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Development of Hepatocyte Spheroids Immobilization Technique Using Alternative Encapsulation Method

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Primary hepatocytes of small animals such as rat and rabbit were often used for the study of extracorporeal liver support systems. Freshly isolated rat hepatocytes form spheroids within two days when cultivated as suspension in spinner vessels. These spheroids showed enhanced liver specific functions and more differentiated morphology compared to hepatocytes cultured as monolayers. However, shear stress caused by continuous agitation deteriorated spheroids gradually. In this work we immobilized spheroids to prolong liver specific activities. First, hepatocyte spheroids were suspended in collagen solution containing calcium chloride and then dropped into alginate solution. A thin layer of calcium alginate was formed around the droplet and then was removed after the inner collagen was gelled by treatment of sodium citrate buffer. Spheroids embedded in collagen-gel bead maintained liver specific functions such as albumin secretion rate longer than hepatocyte spheroids exposed to shear stress. Therefore, we suggest that this immobilization technique may offer an effective long-term hepatocyte cultivation and facilitate the development of a bioartificial liver support device.

Key words : Bioartificial liver, hepatocyte spheroid, collagen, encapsulation.

INTRODUCTION

The liver is an organ with complex functions such as liver specific metabolism and detoxification [1]. Although both acute and chronic liver failure patients can be treated effectively by whole organ transplantation, there are only about 3000 donor livers available annually in the United States, while approximately 30,000 patients die from liver failure [2]. Despite recent advances in medical supportive therapy, patients with severe fulminant hepatic failure (FHF) have mortality rate approaching 90%. To treat such patients, investigators have attempted to improve survival rate by using various extracorporeal bioartificial liver support systems [3-6].

Several requirements should be met in bioartificial liver support systems (BLSS): (1) hepatocytes should be cultured in a sufficiently high density; (2) their metabolic functions should be of a sufficiently high level and duration; and (3) the BLSS module should permit scaling-up and aseptic handling [7].

Other investigators have previously reported that hepatocytes cultured as spheroid have a higher functional activity and more differentiated morphology comparing to monolayer culture [8-10] and we also demonstrated it [11, 12]. But these techniques finally require scale-up for development of bioartificial liver support device, therefore in case of using general suspension culture, shear stress caused by agitation or circulation damaged spheroids gradually. Thus cell immobilization methods are required for this purpose.

Numerous methods on immobilizing cells have been described in the literature [13]. Systems such as microcarriers, gel entrapment, and microencapsulation in which the cells are retained, associate with, or adhere to small particles have been extensively studied for more than 10 years. Particularly microencapsulation provides a convenient method of cell handling but most of capsules based on the formation of calcium-alginate shells are unstable in physiological solutions containing calcium chelating agents [14]. In addition, significant resistance of mass transfer was observed through the calcium alginate shell and we attempted to remove it. Since hepatocytes are anchorage-dependent cells, the appropriate attachment substratum is required for their biological function. It is well known that hepatocytes attach and function better with specific attachment substrata in vitro. As a specific attachment substrata, several investigators have previously reported the appropriateness of collagen gel culture method [15, 16]. Another study explicitly demonstrated that hepatocytes cultured as spheroids exhibit greatly enhanced albumin production compared to that a monolayer of hepatocytes cultured on collagen [9-11]. In the present study, we attempted to establish an *in-vitro* model of scale-up by combining a spheroid culture and a type I collagen gel culture using alternative encapsulation method.

In all tests the widely used alginate capsules

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were employed as a comparative standard control system.

MATERIALS AND METHODS

Hepatocyte Isolation

Hepatocytes were isolated from adult Sprague-Dawley rats weighing 180-200 g by a two-step *in situ* collagenase perfusion technique modified from the method described by Seglen [17-23]. The rat was first anesthetized and the abdomen was entered through a bilateral subcostal chevron incision, and then livers were perfused *in situ* for 10 min via portal vein with 250 mL of perfusion buffer Ⅰ. The perfusate was oxygenated while passing through inside the oxygen-permeable silicone tubing, which was put in an appropriate container with carbogen gas (90% O_2 and 10% CO_2) at a flow rate of 25 mL/min. Air bubbles in the buffer solution were removed by bubble trapper.

After the initial flushout, a perfusion buffer Ⅱ supplemented with collagenase (hepatocyte-qualified, Gibco BRL) and trypsin inhibitor (Gibco BRL) was perfused at a flow rate of 20 mL/min for 10 min.

