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Short communication

Creation of macropores in three-dimensional bacterial cellulose scaffold for potential cancer cell culture

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ABSTRACT

There is an increasing need for an effective *in vitro* model that can resemble the 3-D nature of tumor microenvironments. In this work, a 3-D bacterial cellulose (BC) scaffold with macropores was fabricated by a facile freeze drying method for potential culture of cancer cells. This *in vitro* study reported, for the first time, the role of macropores in the adjustment of cancer cell behavior when compared with previous results cultured in BC scaffolds without macropores. The scaffold was characterized by SEM and mercury intrusion porosimeter. A human breast cancer cell line (MDA-MB-231) cultured in the macroporous BC scaffold was examined *via* cell proliferation, histological and SEM analyses. The results demonstrated that the macroporous scaffold provided a good environment for cell viability, adhesion, proliferation, and infiltration. These findings suggested that the macroporous BC scaffold might have great potential for use in the *in vitro* culture of cancer cells.

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1. Introduction

The standard *in vitro* approaches for cancer research and evaluation of anticancer drugs primarily involve the culture of tumor cells in Petri dishes. However, it is clear now that these two-dimensional (2-D) culture systems are incapable of accurately reflecting the microenvironment of tumors *in vivo*. Recently, three-dimensional (3-D) culture, as compared to 2-D culture, has been suggested as a much better *in vitro* cancer model to study the complex biological processes (Hutmacher, 2010; Hutmacher et al., 2010; Prewitz, Seib, Pompe, & Werner, 2012; Verbridge, Chandler, & Fischbach, 2010; Wang et al., 2014). 3-D scaffolds are preferable in tissue engineering. Similarly, 3-D *in vitro* model systems that are able to mimic the *in vivo* microenvironment are highly sought after in tumor engineering (defined as the construction of complex culture models that recapitulate aspects of the *in vivo* tumor microenvironment to study the dynamics of tumor development, progression, and therapy on multiple scales (Hutmacher et al., 2010). Numerous 3-D tumor engineering scaffolds made from collagen (Chen et al., 2012; Liu et al., 2014; Szot, Buchanan, Freeman, & Rylander, 2011a), chitosan-alginate (Kievit et al., 2010), poly(lactic acid) (PLA) (Sahoo, Panda, & Labhasetwar, 2005), poly(lactic-*co*-glycolide) (PLGA) (Sahoo et al., 2005), hyaluronan (Rhodes, Srivastava, Smith, & Longinotti, 2004), and silk fibroin protein (Talukdar et al., 2010) have been reported. Compared with aforementioned biomaterials, bacterial cellulose (BC), a natural nanofibrous polymer, shows some distinct properties. BC is synthesized extracellularly by the bacterium *Acetobacter xylinum*. In addition to such appealing properties as ultrahigh mechanical strength and modulus, high water holding capacity and porosity, and good biocompatibility (Pertile et al., 2012; Shi et al., 2012), BC displays intrinsic 3D network structure, and, in particular, BC fibers are at the nanometer scale which is the low limit of natural ECM fibers. Therefore, BC has been believed to be a promising material for tissue engineering scaffolds (Klemm et al., 2011; Petersen & Gatenholm, 2011). As suggested by a previous study (Hutmacher et al., 2010), the strategies developed originally for tissue engineering could be used in tumor engineering to enhance cancer research. It is thus reasonable to speculate that BC might be a promising material for tumor engineering scaffolds. However, investigation on BC scaffolds for tumor engineering has been very limited while extensive research has been carried out to develop BC scaffolds for tissue engineering. The only pioneering study on the *in vitro* culture of cancer cells on BC scaffolds demonstrated that these cancer cells cultured on BC did not have observable protrusions indicating undesirable cancer cell responses due to the absence of manufactured large porosity (Szot, Buchanan, Gatenholm, Rylander, & Freeman, 2011b). Furthermore, it has been accepted that pore structure is an

