

Improvement in Mechanical Properties and Biocompatibility of Biosynthetic Bacterial Cellulose/Lotus Root Starch Composites*

Na Yin^a, Shi-yan Chen^{a**}, Yi-meng Cao^b, Hua-ping Wang^a and Qing-kai Wu^{b**}

^a State Key Laboratory for Modification of Chemical Fibers and Polymer Materials, College of Materials Science and Engineering, Donghua University, Shanghai 201620, China

^b Department of Obstetrics and Gynecology, Affiliated Sixth People's Hospital, Shanghai Jiaotong University, Shanghai 200233, China

Abstract Bacterial cellulose/lotus root starch (BC/LRS) composites were prepared by cultivating *Acetobacter xylinum* in nutrient media containing gelatinized lotus root starch. Low concentrations of gelatinized LRS had increased BC production with the maximum value at 6.67 g/L when 5 g/L of LRS was added in the culture media and the composites had thicker and denser fibrils compared with those of BC with low concentrations of LRS (2.5 and 5 g/L). When the concentration of LRS was increased above 7.5 g/L, the morphology of the BC/LRS composites contained more fibril layers that were linked with LRS. The results from X-ray diffraction (XRD) demonstrated that there was no significant difference in structure between BC and BC/LRS composites except a slight increase in crystallinity for BC/LRS composites as the concentration of LRS was lifted up. The tensile tests were performed to display BC/LRS composites prepared with LRS concentration at 2.5 and 5 g/L in media had the tensile strength of 54 and 60 MPa, respectively, which indicated an improvement in mechanical property compared to the unmodified BC (45 MPa). Live/dead assay with chondrocytes seeded on BC/LRS composite revealed higher cell viability ranging from 85% to 90% than BC. Furthermore, cell morphology with typical spindle shape was observed on the surfaces of BC/LRS composite by confocal microscope. Through the overall results, it shows that this study has provided a guidance to prepare BC/LRS composites with better cell biocompatibility and higher mechanical strength than those of BC for the potential use in cartilage tissue engineering.

Keywords Bacterial cellulose; Lotus root starch; Composites; Biosynthesis; Cell biocompatibility

INTRODUCTION

Cellulose is one of the most abundant renewable materials on earth that is synthesized by chemical reagents and enzymes or diverse groups of organisms ranging from multicellular and unicellular plants to bacteria^[1–5]. *Acetobacter xylinum*, a Gram-negative and obligate aerobe, has become a model bacterium for producing bacterial cellulose (BC)^[4–6]. BC is a hydrogel that possesses many desirable properties, including high crystallinity, high purity, and high water content^[7–9]. In addition, BC has excellent mechanical properties that arise from an ultra-fine three-dimensional fiber network with fibril diameters between 10 and 100 nm. These characteristics, combined with BC's biocompatibility and moldability, have attracted a great deal of attention toward BC as a biomedical material. BC materials are currently used for wound dressing, vascular grafts, and

* This work was financially supported by the National Natural Science Foundation of China (Nos. 51273043, 51573024 and 81550008) and the Fundamental Research Funds for the Central Universities and DHU Distinguished Young Professor Program.

** Corresponding authors: Shi-yan Chen (陈仕艳), E-mail: chensy@dhu.edu.cn

Qing-kai Wu (吴氢凯), E-mail: wuqingkai@sjtu.edu.cn

Received July 28, 2016; Revised October 18, 2016; Accepted October 18, 2016

doi: 10.1007/s10118-017-1903-z

cartilage implants^[6, 10, 11]. Among them, cartilage implants were actually insufficient since every year over one million patients require cartilage-grafts procedures. To extend the utility of BC in cartilage tissue engineering, a great deal of effort has been made to modify the physical and biocompatible properties of BC in the past decade. For instance, the combination of poly(3,4-ethylenedioxythiophene) and BC has been reported to give BC an improved performance in mechanical and electrical property as a simulative extracellular matrix^[12, 13]. Nevertheless, composite materials that combine BC with naturally derived biomaterials have been more commonly used as effective strategies to improve the characteristics and modify the structure of BC scaffolds^[14–17].

Starch is one of the most abundant natural materials and readily extracted from a variety of agricultural crops, such as wheat, corn, rice, and potato^[18]. Starch is also a renewable and biodegradable polymer that can be converted into thermoplastic materials through a gelatinization processing^[19]. Starch has been developed as a promising candidate for biomedical applications including drug delivery and tissue engineering^[20–22]. In an earlier study, researchers combined corn or potato starch with BC to generate composites that were aimed to improve the biocompatibility and biodegradability of BC for biomedical applications^[19, 23, 24]. The composites in these studies were made by either adding starch to fully formed BC membranes (solution impregnation) or by including the starch in the culture media while the BC was actively synthesized (biosynthesis technique). Among these methods, the biosynthesis technique is superior for preserving the naturally ordered structure of BC by adding starch with gelatinization processing into the static fermentation medium^[23, 25]. However, the existence of gelatinized starch can change the viscosity of media which may affect mass or oxygen transfer for BC growth and change microorganism movement for the arrangement of BC fibrils. Therefore, one of the main goals in our study is to investigate the impact of gelatinized starch on the efficiency of BC production and the microstructures of BC/starch composites in the presence of various concentrations of starch so as to ensure the quantity and quality for their application in tissue engineering.

Lotus root starch (LRS) is a natural starch isolated from lotus rhizomes that is primarily composed of amylose (20%–30%) and amylopectin (70%–80%). It has better performance in nutritional and medicinal properties than common starch^[26, 27] and can be partially gelatinized in hot aqueous solutions to form a well-distributed mixture with low viscosity^[28]. Hence, it is worthwhile to construct a biocomposite that consists of LRS and BC through culturing *Acetobacter xylinum* in the media that contains gelatinized LRS. In this report, we demonstrated that gelatinized LRS can be introduced into BC culture medium to generate BC/LRS composites. The low viscosity of LRS-containing culture medium not only guarantees efficient BC production, but also allows unique micro-morphologies with enhanced tensile strength to be generated within BC/LRS composites. By conducting *in vitro* study with cartilage cells on BC/LRS membranes, we showed that the composites presented superior biocompatibility to chondrocytes, which promoted cell viability and accelerated normal cell morphology formation in comparison to BC, a further step towards the promising application in cartilage tissue engineering.

