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Fabrication and characterization of chitosan microcarrier for hepatocyte culture

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Abstract Using chitosan as raw materials, a suitable size (300–500 μm) of porous microcarrier was fabricated by suspension crosslinking and lyophilizing method, which made the carrier has an average pore size of 50 μm and 86% porosity. The microcarrier was modified with lactose and maltose respectively. Various factors that influenced the preparation of microcarrier were studied and the reaction conditions were optimized. Rat hepatocytes cultured on modified microcarrier retained a spherical shape which is similar to those in vivo and formed aggregates. The metabolic activities of cells on lactose-modified were higher than those on maltose-modified microcarrier. The highest albumin secretion reached 54.8 μg/10^6 cells/d, and the highest urea synthesis reached 4.65 μmol/10^6cells/d, which may be promoted by the formation of cellular aggregates. In conclusion, lactose-modified porous microcarrier is promising scaffold for hepatocytes culture.

Introduction

Bioartificial liver support system (BALS) is a promising application of tissue engineering for the treatment of fulminant hepatic failure (FHF) because of its effective treatments which might lead to complete recovery [1, 2]. The development of BALS requires a large number of cells to be cultured for the replacement of the damaged tissue. For this application, porous structures microcarrier along with well-interconnected open pores are required for allowing high density of cell seeding and efficient nutrient and oxygen supply to the seeded cells [3].

Chitosan, the partially deacetylated derivative of chitin, is a suitable biomaterial to be used as a scaffold for various biomedical and pharmaceutical uses because it is biocompatible and biodegradable [4–7]. An additional reason for selecting chitosan as a scaffold for hepatocytes culture is that its structure is similar to glycosaminoglycans (GAGs), which are components of the liver extracellular matrix [8, 9]. Lactose is a specific ligand for asialoglycoprotein receptor on hepatocytes. Chitosan has a large number of suitable functional groups, which can be covalently linked with lactose [10]. In this study, lactose modified porous chitosan microcarriers was fabricated and characterized. The morphology of cultured hepatocytes was examined. In addition, the abilities of hepatocytes to secrete metabolites such as albumin and urea were evaluated for 6 days.

Materials and methods

Materials

Chitosan (molecular weight 1.2×10^6, deacetylation degree 90%) was purchased from Haiyang Biochemical Co.Ltd, China; Male Wistar rat and fetal calf serum were purchased from Academy of Military Science, Tianjin, China; Reagent kit for albumin was purchased from Beijing Zhongsheng Com and Reagent kit for urea analysis from Baoding Great Wall Co. Ltd., China.

Unless otherwise stated, all other chemicals were analytical grade made in China.

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Fabrication of Macroporous Chitosan Microcarrier (MCM)

Around 50 mL of chitosan solution (dissolved in 2.5% acetic acid) was added to 200 mL liquid paraffin containing 2% w/v span 80. The mixture was mechanically stirred at 500 rpm for 30 min at 40 °C. A certain amount of glutaraldehyde was added to the reaction system and maintained for half an hour. The system was then adjusted to alkaline by 6% NaOH solution and maintained for another 2 h. When the reaction was completed, the microcarrier was washed with petroleum ether, alcohol and distilled water successively to elute the liquid paraffin and excessive glutaraldehyde. Then the carrier was freezed and MCM was obtained by the lyophilizing method.

The reduction of the Schiff's base was performed by adding sodium borohydride to the MCM in the presence of 30 mmol/L sodium acetate. The reaction was maintained overnight at room temperature under dark condition.

Modification of MCM

Around 2 mL 10% (w/v) sodium hydroxide solution and 1.6 mL epichlorohydrin (EPI) was added to 1 g MCM. The reaction was carried out at 30 °C for 4 h. The microcarrier was washed by distilled water successively to elute the excessive EPI. Then 1 g microcarrier was added to 4 mL pH 4.5 sugar solution, after 12 h reaction at 40 °C, sugar modified MCM were obtained. The reacted lactose or maltose was determined by measuring the sugar concentration by using the method of 3.5-dinitrosalicylic acid (DNS) [11].

Measurement of the porosity

Porosity of the MCM was determined by liquid displacement method [12].

