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# Morphology and metabolism of Ba-alginate encapsulated hepatocytes with galactosylated poly(allyl amine) and poly(vinyl alcohol) as extracellular matrices

INN-KYU KANG[1,*], JONG-SIK MOON[1], HONG MYEONG JEON[1], WAN MENG[1], YANG IL KIM[2], YOON JIN HWANG[2], SUKYOUNG KIM[3]
[1]*Department of Polymer Science, Kyungpook National University, Taegu 702-701, South Korea*
*E-mail: ikknag@bh.knu.ac.kr*
[2]*School of Medicine, Kyungpook National University, Taegu 700-091, South Korea*
[3]*School of Metallurgical & Materials Engineering, Yeungnam University, Kyungbuk 712-749, South Korea*

Lactobionic acid, bearing a $\beta$-galactose group, was coupled with poly(allyl amine) to provide synthetic extracellular matrices together with poly(vinyl alcohol) (PVA). The hepatocytes were encapsulated in Ba-alginate capsules with galactosylated poly(allyl amine) (GA) and PVA as extracellular matrices. From microscopic observation, it was revealed that the microcapsule prepared has a highly porous structure with interconnected pores and pore sizes ranging between 50–150 nm on both the surface and the cross-section. It was found, from the permeability experiment of microcapsules using FITC-dextrans with different molecular weights, that the capsule has a molecular weight cut off (MWCO) of 120 kDa, showing the potential that it can function as an immunoprotecting wall. The hepatocytes, cultured with GA and PVA in the core of the microcapsule, rapidly aggregated within a day, thus resulting in good metabolic functions such as albumin synthesis and ammonia removal.


## 1. Introduction

In recent years, many attempts have been made to immobilize the primary hepatocytes on matrices for use in the bio-artificial liver (BAL) system. Encapsulation is one of the most important techniques used in cell immobilization for biomedical applications [1]. Of the many microcapsules derived from various materials, the one formed by alginate is probably the most common. Alginate can form gels very rapidly in the presence of calcium or barium ions via ionic interaction between the alginate backbone and the chelating cation under mild conditions [2].

The characterization of microcapsules from a permeability view-point have often been connected with the encapsulation of mammalian cells, namely the islets of Langerhans [3] and hepatocytes [4]. The idea is to enclose the cells with a semipermeable and biocompatible membrane that would protect the cells from an attack by the host immune system. In order to meet the requirements of immunoisolation, the capsule wall is supposed to prevent cellular immune rejection as well as exclude antibodies to encapsulated cells. Alginate-polylysine membranes have been used for immunoprotection of hepatocytes after being tested with islet cells [5, 6]. A high-water content and highly permeable anionic polyelectrolyte hydrogel have been synthesized and fabricated into hollow fibers that had an internal diameter of 800 $\mu$m and a wall thickness of 100 $\mu$m [1]. The experimental results showed that the hydrogel-based hollow fibers were permeable by albumin and human immunoglobulin G (150,000), but were imperable by immunoglobulin A (170,000) and M (900,000) after 24 h of diffusion. Gaserod *et al*. [7] prepared microcapsules with high mechanical strength by using the low molecular weight (15,000) of chitosan and reducing the capsule diameter (300 $\mu$m). In the permeability study with these capsules, they demonstrated that the addition of several layers of alginate and chitosan resulted in capsules that were virtually impermeable by IgG [7].

It has been shown that the existence of cell-specific ligands or extracellular signaling molecules can facilitate the interaction of cells with biomaterials and control the growth and differentiation of cells in culture [8, 9]. The $\beta$-galactose moieties are recognized

*Author to whom all correspondence should be addressed.

by asialoglycoprotein receptors (ASGP-R) on the hepatocyte membrane as well as being able to promote hepatocyte attachment [10, 11]. Several researchers have synthesized $\beta$-galactose derivatives through the incorporation of $\beta$-galactose residues into alginate or chitosan for use as synthetic extracellular matrices. Yang *et al*. [12] coupled galactose moieties with alginate through an ethylenediamine spacer to enhance the interaction of hepatocytes with alginate. They reported that higher cell viability and more spheroid formations of hepatocytes were obtained in the galactosylated alginate/$Ca^{2+}$ capsules (GAC) than in the alginate/$Ca^{2+}$capsules (AC). Guo *et al*. [13] prepared the galactosylated chitosan (GC) and used it as a synthetic extracellular matrix together with poly(vinyl alcohol). They reported that the trace amount of GC in the core matrices enabled encapsulated cells to enhance their ammonia removal and albumin secretion ability.

