Preparation of Biocompatible Hydrogel from Lignin-Carbohydrate Complex (LCC) as Cell Carriers

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Two poplar lignin-carbohydrate complexes (LCCs), LCC-48 and LCC-72, were isolated at different milling times, 48 h and 72 h, respectively. A new hydrogel carrier was prepared from these LCCs in the presence of polyethylene glycol diglycidyl ether as the crosslinker for cell (human hepatocyte (L-02)) culture. The effects of the structure of LCC on the carrier were investigated. The FT-IR spectra indicate that the two LCC samples were composed of lignin and polysaccharide, and showed a typical LCC structure. The galactose contents of LCC-48 and LCC-72 were 3.02% and 5.67%, respectively. The results of cell culture show that a large number of hepatocytes adhered to the porous carriers. Hepatocytes grown on the LCC carriers outperformed the control group in every observed category, including cell proliferation rate and metabolic activity. These results indicate that poplar LCC might be a great potential precursor of biological carriers for human hepatocytes culture.

Keywords: Lignin-carbohydrate complexes; Galactose; Hydrogel carrier; Hepatocytes; Biocompatibility

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INTRODUCTION

Plant cell walls in higher plants are composed primarily of cellulose, hemicelluloses, and lignin (Vanholme *et al*. 2010). Some polysaccharides in the cell walls of lignified plants are linked to lignin to form lignin-carbohydrate complexes (LCCs). The lignin-carbohydrate covalent linkages are the most probable cause for residual lignin, which resists delignification during kraft pulping and bleaching (Lawoko *et al.* 2004). Many experiments with LCCs strongly confirm that such bonds exist between lignin and hemicelluloses (Eriksson *et al*. 1980; Xie *et al.* 2000). The proposed linkage type is classified into the following six groups: ether linkage of the hydroxyl group at the position of the lignin side chain with alcoholic hydroxyl of sugar residue (Freudenberg 1965), ester linkage of the alcoholic OH of lignin with the carboxylic group of uronic acid (Yaku *et al*. 1976), hemiacetal or acetal linkage of the carbonyl group located at β-position of lignin with carbohydrates (Bolker 1963), glycoside linkage with the primary alcoholic OH at the γ-position of the phenylpropane unit (Takahashi and Koshijima 1988), glycosidic linkage at the phenolic OH of lignins (Smelstorius 1974), and ester linkage of the carboxylic group of the cinnamic acid unit in lignin with the alcoholic OH of carbohydrates (Lam *et al.* 1992). LCCs are a promising, eco-friendly, and renewable source for generating energy, fuels, and chemicals that could partially replace fossil fuels to reduce the pressure of environmental pollution problems (Ma *et al.* 2015). However, LCC as a precursor for biological material has rarely been reported.

LCC contains hydrophilic, flexible polysaccharide chains and hydrophobic rigid lignin blocks. This structure makes LCC have good biocompatibility and mechanical strength (Kai *et al.* 2016). The polysaccharide moiety consists of various types of sugars such as galactose, glucose, arabinose, mannose, fucose, and occasionally uronic acid, depending on the plant species (Hiroshi *et al.* 2005). The biocompatibility and biodegradability of LCC are still uncertain and need more study (Sakagami *et al.* 2010). LCCs may be used as good biological carriers for hepatocyte culture. In recent years, LCCs isolated from ginkgo wood (*Ginkgo biloba* L.) have shown satisfactory biocompatibility (Li *et al*. 2014), and the hydrogel prepared from artificial LCCs, dehydrogenation polymer (DHP)-galactose complex, has good biocompatibility with human hepatocytes (Wu *et al.* 2016). However, further investigations of the effect of LCC structure on the biocompatibility of hydrogel carrier, especially the LCC extracted *via* different ball milling time, have not been reported.

In the present work, hydrogel carriers were prepared from poplar LCCs in the presence of polyethylene glycol diglycidyl ether as the crosslinker for cell (human hepatocytes (L-02)) cultures. The LCCs were investigated in terms of its structure, components, and relative molecular weight. The growth state of human hepatocytes on the biological carriers was observed by inverted microscope and scanning electron microscopy (SEM). The effects of the LCC structure of the carrier on the metabolic activity of hepatocytes were investigated by determining albumin in culture medium, blood urea nitrogen content, and glucose consumption.

EXPERIMENTAL

Materials

Poplar tree (*Populus euramericana*) wood was obtained from Wuhan botanical garden (Wuhan, China). Human hepatocytes were provided by the Pricells company (Wuhan, China). Polyethylene glycol diglycidyl ether, average M_n 500 (500g/mol), was purchased from Sigma Chemistry Co. (Shanghai, China). HPLC-grade N,N-dimethylformamide was purchased from Aladdin (Shanghai, China). Insulin was obtained from Biosharp (Beijing, China). Fetal bovine serum was provided by MP Biomedicals (California, USA). All other reagents were obtained from Sangon Biotechnology (Shanghai, China) or as indicated in the methods.

