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*Research Article*

# Lignin-Carbohydrate Complexes Based Spherical Biocarriers: Preparation, Characterization, and Biocompatibility

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Spherical biocarriers were prepared with lignin-carbohydrate complexes isolated from ginkgo (*Ginkgo biloba* L.) xylem. The specific surface and average pore size of the biocarriers were 17.15 $m^2 g^{-1}$ and 21.59 nm, respectively. The carriers were stable in solution at pH 4.0~9.5. Fourier transform infrared (FT-IR) spectrum indicated that the spherical carrier was composed of lignin and polysaccharides and had a typical lignin-carbohydrate complex (LCC) structure. The contents of galactose, lignin, and total sugar were 3.30%, 23.9%, and 64.62%, respectively, making the spherical biocarriers have good physical strength and compatible with hepatocytes. It was observed using a scanning electron microscopy (SEM) that liver cells adhered to the spherical biocarriers during culture. Cell counting indicated that the proliferation of liver cells in the experimental group was significantly higher than that of the control group. The albumin secretion (ALB) value and glucose consumption of the human hepatocytes were increased by 51.7% and 38.6%, respectively, by the fourth day when cultivated on the biocarriers. The results indicate that ginkgo LCC is very biocompatible and shows promise for the use as a biomaterial in the culture of human hepatocytes.

## 1. Introduction

Lignin is one of the most abundant natural products in the land-plant kingdom and is formed through phenolic oxidative coupling processes [1]. Lignin macromolecules are formed by the dehydrogenative polymerization of three monolignols: *p*-coumaryl, coniferyl, and sinapyl alcohols [2]. Some hemicellulose in the cell walls of lignified plants is linked to lignin to form lignin-carbohydrate complexes (LCC) [3]. With the development of analysis technology, more information has been reported in the literature describing the structure and properties of LCC, especially lignin-carbohydrate linkage (LC bond) [4–8]. As shown in Figure 1, (Galacto)glucomannan is the most common one in the softwood hemicelluloses, which is considered to be linked to lignin moieties by chemical bonds [9]. It is a branched heteropolysaccharide consisting of two glucose epimers, $\beta$-D-glucopyranose and $\beta$-D-mannopyranose, and galactose units which are bioactive for hepatocytes. Recently, LCC as a biological material has attracted attention. Many researchers have found that lignin-carbohydrate complexes are a good natural biodegradable material [10, 11]. In addition, LCC contains hydrophobic rigid lignin blocks and hydrophilic flexible polysaccharide blocks, making lignin-carbohydrate complexes have good amphipathy, biocompatibility, and mechanical strength [12]. The lignin and carbohydrate blocks in the LCC copolymer not only have an ideal structure for biomaterials, but also have good compatibility with animal cells [13]. The rigid lignin blocks can form lignin-protein complexes with membrane bound proteins in animal cells, enabling cells to grow [14]. Furthermore, the flexible polysaccharide blocks containing 2–5% galactan have the ability to recognize hepatocytes due to the presence of asialoglycoprotein receptors (ASGPR) on which galactose acts as a specific adhesive ligand on the hepatocytes [15–19] (Figure 2). Galactosylated substrates are useful biomaterials in the preparation of scaffolds for hepatocyte cultivation because of their specific interaction of the galactose moiety with the cell surface ASGPR [20].

In the literature describing lignin as a biomaterial, there have been two opinions which have been argued for many years. Some researchers think that although lignin has great strength, its hydrophobicity may affect animal cell adhesion

