

## Preparation and characterization of degradable oxidized bacterial cellulose reacted with nitrogen dioxide

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**Abstract** Bacterial cellulose (BC) is regarded as one of the most attractive and promising biomaterials in medical applications owing to its high purity and mechanical properties and excellent biocompatibility. In this study, a new kind of degradable oxidized bacterial cellulose (OBC) was prepared by oxidizing BC in the presence of nitrogen dioxide ( $\text{NO}_2$ ), which could be used as a scaffold for tissue engineering and tissue regeneration. The chemical structures, micromorphology, and in vitro degradability of OBC were characterized. The results demonstrated that the oxidation reaction was controllable for tailoring the degree of degradation of BC. After oxidation, the degradation of BC was accelerated, depending on the oxidation time. In the case of oxidation for 12 days, the mass loss rate of OBC increased sharply, up to 45% after being immersed in phosphate buffered saline (PBS) for 60 days, compared with only 10% for original BC in the same condition. The oxidation did not affect the crystal structure of BC, however, changed the morphology of its network. The original dense microfibril network of BC could be gradually degraded and disappeared within a desirable period of time in vitro through controlling the degree of the selective oxidation.

**Keywords** Bacterial cellulose · Oxidation · Degradation · Nanomaterials

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## Introduction

Bacterial cellulose (BC), synthesized by *Acetobacter xylinum*, is an interesting material for medical applications [1]. Because of its unique high purity and crystallinity, it has remarkable mechanical properties and excellent biocompatibility [2, 3]. One of the most significant features is that BC has a fine fibril three-dimensional (3D) network structure which is similar to natural extracellular matrix (ECM), compared with plant cellulose. Therefore, BC is promising for a wide range of medical applications, such as wound dressings, artificial skin, artificial blood vessels, and scaffolds for tissue-engineered bone [4–7]. However, it is difficult to degrade BC in a controlled manner in vivo which would limit its potential applications in scaffolds for tissue engineering.

Oxidation is an effective way to modify the chemical structure of cellulose so as to control its degradation ability in human body for biomedical applications [8]. The oxidation of cellulose with periodate was reported to obtain 2,3-dialdehyde cellulose which could degrade in vivo [9]. Cellulose was also oxidized using a compound oxidant, such as TEMPO–NaClO–NaBr, by which, the primary alcohol groups at C6 were selectively oxidized into carboxyl during the process [10, 11]. Recently, Li et al. [12] reported that BC could be oxidized by periodate to obtain 2,3-dialdehyde bacterial cellulose (DABC). The degradation rate of DABC in PBS solution was faster than that of BC, and its crystallinity was also lower than the original one. In addition, among the oxidants, nitrogen dioxide was considered as a more suitable oxidant for cellulose [13], because in this condition, secondary reactions of oxidizing secondary hydroxyl groups of the cellulose were avoided so that cellulose could maintain its mechanical strength. For nanostructured BC, to the best of our knowledge, there has been no report so far about the research on the oxidation of BC using NO<sub>2</sub> as the oxidant.

In this study, a degradable oxidized bacterial cellulose (OBC) was firstly prepared by oxidizing BC with NO<sub>2</sub>. The chemical and crystal structures and microstructures of OBC and BC were characterized, and in vitro degradable properties of BC and OBC in vitro were also investigated and compared.

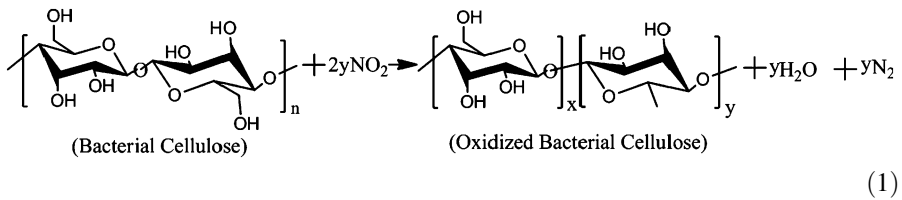
## Materials and methods

### Preparation of OBC

BC was supplied by Hainan Yida Food Co., Ltd. A pre-treatment was used for purifying BC as follows: BC membranes were immersed in 10% NaOH solution for 30 min at 80 °C to remove the bacterial cell debris, then thoroughly washed to neutral by de-ionized water to eliminate the residual NaOH. The purified BC membrane still contained saturated water (about 85 wt%) and was ready for oxidation.

