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MIN6 cells-enclosing aminopropyl-silicate membrane templated by alginate gels differences in guluronic acid content

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Abstract

Mouse insulinoma (MIN6) cells were encapsulated into aminopropyl-silicate membrane deposited on calcium alginate gel beads via the sol–gel synthesis. Two alginates with different guluronic acid (G) contents, high and intermediate, but with the same molecular weights were used. Viability of the cells in the membrane templated by the alginate with an intermediate content of guluronic acid (intermediate-G) was approximately 10% higher than those in the membrane templated by the alginate with a high content of guluronic acid immediately after encapsulation. Growth of cells in vitro was hindered in case of encapsulation in the aminopropyl-silicate membrane deposited on the high-G alginate gel. The MIN6 cells in the microcapsule made from high-G alginate needed a longer period to establish a normoglycemic in recipients than those in the microcapsule made from intermediate-G alginate despite the same number of viable cells implantation. Recipients of the microcapsule with the core made from the intermediate-G alginate maintained their blood glucose values less than 300 mg/dl for a longer period.

Keywords: Alginate; Cell encapsulation; Microcapsule; Silicate; MIN6 cell; Sol–gel process

1. Introduction

Transplantation of cells and tissues encapsulated in immunoisolatable vehicles obviates the need for immunosuppression because it blocks the immune attacks to the grafts. The membrane of the vehicle excludes a permeation of immune molecules such as antibodies, complement proteins, and lymphocytes. On the contrary, low-molecular-weight substances such as nutrients, electrolytes, oxygen, therapeutic secretory products, and cellular waste products have to allow the diffusion across the membrane. Microcapsule is one of the vehicles of the immunoisolatable device. It is spherical shape and the diameter is 300–800 μm. Lim and Sun reported its efficiency in 1980 (Lim and Sun, 1980) by implanting microencapsulated allogenic rat islets. Since then, a number of microcapsules have been reported (Cruise et al., 1998; Iwata et al., 1999; Sakai et al., 2000; Sawhney and Hubbell, 1992). Recent advances in genetic engineering technology have enabled us to make even mammalian cells to secrete any desired products (Chang et al., 1999; Chang and Prakash, 1998). The advances have increased the potential benefits of the cell-enclosing microcapsules for treating a number of diseases. For example, tumors therapies using microencapsulated genetically engineered cells has been performed (Joki et al., 2001; Lohr et al., 2001; Read et al., 2001).

Alginate is the most frequently used material for the microcapsules. It is composed of mannuronic (M)

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and guluronic acids (G) and can be connected by Ca^{2+} through binding of consecutive blocks of G-molecules. The content of guluronic acid plays a vital role in a microscopic structure and a mechanical strength of the resultant Ca-alginate gel (Draget et al., 1994; Smidsrod and Skjak-Braek, 1990). Stabler et al have reported that murine insulinoma βTC3 cells encapsulated in alginate/poly-L-lysine/alginate (Alg/PLL/Alg) microcapsule made from high guluronic acid content experienced a transient hindrance in their metabolic and secretary activity as a consequence of a growth inhibition (Stabler et al., 2001).

In previous study, we have fabricated an alginate/aminopropyl-silicate/alginate (Alg/AS/Alg) microcapsule via sol–gel synthesis (Sakai et al., 2001). The sol–gel matrix was made from 3-aminopropyltrimethoxysilane (APTrMOS) and tetramethoxysilane (TMOS). It was deposited on cell-enclosing Ca-alginate micro gel bead. The crucial difference between the Alg/AS membrane and the well-known alginate/poly-L-lysine membrane is that the former membrane is synthesized via the sol–gel process. The latter membrane is composed of only electrostatic interaction between alginate and poly-L-lysine. The sol–gel process is based on the hydrolysis and condensation of silicone alkoxides. In this process, water is consumed and alcohol, harmful for mammalian cell, is produced as a by-product. Chemical composition of alginate influenced the permeability of the membrane and the quantity of silicate deposition on the gel bead (Sakai et al., 2002a). Thus, understanding the effect of the alginates on the cells after the sol–gel synthesis is important to develop an optimal cell-enclosing Alg/AS/Alg microcapsule. Two alginates with molar ratios of mannuronic acid to guluronic acid (M/G) = 0.65 and 1.30 were used for the encapsulation of mouse insulinoma (MIN6) cells. A growth profile of the enclosed cells and a blood glucose control function of the device were examined.

