Succinylated Bacterial Cellulose Induce Carbonated Hydroxyapatite Deposition in a Solution Mimicking Body Fluid

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Abstract: *Incorporation of bone-like hydroxyapatite into bacterial cellulose (BC) is an attractive approach for the fabrication of a bioactive three-dimensional (3D) scaffold for bone tissue regeneration. This study investigates the influence of the succinylation of BC on its ability to incorporate bone-like hydroxyapatite. A biomimetic process using a 1.5 × Simulated Body Fluid (SBF) was used to deposit the hydroxyapatite into the succinylated-BC. After soaking the succinylated-BC in the 1.5 × SBF for six days, Scanning Electron Microscope (SEM) images were taken and the composition of the succinylated-BC was analyzed by energy dispersive X-ray spectrometry. The biocompatibility of the scaffolds was tested* in vitro *using rat Bone Marrow Stromal Cells (rBMSCs). The SEM images and Fourier Transform Infrared Spectroscopy (FTIR) spectra showed that carbonated hydroxyapatite was deposited on the succinylated-BC. In contrast, only a small amount of carbonated hydroxyapatite deposition was observed on unmodified BC, indicating that the succinyl group in the BC is effective for inducing hydroxyapatite deposition.* In vitro *studies using rBMSCs revealed the biocompatibility of the scaffold. Combining with the ability of the cells to differentiate into bone cells, the succinylated-BC scaffold is a promising 3D scaffold for bone tissue regeneration.*

Keywords: *bacterial cellulose; carbonated hydroxyapatite; 1.5 × simulated body fluid; succinylation; 3D scaffolds*

■ INTRODUCTION

Bone damage is usually repaired using implants, such as allografts or xenografts [1-5]. However, the amount of those grafts available for transplantation may be limited by donor shortage and a possibility of disease transfer [1,3,5]. To overcome these problems, polymeric three-dimensional (3D) scaffolds have gained interest in the reconstruction of damaged bone because of their abundant availability and ease of modification of their chemical and physical properties [3,5].

Bacterial cellulose (BC) has shown promise as a 3D scaffold for bone regeneration because of its high mechanical properties and excellent biocompatibility [6-11]. It has been reported that microporous BC supports MC3T3-E1 osteoprogenitor cells to form cell clusters and deposit mineral on the BC, indicating the potential of BC as a 3D scaffold for bone tissue regeneration [6]. However, BC shows poor bioactivity, rendering it difficult to make bonding with living bone. To design bioactive 3D scaffolds that have a high biological affinity to living bone, it is important to incorporate bone-like hydroxyapatite onto the 3D scaffold [2,4-5]. Bone-like hydroxyapatite can be incorporated into 3D scaffolds by the biomimetic process using a Simulated Body Fluid (SBF) solution that has similar pH and ion concentration with human blood plasma [2,4-5,12]. Some studies have reported the incorporation of bone-like hydroxyapatite onto BC by a biomimetic process [13-14]. However, there is little hydroxyapatite found on the unmodified BC surface after the biomimetic process,

because its hydroxy group is not reactive enough to induce hydroxyapatite deposition [13-14]. Therefore, modification of BC with a functional group that can accelerate hydroxyapatite deposition may be necessary to achieve its bioactivity.

Previous studies reported that 3D scaffolds that contain functional groups such as carboxy and silanol groups could accelerate the deposition of calcium hydroxyapatite into scaffolds [15-16]. In this study, we modified BC with the succinyl group through a simple reaction with succinic anhydride. Succinylation may enhance the bioactivity of BC to deposit bone-like hydroxyapatite. The influence of succinylation of BC on its ability to incorporate bone-like apatite was then investigated.

■ EXPERIMENTAL SECTION

Materials

Ammonium sulfate, sucrose, glacial acetic acid and *N,N*-dimethylformamide were purchased from Merck GmbH, Germany. *N,N*-Diisopropylethylamine (DIPEA) was purchased from Applied Biosystems (Carlsbad, CA, USA). Succinic anhydride was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and recrystallized from 2-propanol prior to use. Other reagents were purchased from Wako Pure Chemical Industries Ltd.