Rat hepatocytes isolation and culture

Hepatocytes were isolated from male Wistar rat (200–300 g) by modified two-step collagenase perfusion according to the method of Seglen [13, 14]. Assessed by trypan blue exclusion, hepatocytes with more than 90% viability were suspended in complete WE medium (basal WE medium supplemented with 10% fetal calf serum, 0.292 mg/mL L-glutamine, 0.2 U/mL insulin, 80 U/mL gentamicin sulphate) and used for further experiments. MCM was placed into 6-well culture plates (Costar co.) (2 mg/each well with 2 mL WE medium). After MCM was settled, the supernatant was removed carefully and 2 mL complete WE medium was added to each well. Hepatocytes suspension in 2 mL complete WE medium containing 1×10^6cells was seeded to MCM in the 6 well culture plates and incubated at 37 °C in 5% CO_2 atmosphere with humidity. After 6 h the time which was necessary for cell attachment, the medium was removed and 3 mL fresh medium was added. The medium was changed every day thereafter and the cells on MCM were observed by light and scanning electron microscope.

Morphology of scaffolds and cultured hepatocytes

Cell-seeded scaffold was fixed with 2.5% glutaraldehyde in PBS at 4 °C for 12 h, and then carefully washed twice with PBS. Cells were dehydrated in a graded ethanol solution, and finally in pure ethanol. Samples were then dried and coated with ultrathin layer of gold in a coating apparatus. Visualization was carried out by using a scanning electron microscope (Hitachi X-650, Japan).

Determination of albumin secretion and urea synthesis

During the culture of hepatocytes, samples were collected every 24 h and assayed. The amount of albumin secreted was determined by the method following the instruction of the kit. Briefly, in 24-well plates, 5 μl standard albumin or samples were added respectively, after the addition of 1.5 mL succinate buffer (pH 4.2) containing bromocresol green (0.15 mmol/l) and polyxyethylene lauroyl ether Brij-35 (30%), the samples were shaken evenly. The reaction was carried out for 5 min at 25 °C, the color change was monitored spectrophotometrically at 630 nm.

Urea synthesis determination was also conducted according to the instruction of the kit. Briefly, in 24-well plates, 25 μl standard urea or samples were added, followed by the addition of 1 mL A liquid (containing H_2SO_4, H_3PO_4, benzaldehyde, ammonium metavanadate) and 0.1 mL B liquid (iodopyrine). After reaction for 15 min at 37 °C, the color change was monitored spectrophotometrically at 600 nm. The rate of urea synthesis was calculated by Lambert–Bill equation.

Table 1 Effect of the concentration of chitosan to the characters of microcarrier

Concentration of chitosan (%)	Surface and rigidity	Porosity (%)
1.5	Irregular surface, bad rigidity, irregular pore	–
2.5	Regular surface, good rigidity and high porosity	86
3.5	Regular surface, good rigidity and lower porosity	73

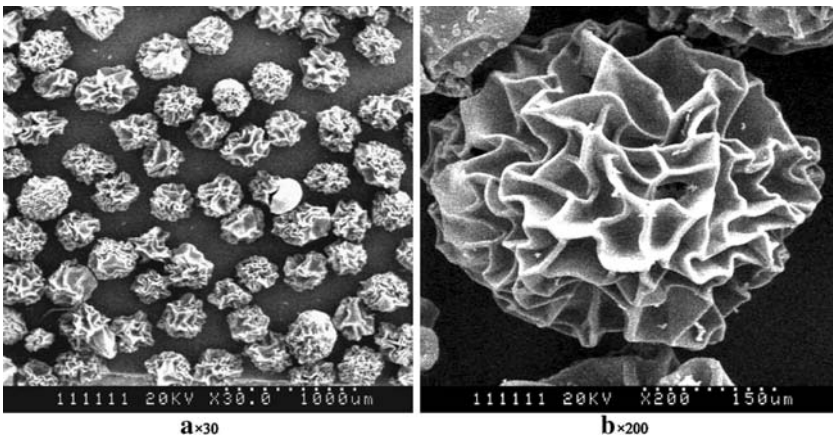

Fig. 1 SEM images of porous chitosan microcarrier

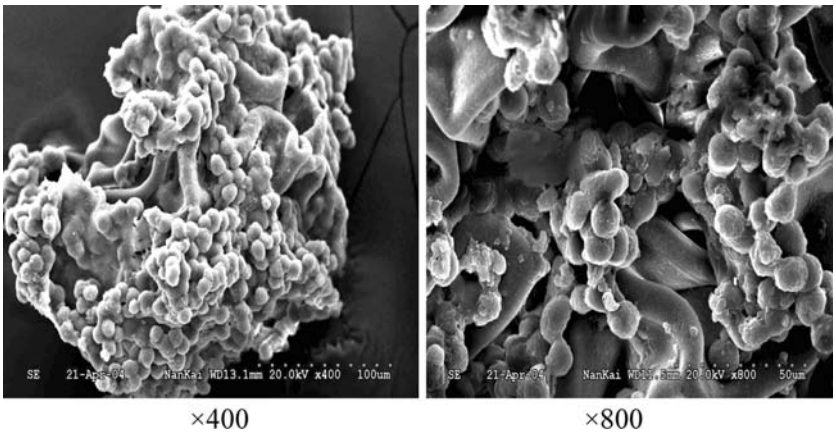

Fig. 2 SEM images of hepatocytes cultured on lactose modified MCM

Results and discussion

Fabrication of Macroporous Chitosan Microcarrier (MCM)