NO<sub>2</sub> was produced by adding a suitable amount of Cu to superfluous HNO<sub>3</sub> (65 wt%) solution. The resulting NO<sub>2</sub> gas was introduced to a separate sealed

device, a gas collecting bottle, in which ten pieces of purified BC (15 mm in diameter and 1 mm in thickness) was placed and reacted with  $\text{NO}_2$  for 3, 6, 9 and 12 days, and the stoichiometric ratio  $[\text{NO}_2:\text{cellulose}]$  was about 0.2. The pressure in the sealed device was 1 atm (atmosphere). The device was stayed at room temperature of 25 °C in the aphotic condition [14]. Equation 1 describes that the primary alcohol groups at C6 of BC were selectively oxidized into carboxyl during the oxidation reaction [13]. A series of OBC samples were produced after BC reacted with  $\text{NO}_2$  for 3, 6, 9, and 12 days, respectively, and then washed by de-ionized water. By-product,  $\text{HNO}_3$ , which resulted from the reaction between water and residual  $\text{NO}_2$  was separated from OBC through extensive wash to neutral by de-ironized water.



### In vitro degradation

Pellicles of BC and OBC oxidized for 12 days were cut into circular slices with a diameter of 15 mm, and immersed in phosphate buffered saline solution (PBS, pH = 7.4) at 37 °C. The samples were taken out separately after different immersion time, washed by de-ionized water, and then freeze-dried and weighed. The original mass of each sample was designated as  $m_0$ . The mass of the sample after degradation was characterized as  $m_1$ . The percentage of the mass loss was calculated by Eq. 2. The mass loss was obtained by averaging the measurements of five specimens in the same degradation condition, and its corresponding standard deviation was also calculated.

$$\text{Mass loss} = \frac{m_1 - m_0}{m_0} \times 100\%.
 \tag{2}$$

### Characterizations

Infrared spectra of OBC with different oxidation time were tested using a FT-IR spectrometer (Perkin-Elmer, model spectrum one). FTIR spectra were obtained in a range of frequency from 4000 to 450  $\text{cm}^{-1}$  with a resolution of 4  $\text{cm}^{-1}$ .

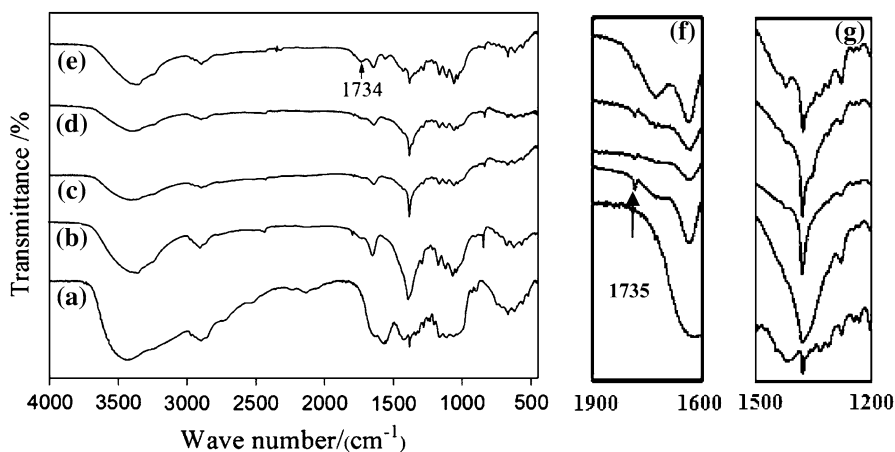
The microstructure of samples was observed by Scanning Electron Microscopy (SEM, Apollo 300). All of the samples were freeze-dried and coated with a thin layer of gold in a sputter coater in advance.

X-ray diffractometry (XRD) (D/MAX-RB) (20 kV, 40 mA) with Cu  $k\alpha$  radiation ( $\lambda = 0.154 \text{ nm}$ ) was used to examine the crystal structure of the samples. The range of diffraction angles ( $2\theta$ ) was 10°–40°.