Procedure

Preparation of bacterial cellulose (BC)

BC was prepared using coconut water as a fermentation medium [9-10]. Briefly, the coconut water medium containing 5% (w/v) sucrose and 0.5% (w/v) ammonium sulfate was boiled, and the pH of the medium was adjusted to pH 4 using glacial acetic acid. The medium was equilibrated to room temperature before use. Ten percent of pre-culture medium containing *Acetobacter xylinum* was then added into the medium. The mixture was incubated at room temperature for 2–3 days. The obtained bacterial cellulose was washed with demineralized water and immersed in 0.1 M sodium hydroxide at 60 °C for 4 h to remove any residual bacteria. Finally, the pellicle was rinsed with demineralized water at room temperature to achieve a neutral pH and freeze-dried to obtain dried BC.

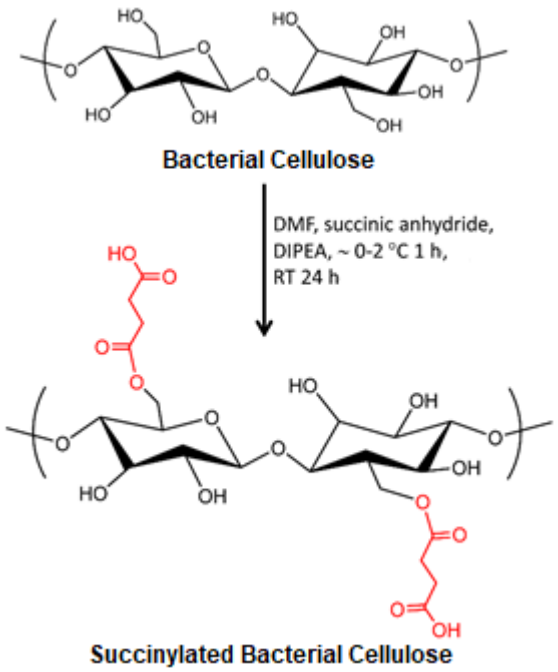

Fig 1. Reaction scheme of bacterial cellulose succinylation

Succinylation of BC

Succinylated-BC was synthesized by reacting the BC with a 10-fold molar excess of succinic anhydride and DIPEA relative to the hydroxy groups of the BC in DMF on ice for 2 h and then for 24 h at room temperature (Fig. 1).

The succinylated-BC was then washed with demineralized water extensively to remove the DMF and unreacted substances, and freeze-dried. Succinylation of the BC was confirmed using a Spectrum One FTIR spectrometer (PerkinElmer, Wellesley, MA, USA) in the range of 400–4000 cm^{-1} based on the KBr method with 16 scans and a resolution of 1 cm^{-1}.

Biomimetic soaking of BC and succinylated-BC

To induce bone-like apatite deposition onto the BC, we soaked the BC and succinylated-BC in 1.5 × SBF solution. The SBF solution was prepared as described previously [16]. BC and succinylated-BC were soaked in 0.2 M $CaCl_2$ for 24 h and followed by soaking in 1.5 × SBF solution for six days at 37 °C. The SBF solution was changed with fresh 1.5 × SBF solution every two or three

days. After six days soaking, the BC and succinylated-BC were washed with demineralized water three times and dried at room temperature.

The BC and succinylated-BC were coated with gold and their microstructure and biological hydroxyapatite formation were analyzed using a scanning electron microscope (SEM; Model S-4800, Hitachi, Tokyo, Japan), and their composition using an energy dispersive X-ray spectrophotometer (EDX; Octane T Ultra W, EDAX Inc.). Furthermore, its functional groups were analyzed using a Spectrum One FTIR spectrometer (PerkinElmer) in the range of 400–4000 cm^{-1} based on the KBr method with 16 scans and a resolution of 1 cm^{-1}.

Biocompatibility of BC and succinylated-BC

Rat bone marrow stromal cells (rBMSCs) were obtained as described previously [17]. The rBMSCs were suspended in α-minimum essential medium (α-MEM; Gibco Invitrogen Corp. Grand Island, NY, USA) containing 20% fetal calf serum (FCS; HyClone, Logan, UT, USA) and cultured in an 80 cm^2 tissue culture flask (153732; Nalge Nunc International) at 37 °C under 5% CO_2 atmosphere. After three days, the attached cells were washed with PBS, treated with 0.02% ethylenediaminetetraacetic acid and 0.25% trypsin, and centrifuged at 1200 rpm for 5 min. After centrifugation, the cells were then re-suspended in 20% FCS/α-MEM at a density of 5×10^6 cells/mL.

The rBMSC suspension was seeded on the BC scaffolds in a 24-well plate (142475; Nalge Nunc International) at a density of 5×10^4 cells/scaffold and incubated at 37 °C under 5% CO_2. The same number of rBMSCs without any samples was cultured in another well of the 24-well plate as a control. Every two or three days, half of the medium was replaced with fresh medium.