EXPERIMENTAL

Materials and Characterization

The cellulose producing bacterial strain, *Acetobacter xylinum* 1.1812, was provided by the Institute of Microbiology, Chinese Academy of Sciences. LRS was purchased from Hangzhou Paradise food Co. Ltd. α -Amylase from *Bacillus subtilis* was supplied by Sigma Aldrich Co. Ltd. All other reagents were analytical grade and were used without further purification from Sinopharm Chemical Reagent Co. Ltd.

The morphologies of freeze-dried BC and BC/LRS composite samples were visualized using Hitachi S-4800 field emission scanning electron microscope (FE-SEM) (Hitachi Ltd., Japan). The samples were cut into 0.5 cm × 0.5 cm squares and coated with a thin layer of evaporated gold prior to analysis. FTIR-ATR spectra of BC and BC/LRS composites in dry form were recorded using a Nicolet NEXUS-670 instrument (Thermo Nicolet Ltd., USA) in the absorption mode, covering a wavenumber range of 4000–600 cm⁻¹. XRD in reflection

mode for all the test samples was carried over a range of 2° – 70° (2θ) using a diffractometer type D/max-rA (Rigaku Co., Japan) with a Cu target and $K\alpha$ radiation ($\lambda = 0.154$ nm). The tensile strengths of BC and BC/LRS composites were compared by measuring the stress and the strain at break and the Young's modulus using a WDW 3020 Universal Testing Machine at room temperature with a cross-head speed of 5 mm/min.

BC production was monitored using a previously reported method through the hydrolysis of starch in the composite using α -amylase from *Bacillus subtilis*^[29]. Briefly, prior to freeze-drying, clean BC/LRS composites were incubated in a beaker with 0.75 units of α -amylase per mL in a buffer containing 0.1 mol/L phosphate buffer (pH 6.9) at 60 °C for 24 h with constant shaking. The reaction was terminated by immersing the beaker in boiling water for 5 min. The membranes were then suspended in 50% ethanol for 10 h and then rinsed extensively with distilled water to remove the residual ethanol. The dry weight of each sample was recorded after drying the membranes in an oven at 80 °C until no change in weight could be detected. BC production was determined in g/L (grams of dried BC per liter of culture media). The LRS content (W_{LRS}) in the composites was calculated from the following equation: $W_{\text{LRS}} = (W - W_{\text{BC}})/W$. Where W is the composite's dry weight and W_{BC} is the dry weight of the composite after α -amylase treatment. For each sample, three measurements were recorded and then averaged.

We used a previously published method of staining BC/LRS composites to identify the presence of gelatinized LRS. The membranes were briefly immersed in KI-I₂ solution that is used as an indicator for the presence of starch at room temperature for 10 min. If they leave blue stain, it can be inferred that gelatinized LRS is present in BC/LRS composites^[30]. The viscosities of fermentation media supplemented with LRS were measured by Physical MCR301 Rheometer (Anton Paar, Austria) at 30 °C. The relative viscosities were calculated relative to the viscosity of distilled water at 30 °C. In this study, the viscosity of distilled water was set to 0.9 mPa·s.

Production and Purification of BC/LRS Composites

The fermentation medium for BC production contains 60 g/L glucose, 6 g/L peptone, 6 g/L yeast extract, 1 g/L citric acid, 1 g/L Na₂HPO₄, 2 g/L KH₂PO₄, and different concentrations of LRS. LRS was added to the fermentation media as the final reagent and caused the media to be highly turbid due to its low solubility. To solubilize the LRS, the fermentation media was heated with constant stirring at 100 °C for 30 min until the turbid media became transparent. This heat treatment effectively gelatinized the LRS. The starter broth was inoculated in 50 mL of fermentation media in a 250 mL Erlenmeyer flask, which was statically cultivated at 30 °C for 7 days. After incubation, the membranes were withdrawn from the culture medium and treated with 0.01 g/mL NaOH at 90 °C for 30 min to lyse and dissolve any microbial cells that remained within the BC membrane. After NaOH treatment, all the membranes were rinsed extensively with distilled water until the pH was 7.0 and followed by being freeze-dried and used for downstream analyses. The membranes that were prepared with 0, 2.5, 5, 7.5, 10, 12.5 and 15 g/L of LRS in the fermentation media are referred to in the text as BC, BC/LRS-1, BC/LRS-2, BC/LRS-3, BC/LRS-4, BC/LRS-5 and BC/LRS-6, respectively.

The Counting for Free Bacterial Cell Number

The free bacterial cell number was determined by only the cells suspended in the fermentation medium and counted by the plate dilution method as colony uniform units (CFU) after removing the BC or BC/LRS membranes from the culture broth. Briefly, 1 mL culture broth was withdrawn from the fermentation medium with static incubation of one day to seven days after removing the BC membranes. The culture broth was diluted serially with sterile 1× phosphate buffered saline (PBS) solution (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na₂HPO₄, 1.76 mmol/L KH₂PO₄, pH = 7.4). We divided the surface of agar plates into eight quadrants in which each quadrant corresponded to one dilution in the series. Three, 10 μ L drops of each dilution of the cell suspension were spaced evenly on the agar surface of each quadrant. This process was repeated for each day culture broth in the dilution series. After the drops had dried on the plates, we inverted the plates and incubated them at 37 °C for 3 days. We counted colonies in each quadrant, determined the number of CFU for each cell dilution.