2. Materials and methods

2.1. Materials

Intermediate-G (Kimica I-1, M/G = 1.30) and high-G (Kimica I-1G, M/G = 0.65) sodium alginates with the same molecular weight (molecular weight = 70,000) were obtained from Kimica (Tokyo, Japan). Aminopropyltrimethoxysilane and tetramethoxysilane were obtained from Tokyo Kasei (Tokyo, Japan). Murine pancreatic β-cell line (MIN6) (Miyazaki et al., 1990) was kindly donated by Prof. Miyazaki (Osaka University, Japan). The MIN6 cells (passage no. 16–20) were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma) containing 15 vol.% heat-inactivated fetal bovine serum, 25 mM glucose, 50 μM β-mercaptoethanol, 75 mg/l penicillin, and 50 mg/l streptomycin. Cells were cultured in a humidified atmosphere at 37 °C with 5% CO_2. DDY mice were obtained from Kyudo (Saga, Japan).

2.2. Cell encapsulation

MIN6 cells were encapsulated in the Alg/AS/Alg microcapsules prepared under the condition resulting in the membrane with immunoisolatable molecular weight cut-off point (Sakai et al., 2002a). Briefly, MIN6 cells were suspended at a concentration of 3.7×10^7 cells/ml in 1.5 wt.% sodium alginate (intermediate-G and high-G) in Ca^{2+}-free Krebs Ringer Hepes buffer solution (CF–KRH, pH 7.4). The suspension was dropped into 100 mM $CaCl_2$ solution buffered with 10 mM Hepes (pH 7.4) using an electrostatic droplet generator. The resultant cell-enclosing gel beads were suspended in n-hexane (14 ml/10 ml Ca-alginate) and cooled in an ice bath. APTrMOS (4.55 mmol/10 ml Ca-alginate) was added into the suspension and stirred for 1 min. Subsequently, TMOS (2.03 mmol/10 ml Ca-alginate) was added and stirred for another 1 min. The Alg/AS microcapsules were washed and incubated in 0.05 wt.% sodium alginate aqueous solution for 10 min to coat the external surface by alginate. All the external alginate layers were prepared from the intermediate-G alginate to eliminate the effect of external alginate on the experiments. The MIN6 cells enclosed in the Alg/AS/Alg microcapsules were cultured with fully supplemented DMEM. The morphology of the encapsulated MIN6 cells was observed with an optical microscope. The microcapsules made from the intermediate-G and high-G alginates were dominated high-G_{core} and intermediate-G_{core}, respectively. There was no significant difference between the diameters of the resultant microcapsules made from the intermediate-G (500 ± 53 μm) and the high-G alginates (508 ± 51 μm).

2.3. Encapsulated cell viability

After liquefying the core alginate gel with 55 mM sodium citrate aqueous solution, the microcapsules were pressed moderately using a nylon mesh with 200 μm pores to break them. The viability of the recovered cells was determined by trypan blue exclusion.

2.4. Microcapsules implantation

Male DDY mice (6–8 weeks old) were made diabetic by a single intraperitoneal injection of streptozotocin (200 mg/kg body weight). The mice with non-fasting blood glucose level exceeding 400 mg/dl in a two consecutive measurements after 1 weeks of the injection were used as recipients. Immediately after encapsulation, the MIN6 cells-enclosing intermediate-G_{core} and high-G_{core} microcapsules were implanted into the peritoneal cavities of the recipients ($n = 4$ for each microcapsule). In the control group, free MIN6 cells were implanted into the peritoneal cavity of each recipient ($n = 4$). The same numbers of viable cells (3.0×10^7 cells) were implanted per animal. Non-fasting blood glucose samples were taken from the lateral tail vein at regular interval, and blood glucose level was measured using glucose analyzer (Sanwa Chem., Nagoya, Japan). The recipients of the intermediate-G_{core} and the high-G_{core} microcapsules were sacrificed 62 and 25 days after implantation, respectively, by deep anesthesia with ether to retrieve the implanted microcapsules. The retrieved microcapsules were observed with an optical microscope.

