

Preparation and characterization of a 3-dimensional macroporous bacterial cellulose scaffold for *in vitro* tissue engineering applications

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The increasing need for a new tissue engineering scaffold has revived interest in bacterial cellulose (BC) and its water holding capacity (WHC), porosity, and biocompatibility. Bacterial cellulose was produced using *Gluconacetobacter xylinus*, and bacterial cellulose film (BCF) was prepared by lyophilization. To obtain macroporous bacterial cellulose (MBCS), BCF was rinsed with poly(ethylene glycol) (PEG-400), adjusted to 0.25% BC concentration, and freeze-dried. The objective of this study to compare BCF and MBCS in terms of porosity, *in vitro* degradation, WHC, and the cell viability of mouse fibroblast cell line (NIH-3T3). The results showed that the MBCS has great potential for tissue engineering.

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1. Introduction

Cellulose is the most plentiful organic biopolymer in nature and its best known source is plants. In addition, microorganisms can synthesize cellulose, such as some bacteria, algae, and fungi. A well-known cellulose-synthesizing microorganism is the Gram-negative bacteria *Gluconacetobacter xylinus* (previously named *Acetobacter xylinum*). The cellulose of *Gluconacetobacter xylinus* is named bacterial cellulose (BC) (also known as microbial cellulose). BC has the same chemical structure as plant-derived cellulose, although it is strictly different in terms of purity, because it does not contain pectin, lignin, or hemicellulose [1, 2]. Due to some notable properties of BC including its three-dimensional (3D) nanoporous network structure, chemical purity, superior water holding capacity (WHC), excellent mechanical properties, flexibility, and biocompatibility, many industries focus on BC. Especially the paper, food, health, textile, bioengineering, and medical industries have studied a wide diversity of implementations of BC [3, 4].

These kinds of biocompatible biopolymers could be used as tissue engineering scaffolds. For instance, BC can be used in manufacturing as a scaffold of artificial blood vessels, bone, cartilage, and skin [5- 7]. A candidate for a tissue engineering scaffold should have suitable porosity and WHC and biodegradation capacity [8, 9].

Porosity and WHC are major characteristics determining the potential implementation of BC in tissue engineering, because these parameters directly affect nutrition flow and cell viability. Moreover, degradation is the most important parameter for supporting new tissue formation as soon as possible [10].

In spite of the excellent properties of BC, it has two limitations for tissue engineering applications. These are biodegradation rate and nanopore size. To solve these problems BC requires some *in situ* or *ex situ* modifications. Freeze-drying is a physical *ex situ* modification method and treatment with poly(ethylene glycol) 400 (PEG 400) is the most common way to make a three-dimensional (3-D) macroporous BC scaffold (MBCS) [9, 11, 12]. The results of these studies showed that PEG administered MBCS had high porosity, high surface area, and excellent

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biocompatibility. Thus, the freeze-drying technique minimally affects the purity, nontoxicity, and biocompatibility of BC [9, 12, 13].

In nature, the degradation of cellulose occurs by hydrolysis. However, the human body does not possess the enzyme that degrades the linkage of β -1,4 glucose. Yet, according to a non-investigated assumption, BC may be degraded by non-enzymatic, spontaneous, and slow breakdown. Therefore, the *in vitro* degradation of BC can give an estimation about the biodegradation of potential BC scaffolds [6]. On the other hand, there is no report in the literature about the *in vitro* degradation and WHC of BC at human body temperature.

In previous research by Cai et al., PEG immersed BCF was evaluated in terms of the viability of mouse 3T3 cells. The research group reported that the BCF/PEG composite had much preferable biocompatibility for 3T3 cells adhesion compared with the pure BCF [13]. Later studies investigated BCF for making a macroporous 3-D scaffold using freeze-drying. Except for mesenchymal stem cells (MSCs) and a human breast cancer cell line (MDA-MB-231), no mammalian cells have been investigated yet [9, 12]. MBCS has huge potential as a tissue engineering scaffold for 3-D tissues. For these reasons, the aim of the present study was to compare BCF and MBCS in terms of stability, *in vitro* degradation, and WHC at human body temperature. Moreover, this study evaluated the cell viability of a mouse fibroblast cell line (NIH-3T3) in MBCS and investigated the biocompatibility of mammalian fibroblast cells for the first time.

