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Non-resorbable Biocellulose Membrane with Biological Safety and Functionality

朝鮮大學校 大學院

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2015年 2月 25日

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List of Abbreviations

GBR	Guided Bone Regeneration
e-PTFE	Polytetrafluroethylene
d-PTFE	High-density polytetrafluroethylene
GM	Gore-tex Membrane
G. xylinus	*Gluconacetobacter xylinus*
KCCM	Korean Culture Center for Microorganisms
BCM	BioCellulose Membrane
SEM	Scanning Electron Microscope
ASTM	American Society for Testing and Materials
PBS	Phosphate Buffered Saline
MG-63 cells	Human MG-63 osteosarcoma cells
DMEM	Dulbecco's Modified Eagle's Medium
KCLB	Korea Cell Line Bank
FBS	Fetal Bovine Serum
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
DMSO	Dimethyl Sulfoxide
IACUC	Institutional Animal Care and Use Committee
Micro-CT	Micro-Computed Tomography
H&E	Hematoxylin & Eosin
SD	Standard Deviation

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국문초록

생체적합성을 지닌 비흡수성 바이오셀룰로오스 차단막

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생활수준 향상에 따른 임플란트 (Implant)시술 환자의 증가로, 구강 내 매식 재료 개발에 대한 수요가 급격히 증가하고 있는 실정이다. 임플란트 시술 시 구강 내 소실된 골의 재생 및 시술 방법으로는 주로 골 이식재 (Bone grafting materials)와 차단막 (Barrier membrane)을 이용한 골유도재생술 (Guided Bone Regeneration, GBR)이 시행되고 있다. 골유도재생술은 임플란트 식립이 요구되는 골 결손 부위에 골 이식재를 식립한 후 차단막을 덮어 뼈가 자라도록 유도해 주는 시술로써 임플란트 이식 성공률을 결정하게 된다. 따라서, 임플란트 시술 시 필수적으로 요구되는 매식재료 중 하나인 차단막은 골 결손부위에 이식된 골 이식재의 이동을 억제하고 불필요한 연조직 세포의 증식은 배제하며 골 재생에 필요한 세포를 선택적으로 접근시킴으로써 골 결손부위의 골 재생과 골 침착을 돕는 지지체 역할을 한다.

치과의료용 차단막은 분해되는 특성에 따라 흡수성과 비흡수성 차단막으로 구분되어진다. 흡수성 차단막은 골 결손부위에 이식 후 자연적으로 구강 내에서 생분해됨으로써 차단막 제거를 위한 2차적 수술이 필요 없다는 점과 조작이 비교적 용이하다는 장점을 가지고 있는 반면, 비흡수성 차단막에 비하여 견고성이 낮아 공간 유지력이 저하

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되거나 결손부의 외형이 좋지 않고 골 이식재 등의 도움이 없다면 조직 재생의 양이 제한될 수 있다는 한계점을 가지고 있다. 또한 구강 내 이식 후 빠른 생분해는 완전한 골 회복이 어려울 가능성이 존재하고 중간 부산물이 국소적인 조직반응을 일으킬 수 있다는 한계점을 지닌다. 한편 비흡수성 차단막은 흡수가 되지 않음으로써 충분한 조직 재생을 유도하지만 차단막을 제거하는 2차 수술이 필요하다는 단점을 지닌다. 비흡수성 차단막은 골 회복까지 완벽히 제 기능을 수행하여 임상적 예후가 좋다는 측면으로 인해 임상에서 선호하는 차단막 종류이다. 그러나 비흡수성 차단막은 합성공정이 매우 복잡한 고가의 고어텍스 제품소재로, 임플란트 이식이 필요한 환자에게 치과치료 비용 상승 원인으로 작용한다는 한계점을 지닌다.

따라서 본 연구에서는 비흡수성 차단막으로써 사용되고 있는 고가의 고어텍스 소재의 치과의료용 차단막을 대체하기 위하여 우수한 생체적합성, 조직접합성, 세포차단능, 공간유지능, 우수한 영양분 전달능, 다공성 및 임상적 조작 용이성 등과 더불어 제조공정 단순화 및 대량생산이 가능한 천연소재 기반 경제적인 치과의료용 차단막을 개발하고자 한다. 이를 위하여 본 연구에서는 단순한 공정과정으로 경제적 대량생산이 용이하고, 나노 섬유로 구성된 3차원적 망상 구조를 형성함으로써 다공성을 확보하고, 치과의료용 매식재료로써 우수한 물리 · 화학적 특성 및 생물학적 안전성을 지녀 식품, 화장품 및 의약품 산업의 신소재로 각광받고 있는 미생물 바이오셀룰로오스 (Microbial cellulose)를 기반으로 치과의료용 비흡수성 차단막 제조 기술을 개발하였다. 또한 개발된 미생물 바이오셀룰로오스 기반 치과의료용 비흡수성 차단막을 한국식품의약품 안전처 (Ministry of Food and Drug Safety)와 한국식품의약품 안전평가원 (National Institute of Food and Drug Safety Evaluation)에서 제공한 의료기기 평가가이드라인에 따라 치과의료용 매식재료로써 요구되는 생물학적 안전성, 골 유도능 및 물리 · 화학적 특성분석을 시행하였고, 본 연구를 통하여 바이오셀룰로오스 합성균주인 *Gluconacetobacter xylinus*로부터 합성된 바이오셀룰로오스를 기반으로 제조된 치과의료용 바이셀룰로오스차단막은 치과의료용 차단막으로써 요구되는 우수한 생체적합성, 조직접합성, 외부세포차단능, 영양분의 전달력 및 장기간 공간확보능과 임상적 조작 용이성 등을 확보하고 있음을 확인하였다. 또한 미생물로부터 간단한 공정과정을 통한 경제적인 대량생산 가능성을 제시함에 따라 향후 치과의료용 비흡수성 차단막으로써 구강 내 임플란트 이식이 요구되는 환자들의 치과치료 비용을 완화시킬 수 있는 경제적인 구강 내 매식재료로써 활용이 가능하리라 사료된다.

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Ⅰ. Introduction

According to improvement of living standards, there are increasing the social interest for maintaining the healthy living including oral health. Furthermore, as the rapidly entering into aging society, patients with oral disease have being increased annually in elderly population. Therefore, the market of dental implant materials has not only being expended rapidly in both domestic and international, but also being demanded for the development of new medical implanting materials with compatibility and effectiveness [1]. However, because patients who required implant placement at oral cavity have usually the inadequate quality and insufficient residual quantity of bone at the recipient site, the bone regeneration of recipient site is needed before implant placement.