In Vitro Biocompatibility of BC/LRS Composites

Chondrocyte P1 cell line was revived in Corning flask in growth medium. DMEM nutrient mixed F12 (Invitrogen), with 10% fetal bovine serum (FBS) (Invitrogen) and 1% penicillin/streptomycin solution (Invitrogen). BC and BC/LRS composites were rinsed in double distilled water and autoclaved for 30 min. After cooling down, the specimens were placed in 24 well plates which contained 1 mL growth medium to facilitate cell adhesion that later sat in an incubator at 37 °C for 1 h. Chondrocytes P1 were washed twice with PBS and incubated with trypsin/EDTA for 2 min at 37 °C to detach the cells from the culture flask. 200 µL of cell suspension at 5×10^4 cells/mL was seeded in each scaffold before the addition of 1 mL of growth media. In the next day, the growth media was replaced by the differentiation medium (DMEM nutrient mixed F12 (Invitrogen), ITS+ (Life Technologies), linoleic acid (5.0 µg/mL; Sigma-Aldrich), human serum albumin (HSA) (1.0 mg/mL; Sigma Aldrich), TGF- β 1 (10 ng/mL; Sigma Aldrich), dexamethasone (10^{-7} mol/L; Sigma-Aldrich), ascorbic acid (14 µg/mL; Sigma-Aldrich), and $1 \times$ penicillin-streptomycin (Life Technologies). The scaffolds were then incubated for 1, 7 and 14 days at 37 °C in 5% CO₂ and 95% relative humidity and the differentiation medium was changed every third day.

Cell viability of chondrocytes on BC and BC/LRS composites was determined by using a live/dead assay kit (Life Technologies). The polyanionic dye calcein AM was well retained within live cells, producing an intense uniform green fluorescence in live cells. Ethidium homodimer-1 entered cells with damaged membranes to bind with nucleic acids, producing a bright red fluorescence in dead cells. The viability of chondrocytes was visualized under a Nikon Eclipse TE2000 inverted fluorescence microscope (Nikon Instrument Inc.) at $10\times$ lens. The percentage of viability was determined by calculating the number of live cells (green) normalized to the total number of cells (green and red).

Nikon C1 laser scanning confocal microscope was used for cell morphology observation. Briefly, chondrocyte cells were fixed in 4% formaldehyde and permeabilized with 0.1% Triton X-100 in PBS. Cells actin filaments were then stained with rhodamine conjugated phalloidin (Life Technologies) and the nuclei were stained with DAPI (Thermo fisher). The scaffolds were soaked in PBS until analysis.

RESULTS AND DISCUSSION

Preparation and the Effects of LRS Concentration on BC Production

A variety of biomaterials have been introduced into the BC culture media for the preparation of BC composites. Several reports have demonstrated that the addition of starch-based materials can affect cellulose production by modifying the behavior of the cellulose producing strain, or by changing the physical properties of the culture media^[31, 32]. Schematic representation of the synthesis pathway of BC/LRS composites is shown in Fig. 1, while the synthesis pathway of pure BC is considered as a control. Initially, LRS granules were introduced into the culture media and gelatinized by heating. During this gelatinization process, the LRS granules swell, leach amylose into the media and lose their crystalline structure^[33]. Hence, a more viscous culture media was formed relative to the original media due to the presence of gelatinized LRS. Then *Acetobacter xylinum* was incubated in the fermentation medium, allowing the development of BC/LRS composites. Based on our KI-I₂ assay (Fig. 1), we confirmed that gelatinized LRS molecules could intercalate within the dense BC fiber network and surround the cellulose fibers, which could not be totally removed by the purification process.

As previously reported, LRS is an additive that is capable of increasing the viscosity after gelatinizing in solution^[28]. It is known that increasing media viscosity may be a factor that affects BC biosynthesis. Therefore, the effects of different concentrations of LRS on the production of BC were investigated. The production values of BC by incubating *Aectobacter xylinum* after 7 days in media containing gelatinized LRS ranging from 0 g/L to 15 g/L are presented in Table 1. After 7 days of static incubation, the total BC production was about 6.45, 6.67, 5.76 and 5.49 g/L at concentrations of 2.5, 5, 7.5 and 10 g/L of LRS, respectively. These values are all above the production in the absence of LRS (5.02 g/L). However, a further increase in LRS concentration from 12.5 g/L to 15 g/L led to reduce BC production with values of 4.03 g/L and 2.66 g/L, respectively. Taken

together, our results show that LRS incorporation in the culture media may give the positive impacts on BC production in a proper concentration.

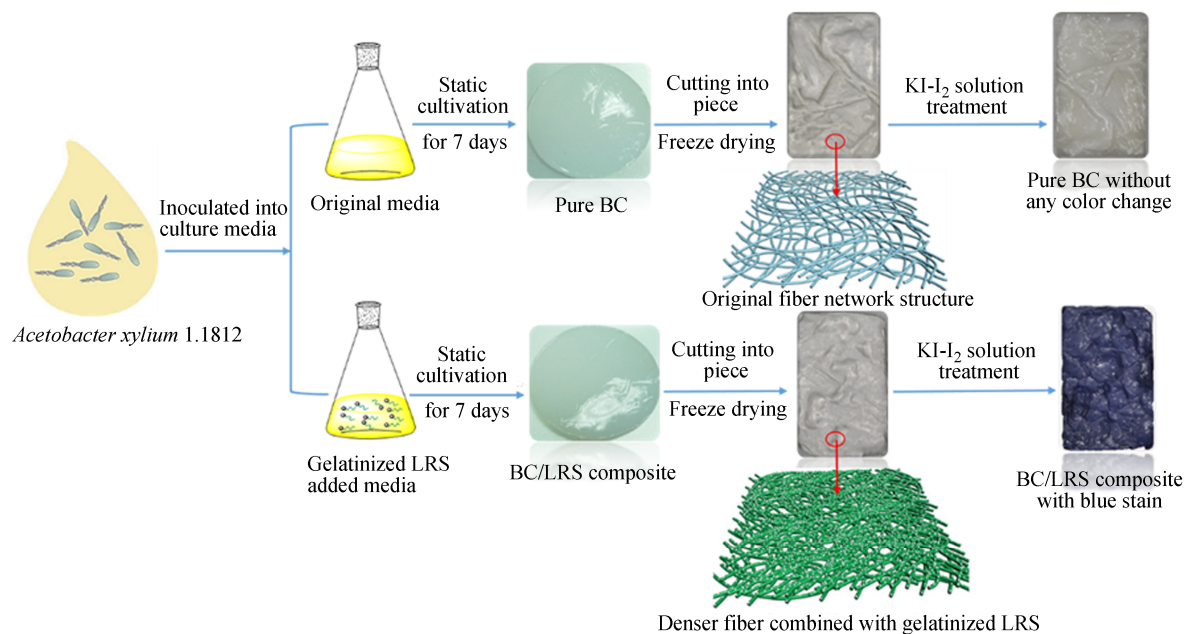


Fig. 1 Schematic representation of the synthesis pathway of BC and BC/LRS composites