All the protocols for using animals were according to the recommendations of Kyushu University entitled "Guide for the care and use of laboratory animals."

3. Results

The viability of the cells immediately after encapsulation in the intermediate-G_{core} microcapsules was 82.3 ± 2.2% (mean ± S.D., $n = 3$, Fig. 1). It was about 10% higher than those in the high-G_{core} microcapsules (72.9 ± 1.7%, mean ± S.D., $n = 3$). Fig. 2 shows representative photographs of the MIN6 cells-enclosing Alg/AS/Alg microcapsules made from the alginates with high-G (a, b, c) and intermediate-G (d, e, f) after 1 (a, d), 14 (b, e), and 45 days (c, f) of encapsulation. For the first few days after encapsulation, the MIN6 cells were uniformly distributed through out the microcapsules as shown in Fig. 2a and d. The MIN6 cells formed small cell clusters in only the microcapsules prepared from the intermediate-G alginate approximately 7 days after encapsulation. Fourteen days after encapsulation, a number of small cell clusters, several dozen micrometers in diameter, were observed in the intermediate-G_{core} microcapsules (Fig. 2e). The diameter of the cell clusters in the intermediate-G_{core} microcapsules continued to increase until 35 days after encapsulation via aggregation of themselves and proliferation of the cells. The average diameter of the clusters after 49 days of encapsulation in the intermediate-G_{core} microcapsules was 255 ± 91 μm. Histology cross-section of the clusters stained with hematoxylin eosin after 49 days of encapsulation in the intermediate-G_{core} microcapsule showed an existence of central necrosis region (Fig. 3, light purple area surrounded by dark purple area). The MIN6 cells in the high-G_{core} microcapsules did not form apparent cell clusters even after 45 days of encapsulation (Fig. 2c).

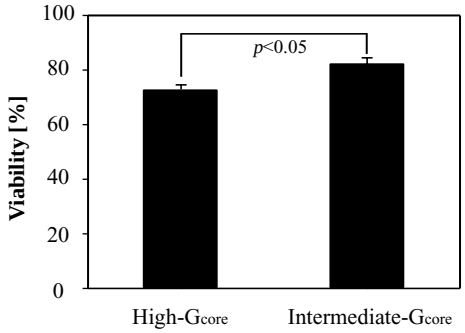

Fig. 1. Viabilites of MIN6 cells encapsulated in high-G_{core} and intermediate-G_{core} microcapsules. Error bar represents mean ± S.D. *P*-value was calculated using the two-tailed Student's unpaired *t*-test.

Fig. 4 shows non-fasting blood glucose concentrations in streptozotocin-induced diabetic DDY mice implanted the same number of the encapsulated and free viable MIN6 cells (3.0×10^7 viable cells/animal). The MIN6 cells encapsulated in the intermediate-G_{core} microcapsules established normoglycemia within 24-h of implantation ($n = 4$, Fig. 4b). On the contrary, the MIN6 cells encapsulated in the high-G_{core} mi-