Guided Bone Regeneration (GBR), representatively well established clinical technique, has being performed frequently for patient to require the enhancement of quality and quantity of bone at the recipient site [2]. In the GBR technique, barrier membrane has been used to reduce the micro-mobility of the particular grafting materials, but also provide proper space for bone regeneration at recipient site which have the insufficient bone volume and quality [3,4]. Therefore, the barrier membrane with bone grafting materials have being used in GBR to promote bone healing and create of new bone formation, at recipient site [5].

Numerous studies have being reported about the importance of barrier membrane, used in GBR [6]. Functions of barrier membrane are that protecting adjacent soft tissues from cells to impede bone formation and improving the mechanical stability of bone grafting materials [7,8]. Therefore, as the prerequisites of barrier membrane, it is needed to satisfy the following general requirements : high biocompatibility; a high flexibility; adequate mechanical strength to maintain the function of barrier membrane; suitable adhesiveness characteristic to prevent the movement of the

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membrane at between the membrane and surrounding bone tissues [9,10].

Also, the barrier membrane is classified by either resorbable or non-resorbable membrane. Resorbable barrier membrane has the advantage that relieves the psychological and financial load of patients by avoiding additional surgery. However, due to its low rigidity, it is a poor to maintain the space and then is biodegraded at a rate more rapid than the required level [1]. Whereas, non-resorbable barrier membrane is a non-degradable materials. But it has the disadvantages that is required additional surgery to remove membrane from the alveolar bone [11]. Despite of non-absorbable barrier membrane with disadvantages, it has been used extensively in dental surgery because maintaining a space for bone formation and introducing the underlying tissues regeneration [12]. Non-resorbable membranes are mainly classified into expanded polytetrafluoroethylene (e-PTFE) or high-density polytetrafluroethylene (d-PTFE) [13]. However, these are resin-based polymer (Gore-tex) and are complex and costly to manufacture. Therefore, to overcome these limitations of current non-resorbable barrier membrane, it is needed to develop the simple manufacturing process using economic natural materials. In the GBR technique, a further improvement can be achieved by using a bioactive membrane capable of accelerating bone formation.

Biocellulose is obtained mainly from the cultures of gram-negative bacteria *Gluconacetobacter* strains as a highly hydrated membrane (up to 99% water) or pellicle at the air-medium interface. The biocellulose is a polymer of higher degree which is consisted pure forms of microfibers and higher structural crystallinity [14]. Biocellulose has attained unique physical and biological properties due to the exceptional structure, such as optical transparency, ductility, high tensile strength, oxygen permeability and biocompatibility [15,16]. In addition, the biocellulose has properties that are a high of elastic modulus and tensile strength under hydrated condition [10].

Since its discovery, biocellulose has being shown the tremendous potentials as an

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effective biopolymer in various fields owing to its impressive physiomechanical properties. The biocellulose with variety characteristics has being boosted up the utilization in biomedical and other related fields as wound dressing, burns, tissue regeneration, skin substitute, electronic and optic devices [17]. Hence, biocellulose has being represented a promising biomaterial.

Therefore, we hypothesized that biocellulose with high biocompatibility, high tensile, and oxygen permeability could be used as the biomaterial for producing the barrier membrane. In present study, the aim of present study, is to develop the non-resorbable biocellulose membrane (BCM) synthesized from *Gluconacetobacter xylinus (G. xylinus)* and is to evaluate the biological effectiveness and physiomechanical properties of synthesized barrier membrane.

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Ⅱ. Materials and Methods

Ⅱ-A. Synthesis of non-resorbable barrier membrane based on biocellulose

The processing steps for synthesizing the barrier membrane based on biocellulose were described briefly in Figure 1.

Ⅱ-A-1. Culture of *G. xylinus* for the synthesis of biocellulose

G. xylinus (KCCM 41431) were purchased from Korean Culture Center for Microorganisms (KCCM, Seoul, Republic of Korea) and were grown according to the culture protocol provided by the KCCM. Briefly, *G. xylinus* were grown on a basal medium containing Yeast extract 1 g/L, Peptone 0.6 g/L, Mannitol 5 g/L in 1 L of distilled water. The prepared medium was sterilized for 15 min at 121℃. Colonies of *G. xylinus* were inoculated into a 100 mL medium in a 250 mL flask shaken at 100 rpm and cultured at 26℃ for 3 days in Shaking incubator (HST201SF, Hanbeck ST, Seoul, Republic of Korea). After agitated culture to propagate the *G. xylinus,* the medium flask cultured in static conditions at 26℃ for 7 days to synthesize the microbial cellulose.

Ⅱ-A-2. Preparation of biocellulose membrane

The prepared biocellulose was sterilized for 15 min at 121℃. The biocellulose was immersed in distilled water and was washed with distilled water several times for 7

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days. The biocellulose was reacted to 200 mL of 0.1 N NaOH at 100℃ for 30 min in order to disrupt and dissolve the microbial cells [18]. The biocellulose was washed throughly with distilled water by pH 7.0 as the neutral condition and was to remove the residual NaOH and culture medium components from the biocellulose. The moisture of biocellulose membrane (BCM) was sucked by an suction master (TB-SM1, Infobiotech, Daejeon, Republic of Korea) and was dehydrated on dry oven (HB-501M, Hanbeck ST, Seoul, Republic of Korea) at 50℃.

Ⅱ-A-3. Surface modification of biocellulose membrane for using barrier membrane by oxygen plasma treatment

To prevent the cell attachment on the surface of BCM, it was treated on oxygen gas using plasma cleaner (PDC-002, Harrick plasma Inc., NY). The synthesized BCM was placed in the chamber (plasma cleaner). After being created a vacuum at the inside of reactor, high-purity oxygen (99.9%) purged into reactor maintained at a gas pressure of 200 mTorr for 3 min. Sequentially, the surface of BCM was treated with oxygen plasma for 1 min. Finally, after surface modification, it was used as non-resorbable barrier membrane based on biocellulose in present study.