In this study, galactosylated poly(allyl amine) (GA) was prepared by the reaction of poly(allyl amine) and lactobionic acid. GA and poly(vinyl alcohol) were used as synthetic extracellular matrices in the core when encapsulating hepatocytes in sodium alginates. The morphology and permeability of the capsules obtained were investigated and their metabolic functions were also examined.

## 2. Materials and methods

### 2.1. Materials

Sodium alginate (from macrocystis pyrifera, medium viscosity), fluoresceinisothiocyanate (FITC)-dextran, Williams' medium, and barium chloride were purchased from the Sigma Chemical Co. (USA). Poly(allyl amine) hydrochloride (Mw: 65,000) and poly(vinyl alcohol) (97% hydrolyzed, average Mw: 50,000–85,000) were purchased from the Nitto Co. (Japan) and the Adrich Chemical Co. (USA), respectively.

### 2.2. GA preparation

Lactose 1-hydrate (12 g, 33 mmol) was dissolved in hot water (9 ml) and diluted with methanol (25 ml). The solution was then mixed with methanol (240 ml) containing 17.1 g of iodine. To the mixed solution, 4 wt% of potassium hydroxide methanol solution (400 ml) was added until the iodine color disappeared. The solution was then recrystallized using a methanolic aqueous solution (methanol: water = 12:5, 600 ml) to obtain potassium lactonate (9.4 g, yield: 68%). The resulting potassium lactonates were dissolved in distilled water and subsequently passed through an amberlite IR-120 column in order to obtain lactobionic acid [14].

Poly(allyl amine) hydrochloride (0.4725 g, 0.005 mol) was dissolved in distilled water (2 ml), mixed with triethylamine (1.01 g, 0.01 mol), and incubated with stirring for 12 h to convert amine salts (-$NH_2$·HCl) into free amine (-$NH_2$). Unused triethylamine was removed by washing with an ammonia aqueous solution. A synthetic scheme of a poly(allyl amine) derivative is illustrated in Fig. 1. Lactobionic acid (L, 1.8 g, 0.005 mmol) was dissolved in a 0.75N sodium citrate buffer solution (pH 4.7) containing 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (WSC) and incubated for 2 h. The solution was then mixed with a poly(allyl amine) aqueous solution and stirred for 48 h at room temperature in order to obtain galactosylated poly(allyl amine) (GA). The reaction solution was dialyzed for two days using distilled water and subjected to the encapsulation experiment after lyophilization (yield: 65%). The elemental composition of the derivatives was examined using a C.H.N and an O analyzer (FISONS, EA 1110, Italy). The introduction of lactobionic acid to poly(allyl amine) was confirmed by observing the $^1$HNMR spectrum of the derivatives in deuterium oxide.

### 2.3. Hepatocyte isolation

Primary hepatocytes were isolated from a SD rat (5–6 weeks old, male, Daehan Biolink Co., Korea) using the modified in situ perfusion method [15]. The dead hepatocytes were removed by density gradient centrifugation on Percoll (Amersham Pharmacia Biotech, Sweden). The Percoll solution (1.065 g/ml), used to recover hepatocytes, was prepared by diluting Percoll (1.13 g/ml) with Hank's solution. The viable primary hepatocytes were suspended in William's E medium, which contained penicillin (50 $\mu$g/ml) and HEPES (10 mM). Isolated hepatocytes that had a viability of more than 90% by trypan blue dye exclusion, were used for the experiments.

### 2.4. Hepatocyte encapsulation

A schematic diagram showing the preparation of encapsulation beads is represented in Fig. 2. Shortly,

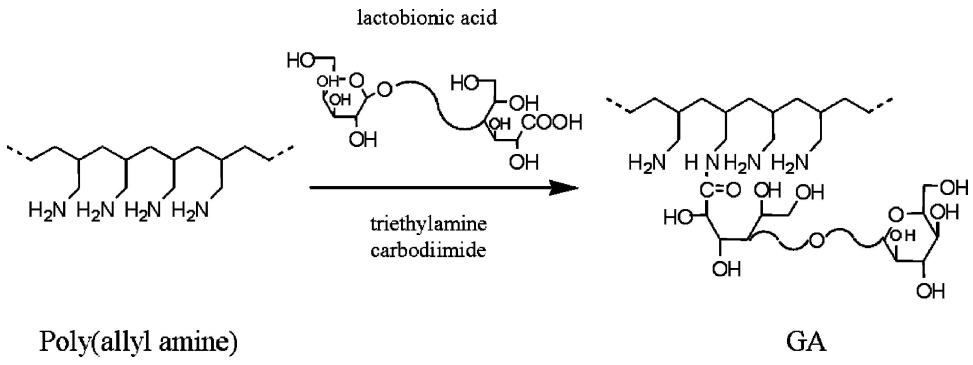


*Figure 1* Synthetic scheme of poly(allyl amine) containing $\beta$-galactose residue in the side chain (GA).

