tissues [28,29]. Inaba et al. reported the cryopreservation of alginate-based microencapsulated islets by slow-freezing [28]. They demonstrated that microencapsulated islets preserved under liquid nitrogen can normalize diabetic mice. Also Stiegler et al. succeeded in the cryopreservation of sodium cellulose sulfate-based microencapsulated single cells [29]. Recently, we examined the cryopreservation of Mic islets using a newly developed vitrification solution that consists of 5.38 M ethylene glycol, 2 M DMSO, 0.1 M PEG 1000, and 0.00175 M PVP K10 (MW 10,000) in Euro-Collins (34.95 g/L dextrose, 7.3 g/L K_2HPO_4, 2.04 g/L KH_2PO_4, 1.12 g/L KCl, and 0.84 g/L $NaHCO_3$), assessing the morphology and insulin secretion function before and after cryopreservation [30,31]. The Mic islets were viable and restored the insulin secretion capability after vitrification and warming; in addition, the mechanical and physico-chemical properties of the agarose hydrogel were well preserved after vitrification and warming. Fig. 3 shows phase-contrast images and immunohistochemically stained images of non-cryopreserved Mic islets after 7 days in culture and cryopreserved Mic islets after one day and seven days in culture. There were no significant differences in the morphology of the islets or agarose microcapsules.

We also evaluated the *in vivo* functions of cryopreserved Mic islets. The Mic islets were transplanted into the intraperitoneal cavities of STZ-induced diabetic mice to evaluate their ability to control blood glucose metabolism and evaluate their immunoisolation properties *in vivo* compared to non-cryopreserved agarose-islets. We employed a xenogeneic transplantation model [31] in which Mic hamster islets were transplanted into the diabetic mice. The mice received 1000, 2000, or 3000 Mic islets intraperitoneally following cryopreservation in a vitrification solution (Fig. 4). Transplantation of cryopreserved Mic islets restored normoglycemia in the STZ-induced diabetic mice. The mean (± SD) normoglycemic period was 32.0 ± 13.2 days, 46.3 ± 13.3 days, and 47 days for recipients of 1000 ($n=5$), 2000 ($n=4$), and 3000 ($n=2$) cryopreserved agarose-islets, respectively, whereas the mean normoglycemic period was 53.2 ± 16.7 days for recipients of 1000 non-cryopreserved Mic islets ($n=7$). The hamster islets within the capsules were completely isolated from the mouse tissues 41 days after transplantation (Fig. 5). Immunohistochemical analysis showed that the islets in the microcapsules expressed insulin. These data indicate that cryopreserved agarose-encapsulated islets transplanted as a bioartificial pancreas successfully controlled blood glucose levels for extended periods. Our results indicated that banking of microencapsulated islets in agarose hydrogel is feasible.

3.4. Clinical application of the bioartificial pancreas

There have been a few reports of the clinical application of a bioartificial pancreas. More than 10 years ago, in 1994, Soon-Shiong et al. reported the first clinical trials of alginate-encapsulated islets in two type 1 diabetes patients [32]. Guluronic acid-rich purified alginate was used to improve the stability and biocompatibility of the alginate microcapsules. One patient took low doses of cyclosporine and azathiopine because they had already received living-related kidney transplantation. At first, 10,000 microcapsules per kg of body weight were transplanted into the peritoneal cavity. Then, an additional 5000 microcapsules per kg were transplanted 6 months after the first transplant. The dosage of exogenous insulin required decreased, and the level of C-peptide increased. Nine months after the first transplantation, the recipient was insulin-free.

In 2005, Valdés-González et al. described a device in which neonatal islets and Sertoli cells were encapsulated and transplanted into 12 patients with type 1 diabetes aged 11–17 years [33]. The device consisted of 6 × 0.8 cm surgical grade stainless steel mesh tubes, with an interior PTFE rod. The device was left in place for 2 months to allow vascularized collagen tissue to completely surround and penetrate it. After this time, the PTFE rod was removed and the device implanted in a subcutaneous site in the patient's upper anterior abdomen. Porcine islets (250,000) and Sertoli cells (7.5×10^6–2.5×10^7) were infused into the space. The device was then sealed with a small PTFE cap. No immunosuppressive therapy was given to any of the recipients, though the donor and recipient combination was xenogeneic. An additional device transplantation was performed in four patients after 3 years. For 12 patients, the required amount of exogenous insulin was reduced by half. Two recipients were insulin-free for several months. Insulin derived from the porcine islets was detected in three recipients 4 years after transplantation. No porcine endogenous retrovirus DNA or RNA was detected in the recipient blood by PCR or RT-PCR.

In 2006, Calafiore et al. reported the transplantation of alginate microcapsules of human islets into 10 patients with type 1 diabetes [34]. No immunosuppressive therapy was given to any of the recipients. Microcapsules (400,000–600,000; total volume approximately 50 mL) were suspended in 100 mL saline and transplanted into the peritoneal cavity. Six months after transplantation, the blood glucose levels in two recipients were decreased, though normoglycemia was not achieved. For one of the two recipients in Calafiore et al.'s report, C-peptide was detected at 1 year. The results showed that the transplantation of microencapsulated islets enabled the stabilization of blood glucose metabolism.

4. Conformal coating of cell surfaces

There is still a serious issue remaining for microcapsule type bioartificial pancreases: the increase in the total volume of the implant after microencapsulation. The average diameter of islets is roughly 150 μm. The diameter of capsules would be approximately three times as large as the original islets. The total volume of the microcapsules is estimated to be 27 times as large as the islets due to increasing by a third power of the radius. In clinical settings, the volume of islet suspensions is 10 mL, and it would be more than 270 mL after microencapsulation. A site to implant such a large volume is difficult to find.

Much effort has been made to reduce the size of the capsules. For example, Calafiore et al. reported much smaller microcapsules, 300 μm in diameter [35], and claimed they would create a reasonable total volume for clinical application. Islets, however, are clinically transplanted into the liver through the portal veins, and capsules with a diameter larger than the islet itself are expected to plug larger blood vessels. This situation imposes harmful effects on the patient's liver. The diameter of the encapsulated islets must be much smaller than that currently attained to allow for the transplantation of the islets through the portal veins. Development of coating technique of islets with a very thin membrane, or conformal coating should be important to reduce the diameter of microcapsules and it realizes their transplantation into liver through the portal veins. In the following, we will introduce new techniques for conformal coating of islets and discuss their advantages and disadvantages.

4.1. Polyion complex formation

Enclosing the cell surface with polymer chains has been applied to the preparation of the bioartificial pancreas (Figs. 6 and 7). Several groups have reported islet surface coverage with a thin polyion complex membrane using the layer-by-layer method [36,37]. The negatively charged cell surface is treated with a cationic polymer solution, and the surface is further exposed to an anionic polymer solution to form a layer-by-layer membrane. Krol et al. attempted to encapsulate human pancreatic islets and demonstrated a minimal loss of islet function and viability by coating them with a poly(allylamine hydrochloride) (PAH)/poly(styrene) sulfate (PSS)/PAH multilayer membrane [36]. Our group also examined an alginate/PLL/alginate multilayer coating on the surface of islets. An NH_2-PEG-lipid was introduced into the islet cell membrane, forming a positively charged islet surface to facilitate the electrostatic binding of negatively

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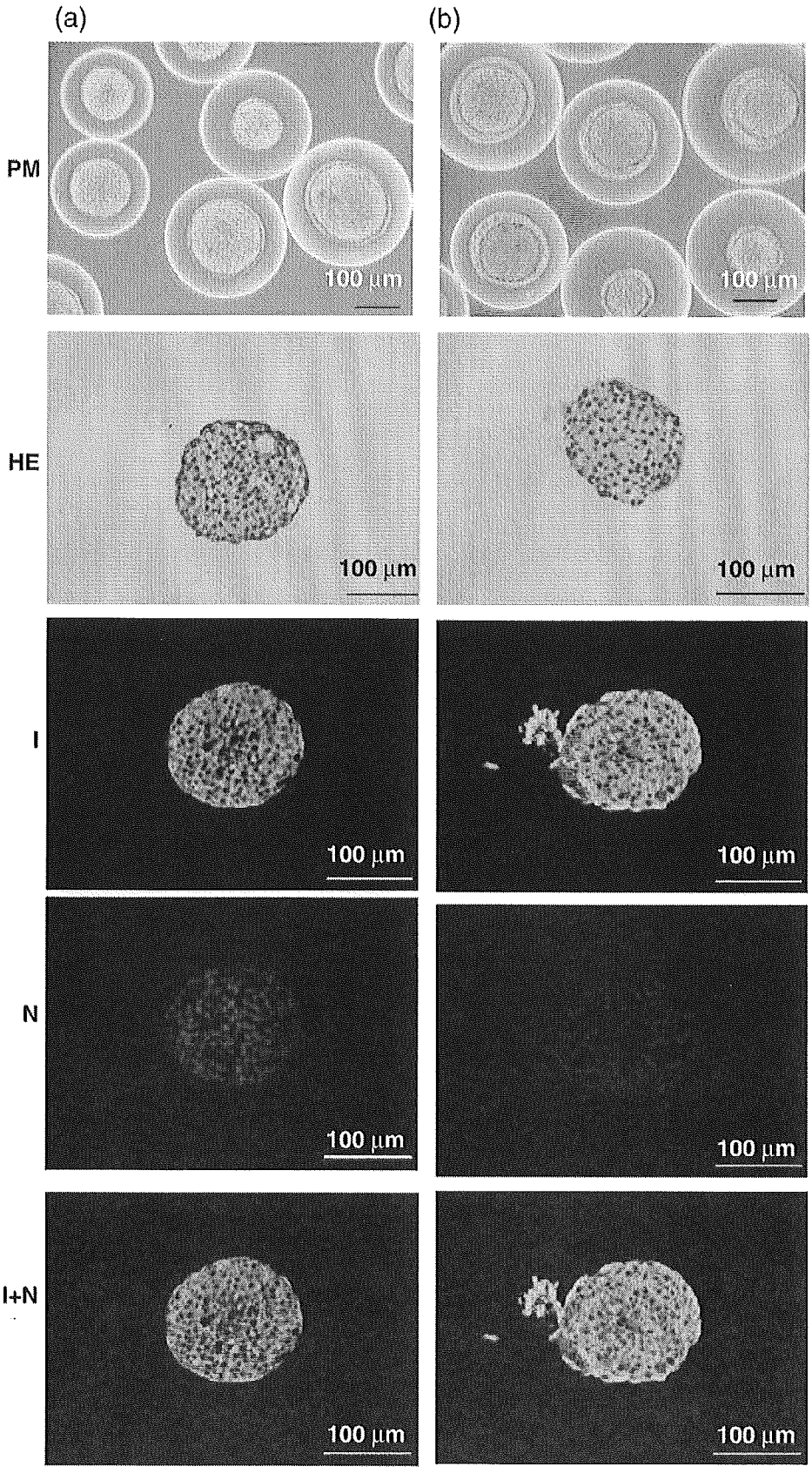

Fig. 3. Morphology and histology of agarose-islets following cryopreservation, thawing, and culture for one week. (a) Control, no cryopreservation. (b) The morphology of the cultured cryopreserved agarose-islets was examined by phase-contrast microscopy (PM). The samples were stained with hematoxylin–eosin (HE), Alexa 488-labeled anti-insulin antibody (I), and Hoechst 33342 for nuclear staining (N). Insulin and Hoechst 33342 stainings were also merged (I + N).