Table 1 Relative viscosities of media, total BC production, the LRS content of composites produced after 7 days static incubation with various LRS concentrations added

	Starch concentration (g/L)	Relative viscosity	Total BC production (g/L)	LRS content (%)
LRS	0.0	1.10	5.02 ± 0.35	0
	2.5	1.71	6.45 ± 0.23	1.8
	5.0	2.66	6.67 ± 0.33	4.3
	7.5	3.85	5.76 ± 0.47	6.4
	10.0	5.11	5.49 ± 0.39	8.1
	12.5	7.16	4.03 ± 0.6	10.2
	15.0	11.46	2.66 ± 0.75	12.6

To our knowledge, the introduction of various materials into culture media can cause significant impacts on BC production by either providing a supplementary nutritional source or changing media viscosity to affect the activities of *Acetobacter xylium*. As we inoculated bacteria into the glucose-free media but containing 15 g/L LRS and observed no BC membrane formed on the top surface of media after 7 days of fermentation, we had confirmed that LRS was not a carbon source to facilitate the production of BC. The media viscosity was then hypothesized to be a key factor to affect BC production. It has been reported that the presence of acetan can improve BC production by increasing the viscosity of culture media. This physicochemical effect of acetan is thought to occur because the addition of acetan leads to a higher viscosity and more dispersed culture broth where the free bacterial cells number increased^[34]. Materials that are chemically similar, but less expensive and more available than acetan (such as agar and xanthan), have been introduced to the culture media to significantly increase the production of BC by changing the rheological properties of the medium^[31, 35]. However, if the viscosity of the medium is too high, the growth, movement, and activity of *Acetobacter xylium* will slow down, resulting in reduced BC production which is just consistent with our results. Table 1 shows the relative viscosity of fermentation media containing different amounts of LRS (ranging from 1.10 to 11.46). As the concentration

of LRS increases, the relative viscosity values exhibit a positive correlation. As shown in Table 1, the addition of LRS at low concentration (2.5, 5, 7.5 and 10 g/L) may lessen the aggregation of water insoluble BC, thus enhancing the positive effects on BC production. It is indicated that the addition of an appropriate concentration of gelatinized LRS at the start of the static cultivation can enhance BC production. However, at high concentrations of LRS, a threshold viscosity is exceeded where BC production becomes lower. The free bacteria cell number of *Acetobacter xylinum* in the culture media with different concentrations of LRS shown in Fig. 2 confirmed the results. When the viscosity of culture media is at lower condition (LRS concentration lower than 10 g/L), we observed the higher number of free bacterial cells in the culture media than that of BC, the higher production of BC. At excessive concentrations of LRS, high viscosity increased resistance to mass transfer for the growth of free bacterial cells and finally led to reduction in the BC production. Therefore, the concentration of the additives into the culture media should be recognized as an important factor when preparing BC-related composites as cartilage scaffolds by using the biosynthesis technique.

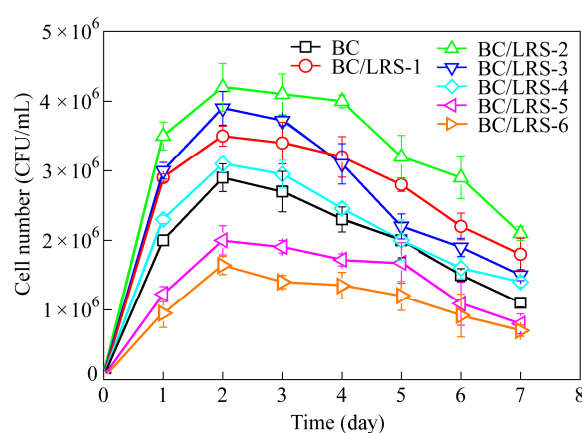


Fig. 2 Free cell number of *Acetobacter xylinum* in the culture with and without the addition of LRS

Structure Analysis

The prepared BC/LRS composites with different concentrations of LRS in the culture media were characterized and FE-SEM images are shown in Fig. 3. It can be seen that pure BC exhibited a loose fibril network just like those previously reported^[36]. Compared with the mean fiber diameter of BC (35 ± 10 nm), BC/LRS composites grown in the presence of 2.5 and 5 g/L LRS appeared to contain a denser and more compact fiber arrangement and the size of cellulosic ribbons in BC/LRS composites increased to (60 ± 15) nm for BC/LRS-1 and (100 ± 20) nm for BC/LRS-2, respectively. The thicker fiber diameters for BC/LRS composites indicated that LRS prompted the secreted cellulose fibrils to associate into larger cellulosic ribbons. However, in the case of BC/LRS-3, BC/LRS-4, BC/LRS-5 and BC/LRS-6, with larger LRS concentrations, the hierarchical structure was formed to contain compact fiber layers with loose single fibers filling the gaps between compact layers. Under low concentrations of LRS, gelatinized LRS easily bound to the surface of cellulose fibrils during their biosynthesis; however, further increasing the LRS concentration raised the media viscosity to a non-optimal level. This highly viscous medium might retard the movement of bacterial cells, which reduced the rate of BC production. The LRS content of all BC/LRS composites was measured and the data are listed in Table 1. The LRS content in the composites scaled up from 1.8% to 12.6% with increasing LRS concentration, which is consistent with the above hypothesis that more LRS is needed for higher levels of BC compaction. The addition of various amounts of LRS in the media yielded composites with different morphologies, which may yield BC/LRS composites with superior physio-mechanical properties for the future use in cartilage implants.