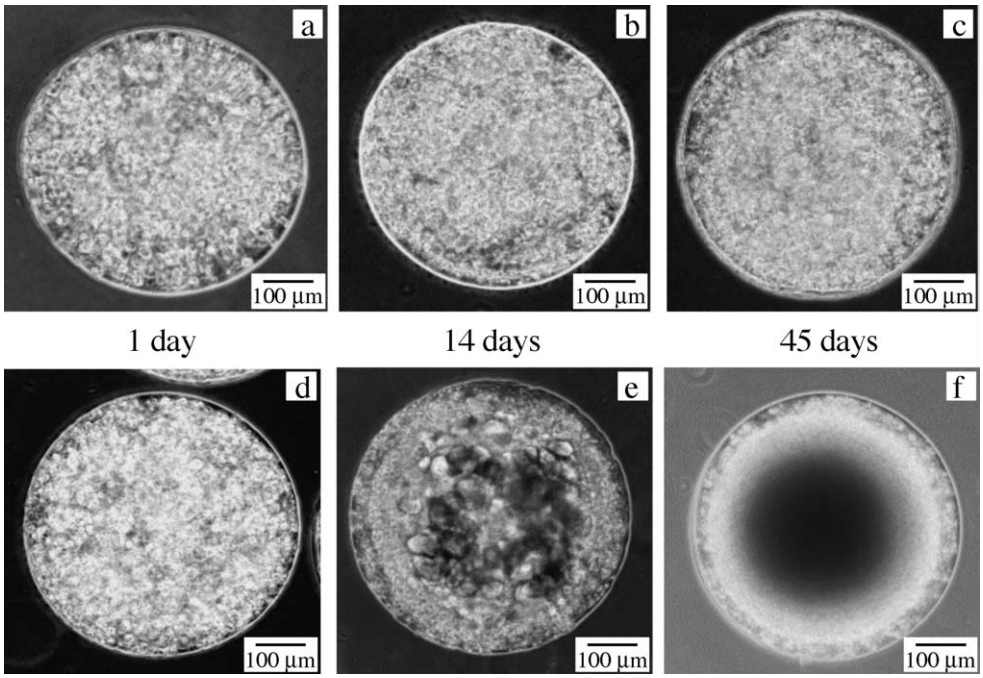

Fig. 2. Photographs of MIN6 cells-enclosing (a, b, c) high-G_{core} and (d, e, f) intermediate-G_{core} microcapsules after (a, d) 1, (b, e) 14, and (c, f) 45 days of encapsulation. The enclosed MIN6 cells were incubated in fully supplemented DMEM. MIN6 cell clusters can be seen in the intermediate-G_{core} microcapsules after (e) 14 days and (f) 45 days of encapsulation. Original magnification was 200×.

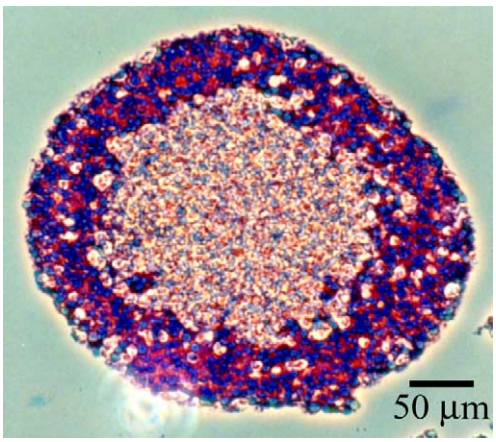

Fig. 3. Cross-section of MIN6 cell cluster stained with hematoxylin and eosin after 49 days of encapsulation in intermediate-G_{core} microcapsule. The enclosed MIN6 cells were incubated in fully supplemented DMEM. Necrotic figure (light purple area surrounded by dark purple area) can be seen in the center part of the MIN6 cell cluster. Original magnification was 200×.

crocapsules spent several days to establish normoglycemia ($n = 4$, Fig. 4a). Three of four recipients of the intermediate-G_{core} microcapsules maintained their blood glucose level less than 300 mg/dl for more than 2 months. The remaining one recipient maintained the state for about 3 weeks. As compared with the result of the recipients of the intermediate-G_{core} microcapsules, the periods of $<$300 mg/dl blood glucose concentration were shorter in the recipients of the high-G_{core} microcapsules. All the recipients of the high-G_{core} microcapsules returned to hyperglycemia approximately 3 weeks after implantation. As shown in Fig. 4c, the blood glucose concentrations of all the recipients of free MIN6 cells decreased slightly on the day following implantation ($n = 4$). The values continued to decrease gradually, and fell into hypoglycemia, $<$45 mg/dl, within 31 days after implantation. Then, all the recipients died by the 50-day.