## Results and discussion

### FTIR analysis

In the presence of  $\text{NO}_2$ , the hydroxyl groups at C6 of cellulose could be converted to carboxylate groups through the selective oxidation reaction [15]. The characteristic band at  $1720\text{--}1760\text{ cm}^{-1}$  in FTIR spectra of the oxidized cellulose indicated the existence of carboxylate groups due to the  $\text{C}=\text{O}$  stretching vibration [16]. Figure 1 shows the FTIR spectra of BC oxidized by  $\text{NO}_2$  for different times. Original BC has no absorption band at  $1720\text{--}1760\text{ cm}^{-1}$  (Fig. 1a). For the short periods of oxidation from 3, 6 to 9 days, a weak absorption peak at the range  $1720\text{--}1760\text{ cm}^{-1}$  (Fig. 1b–d), implying a low oxidation degree of BC. The most important parameter influencing the oxidation degree of BC is the amount of  $\text{NO}_2$  that reacted with BC. Having mentioned above, BC used in this study was in a form of membrane containing plenty of water. When  $\text{NO}_2$  contacted with BC, it reacted with BC and water simultaneously. As a result, the presence of water reduced the degree of oxidation, meanwhile maintaining the 3D structure of BC. After oxidation long enough, for 12 days in this case, the absorption peak at  $1734\text{ cm}^{-1}$  was enhanced, indicating the formation of more carboxylate groups. At the same time, the intensity of band near  $1320\text{--}1210\text{ cm}^{-1}$  associated with the bond stretching of the  $\text{C}\text{--}\text{O}$  group decreased obviously (Fig. 1e), which further proved the formation of OBC. Therefore, the oxidation of BC by  $\text{NO}_2$  is a controllable slow reaction, which would possibly allow to tailor the degree of oxidation of BC by controlling the kinetics or dynamics of the oxidation, such as the stoichiometric ratio of  $\text{NO}_2$ :cellulose, oxidation time, temperature, and pressure. The following sections further demonstrate the case of modification of the degradability of BC by varying the oxidation time.



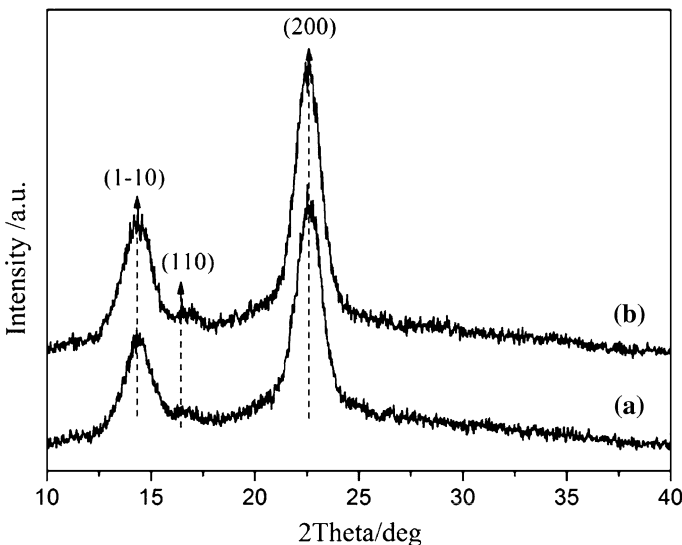
**Fig. 1** FTIR spectra of OBC with different oxidized times (a) 0 day, (b) 3 days, (c) 6 days, (d) 9 days, (e) 12 days. Two enlarged views of the spectral regions from (f)  $1900$  to  $1600\text{ cm}^{-1}$  and (g)  $1500$  to  $1200\text{ cm}^{-1}$

## XRD analysis

Figure 2 shows the XRD patterns of BC and OBC oxidized for 12 days. For original BC (Fig. 2a), three peaks were observed and located at diffraction angles of  $14.6^\circ$ ,  $16.5^\circ$ , and  $22.5^\circ$ , respectively. These peaks corresponded to the primary diffraction of the crystal plane as (1–10), (110), and (200), attributed to the structure of well-defined cellulose I crystal [16, 17]. From Fig. 2b, the diffraction peaks of OBC were almost the same as those of BC, which implied that OBC still retained the crystal structure of cellulose I and the oxidation had little effect on the crystal structure of the cellulose microfibrils. The result indicated that crystallinity of BC before and after oxidation has little statistical difference. As discussed by Goelzer et al. [18], crystalline region contributed greatly to the strength of cellulose. Mechanical properties of cellulose were often deteriorated by other oxidation processes [12]. Hence, it is worthwhile to further investigate mechanical properties and other physical and biocompatible properties of OBC by  $\text{NO}_2$ , which is an ongoing study.