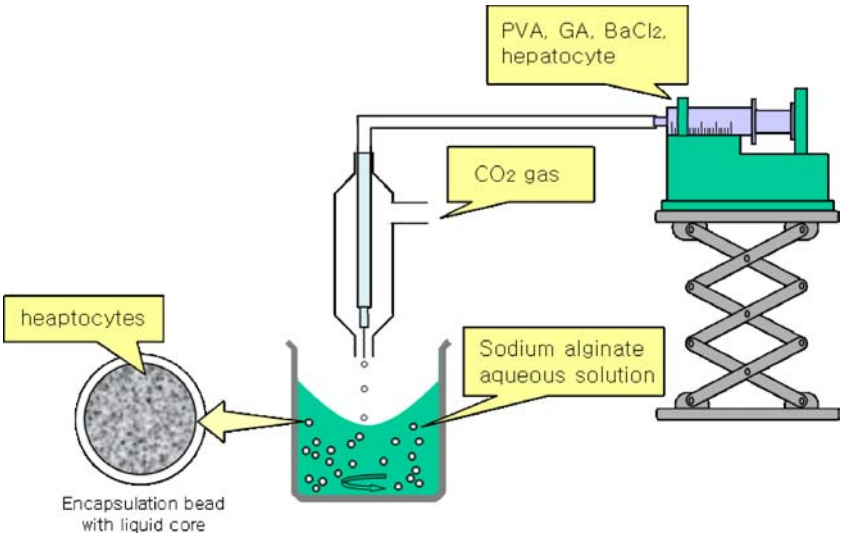


*Figure 2* Schematic diagram showing the preparation of encapsulation beads. Hepatocytes were suspended in PBS containing GA, PVA and $BaCl_2$, and dropped in an sodium alginate aqueous solution using a 22G hypodermic needle mounted coaxially inside a 2 mm tube for concentric gas flow in order to obtain spherical encapsulation beads (diameter approx. 700–800 $\mu$m).

GA (0.7 g) was dissolved in a phosphate buffer solution (10 ml) and subsequently sterilized by passing the GA solution through a membrane filter (pore size: 0.2 $\mu$m, Advantec MFS Inc.). Poly(vinyl alcohol) (PVA, 0.7 g) and $BaCl_2$ (0.15 g) were dissolved in distilled water (10 ml) and then sterilized by using an autoclave (120 °C, 20 min, 1.5 kgf/cm$^2$). The solution (2 ml) was then mixed with a GA solution (1 ml) and then hepatocytes ($1 \times 10^7$ cells). The mixed cell solution was filled into a syringe and compressed air was used to push the solution through a 22G hypodermic needle mounted coaxially inside a 2 mm tube for concentric gas flow. The coaxial tube was used to spray the mixed cell solution into 100 ml of sterile 0.5 wt% sodium alginate solution stirred by a magnetic bar. The size of the encapsulation beads was controlled by changing the flow rate of the mixed cell solution and the air flow. The capsules formed were washed with sterile saline to remove excess sodium alginate and treated with a 0.5% of a chitosan aqueous solution for 5 min.

## 2.5. Morphological observations

Images of the encapsulated hepatocytes were observed in an inverted microscope (Nikon ECLIPSE TS100, Tokyo, Japan) with a phase contrast optic. The microcapsules, prepared by dropping a barium chloride aqueous solution containing GA and PVA into a sodium alginate solution, were placed on a coverglass. The microcapsules were then frozen in a deep freezer (−70 °C) for 24 h. For cross-section analysis, the microcapsule was cut in two using a surgical blade. Both the microcapsule and the cross-sectioned capsule were freeze-dried for 2 days. The microcapsules were mounted onto an aluminium stub and sputter coated with gold using the Multipurpose Evaporation System (CI-2000S, Cluster Instrument, Korea) for 90 s. The morphology of the capsule surface and cross-section was observed using a field emission scanning electron microscope (FE-SEM, S-4300, Hitachi, Japan).