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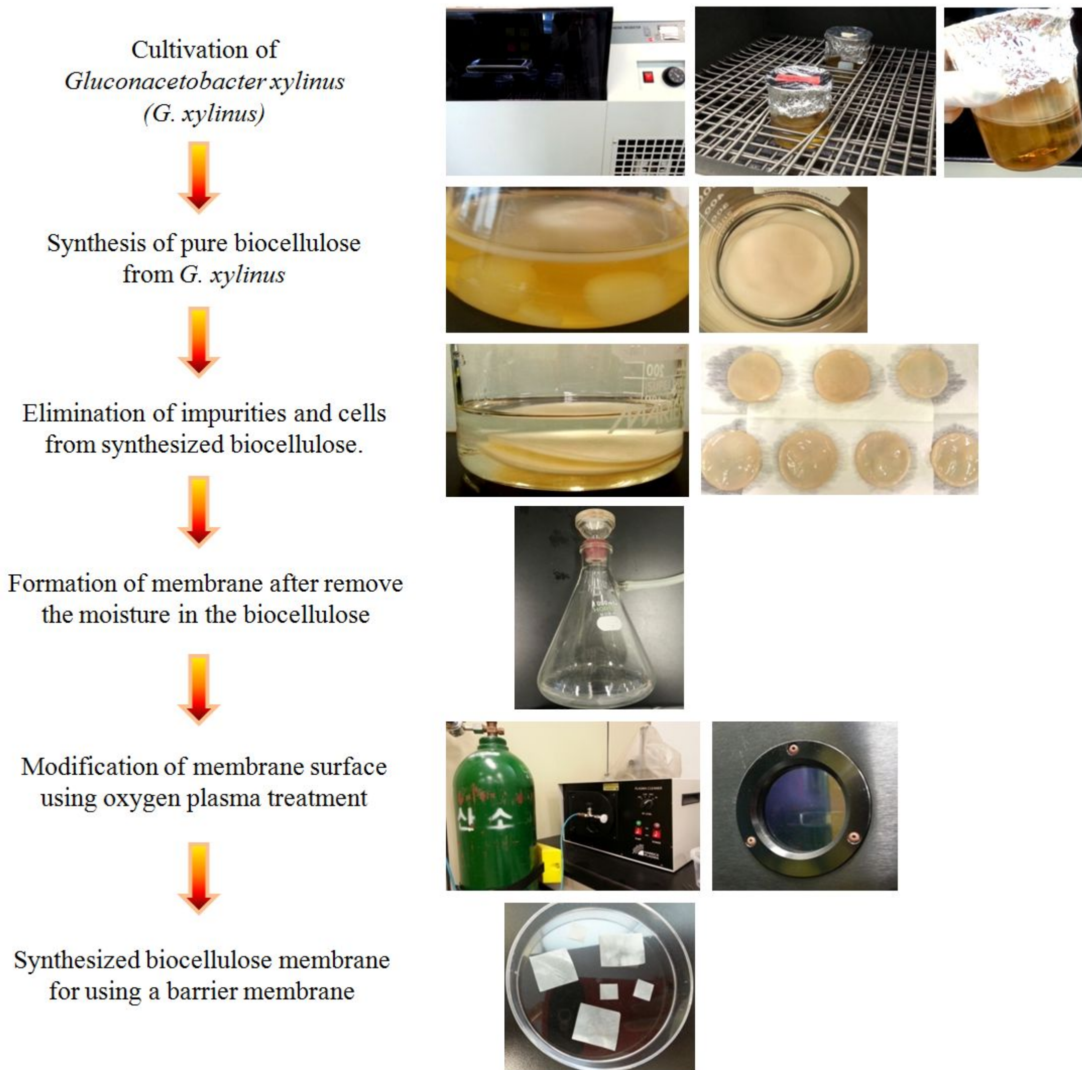

Figure 1. The schematic diagram for the development of biocellulose membrane synthesized from *G. xylinus.*

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Ⅱ-B. Evaluation of physical and chemical properties

Ⅱ-B-1. Analysis of scanning electron microscope

To observe the morphology of the synthesized BCM, I performed analysis of electron microscopic. Briefly, BCM were coated by sputter-coated (Emitech K550 sputter coater, Emitech Ltd, UK) and were observed by scanning electron microscopy (SEM, JSM 840-A, JEOL co., Japan) at 10,000 X, 20,000 X, and 50,000 X magnifications.

Ⅱ-B-2. Analysis of thickness measurement

To measure the thickness of the synthesized BCMs, these were measured by a digital caliper (KOIKO, China). Before the thickness measuring, the membranes were carefully cut into size of 10 mm width and 10 mm length. In present study, thickness measurement was performed on both dried and moisture saturated condition of BCMs.

Ⅱ-B-3. Analysis of tensile strength

In order to obtain significant tensile strength, BCMs with uniformed thickness were prepared as size with 20 mm X 50 mm. Prepared samples were examined their mechanical properties by a Tensile Tester Instron 5543 (High Wycombe, England) according to the procedure of th American Society for Testing and Materials (ASTM D 882) [19]. The samples were stretched at a crosshead speed of 5 mm/min to reach a constant strain rate.

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Ⅱ-C. *In vitro* cytocompatibility

Ⅱ-C-1. Culture of human MG-63 osteosarcoma cells

Human MG-63 osteosarcoma cells (MG-63 cells) were obtained from Korea cell line bank (KCLB, Seoul, Republic of Korea). MG-63 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, GRAND Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Life Technologies, Grand Island, NY, USA) in humidified atmosphere containing 5% CO_2 at 37℃.

Ⅱ-C-2. Analysis of cell viability

The cell viability was assessed using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Life Technologies, Grand Island, NY, USA). The MG-63 cells were seeded at a density of 1×10^5 cells per well in 96 well plates and allowed to attach to the well overnight. After incubation, cultured cells were treated with effluent of the BCMs were released in DMEM with 10% FBS for 24, 48, and 72 h at 37℃. After incubation under the defined conditions, cells were incubated for another 4 h in 20 μL of 5 mg/mL MTT. The supernatant was subsequently removed, and MTT crystals were dissolved in 200 μL/well dimethylsulfoxide (DMSO). Finally, solution was transferred into the 96 well plate and the optical density was measured at 540 nm using a spectrometer (Epoch Micro-volume Spectrophotometer System, BioTek, VT, USA).

Ⅱ-C-3. Analysis of cell attachment

Cell attachment was measured as previously described cell live & dead assay,

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using green calcein-AM and ethidium homodimer-1 (Life Technologies, Grand Island, NY, USA) to stain live and dead cells, respectively. MG-63 cells were plated on membranes (non-treated and treated BCM samples with oxygen plasma, commercial Cytoplast PTFE membrane ; Osteogenics, USA) in chamber slides, stimulated for 24 h, and then stained with green calcein-AM and ethidium homodimer-1 as according to the manufacturer's protocol. The data of cell attachment was photographed using fluorescence microscopy (Eclipse TE200, Nikon Instruments, Melville, NY).

Ⅱ-D. *In vivo* osteoinductivity

The experimental procedure to evaluate the biological safety and osteoinduction of BCM in animal model with calvarial bone defection were briefly described in Figure 2.

Ⅱ-D-1. Housing conditions of experimental animals

Experiment animal selection and management, surgical procedure, and preparation were performed by protocol (CDMDIRB 1008A60) approved by the Institutional Animal Care and Use Committee (IACUC) of Chosun University. In present study, Sprague-Dawley rats (weight 200 to 300 g) were maintained in a clean room controled with 12 h day/night cycles, an ambient temperature of 21℃, and *ad libitum* access to water and a standard laboratory pellet diet.