Cell viability was tested using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer instruction. Optical density (OD) at 450 nm of samples, control, and the medium was then measured using a SpectraFluor Plus microplate reader (Tecan, *Männedorf,* Switzerland).

$$\text{Cell viability}(\%) = \frac{(OD_{450}\,\text{Sample} - OD_{450}\,\text{Medium})}{OD_{450}\,\text{Control} - OD_{450}\,\text{Medium}} \times 100\%$$

Statistical analysis

All statistical evaluations were performed using the one-way analysis of variance routine of KaleidaGraph 4.5 (Synergy Software, Reading, PA, USA) followed by Tukey's honest significant difference test. A value of $p < 0.05$ was accepted as statistically significant. All data were presented as mean ± standard deviation, with n = 3.

■ RESULTS AND DISCUSSION

Synthesis and Characterization of Bacterial Cellulose (BC) and Succinylated-BC

In this study, BC was reacted with succinic anhydride in the presence of DIPEA as a base catalyst. The catalyst facilitates the deprotonation of BC hydroxy groups and enables them to react with succinic anhydride to form succinylated-BC. Fig. 2 shows the FTIR spectra of unmodified BC and succinylated-BC.

Peaks at 3432 and 2900 cm^{-1} in the spectra correspond to O–H stretching and C–H stretching of CH_2 in the BC structure, respectively. A new peak at 1735 cm^{-1} observed in the FTIR spectrum of succinylated-BC was assigned to the carbonyl (–C=O) ester stretching of the succinyl group. These results suggest that the succinyl groups were successfully conjugated to the BC. No peak that could be attributed to unreacted succinic anhydride was detected in the FTIR spectrum of succinylated-BC, indicating the high purity of the obtained succinylated-BC.

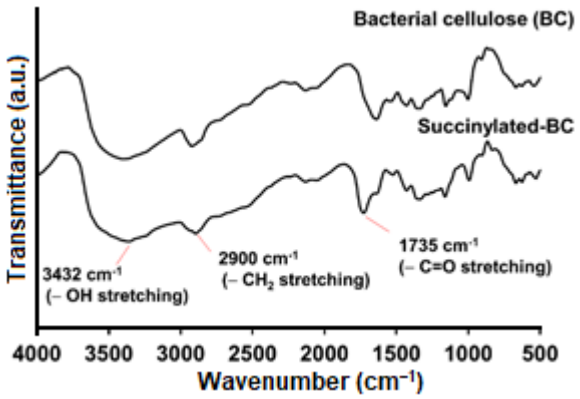

Fig 2. FTIR spectra of bacterial cellulose (BC) and succinylated-BC. FTIR measurement was conducted using the KBr method with 1 cm^{-1} resolution and 16 scans at room temperature

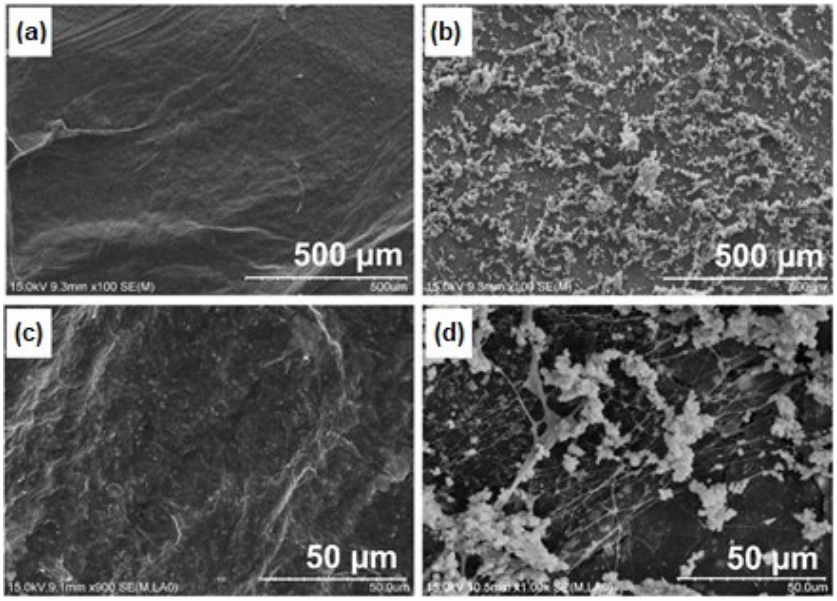

Fig 3. SEM images of bacterial cellulose (a and c) and succinylated bacterial cellulose (b and d) after 6 days immersion in 1.5 × SBF solution