Methods

Preparation of poplar LCC

Poplar wood meal was extracted with a 2:1 mixture (by volume) of benzene and ethyl alcohol, followed by hot water extraction and vacuum drying for 7 days. The extractive-free wood meal was further ground for 48 h and 72 h in a vibration ball mill with water cooling. The LCC obtained by ball milling for 48 h was named LCC-48. The LCC obtained by ball milling for 72 h was called LCC-72. The poplar LCC was then purified as described previously (Björkman 1957), as shown in Fig. 1. The yield of LCC-48 and LCC-72 were 13.8% and 17.3%, respectively.

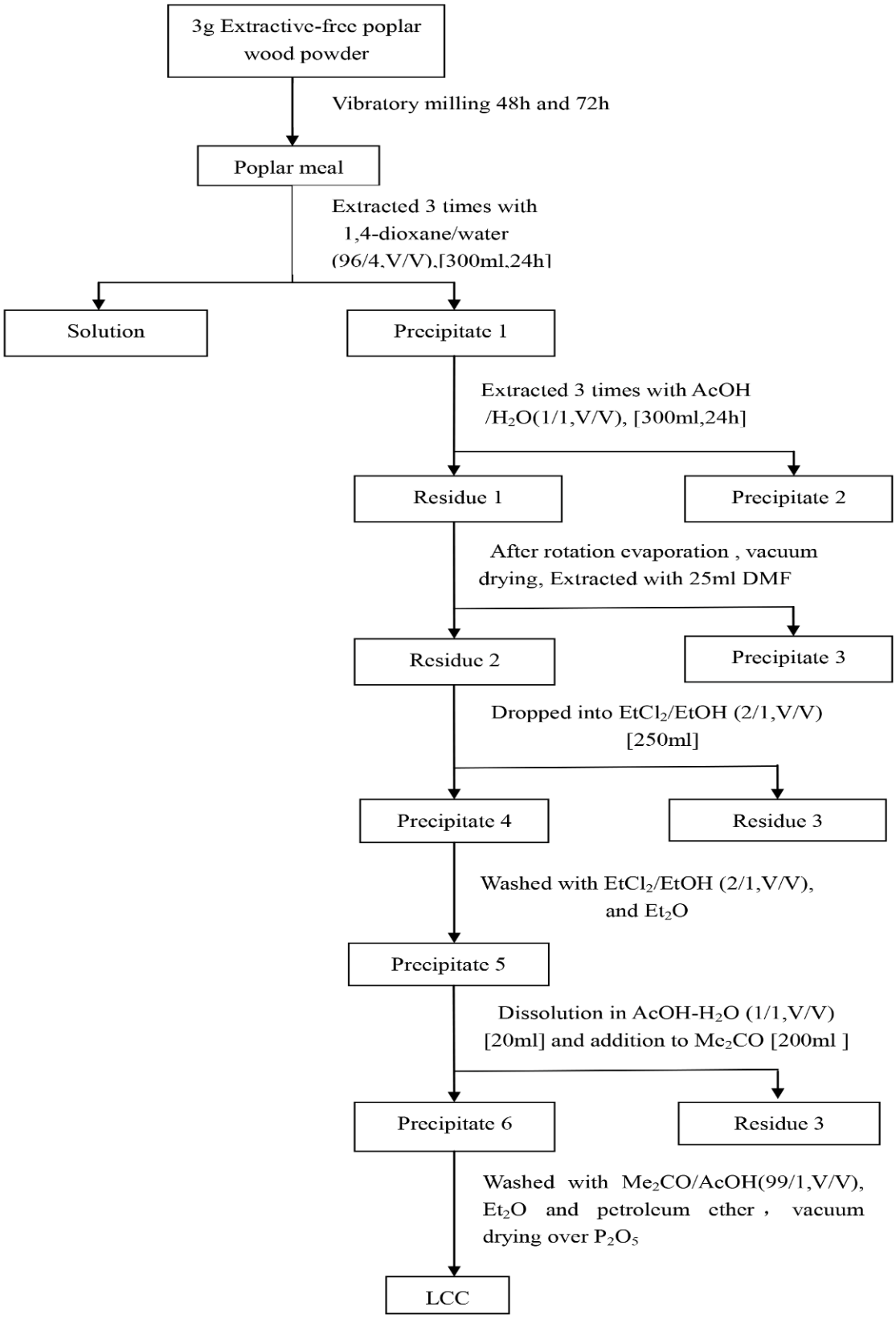

Fig. 1. Isolation of LCC from popular wood by vibratory ball milling for 48 h or 72 h