## 2.6. Permeability of the microcapsule

FITC-dextran molecules, with a molecular weight of 10 to 120 kDa, were dissolved in a PBS (−) at a concentration of 3 mg/ml. The solution (0.5 ml) was mixed with 1 ml of an aqueous solution containing PVA (7 wt%) and $BaCl_2$(1.5 wt%). The mixed solution was dropped into 0.5 wt% of a sodium alginate solution and stirred by a magnetic bar. The capsules formed were washed with sterile saline three times in order to remove excess sodium alginate. The microcapsules were transferred to a dish containing a phosphate buffer solution (PBS), and incubated for 4 h. The dish was further incubated for two days after the PBS was replaced with a fresh one. The microcapsule suspension was then centrifuged at 750 rpm for 3 min so as to precipitate the capsules. The medium (0.2 ml) was taken from the upper layer to examine the amount of FITC-dextran released from the microcapsules. The microcapsules, that had precipitated into the bottom layer, were destroyed using a tissue grinder and the medium (0.2 ml) was taken from the upper layer after being centrifuged at 750 rpm for 3 min. Absorbance of the medium containing FITC-dextran at 495 nm was measured using a kinetic microplate reader, the Multiskan MS (Labsystems, Helsinki, Finland). The amount of FITC-dextran remaining in the core part of the microcapsules was calculated. A percentage was calculated from the calibration curve constructed previously by using the known amount of FITC-dextrans. The experiment was repeated three times using the same microcapsules and a mean value was taken.

## 2.7. Fluidized-bed type bioartificial liver

Column-type bioartificial liver (BAL, 25 ml), as shown in Fig. 3, was designed to culture rat hepatocytes. Hepatocytes encapsulated within a Ba-alginate capsule were placed inside the column, and the media was introduced at the bottom of the column. For the in vitro experiment, a 50 ml reservoir was installed between the peristaltic pump and the BAL module. Oxygen was supplied by the media came into contact with mixed gas of 95% $O_2$

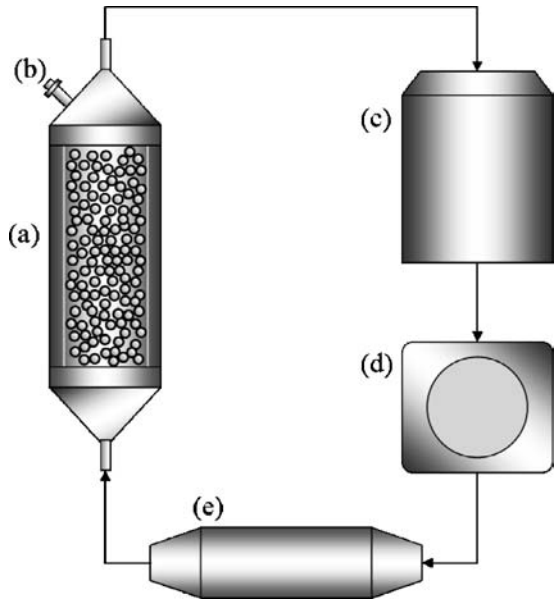


*Figure 3* Schematic diagram of a fluidized-bed reactor system using encapsulation beads: (a) column packed with microcapsules, (b) sampling cock, (c) reservoir, (d) peristaltic pump and (e) oxygenator.

and 5% $CO_2$ using silastic tubing (thickness 0.2 mm). The optimal flow rate of fluid, for the proper fluidization of the encapsulated hepatocytes, was 20 ml/min and the temperature was maintained at 37 °C.

### 2.8. Ammonia removal test

To assess the ammonium metabolism of the cultured hepatocytes, a medium loaded with 1 mmol/l ammonium chloride was used on day 2. The culture medium was sampled before medium exchange began and at 2, 4, 6 and 8 h after medium exchange. The ammonium concentration in the medium was measured using a commercially available test kit, the Wako Ammonia Kit (indophenol method; Wako Pure Chemical Industries, Osaka, Japan). The experiment was carried out in triplicate and the mean value was taken.

### 2.9. Albumin synthesis

The release of albumin into the culture medium was measured by means of enzyme-linked immunosorbent assay (ELISA) [16] using rabbit anti-rat albumin (ICN Pharmaceutical Inc., Ohio, USA) as the primary antibody. Peroxidase-conjugated sheep anti-rat albumin (ICN Pharmaceutical Inc., Ohio, USA) was used as a secondary antibody. The orthophenylenediamine (OPD, Sigma) solution (3 mg/ml OPD and 0.02% $H_2O_2$ in citrate-phosphate buffer, pH 4.8) was used as a substrate of peroxidase. Absorbance at 492 nm was measured using a kinetic microplate reader. The experiment was carried out in triplicate and the mean value was taken.