Fig. 5 shows photographs of the retrieved microcapsules from the peritoneal cavities of the recipients of the (a) intermediate-G_{core} and the (b) high-G_{core} microcapsules after 62 and 25 days of implantation,

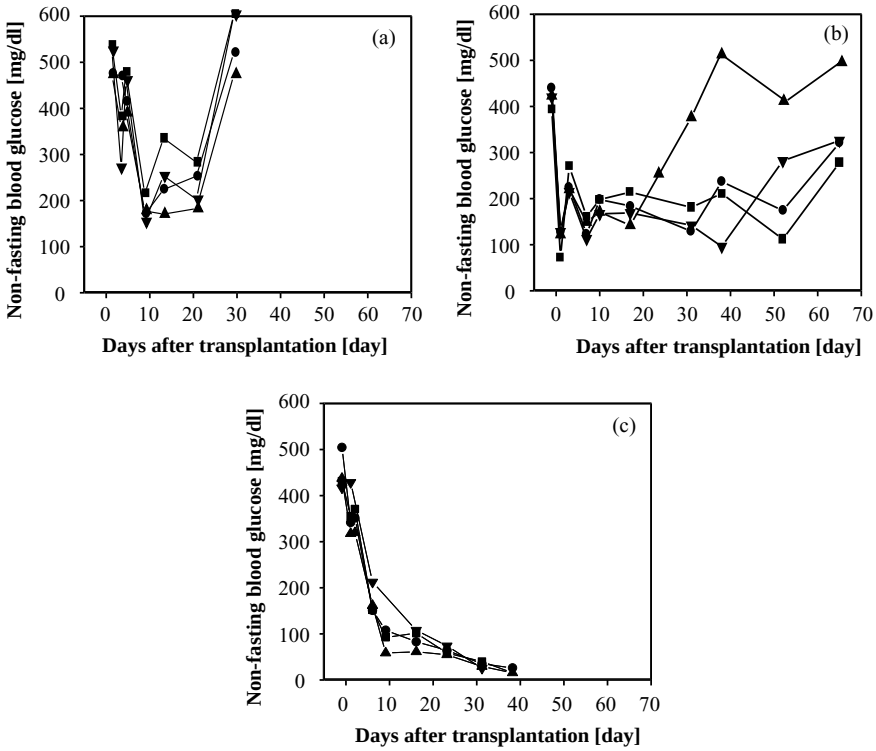

Fig. 4. Non-fasting blood glucose level of recipients implanted MIN6 cells-enclosing: (a) high-G_{core}; (b) intermediate-G_{core} microcapsules; and (c) free MIN6 cells. The same number of viable MIN6 cells (3.0×10^7 cells/recipient) was implanted for all the recipients.

respectively. The intermediate-G_{core} microcapsules were retrieved from the recipients maintained their blood glucose level less than 300 mg/dl for more than 2 months. Apparent cell clusters, which observed in the in vitro culture of the intermediate-G_{core} microcapsule after 1 week of encapsulation (Fig. 2e and f), were not observed in both the microcapsules. Less than 5% of overgrowth was observed for the each microcapsule retrieved from all the recipients.

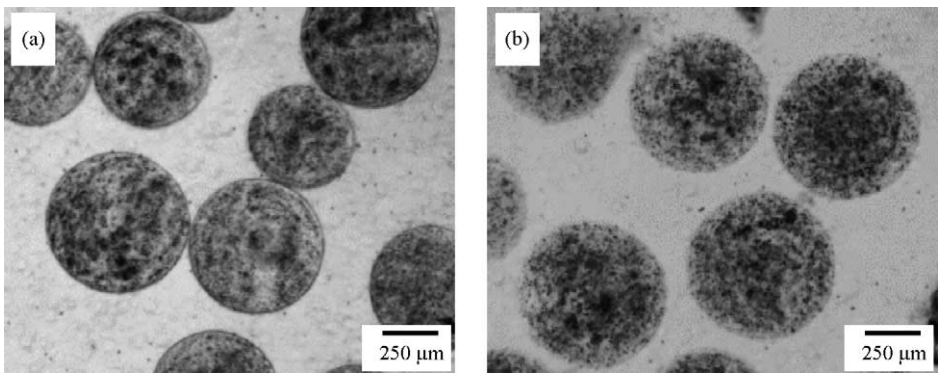

Fig. 5. Photographs of MIN6 cells-enclosing: (a) high-G_{core}; and (b) intermediate-G_{core} microcapsules retrieved from the peritoneal cavities of recipient. The high-G_{core} and the intermediate-G_{core} microcapsules were retrieved 25 and 62 days after the implantation, respectively.

