J. Microbiol. Biotechnol. (2001), **11**(3), 423–427

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

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Effect of Collagen Concentration on the Viability and Metabolic Function of Encapsulated Hepatocytes

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Received: December 7, 2000
Accepted: May 15, 2001

Abstract Chitosan/alginate capsules were formed by electrostatic interactions and had appropriate mechanical strength, permeability to albumin, and stability to hepatocytes. Rat hepatocytes were isolated and immobilized in chitosan/alginate capsules. During the encapsulation process with hepatocyte, 10% of viability was decreased mainly due to the low pH of the chitosan solution. Among various capsule fabrication methods, the chitosan-alginate capsule showed the highest mechanical strength. Addition of collagen in the capsule with hepatocytes enhanced hepatic metabolism as well as the cell viability for 2 weeks of culture. The hepatocyte in the capsule without collagen decreased the viability to 10% for 2-week cultures.

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

Encapsulation of living cells and tissue in polymeric membranes has been developed as a potential tool in the field of bioartificial organ development [1, 8, 9, 14]. When encapsulated hepatocytes were used for transplantation or as a extracorporeal device, the capsule membrane prevented the passage of cellular components and humoral immune molecules, but permitted passage of the secreted metabolic products, and nutrients such as glucose and O_2 [1, 3, 4, 5, 6, 7, 10].

For the application of encapsulation technology, features such as a gentle process protocol, adequate permeability to all essential nutrients, substrates, and wastes, the membrane material suitable for cell attachment, retaining of active metabolic state, and sufficient strength of capsule for fluidized bed perfusion culture should be considered. A number of methods have been described for the encapsulation of mammalian cells. However, most of the methods are not appropriate to satisfy these criteria. Encapsulation using chitosan and alginate has been attractive because of its simplicity, mild condition, and the selective permeation by controlling pore size [5, 6, 13, 14].

Since hepatocytes are anchorage-dependent cells, the appropriate attachment substratum is required for their biological functions. The collagen as a specific attachment substrate has been reported for hepatocytes culture [7, 8, 12]. Hepatocytes *in vivo* contact indirectly with blood through collagen-based extracellular matrix gel layers. Various culture methods mimick the *in vivo* cellular environment in which collagen plays an important role for the maintenance of the cellular function of hepatocytes. In this study, hepatocytes mixed with collagen type I were immobilized in capsules formed by the electrostatic interactions between chitosan and alginate. We observed the viability and metabolic activities of encapsulated hepatocytes and evaluated the effects of collagen addition to encapsulated hepatocytes culture.

MATERIALS AND METHODS

Preparation of Cells for Encapsulation

Hepatocytes were harvested from male Sprague-Dawley rats, weighing approximately 250 g, by a two-step *in situ* collagenase perfusion technique modified from the method described by Seglen [11]. The liver was perfused *in situ* for 10 min via the portal vein with 250 ml of perfusion buffer (NaCl 9 g/l, KCl 0.42 g/l, glucose 0.99 g/l, $NaHCO_3$ 2.1 g/l, HEPES 4.77 g/l). After the initial flushout, a perfusion buffer supplemented with collagenase (0.5 g/l) was perfused at a flow rate of 20 ml/min for 10 min. The liver was filtered through a filter gauge and the hepatocytes were obtained by percoll gradient centrifugation. The isolated hepatocytes showed more than 85% of viability by the trypan blue exclusion method and freshly isolated hepatocytes were used for the encapsulation.