XRD Analysis

The impact of LRS concentration on the structure and crystallinity of the final composites was further monitored by XRD. The diffraction profiles of BC/LRS composites media are shown in Fig. 4. The peak positions in

Fig. 4(a) at 14.6° , 16.8° and 22.8° are characteristic of the interplane distances of BC's I_α and I_β phases^[37, 38]. The XRD pattern of LRS with a B -type crystalline structure has characteristic reflections centered at 5.5° , 15.0° , 17.0° , 19.7° , 22.2° and 24.0° ^[28]. In comparison, the results in Figs. 4(b)–4(g) demonstrated that all the curves of BC/LRS composites with different LRS addition only displayed three distinct peaks similar to the ones observed in native BC. There was no featured signal that could be attributed to LRS crystals in the XRD patterns of BC/LRS composites. This is consistent with a report from Grande *et al.*, who found that starches can lose their characteristic crystalline signals after the starch undergoes a process of gelatinization^[25].

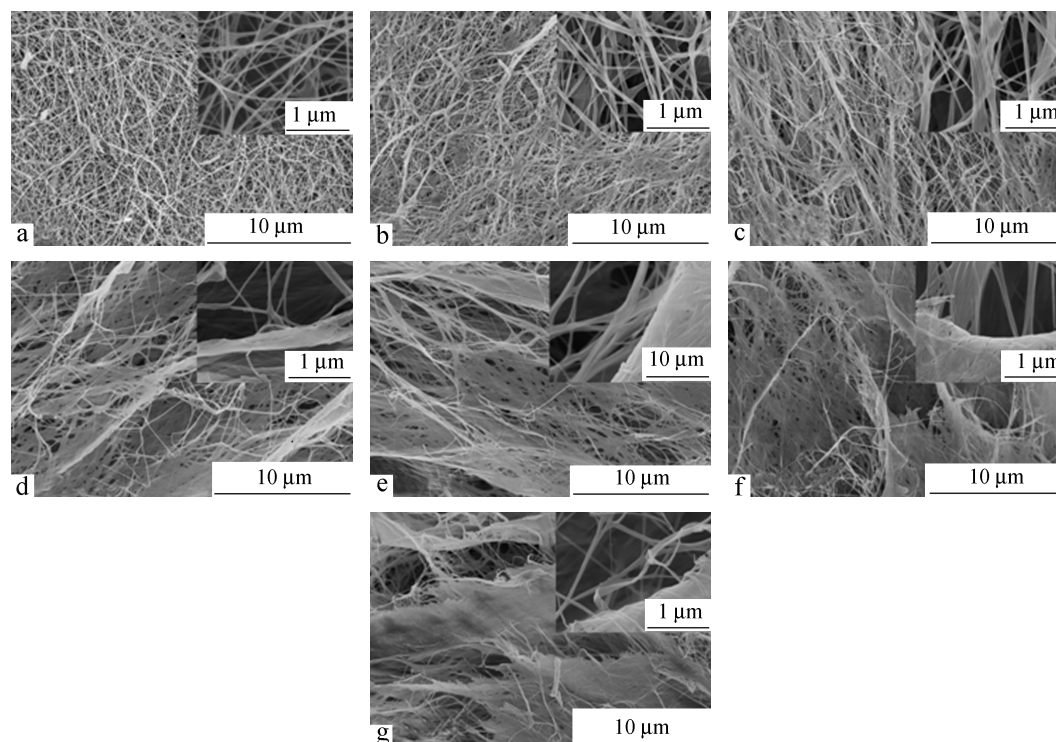


Fig. 3 The FE-SEM images of (a) BC, (b) BC/LRS-1, (c) BC/LRS-2, (d) BC/LRS-3, (e) BC/LRS-4, (f) BC/LRS-5 and (g) BC/LRS-6

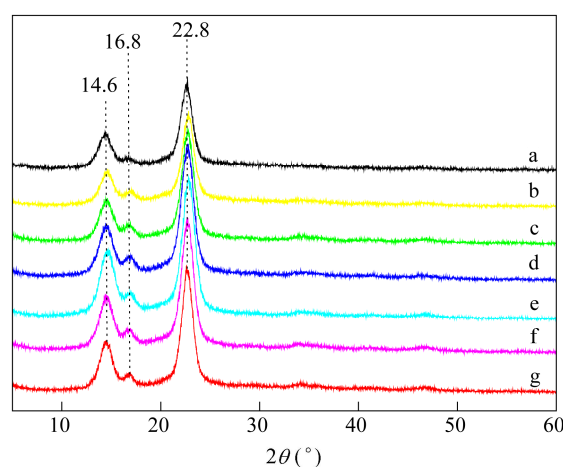


Fig. 4 The XRD patterns of (a) BC, (b) BC/LRS-1, (c) BC/LRS-2, (d) BC/LRS-3, (e) BC/LRS-4, (f) BC/LRS-5 and (g) BC/LRS-6

However, our XRD patterns did reveal that the presence of LRS in the culture media altered the crystalline index of BC (the crystalline index values of all samples are listed in Table 2). When compared to pure BC, there was a slight increase in the crystallinity of the BC/LRS composites. This increase in crystallinity scaled up with increasing LRS concentrations. Pure BC gave a crystallinity of 70.8%, while the largest crystallinity measured was 79.1% for BC/LRS-6 composite produced with the highest tested concentration of LRS. Our combined findings show that the presence of gelatinized LRS in the media favored the crystallization process and reduced the relative amorphous region of BC, which is in accordance with the previous report that the presence of additives in the media has caused the increase in the crystallinity of BC^[13, 32].

BC possesses the typical crystalline forms of cellulose I^[39, 40]. The relative amounts of the two cellulose allomorphs (I α and I β) from BC/LRS composites were determined from our XRD analysis to investigate the impact of LRS in the media on their relative distribution. The d -spacing (nm) values (d_1 and d_2) from XRD patterns are reported to reflect the cellulose I α and I β fractional ratio, since BC's characteristic peaks are actually the combination of the I α and I β diffractions^[32, 41]. Following the observations of Wada *et al.*, the two parameters, d_1 and d_2 in Table 2 show that BC in all BC/LRS samples were I β -rich^[41]. Furthermore, the d_1 values decreased with increasing LRS concentration in the media. This indicates that there was a decrease in the I β component of BC/LRS composites as LRS was increased. The presence of LRS in the media appears to influence the process of microfibril crystallization in a thermodynamically unfavorable direction, away from the stable I β allomorph.