The constitutions of perfusion buffer Ⅰ and perfusion buffer Ⅱ are given in Table 1.

After 10-20 min, upon visual and palpable evidence of liver dissolving, the capsule was broken and the liver substance was raked and irrigated with cold William's E medium (Gibco BRL).

The released cells were filtered through nylon mesh with 100 μm openings and resuspended in fresh Williams' medium E.

The cell suspension was centrifuged (500 rpm, 50 g, 2 min) and the pellet was resuspened twice in Williams' medium E. Finally, cells were counted in a hemacytometer and viability was assessed by trypan blue exclusion method. Their average yield was $2\text{-}3\times10^8$ cells with a viability in excess of 85%.

Table 1. Compositions of perfusion buffer used for rat hepatocyte isolation.

	Buffer Ⅰ (g/L)	Buffer Ⅱ (g/L)
NaCl	8	8
KCl	0.4	0.4
$CaCl_2$		0.56
$NaH_2PO_4 \cdot 2H_2O$	0.078	0.078
$Na_2HPO_4 \cdot 12H_2O$	0.151	0.151
HEPES	2.38	2.38
Collagenase	-	0.5
Trypsin inhibitor	-	0.05
EDTA(EGTA)	0.19	-
Sodium bicarbonate	0.35	0.35
Glucose	0.9	-
Penicillin	100 Unit/mL	100 Unit/mL
Streptomycin	10 mg/mL	10 mg/mL
Amphotericin B	25 μg/mL	25 μg/mL

Culture Condition

The culture medium (HDM) employed for all cultures was William's medium E(Gibco BRL) supplemented with the following additives: epidermal growth factor(20 μg/L, Gibco BRL), insulin(10 mg/L, Gibco BRL), $CuSO_4 \cdot 5H_2O$(0.1 μM, Sigma Chem. Co.), $ZnSO_4 \cdot 7H_2O$(50 pM, Sigma Chem. Co.), H_2SeO_3 (3 μg/L, Sigma Chem. Co.), linoleic acid(50 mg/L, Sigma Chem. Co.), $NaHCO_3$(1.05 g/L, Gibco BRL), HEPES(1.19 g/L, Sigma Chem. Co), penicillin(100 Unit/mL, Gibco BRL), streptomycin(10 mg/mL, Gibco BRL), and amphotericin B(25 μg/mL, Gibco BRL).

After 8 hr of culture, the medium was changed daily and cultured under a humidified atmosphere of 5% CO_2 and 95% air at 37℃.

Culturing of Hepatocyte as Spheroids

The freshly isolated hepatocytes were resuspened in hormonally defined culture medium (HDM) at a concentration of 5×10^5 cells/mL. The cell suspension was placed in siliconized spinner flasks (250 mL, vertical paddle, Bellco Co.) and stirred by a magetic stirrer (Bellco Co.) at 60 rpm in a humidified 95% air/ 5% CO_2 incubator at 37℃.

Medium was changed 8 hr after cell innoculation and daily thereafter by stopping agitation, allowing gravity sedimentation of the spheroids at room temperature, followed by aspiration of the spent medium and replacement by fresh medium. The spent medium was stored at -20℃ prior to analysis. The average of lengths along two perpendicular axes of the spheroid was defined as the spheroid