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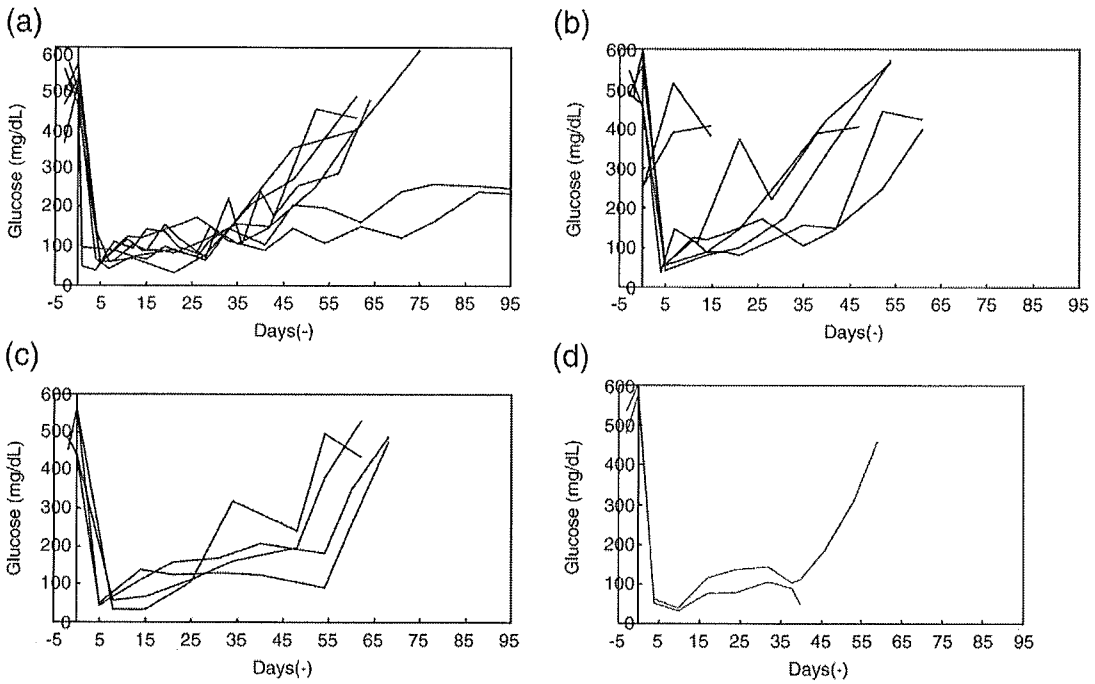

Fig. 4. Changes in the non-fasting blood glucose levels of streptozotocin-induced diabetic BALB/c mice after intraperitoneal implantation of (a) agarose-islets (1000 capsules, $n=7$) or cryopreserved agarose-islets with 15-deoxysperguarin: (b) 1000 capsules, $n=5$; (c) 2000 capsules, $n=4$; (d) 3000 capsules, $n=2$. (d) Forty-one days after implantation, the mice were sacrificed for HE and insulin staining.

charged alginate [37]. Islets coated with this membrane respond normally in a static glucose stimulation assay. Although a layer-by-layer membrane using a polyion complex can be achieved on the islet surface, direct interactions between the cationic polymer and cell surface should be avoided because the cell membrane would be gradually destroyed. The problem is that most cationic polymers, such as poly-L-lysine (PLL) and poly(ethyleneimine), are extremely cytotoxic, and treated cells are severely damaged.

4.2. PEG coating through chemical reaction

Surface modification with ultra thin polymer membranes was originally examined by chemically treating red blood cells (RBC) to enclose surface antigen [38–42]. Islets were also covered by a PEG layer. PEG carrying an activated ester, *N*-hydroxyl-succinimidyl ester (NHS), group at one end is employed to react with an amino group of the membrane proteins or collagen layer on the islet surface [43–45].

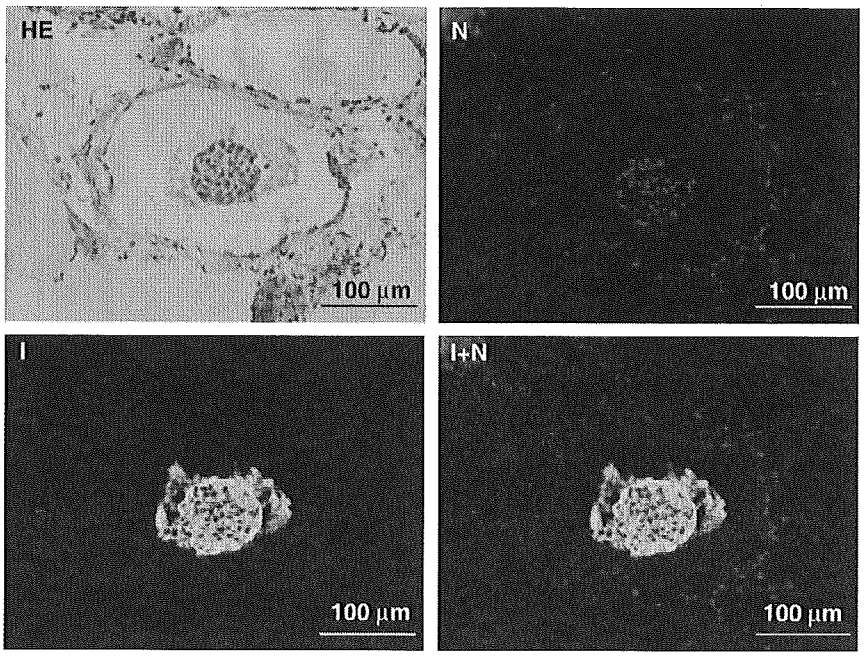

Fig. 5. Histological analysis of a cryopreserved agarose-islet implanted in the intraperitoneal cavity of a diabetic mouse. The graft was retrieved 41 days post-transplantation. HE, hematoxylin–eosin staining; N, Hoechst 33342 dye staining; I, insulin staining; I + N, merged Hoechst 33342 dye and insulin staining images.

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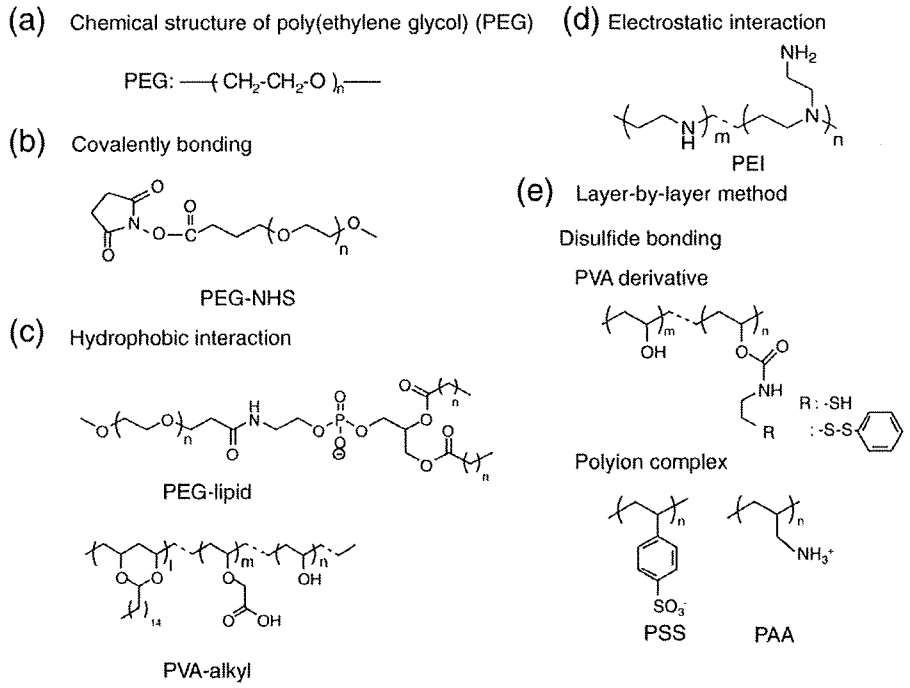

Fig. 6. Chemical structure of (a) poly(ethylene glycol) (PEG), (b) PEG carrying *N*-hydroxyl-succinimidyl ester (PEG-NHS), (c) PEG-conjugated phospholipid (PEG-lipid) and polyvinyl alcohol carrying side alkyl chains (PVA-alkyl), (d) poly(ethyleneimine) (PEI), and (e) poly(allylamine) (PAA), poly(styrene) sulfate (PSS), and PVA derivative (PVA-SH, PVA-PD).