Bioactivity of BC and Succinylated-BC

Bioactivity such as the capability to form direct bonds with natural bones is an important property for 3D scaffolds as bone repairing materials. In this study, an ability to form bone-like hydroxyapatite on surfaces was evaluated by soaking BC and succinylated-BC in 1.5 × SBF for 6 days. Before the soaking treatment, the BC and succinylated-BC were soaked in 0.2 M $CaCl_2$ solution for 24 h. Increasing the Ca^{2+} content in the scaffolds is effective to induce heterogeneous and homogeneous hydroxyapatite nucleation [16].

SEM images of BC and succinylated-BC after six days of soaking in 1.5 × SBF are shown in Fig. 3. The SEM Images show the appearance of particle deposition on the surface of succinylated-BC (Fig. 3(b) and (d)). The morphology of the particles is similar to that of bone-like apatite [16]. Further analysis by EDX confirmed the presence of calcium and phosphorus in the particles, indicating the formation of hydroxyapatite on the BC and succinylated-BC surfaces (Fig. 4(a) and (b)). High intensities of calcium and phosphorus peaks are found on the EDX spectrum of succinylated-BC. In contrast, only small intensities of calcium and phosphorus peaks are observed in the EDX spectrum of unmodified BC. These results indicate that succinyl groups in succinylated-BC could accelerate hydroxyapatite formation on the BC surface.

The ability of the succinylated-BC to induce hydroxyapatite deposition may be caused by its succinyl group. The carboxy moiety (–COOH) of the succinyl groups in the succinylated-BC are negatively charged at physiological environment (pH = 7.4), allowing it to make an ionic interaction with calcium ion (Ca^{2+}) in solution to form $–COOCa^{+}$ and $(–COO)_2Ca$ complexes that induce hydroxyapatite nucleation [16].

It has been reported that polyamide films containing carboxy groups can deposit bone-like hydroxyapatite on their surfaces after soaking in 1.5 × SBF solution when they contain calcium chloride [16]. Hydroxyapatite deposition was induced by an increase of Ca^{2+} concentration on the surfaces of succinylated-BC which may increase the degree of supersaturation of the solution [16]. Further evidence of hydroxyapatite deposition on BC and succinylated-BC was confirmed by FTIR analysis. FTIR spectra of BC and succinylated-BC after soaking in 1.5 × SBF solution for six days were shown in Fig. 5.

Peaks at 564 and 605 cm^{-1} in the FTIR spectrum of succinylated-BC were ascribed to PO_4^{3-} bending vibration (O–P–O bond) [18-19]. Peaks at 1035 and 1058 cm^{-1} were

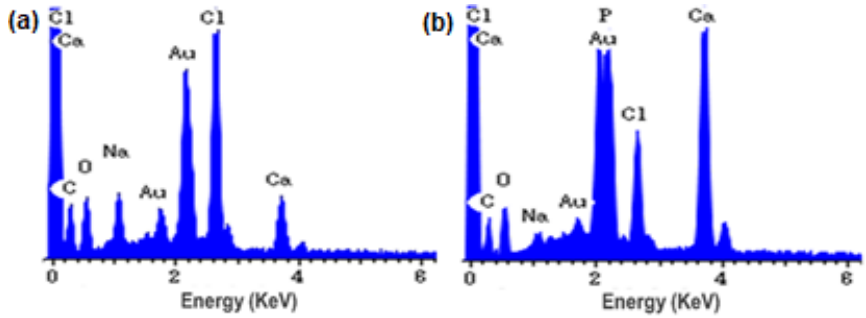

Fig 4. EDX spectra of bacterial cellulose (a) and succinylated bacterial cellulose (b) after 6-day immersion in 1.5 × SBF solution