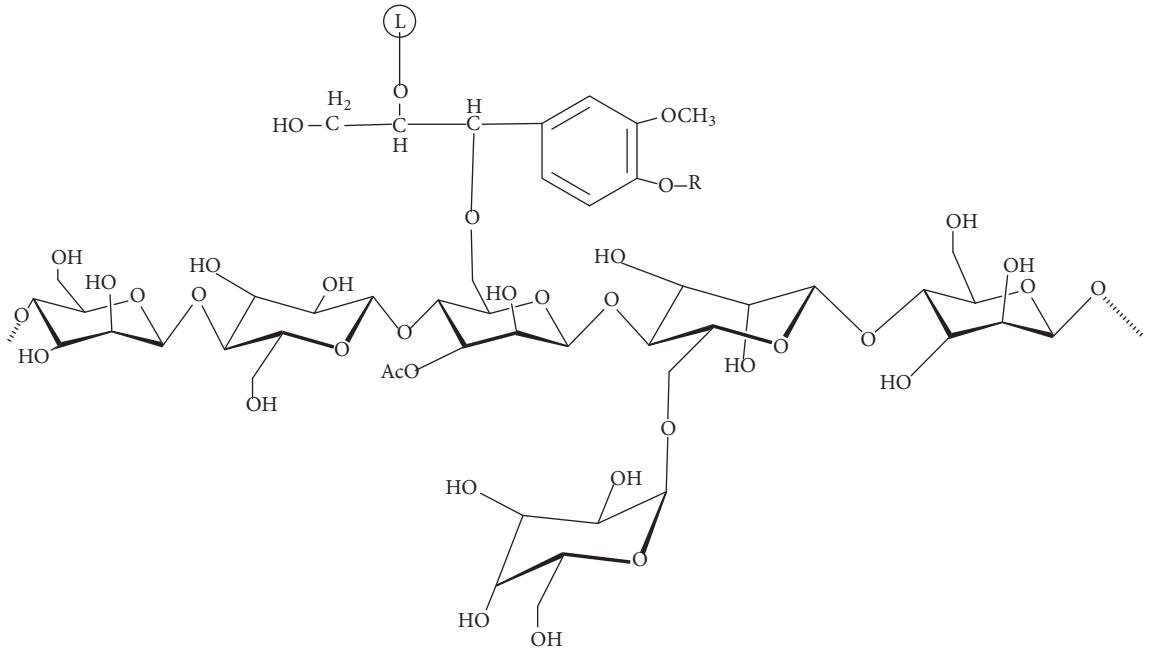



Figure 1: (Galacto)glucomannan in softwood hemicelluloses with linkage to lignin moieties (R = H or polylignol; L = polylignol).

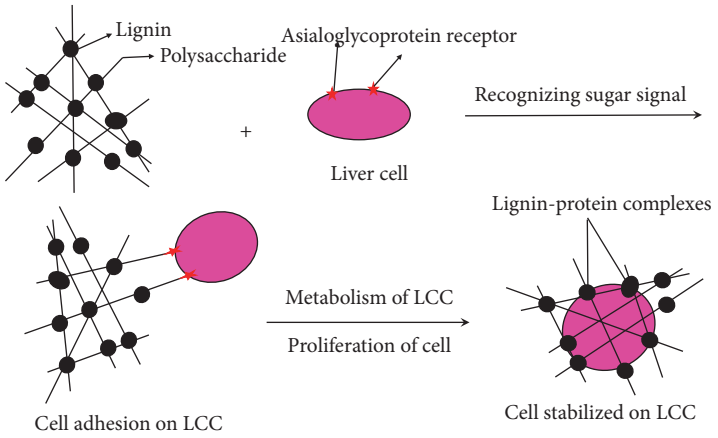



Figure 2: Binding of LCC material to hepatocyte.

on the lignin-based biocarriers. Other researchers suggest that lignin has many functional groups that possess physiological activity, such as methoxyl, phenolic hydroxyl, alcoholic hydroxyl, carboxyl, and carbonyl, which may promote normal metabolism of animal cells. Recently, Erakovic et al. studied the biocompatibility of modified lignin scaffold material and demonstrated that the lignin fragments in lignin-carbohydrate complexes not only have great strength, but also have good biocompatibility [21–23]. Moreover, Chung et al. [24] and Park et al. [25] demonstrated that the scaffolds immobilized by galactose retained a greater number of hepatocytes than those scaffolds which were unmodified or immobilized with galactose, due to specific interactions between hepatocytes and galactose moieties. Yang et al. [26] and Wang et al. [27] investigated the ability of hydrogels prepared with galactosylated acrylate (GAC) and poly (N-isopropylacrylamide) (NIPAAm) as scaffolds to proliferate hepatocytes and maintain the function of albumin and urea synthesis. They found that the cell adhesion and proliferation of hepatocytes occurred primarily on the surface of the hydrogels, suggesting that the incorporation of GAC containing galactose units could stimulate cell adsorption and growth, as compared with conventional PNIPAAm hydrogel. In our previous research, Wu et al. reported that hydrogel prepared from artificial LCC, that is, dehydrogenation polymer-(DHP-) galactose complex, has good biocompatibility with human hepatocytes [28]. However, the biocompatibility of natural LCC with hepatocytes is still open to investigation.