PEG carrying an activated ester group at both ends is also used for surface modification. One of the molecules is used for covalent bonding to the surface and the other for the immobilization of albumin to mask the surface antigen. PEG-NHS is shown in Fig. 6(b), and a mode of reaction is schematically shown in Fig. 7(a). When the molecules were applied to the surface modification of porcine islets,

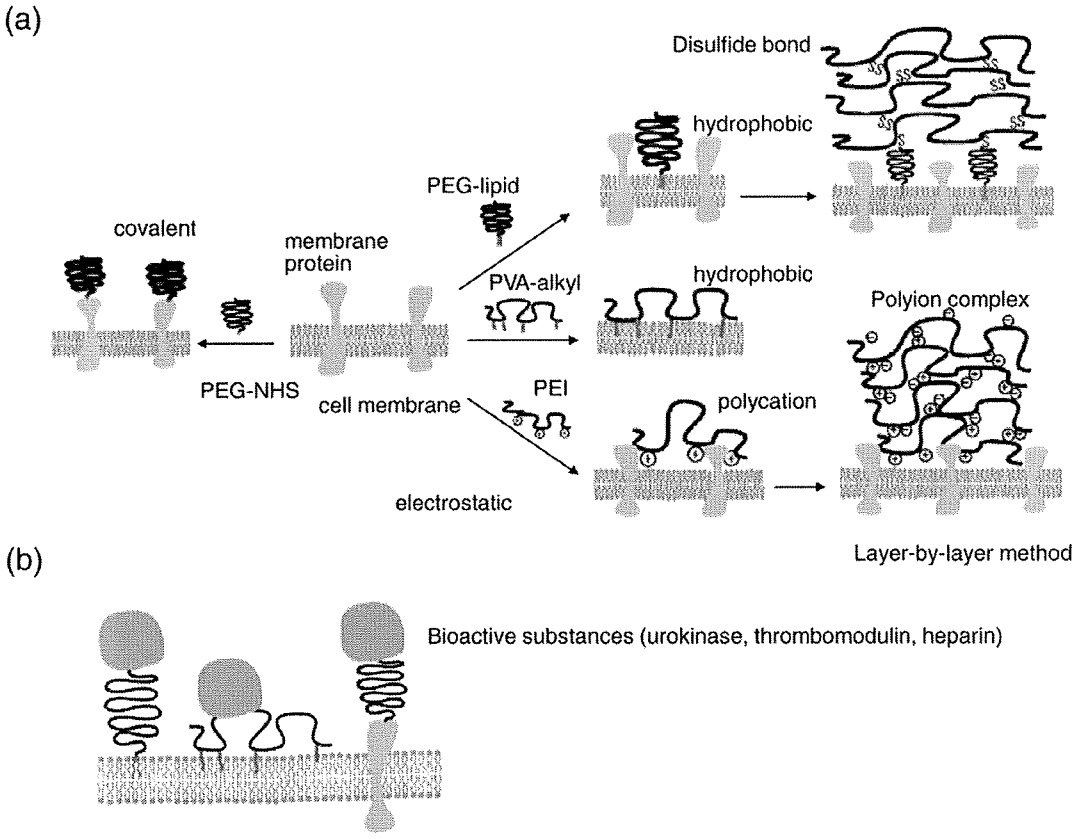

Fig. 7. (a) Cell surface modification with synthetic polymers by covalent bonding, hydrophobic interaction, electrostatic interaction, and the layer-by-layer method. (b) The immobilization of bioactive substances to the cell surface via polymers.

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no change was observed in their morphology or viability [43]. Five thousand porcine islets modified with PEG-NHS were transplanted into the liver of NOD-SCID mice through the portal vein. The mice were previously transplanted with human lymph cells to reconstitute the human immune system, providing a xenotransplantation model of porcine to human. In this study, the porcine islets were previously transfected with adenovirus expressing *Bcl-2* to suppress apoptosis. When naïve islets and naked *Bcl-2* expressing islets were transplanted, the blood glucose level was not normalized for either case where it was reduced to approximately 150 mg/dL for 15 days. On the other hand, normoglycemia could be achieved by transplanting islets that expressed *Bcl-2* and were modified with PEG and albumin for 15 days. It seems that PEG on the islet surface is supplemental against the immune rejection reaction. In fact, when PEG modified islets that did not express *Bcl-2* were transplanted, the blood glucose level was transiently normalized but increased again.

Byun's group reported that the surface of islets covered with PEG reacted with the amino groups of the collagen layer remaining on the islet surface. Islets from a Sprague–Dawley rat (1200 islets) were transplanted under the kidney capsule of an STZ-induced diabetic Fisher 344 rat [44,45]. When naked islets were transplanted without cyclosporine A, an immunosuppressive drug, normoglycemia was not maintained for more than 5 days. With low dose of cyclosporine A, normoglycemia was prolonged up to 12 days. On the other hand, when islets with a PEG-NHS modified surface were transplanted into recipient rats treated with low dose cyclosporine A, normoglycemia was maintained for 1 year. Without cyclosporine A, the period of normoglycemia was at least 11 days, even though the islet surface was modified with PEG. It seems that the immune rejection reaction can be effectively suppressed with a combination of PEG surface modification and cyclosporine A administration.

4.3. Polymer coating through hydrophobic interaction

Our group has been studying the surface modification of cells and islets with thin polymer membranes using various amphiphilic polymers, such as PEG-conjugated phospholipid (PEG-lipid) and polyvinyl alcohol (PVA), carrying long alkyl chains [37,46–51]. PEG-lipid has been widely used for modifying the surface of liposomes [52,53]. Surface modification with PEG-lipid improves the biocompatibility, thus prolonging the circulation time of liposomes *in vivo*. Bertozzi et al. also studied the non-covalent cell surface modification with phospholipid derivatives bearing polysaccharides and proteins [54,55].

The chemical structure of PEG-lipid is shown in Fig. 6(c), and its interaction with the cell membrane is shown schematically in Fig. 7(a). PEG-lipid is an amphiphilic molecule composed of a hydrophilic PEG chain and hydrophobic phospholipids. The PEG chain can be anchored to the cell surface because the hydrophobic portion of the PEG-lipid is spontaneously incorporated into the lipid bilayer of the cell membrane when PEG-lipid is mixed with cells. The thickness of the PEG layer formed on the cell surface is several nanometers, depending on the molecular weight of the PEG used.

To see the surface modification with PEG-lipid, islets were modified with PEG-lipid labeled with the fluorescent dye FITC. Fig. 8(b) shows the FITC-PEG-lipid-modified hamster islets observed by confocal laser scanning microscopy. Clear fluorescence at the periphery of each islet indicates a PEG layer formed on the islets. Although a conformal PEG layer could be formed on the cell or islet surface at the nanometer level and the method makes it possible to drastically decrease the total volume of a graft compared to conventional microcapsules, fluorescent microscopic analyses indicated that the PEG layer was not stable on the islets and disappeared from the cell surface over 3 days. Additional

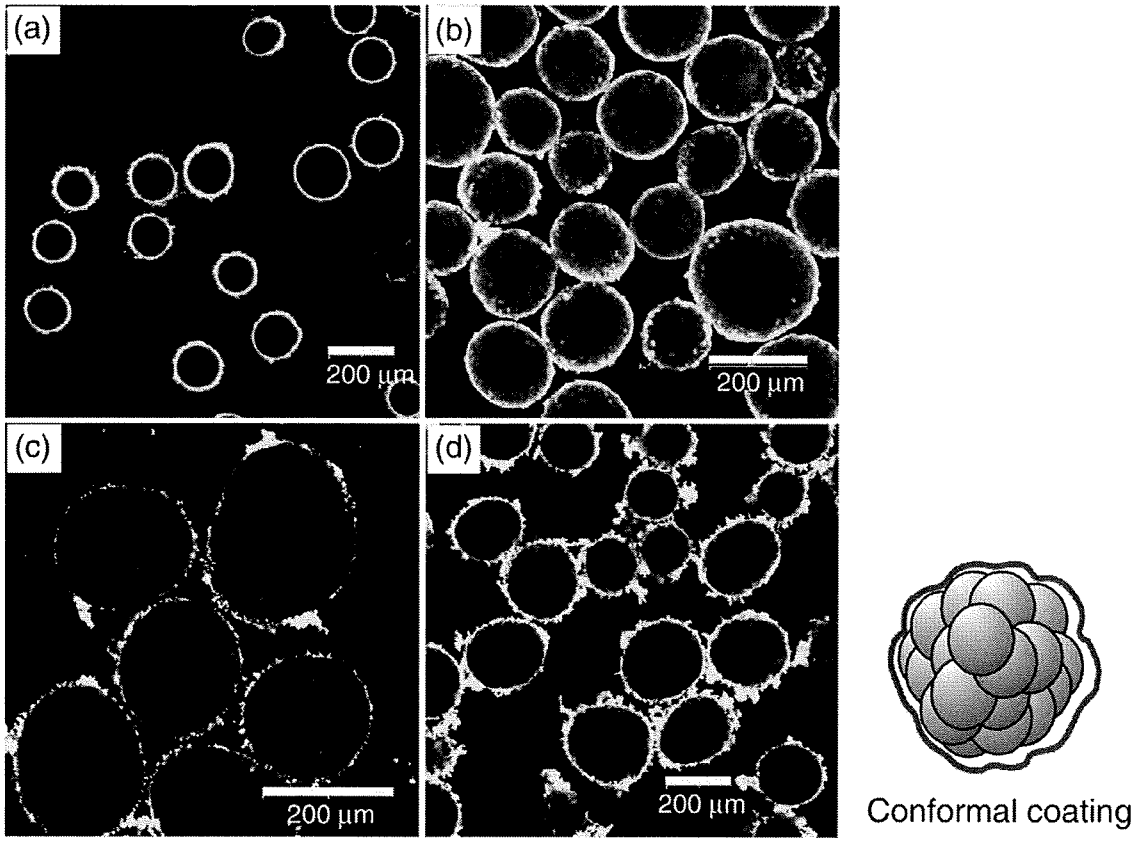

Fig. 8. Confocal laser scanning microscope images of the surface modification of CCRF-CEM cells and the enclosure of islets within polymer membranes via PEG-lipid derivatives. (a) FITC-PEG-lipid-modified CCRF-CEM cells. (b) FITC-PEG-lipid-modified islets. (c) Layer-by-layer membrane PVA-coated islets. PVA was labeled with FITC. (d) Layer-by-layer membrane biotin-BSA and FITC-streptavidin-coated islets.

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improvements should be made with the long-term functioning of the PEG layer for immunoisolation.

