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Optimization of Chitosan-Alginate Encapsulation Process Using Pig Hepatocytes for Development of Bioartificial Liver

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Abstract Chitosan-alginate capsules were formed by electrostatic interactions and exhibited an appropriate mechanical strength, permeability, and stability for the culture of hepatocytes. Pig hepatocytes were isolated and hepatocyte spheroids formed and immobilized in chitosan-alginate capsules. An encapsulation procedure of 3 min and spheroid formation period of 24 h were the optimum conditions for the best liver functions. Pig hepatocytes with a cell density of 6.0×10^6 cells/ml in the capsules were found to be most suitable for application in a bioartificial liver support system. The encapsulated pig hepatocyte spheroids exhibited stable ammonia removal and urea secretion rates in a bioreactor for 2 weeks. Accordingly, chitosan-alginate encapsulated hepatocyte spheroids in a packed-bed bioreactor would appear to have potential as a bioartificial liver.

Key words: Encapsulation, alginate, chitosan, collagen, hepatocyte, bioartificial liver

To treat fulminant hepatic failure (FHF) patients, various extracorporeal bioartificial liver support systems have already been developed. Yet, several requirements need to be met for the development of a bioartificial liver support system: hepatocytes should be cultured in a sufficiently high density, their metabolic functions should be of a sufficiently high level and duration, and the bioartificial liver support system module should permit scaling-up and aseptic handling [19, 21, 22]. As such, the encapsulation of hepatocytes into semipermeable membranes is a promising technique for the development of extracorporeal bioreactors or the transplantation of immunoisolated hepatocytes for the treatment of liver failure [1, 18, 27].

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Microencapsulation allows the diffusion of nutrients to the hepatocytes and diffusion of metabolic products, such as albumin and clotting factors, from the hepatocytes. The capsule membrane is permeable to small molecules, such as glucose, oxygen, and insulin, yet totally impermeable to large molecules, such as immunoglobulin [2, 7, 8, 10, 13, 16, 17]. However, if encapsulated hepatocytes are to be used for the development of a bioartificial liver system, the mechanical strength of the capsules must be high enough to maintain its shape in the shear of a bioreactor. Among the various capsule fabrication methods, chitosan-alginate capsules have been reported to show the highest mechanical strength [12]. Chitosan and alginate are readily available, biocompatible, and have been used to encapsulate various plant cells and mammalian cells for effective secondary metabolite and antibody production [3, 23]. Furthermore, chitosan offers the potential to control the pore size of the membrane and membrane diffusional properties through the influence of the solution concentration, ionic strength, and pH [9, 11, 24, 26, 28].

Several investigators have already found that freshly isolated primary hepatocytes can be cultured into three-dimensional, tightly packed, freely suspended, multicellular aggregates or spheroids [6, 15, 25, 28]. These specialized cell structures exhibit enhanced liver-specific functions and a prolonged differentiated state compared to cells maintained in a monolayer culture. Extensive cell-cell contacts and tight junctions have also been observed in the specialized cell structure, along with microvilli-lined channels reminiscent of bile canaliculi. Plus, the cells in spheroids appear to mimic the morphology and ultrastructure of the *in vivo* liver lobule. The ability of hepatocytes to organize into three-dimensional structures has been hypothesized to contribute to their enhanced liver-specific activities [25].

Accordingly, this study examined the ammonia removal rate and urea secretion rate of pig hepatocyte spheroids

encapsulated in chitosan-alginate capsules to determine the optimum conditions for the encapsulation procedure. As such, a packed-bed bioreactor with encapsulated pig hepatocytes was devised as a bioartificial liver (BAL) support system, and the efficacy of the system was evaluated *in vitro*.