In order to understand the possibility of the application of natural LCC in the tissue engineering area, LCC

was isolated from ginkgo wood (*Ginkgo biloba* L.), in the group of gymnosperm. Spherical biological carriers were prepared using liquid nitrogen freeze-drying method. The spherical biocarriers with relative large specific surface area may provide more growth area for cell culture *in vitro*. Hepatocytes are difficult to proliferate in monolayer cultures and can be easily damaged by trypsin digestion and may be resolved using spherical biocarriers [29]. In this work, Fourier transform infrared spectroscopy (FT-IR), optical microscopy, high precision surface area, and pore size analyzer were used to characterize the structure and morphology of spherical biocarriers. The biocarriers were then used in the culture of human hepatocytes. The growth of the hepatocytes on the biocarriers was observed using an inverted biological microscope and scanning electron microscopy (SEM). The metabolic activities of the cells, including albumin secretion and glucose consumption, were also determined.

## 2. Materials and Methods

*2.1. Materials.* Gingko tree was obtained from Wuhan Botanical Garden. Human liver cells (L-02) were provided by Pricells Company (Wuhan, China).

*2.2. Preparation of Gingko LCC.* Gingko wood meal (40–60 mesh) was extracted using benzene/ethyl alcohol (2/1, V/V) followed by a hot water treatment and then dried in vacuo for 7 days. The extractives-free wood mill was further ground for 72 h in a vibration ball mill with water cooling. The gingko LCC was then extracted and purified using the Björkman method [30].

*2.3. Posttreatment of the LCC.* The LCC were further treated with hot water at 50°C in order to remove the water-soluble fraction which will cause the swelling of the LCC-based spherical biocarriers. The ginkgo LCC and distilled water were put into an Erlenmeyer flask. The mixture was stirred at 50°C for 8 h and then filtered. The water-insoluble LCC fraction was obtained by freeze-drying, yield: 21.7%.

*2.4. Preparation of LCC-Based Spherical Biocarriers.* The ginkgo LCC-based spherical biocarriers were obtained using the freeze-drying method, as shown in Figure 3. Firstly, 1 g water-insoluble ginkgo LCC was dissolved in 5 mL 90% (V/V) acetic acid under magnetic stirring. Secondly, the solution was dropped into liquid nitrogen using a 1 mL injector. Thirdly, the spherical biocarriers were obtained by drying the frozen beads using a freeze dryer (Labconco 195, USA). The morphology of spherical biocarriers was characterized using optical microscope and SEM.

*2.5. FT-IR Spectroscopy.* KBr pellets were prepared from 1 mg ground sample and 60 mg predried KBr. The spectrum was recorded in the range of 4000 $cm^{-1}$–400 $cm^{-1}$ using a FT-IR spectrometer (Thermo Fisher 6700, USA).

*2.6. Composition of the Spherical Biocarriers Material.* Three-milligram spherical biocarrier samples were hydrolyzed using 3 mL 72% sulfuric acid at room temperature for 60 min. The sulfuric acid was then diluted to 4% using distilled water and sample hydrolyzed for 45 min at 121°C in an autoclave. The mixture was filtered through 1G4 glass filter. The filtrate was used to detect the sugar composition of the sample using HPLC (20AT, Shimadzu) equipped with a Aminex HPX-87P column at 85°C using water as the eluent at speed of 0.6 mL $min^{-1}$. The water-insoluble fraction was used to determine acid insoluble lignin [31].

*2.6.1. SEM Observation of Spherical Biocarriers.* The spherical biocarriers were put on a silicon wafer and sprayed with gold ions in vacuo. The morphological structures of surface and cross sections were observed using a JSM-6390LV SEM.

*2.6.2. Diameter of the Spherical Biocarriers.* The samples were examined using a stereomicroscopy (Olympus SZX16, Japan) equipped with a scale. The average diameter of the spherical biocarriers was calculated.

*2.6.3. Pretreatment for Specific Surface Determination of Spherical Biocarriers.* An empty tube was weighed and marked as $m_0$. The spherical biocarriers were put into the empty tube and treated at 120°C for 4.5 h. The dried spherical biocarriers were cooled to 25°C in a cooling bath. The tube containing the biocarriers was weighed and marked as $m_1$. The value of $m_1$ minus $m_0$ was the weight of the spherical biocarriers.

*2.6.4. Determination of Specific Surface.* The nitrogen adsorption method [32, 33] was used in the determination of the specific surface using a BELSORP-mini II type high precision surface area and pore size analyzer (Ankersmid, Netherland). The Brunauer–Emmett–Teller (BET) specific surface determination is based on the gas adsorption characteristics on a solid surface. In addition, corresponding to the defined pressure, the equilibrium adsorption was definite. The equilibrium adsorption determined was equivalent to the specific surface of the sample. The formulae to calculate these are

$$Sg = 4.36 \times \frac{V_m}{m},$$
$$\frac{P}{Va\left(P_0 - P\right)} = \frac{1}{V_m \bullet C} + \frac{C-1}{V_m \bullet C} \bullet \left(\frac{P}{P_0}\right), \quad (1)$$

where Sg is specific surface area of the sample ($m^2\ g^{-1}$); $V_m$ is saturated nitrogen molecular monolayer adsorption (mL); $Va$ is the actual adsorption of the sample (mL); $m$ is the weight of the sample (g); $C$ is constant related to adsorption capacity of the sample; $P$ is adsorbent partial pressure; $P_0$ is adsorbent saturated vapor pressure.