The layer-by-layer method was employed to increase the stability of the PEG-lipid membrane on the cell surface. Various functional groups, such as maleimide and biotin, can be easily introduced to the end of the PEG chain of PEG-lipids. These groups can be used as the reaction points for forming multilayer membranes on the cell surface. For example, it is possible to form a layer-by-layer membrane of PVA carrying thiol groups on a PEG-lipid carrying a maleimide group on the surface of islets using the reaction between the thiol and maleimide groups (Fig. 8(c)) [46]. PVA chains are immobilized on the islet surface through a stable thioether bond. This thiol/maleimide bond forms readily under mild conditions. To form a third layer of PVA, a thiol/disulfide exchange reaction between the pyridyldithio group and thiol group is used.

A layer-by-layer membrane can also be formed by the reaction between biotin and streptavidin. Biotin-PEG-lipids were anchored to the cell membranes of islets and further covered by streptavidin. The modified islets were alternatively exposed to a biotin–bovine serum albumin conjugate solution and a streptavidin solution to form 20 layers (Fig. 8(d)) [48]. The thickness of the membrane was approximately 30 nm based on the calculation from surface plasmon resonance (SPR) analysis. A glucose stimulation test was performed in order to examine the ability of the modified islets to control insulin release in response to changes in the glucose level. No significant difference in insulin release was observed between groups of islets with different surface modifications.

We also analyzed the effect of PEG modifications on graft survival immediately after intraportal transplantation into STZ-induced diabetic mice [50]. Hamster islets were surface modified with PEG-lipid (PEG-islets) and transplanted (500 islets) into the liver of STZ-induced diabetic mice (BALB/c) through the portal vein. Graft survival was significantly prolonged compared to bare islets in the livers of diabetic mice (Fig. 9(a) and (b)). Livers were retrieved from the recipient mice 1 h or 1 day after transplanting naïve or PEG-islets through the portal vein and subjected to histochemical analyses. One hour after naïve islet transplantation (Fig. 9(c)), the islets were damaged and destroyed in the blood vessels of the liver. The aggregation of RBC was observed around destroyed islets, indicating that platelet aggregation and blood coagulation occurred on the islet surface. Cell damage to some islets was also observed after 1 day. However, in the livers of mice transplanted with PEG-islets, most islets were not damaged and remained intact in the blood vessels of the liver 1 h after transplantation (Fig. 9(d)). The same results were

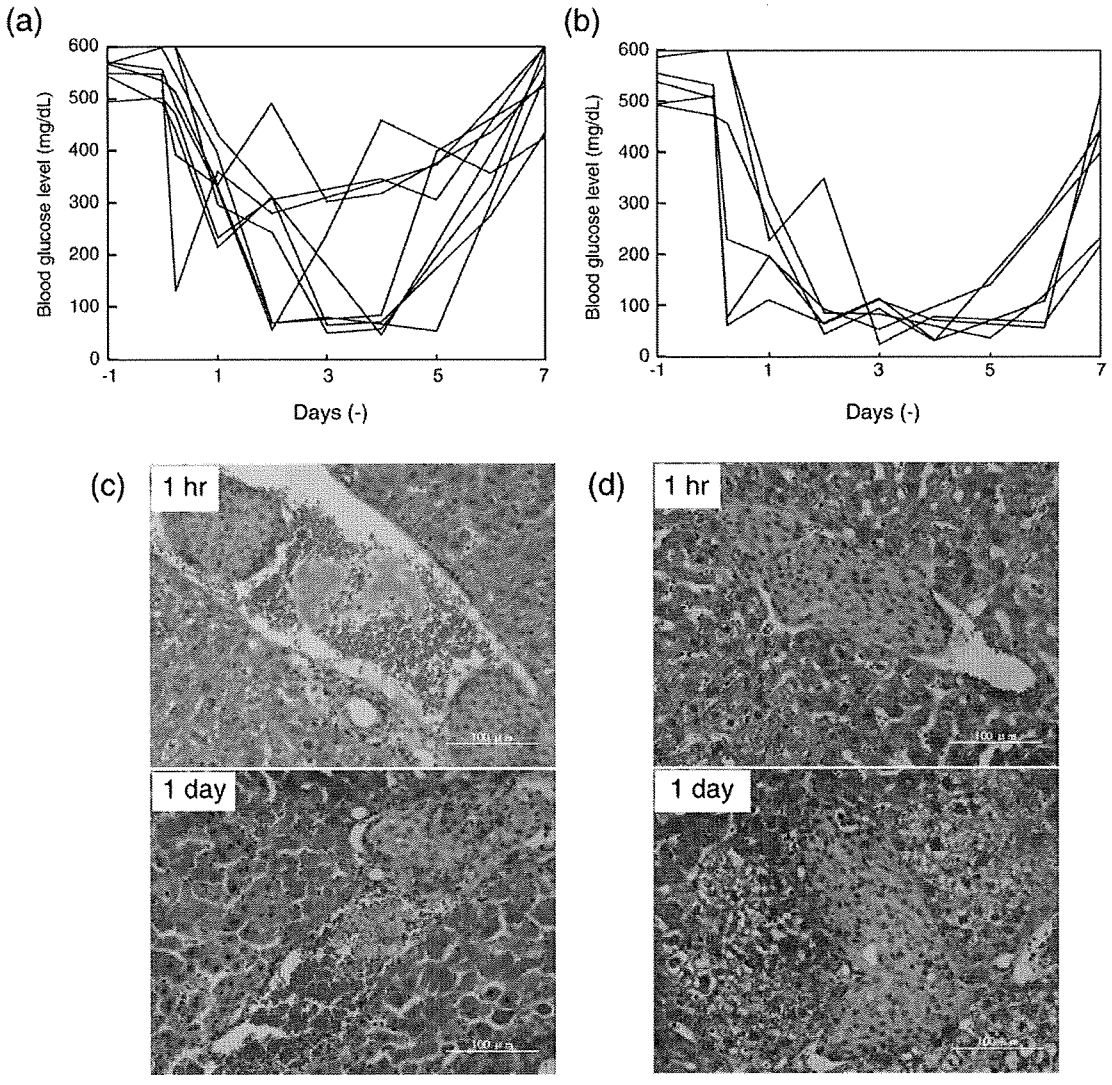

Fig. 9. Changes in the non-fasting blood glucose levels of streptozotocin-induced diabetic BALB/c mice after intraportal transplantation of (a) 500 control islets ($n = 9$) or (b) 500 PEG-islets ($n = 6$). Graft failure was defined as two consecutive plasma glucose determinations ≥ 200 mg/dL. (c) Histochemical analysis (HE stain) of control islets and (d) PEG-islets transplanted into the liver of diabetic mice through the portal vein. The grafts were retrieved at 1 h or 1 day post-transplantation as noted.

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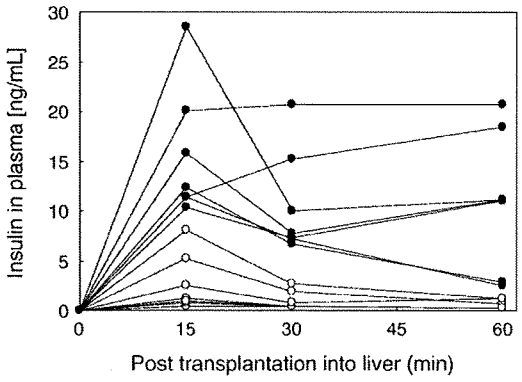

Fig. 10. Changes in the blood insulin levels determined by ELISA after intraportal transplantation of control islets (closed circles, $n = 7$) or PEG-islets (open circles, $n = 7$) in diabetic mice.

observed 1 day after transplantation. These histochemical analyses support the finding that graft survival is prolonged after transplanting PEG-islets. The overshoot reduction of the blood insulin level within 60 min after naïve islet transplantation suggests cell damage immediately after naïve islet transplantation, which could be suppressed by surface modification with PEG (Fig. 10). We showed that surface modification with PEG-lipid is useful for improving graft survival.

4.4. Improvement of islet blood compatibility

Islets are exposed to fresh blood when they are transfused into the liver through the portal vein. The coagulation and complement systems are activated by the islet surface during the early post-transplantation phase, leading to the release of chemotactic factors, tissue factor, chemokines, and other inflammatory mediators. The transplanted islets are destroyed by the innate immune reactions [56]. Some methods of regulating early coagulation and blood-mediated inflammatory reactions have been investigated, such as administering the thrombin inhibitor melagatran [57], activated protein C [58], low molecular weight dextran sulfate [59], and the water-soluble domain of complement receptor I (sCR1) [60,61]. Successful transplantation has been achieved in animal models by reducing islet loss. However, it is difficult to apply these methods in the clinical setting because systemic administration is associated with an increased a risk of bleeding. The new idea to overcome these issues is the immobilization of bioactive substances, such as heparin, sCR1, urokinase, and thrombomodulin, to the islet surface to suppress blood-mediated inflammatory reactions [48,51,62,63].

Nilsson et al. reported a method for preventing instant blood-mediated inflammatory reactions by coating porcine islets with heparin which is expected to inhibit surface thrombosis formation on islets after their transplantation into the liver [62]. Briefly, biotin was immobilized on the surface of porcine islets through the activated ester method, and the islet surface was further treated with avidin. The islet surface was coated with macromolecular conjugates of heparin consisting of roughly 70 heparin molecules covalently linked

