immunized. Two weeks later, 5×10^6 immune or control spleen cells were stimulated in vitro at 37°C with 2×10^5 cells of SSP2 clone 3.9 or CS clone 1.5 (*15*) that had been treated with mitomycin C (50 μg/ml, 30 min) in 2 ml of Dulbecco's modified Eagle's medium complete medium (*23*). After 6 days, cells were harvested, counted, and incubated for 6 hours at 37°C at various effector/target ratios with 5×10^3 ^{51}Cr-labeled cells of SSP2 clone 3.9, CS clone 1.5, pSV2neo-transfected P815 cells, or nontransfected P815 cells. Percent specific lysis was determined as 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). The spontaneous release values (medium control counts per minute/detergent-release counts per minute) for all targets of all experiments reported in this paper were similar (mean ± SEM, 10.34 ± 1.02%).
14. S. Khusmith *et al.*, unpublished data.
15. The entire coding sequence of the CS gene of the 17XNL strain of *P. yoelii* [A. A. Lal *et al.*, *J. Biol. Chem.* **262**, 2937 (1987)] was cloned into the Pst I–Eco RI restriction site of the expression vector pcDL-SR-α-296 [Y. Takebe *et al.*, *Mol. Cell. Biol.* **8**, 466 (1988)]. This vector contains an SR-α-promotor, a splicing site for the SV40 late gene, and a polyadenylation signal from the SV40 late region. We cotransfected mouse P815 cells (H-2^d) with plasmid DNA and pSV2neo DNA by electroporation using a Gene Pulser (Bio-Rad) and standard conditions [G. Chu, H. Mayakawa, P. Berg, *Nucleic Acids Res.* **15**, 1311 (1987)]. Stable cell clones were derived as above (*11*) and screened with MAb NYS1 (*8*) to detect CS protein.
16. We achieved depletion of either CD4$^+$ or CD8$^+$ T cells by incubating cells that had been stimulated in vitro for 6 days with SSP2 clone 3.9 with either anti-CD8 MAb 19/178 (*24*) or an antibody to CD4 (anti-CD4), MAb RL172 [R. Ceredig, J. W. Lowenthal, M. Nabhoiz, H. R. Macdonald, *Nature* **314**, 98 (1985)], and complement (C).
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25. We note with sadness that R. L. Beaudoin, our friend and colleague, passed away on 22 May 1990.
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Biohybrid Artificial Pancreas: Long-Term Implantation Studies in Diabetic, Pancreatectomized Dogs

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Diabetic complications such as neuropathy, retinopathy, and renal and cardiovascular disease continue to pose major health risks for diabetic patients. Consequently, much effort has focused on approaches that could replace conventional insulin therapy and provide more precise regulation of blood glucose levels. The biohybrid perfused artificial pancreas was designed to incorporate islet tissue and a selectively permeable membrane that isolates this tissue from the immune system of the recipient. Biohybrid pancreas devices containing canine islet allografts were implanted in ten pancreatectomized dogs requiring 18 to 32 units of injected insulin daily. These implants resulted in good control of fasting glucose levels in six of these animals without further exogenous insulin for periods of up to 5 months.

THE USE OF CONVENTIONAL INSULIN therapy for control of glucose levels in diabetic patients is associated with a number of drawbacks. These include the need for daily injections and a high degree of patient compliance with a strict regimen of diet and exercise. Furthermore, normoglycemia may still be difficult to achieve, particularly in Type I diabetics. Although controversial, data suggest that the long-term complications associated with diabetes may result from this lack of optimal glycemic control (*1*).

Studies of diabetic patients with pancreatic transplants indicate that normoglycemia can be achieved without administration of exogenous insulin (*2*). However, many potential problems are associated with the use of whole or segmental pancreatic transplants. These include the limited availability of donor organs, the need to provide drainage for exocrine systems, and the requirement for generalized immunosuppression. Transplantation of isolated human islet allografts offers a partial solution to some of these problems, but still involves the use of immunosuppression and the difficulties in obtaining significant numbers of purified human islets. A number of studies have focused on approaches designed to decrease the immune response to transplanted islet tissue. These include modification of the donor islet tissue by ultraviolet radiation, prolonged tissue culture or use of antisera against immunocytes (*3*), treatment of the recipient with antisera targeted at subpopulations of immunocytes (*4*), and the use of immunologically privileged transplantation sites such as the brain, testicle, or thymus (*5*). Although these approaches have shown varying degrees of success when used alone and in combination in diabetic laboratory rodents, convincing proof of long-term efficacy in larger animals is generally lacking.