## 3. Results

Fig. 4 shows the $^1$H nuclear magnetic resonance (NMR) spectrum of galactosylated poly(allyl amine) in deuteriated water. The peaks at 0.98 and 2.8 ppm are based on methylene groups of the main chain and side chain of poly(allylamine), respectively. The peaks, based on methylene and the methine group of $\beta$-galactose residue, appeared at around 3.5–4.0 ppm, showing the successful coupling reaction of poly(allylamine) and lactobionic acid. The synthesis of GA was also confirmed by observing the infrared absorption at 1635 $cm^{-1}$, which is based on the carbonyl stretching vibration of amide bond (-CONH-). It could be calculated, from elementary analysis, that 33% of primary amine in poly(allylamine) is substituted with lactobionic acid.

In this study, poly(allyl amine), containing $\beta$-galactose residue in the side chain, was used as a synthetic extracellular matrix when preparing encapsulation beads. The micro-structure of the capsule beads obtained was examined using a field emission scanning electron microscope and the results are shown in Fig. 5. In the present study, the alginate microcapsule displayed a highly porous structure with interconnected pores and pore sizes ranging between 50–150 nm in diameter. Image of the capsule surface (a) was almost the same as that of the cross-section (b).

For ensuring long term viability of the cells, nutrients, oxygen and metabolic wastes needed to be able to permeate the microcapsules. Some applications require selective permeability to allow nutrient exchange and at the same time to prevent the passage of large molecules such as immunoglobulins into the microcapsules. To assess the suitability of microcapsules for use in

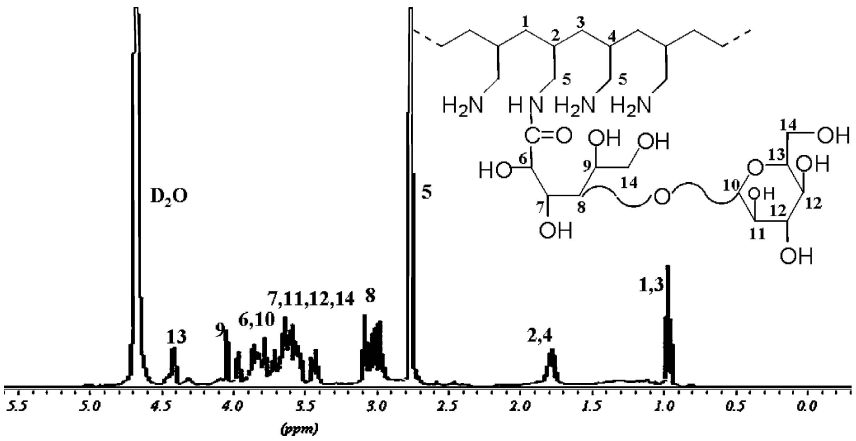


*Figure 4* $^1$H-NMR spectrum of a poly(allyl amine) containing $\beta$-galactose residue in the side chain in $D_2O$.