## Mass loss curves

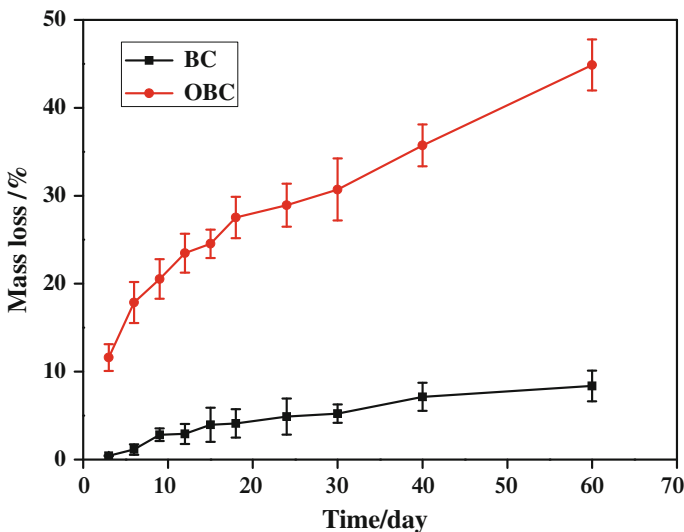
Figure 3 shows the mass loss of BC and OBC oxidized for 12 days after being immersed in PBS solution. The mass loss rate is often used to assess the in vitro degradation of biomaterials [19]. It is well known that BC degrades very slowly in the absence of cellulase [20]. As expected, the mass loss rate of BC measured was lower than 10 wt% after 60 days in PBS solution, in consistency with the results reported by Li et al. [12]. While after oxidation with  $\text{NO}_2$ , the mass loss of OBC increased greatly, with an increase of the degradation time. By the end of 60 days, the mass loss of OBC was up to 45 wt%. Apparently, the mass loss rate of OBC in



**Fig. 2** The XRD patterns of samples. (a) BC, (b) OBC oxidized for 12 days

Fig. 3 is slower than that of DABC (oxidized by periodate) [12], which is most likely due to mild selective oxidation of  $\text{NO}_2$  [13] compared with periodate.

The degradation of BC in PBS solution was triggered by the hydroxyl groups in the glucose chain which converted to new hydrogen bonds by combining with water, and this led to the swelling and degradation of cellulose macromolecular chain [21]. The higher mass loss of OBC was due to the primary hydroxyls on C6 in the glucose chain was oxidized selectively to carboxylate, which was inclined to hydrolyze easily. Therefore, OBC became to swell and degrade faster in PBS solution compared with BC. Even so, it was noticed that OBC with 45% mass loss after in vitro degradation for 60 days still maintained its initial shape and partial mechanical properties, implying more or less uniform selective oxidation might have occurred in the BC membrane. Furthermore, BC is distinguished from its plant counterpart by its microfibril structure consisting of finer nanofibrils (about 1.5 nm in diameter) with a high degree of crystallinity (about 83.5%, calculated from the XRD diffraction peak's area), which is similar to Park S's results [22]. This unique nanostructured fibril network provides abundant surface and interface between amorphous and crystalline structures. Compared with 10% mass loss of BC at the same condition, the high mass loss of OBC with high crystallinity similar to BC (Fig. 2) suggests that cellulose chains in amorphous parts, surface, and interface between micro- and nano-fibrils as well as crystal defects of fibrils would be vulnerable to be oxidized. The high crystalline degree and less crystal defects of OBC may also help to interpret its slow degradation in comparison with electrospun plant cellulose oxidized by  $\text{NO}_2$  [23]. In addition, the fast mass loss rate of OBC observed in Fig. 3 indicates that the degradation products, such as reducing sugar, could leave the cellulose mats and diffuse to PBS media easily as soon as when