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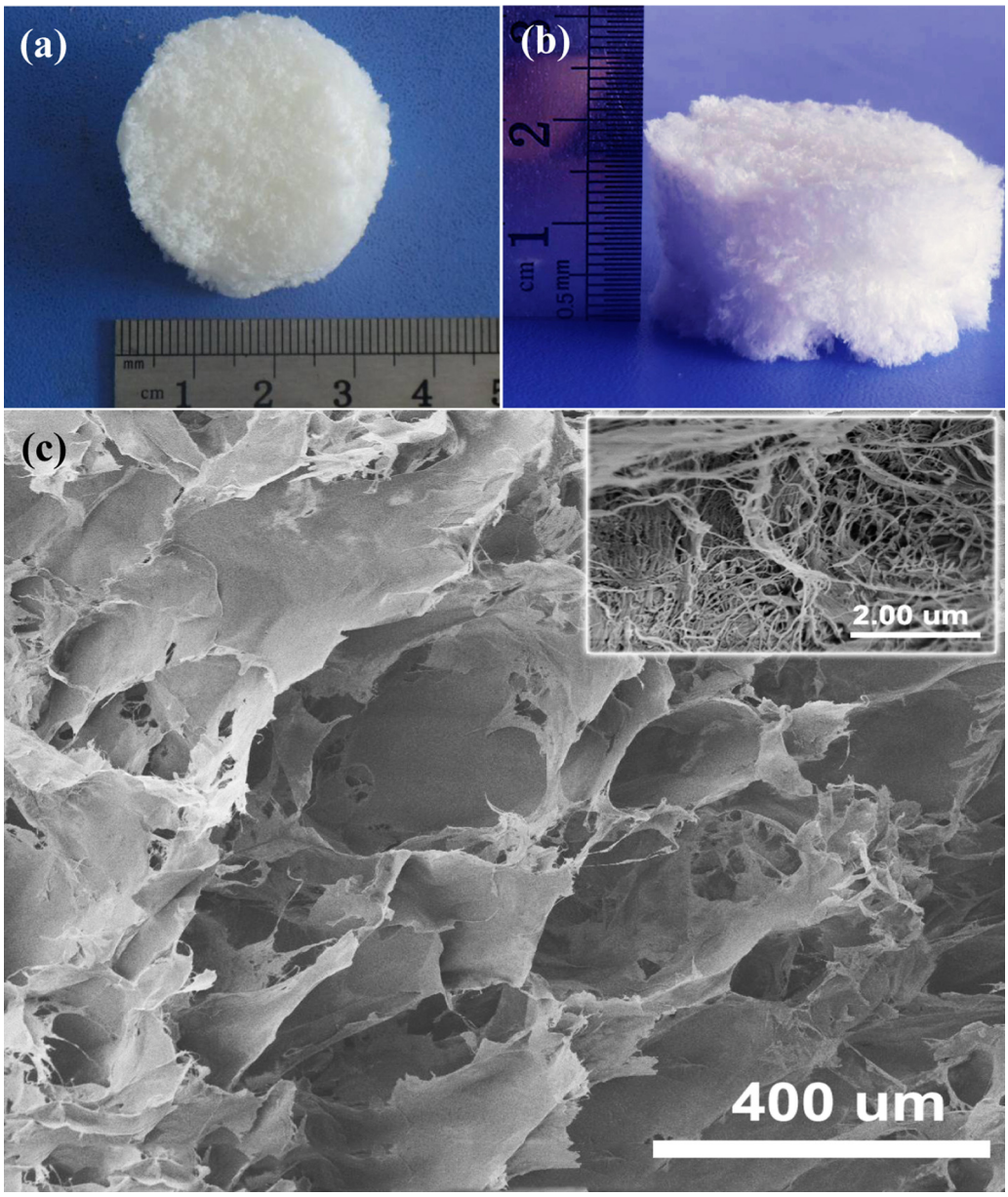

Fig. 1. Photos ((a) and (b)) and SEM image (c) of 3-D macroporous BC scaffold (inset presents a typical image of pore wall).

essential consideration in the development of scaffolds for tissue engineering and pores must be large enough to allow cell growth, migration and nutrient flow, vascularization, and new tissue formation and remodeling so as to facilitate host tissue integration upon implantation. Our previous work indicated that synovium-derived mesenchymal stem cells (MSCs) could proliferate deep (150 μm) into a BC scaffold with macropores (Gao et al., 2011). However, how the macropores affect cancer cell behavior has not been reported.