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Encapsulation Procedure

Chitosan (0.5%, w/v) was dissolved in 0.5% (w/v) glutamic acid and 0.1 M $CaCl_2$ was added to the chitosan solution. The pH of the chitosan solution was adjusted to 6.0 to provide a barrier between exogenous cells and immune system components. Alginate (0.5%, w/v) was dissolved in deionized water. Phosphate-buffered saline (PBS, pH 7.4) was used to wash the capsules. Harvested hepatocytes were suspended in the chitosan solution containing 0, 0.05, or 0.5 mg/ml of collagen. Capsules were prepared according to the previous report [14]. Encapsulated hepatocytes were cultured in Dulbecco's modified Eagle's medium (DMEM, pH 7.4) supplemented with 10% fetal bovine serum (FBS) in a T-flask and 1 mM NH_4Cl was added to measure ammonia removal rate. The medium was changed daily and cultured under a humidified atmosphere of 10% CO_2 and 90% air at 37°C. Spent media were replaced completely everyday with fresh culture media and stored at 4°C for the analyses.

Determination of Capsule Strength

The mechanical strengths were determined by measuring the force required to rupture a 2.5-mm diameter capsule using uniaxial compression between parallel plates. Capsules of approximately 2.5 mm in diameter were formed using the optimized protocols and allowed to equilibrate with the culture medium (DMEM, pH 7.4). Ten capsules from the batches of each formulation were tested using a texture analyzer (TA-XT2i, Stable Micro Systems, U.K.). After making contact with the capsule, the probe was moved downward at a speed of 1.0 mm/s until the capsule was ruptured. The average rupture force was recorded in Newtons.

Determination of Viability

The viability of the encapsulated hepatocytes was measured by MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay based on the capacity of mitochondrial dehydrogenase proportional to the living cell number as described by Yu *et al.* [14].

Ten to 20 capsules were taken from the T-flasks and mixed with a solution of MTT in PBS (5 mg/ml) under sterile conditions. One part of MTT solution was added to 4 parts capsules and medium, and incubated at 37°C for 4 h. After incubation, capsules were ruptured by intensive shaking, and 1 ml of the ruptured capsule solution was added to 4 ml of lysis buffer prepared by mixing 405 ml 2-propanol, 20 ml 1 N HCl, and 75 ml 20% SDS and the mixture was sonicated for 90 min. Debris of the capsules or cells was removed by centrifugation (1,000 ×g, 15 min) and absorbance of the supernatant measured at 570 nm. The cell number of hepatocytes could be calculated from the standard curve.

Measurement of Liver Specific Functions

The ammonia removal rate, the urea production rate, and the albumin synthesis rate were measured by the procedures described by Yu *et al.* [14]. 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 color reagent I containing phenol (10 g/l) and sodium nitroprusside (50 mg/l), and 1.0 ml color reagent II containing NaOH (5 g/l), $Na_2HPO_4 \cdot 12H_2O$ (53.6 g/l), and 1% sodium hypochlorite. After gentle vortexing, tubes were allowed to stand at 37°C for 20 min and samples were analyzed for the absorbance at 630 nm. To determine the urea production rate, the test tubes were filled with 3 ml of color reagent that was the mixture of 20 ml of solution containing diacetylmonoxime (6 g/l) and thiosemicarbazide (0.3 g/l) and 100 ml of 5 M H_3PO_4, and followed by the addition of 100 µl of the sample. After gentle mixing, tubes were allowed to stand at 100°C for 10 min and samples were analyzed for the absorbance at 540 nm. Albumin concentrations in the medium were determined by an enzyme-linked immunosorbent assay (ELISA), using IgG to rat albumin, peroxidase-conjugated IgG to rat albumin, and purified rat albumin as the standard. These rates, in all the culture experiments noted, were calculated from each concentration in a 24-h culture supernatant and divided by the inoculated cell number and all points were the average of triplicate experiments.