4. Discussion

Microencapsulation of cells has been developed for transplantation of cells without immunosuppression for nearly two decades. Recent advances in genetic engineering technology have raised the importance of the microencapsulation techniques. We have developed an Alg/AS/Alg microcapsule (Sakai et al., 2001) and revealed its feasibility for an islet-enclosing bioartificial pancreas by using the alginate with intermediate content of guluronic acid (Sakai et al., 2002b). We have also shown that the chemical composition of the core alginate affected permeability of the membrane and quantity of the silicate deposition on the alginate gel (Sakai et al., 2002a). It is important to reveal these influences on the enclosed cells for developing optimal preparation conditions of the microcapsule. Low-G alginate was not used in the present study because the aminopropyl-silicate membrane prepared on the gel bead made from low-G alginate had no immunoisolatable permeability (Sakai et al., 2002a).

In vitro culture, only the MIN6 cells encapsulated in the microcapsule made from the intermediate-G alginate formed cell clusters (Fig. 2e and f). The inhibition of cell growth in the high-G alginate gel reported here are in agreement with those of Constantinidis et al. (Constantinidis et al., 1999; Stabler et al., 2001) for βTC3 cells encapsulated in Alg/PLL/Alg microcapsules. They have explained that the inhibition was induced by not simply the overall rigidity of the matrix, but rather the strength of the alginate gel network at the microstructure level (Stabler et al., 2001). Helmlinger et al. have evidenced the effect of stress on the growth of spheroid in matrices using agarose gel (Helmlinger et al., 1997). The content of guluronic acid of alginate plays a vital role in the strength because the gelation of alginate with Ca^{2+} results from the formation of "egg-box junction" of guluronic residues and Ca^{2+} (Draget et al., 1997; Smidsrod and Skjak-Braek, 1990). Thus, one reason of the inhibition of the cell growth in the high-G_{core} microcapsule can be interpreted in terms of the higher stress at the microscopic level. The difference in the degree of the harmful effects accompanied by the sol–gel synthesis, consumption of water and production of alcohol as a by-product during the sol–gel process, is interpreted as another reason. The quantity of silicate deposition on the gel bead made from the high-G alginate was about 16% larger than that on the gel bead made from the intermediate-G alginate (Sakai et al., 2002a). An increasing in the quantity of silicate deposition equates with an increasing in the quantities of alcohol produced as a by-product and of water consumed during the sol–gel process. In fact, the viabilities of the encapsulated cells before the deposition of the aminopropyl-silicate were almost the same and they were about 95%, yet, the viability of the MIN6 cells in the intermediate-G_{core} microcapsules was approximately 10% higher than those in the high-G_{core} microcapsules (Fig. 1).

Cellular overgrowth on the surface of implanted microcapsule is a major factor inducing graft failure because it results in an insufficient oxygenation to the enclosed cells (De Vos et al., 1997; Uludag et al., 2000). In present study, it does not contribute to the shorter period of normoglycemia of the high-G_{core} recipients because more than 95% of the retrieved microcapsules were not overgrown. The phenomenon of graft failure in the absence of overgrowth on the microcapsule under the sufficient volume of islets transplantation has been reported previously (De Vos et al., 1997) and is explained by functional limitation and lifespan limitation of the enclosed islets. Lower insulin secretion function of the cells in the high-G_{core} microcapsule is interpreted as a major factor which resulted in the shorter period of normoglycemia of the high-G_{core} recipients than those of the intermediate-G_{core} recipients (Fig. 4a and b). The lower insulin secretion function of the enclosed cells than those in the intermediate-G_{core} microcapsule was shown in the result that the high-G_{core} recipients showed higher blood glucose level after 1-day of the implantation. In previous study, insulin secretion function of the islets enclosed in the Alg/AS/Alg microcapsule made from intermediate-G alginate decreased with increasing the quantity of the silicate deposition (Sakai et al., 2002c). Take into consideration this result and the larger quantity of silicate deposition on high-G alginate gel (Sakai et al., 2002a), it appears that the lower insulin secretion function resulted from the larger harmful effects accompanied by the sol–gel process. The enclosed cells in the high-G_{core} microcapsule showed a lower viability than those in the intermediate-G_{core} microcapsule (Fig. 1). Thus,