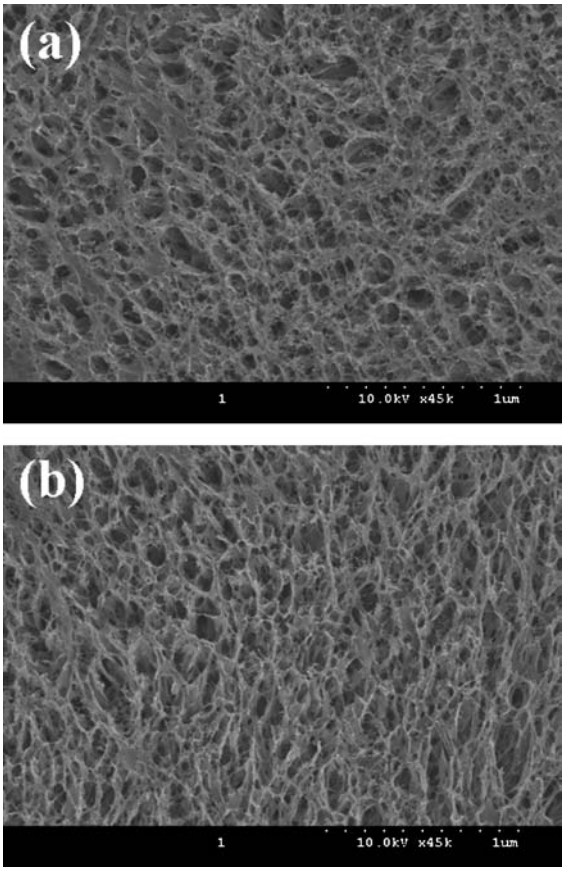


*Figure 5* Field emission scanning electron microscopes of (a) surface and (b) cross-section of the microcapsule. The PBS solution (0.5 ml) was mixed with 1ml of an aqueous solution containing PVA and $BaCl_2$. The mixed solution was dropped into a 0.5 wt% sodium alginate solution. The capsules were washed with sterile saline three times, frozen in a deep freezer, and subsequently cut in two using a surgical blade for microscopic observation.

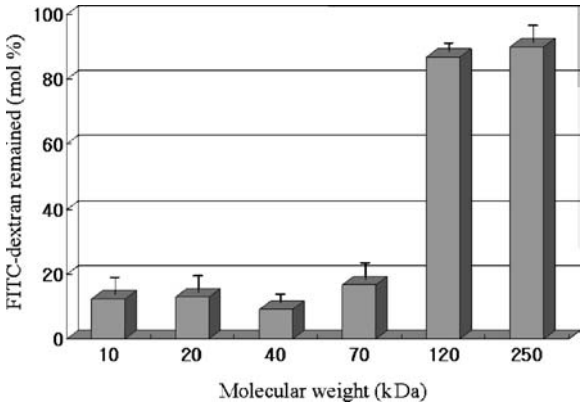


*Figure 6* Permeability of the FITC-labeled dextran to the encapsulation beads. FITC-dextrans with a molecular weight of 4–120 kDa were dissolved in a PBS(−) with a concentration of 3 mg/ml. The solution, mixed with an aqueous solution containing PVA and $BaCl_2$, was dropped into a 0.5 wt% sodium alginate solution. The capsules formed were washed with sterile saline three times and transferred to a dish containing a PBS for two days. The amount of FITC-dextran released from the microcapsules was calculated by measuring the absorbance of the medium at 495 nm.

bio-artificial liver applications, we have characterized the permeability profiles of microcapsules. FITC-dextran, with a molecular weight of 10–250 kDa, was encapsulated inside the microcapsules and the rate of its release from the microcapsules was measured by spectrophotometry. In the case of FITC-dextrans with a molecular weight of less than 70 kDa, 15–20% of the dextran remained in the core of the capsules. While in the case of FITC-dextrans with a molecular weight greater than 120 kDa, 80–90% of the dextran remained in the core of the capsules. Since molecules larger than 120 kDa were not able to permeate the microcapsules, large molecules such as immunoglobulins, which mediate the immune response, should not be able to permeate the microcapsules. The morphology of hepatocytes within the encapsulated alginate beads was observed as a function of culture time using an optical microscope. After a 6 h incubation period (Fig. 7(a)), hepatocytes are dispersed into the core part. The cells, however, flocked to the center position and then completely aggregated after 24 h (Fig. 7(d)). The photo of the encapsulation beads showed an obvious liquid core-shell structure. The encapsulation beads obtained in this study consisted of a shell and liquid core.

In order to estimate the metabolic function of the encapsulated hepatocytes, the ammonia removal and albumin secretion ability of hepatocytes in the two culture conditions were examined. The two culture conditions were monolayered and encapsulated with GA. Fig. 8 shows a result of ammonia removal by encapsulated hepatocytes cultured in a W.E. medium for two days. When the medium is replaced with 1 mM of $NH_4Cl$, some ammonia-free medium exists in the capsule for a certain time due to the barrier of capsule wall. This free medium can dilute the outer ammonia concentration. The ability to remove ammonia by the monolayered hepatocytes (▼) was lower than that of the encapsulated hepatocytes (●), as shown in Fig. 8. The amount of albumin secreted by the encapsulated hepatocytes with GA in the liquid core increased up to the three-day period and, thereafter, slowly decreased (Fig. 9). On the other hand, the amount of albumin secreted by the monolayered hepatocytes decreased gradually and the amount was less than that of the encapsulated hepatocytes with GA.