RESULTS AND DISCUSSION

With the growing interest in using encapsulated hepatocytes for applications in developing artificial liver [6], recent

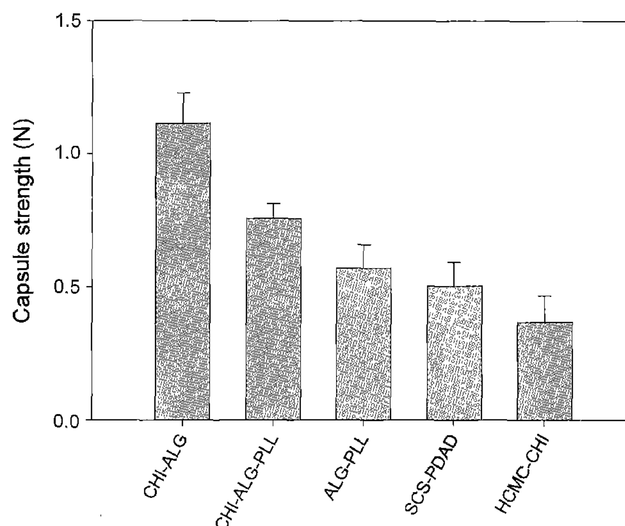


Fig. 1. Mechanical strength of various capsules used for the animal cell culture.

CHI-ALG (Chitosan-Sodium alginate) [14], CHI-ALG-PLL (Chitosan-Sodium alginate-Poly-L-lysine) [9, 14], ALG-PLL (Sodium alginate-Poly-L-lysine) [9], SCS-PDAD (Sodium cellulosesulfate-Poly-diallyldimethy-ammonium chloride) [2], HCMC-CHI (High viscosity carboxylmethylcellulose-Chitosan) [10], Distance (Strain): 70%.

studies have focused on choosing suitable materials and protocol for the encapsulation, and prolonging the viability and functions of primary hepatocytes. The encapsulated hepatocytes can be used for the development of a bioartificial liver device, thus the mechanical strength of capsules must be high enough to maintain its shape in the shear in the bioreactor. Figure 1 shows the mechanical strength of various capsules prepared for liver cell [6, 14], insect cell [2], and mammalian cell [10] culture with various natural and synthetic polymers. Among those capsules, the chitosan/alginate capsule exhibited a significantly higher rupture strength.

The influence of the encapsulation process on the cell viability was evaluated and shown in Fig. 2. The first sample was taken from the cell dispersion mixed with chitosan solution at pH 6. Low pH of chitosan solution decreased 7% of the cell viability. The second sample was taken from the outlet of a silicone tube connected to the droplet apparatus and 1% of viability was decreased. The next sample was taken from the inner capillary of the encapsulation apparatus. There was no viability decrease. Within the precipitation bath, the viability of the cells decreased about 2%. Hence, the overall viability decrease was about 10% during the whole encapsulation process. Comparing to other methods using synthetic polymers and long periods of process, the chitosan/alginate encapsulation method was mild and biocompatible due to the short process time.

Based on the evaluation of encapsulation methods, we chose the chitosan and alginate method for the encapsulation of hepatocyte. Because hepatocytes are anchorage-dependent cells, collagen was added as the appropriate attachment substratum for their biological function, and the effect of the collagen was evaluated for the viability and metabolic activities of hepatocytes in the capsules. Encapsulation was carried out at a cell density of 1.5×10^6 cells/ml of chitosan solution. The average diameter of the capsules was 3±0.2 mm, which was the size without O_2 limitation [7]. The photograph of hepatocytes isolated from rat and encapsulated hepatocytes are shown in Fig. 3.

The viabilities of encapsulated hepatocytes with and without collagen were determined and are shown in Fig. 4.