*2.6.5. Determination of the Pore Size.* The pore size was also determined using a BELSORP-mini II type high precision surface area and pore size analyzer (Ankersmid, Netherland). The gas adsorption method was used to determine the pore size. This method is based on capillary condensation and the

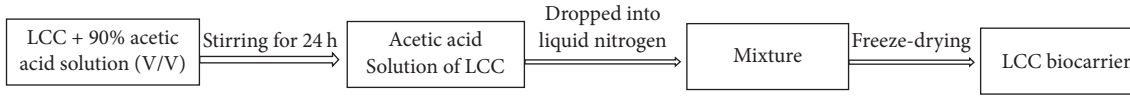



Figure 3: Preparation of LCC-based spherical biocarriers.

volume equivalent substitution. Corresponding to the ratio of $P/P_0$, there is a critical radius, Rk. The critical radius is calculated using the Kelvin equation as follows:

$$\mathrm{Rk} = \frac{-0.414}{\log\left(P/P_0\right)}. \quad (2)$$

*2.6.6. Size Stability of Spherical Biocarriers.* Five milligrams of spherical biocarriers was suspended in 5 ml of buffering solutions at various pH (acetate buffer pH 4~5, phosphate buffer pH 6.8~7.4, and sodium bicarbonate buffer pH 9.5). The size and morphology of spherical biocarriers in different buffering solutions were determined using a stereomicroscopy (Olympus SZX16, Japan) equipped with a scale after 15 days.

*2.7. Culture of Human Hepatocytes.* Human hepatocytes (L-02), obtained from Pricells Company (Wuhan, China), were rinsed with phosphate buffer. The hepatocytes were inoculated into 12-well culture plates at a density of $3 \times 10^5$ cells $\mathrm{mL}^{-1}$. The spherical biocarriers were then sterilized at 120°C for 4 h. These were then added to the wells at a concentration of 2.5 mg $\mathrm{mL}^{-1}$. The hepatocytes together with the spherical biocarriers were incubated at 37°C, 5% $CO_2$, and 100% relative humidity. The culture medium used was RPMI-1640 supplemented with 20% FBS, 1% penicillin, and streptomycin solution. The adhesion of human hepatocytes to the spherical biocarriers was observed daily using a XDS-1 inverted biological microscope. Cell-free supernatant was collected daily to detect the metabolic activity of the cells.

*2.7.1. Cell Number Counting.* On the 1~5th days of culture liquid media were collected every day. The cells were then washed twice with phosphate buffer, followed by 0.25% trypsin for 1~2 min. The media containing trypsin were then discarded. Then the new media were added to terminate the digestion. The cells were no longer adhered to the well but in a cell suspension. Two hundred mL of suspension liquid was taken and mixed with equal volume of 0.4% trypan blue staining solution. Some of the stained cells were put onto a blood cell counting plate. The number of stained cells was counted using a microscope.

*2.7.2. Observation of the Cells Adhesion on the Carrier by SEM.* On the 3rd day of cell culture, some spherical biocarriers were removed. The spherical biocarriers were fixed using 2.5% glutaraldehyde (GA) for 12 h at 4°C. The spherical biocarriers were then further fixed using 0.1% osmic acid for 30 min. After the spherical biocarriers were washed using phosphate buffer, a gradient dehydration was carried out using ethanol at concentrations of 30%, 50%, 70%, 80%, 90%, and 100%. The dried spherical biocarriers were put onto silicon wafer and sprayed with gold ions in vacuo. The hepatocytes adhered to the spherical biocarriers were observed using a JSM-6390LV SEM.