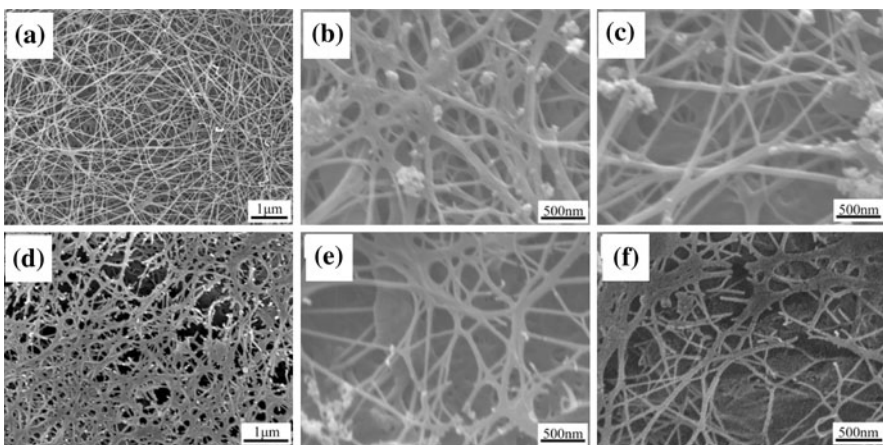
**Fig. 3** The mass loss curves of BC and OBC oxidized for 12 days in PBS

formed [23], attributed to the high solubility of the reducing sugar and the large surface area of the fibril network structure.

### SEM analysis

SEM images showed in Fig. 4 display the surface morphology of BC and OBC before and after immersed in PBS solution. The original BC presented a dense 3D network structure of the microfibrils (Fig. 4a). After oxidation, the 3D microfibril networks of OBC were still more or less retained (Fig. 4d). However, some microfibrils appeared to aggregate or fuse into bundles resulting in the network of OBC consisting of more microfibrils with a relatively larger diameter, and a few fibrils appeared to be fragmented by the oxidation process (Fig. 4a and d). As a result, larger pores were found in OBC fibrous network.

The morphology of BC and OBC after degradation in PBS for different times was shown in Fig. 4b, c, e, and f, respectively. After being immersed in PBS solution for 9 and 18 days, BC maintained the original microfibril networks with a few fibrils ruptured (Fig. 4b and c). In contrast, for OBC, after being immersed for 9 days, the 3D microfibril network partly disappeared, and many fibrils fractured and fell into pieces (Fig. 4e) because of the accelerated degradation of OBC. After 18 days, more OBC fibers were eroded, and some local microfibril network was almost collapsed, but with larger microfibrils retained (Fig. 4f). In agreement with the above results of degradation in vitro, the selective oxidation of glucose chain sped up the degradation of OBC, and could gradually destruct local microfibril network of OBC at a desirable degradation rate by controlling the oxidation time. The formation of thicker microfibrils appeared to slow down the degradation, while to retain the shape and mechanical properties of OBC to some extent. The observation from Fig. 4 further supports our above speculation about the mechanisms of oxidation and degradation.



**Fig. 4** SEM micrographs of **a** BC, **b** BC in PBS for 9 days, **c** BC in PBS for 18 days, **d** OBC, **e** OBC in PBS for 9 days, and **f** OBC in PBS for 18 days

## Conclusion

BC could be selectively oxidated by  $\text{NO}_2$  in the aphotic condition, with the hydroxyl groups at C6 of BC chain substituted by the carboxylate groups. The oxidation time is a controllable factor which would allow controlling the level of oxidation process easily. OBC maintained the original crystal structure of BC and 3D microfibril network structure but with thicker fibrils and relatively larger pores. After oxidation by  $\text{NO}_2$ , OBC is prone to degrade in PBS solution more easily with a sharp increase of the mass loss rate compared with BC. For OBC oxidized for 12 days, the mass loss rate was achieved up to 45% *in vitro* after 60 days in contrast to only 10% of the original BC. The swelling and fragmentation of microfibrils were observed during OBC degradation over different periods of time. The microfibril network of OBC was gradually destroyed and partly disappeared at a high degree of degradation. The stoichiometric ratio of  $\text{NO}_2$ :cellulose is another critical parameter for controlling the oxidation. During oxidation, the presence of water in BC membrane assisted to maintain 3D structure of BC membrane, but reduced the degree and efficiency of oxidation due to the reaction between  $\text{NO}_2$  and water. Overall, the selective oxidation by  $\text{NO}_2$  has been proven to be a feasible method of modifying the degradability of BC. Further systematic study on the controllability of degradation and resulting structure, mechanical properties, surface chemistry, and biocompatibility will be featured in a future article.

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