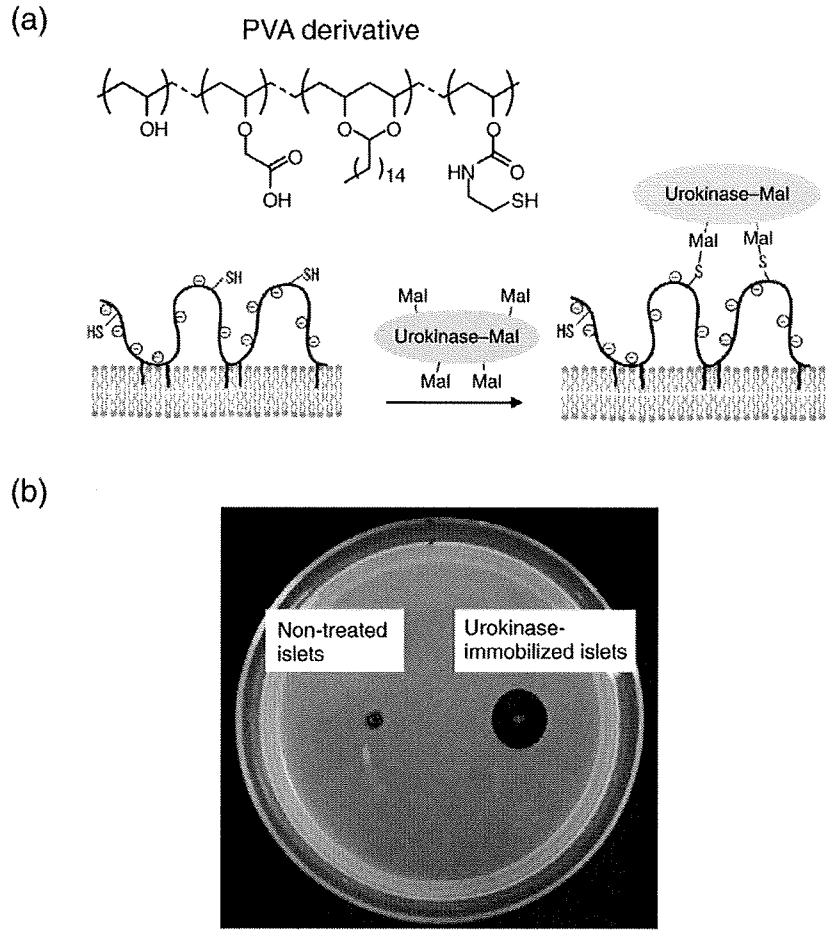

Fig. 11. (a) Method for immobilization of urokinase on the islet surface using amphiphilic PVA-alkyl. Maleimide groups were introduced onto urokinase using sulfo-EMCS. (b) Fibrin plate assay. Urokinase-immobilized islets and non-treated islets (100 islets each) were placed on a fibrin gel plate and incubated at 37 °C for 13 h. A large transparent area formed around the urokinase-immobilized islets by the dissolution of the fibrin gel by plasmin, which is produced from plasminogen by urokinase.

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to an inert carrier chain through electrostatic interaction with avidin. In allotransplantation between porcine, increase of plasma concentrations of thrombin–antithrombin (TAT) complexes and complement activation (C3a) were suppressed when heparin-coated islets were transplanted. The transient increase in insulin released from destroyed porcine islets was also suppressed. Heparinization of the islet surface prevented early islet destruction in the liver. Chaikof et al. reported the immobilization of recombinant thrombomodulin on the surface of islets [63]. Phosphine molecules were covalently conjugated to the amino groups of membrane proteins by Staudinger ligation for immobilization of thrombomodulin. The presence of thrombomodulin on the islet surface resulted in a significant increase in the production of activated protein C, indicating a reduction in islet-mediated thrombogenicity.

Our group has already succeeded in immobilizing fibrinolytic urokinase on the islet surface [48,51]. Urokinase is a serine protease that activates plasminogen. The activation of plasminogen triggers a proteolysis cascade that participates in thrombolysis. Immobilizing urokinase on the islet surface is expected to help dissolve small blood clots that may form on islets, thereby suppressing blood-mediated inflammatory reactions. An amphiphilic PVA derivative carrying long alkyl side chains and thiol carboxylic groups was synthesized and used for modifying the surface of islets; it was immobilized on the cell surface through the hydrophobic interaction between the alkyl side chains and lipid bilayer of the cell membrane (Fig. 11(a)). Thiol groups were introduced to the islet surface, and urokinase carried maleimide groups by conjugation with a hetero-bifunctional cross-linker (sulfo-EMCS, *N*-(6-maleimidocaproyloxy)sulfosuccinimide). The urokinase was immobilized on the islet surface by the maleimide/thiol reaction. A fibrin plate-based assay was performed to assess the function of the urokinase on the islets. Urokinase-islets and naïve islets (100 islets, respectively) were spotted on a fibrin gel and the area of dissolved fibrin around the spotted urokinase-islets determined. Fig. 11(b) shows the fibrin plate 24 h after spotting. A large transparent area (1.8 cm diameter) was observed around the urokinase-islets, indicating urokinase activity. On the other hand, the transparent area was small around the naïve islet spots. These results suggest that the immobilization of bioactive substances on the islet surface is a promising method of inhibiting the islet destruction caused by blood-mediated inflammatory reactions, improving graft survival following intraportal transplantation.

4.5. Enclosure of islets with living cells

Finally, we introduce our recent attempts to enclose islets with living cells. If the surface of islets can be covered with a patient's own

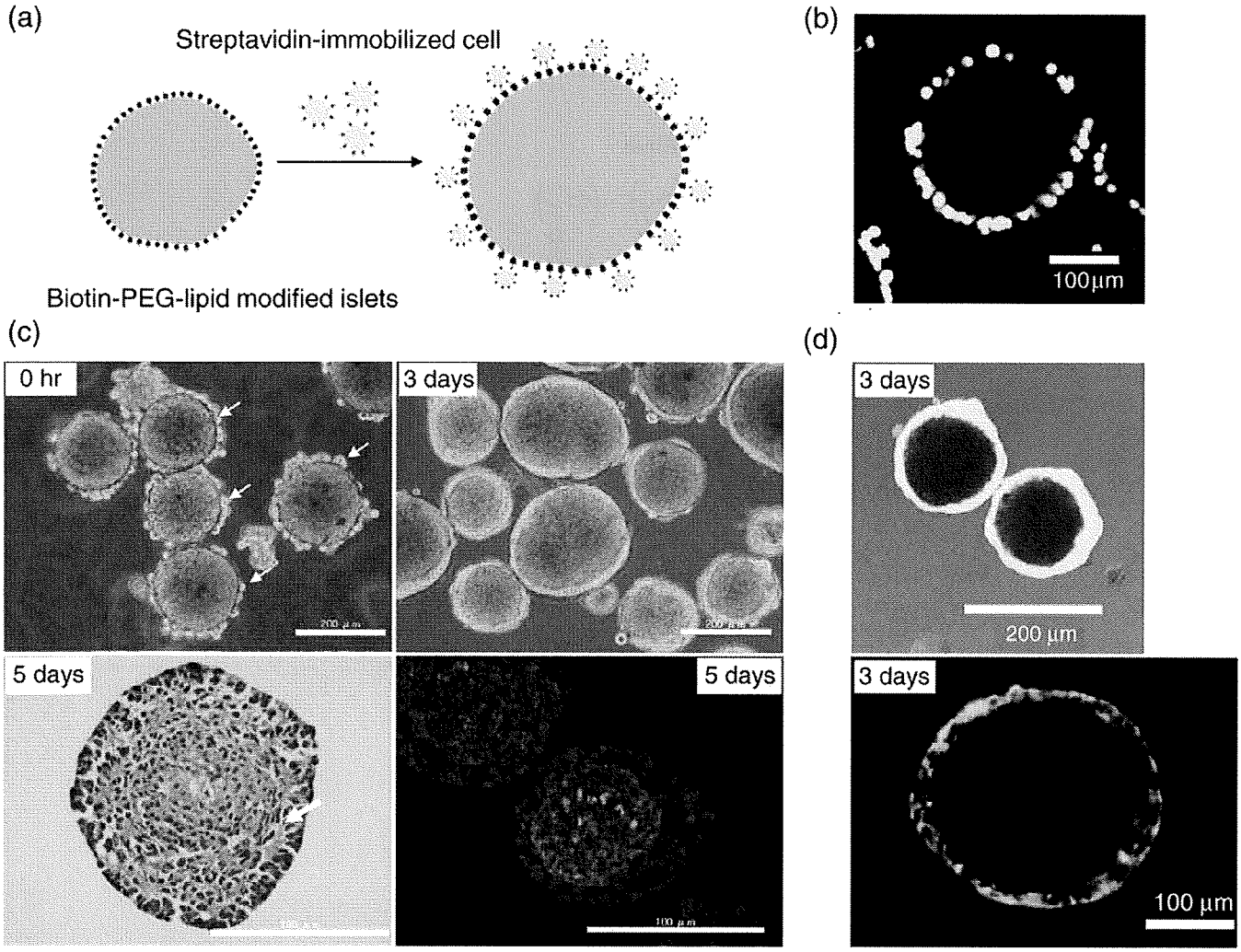

Fig. 12. Encapsulation of islets with living cells. (a) Schematic illustration of how to enclose an islet with living cells utilizing avidin–biotin interactions. (b) Hamster islets modified with biotin-PEG-lipid were immobilized with streptavidin-immobilized HEK293 cells. The HEK293 cells were first labeled with CellTracker®. (c) Phase-contrast microscopy (top) and histochemical analyses (HE staining, lower left) of HEK293 cell-immobilized islets in culture. Lower right: merged Alexa 488-labeled anti-insulin antibody and Hoechst 33342 dye for nuclear staining. (d) GFP-expressing HEK293-encapsulated islets after 3 days of culture, and a sliced section at 3 days.

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vascular endothelial cells, histocompatibility and blood compatibility will be significantly improved, inhibiting graft rejection and the destruction caused by blood-mediated inflammatory reactions. There were some preceding trials of islet enclosure with living cells. For example, Pollok et al. first reported that it is possible to macro-encapsulate rat islets with porcine chondrocyte membrane [64]. Porcine chondrocytes were cultured to form a confluent monolayer and deposit matrix. After the islets were attached to the poly(glycolic acid) polymer fibers, the hybrid was wrapped by hand with chondrocyte membrane. In such macroencapsulation, islet fusion inside the chondrocyte membrane causes the necrosis of the islet cells at the center. Lee et al. also demonstrated islet macroencapsulation with chondrocyte membrane using the cell sheet engineering technique [65]. However, all issues have not yet been solved; therefore, some aspects of the cell-based encapsulation methods should be improved. We used amphiphilic PEG-lipid and the biotin/streptavidin reaction to immobilize HEK293 cells on the surface of islets (Fig. 12(a)) [49]. After biotin molecules were introduced on the surface of HEK293 cells by biotin-PEG-lipid, the surface was further treated with streptavidin. The biotin-PEG-lipid-modified islets were mixed with streptavidin-immobilized HEK293 cells to immobilize the cells on the islet surface (Fig. 12(b)). The surface of the islets was completely covered with a cell layer after 3 to 5 days in culture, without central necrosis of the islet cells (Fig. 12(c) and (d)). Insulin secretion upon glucose stimulation was well maintained after HEK293 cell encapsulation. Using our technique, it will be possible to microencapsulate islets with cells derived from recipients with type 1 diabetes. We expect that this novel method will enable the preparation of self-adjusting bioartificial pancreases in control host reactions.