*2.7.3. Detection of Metabolic Activity.* The amount of albumin secreted was determined using the method according to the instructions of kit number A028 (provided by Nanjing Jiancheng Bioengineering Institute, China). Briefly, 10 $\mu$L distilled water, standard albumin, and cell-free supernatant were added to a test tube. After the addition of 2.5 mL bromocresol green buffer, the samples were shaken. After the reaction was carried out for 10 min at room temperature, the absorption values were monitored at 628 nm using a UV-Vis spectrophotometer (Shimadzu 2550, Japan).

$$\mathrm{ALB}\,(\mathrm{g/L}) = \frac{A_1 - A_2}{A_0 - A_2} \times C_0, \quad (3)$$

where ALB is the content (g $\mathrm{L}^{-1}$) of albumin; $A_0$, $A_1$, and $A_2$ are the absorbance values of standard tubes, sample tubes, and control groups, respectively. $C_0$ (g $\mathrm{L}^{-1}$) is the concentration of the standard.

The amount of glucose consumed by the hepatocytes was determined using the method according to the instruction of kit number CAT361500 (provided by Nanjing Jiancheng Bioengineering Institute, China). In test tubes, 10 $\mu$L distilled water, standard glucose, and cell-free supernatant were added. After the addition of 1 mL of a solution containing phosphate buffer (pH 7.0), phenol 10.6 mmol $\mathrm{L}^{-1}$, and aminoantipyrine of 70 mmol $\mathrm{L}^{-1}$, the samples were shaken. After the reaction was carried out for 15 min at 37°C, the absorption values were determined at 505 nm using a UV-VIS spectrophotometer.

$$C\,(\mathrm{mmol/L}) = \frac{A_1}{A_0} \times C_0, \quad (4)$$

where $C$ is the content (mmol $\mathrm{L}^{-1}$) of glucose. $A_0$ and $A_1$ are the absorbance values of standard tubes and sample tubes, respectively. $C_0$ (g $\mathrm{L}^{-1}$) is the concentration of the standard.

## 3. Results and Discussion

*3.1. FT-IR Analysis.* A peak of 3419.2 $\mathrm{cm}^{-1}$ was assigned to the hydroxyl groups of gingko LCC, as shown in Figure 4. The strong absorption of C-O stretch at 1035.6 $\mathrm{cm}^{-1}$ indicated the presence of polysaccharides [34]. The peaks at 1510 $\mathrm{cm}^{-1}$ and 1423.2 $\mathrm{cm}^{-1}$ were related to the vibration of aromatic structures in the lignin moieties [35]. FT-IR analysis confirmed that the spherical biocarriers had a typical LCC structure composed of lignin and polysaccharides. Rigid hydrophobic lignin and flexible hydrophilic polysaccharide fragments gave

Table 1: Composition of ginkgo LCC-based biocarrier.

| Composition | Lignin | Glucose | Xylose | Galactose | Arabinose | Mannose | Total sugars |
|---|---|---|---|---|---|---|---|
| Content % | 25.5 | 22.30 | 10.93 | 3.30 | 6.15 | 21.94 | 64.62 |

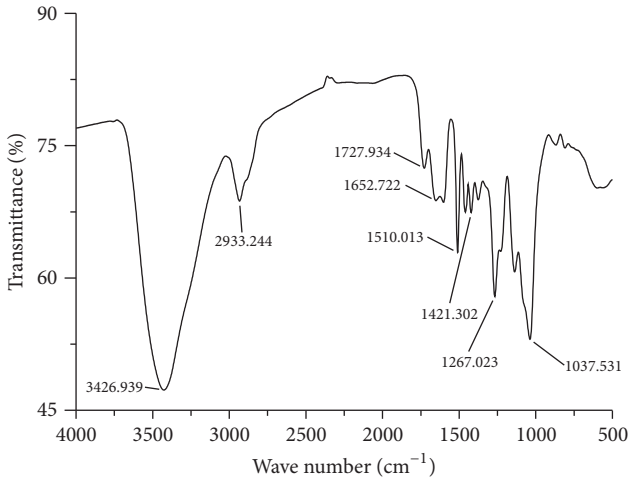



Figure 4: FT-IR spectroscopy of the LCC-based spherical biocarriers.

the spherical biocarriers good amphipathy properties and high strength, which are essential requirements of natural medical materials.

*3.2. Composition Analysis.* Galactose can be recognized by a receptor on hepatocytes and has a high physiological activity for hepatocytes [36]. Galactose was used to enhance the selective interactions between biocarriers and hepatocytes. As shown in Table 1, the content of galactose units in the LCC macromolecule was 3.30%. The contents of lignin and total sugars were 25.5% and 64.62%, respectively, which gave the spherical biocarriers good physical strength [37]. The results suggest that spherical biocarriers are suitable for use as biocarriers of human hepatocytes.