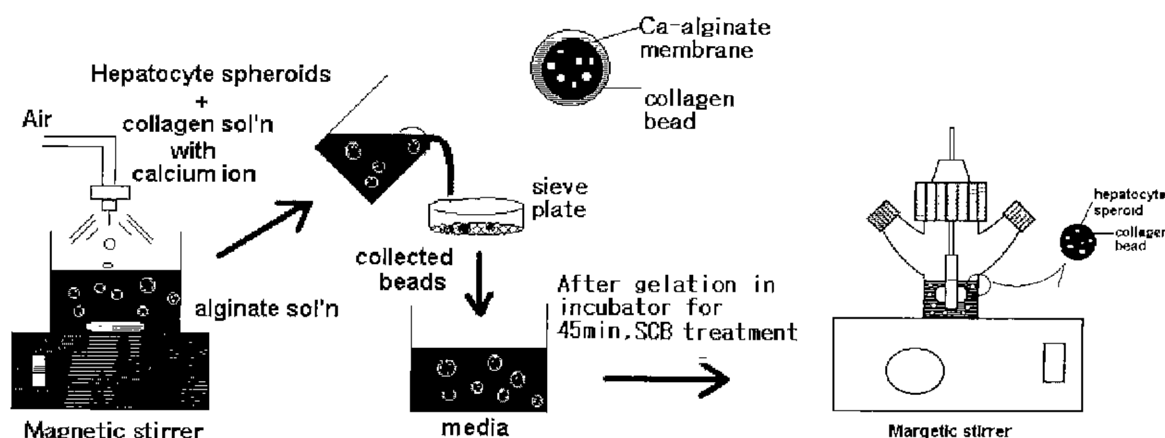


Fig. 1. Schematic diagram of a process for collagen-gel bead preparation.

diameter. Between 40 and 50 spheroids were evaluated to obtain the representative average diameter.

Encapsulation of Hepatocytes Cultured as Spheroid

This procedure was conducted at room temperature using aqueous buffers at physiological pH (Fig. 1). Within 48 hr, hepatocytes cultured as spheroid were prepared for encapsulation and resuspended into 0.8% (w/v) collagen (Sigma. calf skin) solution containing 60 mM $CaCl_2$ Eight volumes of the collagen solution were mixed with 1 volume of 10× Williams' medium E and 1 volume of neutralizing buffer (50 mM NaOH, 20 mM HEPES, 25 mM $NaHCO_3$) on ice. The cell-loading density was 2.5×10^6 cells/mL of collagen solution.

The resulting spheroid solution is dropped into a vigorouly stirred 0.75% sodium alginate solution with 0.08%(v/v) Tween 80 using 21G bluntly ended needle and air flow rate of 16 ℓ/min. The introduction of surfactant Tween 80 facilitated the formation of spherical capsules.

A layer of calcium alginate is formed around the droplet during a reaction period of 3 min. The formed capsules were meshed and kept in medium for gellation of the collagen in a 37℃ incubator for 45 min. After 45 min, the gelled collagen beads were collected and immersed in 50 mM sodium citrate buffer (50 mM sodium citrate, 0.47% NaCl, 20 mM D-fructose, pH 7.4) for 10min to remove the layer of calcium alginate.

Bead diameters are approximately 1 mm. The resulting collagen-gel beads without calcium alginate shell were washed three times with PBS to remove excess citrate before they were introduced into spinner flasks. Alginate capsules employed as a comparative standard were formed by a conventional process [24-26].

Encapsulation process caused less than 5% loss in hepatocyte viability.

Measurement of Ammonia Removal and Urea Synthetic Activity

These metabolic functions of hepatocytes were chosen as minimum representative metabolic functions of the liver. To assess the ammomium metabolism and urea synthesis activity of the cultured hepatocytes, medium loaded with 1 mM ammonium chloride was used daily after 2 days of innoculation.

Ammonium and urea-N concentration in the media were measured colorimetrically using commercially available test kits, i.e., the Asan blood ammonia assay kit (Asan Pharma, KOREA) [27] and Blood urea nitrogen assay kit (Sigma Co. No636) [28] respectively. Ammonia and urea production rates in all culture experiments were calculated from each levels in a 24 hr culture supernatant divided by the inoculated cell number.

Albumin Concentration Measurement

Collected media samples were analyzed for rat albumin content by enzyme-linked immunosorbent assay(ELISA).

Antibodies to the albumin were purchased from Cappel. The 96-well plates (NUNC-Immuno plate, Maxisorp) were coated with 100 μℓ of IgG to rat albumin at 2 μg/mL in bicarbonate coating buffer (0.04M Na_2CO_3, 0.06M $NaHCO_3$), overnight at 4℃. The wells were washed three times with PBS plus 0.05%(v/v) Tween20 (PBS-Tween), blocked for 1hr at room temperature with 3% casein in PBS and then rewashed with PBS-Tween. Sample and albumin standard were transferred to the precoated plates and allowed to incubate for 1 hr at room temperature before rewashing. Subsequently, 100 μℓ/well of peroxidase-conjugated IgG fraction to rat albumin (Cappel.55776) diluted 1:5000 in PBS was added, and the plates were incubated for 1hr at room temperature before being rewashed and were developed with 100 μℓ of 25 mM citrate and 50 mM phosphate, containing 0.4 mg/ml. o-phenylenediamine and 0.012%(v/v) hydrogen peroxide at room temperature. The reaction was stopped with 100 μℓ/well of 2 M sulfuric acid after 7 min of incubation. The presence of bound antibodies was detected by the conversion of o-phenylenediamine by the conjugated peroxidase. The absorbance was measured at 490 nm wave length with the ELISA reader (Ceres UV900 HDi, Bio-Tek Instrument). Concentration of samples were determined from a standard curve generated for each ELISA plate. Generally, two duplicate wells were averaged for each sample.