MATERIALS AND METHODS

Hepatocytes Isolation and Spheroids Formation

A pig (weighing 120 kg) was slaughtered at a slaughterhouse (Dongwon Industries Co., Ltd., Busan, Korea) using the normal slaughtering process (electric shock and death through exsanguination). The carcass was then transferred to a boiling water bath for cleaning, the abdominal cavity opened, and the liver excised. Next, the liver was transported to the laboratory in an ice-box filled with perfusion buffers (30±10 min), then the left medial liver lobe was carefully excised [14]. The anatomic site of perfusion, as well as the following details on the perfusion buffers, were derived from practical experience: the tissue was perfused at a flow rate of 100 ml/min for 10 min via a left hepatic vein using an oxygenated perfusion buffer (NaCl 8 g/l, KCl 0.4 g/l, $NaH_2PO_4 \cdot 2H_2O$ 0.078 g/l, $Na_2HPO_4 \cdot 12H_2O$ 0.151 g/l, glucose 0.9 g/l, $NaHCO_3$ 0.35 g/l, HEPES 2.38 g/l, EDTA 0.19 g/l). Meanwhile, the liver lobe was perfused at a flow rate of 100 ml/min for 20 min using a perfusion buffer supplemented with collagenase (0.5 g/l) and calcium chloride (0.56 g/l). The liver was then filtered through two nylon meshes (grid size 200 μm) and the cell pellets washed twice with a William's E medium at 500 rpm for 5 min. The isolated hepatocytes that exhibited a viability of more than 95% using the trypan blue exclusion method and freshly isolated hepatocytes were used for the encapsulation and spheroid formation.

For the spheroids formation, the isolated pig hepatocytes were inoculated at a cell density of 5×10^5 cells/ml into a spinner flask containing 100 ml of a culture medium and stirred using a magnetic stirrer at 60 rpm. After 24 h of culture, the hepatocyte spheroids were harvested and encapsulated.

Culture Conditions

A hormonally defined medium (HDM) was used for the culture medium, which was William's E medium supplemented with an epidermal growth factor (EGF, 20 μg/l), insulin (10 mg/l), hydrocortisone (1.7 mg/l), $CuSO_4 \cdot 5H_2O$ (24.97 μg/l), $ZnSO_4 \cdot 7H_2O$ (14.38 pg/l), H_2SeO_3 (3 μg/l), linoleic acid (50 mg/l), $NaHCO_3$ (1.05 g/l), HEPES (1.19 g/l), penicillin (58.8 mg/l), and streptomycin (0.1 g/l). The encapsulated hepatocytes were cultured in the HDM at pH 7.4 on a 6-well plate, and 1 mM NH_4Cl was added to measure the ammonia removal rate. The medium was changed daily and cultured under a humidified atmosphere of 10% CO_2 and 90% air at 37°C. The spent media were completely replaced with fresh media everyday. In addition, to evaluate the liver-specific functions, the discarded media were also collected daily and stored at 4°C prior to analysis.

Encapsulation Procedure

The chitosan (0.5%, w/v) was dissolved in a 0.5% (w/v) glutamic acid solution containing $CaCl_2$ (11.09 g/l). The pH of the chitosan solution was adjusted to 6.0 to provide a membrane pore size that would prevent the diffusion of exogenous cells and immune system components. Meanwhile, the alginate (0.5%, w/v) was dissolved in deionized water. Phosphate-buffered saline (PBS, pH 7.4) was used to wash the capsules. The harvested hepatocytes cells or spheroids were suspended in the chitosan solution containing 0.05 mg/ml of collagen. The procedure used to prepare the capsules is shown in Fig. 1. The encapsulation procedure was also performed under aseptic conditions. The encapsulated hepatocytes were cultured in the HDM on a 6-well plate, and 1 mM NH_4Cl was added to measure the ammonia removal rate.

Bioreactor and Bioartificial Liver (BAL) Support System

The BAL support system consisted of a medium reservoir, pump, bubble trapper, and cylindrical-type chitosan-alginate