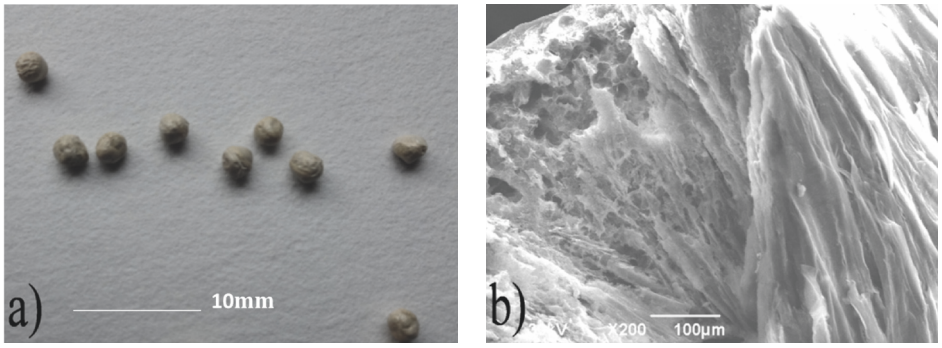
*3.3. Morphology of the Spherical Biocarriers from Gingko LCC.* The spherical biocarriers prepared with gingko LCC were porous material as observed by optical microscopy and SEM. As shown in Figure 5(a), the spherical biocarrier is seen in light gray. The morphology of the spherical biocarriers demonstrated that the LCC particles were porous and suitable for cell biocarriers under culture conditions as shown in Figure 5(b).

*3.4. Diameter Determination and the Stability.* As a biomaterial, the large diameter of the biocarriers will decrease the specific surface area, whereas small diameters will increase the density of biocarriers. Both could impact the growth of cells. Therefore the diameter of biocarriers must be in the appropriate range for the particular cells. In this study, the dry and wet diameters of the spherical biocarriers are 1.8–2.0 mm and 1.9–2.1 mm, respectively. There are some differences between dry and wet spherical biocarriers. The results indicated that the diameters of the spherical biocarriers were stable within a range that was suitable for cell culture. The stability of spherical biocarriers was determined when high concentrations of spherical biocarriers were suspended in a weak acid of pH 4-5, a neutral solution of pH 7.4, and an alkaline solution of pH 9.5. In Figure 6, it was found that the spherical biocarriers kept their intact diameter and style after 15 days in suspension. It was found that the spherical biocarriers had good stability and were suitable for cultivation at different pH.

*3.5. Specific Surface Area and Average Pore Size of the Spherical Biocarriers.* Specific surface area and average pore size of spherical biocarriers were measured using a high precision surface tester. The BET curve is shown in Figure 7. The $V_m$ value can be calculated using the linear slope and intercept in Figure 7. According to (1) and Kelvin equation (2), the specific surface area and average pore size of spherical biocarriers were calculated to be 17.15 $m^2 g^{-1}$ and 21.59 nm, respectively. The results revealed that the spherical biocarriers had a high specific surface area. Thus, the spherical biocarriers can provide enough surface for cell growth and increase the cell density. When the monolayer of cells undergoes a trypsin digestion process, the damage to cells can be reduced by the cells being easily removed from the medium, compared with conventional monolayer cell culture, because of the 3D culture structure with the use of spherical biocarriers.

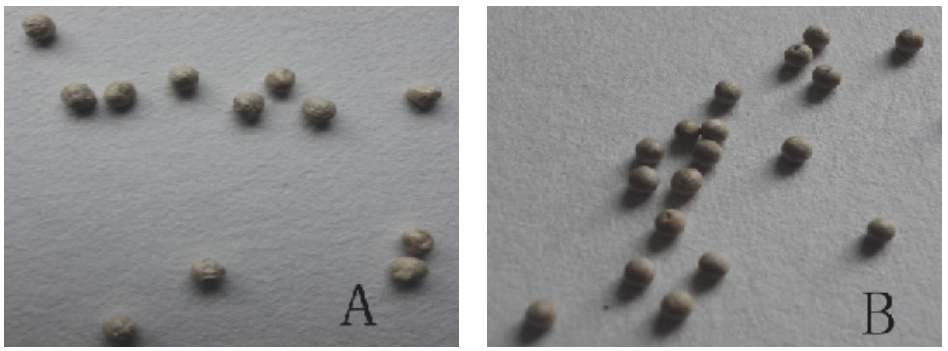
*3.6. Cell Growth and Metabolic Activities of Hepatocytes Adhered to Spherical Biocarriers.* In the presence of the biocarriers, human hepatocytes (L-02) were cultured statically. As shown in Figure 8, it was found that the majority of hepatocytes adhered to the spherical biocarriers, indicating that the LCC is nontoxic, biocompatible, and suitable for the hepatocyte culture. Using cell counting, the cell growth conditions of experimental and control groups in suspension during days 1 to 5 were observed in Figure 9. The cultured cells of the experimental and control groups during the first 3 days grew slowly. On the 4th day, the proliferation rate of the cells increased, which was greater in the experimental group compared to that of the control group.