The concentration of chitosan solution and the amount of glutaraldehyde are two important factors that influence the appearance, rigidity, and porosity of MCM. In the preparation of MCM, when the concentration of chitosan solution reached to 2.5%, the MCM has a regular surface and good rigidity with a porosity of 86% (Table 1).

Optimization of the reaction conditions of sugar modification

In the sugar modified reaction, pH and time are two important factors that can influence the modification of MCM. After the modification reaction for12 h at pH 4.5 the reacted lactose reached the highst value.

Morphology of MCM and cultured hepatocytes

SEM result shows the MCM having a pore diameter in the range of 30–70 μm (Fig. 1). Hepatocytes cultured on lactose modified microcarrier exhibited a round cellular morphology similar to the cells in vivo (Fig. 2). There are many microvilli on the surface of the cells, indicating a healthy state.

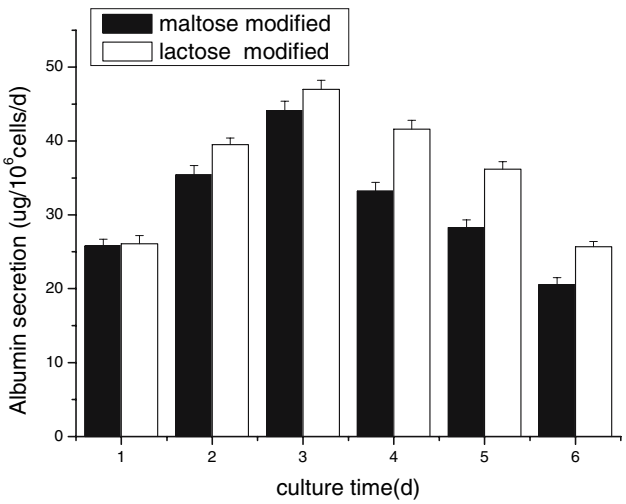

Fig. 3 Albumin secretions of hepatocytes cultured within MCMs modified with lactose and maltose

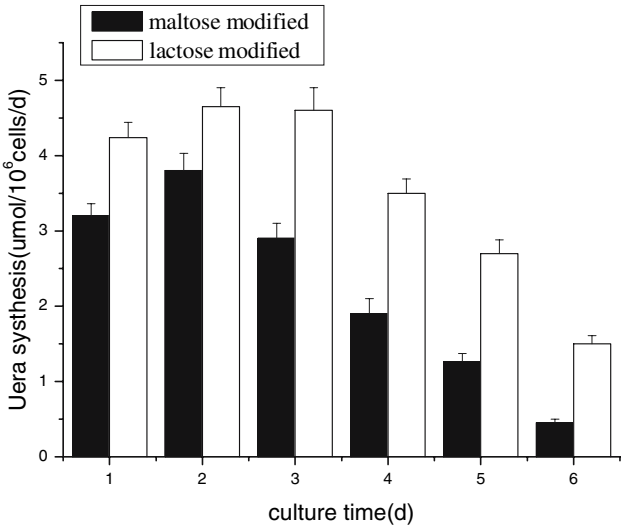

Fig. 4 Urea syntheses of hepatocytes cultured within MCMs modified with lactose and maltose

Metabolic activities

The ability of cultured hepatocytes to perform liver-specific functions was evaluated. Albumin secretion and urea synthesis results are depicted in Figs. 3 and 4 respectively. Generally, albumin secretion was maintained over the period of 6 days. On lactose modified MCM, albumin production was higher than that on maltose modified carrier.

As shown in Fig. 4, cells on lactose modified MCM has much higher ability for urea synthesis than that on maltose modified carrier, which maintained a higher level in the first four days.

Conclusion

In this study, lactose and maltose were linked on the porous chitosan microcarrier respectively. The results of cells morphology and metabolic activities showed that lactose modified chitosan microcarrier is a better scaffold than the maltose one. The highest albumin secretion of lactose modified MCM reached 54.8 μg/10^6cells/d, and the highest urea synthesis reached 4.65 μmol/10^6cells/d.

In conclusion, lactose modified MCM may be promising scaffold in the development of the hybrid bioartificial liver support system.

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