The present study, for the first time, examined whether a BC scaffold with macropores (defined as a pore diameter larger than 100 μm (Le Huec, Schaeverbeke, Clement, Faber, & Le Rebeller, 1995; Taboas, Maddox, Krebsbach, & Hollister, 2003) could be a good *in vitro* cancer model. These findings could then support the notion that alteration of scaffold pore structure might be able to modify the behavior of cancer cells and thus offer an effective strategy for the design and fabrication of good *in vitro* models for the study of cancer biology and the development of cancer therapeutics.

To this end, a novel BC scaffold with macropores was fabricated by a facile freeze drying process. A human breast cancer cell line (MDA-MB-231) was cultured in the macroporous BC scaffold and cell viability, adhesion, proliferation and infiltration were evaluated.

2. Materials and methods

2.1. Preparation of 3-D macroporous BC scaffold

BC pellicles were prepared according to the procedures described previously (Hong et al., 2006). The freeze–drying technique (Gao et al., 2011) with minor modifications was used to prepare the 3-D BC scaffold with macropores in this work. Briefly, the BC pellicles were crushed and mixed with deionized water using a home-made blender at 4000 rpm for 5 min to prepare an optimized 0.25 wt% BC emulsion. The BC emulsion was then poured into appropriate molds and freeze-dried at −50 °C for 1 day and the scaffold with a dimension of Φ10 × 1 mm was obtained.

The resultant macroporous BC scaffold was used in further analysis.

2.2. Scaffold characterization

The morphology of the 3-D macroporous BC scaffold was observed using a Nano 430 field emission scanning electron microscope (FESEM), FEI, USA. For FESEM observations, samples were sputter-coated with gold and were observed at an accelerating voltage of 10 kV. The pore size distribution, porosity and surface area of the scaffold were determined by a mercury intrusion porosimeter (PoreMaster 60 GT, Quantachrome Instruments).

2.3. MTT assay

The cell proliferation was evaluated by the colorimetric MTT assay. First, the human breast cancer cell line (MDA-MB-231), obtained from Tianjin Medical University Cancer Institute and Hospital, Tianjin, China, was maintained in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 incubator. Monolayer MDA-MB-231 cells were harvested by trypsin/EDTA treatment. Before cell seeding, a circular BC scaffold ($\Phi 10 \times 1$ mm) was sterilized with UV radiation. After sterilization, the scaffold was pre-soaked in DMEM for at least 12 h. Then, the sterilized macroporous scaffold was placed into 24-well culture plates and seeded with a cell suspension with a cell density of 2×10^5 cells/mL, followed by culturing for 1, 3, 5, 7 days at 37 °C in a 5% CO_2 incubator. After incubation, the cell-scaffold constructs were rinsed with PBS, followed by incubation in the culture medium containing 50 μL MTT reagent for 4 h. After removal of the medium, 500 μL of DMSO was added to the wells. The solution (150 μL) from each sample was transferred to 96-well plates and the optical density (O.D.) was measured at an absorbance of 490 nm.

2.4. Cell imaging

After 7 days culture, the cell-scaffold samples were fixed using 4% glutaraldehyde for 12 h, dehydrated through a series of graded alcohols, air-dried, sputter-coated with gold and then observed using the aforementioned SEM.