5. Perspective

A shortage of human donors is one of the major obstacles to cell transplantation therapy for type 1 diabetes. In the near future, we will be able to obtain insulin secreting β cells or tissues from embryonic stem (ES) cells or induced pluripotent stem (iPS) cells. A shortage of human donors will not be a limiting factor for cell therapy in type 1 diabetes. Immune reactions against grafts, however, still need to be overcome. Cells derived from ES cells are allogeneic and should be protected from the recipient's immune system. Cell and tissue transplantation will be possible without the administration of immunosuppressive drugs when cells or tissue can be derived from a patient's iPS cells. Type 1 diabetes, however, is an autoimmune disease, and β cells derived from iPS cells will be destroyed by the recurrent autoimmune reactions, which are hardly controlled, even by the administration of immunosuppressive drugs. The results obtained using NOD mice as mentioned above [22] indicate that the agarose microcapsule that effectively protects islets from autoimmune destruction will be useful in the transplantation of β cells derived from a patient's own iPS cells. A bioartificial pancreas would be promising for success with transplanting β cells derived from ES cells and iPS cells.

Three major types of bioartificial pancreas have been studied: diffusion chamber, hollow fiber unit, and microcapsule (Fig. 2). In the past 10 years, most research groups have concentrated their efforts on the development of the microcapsule type. Although many groups have reported that the bioartificial pancreas functions very well in animal models, some of the studies, especially those using large animal models, are hardly reproduced. In addition, the clinical applications were few [32,34] and the clinical outcomes were not clear. Various factors limit the application of a microcapsule type bioartificial pancreas in human patients. As discussed previously, the size of the microcapsules is one of the major obstacles. The microencapsulated islets with a larger radius than that of the islets are hardly infused into the liver through the portal vein, and their total volume increases to several hundreds of milliliters due to increasing by a third power of the radius. In recent years, new techniques, such as conformal coating and islet enclosure, have been developed. Using these techniques, the size and total volume can be greatly reduced.

The required functions for conformal coating depend on their applications. To suppress early islet loss due to instant blood-mediated inflammatory reactions (IBMIR), the coating should carry biomolecules, such as heparin, thrombomodulin, urokinase, and complement receptor 1, which can suppress the activation of the blood coagulation cascades and the complement system, respectively. That the coating functions for a few days after infusion of islets into the liver is enough. For immunoisolation, the conformal coating should be stable for a long time. The coating membrane covers islets and isolates them from the recipient's immune system during islet functioning. Most conformal coatings function for several hours to several days, easily dissociating and disappearing from the islet surface, or they deteriorate in a few days due to the dynamic movement of the cell membrane. Reported conformal coatings are expected to be applicable to the suppression of early islet loss but not stable enough for immunoisolation. Much effort is needed to improve the conformal coating so it becomes applicable to long-term immunoisolation.

The *in vivo* life span of microencapsulated islets is determined by various factors in addition to IBMIR and immune reactions. Edmonton's group [66] reported a graft survival rate in clinical islet transplantation of less than 10% at 5 years post-transplantation. Clinicians and researchers working on islet transplantation were disappointed in the clinical outcomes of islet allotransplantation; they expected the function to last longer because an autograft of islets that maintained function more than 13 years was reported [67] and steroids, which are toxic to islets, were not used in the Edmonton protocol. Although a major cause of graft loss is IBMIR, the researchers pointed out vascular insufficiency and toxic side effects of immunosuppressive drugs on β cells as causes of short islet functioning. For the transplantation of microencapsulated or conformal coated islets, neo-vasculature into islets could not be expected. Oxygen and nutrients are supplied to islet cells by diffusion, and this effect is not clear in the life span of grafts. When grafts are transplanted into the peritoneal cavity, they form a large aggregate. When the microencapsulated islets are transfused into the portal vein, they block blood flow due to their larger radius compared to islets. Both impose limitations on the oxygen supply to islet cells by diffusion. These circumstances should be carefully studied using large animal models.

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Microencapsulation of Islets with Living Cells Using PolyDNA-PEG-Lipid Conjugate

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Microencapsulation of islets with a semipermeable membrane, i.e., bioartificial pancreas, is a promising way to transplant islets without the need for immunosuppressive therapy for insulin-dependent diabetes mellitus (type I diabetes). However, materials composing a bioartificial pancreas are not ideal and might activate defense reactions against foreign materials. In this study, we propose an original method for microencapsulation of islets with living cells using an amphiphilic poly(ethylene glyocol)-conjugated phospholipid derivative (PEG-lipid) and DNA hybridization. PolyA and polyT were introduced onto the surfaces of the islets and HEK 293 cells, respectively, using amphiphilic PEG-lipid derivatives. PolyA20 modified HEK cells were immobilized onto the islet surface where polyT20-PEG-lipid was incorporated. The cells spread and proliferated on the islet surface, and the islet surface was completely encapsulated with a cell layer after culture. The encapsulated islets retained the ability to control insulin release in response to glucose concentration changes.

INTRODUCTION

The bioartificial pancreas, which encapsulates islets of Langerhans (islets) within a semipermeable membrane, is one of the therapeutic devices for patients with insulin-dependent diabetes mellitus (type I diabetes). It is a safe and simple way to transplant islets without the need for immunosuppressive therapy. The semipermeable membrane protects the islets from the immune system of a recipient patient, and thus, the islets are expected to survive and release insulin for a long period of time and thereby control glucose metabolism. Various types of bioartificial pancreas have been proposed and developed (*1*–*3*), with the microencapsulated type being a promising model. In this example, islets are microencapsulated within an alginate/poly(L-lysine) polyion complex membrane (*4*) or an agarose hydrogel (*5*–*8*). Our group has used the agarose system and has demonstrated its efficacy in diabetic animals (*5*–*8*). Recently, we also have developed an original design for a bioartificial pancreas for transplantation into the liver through the portal vein (*9*–*15*). However, materials composing a bioartificial pancreas have not been ideal and might activate defense reactions against foreign materials. Compatibility of the membrane with the recipient patient should be improved.

We proposed to enclose islets from a donor under a layer of cells from a recipient to increase compatibility with the patient. Since the outermost surface is the recipient's own cells, the host immune defense system will not be provoked. In our previous study, we developed a method to enclose islets with living cells (*15*). Amphiphilic poly(ethylene glycol)-conjugated phospholipid derivatives (PEG-lipid) and biotin/streptavidin reactions were employed (*15*). Although the biotin/streptavidin reaction worked well to cover the islets with living cells, streptavidin is a xenogeneic protein and is expected to activate the host immune system. We sought to improve this technique with the use of biocompatible materials.

In this study, we employed DNA hybridization instead of the biotin/streptavidin reaction. Polyadenine (polyA) and polythymine (polyT) were introduced onto the surfaces of the islets and HEK 293 cells, respectively, by using polyA or polyT-conjugated PEG-lipid. We already succeeded in the cell–cell attachment induced by hybridization of DNA-conjugated PEG-lipid (*18*). The hybridization of DNA-conjugated PEG-lipid was effectively used for the cell–cell attachment and cell-immobilization to the substrate. The HEK 293 cells were thereby immobilized on the surface of the islets through hybridization of polyA and polyT. A layer of living HEK cells was formed on the surface of islets.

EXPERIMENTAL PROCEDURES

Materials. α-*N*-Hydroxysuccinimidyl-ω-maleimidyl poly(ethylene glycol) (NHS-PEG-Mal, M_w: 5000) was purchased from Nektar Therapeutics (San Carlos, USA). 1, 2-Dipalmitoyl-*sn*-glycerol-3-phosphatidylethanolamine (DPPE) was purchased from NOF Corporation (Tokyo, Japan). Dichloromethane, triethylamine, sucrose, and diethyl ether were purchased from Nacalai Tesuque (Kyoto, Japan). Hoechst 33342 nuclear stain was purchased from Dojindo Laboratories (Kumamoto, Japan). Alexa 488-labeled goat antiguinea pig IgG, minimum essential medium (MEM), HEPES buffer solution, Hanks' balanced salt solution(HBSS), Medium 199, RPMI-1640 medium, penicillin, and streptomycin were purchased from Invitrogen Co. (Carlsbad, CA, USA). Fetal bovine serum (FBS) was obtained from Equitech-Bio, Inc. (TX, USA), and phosphate-buffered saline (PBS) from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). Enzyme-linked immunosorbent assay (ELISA) kits for the insulin assay were purchased from Shibayagi Co., Ltd. (Gunma, Japan). Tissue-Tek was purchased from Sakura Fine Technical Co. Ltd. (Tokyo, Japan). Goat normal serum and polyclonal guinea pig anti-insulin serum were purchased from Dako (Glostrup, Denmark). The 10% formalin solution, dithiothreitol (DTT), and Triton X-100 were purchased from Wako Pure Chemical (Osaka, Japan). NAP-5 column was purchased from GE health care (GE Healthcare UK Ltd., Buckinghamshire, UK).