Scanning Electron Microscopy(SEM)

After the medium was aspirated, the beads were fixed with 2.5% glutaraldehyde in 0.1 M PBS, pH 7.2 and they were post-fixed with 2% osmium tetroxide in PBS for 1 hr, at 4℃, dehydrated through a graded series of ethanol, and finally immersed in isoamyl acetate for 30 min. These specimens in isoamyl acetate were dried in a critical point dryer (Hitachi HPC-1) and then coated with Pt-Pd in an Eiko Ion coater.

RESULTS AND DISCUSSION

Cell Encapsulation and Culture

Hepatocytes cultured in stirred conditions formed multicellular spheroids within 24 hr and grew in size from 70-110 μm in diameter in the first day to 170-240 μm after two days in culture. These spheroids exhibit compact morphology and smoothy boundaries. Later on, little increase in size was observed by microscopic measurements. We have previously reported that hepatocytes cultured as spheroid have

Table 2. Effect of collagen and alginate concentration on bead formation

Collagen conc. / Alginate conc.	0 210%	0.350%	0 525%	0.700%	0.800%
0.2%	×	×	×	×	×
0.5%	×	×	×	△	O
0.7%	×	×	×	△	O
1%	×	×	×	△	O

※ O : well formed, △ : unstable, ×. did not formed

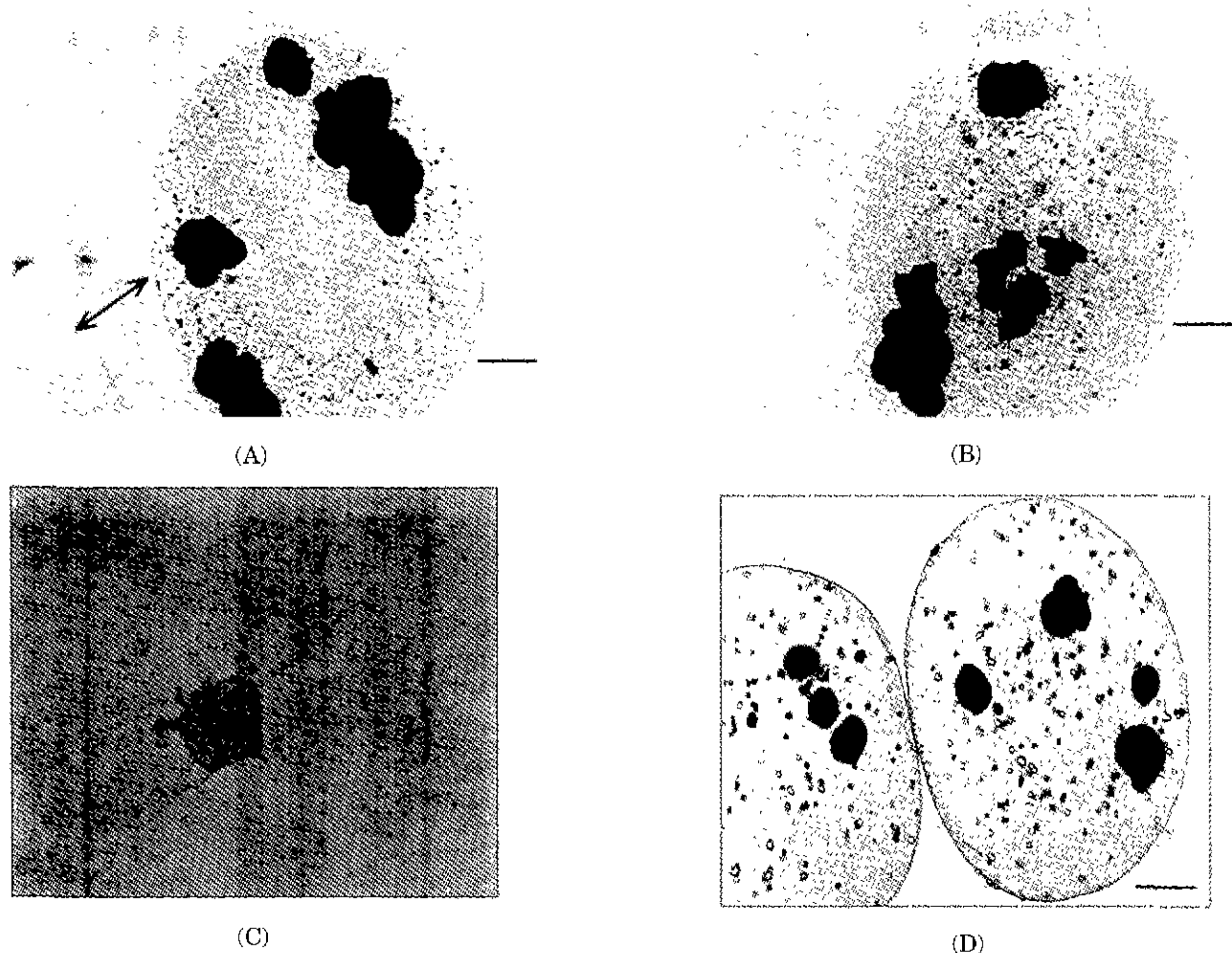


Fig. 2. Light microscopic photographs of hepatocyte spheroids encapsulated in collagen-gel bead. Bar indicate 400 μm. (A) Hepatocyte spheroids in collagen bead with Ca-alginate shell (↔), 40× (B) Collagen bead after removal of Ca-alginate shell. 40× (C) Hepatocyte spheroids in collagen-gel bead, 20 days after encapsulation. 100× (D) Hepatocyte spheroids in Ca-alginate bead, 40×.

a higher functional activity and more differentiated morphology comparing to monolayer culture [11, 12].