In Figure 10, it was found that the content of albumin secretion (ALB) from the hepatocytes cultured on the porous biocarriers was significantly greater compared to the control group (without biocarriers). On the 4th day of cultivation the ALB value reached the highest level at 10.45 $g d^{-1} L^{-1}$, while the control group had a high value of 6.89 $g d^{-1} L^{-1}$. Therefore, The ALB value of the sample with the use of biocarriers was increased by 51.7% as compared with the control. As shown in Figure 11, the glucose consumption of the hepatocytes increased significantly with the use of




(a) (b)

Figure 5: Morphological observations by stereomicroscopy (a) and SEM (b) of the spherical biocarriers prepared from gingko LCC.




(a) (b)

Figure 6: The morphology of spherical biocarriers before (a) and after 15 days (b) suspended in pH 7.4.

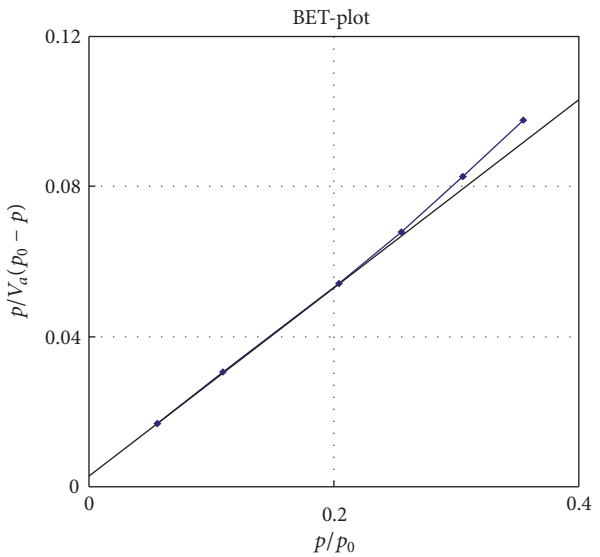


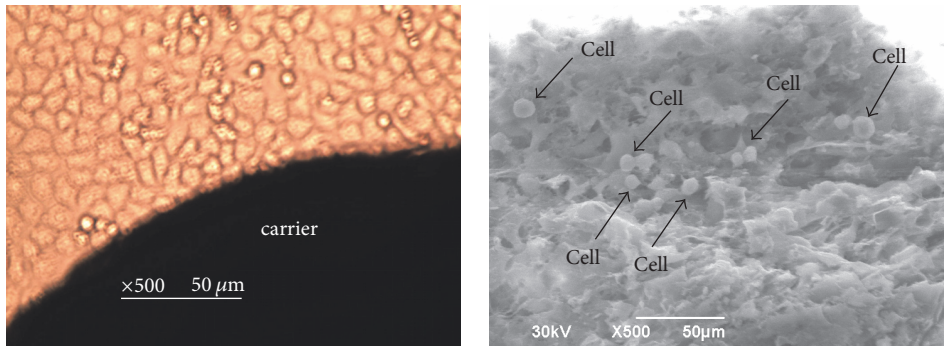

Figure 7: BET adsorption isotherm of the spherical biocarriers.

biocarriers when compared to the control experiment. In the 4th day of cultivation, glucose consumption reached the highest value of 14.0 mmol $d^{-1}$ $L^{-1}$, while the highest value was 10.1 mmol $d^{-1}$ $L^{-1}$ in the control group. This suggests that the glucose consumption value of sample using biocarriers is enhanced by 38.6% as compared with the control. These results indicated that the biocarriers using ginkgo LCC had biocompatibility with human hepatocytes. The LCC are a promising biomedical polymers that could be used in the tissue engineering of culture hepatocytes to create hepatic organs.

## 4. Conclusions

(1) The spherical carriers were prepared using lignin-carbohydrate complexes from poplar wood with the liquid nitrogen freeze-drying method. It was found that the carriers were stable in aqueous solution. The specific surface area and average pore size were 17.154 $m^2$ $g^{-1}$ and 21.59 nm, respectively. The specific surface analysis and SEM results indicated that the spherical carriers prepared from gingko LCC could provide a large cell growth platform.