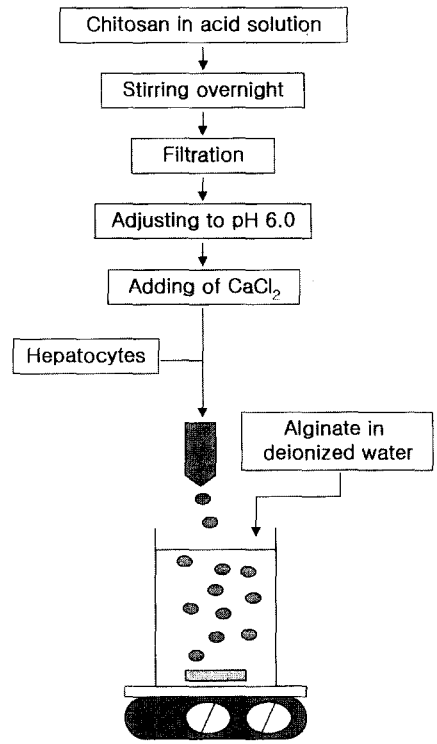


Fig. 1. Encapsulation procedure. Chitosan/cell suspension was dropped into 0.5% alginate solution.
After 3 min, the prepared capsules were collected by retention on a mesh screen and washed with a PBS solution. The capsules were then transferred to the HDM for incubation.

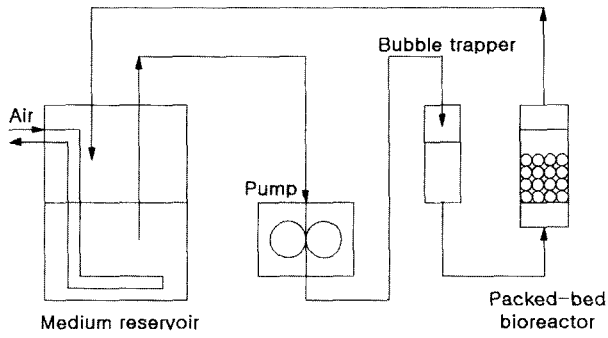


Fig. 2. Schematic diagram of bioartificial liver system.
Pig hepatocytes spheroids immobilized in chitosan-alginate capsules were used in the bioartificial liver. The BAL consisted of a medium reservoir (80 ml), bubble trapper, and cylindrical-type packed-bed bioreactor (20 ml) containing encapsulated hepatocytes.

packed-bed bioreactor containing the encapsulated hepatocytes. The medium flow rate was about 20 ml/min and the total volume of the circulation system, including the circulation line, was 100 ml; 80 ml for the reservoir and 20 ml for the reactor, including the tubing space. A schematic diagram of the bioreactor is shown in Fig. 2. The total cell number in the BAL support system was 5×10^7 cells seeded at 1×10^7 cells/ml in a capsule solution of 5 ml, as shown in the schematic diagram of the capsule and the reactor (Fig. 3).

Measurement of Liver-Specific Functions

The ammonia removal rate and urea production rate were measured using the procedures described by Yu *et al.* [28]. To determine the ammonia removal rate, 640 μl of sodium tungstate (100 g/l) and 160 μl of the sample were added to each test tube, followed by the addition of 1.0 ml of a color reagent I containing phenol (10 g/l) and sodium nitroprusside (50 mg/l), and 1.0 ml of a color reagent II containing NaOH (5 g/l), $Na_2HPO_4\cdot12H_2O$ (53.6 g/l), and 1% sodium hypochlorite. After gentle vortexing, the tubes were allowed to stand at 37°C for 20 min and the samples analyzed for the absorbance at 630 nm. To determine the urea production rate, the test tubes were filled with 3 ml of a color reagent based on a mixture of a 20 ml solution of diacetylmonoxime (6 g/l) and thiosemicarbazide (0.3 g/l) and 100 ml of 34% H_3PO_4, followed by the addition of 100 μl of the sample. After gentle mixing, the tubes were allowed to stand at 100°C for 10 min and the samples analyzed for the absorbance at 540 nm. The rates for all the culture experiments noted were calculated from each concentration in a 24-h culture supernatant and divided by the inoculated cell number. All points were the average of triplicate tests.

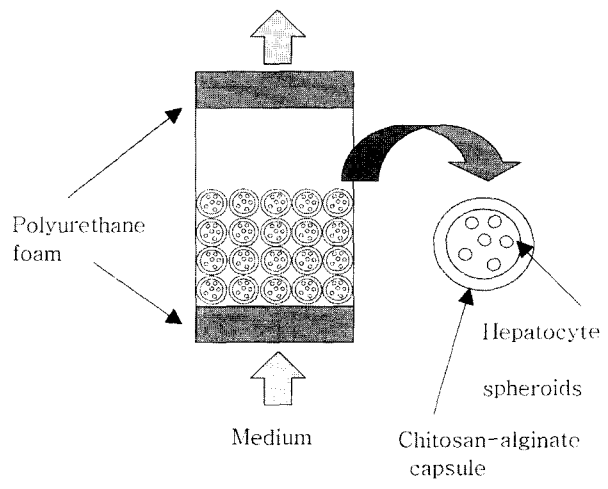


Fig. 3. Bioreactor of bioartificial liver system for *in vitro* test.
A total of 5×10^7 hepatocytes were immobilized with a density of 1.0×10^7 cells/ml chitosan-alginate capsules. Porous supports were placed at the top and bottom of the bioreactor for capsule retention.