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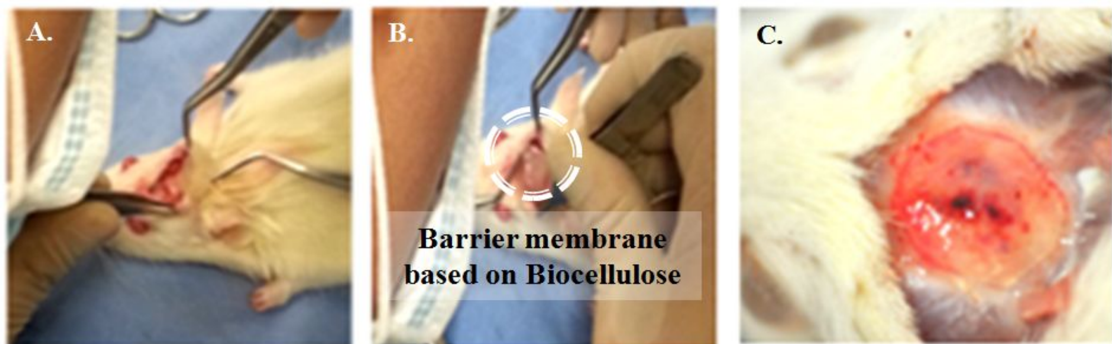

Figure 2. The experimental procedure to evaluate the biological safety and osteoinduction of synthesized biocellulose membrane in animal model with calvarial bone defection. Experiment animal selection and management, surgical procedure, and preparation were performed as follow as experimental protocol (CDMDIRB 1008A60) approved by the IACUC of Chosun University. (A) Generation of animal model with calvarial bone defection. (B) Transplantation of barrier membrane into defecting area in animal model with calvarial bone defection. (C) The view of defecting area at 8 weeks after the transplantation of barrier membrane.

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Ⅱ-D-2. Surgical generation of experimental animals with calvarial bone defection

The animals were anaesthetized by an intramuscular injection using Zoletile (15 mg/kg; Virbac, Virbac Korea, Republic of Korea). Routine infiltration anaesthesia using 2% Lidocaine (epinephrine 1:100000, lidocaine HCl, Huons, Republic of Korea) was performed at the surgical site. Sequentially, surgical site was shaved to remove hair and was sterilized using betadine solution to prevent contamination. Briefly, an incision was made in the sagittal plane across the cranium and a full thickness flap reflected exposing the calvarial bone of experimental animal. A standardized, circular, transosseous defect, 8 mm in diameter, was created on the cranium with the use of a saline cooled trephine bur. After removal of the trephined calvarial disk, sterilized BCMs were covered on the defecting area of calvarial bone evenly without suturing. The perosteum and skin were then closed and sutured with 4-0 coated polyglactin.

Experimental animals were sacrificed at 8 weeks post-surgery by CO_2 asphyxiation. Calvarial bones dissected from each experimental animals were fixed with 4% paraformaldehyde for 10 days at 4℃ to perform the histological and radiographic evaluation.

Ⅱ-D-3. Radiographic evaluation

Ⅱ-D-3-a. Analysis of x-ray image

Dissected calvarial bone were determined using the MX-20 Specimen Radiography System (Faxitron Bioptics LLC, Lincolnshire, IL, USA). The regeneration of bone at defecting site applied with BCM was radiographied by placing the calvarial bones

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dissected directly on the X-ray film for 5 min with the energy for 10 kV. The image was digitized, and the bone regeneration were evaluated.

Ⅱ-D-3-b. Analysis of micro-CT image

Bone regeneration at the defecting site on the calvarial bone were evaluated by microscopic examination and micro-Computed Tomography (micro-CT) scanning. Freshly dissected calvarial bone were immediately fixed in 4% paraformaldehyde, followed by micro-CT imaging analysis performed at the Center for University-Wide Research Facilities of Chonbuk National University, using a SkyScan 1076 (SkyScan, Konitch, Belguim) with the energy for 130 kV and rotation step 0.2°.

Ⅱ-D-4. Histological evaluation

Skull of experimental animals were excised, post-fixed in 4% paraformaldehyde for 7 days, dehydrated in a series of ethanol solutions (50, 70, 95, and 100%) at 15 min per step, and then submerged in xylene. For conventional histological staining, paraffin-embedded tissue blocks were prepared and sectioned at 10 μm thickness with a Leica RM2135 microtome (Leica Microsystems AG, Wetzler, Germany) on superfrost plus slides (Menzel, Braunschweig, Germany). After deparaffinization in xylene for 30 min, sections were hydrated through a gradient with decreasing proportions of ethanol. The bone paraffin sections stained with hematoxylin & eosin (H&E) staining and safranin-O & fast green staining at x4 modifications to show the calvarial defect zones.

Ⅱ-D-4-a. Hematoxylin & Eosin staining

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Bone morphology was analyzed after conventional H&E staining (Sigma-Aldrich, Gillingham, UK). The bone paraffin sections stained with H&E staining at x4 modifications to show the calvarial defect zones by microscopy.

Ⅱ-D-4-b. Safranin-O & fast green staining

Proteoglycan content of the bone was assessed following safranin-O & Fast green staining (Sigma-Aldrich, Gillingham, UK). The bone paraffin sections stained with safranin-O & fast green staining at x4 modifications to show the calvarial defect zones by microscopy.

Ⅱ-E. Statistical analysis

The experimental data are presented as the mean ± standard deviation (SD) from at least three independent experiments and were compared using analysis of variance, followed by Student's *t*-test. A *p*-value of less than 0.05 was considered statistically significant.

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Ⅲ. Results

Ⅲ-A. Synthesis of non-resorbable barrier membrane based on biocellulose

To synthesize pure biocellulose for using source of a non-resorbable barrier membrane, *G. xylinus* was inoculated into 200 mL liquid media and was incubated to propagate on the orbital shaking platforms at 26℃ till the formation of pellicle. Pellicle composed of propagated *G. xylinus* was cultured in static conditions at 26 ℃ till the formation of biocellulose with 0.5 mm thickness. According to previously established method to dissociate the *G. xylinus* from synthesized biocellulose, collected biocellulose was processed in 0.1N NaOH at 100℃ for 30 min [18]. Sequentially, biocellulose was washed throughly with distilled water by pH 7.0 as the neutral condition for removing the residual NaOH and culture medium components from the biocellulose. The moisture of biocellulose membrane (BCM) was sucked by an suction master (TB-SM1, Infobiotech, Daejeon, Republic of Korea) and was dehydrated on dry oven (HB-501M, Hanbeck ST, Seoul, Republic of Korea) at 50℃. As a result, membrane of film-type was manufactured from the hydrated cellulose. The processed BCM was treated on oxygen plasma to prevent the external cell attachment. The BCMs that are produced from *G. xylinus* and are modified a surface with oxygen plasma treatment are shown in Figure 3. The completely synthesized BCMs generally have a pH in the range of 7.0 ± 0.2 and a weight in the range of 0.01 ± 0.02 g.