The concept of the biohybrid artificial implantable pancreas was developed so that these problems associated with conventional pancreas or islet transplantation could be overcome. The device utilizes a selectively permeable membrane with a nominal molecular mass cut-off of 50 kD (*6*). The tubular membrane is coiled inside a protective housing that provides a compartment for the islet cells. The membrane is connected at each end to a standard (6 mm) arterial polytetrafluoroethylene (PTFE) graft that extends beyond the housing and is used to connect the device to the vascular system as an arteriovenous shunt. Blood flow through the device from the graft and tubular membrane results in exchange of glucose, insulin, and factors necessary for sustained islet viability across the membrane between the circulating blood and the cell compartment. Antibodies and lymphocytes responsible for immune rejection are, however, excluded from the cell compartment (*7*). This immunoisolation facilitates the use of xenogeneic islets, which can be isolated in large numbers.

Various approaches to the biohybrid pancreas have been described (*8*). To establish the potential of this type of device as a viable therapy for diabetic patients, several critical elements must be demonstrated. These include (i) the ability to support long-term islet viability and function; (ii) long-term patency of the vascular shunt and biocompatibility with respect to thrombosis; (iii) protection of islet allografts or xenografts from immune rejection; (iv) elimination of the need for exogenous insulin therapy in

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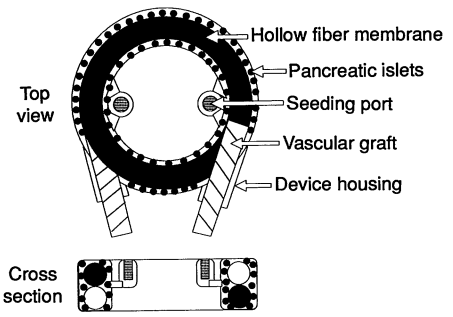

Fig. 1. Schematic diagram of the biohybrid pancreas device. The biohybrid pancreas device consists of an annular-shaped acrylic housing containing 30 to 35 cm of coiled, tubular membrane with an inner diameter of 5 to 6 mm and a wall thickness of 120 to 140 μm. This provides approximately 60 cm^2 of membrane surface area and a cell compartment volume around the membrane of 5 to 6 ml. These devices were 9 cm in diameter and 2 cm high and weighed 50 g. Islets were seeded into the cell compartment through silicone rubber ports that could be accessed for reseeding. So that the islets remained distributed throughout this compartment, the islets were seeded as a suspension in nutrient media containing 1% bacto-agar (Difco, Detroit, Michigan).

diabetic animals as a result of implantation.

For our studies, islets were prepared from either adult mongrel dogs or bovine calves (0 to 2 weeks old) by modified versions of previously described methods (*9*). Pancreatic tissue was dissociated by a collagenase digestion procedure, and the islets were separated from exocrine tissue on a discontinuous Ficoll density gradient. The purity of the isolated islets, as assessed by diphenylthiocarbazone staining, amylase activity, and light microscopy, exceeded 90 to 95% in all preparations (*10*). Isolated islets were then seeded into devices (Fig. 1) for implantation in vivo or for in vitro perfusion culture.

A number of experiments were carried out in vitro for evaluation of the long-term secretion of insulin from islets seeded within the device. Assessment of the insulin output in response to stimulatory levels of glucose (200 mg/dl) from an in vitro device is illustrated (Fig. 2A). In perfusion culture, the device could support islet function for over 9 months, although the level of insulin output did show a decrease over the course of this experiment. Most in vitro experiments did not extend this long because of technical difficulties in maintaining sterility during the large number of manipulations. However, data from 17 other devices obtained for periods ranging from 60 to 156 days (80 ± 5, mean ± SEM) were similar to the pattern in Fig. 2A in that the secretion of insulin peaked within the first 2 months of culture and then decreased slowly.

The ability of these seeded devices to maintain insulin secretory activity during long-term perfusion in vitro suggested they had the potential to provide an in vivo implant with reasonable longevity. Furthermore, the results from the experiments with the device were consistent with other data from islets cultured in a static incubation system that secreted insulin in response to glucose for periods exceeding a year (*11*).

In addition to evaluation of the insulin output in the presence of a constant stimulatory glucose level, acute glucose responsiveness was also investigated. Islets responded to a shift in glucose concentration from 100 to 300 mg/dl with an increase in insulin secretion (Fig. 2B). The insulin output began to increase within 15 min, and the peak level, which represented more than a tenfold increase in insulin output, was reached approximately 60 min after the glucose level rose to 300 mg/dl. Three separate experiments in which this protocol was used indicated a 12 ± 3-fold (mean ± SEM) increase in insulin output with a delay of 21 ± 5 min before the insulin concentration in the perfusate began to increase. An important feature of an artificial pancreas device is that insulin responses to glucose are rapid. Mathematical modeling of the kinetics of the insulin response suggest that the lag should be less than 15 min for achievement of normal glucose homeostasis (*12*), so further efforts to reduce the response time may be necessary.