Results and Discussion

Effects of Encapsulation Time on Liver Functions of Pig Hepatocytes in Chitosan-Alginate Capsules

Isolated primary pig hepatocytes were encapsulated and cultured in capsules using an HDM. The encapsulation

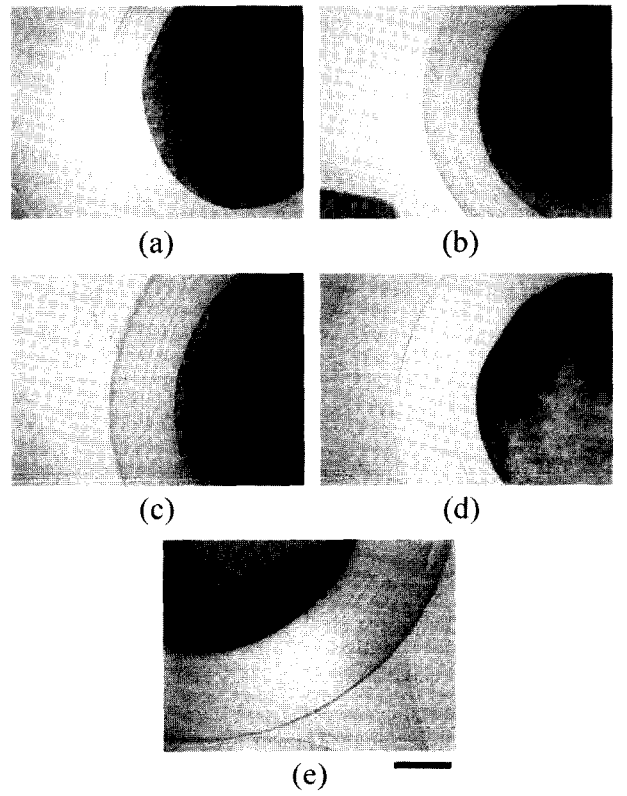
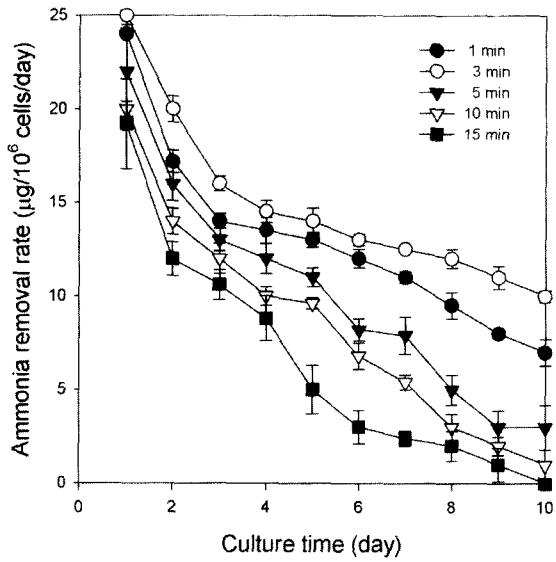
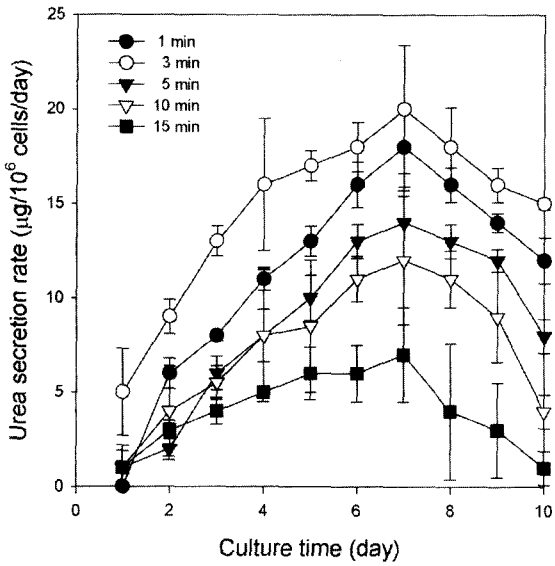