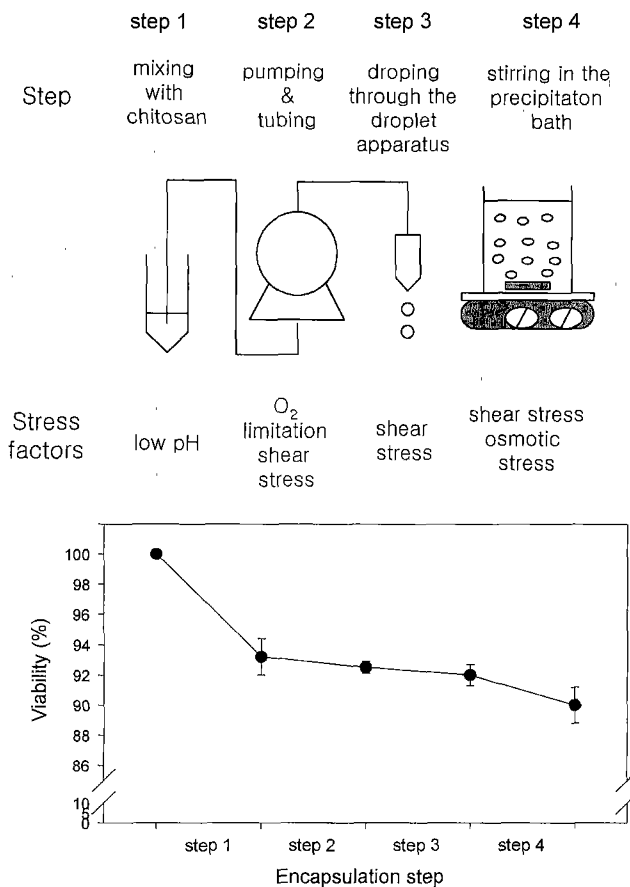
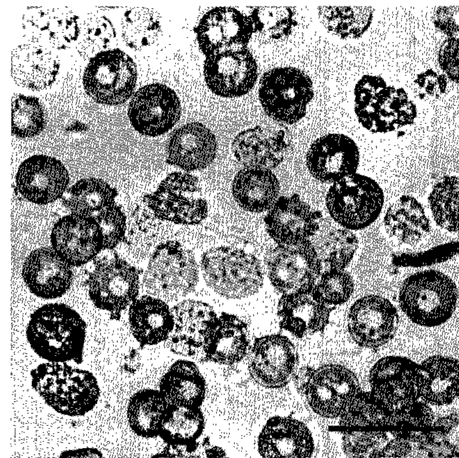
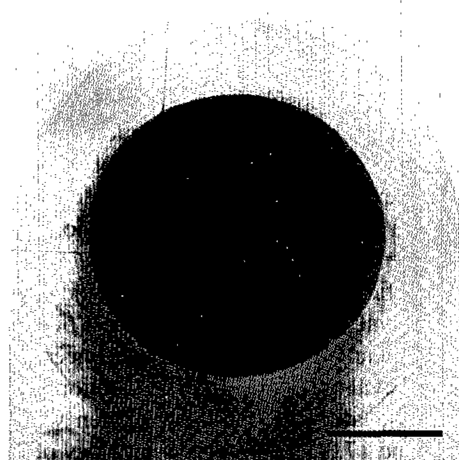


Fig. 2. The influence of the encapsulation process on viability of hepatocytes.



(a)



(b)

Fig. 3. Photographs of hepatocytes isolated from rat.
(a) Hepatocytes isolated from rat. (b) Encapsulated hepatocytes as single cells. Bar scale: 50 μm for (a) and 1 mm for (b).

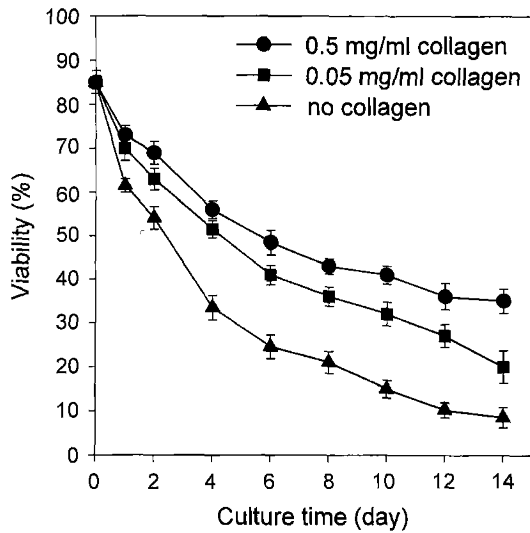


Fig. 4. Effect of collagen on the viability of rat hepatocytes in chitosan-alginate capsules.