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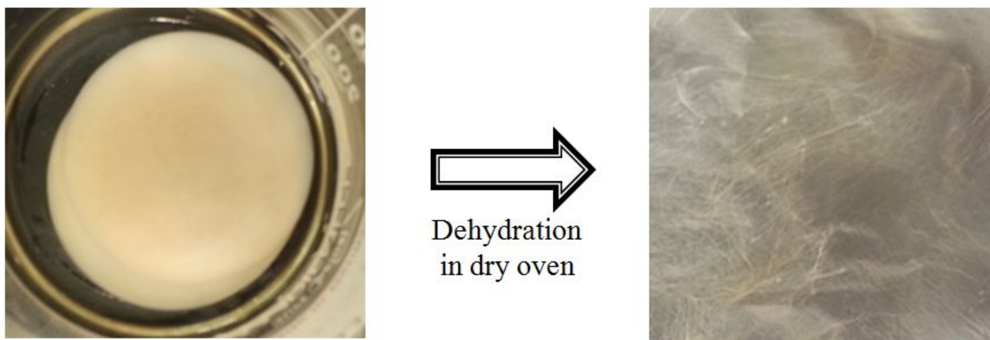

Figure 3. The non-resorbable barrier membrane based on the biocellulose synthesized from *G. xylinus*

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Ⅲ-B. Physical and chemical properties of synthesized biocellulose membrane

Biocellulose with various physic-chemical properties such as microporous structure, structural integrity and biocompatibility can be considered as a biopolymer to develop the non-resorbable barrier membrane [14, 20]. According to guideline of Ministry of Food and Drug Safety, non-resorbable barrier membrane based on biocellulose was confirmed whether or not having with the proper physical and chemical properties for using medical materials.

Ⅲ-B-1. Observation of surface structure and pore numbers to modified biocellulose membrane

To observe the surface structure of synthesized BCMs, we had performed the electron microscopic analysis using SEM. The SEM results showed that the synthesized BCMs had a network arrangements composed of microfibers and micro pores. Furthermore, the comparative assessment between BCMs with and without surface modification by using oxygen plasma treatment was illustrated as shown in Figure 4. The electron microscopic surface structure of BCM without surface modification showed a dense amorphous structure of microfibers and micropores. On the other hand, the electron microscopic surface structure of BCM with surface modification had a regular microstructure of microfibers and micropores compared with BCM without surface modification. It might be induced by electrical repulsion formed between microfibers with negative charge caused by oxygen plasma treatment as a surface modification. Therefore, surface modification using oxygen plasma treatment might a potent processing methods to induce the regular microstructure of BCM.

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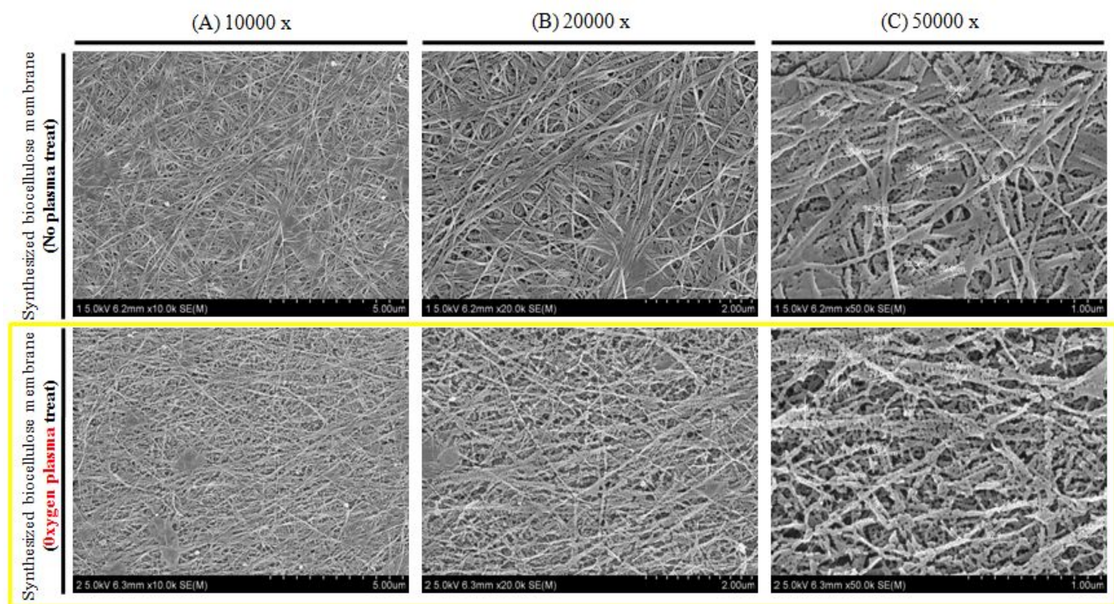

Figure 4. The electron microscopic comparison of surface structure and pore size between synthesized biocellulose membrane without (top) or with (bottom) surface modification using oxygen plasma treatment by scanning electron microscope (SEM). (A) 10000 x, (B) 20000 x, (C) 50000 x.

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Ⅲ-B-2. Thickness of synthesized biocellulose membrane

To assess the thickness and the moisture absorption factor of synthesized BCMs, the assessment of thickness using a digital caliper was performed in both hydrating and dehydrating condition of synthesized BCMs. The thickness of synthesized BCMs was presented in Table 1. The thickness of BCMs in dehydrating condition was calculated as the average values of 0.04 ± 0.02 mm. Whileas, the thickness of BCMs in hydrating condition was calculated as the average values of 0.06 ± 0.02 mm thickness. Subsequently, the moisture absorption factor of synthesized BCMs was calculated as an approximately 56% more.

Ⅲ-B-3. Tensile strength of synthesized biocellulose membrane

Mechanical property of biocellulose is a major factor for using as a scaffold material and biomedical fields like wound dressing materials, tissue engineering and etc [21]. A tensile strength test was performed to investigate the intrinsic mechanical properties of the synthesized BCM with surface modification according to the procedure of the ASTM D 882 [22]. The tensile strength of synthesized BCM with surface modification was described in Table 2. For synthesized BCM with surface modification, the tensile strength was evaluated approximately 16.94 ± 1.2 MPa, and the elastic modulus obtained from the stress-strain curve was evaluated approximately 654.89 ± 10 MPa.

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Table 1. The thickness of non-resorbable barrier membrane based on biocellulose

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Average
Dehydrating samples (mm)	0.02	0.03	0.03	0.04	0.06	**0.04 ± 0.02**
Hydrating samples (mm)	0.04	0.04	0.05	0.06	0.09	**0.06 ± 0.02**

The thickness of biocellulose membrane was assessed by a digital caliper. Each value in the table is presented as the mean ± SD.