## 4. Discussion

Alginate bead-entrapped hepatocytes have been used in an extracorporeal bioartificial liver [17, 18]. Dixit *et al*. [19] prepared microencapsulated hepatocytes with Type 1 collagen within an alginate-poly-L-lysine composite membrane for use in a hybrid bioartificial liver support system. Gaserod *et al*. [7] prepared alginate-chitosan macrocapsules with high mechanical strength by using the low molecular weight of chitosan or by reducing the capsule diameter. Guo *et al*. [13] synthesized galactosylated chitosan (GC) and used it as synthetic extracellular matrices together with poly(vinyl alcohol). They reported that Ba-alginate-encapsulated hepatocytes with GC exhibited a higher metabolic function in a albumin secretion compared to those entrapped in Ba-alginate beads and monolayer-cultured onto a collagen-immobilized polystyrene dish.

In the present study, we synthesized poly(allyl amine) containing $\beta$-galactose residue in the side chain (GA) and used it as synthetic extracellular matrices when preparing Ba-alginate encapsulated hepatocytes. Alginate can be used in the state of both entrapment

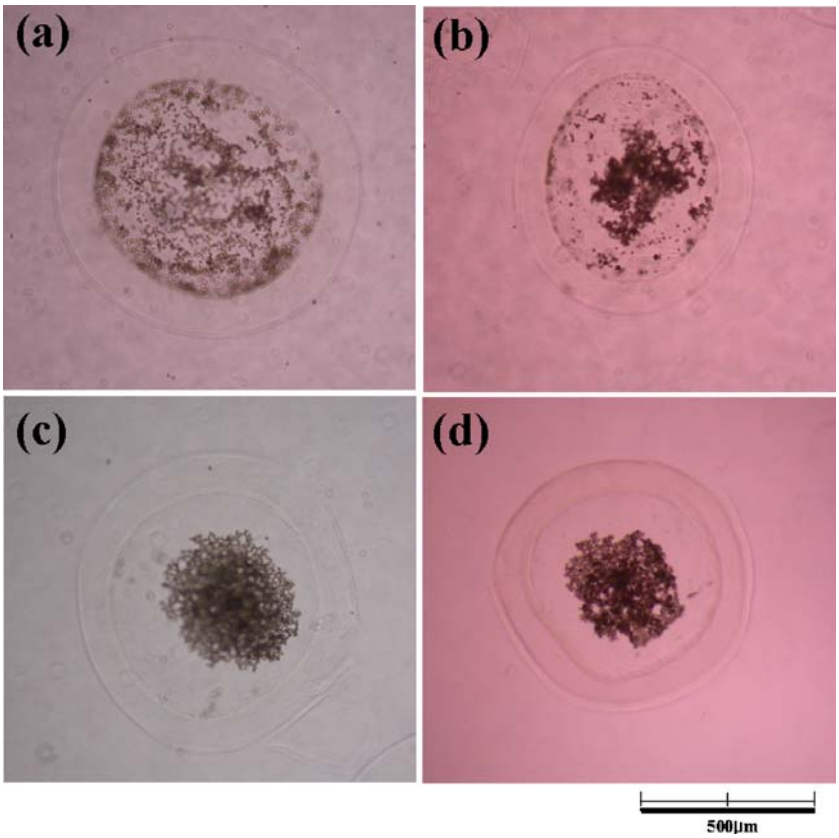


*Figure 7* Optical micrographs of encapsulated hepatocytes after 6 (a), 12 (b), 18 (c) and 24 h (d) of culturing. The hepatocytes gradually aggregated as the incubation time increased to 24 h.

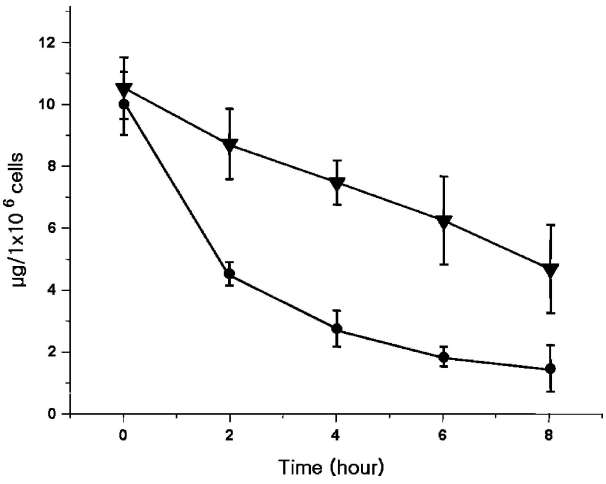


*Figure 8* The change of the level of ammonia concentration in the medium caused by rat hepatocytes in encapsulation beads with GA (●) and on a collagen monolayer (▼) after two days. When performing the assay, the beads were cultured in a medium containing 1mmol $NH_4Cl$. The samples were collected every 2 h for 8 h and the medium was changed with a fresh W.E. medium after assay. Data were expressed as the mean ± SD ($n = 3$).