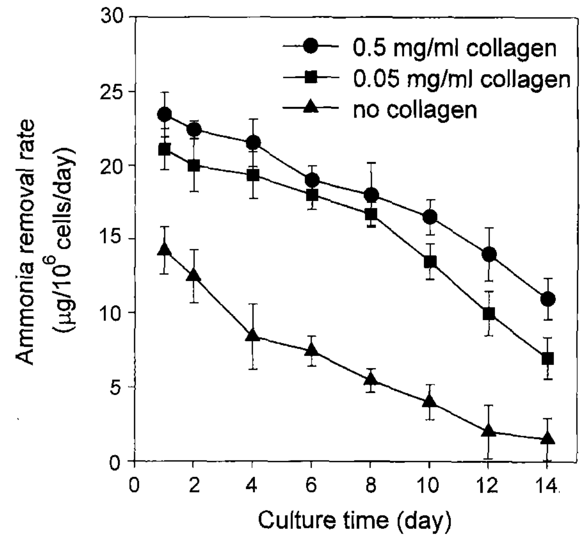


Fig. 5. Effect of collagen on the ammonia removal rate of rat hepatocytes in chitosan-alginate capsules.

Encapsulated hepatocytes without collagen showed a rapid decrease in viability and reached 62% after 1 day of incubation. The viabilities of encapsulated hepatocytes at the collagen concentrations of 0.05 mg/ml and 0.5 mg/ml were 70% and 74% after 1 day of incubation, respectively. As the culture time progressed, the viability of hepatocytes in the capsule decreased at a slow rate when the collagen concentration in the capsule was 0.5 mg/ml. The viability of encapsulated hepatocyte containing 0.5 mg/ml collagen was 38% after 14 days of culture, however, that of capsule without collagen was 9%. The result indicated that hepatocytes could maintain viability in capsules containing collagen, because the culture system mimics the normal environment of liver *in vivo*. Monolayer culture of hepatocytes with collagen coating (0.05 mg/ml) showed the viability of 80% at the beginning of incubation (data not shown), however, the viability was lower than that of capsule culture containing 0.05 mg/ml collagen after 14 days. This indicates that the encapsulation in this study could provide a microenvironment for hepatocytes and prolong their viability.

The ability of hepatocytes to perform liver-specific functions was examined with the encapsulated hepatocytes. The ammonia removal and urea production rates of encapsulated hepatocytes were examined as relevant indicators of liver specific functions. Figures 5 and 6 show the ammonia removal and urea production rates of encapsulated hepatocytes containing different collagen concentrations. The ammonia removal rate and urea production rates of the encapsulated hepatocytes without collagen were 14.2 μg/10^6 cells/day and 17.0 μg/10^6 cells/day at 1 day of incubation. After two weeks, these rates dropped to 1.5 μg/10^6 cells/day and 2.1 μg/10^6 cells/day, respectively. The decrease in the ammonia removal rate and urea production rate could be due to the decline in viability. However, the primary reason for the decreases was due to the lack of cell attachment. The anchorage-dependent primary cell requires the appropriate attachment substratum for biological function *in vitro*. The ammonia removal and urea production rates for the encapsulated hepatocytes containing 0.05 mg/ml collagen were determined as 21.1 μg/10^6 cells/day and 21.3 μg/10^6 cells/day at 1 day of incubation, respectively. After two weeks of incubation, these rates decreased to 7.0 μg/10^6 cells/day and 6.5 μg/10^6 cells/day, respectively. The encapsulated hepatocytes containing 0.5 mg/ml collagen showed high rates of 23.5 μg/10^6 cells/day for ammonia removal and 24.0 μg/10^6 cells/day for urea production at