2. Materials and Methods

2.1. Preparation of BCF and MBCS samples

Gluconacetobacter xylinus (ATCC® 10245™) was used as a producer of BC. The essential nutrient component was provided by Hestrin–Schramm broth (HSB). HSB contains (% w/v) 0.5% yeast extract, 0.5% peptone, 2% glucose, 0.5% Na₂HPO₄, and 0.115% citric acid, pH adjusted to 6.0 [14]. The solution was sterilized by autoclaving. For starting static culture conditions freshly prepared 5% *Gluconacetobacter xylinus* suspension was inoculated in HSB and cultured for 10 days at 30°C. At the end of incubation, the BC pellicle was harvested under aseptic conditions. For removing the bacterial layer from the surface of the BC, the pellicle was boiled in deionized water for 2h, following in 0.5 M NaOH for 15 min. The pellicle was soaked in 1% NaOH for 2 days after the boiling step, and the pellicle was rinsed with deionized water several times until neutral pH was achieved. The BC pellicle was frozen in liquid nitrogen and then lyophilized at -50°C for a day. The samples were preserved in a desiccator until further analysis.

The smoothly shaped and lyophilized BC pellicle was named BC film (BCF) and used as an unmodified BC sample for the control group.

The freeze-drying method described by Gao et al. was administered with some minor modifications for the preparation of the MBCS. The lyophilized BC pellicle was soaked in PEG 400 for a day; then the BC pellicle was rinsed in deionized water several times and lyophilized for another day under the same lyophilization conditions. The PEG administered and lyophilized BC pellicle was blended with deionized water using a homogenizer (Heidolph, Germany) at high speed for 10 min to prepare 0.25% BC suspension. For 1 cm³ of scaffold, the suspension was transferred to a 24-well plate and freeze-dried at the same lyophilization conditions for a day [9, 12]. The scaffolds were sterilized by autoclaving.

All standardization tests were designed considering human body conditions, so the tests were applied with some minor modifications.

2.2. Water holding capacity

The WHC was calculated by the method of water substitution with minor alterations. The dry weight of the same thickness and dimension BC film and MBCS samples were weighed and recorded as W_d. Samples were soaked with PBS (pH 7.4) for 24 h at 37°C. The wet membranes were carried with filter paper and blotting gently to remove surface water. The wet weight of the

samples (W_w) was evaluated after 0, 7, 14, 21, and 30 days. The water holding ratio was calculated according to Eq. (1) [10, 15, 16].

$$\text{Water holding ratio (\%)} = (W_w - W_d)/W_d \times 100 \quad (1)$$

2.3. In vitro degradation study

In vitro degradation was calculated according to the weight loss of samples. Cubes of 1 cm³ MBCS and 1 cm² BCF were soaked in PBS and incubated at 37°C for 1, 7, 14, 21, and 30 days. The wet samples were weighed and recorded as W_w . After that the scaffolds were washed in deionized water several times and dried in an ordinary incubator at 37°C for 2 h. The final dry samples were weighed and were noted as W_d . The percentage of degradation was calculated using Eq. (2) [10, 16].

$$\text{In vitro degradation (\%)} = (W_w - W_d)/W_w \times 100 \quad (2)$$

2.4. Porosity study

The porosity of the MBCS samples was measured by the water displacement method at human body temperature. The MBCS samples were prepared as 1 cm³ and weighed (W_1). Then the scaffolds were immersed in a known volume (V_1) of water for 30 min. The total volume of water and the water absorbed scaffold was recorded as V_2 . The volume difference ($V_2 - V_1$) symbolized the total volume of the MBCS sample (including the volume of BC fibers and pores). The water absorbed scaffolds were put into a petri dish and dried in an oven at 70°C. The completely dried weight of MBCS samples was recorded as W_2 . p represents the density of water at 37°C. The calculation of porosity was obtained by Eq. (3) [10, 17]. And the surface area and porosity measurement was verified by a BET device (Micrometric surface area and porosity, Tristar II, USA).

$$\text{Porosity (\%)} = (W_1 - W_2)/p/(V_2 - V_1) \quad (3)$$

2.5. Scaffold and cell observation by field emission scanning electron microscope (FESEM)

The freeze-dried MBCS samples were coated with platinum (Q150 ES Quorum, UK) and observed with a FESEM (Supra 55, Zeiss, Germany).