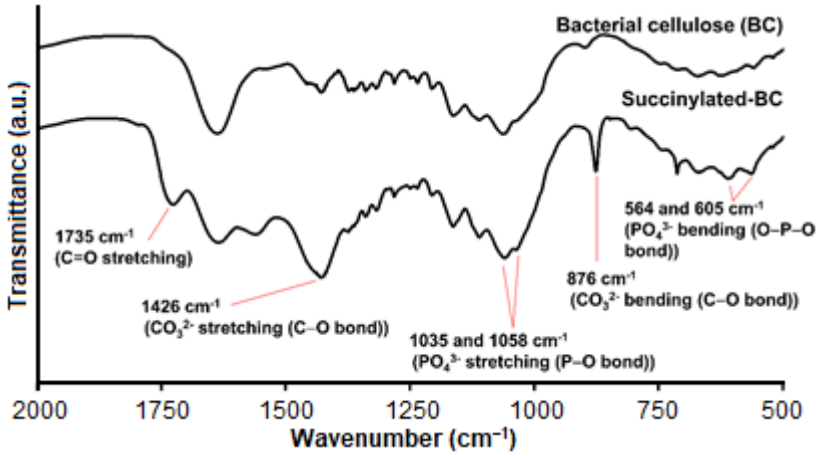

Fig 5. FTIR spectra of bacterial cellulose (BC) and succinylated-BC after 6-day immersion in 1.5 × SBF solution

assigned to PO_4^{3-} stretching vibration (P–O bond). These results show the presence of phosphate groups in the deposited particles. In addition, peaks correlated with carbonate groups were also observed. Strong peaks at 876 and 1425 cm^{-1} are ascribed as CO_3^{2-} bending and stretching vibration (C–O bond), respectively. These peaks are known to be specific for carbonated hydroxyapatite [18].

The results described above show that the deposited particles are carbonated hydroxyapatite. The carbonated hydroxyapatite has low crystallinity, quite similar to those of hydroxyapatite in living bone [16,18]. The formation of the carbonated hydroxyapatite on the succinylated-BC after soaking in 1.5 × SBF solution may enhance the ability of the scaffold to make a direct and strong bonding between the 3D scaffold and living bone [1,5]. The FTIR spectrum of BC shows only low intensities of carbonate peaks, indicating that the surface of unmodified BC is only slightly active towards hydroxyapatite deposition. These results demonstrated that the succinyl group in the succinylated-BC plays an important role in activating the surface of BC to deposit hydroxyapatite. These results also suggest the potential of succinylated-BC as a 3D scaffold for bone tissue regeneration.

Biocompatibility

Biocompatibility is an important characteristic of 3D scaffolds intended for biomedical applications. Furthermore, the 3D scaffolds must have good biocompatibility with surrounding cells to promote cell proliferation and differentiation [3]. The biocompatibility of the scaffolds was tested *in vitro* using rBMSCs. The WST-8 assay results demonstrated that cells in the BC and succinylated-BC were alive and proliferated, indicating the biocompatibility of BC and succinylated-BC (Fig. 6). These results suggest that incorporation of negatively charged groups into BC does not influence cell viability, further indicating the biocompatibility of

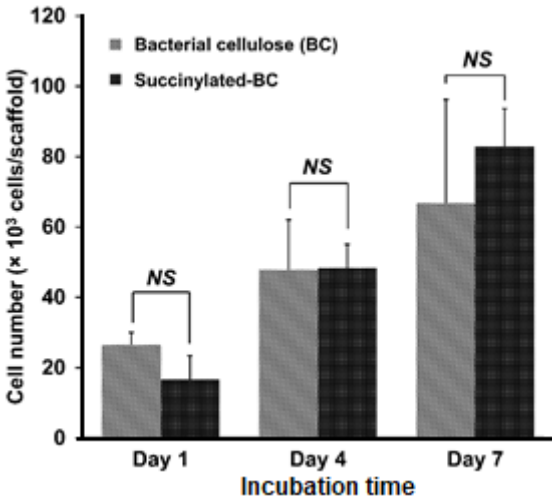

Fig 6. The viability of rat bone marrow stromal cells after seeded on bacterial cellulose (BC) and succinylated-BC and incubated for 7 days. NS = not significant

the succinylated-BC. It has been reported that succinylation of a collagen-like polypeptide similarly did not influence the biocompatibility of the polypeptide for rBMSCs [20-21]. Considering its biocompatibility, bioactivity and the ability of the cells to differentiate into bone cells, the succinylated-BC has potential as a 3D scaffold for reconstructing damaged bone.

■ CONCLUSION

Bacterial cellulose (BC) was successfully modified through simple reaction with succinic anhydride and DIPEA. Succinylation of BC was effective in accelerating bone-like apatite formation on the BC surface after soaking in 0.2 M $CaCl_2$ solution for 1 day and 1.5 × SBF solution for 6 days. These results indicate that modification of an inert 3D scaffold with functional groups containing carboxy groups, such as succinyl group, is effective in inducing bone-like apatite deposition.

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