Preparation of LCC carriers

Poplar LCC-48 (100 mg) and poplar LCC-72 (100 mg) isolated by ball milling were placed in different test tubes, then NaOH solution (200 μL, 3.3 mol/L) was added to the tubes. Subsequently, the mixtures were then stirred in an ice bath for 4 h until the LCC completely dissolved. To the above solutions, polyethylene glycol diglycidyl ether (50 μL) was added and further stirred until all polyethylene glycol diglycidyl ether was dissolved. This suspension was then stirred at 50 °C for 24 h to obtain hydrogel porous biological carriers. These were subsequently added to an Erlenmeyer flask. The material was washed with distilled water until the water became clear and the solution reached pH 7. The samples were then freeze-dried for 24 h. The carrier obtained with LCC-48, which was obtained by ball milling for 48 h is named LCC-48-C, while the carrier prepared with LCC-72 is named LCC-72-C.

FT-IR determination of LCC

Fourier transform infrared (FT-IR) spectrum was recorded on a Thermo Scientific Nicolet 6700 (Waltham, MA, USA) using the KBr pellet technique. For each spectrum, the scans were stored at a resolution of 2 cm^{-1} over the range of 4000 cm^{-1} to 500 cm^{-1}.

Chemical composition of LCC

Sugar contents were measured according to the previous literature (Huang *et al.* 2015; Min *et al.* 2014). A two-step acid hydrolysis procedure was applied to fractionate the biomass into two forms, *i.e.*, acid-soluble sugars and acid-insoluble lignins, that are more easily quantified. Sugars hydrolyzed into the monomeric forms in the hydrolysis liquid were determined by high performance liquid chromatography (HPLC) (Shimadzu LC-20AT, Kyoto, Japan) with a differential refractive index detector (Shimadzu RID-10A) on an Aminex HPX-87P column (Bio-Rad, Hercules, CA, USA) running at a flow rate of 0.6 mL/min at 65 °C, with water as the moving phase.

The acid-insoluble lignin was determined according to the method described by Ibáñez and Bauer (2014). The content of acid-soluble lignin was determined as follows. One gram of woody material was treated with concentrated sulfuric acid (72 wt.%) at 18 °C for 2 h. After water was added to dilute the H_2SO_4 to 3 wt.%, the mixture was heated to boiling for 4 h with a continuous addition of water to keep a stable level of water in the flask. The mixture was then filtered *in vacuo* with a G4 glass filter. The obtained acid-insoluble lignin was dried at 105 °C overnight. The filtrate was used to determine the acid-soluble content by a UV-Vis spectrometer (Shimadzu 2550, Japan) at wavelength of 205nm according to the method described by Lin. (Lin and Dance 1992).

Molecular weight determination

The lignin was dissolved in N,N-dimethylformamide (DMF, 1 mg/ml) and passed through a 0.45 μm membrane filter. The molecular weight of lignin was determined by gel permeation chromatography (GPC) (Shimadzu, Kyoto, Japan) with a refractive index detector with DMF as the eluent (1.0 mL/min), injecting 25 μL at 40 °C (Abdelaziz *et al.* 2016).

SEM observation of the LCC carriers and adhering hepatocytes

The hydrogel bio-carriers in the experimental group were taken out on the 5th day during the culture of human hepatocyte *in vitro*. After being stabilized with 2.5% glutaraldehyde (GA) for 24 h followed by treatment with osmic oxide for 1 h, the carriers

were sequentially dehydrated with 30%, 50%, 70%, 80%, 90%, and 100% ethanol, washed with phosphate-buffered saline (PBS), and vacuum-dried at 40 °C. The dried samples were adhered to a copper with adhesive then sprayed with a layer of metal film in vacuum evaporator. Scanning electron microscopy was conducted on a JSM-6390LV (Electronics Co., Ltd., Tokyo, Japan), which was used to observe the morphological structure of the surface and cutting section.

Cell culture of human hepatocytes

Human hepatocytes (L-02) were purchased from Pricells Company (Wuhan, China). The cell culture was washed with PBS buffer twice and digested with 0.25% trypsin for 1 to 2 min. After the digestive solution was removed, 20% (by volume) fetal bovine serum was further supplemented to the medium. Insulin (0.01 mg/mL), and double-antibody 1× RPMI-164 was added to the medium to culture the hepatocytes. The culture medium in the flask was mixed with a pipette until all of the parietal cells were in solution. The culture medium was transferred to a sterile tube and centrifuged at 800 rpm for 5 min, and the supernatant was decanted (Muhammad *et al.* 2011; Shadforth *et al.* 2012). The cells were resuspended in a complete culture medium.

Human hepatocyte suspension at a density of about 5×10^4 cells/mL was prepared in advance. The LCC carriers were sterilized in dry state at 165 °C for 2 h and seeded in 24-well culture plates. In the control group, the cultured cells were not added to LCC carriers. Cell suspensions (100 μL) were added to each well and then were supplemented with the complete culture solution (900 μL) under a humid atmosphere containing 5% CO_2 at 37 °C.