For observation scaffold-cell complex 24- and 48-hour culture samples were fixed by serial acetone administration and dried with a critical point dryer (K850 Emitech, Quorum, UK) and were sputter-coated with platinum. Then the samples were examined using the aforementioned FESEM.

2.6. Cell seeding and cell viability assay

For detecting cell viability a *Mus musculus* fibroblast cell line (NIH/3T3 (ATCC® CRL-1658™)) was cultured with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in 5% CO₂ in an incubator (8000, WJ, Thermo, USA). Confluent NIH/3T3 cells were collected by trypsin-EDTA administration and 10⁶ cells were resuspended with the complete growth medium.

Each scaffold was soaked with a complete growth medium and inoculated with the cell suspension, which included 10⁶ NIH/3T3 cells. The scaffolds were cultured for 24 and 48 hours at 37°C in 5% CO₂ in an incubator. After the end of incubation, the scaffolds were transferred to a 96-well plate for the determination of cell viability. Cell viability was detected with the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS) (Promega, USA). Next 30 µl of MTS reagent [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] was pipetted on each scaffold followed by incubation for an hour at 37°C in 5% CO₂ in an incubator. The absorbance of samples was measured at 490 nm.

2.7. Statistical analysis

All of the experiments were realized with six copies and the pore size evaluation was conducted with twenty measurements. The statistical analysis was conducted by SPSS. The datas of experiments were introduced as mean value \pm standard deviation (SD). The results of the SPSS analysis with p values of <0.05 were considered statistically significant.

3. Results and discussion

3.1. Water holding capacity

The WHC of tissue engineering scaffolds is very important for the nutrient intake of cells that adhered to scaffold surface and pores [10].

As seen in Fig. 1, all of the BCF and MBCS suspensions had high water holding ability and displayed significant WHC differences. The results are in agreement with the previous study by Lin et al. (2015) [15]. The minor differences between the study by Lin et al. and our study arise from the difference in temperature. Our study showed that BCF could hold water at least 2 times its own weight at 37°C. The WHC value of MBCS was significantly lower compared to BCF, because the macroporous structure of MBCS caused a decrease in cellulose fiber per surface area. Despite the significant decrease in the WHC of MBCS, the WHC values of MBCS samples provided sufficient nutrient intake for NIH-3T3 cell viability and the result was in harmony with the MTS assay results.

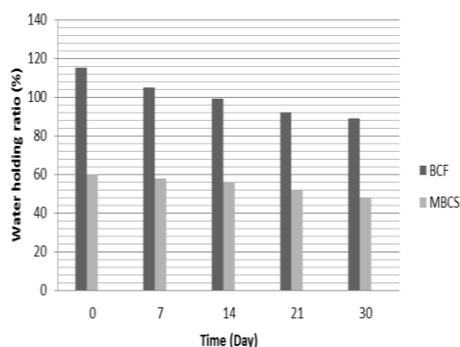


Fig. 1. The average water holding ratio of BCF and MBCS after four weeks' storage at 37°C.

3.2. *In vitro* degradation

Degradation is one of the most important parameters and plays a vital role in new tissue formation. The degradation rate could affect cell viability, cell growth, and migration and even the immune response. For this reason *in vitro* biodegradation of BC is important for tissue engineering applications. Fig. 2 shows the percentage of degradation of BCF and MBCS after four weeks' storage at 37°C.

According to the 30-day *in vitro* degradation period, the weight loss in MBCS is more than the weight loss in BCF. This result indicated that the increased surface area and macroporous structure of MBCS can contribute to the degradation process positively. However, the increased degradation rate of MBCS is still not ideal for tissue engineering applications.