Table 2 The d -spacing and crystallinity index of BC, BC/LRS-1, BC/LRS-2, BC/LRS-3, BC/LRS-4, BC/LRS-5 and BC/LRS-6

Sample	d -Spacing (nm)		Crystallinity (%)
	d_1	d_2	
BC/LRS-0	0.61458	0.53043	70.8
BC/LRS-1	0.61375	0.52359	72.1
BC/LRS-2	0.60914	0.52357	73.2
BC/LRS-3	0.60704	0.52295	75.0
BC/LRS-4	0.60293	0.52052	75.9
BC/LRS-5	0.59889	0.52048	77.0
BC/LRS-6	0.59012	0.52019	79.1

Tensile Strength

The high tensile strength of BC has been a key advantage for the application in tissue engineering^[5, 42]. Figure 5(a) shows the typical stress-strain curves for pure BC versus BC/LRS composite materials. The mechanical properties of all samples are also illustrated in Fig. 5(b). Through analyzing the data, we concluded that the modified morphology in some BC/LRS composites had performed a positive influence in their mechanical strength. The tensile strength of unmodified BC was found to be 45 MPa. The BC/LRS composites made with 2.5 g/L and 5 g/L of LRS in their fermentation media showed higher tensile strength (54 and 60 MPa, respectively) compared to the native BC. However, BC/LRS-3, BC/LRS-4, BC/LRS-5, and BC/LRS-6 which were supplemented with more LRS had reduced tensile strengths to 42, 37, 33 and 27 MPa, respectively. These trends are explained by our analysis in FE-SEM images. The BC/LRS composites with low concentrations of LRS formed denser 3D fiber networks. The glucose chains aggregated together and resulted in thicker and stronger fibers that improved the mechanical strength as previously reported^[43]. However, further increase in the LRS concentration caused the composite to form hierarchical structures composed of compact composite layers cross-linked by sparse single fibers. The decreased tensile strength at high LRS concentrations was likely the result of weakened inter- and intramolecular hydrogen bonding between the composite layers. The Young's modulus of the BC/LRS composites reflects the tensile strength: first increasing, and then decreasing after a threshold LRS concentration is exceeded. The thick and compact fibrils in BC/LRS-1 and BC/LRS-2 appear to enhance the hardness of BC. However, the hierarchical structures found in BC/LRS-3, BC/LRS-4, BC/LRS-5 and BC/LRS-6 led to greater elasticity due to their ability to accommodate more layer-to-layer mobility. Overall, our results indicated that the mechanical properties of composites with low concentrations of LRS added in the media were enhanced relative to native BC. Even though the tensile strength of BC composites decreased at high

LRS concentrations, the increased elasticity of these materials is a highly desirable quality for being used as cartilage scaffolds.

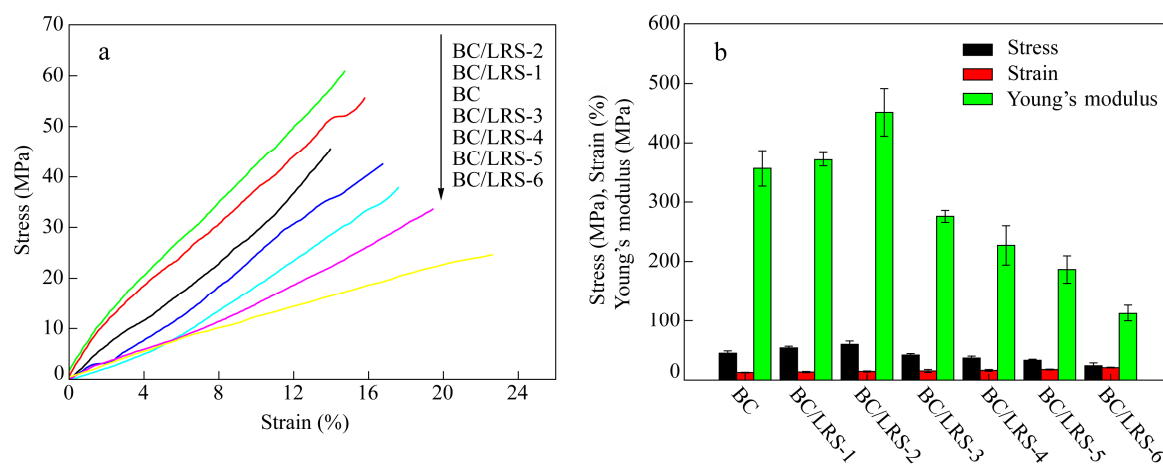


Fig. 5 Mechanical properties of BC, BC/LRS-1, BC/LRS-2, BC/LRS-3, BC/LRS-4, BC/LRS-5 and BC/LRS-6: (a) Tensile stress-strain behaviors; (b) Tensile stress and strain at breaking point, Young's modulus

In Vitro Study

To further test whether BC/LRS composites are feasible to be used in cartilage tissue engineering, we carried out cell culture experiments with chondrocyte to evaluate the biocompatibility of BC/LRS composites. Figure 6 shows cell viability at 1, 7 and 14 days after cultivating chondrocyte cells on pure BC and BC/LRS-2 composites, which was determined by using live/dead assay. We chose BC/LRS-2 composites as a representative sample as it displays the largest cellulose production and the highest tensile strength. Compared to untreated