Table 2 shows the effect of concentration of collagen and alginate on bead formation observed during screening experiments. While many of the combinations produced capsule membranes, a number of them were too fragile to support their own weight when removed from the liquid. The optimized capsule formation, developed by trial and error, are shown in Table 2.

As described above, Fig. 2-A shows collagen-gel bead with calcium-alginate shell and hepatocytes can spread out in the collagen-gel bead as formed large irregularly shaped aggregates within the collagen gel matrix, whereas no spreading was observed in the alginate bead (Fig. 2-D).

A layer of calcium-alginate was removed when collagen-gel beads were treated with sodium citrate buffer for 10 min and collagen-gel bead was maintained by the end of culture without rupturing of bead (Fig. 2-B & C). Spheroids extended many dendritic processes composed of an arrangement of cells when they were embedded within the collagen gel matrix [16]. But in case of embedding in collagen-gel bead, spheroids showed dendritic structure less than that in collagen gel formed in well plates (Fig. 2-C).

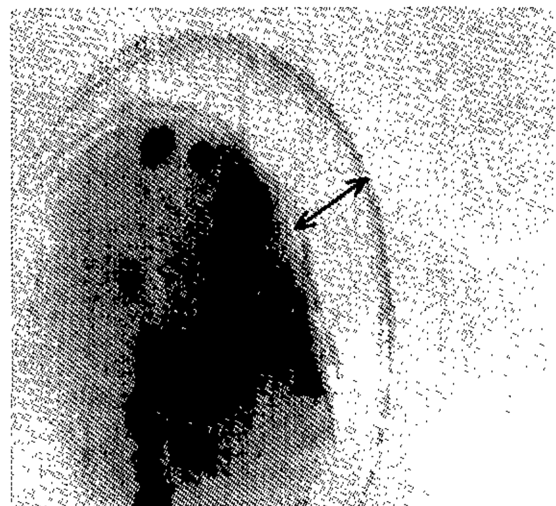


Fig. 3. Light microscopic photograph of hepatocyte spheroids encapsulated in collagen-gel bead. Collagen gel was contracted by hepatocyte spheroids. Arrow indicate the area of contraction.

We attempted to observe the contraction of gel by spheroids embedded in collagen-gel bead. As shown in Fig. 3, it is likely to be affected by concen-

tration and distribution of spheroids in the gel-bead although the mechanism for the contraction of collagen gel is unclear.