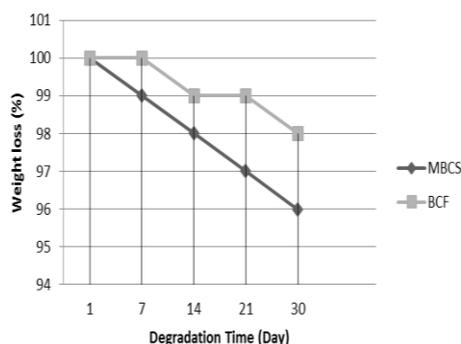


Fig. 2. The percentage of degradation of BCF and MBCS over four weeks at 37°C.

3.3. Porosity, pore size, and surface area evaluation

A potential 3D scaffold morphology should have appropriate pore size and high porosity to support colonization of cells [10].

For cell adhesion in a tissue engineered scaffold, not only does porosity make sense, but also pore size is extremely important for cell viability. Therefore, the parameters should be evaluated together. Table 1 indicates the difference between BCF and MBCS samples in terms of porosity, pore size, and surface area.

The formation of BCF nanopores is random. For this reason, many studies have shown different ranges of pore size diameters between 10 nm and 100 nm [18]. In the present study pore sizes of BCF of between 40 nm and 130 nm were recorded. This is approximately in agreement with the study by Laçin that used the same bacterial species [19]. However, the nanometric pore size limitedly supports the cell migration and colonization into the BCF, resulting in decreased O.D. values in the MTS assay.

The pore size of 0.25% MBCS varies from about 100 to 700 μm and the average pore size is about 500 μm . The pore formation of MBCS via PEG administered freeze-drying is random and for this reason some pore sizes are not ideal for cell colonization. However, the present study showed that the pore size distribution was sufficient for the viability of NIH-3T3 cells. High porosity and large surface area are regarded as a great advantage for the cell adhesion and rapid nutrient exchange required for cell survival [12]. If porosity, surface area, and pore size are evaluated together, MTS assay results can be better understood. The percentage of porosity was not significantly different between BCF and MBCS samples, but the significantly higher pore size and surface area facilitated the survival of NIH-3T3 cells. This argument was supported by the results of previous studies [9, 12].

Table 1. The results of porosity, pore size, and surface area for BCF and MBCS samples.

	BCF	MBCS (0.25%)
Average pore size	98.12 \pm 42.18 nm	530.725 \pm 142.257 μm *
Porosity (%)	92.54 \pm 0.98	93.12 \pm 1.45
Surface area (m^2/g)	84.72 \pm 1.56	94.33 \pm 1.08*

* $p < 0.05$ according to the same value of BCF.

3.4. Scaffold and cell observation by FESEM

The morphology scaffolds and were observed using a FESEM. The measurements of pore size showed that the average pore size of MBCS was markedly different from that of BCF.

The cell morphology of NIH-3T3 cells on BCF and MBCS as observed by FESEM is shown in Fig. 3.c and 3.d, respectively. The images indicated that the NIH-3T3 cells attached to both BCF and MBCS samples. The adherent cells had round morphology and they did not reach their characteristic shape after 2-day culturing. Moreover, filopodia of fibroblast cells were clearly observed on MBCS; this indicated that the cells can be colonized around this microsphere.