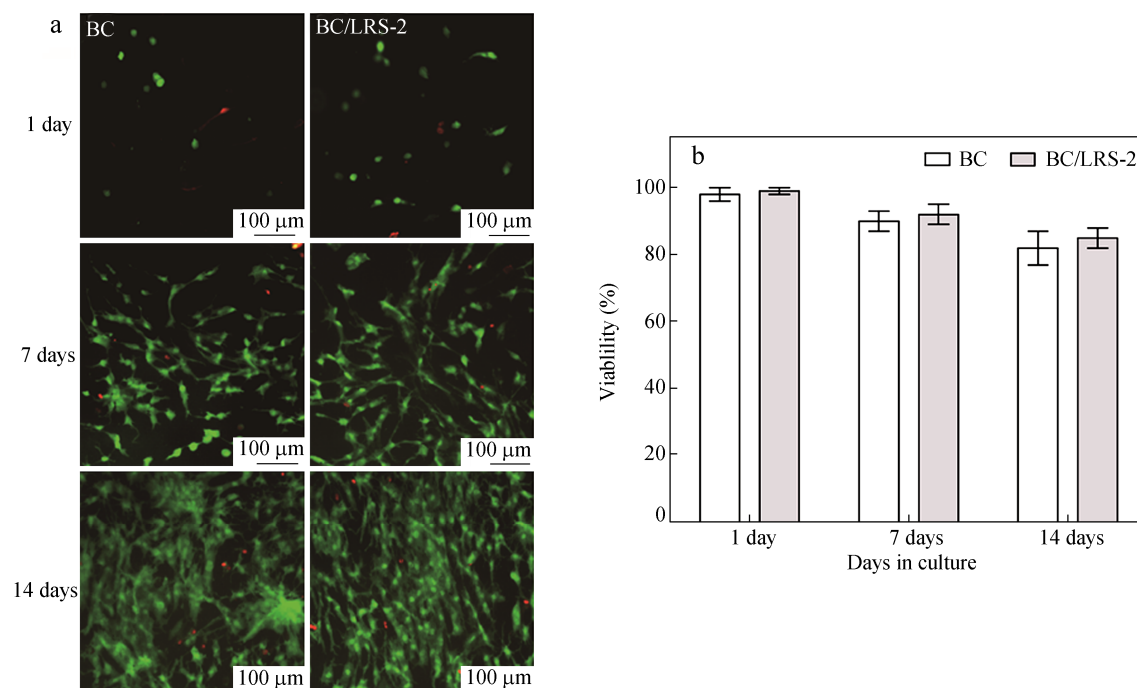


Fig. 6 Cell viability of chondrocytes on BC and BC/LRS-2 composite after 1, 7 and 14 days in culture by live/dead assay: (a) Representative live/dead fluorescent images; (b) Quantitative analysis of chondrocytes viability on BC and BC/LRS-2 composite

BC, BC/LRS-2 composite displayed slightly higher cell viability of 85% to 90% after 14 days in cultivation. Starch is a well-known scaffold material in tissue engineering due to its superior biocompatibility and physical properties. In this study, as the presence of LRS had led to increased cell viability for the composites, it suggested that LRS owned decent biocompatibility to chondrocytes relative to BC and delivered positive influences on promoting cell proliferation as other starch source did.

To further investigate whether the presence of LRS can affect cell morphology, we imaged chondrocytes growing on BC and BC/LRS-2 composites after 1, 7 and 14 days incubation using laser scanning confocal microscope. As shown in Fig. 7, chondrocytes on BC/LRS-2 composites produced earlier cell morphology changes, in which some cells started to spread after 1 d cultivation while cells on BC are still in round shape. With the expectation of higher densities of chondrocytes cells on BC/LRS-2 composites over 14 days culture period, we also find most cells are well distributed and spread on the surface of composite. The results demonstrated that BC/LRS-2 composites created an environment with better biocompatibility, which might facilitate cell growth and accelerate differentiation rate.

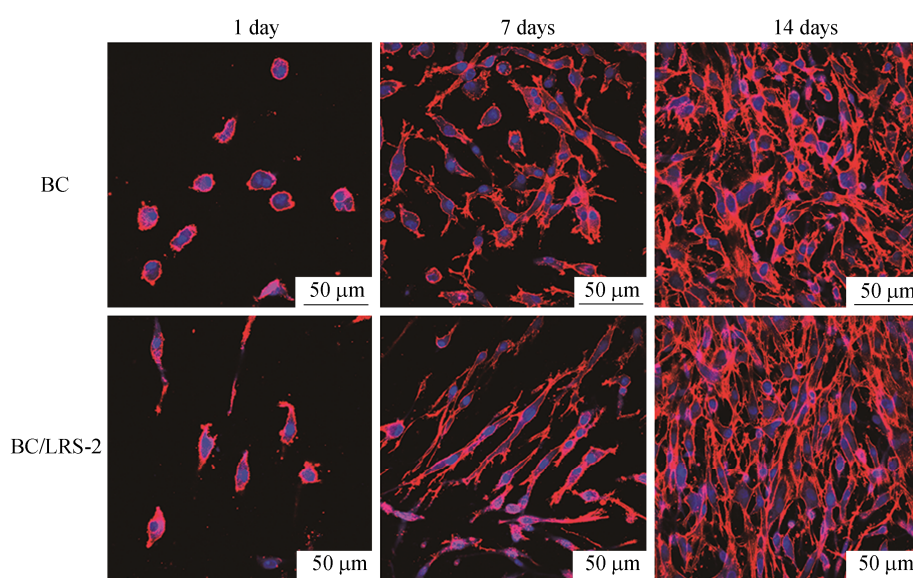


Fig. 7 Morphology of chondrocytes seeded on BC and BC/LRS-2 composite for 1, 7 and 14 days

CONCLUSIONS

In conclusion, a series of BC/LRS composites were prepared and the optimized condition at the LRS concentration of 5 g/L to yield the largest production was given. The microstructure, crystallinity, and mechanical properties of BC/LRS composites could be controlled by tuning the concentration of LRS in the culture media. The vitro study on cell viability and morphology showed that BC/LRS composites were conducive to cells growth and differentiation. The obtained BC/LRS composites with enhanced mechanical properties and controllable morphology may be leveraged in future application of cartilage tissue engineering.