Table 2. The tensile strength of non-resorbable barrier membrane based on biocellulose

Membrane W x S (mm)	Maximum Load (N)	Tensile strength (MPa)	Elastic modulus (MPa)	Tensile strain (%)
20 x 50	25	16.94 ± 1.2	654.89 ± 10	4.26 ± 0.5

The tensile strength of biocellulose membrane was assessed by Tensile Test Instron 5543. All assessment were performed with triplicates. Each value in the table is presented as the mean ± SD.

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Ⅲ-C. *In vitro* cytocompatibility

The biomaterials must have a high degree of biological stability. Biocompatibility of biomaterials has been defined as its ability to perform with an appropriate host response in a specific application [23]. Therefore, we performed the assessment of cell cytotoxicity to evaluate the biological safety of synthesized BCMs in human osteoblast MG-63 cells.

Ⅲ-C-1. MG-63 cell viability of the effluents from synthesized biocellulose membrane

To determine whether synthesized BCMs has a cytotoxicity or not, we performed the MTT assay, a representative method to evaluate the cell cytotoxicity, in MG-63 cells. In addition, to prepare the aimed effluents, synthesized BCM was eluted in DMEM containing 10% FBS for 24, 48, and 72 h. MG-63 cells were treated with collected effluents prepared at different elution periods for 24 h. As shown in Figure 5, the relative cell cytotoxicity was verified as 76.3 ± 14.3% in MG-63 cells treated with BCM effluents eluted for 24 h compared with 102 ± 25.1% in control. Furthermore, the cell cytotoxicity was verified as 90.7 ± 19.4% and 77.8 ± 14.4% in MG-63 cells treated with BCM effluents eluted for 48 and 72 h compared with non-treated control, respectively. Therefore, these are clearly suggesting that synthesized BCM has a high level of biological safety in MG-63 cells.

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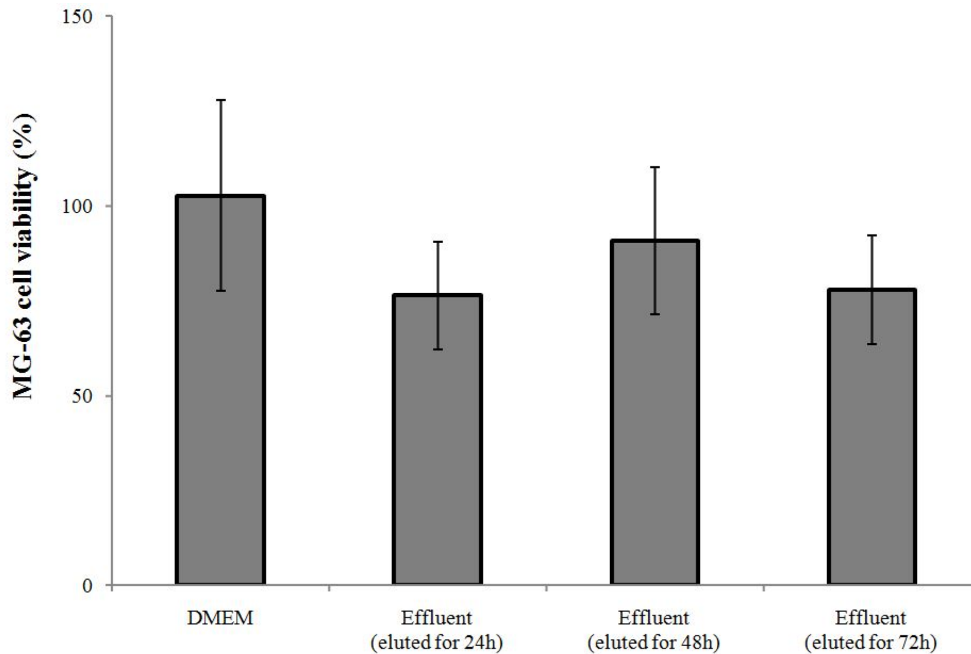

Figure 5. Cell viability of synthesized biocellulose membrane in MG-63 osteoblastic cells. Effluents of the biocellulose membranes were prepared by the guidelines provided from Ministry of Food and Drug Safety (Republic of Korea) for the measurement of cell cytotoxicity. The MG-63 cells were cultured in either DMEM with 10% FBS or the effluent of BCMs. MTT assay was performed to verify the effluents of the BCMs synthesized in MG-63 cell viability.

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Ⅲ-C-2. MG-63 cell attachment of the membranes (untreated or treated by oxygen plasma and commercial) to compare

Next, we hypothesized that electrical repulsion formed between living cells with negative charge and BCM with negative charge might be suppressed the adhesion of external cells on the their surface. Therefore, the surface of synthesized BCM was treated by oxygen plasma as a surface modification. To verify the cell attachment on the surface of synthesized BCM with surface modification using oxygen plasma treatment, we visualized the attached cell on the surface of BCM using cell live & dead assay. As shown in Figure 6, many MG-63 cells were attached on the surface of synthesized BCM without negative charge. On the other hand, the number of MG-63 cells attached on the surface of synthesized BCM with negative charge formed by oxygen plasma treatment were significantly less than compared to synthesized BCM without negative charge and a commercial d-PTFE membrane, Cytoplast.

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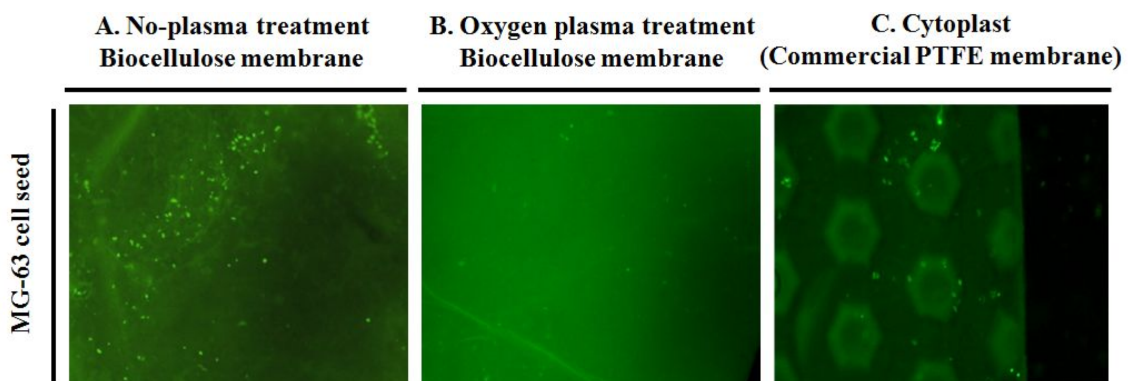

Figure 6. MG-63 cell attachment of the membranes (A,B,C) to compare. prevent the cell attachment on the surface of BCM, the surface of BCM was treated with oxygen plasma. And then, comparison of MG-63 cell attachment between BCM without (A) or with (B) surface modification using oxygen plasma treatment and commercial PTFE membrane (C) used as control were performed by cell live & dead assay.