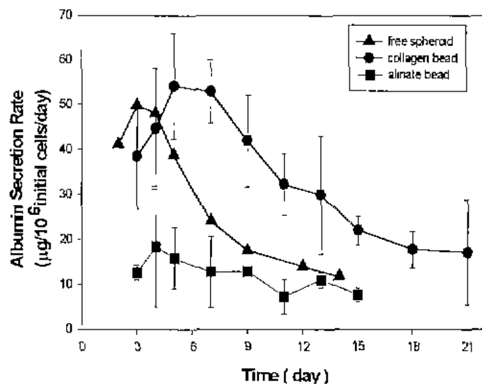
Assays of Hepatocytes Functional Activities

Under spinner culture conditions, hepatocytes lose rapidly their ability to perform differentiated functions by shear stress. In an attempt to evaluate the appropriateness of spheroid supporting system allowing maintenance of hepatocyte functions, the relative rate of albumin production, urea synthesis and ammonia removal were measured as a function of culture time.

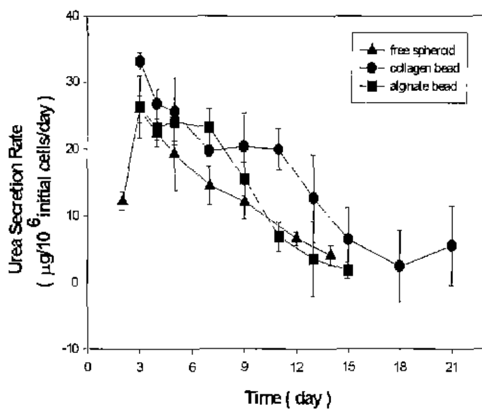
As shown in Fig. 4, these activities were maintained higher than spinner culture exposed to shear stress for up to 21 days (no measurements was performed at later times). Especially, in order to further elucidate the effect of collagen in detail, we compared collagen-gel bead to the general alginate bead.

Spheroids in collagen-gel bead was superior to other culture system on albumin secretion rate (Fig. 4-A). Therefore, we confirmed that albumin synthesis by rat hepatocyte was kept at a high level in culture using collagen-gel system.

The rate of urea production divided by the initial hepatocyte concentration was taken as the specific urea production rate. Fig. 4-B presents urea productivity of spheroids immobilized in collagen-gel bead and alginate bead over a period of 21 days. Specific productivity in both cultures increased for the first two days and then gradually decreased and spheroids immobilized in bead were a more stable than free spheroids exposed to shear stress.



(A)



(B)

Fig. 4. Urea synthesis rate and albumin secretion rate of hepatocyte spheroids cultured as suspension, collagen-gel beads and alginate beads.

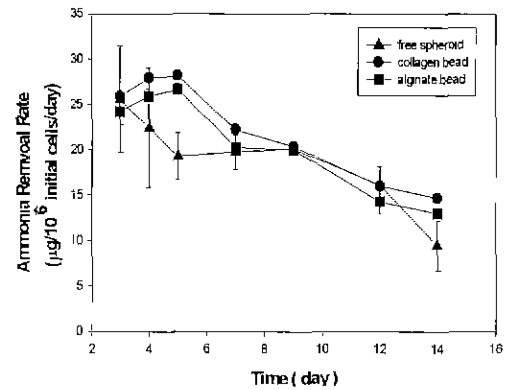
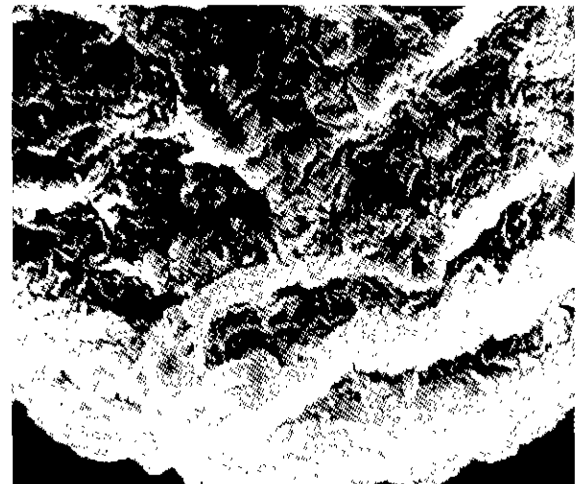
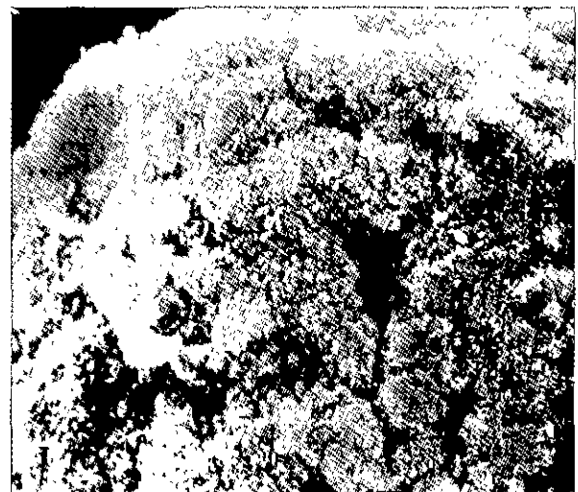