Cell counts

For one week of the culture of human hepatocytes (L-02), the liquid mediums were collected every day in a clean bench, and the cells were washed twice with phosphate buffer followed by 0.25% trypsin digestion for 1 min to 2 min. After the digestive juice was removed, new medium was added to terminate the digestion. The cells were dissociated into a single cell suspension. Cell suspensions (200 μL) were stained with 0.4% trypan blue solution and placed on a hemacytometer for counting on a Leica DM-6000 CS microscope (Leica Instruments Inc., Wetzlar, Germany).

Observation by inverted microscope

After one week of culture, human hepatocytes (L-02), LCC-72-C, LCC-48-C, and the control group were observed with an inverted biological microscope (COXEM EM-30 Plus, Shanghai, China).

Detection of metabolic activity

The content of albumin was determined with a commercially available kit (provided by Nanjing Jiancheng Bioengineering Institute, China). The culture medium of the LCC carriers and the control group was collected every day for one week. In test tubes, 10 μL of the control (distilled water), standard (albumin, 34.8 g/L), or sample (cell supernatant) was mixed with bromocresol green buffer (2.5 mL). The solution was incubated for 10 min at room temperature. The absorbance at 628 nm was measured on an UV-Vis spectrophotometer (Shimadzu 2550, Kyoto, Japan). The albumin content was calculated as follows,

$$ALB = (A_1 - A_2) / (A_0 - A_2) \times C_0 \quad (1)$$

where *ALB* is the content (g/L) of albumin, A_0, A_1, and A_2 are the absorbance values of standard tubes, sample tubes, and control tubes, respectively, and C_0 (g/L) is the concentration of a standard.

The content of urea nitrogen was determined by a commercially available kit (provided by Nanjing Jiancheng Bioengineering Institute, China). The culture medium of the LCC carriers and control group were collected every day for one week. Distilled water (20 μL), standard urea nitrogen (10 mmol/L), and cell-free suspension were mixed with oxime solution (1 g/L, 1 mL) and a solution of sulfuric acid (0.824 mol/L) mixed with phosphoric acid (1.135 mol/L) (1 mL) in test tubes. The solutions were heated in a boiling water bath for 15 min and then rapidly cooled in an ice bath. The absorption at 520 nm were monitored by a UV-Vis spectrophotometer (Shimadzu 2550). The nitrogen content was calculated as follows,

$$BUN = (A_1 - A_2) / (A_0 - A_2) \times C_0 \quad (2)$$

where *BUN* is the content (mmol/L) of nitrogen, A_0, A_1, and A_2 are the absorbance values of standard tubes, sample tubes, and control groups, respectively, and C_0 (g/L) is the concentration of a standard.

The glucose content was determined by a commercially available kit (provided by Nanjing Jiancheng Bioengineering Institute, China). The culture medium of the experimental group was collected every day for one week. In test tubes, 10 μL of distilled water, standard glucose, or cell-free supernatant was mixed with a solution consisting of phosphate buffer (pH 7.0), 10.6 mmol/L phenol, and 70 mmol/L aminoantipyrine. The reaction was incubated for 15 min at 37 °C. The absorption values were measured at 505 nm by an UV-Vis spectrophotometer (Shimadzu 2550). The glucose content was calculated as follows,

$$C = (A_1 / A_0) \times C_0 \quad (3)$$

where *C* is the content (mmol/L) of glucose, A_0 and A_1 are the absorbance values of standard tubes and sample tubes and respectively, and C_0 (g/L) is the concentration of a standard.

RESULTS AND DISCUSSION

FT-IR Analysis

The LCC structure was analyzed by FT-IR spectrometry. As shown in Fig. 2 and Table 1, there was no significant difference between LCC-48 and LCC-72, which are composed of lignin and polysaccharide, with a typical structure of lignin-carbohydrate complexes. The FT-IR spectra of the two samples show a strong absorbance at 3428.2 to 3434.4 cm^{-1} from the -OH and at 2918.7 to 2919.1 cm^{-1} from -CH stretching vibration. The absorption band at 1738.1 to 1732.2 cm^{-1}, which indicated the presence of C=O unconjugated bonds was more intense in the spectrum of LCC-48 because the LCC-48 contained more hemicellulose. The absorption peaks at 1594.9 cm^{-1} and 1506.5 cm^{-1} indicated that these are the vibration peaks of the aromatic ring in a basic lignin structure. A peak appearing at 1376.1 to 1378.3 cm^{-1} in the two samples was assigned to the vibration of aromatic rings (Singh *et al.* 2005; You *et al.* 2015). A strong absorption at 1045.3 to

1045.7 cm^{-1} was attributed to the C-O stretch of polysaccharides. These results indicated that the LCCs are composed of lignin moieties and sugar units of polysachride.