Fig. 4. Micrographs of chitosan-alginate capsule membranes prepared with (a) 1 min, (b) 3 min, (c) 5 min, (d) 10 min, and (e) 15 min process times.
Bar indicates 300 μm.



(a) Ammonia removal rate



(b) Urea secretion rate

Fig. 5. Effect of encapsulation time on ammonia removal and urea secretion rates of pig hepatocytes in chitosan-alginate capsules.

was carried out at a cell density of 1.5×10^6 cells/ml in a chitosan solution. Figure 4 shows the micrographs of the capsule membranes with different encapsulation process times. With a process time of 1, 3, 5, 10, and 15 min, the thickness of membrane was 205, 310, 379, 448, and 500 μm, respectively. As the encapsulation process time increased, the capsule gel layer became thicker.

The ability of the hepatocytes to perform liver-specific functions was evaluated using the encapsulated hepatocytes prepared with different process times. Figure 5(a) shows the ammonia removal rates for the encapsulated hepatocytes with 1, 3, 5, 10, and 15 min process times, which were 17.2, 20.0, 16.0, 14.1, and 12.2 μg/10^6 cells/day, respectively, after 2 days of incubation, then decreased to 7.0, 10.0, 3.0, 1.0, and 0.1 μg/10^6 cells/day, respectively, after 10 days of incubation. Meanwhile, as shown in Fig. 5(b), the urea production rates were 18.0, 20.5, 14.0, 12.0, and 7.1 μg/10^6 cells/day from the hepatocytes encapsulated during 1, 3, 5, 10, and 15 min, respectively, after 7 days of incubation, then decreased to 12.0, 15.0, 8.0, 4.0, and 1.0 μg/10^6 cells/day, respectively, after 10 days of incubation, due to a decline in cell-specific activity after 7 days of incubation.

In the case of the capsules prepared in 1 min, the capsule membrane was weak and easily broken, yet the encapsulated hepatocytes prepared during more than 5 min showed low liver functions, as shown in Fig. 5, indicating that the capsules had a limited diffusion of nutrients and O_2 through the capsule membrane due to the thickness of the Ca^{2+}-alginate gel [10]. Thus, the capsule membrane needs to be permeable to small molecules, such as nutrients and oxygen, but its mechanical strength must also be high enough to maintain its shape against the shear force in the bioreactor [27]. Therefore, the encapsulation of the hepatocytes during 3 min was found to be the optimum process time for the application of encapsulated hepatocytes in a bioartificial liver as regards the liver functions.

Effects of Spheroids Formation Time on Liver Functions of Pig Hepatocytes in Chitosan-Alginate Capsules

Freshly isolated primary pig hepatocytes were aggregated into larger cell clumps that eventually formed into tightly packed spherical cell structures after 24 h. Within 1, 24, 48, and 72 h after inoculation, the hepatocyte spheroids were harvested and encapsulated. The inoculum cell density was 1.5×10^6 cells/ml and the average diameter of the capsules was 2±0.2 mm, allowing unlimited O_2 supply [12].

To evaluate the liver functions of the encapsulated hepatocytes spheroids, the ammonia removal and urea secretion rates were determined, as shown in Figs. 6(a) and 6(b), respectively. The spheroids maintained a compact morphology, with a mean diameter of approximately 200 μm. The ammonia removal rates for the encapsulated spheroids formed during 1 h, 24 h, 48 h, and 72 h were determined as 16.2, 17.0, 11.4, and 6.5 μg/10^6 cells/day, respectively, after 3 days of incubation, then decreased to 6.5, 9.7, 4.1, and 3.0 μg/10^6 cells/day, respectively, after 11 days of incubation. The urea secretion rates were 14.8, 16.9, 13.8, and 13.1 μg/10^6 cells/day for the spheroids formed during 1 h, 24 h, 48 h, and 72 h, respectively, after 2 days of incubation, then increased to 16.9, 20.7, 13.1, and 9.2 μg/10^6 cells/day, respectively, after 11 days of incubation. As such, the spheroids formed during 24 h exhibited the best liver functions.