Fig. 5. Ammonia removal rate of hepatocyte spheroids cultured as suspension, collagen beads and alginate beads.



(A)



(B)

Fig. 6. Scanning electron microscopy of collagen bead and alginate bead. The bead samples were taken on 20 days of suspension culture. A: Collagen bead, 600×, B: alginate bead after SCB treatment. 600×.

The ammonia removal rates by spheroids immobilized in collagen-gel bead and free suspended spheroids were measured for 14 days and shown in Fig. 5. In case of collagen-gel bead, ammonia removal activities increased for the first five days and then gradually decreased, whereas free suspended spheroids continuously decreased from the beginning without initial increasing. However, the difference between collagen-gel bead and alginate bead was little on ammonia removal rates. This result was similar in various gel types (i.e. alginate, gelatin, etc.) in the six-well plate (data not shown).

Fig. 6 is a SEM view of the surface of collagen-gel bead and alginate bead.

Beads were found to shrink during amyl acetate evaporation in critical point dryer process. Therefore beads have a rugged surface. Alginate beads were unstable in solution containing calcium chelating agents, whereas collagen-gel beads were stable despite a long-term culture.

Discussion

We attempted to develop collagen gel bead system as a scaling-up form of collagen-gel or collagen sandwich system [15].

Culturing in a sufficiently high density is required for artificial liver support system. However, when hepatocytes were encapsulated and cultured in high densities in a calcium-alginate or general encapsulation system, significant resistance of mass transfer was observed through the calcium alginate gel layer (data not shown). Therefore, we suggest that our culture system has an advantage of removing calcium-alginate gel layer and that long-term culture with high cell density can be performed. For ammonia removal rate, the spheroids expressed a similiar removal rate irrespective of the difference in immobilizing methods. In order to prove this point, the effect of collagen gel and alginate gel were tested with static culture method in six well plate and the results was similiar in various gel type (data not shown). Whereas, these spheroids embedded in collagen-gel beads maintained liver specific functions, such as albumin secretion rate, longer than spheroids exposed to shear stress.

Because hepatocytes *in vivo* contact indirectly with blood through collagen-based extracellular matrix gel layers, various culture methods mimicking the *in vivo* pericellular environment, in which collagen gel plays an important role, were reported to maintain the cellular function of hepatocytes for a long time. Thus, mimicking of the collagen-based pericellular environment *in vivo* appears to be required for sustaining liver functions. In this work, many dendritic processes composed of an arrangement of cells was observed in collagen-gel bead but not in alginate bead. This phenomenon indicates that the morphology of spheroids was influenced by collagen. We could postulate that collagen affected some activities of spheroid since hepatocyte was anchorage-dependent cell although the details of cell-collagen interaction are not clear. However, we also observed the contraction of collagen gel bead because the reduction of bead size may have a significant effect on considering the type of reactor for scale up. But yet we could not determine whether it affect permeability or not. Future studies will attempt to optimize bead formation composed of other extracellular matrix with collagen and also should culture hepatocyte spheroids with high cell density in collagen-gel bead in order to permit scaling-up. Review of the most recent literature indicates that, in the United States alone, at least four different hollow-fiber bioreactor designs are currently being tested as components of extracorporeal liver support system [5, 29-35].

Considering the above facts, we expect that our system will contribute to numerous tissue engineering applications including the extracoporeal artificial liver support system.

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