The addition of chitosan also causes significant moisture absorption, plasticizing the material, and reducing its tensile strength. The combination of bacterial cellulose membrane with collagen enhances the performance of biomaterials and lowers tensile strength, but still within the standard range. The triple-helix structure of collagen allows for supporting tissue structure and regeneration processes but does not support the formation of strong mechanical intermolecular bonds.

The control sample kombucha scoby showed a high elongation strain value. The addition of glycerol, chitosan, and collagen can decrease the elongation values, but they remain within the standard range of artificial dura mater. The addition of glycerol reduces the material's ability to undergo extensive extension strain before mechanical failure. The addition of chitosan decreases the elongation due to its relatively rigid molecular structure. Collagen enhances mechanical properties and reduces elongation due to its unique three-dimensional nanostructure.

Table 3 Swelling Test Results

Sample	Variations in Membrane Composition	Swelling Ratio (%)
Control	Scoby-Kombucha	50%
A	Kombucha-Gliserol	42%
B	Kombucha-Gliserol-Chitosan)	58%
C	Kombucha-Gliserol-Chitosan-Collagen)	61%

Analysis of the control sample in the form of kombucha scoby showed a swelling ratio of 50%, the swelling ratio of sample A in the form of kombucha-glycerol was 42%, sample B in the form of kombucha-glycerol-chitosan was 58%, and sample C was kombucha-glycerol-chitosan-collagen. amounting to 61% as shown in Table 3. According to Alifiany [17] water absorption that is too high in the membrane >70% will cause the membrane to become soft and the membrane's resistance to decrease. The test results show that Control Samples, A, B, and C have good water absorption values and do not exceed the maximum water absorption limit on the membrane, namely less than 70%.

The addition of glycerol to kombucha scoby could reduce the water absorption value of the membrane because glycerol fills the space between fibers and reduces space for water molecules. The addition of chitosan increases the water absorption value because the amine functional group is polar and forms hydrogen bonds with water molecules [16]. The addition of collagen increases water absorption levels because hydrophilic groups such as amide groups (-CONH2),

carboxyl groups (-COOH), and hydroxyl groups (-OH) form hydrogen bonds with water molecules. Collagen's triple helix structure provides mechanical stability and allows the formation of water-permeable tissue.

Table 4 Degradation Test

Sample Variation	**Initial Weight (g)**	**Final Weight (g)**		**Degradation Percentage (%)**	
		7 days	14 days	7 days	14 days
Control (Scoby-Kombucha)	0.06	0.056	0.049	6.67%	18.3%
A (Kombucha-Gliserol)	0.02	0.019	0.017	5%	15%
B (Kombucha-Gliserol-Chitosan)	0.14	0.13	0.115	7.14%	17.85%
C (Kombucha-Gliserol-Chitosan-Collagen)	0.03	0.027	0.24	10%	20%

According to the observations conducted, the degradation process of the material occurs gradually, not instantaneously disappearing. From Table 4, the degradation percentage of the control sample (kombucha scoby) on day 7 is 6.67% and on day 14 is 18.3%. Then, sample A with the addition of 0.75% glycerol showed a degradation percentage or the amount of weight lost on day 7 at 5% and on day 14 at 15%. For sample B with the addition of 0.5% chitosan solution, it degraded by 7.14% on day 7 and 17.85% on day 14. Whereas sample C with the addition of 0.1% collagen solution degraded by 10% on day 7 and by 20% on day 14. It can be concluded that the control sample, A, B, and C can be used as candidates for artificial dura mater because the minimum degradation standard for artificial dura mater is about 1 to 3 months [17].

The addition of glycerol modification will decrease the rate of degradation due to its high tensile strength and elasticity, and crystalline structure that makes the membrane not easily degraded quickly. Chitosan reduces the rate of degradation by forming hydrogen bonds between the OH groups of kombucha and chitosan, which inhibits molecular mobility [18]. Meanwhile, the rate of degradation will increase with the addition of collagen by forming a porous membrane that allows the integration of connective tissue and minimizes the leakage of CSF. Collagen also supports the proliferation and regeneration of tissue around the artificial dura mater and enhances cell adhesion factors [15].