the larger harmful effects may also have induced the shorter lifespan of the enclosed cells resulting in shorter period of normoglycemia. One may hypothesize that the alginate gel matrices itself hindered the insulin secretion function of the cells in the high-G_{core} microcapsule. This, however, is unlikely since it has been reported that the insulin secretion function of βTC3 cells, both of MIN6 and βTC3 are murine pancreatic β cell lines, in the Alg/PLL/Alg microcapsule was not influenced by the chemical composition of alginate (Stabler et al., 2001). The crucial difference between the Alg/PLL/Alg and Alg/AS/Alg microcapsules is that the latter membrane is synthesized via the sol–gel process. Supplying molecules typified by oxygen to encapsulated cells plays a vital role in the success of cell-enclosing microcapsule implantation (Lee and Bae, 2000; Uludag et al., 2000). Diameter of microcapsule and permeability of capsule affects it. In the present study, however, it had no responsibility for the difference in the results for the two kinds of microcapsules: The diameters of the microcapsules were almost the same, and independent permeability measurements have shown that the membrane prepared on the gel bead made from high-G alginate had a better permeability for smaller substances than that on the gel bead made from intermediate-G alginate (Sakai et al., 2002a).

One of the intermediate-G_{core} recipients showed a significant shorter period of normoglycemia than the other three recipients despite that a serious cellular overgrowth was not observed for the retrieved microcapsules in comparison with those from the other recipients. The shorter period of normoglycemia may have resulted from an individual difference in insulin sensitivity of the recipients, or a decline in insulin secretion function which is attributed to an insufficient oxygenation induced by an unfortunate localization of the implanted microcapsules.

MIN6 cell line was established from β cell tumors that developed in C57BL/6 mice and can proliferate permanently (Miyazaki et al., 1990; Suzuki et al., 2002). It is known that tumor cells can dodge the immune attacks from the host. Thus, the risk of implanting a proliferative cell line is that it has a potential to become a tumor in vivo (Chang et al., 1999). It appears that a certain degree of the implanted free MIN6 cells was killed by the immune attack of the host, because the blood glucose concentrations of the recipients decreased but it was higher than those of the intermediate-G_{core} recipients 1-day after implantation (Fig. 4c). It is reasonable to think that the continuous decrease in blood glucose level leading to the hypoglycemia resulted from the excess growth of the survived cells. The reoccurrence of hyperglycemia of the recipients of the each microcapsule (Fig. 4a and b) and the non-growth of the cells in the implanted microcapsules (Fig. 5) represent that the AS/Alg/AS microcapsule suppressed an excess growth and a leakage of the encapsulated MIN6 cells.

In the intermediate-G_{core} microcapsule, the MIN6 cells clusters showed a constant mean diameter after 35 days of incubation. The value, approximately 250 μm in diameter, was a half of the microcapsules diameter. Necrotic region was observed at the center part of the clusters (Fig. 3, light purple area). The necrotic region would result from the low oxygen tension caused by the oxygen consumption of the cells surrounding the necrotic region. Recently, it has been reported that a cell-to-cell contact in multicellular aggregate induced cell proliferation arrest by up-regulating expression of the cyclin-dependent kinase inhibitor (LaRue et al., 1998). Thus, the reason for the constant diameter of cell clusters in the intermediate-G_{core} microcapsule was interpreted due to either or both of the two reasons: balance between cell proliferation and necrosis of internal cells, and cell cycle arrest induced by cell aggregation. In contrast to the cell cluster formation in vitro, it was not occurred in vivo even after 62 days of implantation into the peritoneal cavities (Fig. 5b). The low oxygen tension and low concentrations of essential minerals in the peritoneal fluid (Colton and Avgoustiniatos, 1991; Schrezenmeir et al., 1994) may contribute to the difference in the growth of the encapsulated cells between in vitro and in vivo. De Vos et al. (De Vos et al., 1997) have explained that the phenomenon of decrease of the size in microencapsulated islets after implantation into the peritoneal cavities of recipients results from the insufficient supply of oxygen and essential minerals.

To conclude, this study demonstrates that the guluronic acid content of alginate plays a vital role in the proliferation and secretory activities of the cells en-

capsulated in the Alg/AS/Alg microcapsule. It was a consequence of the effects accompanied by the sol–gel process as well as the stress given by the alginate gel at a microscopic level.

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