The maintenance of viable cells requires a good mass transfer to supply oxygen and nutrients and remove

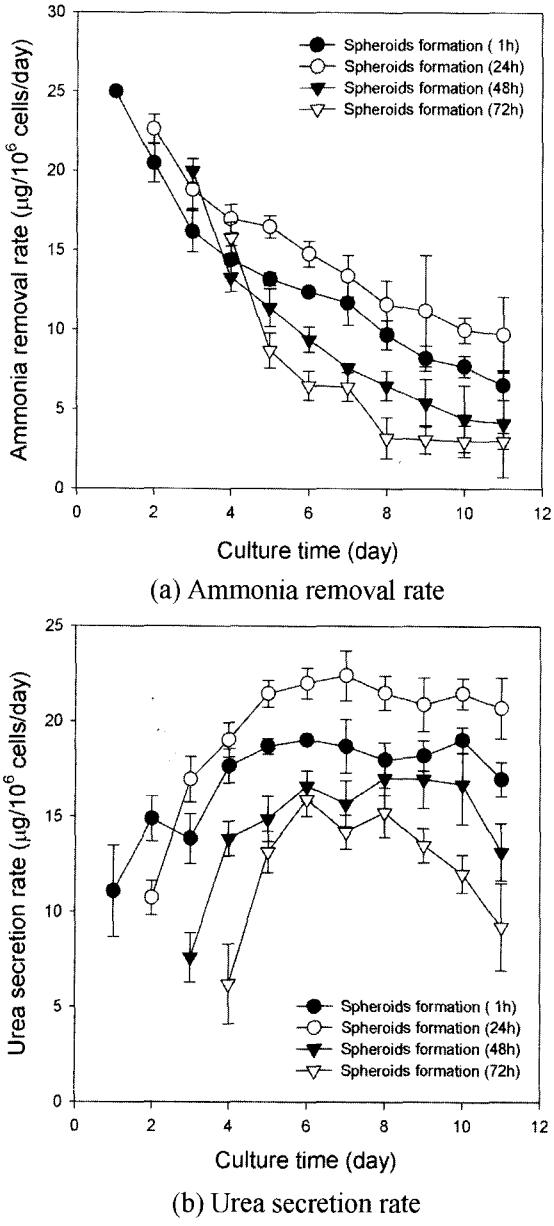


Fig. 6. Effect of spheroids formation time on ammonia removal rate and urea secretion rate of pig hepatocytes in chitosan-alginate capsules.

metabolic wastes. Therefore, the spheroid diameter is important, as the mass transfer in the center of the spheroid is limited by the diffusion. In the spheroid culture, the diameter can be controlled by the inoculation cell density and spheroid formation time. In this study, multicellular spheroids, 100 to 150 μm in diameter, were formed in the spinner flask after 24 h of inoculation. Thus, hepatocyte spheroids prepared based on 24 h of suspension culture were found to be the optimum cell source for a bioartificial liver system as regards the hepatic functions.

Effects of Cell Density on Liver Functions of Pig Hepatocytes in Chitosan-Alginate Capsules

One of the important factors involved in designing a liver support system is the amount of liver tissue required to provide adequate bioactive support to the patient. For a device with a clinical reality, it must be scaled to a size that provides effective therapy. Studies indicate that between 10% and 30% of the normal liver mass is needed to sustain life, which in adults corresponds to 150 g to 450 g of cells. In the case of a 70 kg male, a 10% value corresponds to 1.5×10^{10} hepatocytes. The actual number of cells used in