3.1. Scanning Electron Microscopy (SEM)

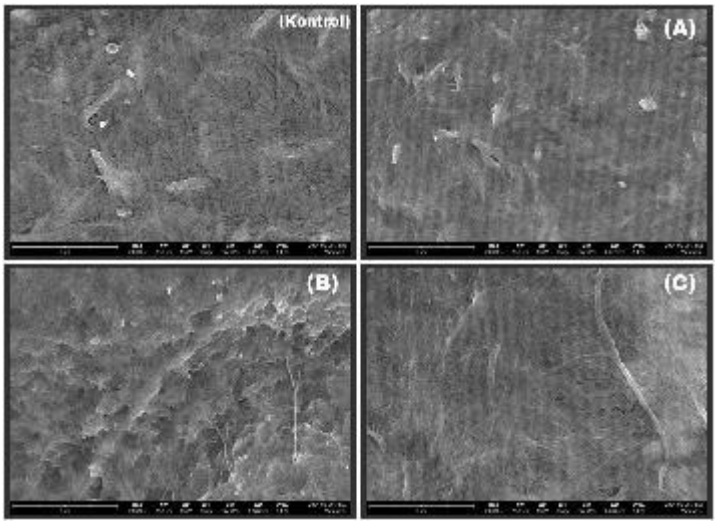

Figure 1 SEM results of Kombucha (Control), Kombucha-Glycerol (A), Kombucha-Glycerol-Chitosan (B), Kombucha-Glycerol-Chitosan-Collagen (C)

Table 5 Scanning Electron Microscopy

Sample	Variations in Membrane Composition	Pore Size (µm)
Control	Scoby-Kombucha	0.2-0.4
A	Kombucha-Gliserol	0.7-1.0
B	Kombucha-Gliserol-Chitosan)	1.2-2.3
C	Kombucha-Gliserol-Chitosan-Collagen)	1.6-2.7

Based on the SEM test results, it was found that the pore size in each sample showed significant variations. In the control (Kombucha), the pore size ranged between 0.2-0.4 µm. The addition of glycerol to sample A (Kombucha-Glycerol) increased the pore size to 0.7-1.0 µm. Furthermore, the Kombucha-Glycerol-Chitosan combination in sample B has a pore size of 1.2-2.3 µm. Sample C consisting of Kombucha-Glycerol-Chitosan-Collagen showed an increase in pore size to 1.6-2.7 µm. This gradual increase indicates that the addition of glycerol, chitosan, and collagen significantly affects the porosity of bacterial cellulose membranes, with the largest pore size found in sample C. This larger pore size can support the growth and migration of fibroblast cells, as well as provide adequate space for healing and tissue regeneration [19].

Table 6 Cytotoxicity Test

Sample	Variations in Membrane Composition	Cell Viability Percentage (%)
Control	Scoby-Kombucha	71%
A	Kombucha-Gliserol	70%
B	Kombucha-Gliserol-Chitosan)	75%
C	Kombucha-Gliserol-Chitosan-Collagen)	77%

Based on the analysis, the results showed that the cell viability percentage for the kombucha scoby (control) sample was 71%, for the kombucha-glycerol sample it was 70%, for the kombucha-glycerol-chitosan sample it was 75%, and for the kombucha-glycerol-chitosan-collagen sample it was 77%. The analysis of all samples indicated that the cytotoxicity calculation values were above the toxicity standard, which is >50%. A sample can be considered non-toxic if the live cell percentage is above 50% [20]. All samples were declared biocompatible because the bacterial cellulose material has a structure similar to the extracellular matrix in humans [21].

4. Conclusion

In the functional group analysis using FTIR testing, each of the four sample compositions had functional groups indicating that the biocomposite was successfully synthesized. The Kombucha-Glycerol sample showed a dominant intensity of the -OH stretching functional group. The Kombucha-Chitosan-Glycerol sample exhibited the characteristic chitosan functional group, the N-H group. The Kombucha-Chitosan-Collagen-Glycerol sample contained the characteristic collagen functional group, C=O Stretching (Amide I). The Ultimate Tensile Strength (UTS) and Elongation of all samples, except for the control, met the artificial dura mater UTS standards (4-20 MPa) and elongation values (7-20%). In the swelling test, all four samples achieved swelling results in accordance with the swelling ratio standard for a composite membrane, which is less than 70%. The degradation test obtained the highest percentage of degradation in the biocomposite sample with the Kombucha-Chitosan-Collagen-Glycerol composition, with a degradation percentage of 20%. Morphology test results for the kombucha-glycerol-chitosan and kombucha-glycerol-chitosan-collagen sample compositions showed pore sizes that meet the standard. The biological characteristics obtained through cytotoxicity testing indicated that all four artificial dura mater composition variations were non-toxic as they had a live cell viability percentage above 50%. The best composite of the four composition variations that meets the standards for use as artificial dura mater is the Kombucha-Chitosan-Collagen composition with Glycerol as a plasticizer, as this composition meets all characterization standards.

Compliance with ethical standards

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Disclosure of Conflict of interest

No conflict of interest to be disclosed.

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