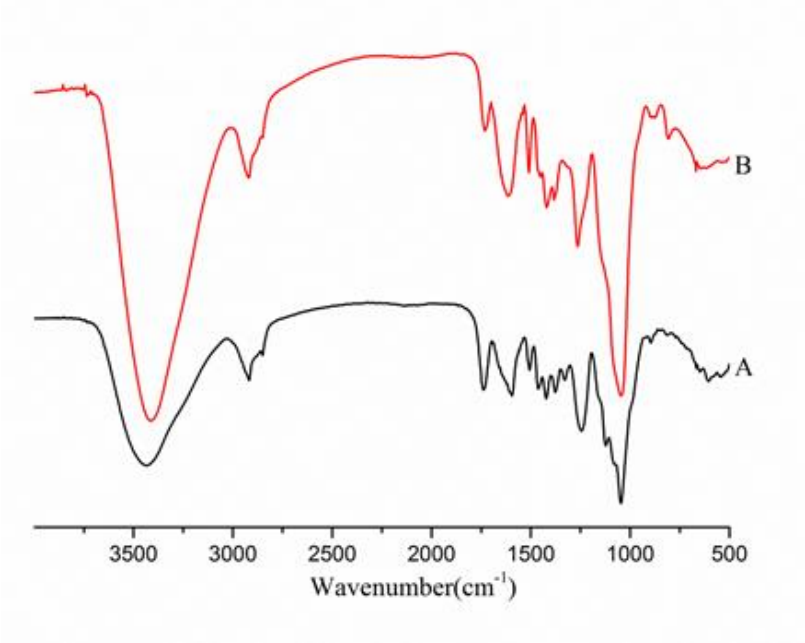

Fig. 2. The FT-IR spectroscopy of LCC-48 (A) and LCC-72 (B)

Table 1. Assignment of Main Characteristic FT-IR bands of LCC-48(A) and LCC-72(B)

Wavenumber (cm^{-1})		Assignment	Reference
A	B		
3434.4	3428.2	O-H stretch	Shivakumar *et al.*2017
2918.7	2919.1	C-H stretch	Zhang *et al.* 2017; Shivakumar *et al.*2017
1738.1	1732.2	Unconjugated C=O stretch and acetyl groups in hemicellulose	Durmaz *et al.* 2016
1594.9	1605.9	Aromatic ring in lignin	Naumann *et al.* 2005
1506.5	1508.8	Aromatic skeletal vibration in lignin	Durmaz *et al.* 2016
1463.2	1458.2	C-H deformation (asymmetric)	Hon *et al.* 2000 Durmaz *et al.* 2016
1376.1	1378.3	C-H deformation vibration	Zhang *et al.* 2017; Durmaz *et al.* 2016
1329.2	—	Syringyl ring breathing with C-O stretch in lignin	Hon *et al.* 2000
1263.7	1263.8	Guaiacyl ring plus C=O stretch	Hon *et al.* 2000 Durmaz *et al.* 2016
1045.7	1045.3	C-O stretch in carbohydrates	Durmaz *et al.* 2016

Chemical Composition of LCC

The compositions of the two LCC samples were analyzed as described previously (Gao *et al.* 2015). The solid received after the two-step acid hydrolysis was acid insoluble lignin. The solution was used for sugar and acid soluble lignin determination. Table 2 shows the effect of different milling times on the composition of the poplar LCC. In this experiment, there was no correction for the lost of sugars during hydrolysis of LCC by H_2SO_4. Some hemicellulose components were degraded by acid in the hydrolysis process. For example, a part of xylan was converted to furfural. Therefore, the sugar analysis of LCC can be regarded as preliminary. Galactose is recognized by the receptors on liver cells with high physiological activity to hepatocytes (Kim *et al.* 2006). Galactose enhances the selective interaction between biological carriers and hepatocytes. As shown in Table 1, the galactose contents in LCC-48 and LCC-72 were 3.0% and 5.7%, respectively. Obviously, LCC-72 contained more galactose units than that of LCC-48. Thus, LCC-72 was more compatible for hepatocytes than LCC-48. The lignin and total sugar contents of LCC-72 were 27.5% and 61.4%, respectively, which will lead to good physical strength of LCC-based porous biological carriers. Because the lignin contents of LCC-72 was lower than that of LCC-48, the carriers prepared from the LCC-72 may be more flexible and suitable than that from LCC-48.