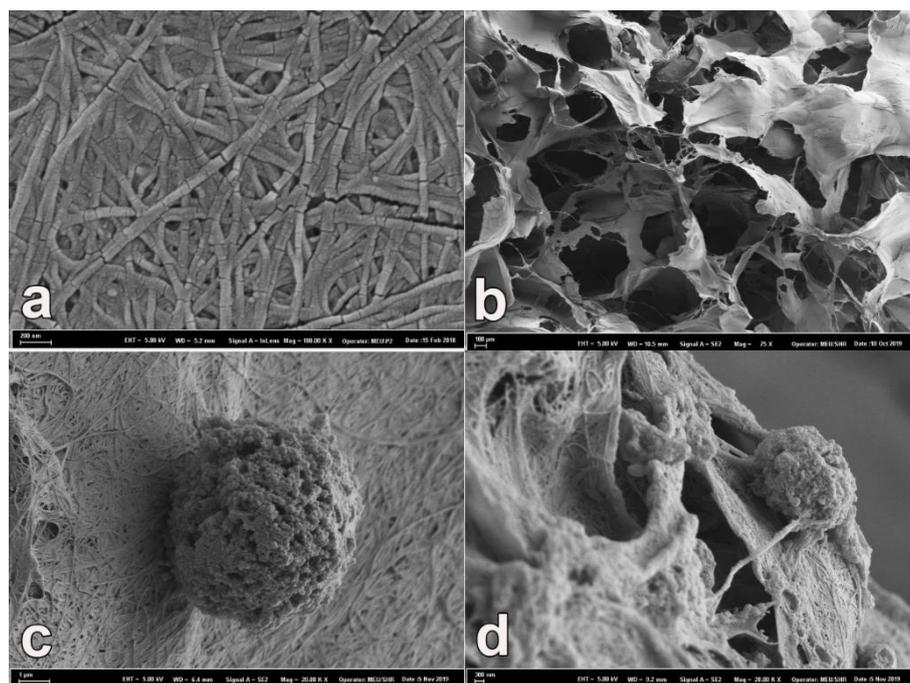


Fig. 3. a. FE-SEM microphotographs of BCF cultured for ten days. b. FE-SEM images of PEG-400 administered and freeze-dried 0.25% MBCS. c. FE-SEM observation of an adherent NIH-3T3 cell on the BCF. d. Photograph of an adherent NIH-3T3 cell on the MBCS.

3.5. Cell viability assay: MTS

This study investigated whether a PEG 400 administered MBCS scaffold was biocompatible with a mouse embryonic fibroblast cell line (NIH-3T3) for the first time. For this reason, the cell viability of NIH-3T3 was tested by MTS assay. The results of the MTS assay are shown in Fig. 4. The results of the MTS assay indicated that BCF supports the viability of NIH-3T3 cells but the number of viable cells is considerably lower than that of MBCS. The results of the unmodified BCF were supported by the previous studies.

According to a previous study by Nwe et al., the number of NIH-3T3 cells on unmodified bacterial cellulose membrane increased approximately twofold after 5 days of cultivation [8]

Similar results have been reported by Volova et al.; NIH-3T3 cells proliferate on BCF after 3 days of cultivation, but the number of cells on BCF was not significantly different than those after polystyrene culture plate cultivation [20].

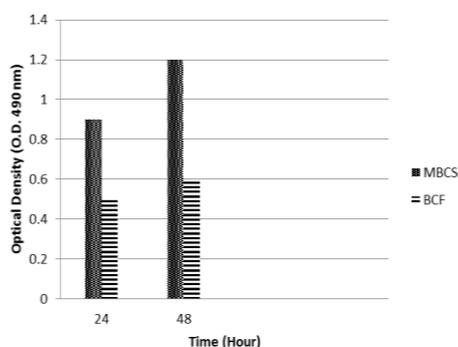


Fig. 4. The results of the MTS assay of NIH-3T3 cells after 24 and 48 hours' incubation on BCF and MBCS.

4. Conclusion

Freeze-drying is a simple and easily applicable method to prepare MBCS. PEG 400 administration to BC enhanced its biocompatibility and our findings indicated that the water holding and porosity properties of MBCS are adequate for the viability of the NIH-3T3 murine fibroblast cell line. Even though the *in vitro* degradation ratio of MBCS was higher compared with that of BCF, this enhancement is not sufficient for a tissue engineering scaffold.

Cell culture studies also showed that NIH-3T3 murine fibroblast cells attached and proliferated on 0.25% MBCS after 2 days of culturing. Moreover, FESEM observations indicated that NIH-3T3 cells can be spread and colonized after 2 days of incubation. Overall, 0.25% MBCS can be a good candidate for 3-D tissue engineering scaffolds in terms of water retention capacity, porosity, and cell support ability.

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