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Dynamic Seeding and Perfusion Culture of Hepatocytes with Galactosylated Vegetable Sponge in Packed-Bed Bioreactor

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A galactose moiety was introduced into the fiber surface of a vegetable sponge by the covalent binding of lactobionic acid. The galactosylated sponge was used as scaffold for the culture of rat hepatocytes in a packed-bed bioreactor. Hepatocytes could be dynamically seeded into and uniformly distributed throughout the scaffold, and the immobilized cells maintained high albumin and urea production rates during long-term perfusion culture. The hepatocytes showed an increasing albumin production rate from 49 to 109 μg/10^6 cells/d over the 7-d culture.

[**Key words:** bioartificial liver, hepatocytes, bioreactor, galactose, vegetable sponge]

A bioartificial liver device using hepatocytes cultured in a bioreactor is expected to be used for treating severe liver insufficiencies, such as fulminant hepatic failure. A clinically applicable bioartificial liver device must achieve efficient, large-scale, and high-density hepatocyte culture. The use of three-dimensional (3D) matrices for hepatocyte culture is more effective in promoting the expression of a differentiated phenotype than that of conventional two-dimensional substrates (1–7). A novel 3D macroporous vegetable sponge from the fibrous interiors of dried fruits of the plant *Luffa cylindrica* can be a compatible bed for hepatocytes (8). In this study, to facilitate its intended use in a bioartificial liver device, a flow system with a vegetable sponge cylinder packed in a column reactor was designed. The system was studied for the dynamic seeding and perfusion culture of rat hepatocytes. Hepatocytes cultured in a flow perfusion system can offer more advantages than those cultured in a traditional static culture system because it mimics hepatic circulation and permits an efficient continuous transport of gas and nutrients through the 3D matrix to the cells. A galactose moiety was introduced into the scaffold to further improve the loading efficiency and metabolic performance of hepatocytes. The resulting galactosylated sponge is expected to facilitate hepatocyte adhesion through the receptor-mediated interaction of asialoglycoprotein receptors (ASGPR) of hepatocytes with galactose moieties along the fiber surface of the 3D macroporous scaffold and to enhance cell functions such as albumin secretion and urea synthesis (9–11).

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MATERIALS AND METHODS

Materials A vegetable sponge was prepared from the peripheral part of a ripened dried fruit of *L. cylindrica*. It had a porosity of 90±10%, a density of 0.92±0.05 g/cm^3, a specific pore volume of 31±6 cm^3/g, a pore size of 335±65 μm, and a fiber diameter of 0.63±0.22 mm. Lactobionic acid (LA) was purchased from TCI (Tokyo). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxylsuccinimide (NHS) were purchased from Pierce (Rockford, IL, USA). All the other chemicals used were purchased from Sigma (St. Louis, MO, USA), unless otherwise stated.

Isolation of hepatocytes and cell culture conditions Hepatocytes were isolated from male SD rats weighing 250 to 300 g by two-step collagenase perfusion. The viability of the isolated hepatocytes was >85%, as assessed by a trypan blue exclusion test. The isolated hepatocytes were cultured in Williams' E medium supplemented with 10% fetal bovine serum, 0.1 μM dexamethasone, 0.1 μM insulin, and antibiotics (200,000 IU/*l* penicillin G, 200 mg/*l* streptomycin, and 500 mg/*l* amphotericin B) at 37°C in a 5% CO_2, 95% air environment with a daily medium change.

Preparation and analysis of galactosylated LS To convert the chitin in the sponge to chitosan by deacetylation, the sponge was boiled in 40% NaOH at 110°C for 5 h. The coupling of lactobionic acid (LA) to the deacetylated sponge was carried out with EDC and NHS as coupling reagents, which catalyze the formation of amide linkages between the carboxyl groups of LA and the amine groups of the sponge. Fine millimolar LA was reacted with the deacetylated sponge in 30 ml 2-morpholinoethane sulfonic acid (MES) buffer (pH 6.0) containing 1 mg/ml NHS and 3 mg/ml EDC at room temperature for 72 h. The amount of LA conjugated was determined by mass balance after subtracting unreacted LA by assaying the residual sugar concentration in the solution. Fourier-transformed infrared (FTIR) spectra were recorded using a Horiba FT-730 FTIR spectrometer (Horiba, Kyoto). Dried samples were ground into fine powder, mixed with KBr powder and compressed into pellets for FTIR spectrometry.

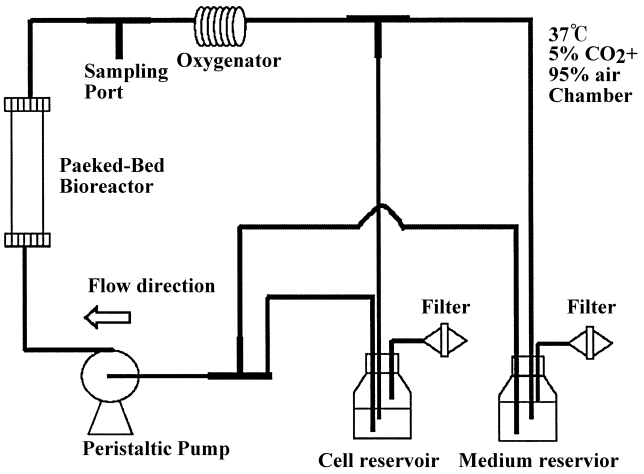

FIG. 1. Schematic diagram of bioreactor system for dynamic seeding and perfusion culture of hepatocytes.