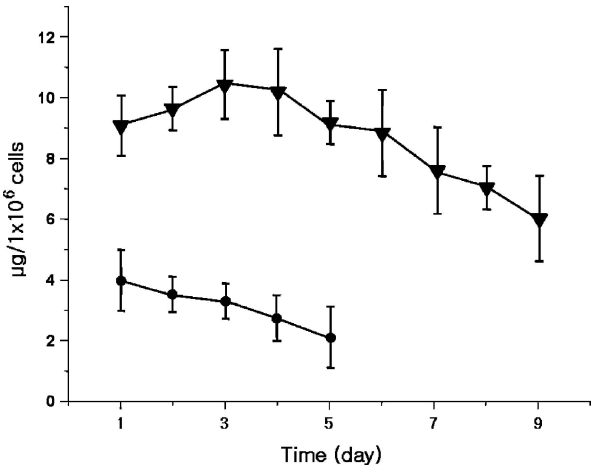


*Figure 9* Albumin secretion by rat hepatocytes cultured in encapsulation with GA (▼) and on a collagen monolayer (●). The culture medium of the fluidized-bed reactor was replaced with the fresh one daily, and the amount of albumin that secreted into the medium by hepatocytes for 24 h was assayed by means of ELISA. Data were expressed as the mean ± SD ($n = 3$).

and encapsulation when enveloping the hepatocytes in polymers. Compared with entrapment beads, encapsulation beads showed relatively low mechanical stability. The methods used to increase the stability of microcapsules have been reported. Zimmermann *et al*. [19] prepared the stable microcapsule by using alginate with ultra high viscosity. Chia *et al*. [20] developed a cell-friendly microcapsulation method by using a terpolymer of 2-hydroxyethyl methacrylate, methacrylic acid and methyl methacrylate. The stability of the microcapsule is also related with the wall thickness and the capsule size [7]. The microcapsules obtained in this study had a mean diameter of 700–800 $\mu$m and an average wall thickness of 120 $\mu$m, as shown in Fig. 7. Capsule permeability and molecular weight cutoff (MWCO) are very important factors in terms of immunoprotection. From the results of capsule permeability (Fig. 6), it could be say that the microcapsules prepared in this study had a MWCO of 120 kDa. Considering that the molecular weight of albumin and immunoglobulin are about 65 and 150 kDa, respectively, the capsules

prepared in this study seemed to be used as an immunoprotective capsule in BAL. It is believed that the ideal molecular cutoff should be around 100 kDa, to allow for the exchange of albumin and albumin-bound substances while preventing the diffusion of immunoglobulins and some complement factors [21]. Mullon [22] reported that a 0.15 $\mu$m pore size polysulfone membrane decreased the risk of porcine endogenous retrovirus transmission by a factor of 100,000. As represented in Fig. 5, the microcapsule displayed a pore size of 0.05–0.15 $\mu$m, showing the potential to be able to function as an immunoprotection barrier. In the previous study, we prepared the hepatocyte-encapsulated capsules with a diameter of 2.5 mm. When culturing the encapsulation beads, the cells in the core of the capsules were tightly aggregated after seven days. The size of the microcapsules prepared in this study are between 700–800 $\mu$m and the hepatocytes in the core rapidly aggregated. The reason for the fast cell aggregation in the capsule possibly may have been due to the smaller size of the capsule (700–800 $\mu$m) compared with the previous ones (2.5 mm). The aggregated cells were expected to maintain a good biological function. As shown in Fig. 9, the amount of albumin produced by the encapsulated hepatocytes increased up to the three day mark, and the amount lasted until 5th day, showing good metabolic function.

## 5. Conclusions

Galactosylated poly(allyl amine) (GA) and poly(vinyl alcohol) (PVA) were used as extracellular matrices (ECM) in the preparation of encapsulated hepatocytes using alginate and barium chloride. It was found, from the microscopic observation, that the microcapsules have a porous structure with interconnected pores, with a size of 50–150 nm. The results of the permeability experiment of microcapsules using FITC-dextrans showed that the capsule has a molecular weight cut off of 120 kDa. The hepatocytes encapsulated in alginate with GA and PVA rapidly aggregated in the core. The aggregated cells showed high albumin synthesis and ammonia removal, suggesting good metabolic function.

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