2.5. Histological analysis

After 3 and 7 days culture, the cell-scaffold constructs were washed with ice-cold normal saline (0.9% NaCl), cut transversely into thin slices (5 μm), and then fixed into 10% neutral-buffered formaldehyde for 24 h. The samples were then transferred into 70% ethanol, processed, paraffin embedded and H&E stained using standard protocols. The cell distribution reported in this paper was from the thin slice cut at a distance of 300 μm from the scaffold surface.

2.6. Statistical analysis

All experiments were performed in triplicate unless otherwise stated. Statistical analysis of data was performed using an SPSS system. All data were presented as mean value ± standard deviation (SD). Results with p-values of <0.05 were considered statistically significant.

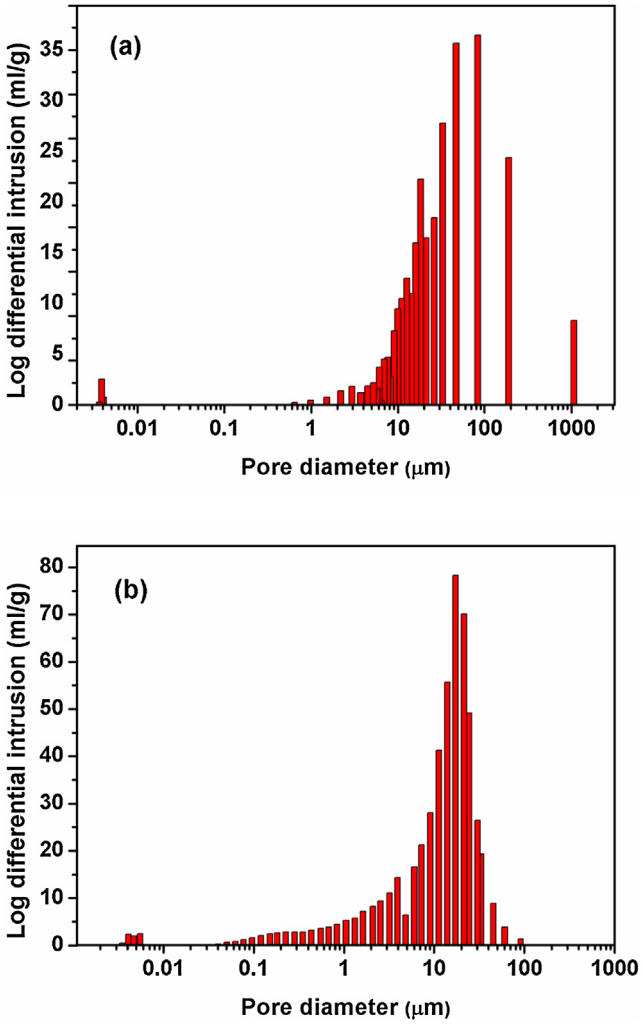

Fig. 2. Pore size distribution of 3-D macroporous (a) and pristine (b) BC scaffolds.

3. Results

3.1. Morphology and pore structure of BC scaffold with macropores

Fig. 1 shows the photographs and SEM images of the 3-D macroporous BC scaffold. Fig. 1a and b shows the size of the scaffold and its three dimensionality. As shown in Fig. 1c, pores were observed throughout the scaffold. Note that the walls of these pores were also porous, consisting of nanofibers and nanopores (see inset in Fig. 1c).

The pore size distribution curves (Fig. 2) indicated that the 3-D nanofibrous BC scaffold fabricated in this work contained macropores (>100 μm), micropores (<100 μm as defined in (Le Huec et al., 1995; Taboas et al., 2003), and nanopores (<100 nm). Thus, this BC scaffold was called macroporous. A comparison of Fig. 2a and b demonstrated that both the macroporous scaffold and the pristine BC scaffold that was prepared in accordance to literature (Szot et al., 2011b) showed hierarchical pore structure but the pore size distribution was significantly different, the former containing macropores, micropores and nanopores but the latter having only micropores and nanopores. In addition, the measured porosity and specific surface area of the macroporous scaffold (90% and 90 m^2/g, respectively) were comparable to the pristine BC scaffold (92% and 91 m^2/g).