REFERENCES

- 1 Cannon, R.E. and Anderson, S.M., Crit. Rev. Microbiol., 1991, 17: 435
- 2 Chan, J.J., Microsc., 2012, 247: 23
- 3 Mizrachi, E., Mansfield, S.D. and Myburg, A.A., New Phytol., 2012, 194: 54
- 4 Ullah, M.W., Ul-Islam, M., Khana, S., Kima, Y. and Park, J.K., Carbohydr. Polym., 2015, 132: 286
- 5 Ullah, M.W., Ul-Islam, M., Khana, S., Kima, Y. and Park, J.K., Carbohydr. Polym., 2016, 136: 908

- 6 Brown, A.J., *J. Chem. Soc. Trans.*, 1886, 49: 432
- 7 White, D.G. and Brown, J.R.M., "Cellulose and Wood: Chemistry and Technology", ed. by Schuerch, C., John Wiley & Sons Inc. New York, 1989, p. 573
- 8 Bäckdahl, H., Helenius, G., Bodin, A., Nannmark, U., Johansson, B.R., Risberg, B. and Gatenholm, P., *Biomaterials*, 2006, 27: 2141
- 9 Yamanaka, S., Watanabe, K., Kitamura, N., Iguchi, M., Mitsuhashi, S., Nishi, Y. and Uryu, M., *J. Mater. Sci.*, 1989, 24: 3141
- 10 Bodin, A., Concaro, S., Brittberg, M. and Gatenholm, P., *J. Tissue. Eng. Regen. Med.*, 2007, 1: 406
- 11 Klemm, D., Schumann, D., Udhardt, U. and Marsch, S., *Prog. Polym. Sci.*, 2001, 26: 1561
- 12 Chen, C., Yu, Y., Li, K., Zhao, M., Liu, L., Yang, J., Liu, J. and Sun, D., *Cellulose*, 2015, 22: 3929
- 13 Chen, C., Zhang, T., Zhang, Q., Feng, Z., Zhu, C., Yu, Y., Li, K., Zhao, M., Yang, J., Liu, J. and Sun, D., *ACS Appl. Mater. Interfaces*, 2015, 7: 28244
- 14 Andrade, F.K., Costa, R., Domingues, L., Soares, R. and Gama, M., *Acta Biomater.*, 2010, 6: 4034
- 15 Saibuatong, O. and Phisalaphong, M., *Carbohydr. Polym.*, 2010, 79: 455
- 16 Siró, I. and Plackett, D., *Cellulose*, 2010, 17: 459
- 17 Wiegand, C., Elsner, P., Hipler, U. and Klemm, D., *Cellulose*, 2006, 13: 689
- 18 Ellis, R.P., Cochrane, M.P., Dale, M.F.B., Duffus, C.M., Lynn, A., Morrison, I.M., Prentice, R.D.M., Swanston, J.S. and Tiller, S.A., *J. Sci. Food Agric.*, 1998, 77: 289
- 19 Martins, I.M.G., Magina, S.P., Oliveira, L., Freire, C.S.R., Silvestre, A.J.D., Neto, C.P. and Ganidi, A., *Compos. Sci. Technol.*, 2009, 69: 2163
- 20 Azevedo, H.S., Gama, F.M. and Reis, R.L., *Biomacromolecules*, 2003, 4: 1703
- 21 Le Corre, D.B., Bras, J. and Dufresne, A., *Biomacromolecules*, 2010, 11: 1139.
- 22 Salgado, A.J., Coutinho, O.P. and Reis, R.L., *Tissue. Eng.*, 2004, 10: 465
- 23 Grande, C.J., Torres, F.G., Gomez, C.M., Troncoso, O.P., Canet-Ferrer, J. and Martinez-Pastor, J., *Polym. Ploym. Compos.*, 2008, 16: 181
- 24 Wan, Y., Luo, H., He, F., Liang, H., Huang, Y. and Li, X., *Compos. Sci. Technol.*, 2009, 69: 1212
- 25 Grande, C.J., Torres, F.G., Gomez, C.M., Troncoso, O.P., Canet-Ferrer, J. and Martinez-Pastor, J., *Mater. Sci. Eng. C: Mater. Biol. Appl.*, 2009, 29: 1098
- 26 Mukherjee, P.K., Mukherjee, D., Maji, A.K., Rai, S. and Heinrich, M.J., *Pharm. Pharmacol.*, 2009, 61: 407
- 27 Sridhar, K.R. and Bhat, R., *J. Agric. Technol.*, 2007, 3: 143
- 28 Zhong, G., Chen, Z. and We, Y., *Int. J. Food Sci. Technol.*, 2007, 42: 1449
- 29 Konsula, Z. and Liakopoulou-Kyriakides, M., *Process Biochem.*, 2004, 39: 1745
- 30 Zhang, Q., Lu, Z., Hu, H., Yang, W. and Marszalek, P.E., *J. Am. Chem. Soc.*, 2006, 128: 9387
- 31 Chao, Y., Mitarai, M., Sugano, Y. and Shoda, M., *Biotechnol. Prog.*, 2001, 17: 781
- 32 Ul-Islam, M., Ha, J.H., Khan, T. and Park, J.K., *Carbohydr. Polym.*, 2013, 92: 360
- 33 Jenkins, P.J. and Donald, A.M., *Carbohydr. Res.*, 1998, 308: 133
- 34 Ishida, T., Sugano, Y., Nakai, T. and Shoda, M., *Biosci. Biotechnol. Biochem.*, 2002, 66: 1677
- 35 Bae, S. and Shoda, M., *Biotechnol. Prog.*, 2004, 20: 1366
- 36 Gea, S., Reynolds, C.T., Roohpour, N., Wirjosentono, B., Soykeabkaew, N., Bilotti, E. and Peijs, T., *Bioresour. Technol.*, 2011, 102: 9105
- 37 de Oliveira, R.L., da Silva Barud, H., de Assunção, R.M.N., da Sliva Meireles, C., Carvalho, G.O., Filho G.R., Messaddeq, Y. and Ribeiro, S.J.L., *J. Therm. Anal. Calorim.*, 2011, 106: 703
- 38 Ul-Islam, M., Khan, T. and Park, J.K., *Carbohydr. Polym.*, 2012, 89: 1189
- 39 Oh, S.Y., Yoo, D.I., Shin, Y., Kim, H.C., Kim, H.Y., Chung, Y.S., Park, W.H. and Youk, J.H., *Carbohydr. Res.*, 2005, 340: 2376
- 40 Yan, Z., Chen, S., Wang, H., Wang, B. and Jiang, J., *Carbohydr. Polym.*, 2008, 74: 659
- 41 Wada, M., Okano, T. and Sugiyama J., *J. Wood Sci.*, 2001, 47: 124
- 42 Wang, J., Wan, Y., Luo, H., Gao, C. and Huang, Y., *Mater. Sci. Eng. C: Mater. Biol. Appl.*, 2012, 32: 536
- 43 Shezad, O., Khan, S., Khan, T. and Park, J.K., *Carbohydr. Polym.*, 2010, 82: 173