(2) The FT-IR spectral analysis indicated that the spherical carriers were composed of lignin and polysaccharides. The spherical biocarriers had a typical LCC structure. The chemical analysis indicated that the contents of galactose, lignin, and total sugars were 3.30%, 23.90%, and 64.62%, respectively, giving good




(a) (b)

Figure 8: An inverted microscope image (a) and SEM image (b) of the human hepatocytes L-02 cultured on the spherical biocarriers.

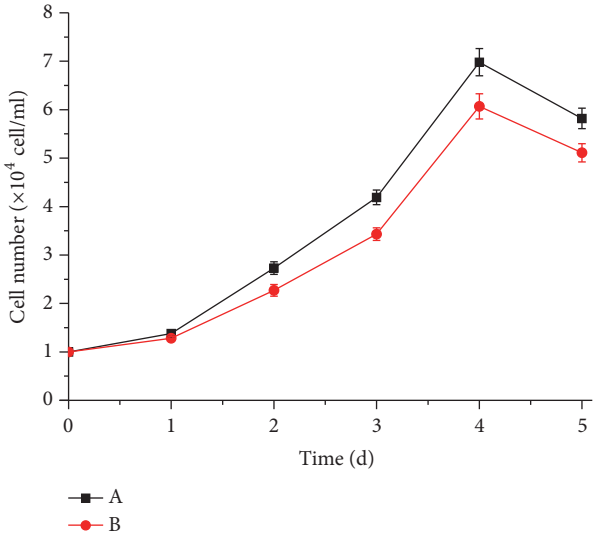



Figure 9: Cell counting of hepatocyte growth on spherical biocarriers (A) and control medium (B).

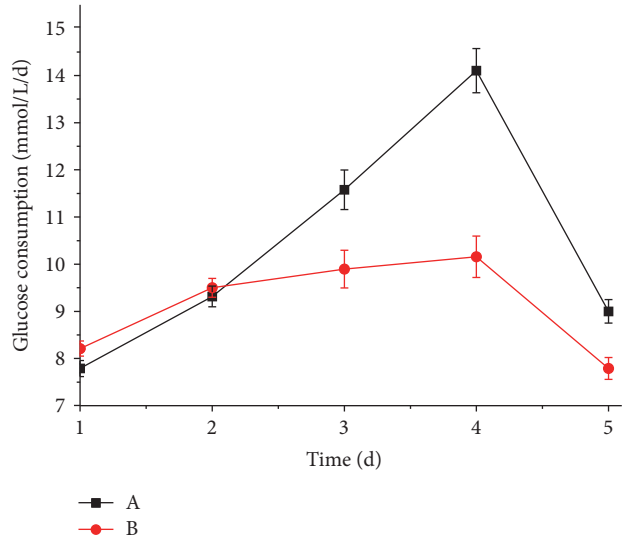



Figure 11: Glucose consumption of human hepatocytes cultured on biocarriers (A) and conventional media (B).

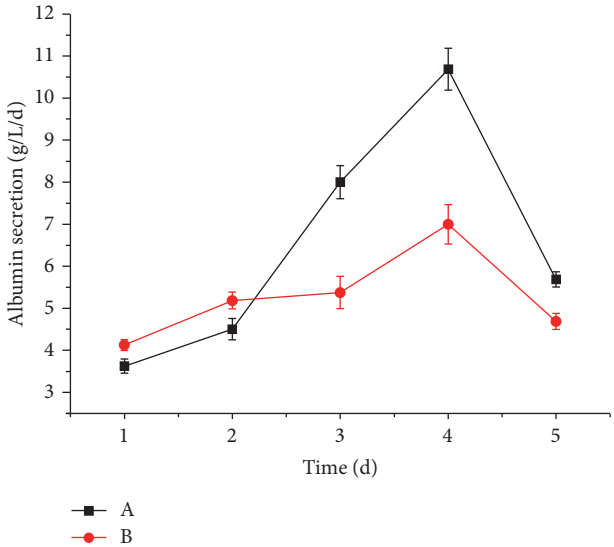



Figure 10: Albumin secretion by human hepatocytes cultured on biocarriers (A) and conventional media (B).

physical strength and compatibility of the biocarriers to hepatocytes.

(3) Cell counting showed that the cells increased faster than those of control group. It was also found that albumin secretion (ALB) value and glucose consumption of the human hepatocytes were enhanced by 51.7% and 38.6%, respectively, when cultivated on the biocarriers. The results indicate that material of ginkgo LCC is very biocompatible and shows promise for use as a biomaterial in the culture of human hepatocytes.

## Conflicts of Interest

The authors declare that they have no conflicts of interest in this work.

## Acknowledgments

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