Synthesis of polyDNA-PEG-Phospholipid Conjugate (polyDNA-PEG-lipid). Mal-PEG-lipid was synthesized by first dissolving NHS-PEG-Mal (180 mg), triethylamine (50 μL) and

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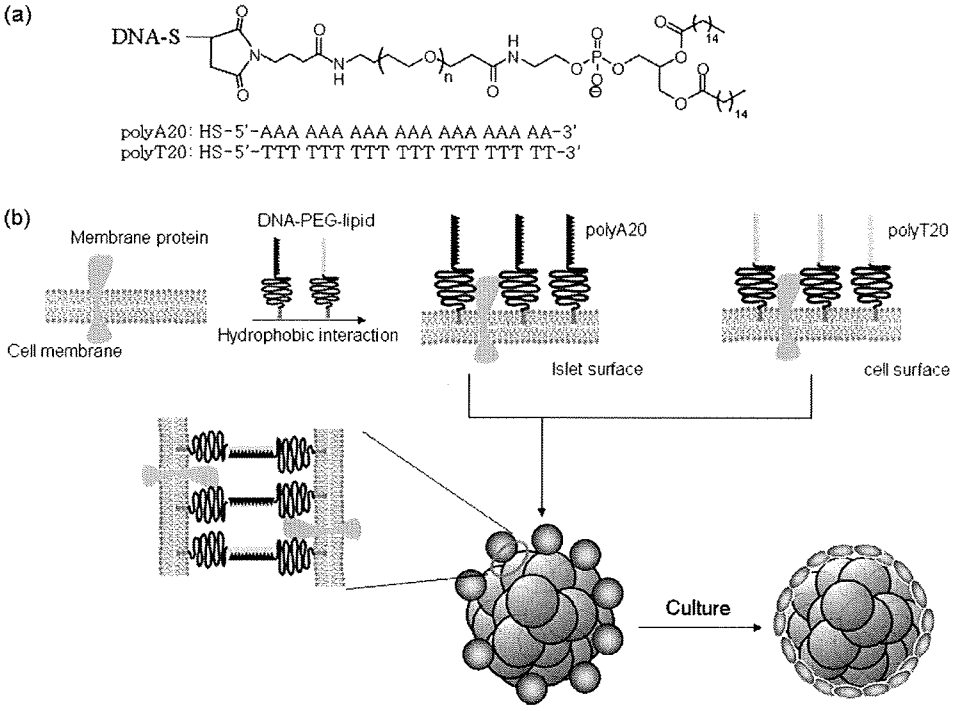
Scheme 1[a]



[a] (a) Chemical structure of DNA-conjugated PEG-DPPE (polyDNA-PEG-lipid) and the DNA sequence of polyA20 and polyT20. (b) Schematic illustration of interaction between polyDNA-PEG-lipid and a lipid bilayer of the cell membrane, and immobilization of living cells to the islet surface. DNA (polyA20 or polyT20)-PEG-lipid has hydrophobic acyl chains which anchor into the cell membrane. After mixing polyT-PEG-lipid-modified GFP-HEK cells and polyA-PEG-lipid-modified islets, they were suspended in medium and cultured at 37 °C and 5% CO_2. During culture, GFP-HEK cells spread and grew on the islet surface.

DPPE (20 mg) in dichloromethane and stirring for 36 h at room temperature as shown in Scheme S1 (Supporting Information) (rt) (*12*). After precipitation with diethyl ether, Mal-PEG-lipid was obtained as a white powder (190 mg, yield 80%). ^{1}H NMR ($CDCl_3$, 400 MHz, δ ppm): 0.88 (t, 6H, $-CH_3$), 1.25 (br, 56H, $-CH_2-$) 3.64 (br, 480H, PEG), 6.71 (s, 2H, —HC=CH—, maleimide).

The structure of polyDNA-PEG-lipid was shown in Scheme 1a. PolyA20 and polyT20 which carry $(CH_2)_6$-SS-$(CH_2)_6$-OH at 5′ end were purchased from Sigma-Aldrich Chemical Co (see Scheme S1 in Supporting Information). PolyDNA-SH was prepared by reduction of the disulfide bond with DTT in accordance with the instructions of the manufacturer. Briefly, polyDNA-disulfide conjugate (in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) was mixed with DTT (0.04 M) for 16 h at rt for removal of protection group for thiol. After purification with NAP-5 column, polyDNA-SH was obtained.

The SH groups at the 5′-ends of polyDNAs were used to form conjugates with the Mal-PEG-lipid. A PBS solution of polyDNA-SH (1.0 mg) was mixed with Mal-PEG-lipid (5.0 mg) and the reaction mixture was left for 24 h at rt to form conjugations. polyDNA-PEG-lipid (500 μg/mL in PBS) was used for surface modification of cells and islets without any further purification.

Encapsulation of Islets with GFP-HEK Cells. HEK293, which stably expressed enhanced green fluorescence protein (EGFP) (GFP-HEK), was kindly supplied by Dr. K. Kato (Institute for Frontier Medical Sciences, Kyoto University). The GFP-HEK cells were routinely maintained in MEM supplemented with 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. GFP-HEK cells were collected by centrifugation (180 × *g*, 5 min, rt) after treatment with trypsin. The cells were washed with HBSS to remove the medium. A cell pellet (2×10^6 cells) was obtained by centrifugation. After the addition of polyT20-PEG-lipid solution (50 μL, 500 μg/mL) to the cell suspension, the suspension was incubated for 1 h with gentle agitation at rt. The cells were then suspended in 10 mL HBSS and collected by centrifugation (180 × *g*, 5 min, 25 °C, twice) to obtain polyT20-PEG-lipid-modified cells.

Islets were isolated from the pancreas of female Syrian hamsters (7−8 weeks old, Japan SLC, Inc., Shizuoka, Japan) using the collagenase digestion method. The islets were cultured for 7 d after isolation to remove cells damaged by the isolation procedure. The islets were maintained in culture medium (Medium 199 with 10% FBS, 8.8 mM HEPES buffer, 100 units/mL penicillin, 100 μg/mL streptomycin, and 8.8 U/mL heparin). A solution of polyA20-PEG-lipid solution (500 μg/mL, 100 μL of PBS) was added to suspension of the islets in serum-free medium (200 islets), and the mixture was incubated at rt for 1 h. After washing three times with serum-free medium, polyA20-PEG-lipid-modified islets were obtained. Finally, polyT20-PEG-lipid-modified GFP-HEK cells (2×10^6) and polyA20-PEG-lipid-modified islets (200 islets) were mixed in serum-free medium (300 μL), and the mixture was incubated for 60 min with gentle agitation at rt. The GFP-HEK cell-immobilized islets were picked up by hand using a Pasteur pipet (inside diameter: 1 mm) under a stereomicroscope and cultured on a nontreated dish at 37 °C under 5% CO_2 in Medium 199 supplemented with 10% FBS. Islets were observed by a confocal laser scanning microscope (FLUOVIEW, FV500, Olympus Optical Co. Ltd., Tokyo, Japan) and a phase-contrast microscope (IX71, Olympus). The FITC and GFP were excited by an argon laser (488 nm) and the fluorescence was detected though a bandpass filter (510−550 nm).

Histochemical Analysis. GFP-HEK cells-immobilized islets were washed with PBS and then transferred to 10% formalin solution and incubated for 1 d at rt. The formalin solution was removed, and the islets were sequentially kept in 3% and 10% sucrose in PBS for 1 d, followed by incubation in 20% sucrose in PBS for an additional 1 d at rt. The islets were embedded in

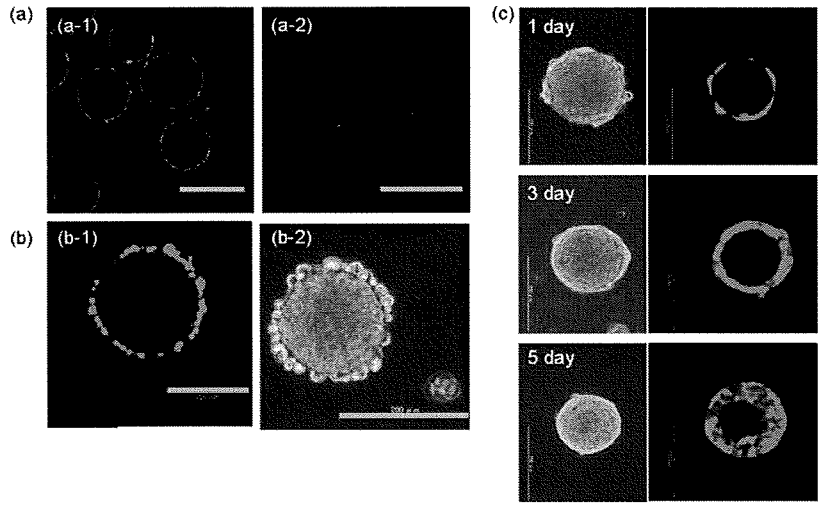


Figure 1. Encapsulation of islets with living cells. (a) Hamster islets modified with polyA20-PEG-lipid were treated with (a-1, FITC) FITC-labeled polyT20. (a-2) Naked islets were treated with FITC-labeled polyT20. These islets were observed by a confocal laser scanning microscope. (b) Attachment of polyT20-PEG-lipid modified GFP-HEK cells onto the surface of polyA20-PEG-lipid modified hamster islets. An islet was observed by a confocal laser scanning microscope for (b-1, GFP) and a phase contrast microscope (b-2). (c) GFP-HEK cells-immobilized islets were cultured in Medium 199 supplemented 10% FBS at 37 °C under 5% CO_2 for 1, 3, and 5 days. Islets were observed by a phase contrast microscope (left panels) and a confocal laser scanning microscope (right panels, GFP). Scale bars: 200 μm.

Tissue-Tek for freezing. The frozen specimens were sliced (6 μm thick) using a cryostat (CM 3050S IV, Leica, Solms, Germany). The sliced sections were permeabilized by treatment with 0.2% Triton X-100 in PBS at rt for 15 min. The samples were first treated with a 10% normal goat serum in PBS for 1 h to block the nonspecific binding of antibodies. The samples were then treated with 1% guinea pig anti-insulin serum in PBS containing 3% goat normal serum for 3.5 h at rt, and then washed with PBS. The samples were incubated with fluorescently labeled secondary antibody, 0.2% Alexa 488 Goat antiguinea pig IgG in PBS containing 3% goat normal serum, at rt for 1.5 h. Cell nuclei were counterstained with Hoechst 33342. The localization of secondary antibodies and the Hoechst dye was analyzed by a fluorescence upright microscope (BX51, Olympus). The sliced sections were also stained with hematoxylin-eosin (HE) using a conventional staining method.

Insulin Secretion from GFP-HEK Cell-Encapsulated Islets on Glucose Stimulation. Static insulin secretion tests were performed on GFP-HEK cell-immobilized islets (50 islets) after culturing for 3 days to evaluate their insulin-secreting ability in response to changes in glucose concentration. As a control experiment, this assay was performed on islets without enclosure with HEK cells (naïve islets). GFP-HEK cell-immobilized islets and naïve islets were exposed to solutions of glucose in Krebs-Ringer's buffer (KRB) at concentrations of 0.1 g/dL, then 0.3 g/dL, and finally 0.1 g/dL glucose for intervals of 1 h in each solution at 37 °C. The supernatants were collected after each 1 h incubation, and the insulin concentrations in the KRB solutions were determined by ELISA.

Statistical Analysis. Comparisons between two groups were made using Student's *t*-tests. $p < 0.05$ was considered statistically significant. All statistical calculations were performed using the software *KaleidaGraph 4.0J*.