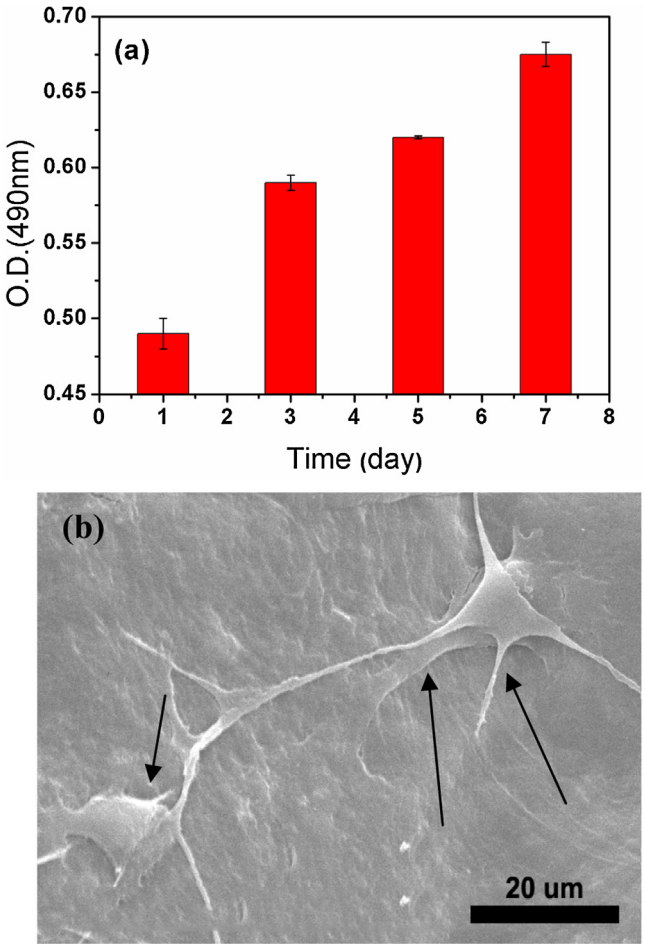

Fig. 3. MTT result showing increase in the number of MDA-MB-231 cells with culture time (a) and SEM image of MDA-MB-231 cells on 3-D macroporous BC scaffold (b).

3.2. Cell studies

The MTT result shown in Fig. 3a demonstrated that the O.D. values obtained at 1, 3, 5, 7 days were significantly different ($p < 0.01$ in all cases), indicating that the cells were viable and proliferated well during 7 days culture. Fig. 3b shows a representative SEM image of the MDA-MB-231 cells after 7 days seeding on the 3-D macroporous BC scaffold. Note that the MDA-MB-231 cells exhibited good attachment to the macroporous BC scaffold. Furthermore, the growing MDA-MB-231 cells on the BC scaffold demonstrated normal cell shape with protruded pseudopodiums covering the scaffold surface (arrows in Fig. 3b), suggesting that the macroporous BC scaffold supported the attachment and spreading of the MDA-MB-231 cell line.

The cell behavior was further evaluated by histological analysis (Fig. 4). As clearly shown in Fig. 4A, several cell clusters were observed within the macroporous BC scaffold suggesting the infiltration of cancer cells into the scaffold. Furthermore, it was inferred that cells penetrated into the macroporous BC scaffold for at least 300 μm since the thin slice was cut at a distance of 300 μm from the scaffold surface. Fig. 4B further demonstrates that cells penetrated into a depth of 300 μm and showed successive cell growth and proliferation there.