Table 2. Chemical Composition (%) of Poplar LCCs

Samples	acid-insoluble lignin	acid-soluble lignin	Total lignin	Glucose	Xylose	Galactose	Arabinose	Mannose	Total sugar
LCC-48	28.9	3.4	32.3	6.8	29.9	3.0	3.8	8.7	52.2
LCC-72	22.3	5.2	27.5	4.8	33.2	5.7	4.3	13.5	61.5

Molecular Weight Determination

As LCC is a kind of homogenous natural polymer in which lignin and carbohydrates connect each other, not a mixture of lignin fraction and carbohydrate composition, the molecular weight can be determined by GPC. The weight-average molecular weight (M_w), number-average molecular weight (M_n), and polydispersity (M_w/M_n) in LCC-48 and LCC-72 with different ball milling times are shown in Table 2. LCC-48 and LCC-72 had a relative narrow polydispersity as shown by Mw/Mn < 1.8. The number-average molecular weights of LCC samples with different ball billing times were between 7,000 g/mol and 10,000 g/mol. The results indicated that LCC-72 had a lower molecular weight. Molecular weight decreased with the extension of ball milling time. Generally, the molecular weight of the LCC samples was relatively low for preparation of bio-carrier. Therefore, it should be cross-linked during the preparation of bio-carrier to increase the strength and stability.

Table 2. Weight-Average (M_w), Number-Average (M_n) Molecular Weights, and Polydispersity Indexes (M_w/M_n) of LCC-48 and LCC-72

Samples	M_w	M_n	M_w/M_n
LCC-48	17558	9836	1.785
LCC-72	9786	7153	1.370

SEM Observation of the Morphology of Biological Carriers and Adhering Hepatocytes

The hydrogel carriers had a good porosity and specific surface area as observed by SEM. As shown in Fig. 3, the pore size of LCC-72-C was more suitable for the growth of human hepatocytes than that of LCC-48-C and control sample (without LCC). As shown in Fig. 3, liver cells adhered to the surface of porous biological carriers. The morphology of the spherical bio-carrier demonstrated that the LCC fraction of poplar wood is a suitable material for cell bio-carriers.

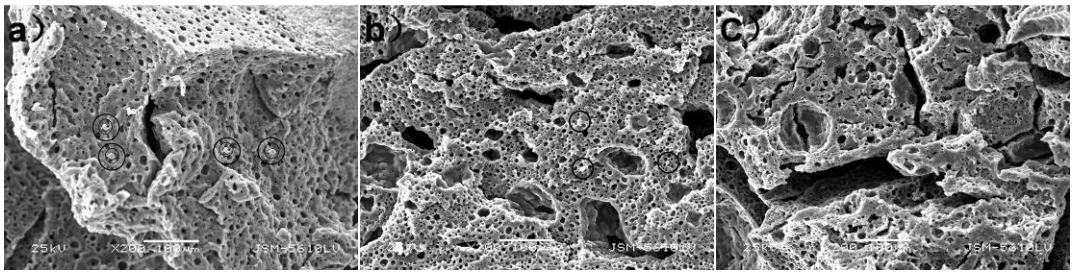

Fig. 3. Adhesion of cells in the porous carriers observed by SEM. (a) LCC-72-C; (b) LCC-48-C; (C) control sample

Cell Counts

As shown in Fig. 4, the cell number reached its highest values on the 5th day. The highest values of cell numbers of LCC-72-C, LCC-48-C, and the control experiment were 1.84 × 10^5 cells, 1.68 × 10^5 cells, and 1.32 ×10^5 cells, respectively. The cell number of LCC-72-C was obviously superior to those of LCC-48-C and control group. Therefore, LCC-72-C has better biological compatibility, and it could be used as a potential carrier for human cell culture *in vitro* (Park *et al.* 2003).

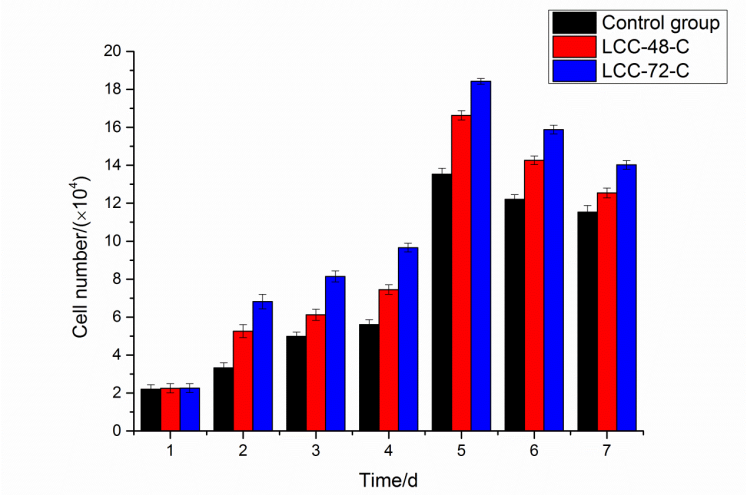

Fig. 4. Cell counting of human hepatocytes cultured in LCC-48-C medium, LCC-72-C medium, and the control group

Observations by Inverted Microscope

Inverted microscopy observations showed that hepatocytes adhered to the surface of porous carriers. As shown in Fig. 5, the number of active cells adhered to LCC-72-C was considerably more than those of LCC-48-C and the control group. These results also indicate that LCC-72-C had better biocompatibility.