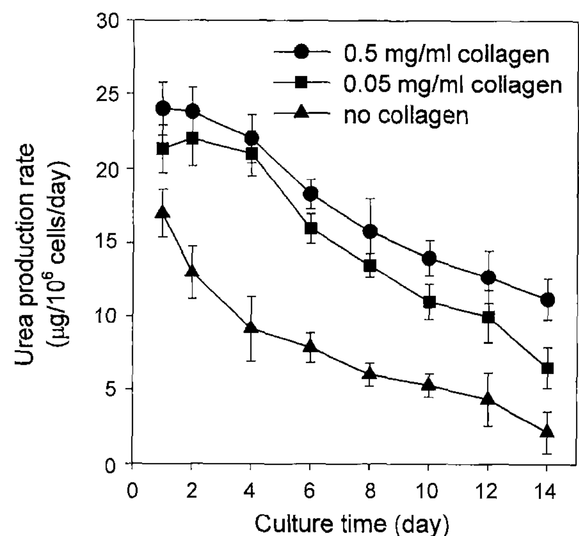


Fig. 6. Effect of collagen on the urea production rate of rat hepatocytes in chitosan-alginate capsules.

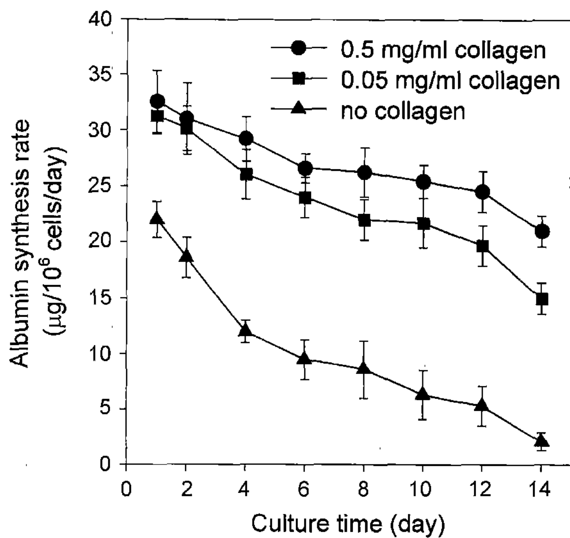


Fig. 7. Effect of collagen on the albumin synthesis rate of rat hepatocytes in chitosan-alginate capsules.

1 day of incubation. After two weeks of incubation, these rates decreased to 11.0 μg/10^6 cells/day and 11.2 μg/10^6 cells/day, respectively. The higher collagen concentration in the encapsulated hepatocytes might provide better cell attachment, thus, the liver function decreased slowly, as well as cell viability, compared to the encapsulated hepatocytes without collagen.

The ability of hepatocytes to perform albumin synthesis was determined during 14 days of incubation and the results are shown in Fig. 7. According to the previous report [5, 14] on the albumin diffusion in the chitosan-alginate membrane, there was no albumin accumulation. Throughout 14 days of culture period, the albumin synthesis rate of encapsulated hepatocyte without collagen decreased continuously and reached 2.1 μg/10^6 cells/day at 14 days of incubation. However, the rates of encapsulated hepatocytes in collagen concentrations of 0.5 mg/ml and 0.05 mg/ml decreased slowly and were 21.0 μg/10^6 cells/day and 16.0 μg/10^6 cells/day at 14 days of culture, respectively.

One of the essential elements for bioartificial liver development is to sustain the liver specific functions of hepatocytes for a long time. We confirmed that liver specific functions were kept at high levels in capsular culture with collagen addition even at very low concentrations. We expect that this system will offer a clue to the design of an artificial liver device system Further works on *in vivo* tests are in progress.

Acknowledgment

This study was supported by a grant of the Korea Health 21 R&D project, Ministry of Health & Welfare, Republic of Korea (HMP-00-B-31400-0159).

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