4. Discussion

It has been accepted that pore structure is an essential consideration in the development of scaffolds for tissue engineering. The pores must be large enough to allow for cell growth, migration and nutrient flow. If the pores are too small, cell migration, the diffusion of nutrients and the removal of waste are limited. Our previous study demonstrated that BC scaffolds with macropores allowed the fibrous synovium-derived mesenchymal stem cells (MSCs) to proliferate and penetrate into the scaffolds (Gao et al., 2011), whereas the pristine BC scaffolds did not allow satisfactory proliferation and penetration. Similarly, the pore structure characterization of the BC scaffold prepared in this work revealed the presence of macropores which were absent in the pristine BC scaffold. More importantly, this work, using the MDA-MB-231 cell line, revealed the novel finding that the macroporous BC scaffold promoted cancer cells adhesion, growth, proliferation, and particularly penetration into the macroporous scaffold for at least 300 μm. These findings were in contrast to cell behavior using the pristine BC scaffold reported previously (Szot et al., 2011b) and suggested that the difference was due to the presence of macropores in the macroporous BC scaffold. These findings implied that a BC scaffold with an appropriate

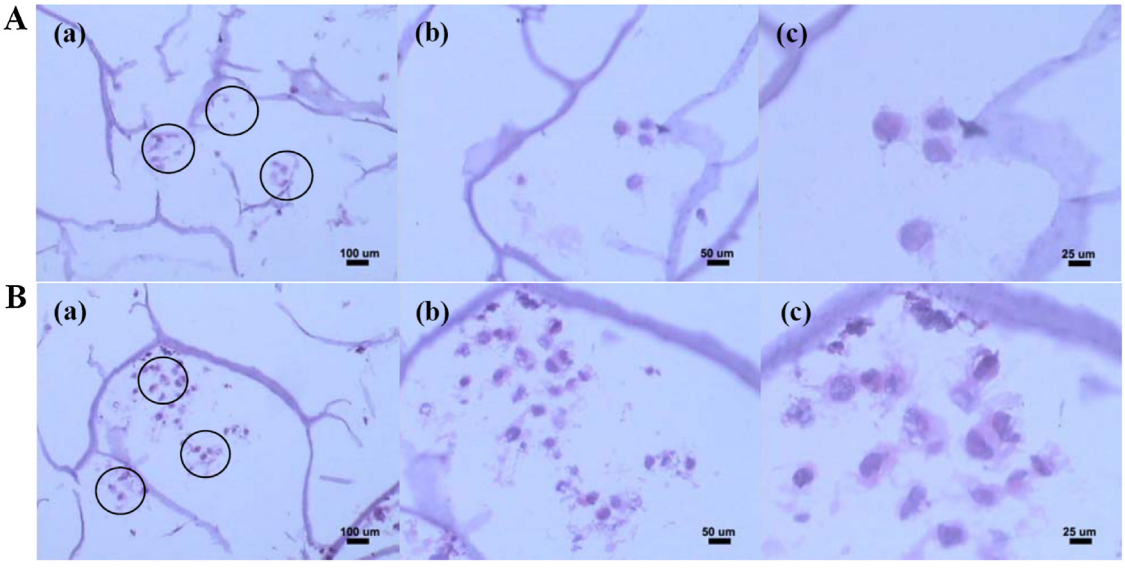

Fig. 4. Histological evaluation of MDA-MB-231 cell line after 3 (A) and (B) 7 days culture in 3-D macroporous BC scaffold.

pore structure might be good culture media for cancer cells and confirmed the importance of pore structure in the scaffolds used in tumor engineering. Therefore, the present study might mark a significant development toward construction of the ideal scaffold for *in vitro* cancer study and evaluation of anticancer drug efficacy. Further work regarding other physical properties of scaffolds (such as pore shape, porosity, fiber diameter and orientation) and more in-depth cell studies are underway.

5. Conclusions

A 3-D nanofibrous BC scaffold with macropores (larger than 100 μm in diameter) was produced by the facile emulsion freeze-drying technique. The MDA-MB-231 cell line cultured in the macroporous BC scaffold demonstrated favorable proliferation and adhesion. Furthermore, histological analysis showed clearly that the cancer cells penetrated into the scaffold. These findings suggested that engineered 3-D macroporous BC scaffold might be well suited as a cancer cell culture model for cancer biology studies and drug screening applications *in vitro*.

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