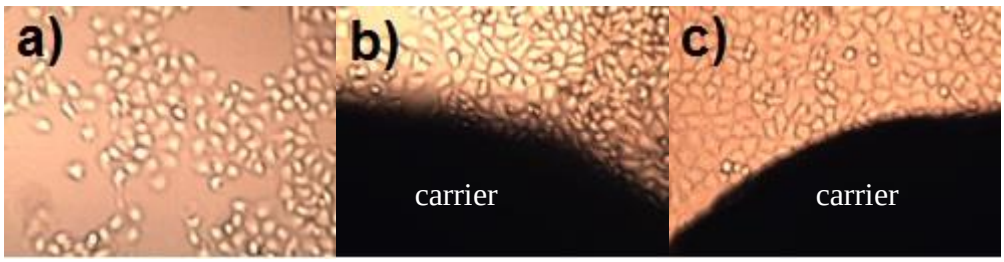

Fig. 5. The hepatocytes cultured on the fifth day of the control group (a), LCC-48-C (b), and LCC-72-C (c)

Detection of Metabolic Activity

Albumin content

As shown in Fig. 6, the activity of albumin secretion (ALB) of human hepatocytes prepared by LCC-72-C, LCC-48-C, and the control group increased during the first 5 days and reached the highest value in the fifth day. The highest values of albumin secretion of LCC-72-C, LCC-48-C, and the control experiment were 16.027 g/L/d, 14.173 g/L/d, and 10.428 g/L/d, respectively. The release of albumin (ALB) from the metabolism of hepatocytes of LCC-72-C medium was considerably superior to those of the LCC-48-C and control group during the culture process when the porous bio-carriers were applied. These results indicated that LCC-72-C has good biocompatibility for human hepatocyte culture.

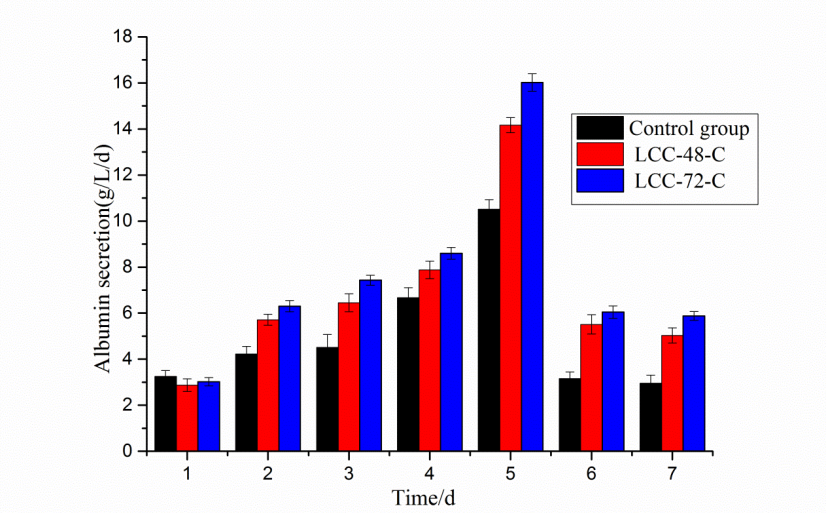

Fig. 6. Albumin secretion of human hepatocytes cultured in the carriers LCC-48-C, LCC-72-C, and the control group

Blood urea nitrogen

As shown in Fig. 7, the blood urea nitrogen (BUN) content released from human hepatocytes in LCC-72-C, LCC-48-C, and the control group increased during the first five days and reached the highest value in the cultivation of the 5th day. The highest values of BUN content of LCC-72-C, LCC-48-C, and the control experiment were 3.156 mmol/L/d, 2.873 mmol/L/d, and 1.268 mmol/L/d, respectively. The BUN content of LCC-72-C was higher than that of LCC-48-C and the control group. These results indicated that human liver cells cultured in LCC-72-C carriers had higher metabolic activity.