Dynamic seeding and flow perfusion culture in bioreactor A homemade flow perfusion culture system was fabricated in our laboratory by adding a dynamic cell-seeding loop in line with the perfusion culture loop (Fig. 1). The packed-bed bioreactor was a glass cylindrical vessel (ID=0.8 cm, length=11.25 cm) end-capped with two PVC end pieces. A sponge with the same dimensions as those of the interior of the bioreactor was packed into the reactor. The culture medium was circulated through the bioreactor from the bottom by a peristaltic pump through 3.1-mm-ID silicon tubings. The loop consisted of a 100-ml medium reservoir and an oxygenator (oxygenation silicon tubing, length=1.66 m, ID=7 mm). The entire seeding/perfusion unit was sterilized using an autoclave and maintained at 37°C with 5% CO_2 inside a CO_2 incubator. The isolated hepatocytes (7×10^7 cells) were suspended in 60 ml of culture medium using a magnetic stir bar and a stirrer at 60 rpm. The hepatocytes were dynamically seeded into the scaffold placed within the bioreactor at 5 ml/min for 12 h. After the flow seeding period, the reservoir bottle containing the cell suspension was replaced with 60 ml of fresh culture medium and the medium was recirculated through the loop at 18 or 34 ml/min for 12 h to flush out any loosely attached cells. This time period was determined beforehand to be adequate for obtaining a cell-free effluent from the bioreactor. After the flush period, the loop was switched to a new reservoir bottle containing 60 ml of fresh culture medium for perfusion culture. The culture medium was replaced daily and the spent medium was analyzed for metabolites.

Analytical Methods The number of hepatocytes was determined by DNA content measurement using Hoechst dye 33258 at excitation and emission wavelengths of 360 and 460 nm, respectively. Prior to DNA measurement, scaffolds with immobilized hepatocytes were frozen and thawed twice to lyse the cells completely. Urea-N concentration was determined by the urease method, albumin concentration by ELISA, and lactate dehydrogenase (LDH) concentration using a commercial kit from Sigma.

RESULTS AND DISCUSSION

Characterization of galactosylated LS The deacetylated sponge was found to exhibit characteristic absorption bands at 1655 and 1585 cm^{-1}, which were attributed to amides I and II of chitosan, respectively, and at 1380 cm^{-1} (–CH_2 bending) (Fig. 2, line 1). The characteristic peaks of the polysaccharide structure of chitosan appeared at 1161 (antisymmetrical stretching of the C–O–C bridge), 1084, and 1040 cm^{-1} (skeleton vibrations involving C–O stretching). LA exhibited a broad –OH absorption in the region between 3000 and 3800 cm^{-1}, and a distinct band at 1740 cm^{-1} due to the carbonyl stretching (C=O) of carboxyl groups (Fig. 2, line 2). After LA was coupled to the sponge, the carbonyl stretching of LA disappeared owing to amide bond formation (Fig. 2, line 3). In addition, the –OH stretching peak of LA shifted from 3400 cm^{-1} to approximately 3460 cm^{-1}, indicating enhanced intermolecular hydrogen bonding after introducing LA into the scaffold.

The degree of deacetylation increased from 33±2% to 82±5% after alkaline treatment (8). The amount of LA coupled was calculated to be 83.4±5.6 mg/cm^3 (N=7), which translated into 1.77 μmol of galactose per cm^3 of scaffold. This value is in agreement with the ligand concentration for enhancing hepatocyte functions in 3D scaffolds (12).

Dynamic seeding of hepatocytes For 3D hepatocyte culture in scaffolds in a bioreactor, the effective cell seeding method must be compatible with scaffold chemistry and

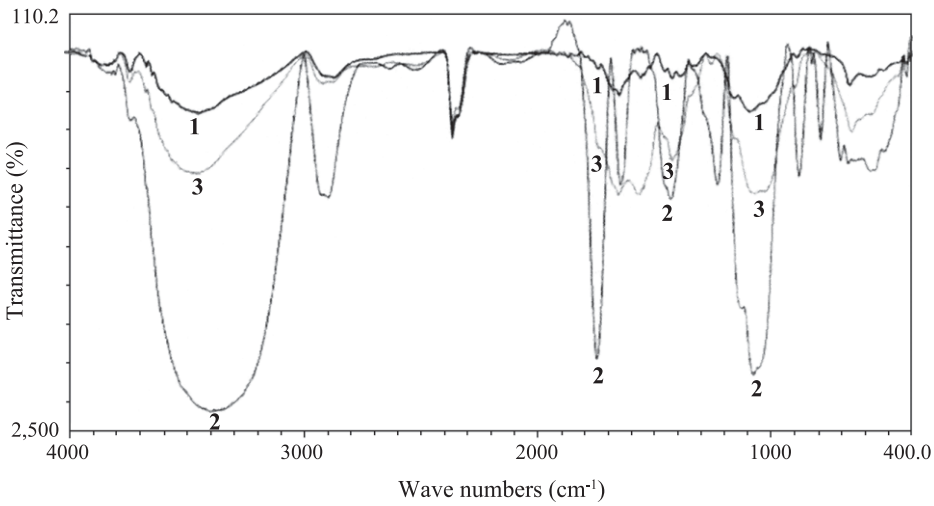

FIG. 2. FTIR spectra of deacetylated vegetable sponge (line 1), lactobionic acid (line 2), and galactosylated vegetable sponge (line 3).