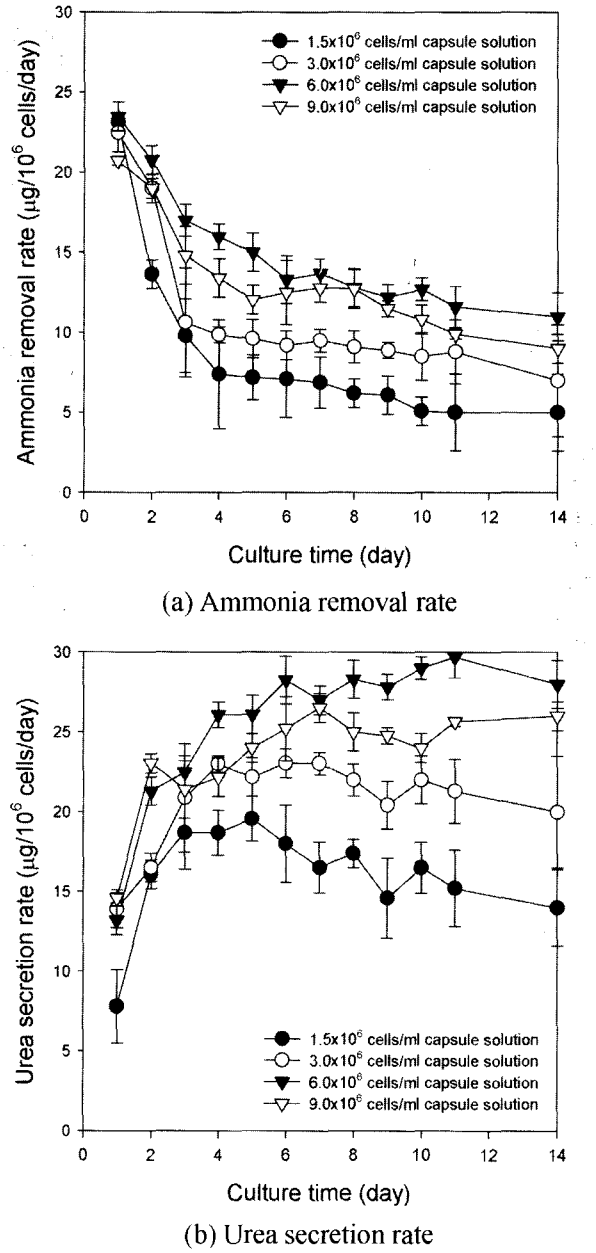


Fig. 7. Effect of cell density on ammonia removal and urea secretion rates of pig hepatocytes in chitosan-alginate capsules.

previous studies of hepatocyte-based systems has varied from 0.5×10^8 to 2.0×10^{10} [5, 20]. Figures 7(a) and 7(b) show the ammonia removal and urea production rates for the encapsulated hepatocytes containing different cell densities. The ammonia removal rates for the encapsulated hepatocytes containing 1.5×10^6, 3.0×10^6, 6.0×10^6, and 9.0×10^6 cells/ml in the capsule solution were determined as 9.8, 10.6, 17.0, and 14.8 μg/10^6 cells/day, respectively, after 3 days of incubation, which then decreased to 5.0, 8.8, 11.6, and 9.9 μg/10^6 cells/day, respectively, after 11 days of incubation. Meanwhile, the urea secretion rates were 15.2, 21.3, 29.7, and 25.7 μg/10^6 cells/day for the encapsulated hepatocytes containing 1.5×10^6, 3.0×10^6, 6.0×10^6, and 9.0×10^6 cells/ml in the capsule solution, respectively, after 11 days of incubation. Therefore, a pig hepatocyte density of 6.0×10^6 cells/ml in the capsule solution was found to be most suitable for application in a bioartificial liver support system when using chitosan-alginate capsules. The encapsulated hepatocyte spheroids with 9.0×10^6 cells/ml exhibited weaker liver functions compared to those with 6.0×10^6 cells/ml, probably due to the diffusion limitation through the capsule membrane. Thus, to satisfy a BAL system for clinical human use, 2 l of encapsulated hepatocyte spheroids with 6.0×10^6 cells/ml are required (more than 10% of total human liver cells).