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Ⅲ-D. *In vivo* osteoinductivity

In vivo test was conducted to verify whether synthesized BCM has a biological safety and osteoinductivity in living animals.

Ⅲ-D-1. Radiographic evaluation of bone regeneration in animal model with calvarial bone defection

Next, to evaluate the biological safety and osteoinductivity of synthesized BCM, the experimental animals with 8 mm bone defection on calvarial were generated according to the protocol (CDMDIRB 1008A60) approved by IACUC at Chosun University. And then synthesized BCMs were transplanted onto the bone defecting region of experimental animals. At 8 weeks after post-transplantation of BCM, calvarial bones were collected to evaluate the ability of BCM as a non-resorbable barrier membrane using radiographic analysis using X-ray and micro-CT. As shown in Figure 7, bone defection was still observed on the defecting region in the calvarial bone without transplantation of BCM. On the other hand, bone defection did not observed on the defecting region in the calvarial bone with transplantation of BCM. Furthermore, the radiographic observation revealed that BCM transplanted onto defecting region of experimental animals induced significantly the new bone formation. Therefore, both visual inspection and radiographic analysis were clearly indicating that synthesized BCM has a excellent osteoinductivity.

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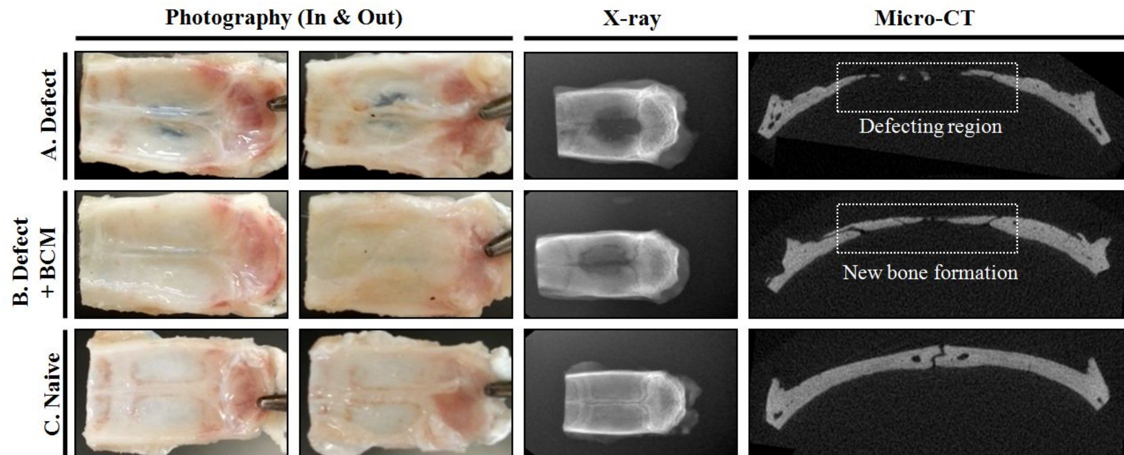

Figure 7. The radiographic evaluation of defecting area at 8 weeks after the transplantation of biocellulose membrane using photography, x-ray and micro-CT image analysis. After 8 weeks post-transplantation of BCMs onto defecting region of animal model with calvarial bone defection, tissues were harvested and were immediately fixed using 4% paraformaldehyde for 4 days. After fixation, tissues were performed x-ray and micro-CT to evaluate the bone regeneration at the defecting region. (A) Tissue without implantation of BCM, (B) tissue with implantation of BCM, (C) naive tissue.

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Ⅲ-D-2. Histological evaluation of bone regeneration in animal model with calvarial bone defection

To evaluate the osteoinductivity of BCM, harvested calvarial bones were decalcified and were performed the histological analysis using H&E staining to observe the morphological alteration of tissues and safranin-O and fast green staining to verify the new bone formation at the defecting region of experimental animals. As shown in Figure 8A, the result of H&E staining showed that transplanted BCM did not only affect the morphological alteration but also did not induce the inflammation in defecting region of experimental animals. Furthermore, the invasion of other connective tissue into defecting region was completely inhibited by transplanted BCM. However, to evaluate the new bone formation in the defecting region transplanted with BCM, safranin-O & fast green staining was performed as shown as Figure 8B. As same as the results of radiographic analysis, the result of safranin-O & fast green staining was revealed that new bones under the transplanted BCM onto defecting region of experimental animal was observed. Therefore, taken together, these are clearly suggesting that synthesized BCM has a potent physio-chemical functions as a non-resorbable barrier membrane.

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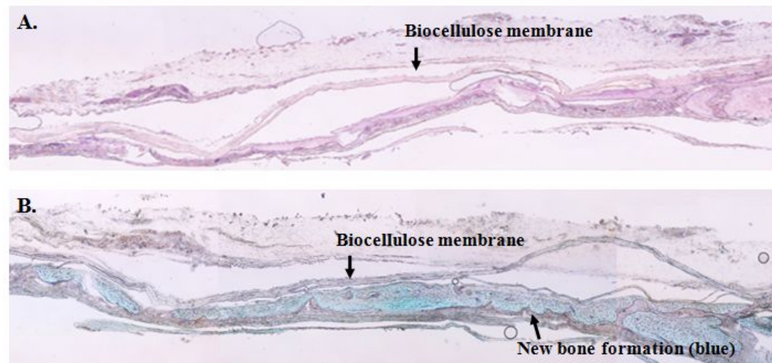

Figure 8. The histological evaluation of defecting area at 8 weeks after the transplantation of biocellulose membrane. Experimental animals were sacrificed at 8 weeks post-surgery. Collected bones were evaluated by histological analysis using H&E staining and safranin-O & fast green staining. (A) Bone morphology was analyzed by the H&E staining, (B) bone regeneration was analyzed by the safranin-O (for proteoglycan) & fast green (for bone matrix) staining.

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Ⅳ. Discussion

GBR is frequently performed to regenerate the bone for implant replacement in dental surgery and is usually required the barrier membrane with bone grafting materials to promote bone healing [5]. Especially, the importance of the barrier membrane in GBR has been reported in numerous studies. The barrier membrane that suppressing infiltration of the soft tissue and increasing physical strength of bone grafting materials is needed the prerequisites as high biocompatibility, flexibility, adequate mechanical strength and suitable adhesiveness to lead the complete bone formation [6,10].

GM which is one of the strongest candidates as non-resorbable barrier membrane has been used most widely in the dental surgery for GBR. It has satisfied all of the properties required for the successful bone regeneration in bone defection region [25,26]. Also, it has various physical properties such as porous structure, biocompatibility and easy clinical applicability [27]. Therefore, GM is used widely in dental implant surgery [17]. Furthermore, its efficacy has being proven. Although GM has been applied for using as barrier membrane in GBR, it has an expensive material and complicated production systems. Because these limitations of GM, surgery cost of implant surgery is on the rise. Therefore, non-resorbable barrier membrane is needed the simple manufacturing process using a economic natural material to replace the GM.