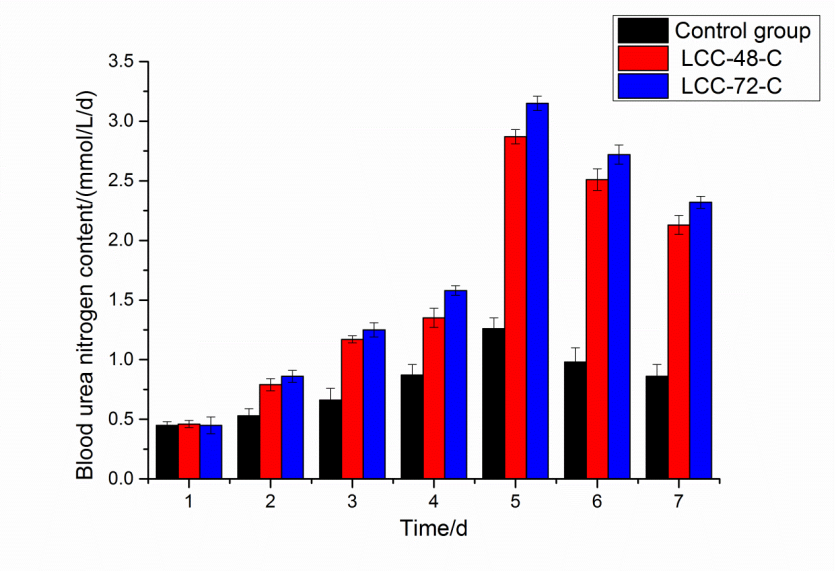

Fig. 7. Blood urea nitrogen of human hepatocytes cultured in the carriers of LCC-48-C, LCC-72-C, and the control group

Glucose consumption determination

As shown in Fig. 8, glucose consumption in human hepatocytes (L-02) cultured in LCC-72-C, LCC-48-C, and the control group reached the highest value on the 5th day of cultivation. The highest values of glucose consumption of the cells cultured in LCC-72-C, LCC-48-C, and the control group were 8.162 mmol/L/d, 7.084 mmol/L/d, and 6.823 mmol/L/d, respectively.

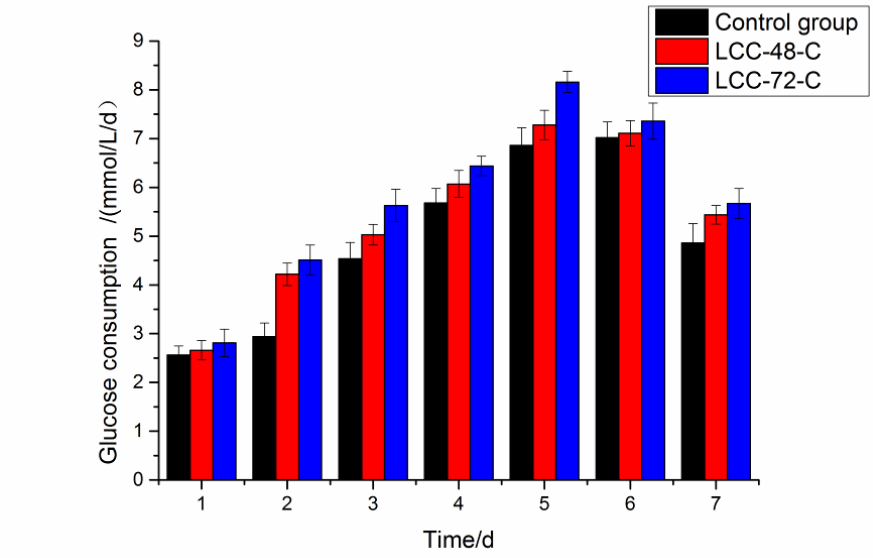

Fig. 8. Glucose consumption of human hepatocytes cultured in the carriers LCC-48-C, LCC-72-C, and the control group

These results showed that LCC-72-C was considerably superior to LCC-48-C and the control group. These results indicated that the carriers prepared with poplar LCC-72-C have satisfactory biocompatibility with human liver cells. Therefore, the LCC-72-C is a

promising biomedical carrier in the tissue engineering of the culture of liver organs from human liver cells.

CONCLUSIONS

1. The porous biological carriers were prepared from lignin-carbohydrate complexes isolated by 48 h and 72 h ball milling of poplar wood and with gelation forming method. Optical and scanning electron microscopy showed that poplar LCC-based porous carriers can provide a biocompatible medium for liver cell growth.
2. FT-IR spectra showed that the two LCCs are composed of lignin and polysaccharide, with a typical structure of lignin-carbohydrate complexes. The flexible hydrophilic polysaccharide fragments and rigid hydrophobic lignin fragments provide porous biological carriers with high strength and good amphipathicity, which meets the basic requirements of natural medical materials. LCC-48 and LCC-72 indicated contained 3.02% and 5.67% galactose, respectively. Thus, carriers prepared with LCC-72 have good compatibility with liver cells because galactose can be recognized by hepatocyte receptors**.**
3. A large number of liver cells adhered to the porous biological carrier. Cell proliferation was the fastest in LCC-72-C. The proliferation of hepatocytes in the LCC-72-C was also substantially higher than that of LCC-48-C and the control group. Albumin secretion (ALB) value, blood urea nitrogen, and glucose consumption of the LCC-72-C was considerably superior to those of the LCC-48-C and control group when the hepatocytes were cultured in the bio-carriers. These results indicate that LCC-72-C was more biocompatible and shows promise for use as a biomaterial in the culture of human hepatocytes.

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