Liver-Specific Functions of Pig Hepatocytes in a Bioartificial Liver (BAL) Support System

The hepatocyte spheroids were encapsulated in chitosan-alginate capsules and packed in the bioreactor of the BAL system. To develop a bioartificial liver support system, the hepatocytes should be cultured in a sufficiently high density, and their metabolic functions be of a sufficiently high level and duration [19]. In the above results, the encapsulated hepatocyte spheroids with 9.0×10^6 cells/ml exhibited weaker or similar liver functions compared to those with 6.0×10^6 cells/ml. Yet, a BAL system for clinical human use requires encapsulated hepatocyte spheroids with a high cell density of 1.0×10^7 cells/ml. Assuming that the minimum cell mass necessary to support a patient undergoing acute liver failure is about 5- 10% of the total liver weight, a bioartificial liver containing about 10^{10} hepatocytes with a priming volume not exceeding 1 l is still a daunting challenge. Therefore, pig hepatocyte spheroids with a cell density of 1.0×10^7 cells/ml were encapsulated during 3 min and packed in the bioreactor.

The metabolic activities of the pig hepatocytes in the packed-bed bioreactor were then evaluated in terms of the ammonia removal and urea secretion rates over a 2-week period as shown in Fig. 8. With a culture medium containing 1 mM ammonium chloride and cell density of 5×10^5 cells/ml, the highest ammonia removal rate was 28 μg ammonia/10^6 cells/day on the first day of operation. After 3 days of operation, the encapsulated hepatocyte spheroids exhibited an ammonia removal rate of above 70%, then after 2 weeks, the encapsulated hepatocytes spheroids maintained a 50% ammonia removal rate

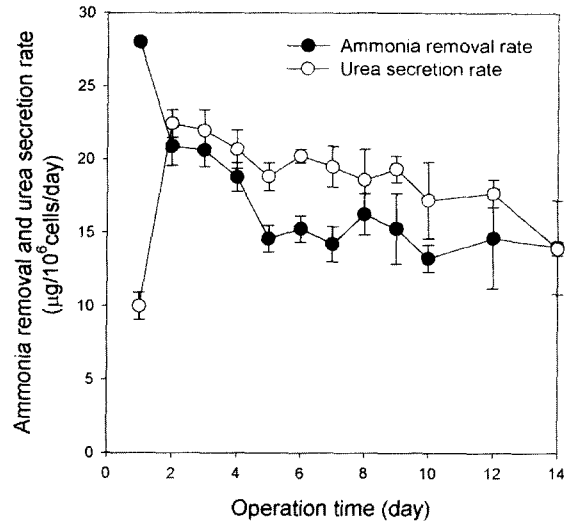


Fig. 8. Ammonia removal and urea secretion rates of chitosan-alginate encapsulated pig hepatocytes in a bioreactor.

compared to that on day 1. Meanwhile, during the 14-day circulation operation period, the urea secretion rate increased to 23 μg/10^6 cells/day after 2 days of operation, then the rate was maintained at around 20 μg/10^6 cells/day until day 12 and decreased to 14 μg/10^6 cells/day after 14 days of incubation.

The most widely investigated bioreactor at present is based on a hollow fiber membrane, however, difficulties remain in building a scale-up module. Moreover, the semipermeable membrane can act as a diffusional barrier between the hepatocytes and the plasma in the patient, plus xenogenic hepatocytes are placed directly in contact with plasma without a secure immunological barrier. Accordingly, this study presented a packed-bed or fluidized-bed bioreactor filled with microencapsulated hepatocyte spheroids as a promising design for a bioartificial liver device, due to the potential advantages of a high mass transport rate and optimal microenvironment for hepatocyte cultures. Furthermore, it was shown that encapsulated hepatocyte spheroids in chitosan-alginate operated with a packed-bed bioreactor have potential as a bioartificial liver on a weekly basis. One of the essential elements for bioartificial liver development is to sustain the liver-specific functions of hepatocytes over a long time. Hepatocytes embedded within alginate beads have been found to retain their functions *in vitro* for up to 1 week and express liver-specific functions at high levels [4]. This study also confirmed that the liver-specific functions were kept at high levels with encapsulated hepatocyte spheroids in a packed-bed bioreactor as a BAL system. In fact, the encapsulated pig hepatocytes remained viable and functional for at least 2 weeks. As such, it is hoped that this system will offer further clues to the design of an artificial liver device system. However, more work on prolonged viability and liver functions in plasma perfusion or *in vivo* is required before clinical trials can be done.

Acknowledgments

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