Biocellulose is a natural polymer synthesized from *Gluconacetobacter genus* and is a composed of extremely pure polysaccharides fibers with a diameter of only 20 nano-meters. Recently, biocellulose with unique physical and mechanical properties due to its tridimensional and branched micro-fibrillar structure have being considered as an interesting natural polymer in the specific technological application such as audio membranes, electronic paper, and transparent nano-composites [15]. Furthermore, biocellulose with various biological compatibilities such as non-toxic,

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swelling property with extremely hydrophilic, and exchange of oxygen and nutrient through micropores composed of microfibers has being considered as an interest natural materials for using as a wound healing membranes, substituting natural skin, and chirurgical implants in biomedical research field [5,18].

Especially, biocellulose with various physic-chemical properties such as microporous structure, structural integrity and biocompatibility can be considered as a biopolymer to develop the non-resorbable barrier membrane [14,20]. Moreover, because the culture media of *G. xylinus* to synthesize biocellulose is able to use a various types of culture media produced from food wastes, it can produce economically through commercial manufacturing process, eco-friendly [14]. Therefore, we hypothesized that the non-resorbable barrier membrane based on biocellulose synthesized from *G. xylinus* can be considered as a biopolymer to replace and overcome the disadvantage of Gore-tex.

According to established our hypothesis, biocellulose synthesized from *G. xylinus* as shown in was processed as the non-resorbable type of barrier membrane by novel dehydrating process. However, as a perquisite for using barrier membrane, candidate must have highly hydrophilic properties to accelerate the bone healing [16]. In present study, the non-resorbable BCM which composed of the pure cellulose microfibers with extremely hydrophilic properties is confirmed that has an amazing fluid-holding capacity. The thickness of synthesized BCM was approximately 0.04 ± 0.02 mm at dehydrated condition. On the other hand, its thickness was verified as approximately 0.06 ± 0.02 mm under hydrated condition. The thickness of synthesized BCM under hydrated condition increased approximately 56% more than that of dehydrated condition. Therefore, the BCM with excellent capacity of moisture retention might be accelerated the bone regeneration through the gradual secretion of various anabolic growth factors included in exudate absorbed into its internal micro-space during GBR surgery. Furthermore, the thickness of commercial non-resorbable barrier membrane was verified as approximately 0.2 mm. Hence, the thickness of commercial non-resorbable barrier

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membrane was higher at least 40 folds compared with that of membrane based on biocellulose. These are indicating that synthesized BCM might be easier more than a commercial that of membrane in a point view of operation. However, barrier membrane for using GBR must have a stable tensile strength to against the mechanical movement of oral cavity during the period of bone healing [15]. According to this prerequisite, the tensile strength of synthesized BCM was measured by Tensile Test Instron and was verified as approximately 16.94 ± 1.2 MPa. While as average tensile strength of commercial non-resorbable barrier membrane was revealed as approximately 6 MPa. The BCM has a tensile strength at least 2.5 folds more compared with commercial non-resorbable barrier membrane. Taken together, these are indicating that BCM has a high degree of physical stability compared with commercial non-resorbable barrier membrane. In addition, the synthesized BCM has a semi-transparent more than that of opaque membrane supplied as commercially. Therefore, this property could be endowed a functional usefulness that barrier membrane can be transplanted to the recipient site correctly.

However, BCM is needed a process to remove after complete regeneration of bone. During the removing process, cells attached on the BCM could be induced the additive injury at the bone healing site [10]. Therefore, BCM is needed to surface modification for preventing the cell attachment on its surface. To prevent the cell attachment on the surface of BCM, oxygen plasma treatment was performed to modify the surface of charge as a negative charge. Because the cytosolic membrane of living cells has a negative charge, repulsive force formed between barrier membrane with negative charge and living cells might be prevented the cell attachment. However, to evaluate whether BCM with negative charge formed by oxygen plasma treatment can prevent the cell attachment, we performed the cell attachment assay using cell live & dead assay kit. The result of cell attachment assay showed that BCM with negative charge prevented the cell attachment on its surface more than BCM without electrical charge and a commercial PTFE membrane (Cytoplast). Therefore, these results are suggesting that surface modifications as negative charge using oxygen plasma treatment might be

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minimized the soft tissue damage during the removing process of BCM. Moreover, electron microscopical analysis of BCM revealed that the negative charge formed by oxygen plasma treatment induced the extension of micropore diameter compared with non-treated BCM. It might be mediated by the repulsive force formed between microfibers with negative charge. Therefore, the extended diameter of micropores on the BCM might be increased the exchange of oxygen and the supplement of nutrient to osteoblast during the period of bone regeneration [6].

Next, barrier membrane used in GBR must have a high degree of biological safeties. Therefore, to verify the biological safety of synthesized BCM, we performed the cell cytotoxicity assay in the human osteoblastic MG-63 cell as follow as the guideline supplied from the Ministry of Food and Drug Safety, Republic of Korea. The effluent prepared from synthesized BCM did not affect the cell viability. Therefore, these are clearly suggesting that synthesized BCM has a high degree of biological safety.

However, to verify whether synthesized BCM has a biological and osteoinductive activity in living animals as *in vivo* test, we generated the animal model with calvarial bone defection and implanted the synthesized BCM into recipient site [24]. After implantation, animals were hosed for 8 weeks and were sacrificed to evaluate the radiographical evaluation using x-ray and micro-CT. x-ray image analysis revealed that defecting site on the calvarial bone did not heal yet in experimental animal without implantation of non-resrobable BCM had been shown the defecting. While as new bone formation had been observed accordance with synthesized BCM implanted into defecting calvarial bone of experimental animal. Furthermore, as same as results of x-ray analysis, micro-CT image analysis revealed that experimental animal with implantation of synthesized BCM into defecting calvarial bone had been shown the excellent new bone formation compared with experimental animals without implantation. Moreover, to verify the new bone formation in the experimental animal model with the implantation of BCM, we performed the histological evaluation using both H&E staining and safranin-O & fast green

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staining. In the H&E staining, BCM implanted into defecting region of calvarial bone did not affect the cell morphology and did non induce the inflammation. Furthermore, in the results of safranin-O & fast green staining, renewal bones located on the under of BCM implanted into defecting region of calvarial bone had been stained as green color by fast green reagent.

Through the following results, it was confirmed that BCM is appropriate to apply as a non-resorbable barrier membrane. Therefore, the BCM having a high biocompatibility and various physical characteristics is expected to overcome the limitation of GM by manufacturing through simple process and to relieve the cost of implant surgery.

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