RESULTS

Islet Encapsulation with Living GFP-HEK Cells. Islets were treated with polyA20-PEG-lipid, followed by treatment with FITC-labeled polyT20 (Figure 1a). Fluorescence from FITC-labeled polyT20 was clearly seen at the periphery of each islet (Figure 1a-1). No fluorescence was observed in naked islets treated with FITC-labeled polyT20 (Figure 1a-2). Thus, polyA20 can be immobilized onto the surface of islets using polyA20-PEG-lipid without damaging islet morphology, and polyA20 on the cell surface can hybridized with polyT20.

GFP-HEK cells were immobilized onto the surface of islets by DNA hybridization between polyA20 on the islets and polyT20 on the GFP-HEK cells. Figure 1b shows a microscopic image of a GFP-HEK cell-islet complex just after preparation. The HEK cells on the islet surface appeared as white cells under a phase contrast microsope (Figure 1b-2) and were more clearly identified as green cells of GFP-HEK under a confocal laser scanning microscope (Figure 1,b-1). Single HEK cells were recognized on the islets, indicating that the GFP-HEK cells had become immobilized on the islet surface by DNA hybridization. The inhibition study was also performed. When polyA20-islets and polyT20-GFP-HEK were previously incubated with polyT20 and polyA20, respectively, no specific immobilization of GFP-HEK cells on islets was observed (see Figure S1 of Supporting Information).

The modified islets were cultured in medium in a culture dish and observed at 1, 3, and 5 days after the complex was formed (Figure 1c). Although GFP-HEK cells were attached and spread on the surface after 1 day of culture, the islet surface was not completely covered with cells. The HEK cells spread and gradually proliferated on the islet surface, and by 3 days in culture, the islet surface was fully covered with a layer of HEK cells (Figure 1c). As seen Figure 1c and Figure 2, HEK cells proliferated continuously and form a thicker multicell layer due to lack of contact inhibition as the culture period proceeded.

Histochemical Analysis. The GFP-HEK cell-encapsulated islets were histochemically analyzed by HE and insulin staining (Figure 2). In HE staining, a layer of GFP-HEK cells was observed around the islet surface after culturing for 3 and 5 days (left panel in Figure 2). The nuclei of GFP-HEK cells were slightly larger than that of islets. The multilayers of GFP-HEK cells were formed on the islet surface after culturing for 3 and 5 days. Necrosis of islet cells was not observed even at the center of the complex at 5 days. Figure 2 also shows images of insulin-stained GFP-HEK cell-encapsulated islets (right panel). Islets stained green with anti-insulin antibody were found in a core cell aggregate. These results indicate that GFP-HEK cell-encapsulation did not impair the morphology of islets.

Insulin Secretion by Glucose Stimulation. We examined the abilities of the islets modified with or without HEK cell-

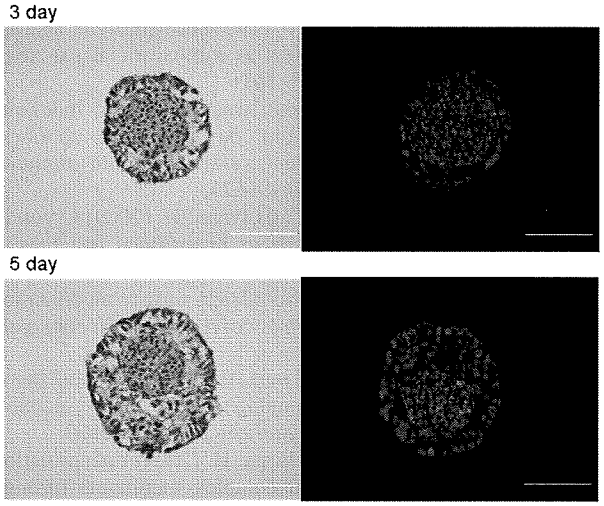


Figure 2. Histochemical analyses of GFP-HEK cell-immobilized islets cultured for 3 and 5 days. Frozen sections of GFP-HEK cell-immobilized islets were stained with (left panels) hematoxylin-eosin (HE) or (right panels) Alexa 488-labeled anti-insulin antibody and Hoechst 33342 dye for nuclear staining. Right panels show merged microscopic images of insulin (green fluorescence) and Hoechst 33342 staining (blue fluorescence). Scale bars: 100 μm.

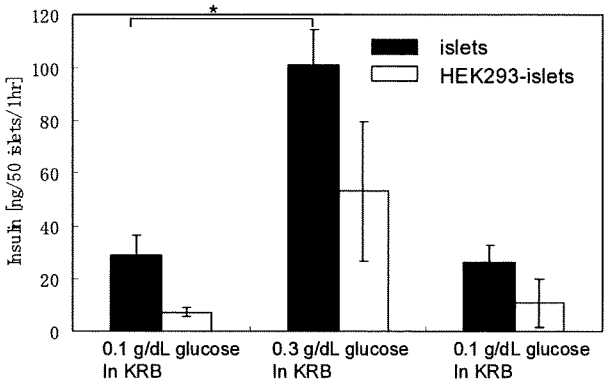


Figure 3. Glucose stimulation test of islets encapsulated HEK cells after 3 days culture. As a control experiment, the assay was performed on naive islets. The amounts of insulin secreted from the islets in response to glucose concentration changes (0.1, 0.3, 0.1 g/dL) were determined by ELISA. Results are expressed as mean ± SD for $n = 3$. KRB: Krebs-Ringer buffer. An asterisk represents a significant difference ($p < 0.05$) between two groups.

encapsulation to release insulin. At the basal glucose concentration, 0.1 g/dL, insulin release by the encapsulated islets was less than that by naïve islets. We also performed a glucose stimulation test to examine the ability of the modified islets to regulate insulin release in response to changes in the glucose level. When the glucose concentration in the medium was increased from 0.1 g/dL to 0.3 g/dL, islets of both groups increased insulin release above basal levels (Figure 3). Insulin release returned to basal levels when the islets were re-exposed to 0.1 g/dL glucose. Glucose stimulation indexes (insulin release at 0.3 g/dL glucose/insulin release at 0.1 g/dL glucose) calculated from these results were 7.3 ± 5.3 and 3.6 ± 1.0 for the encapsulated islets and naïve islets, respectively. Although there was no significant difference between two groups for glucose stimulation index, the amount of insulin secretion of the encapsulated islets tended to be lower than that of native islets at 0.1 g/dL and 0.3 g/dL glucose in KRB. These results indicate that the HEK cell layer did not influence the islets' ability to regulate insulin release in response to glucose concentration, although the cause of decreased insulin release by encapsulated islets was not clear. It might be attributed to the oxygen consumption by the HEK293 cells since low oxygen tension can decrease islet cell function. The decrease of basal insulin release in encapsulated islets is a topic that requires further study.

DISCUSSION

There were some fundamental studies on improvement of biocompatibility by using cells such as chondrocyte (*16, 17*). Pollok et al. first reported the macroencapsulation of rat islets with porcine chondrocyte membrane (*16*). Porcine chondrocytes were cultured to form a confluent monolayer for use as a matrix upon which to deposit islets. After the islets were attached to poly(glycolic acid) polymer fibers, the islet−polymer composite was wrapped with chondrocyte membrane by hand. Lee et al. also demonstrated enclosure of islets with chondrocyte membranes using the cell sheet engineering technique (*17*). However, the increase of total volume after enclosure and aggregate formation of islets inside the membrane led to necrosis of islet cells at the center. Lee et al. also demonstrated enclosure of islets with chondrocyte membranes using the cell sheet engineering technique (*17*). The issue of islet necrosis still remains to be resolved. They expected that chondrocyte membrane would work as an immuno-isolation membrane of bioartificial pancreas, although unfortunately, they have not yet reported results of animal experiments.

To overcome this problem, we studied methods to enclose islets singly with living cells. Herein, we have used amphiphilic polyDNA-PEG-lipid to immobilize GFP-HEK cells on the surface of islets through hybridization between polyA20 and polyT20. The surface of the islets was completely covered with a cell layer after 3 to 5 days in culture without central necrosis of the islet cells (Figure 2). Insulin secretion upon glucose stimulation was well maintained in the cell-encapsulated islets complex, although total insulin secretion was reduced as compared to normal islets (Figure 3). HEK cells which formed a multicell layer on islets consume oxygen and thus islet cells were exposed to low concentration of oxygen. We thought that the reduction of insulin secretion was due to the insufficient oxygen supply to β-cells of islets. The same phenomena were observed in islets encapsulated with HEK cells using the biotin/streptavidin reaction. However, we might evade this issue by encapsulation with endothelial cells because they are expected to form a single cell layer.

We have reported immobilization of cells onto the islet surface by using biotin/streptavidin reaction (*15*). Although the specific biotin/streptavidin reaction worked well to cover the islets with living cells, streptavidin is expected to activate the host immune system because it is a xenogeneic protein isolated from bacteria. Therefore, we improved the technique compatible to future clinical application. There was no difference in islet encapsulation efficiency between these two techniques.

Many shortcomings remain to be overcome prior to clinical application. The cells employed are HEK293 cells, which proliferate rapidly and form a multicellular layer. Most primary cells, that is, cells isolated from normal animals, proliferate much more slowly than HEK cells and cannot form a multicellular layer. Thus, we should select types of primary cells which can effectively form a cell layer on islets. The short- and long-term effects of the covered cells on islet functions should be carefully examined. We also should follow the fate of cells after transplantation. In addition, the efficiency and biocompatibility of cell-based microencapsulation should be examined carefully by using animal experiments early. Although these issues should be addressed, the technology developed in this study will be

useful in preparation of future bioartificial pancreas and studies on cell−cell interaction.

CONCLUSIONS

Islets can be individually encapsulated with living cells through hybridization between polyA and polyT which can be introduced onto the surfaces of islets and cells, respectively, using amphiphilic PEG-lipid derivatives. No central necrosis was observed after encapsulation. The encapsulated islets still retained the ability to control insulin release in response to changes in glucose concentration.

ACKNOWLEDGMENT

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Supporting Information Available: Chemical scheme for preparation of polyDNA-SH and polyDNA-PEG-DPPE is shown in Scheme S1. The result of inhibition experiment for immobilization of polyT20-GFP-HEK onto polyA20-islets is shown in Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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