In vivo function of the biohybrid artificial pancreas was studied with the use of pancreatectomized dogs, a well-established large-animal model for diabetes (*2*). In normal dogs, the fasting glucose levels averaged 91 ± 7 mg/dl (mean ± SEM, n = 16). After pancreatectomy, insulin therapy was designed to maintain the fasting glucose levels below 250 mg/dl. Although not normal, this level of hyperglycemia was tolerated because tighter control risked hypoglycemia. In the initial in vivo studies, single devices seeded with canine islet allografts were implanted

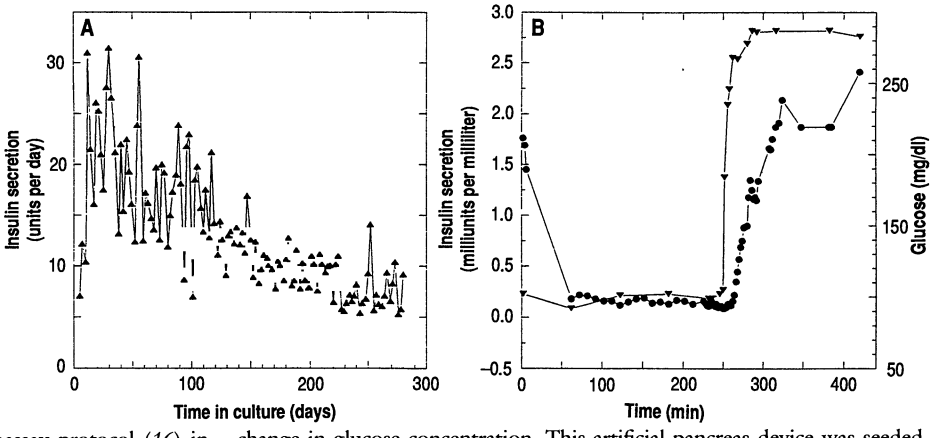

Fig. 2. (**A**) In vitro insulin output from the biohybrid pancreas. Insulin secretion from 1 × 10^5 to 2 × 10^5 canine islets seeded in the artificial pancreas device was evaluated in vitro at 37°C in a humidified atmosphere of 5% CO_2 in air. Perfusion through the lumen of the fiber was through Silastic tubing rather than the PTFE graft material described for the in vivo devices. The Silastic tubing facilitated exchange of oxygen and CO_2 and, in contrast to PTFE, was impermeable to the tissue culture medium used for perfusion. Medium [M199/Earle's supplemented with 5% fetal bovine serum, 20 mM Hepes, penicillin (400 IU/ml), and glucose (200 mg/dl)] was recirculated from a 250-ml reservoir at a rate of 10 ml/min with a peristaltic pump (Rainin Inst.). The medium was changed three times per week and sampled at each change. Determination of insulin levels was done by a standard radioimmunoassay protocol (*16*) in which the binding was allowed to go to equilibrium during a 16-hour incubation at 4°C. ^{125}I-labeled porcine insulin with specific activity of 80 to 120 μCi/mg was obtained from New England Nuclear. Guinea pig antiserum raised against monocomponent porcine insulin was used at a final dilution of 1:80,000. Free antigen was separated from antigen-antibody complex by precipitation with IgGsorb (*Staphylococcus aureus* protein A, The Enzyme Center, Malden, Massachusetts). The limit of assay sensitivity was 25 microunits per milliliter, and data reduction was done by a log-logit transformation of the standard curve by Cobra gamma counter (Packard Inst.). Similar results to those illustrated here were obtained for 17 other devices (see text). (**B**) Responsiveness of the biohybrid pancreas to an acute change in glucose concentration. This artificial pancreas device was seeded with 9.5 × 10^4 canine islets and maintained in perfusion culture for 43 days. To assess responsiveness to acute changes in glucose, the device was removed from long-term culture and incorporated into a perfusion system in which samples of the perfusate from the device were collected every 2 min and assayed for insulin and glucose. The device was perfused with Krebs-Ringer buffer (KRB) containing 0.5% bovine serum albumin and glucose (100 mg/dl) for 4 hours to establish a basal level of secretion. The perfusate was then changed to KRB containing glucose (300 mg/dl), and the experiment was continued for an additional 3 hours. The data shown are representative of three experiments done under these conditions (see text). (●, insulin; ▼, glucose.)

into 12 pancreatectomized dogs (*13*). Six of these implants had a significant effect on the exogenous insulin requirements. On the basis of the decreases in required insulin, these data suggested that the in vivo devices were secreting between 15 and 20 units of insulin per day. However, the insulin output obtained from a single device was not sufficient, in most cases, to replace exogenous insulin therapy completely.

To eliminate the need for exogenous insulin for animals requiring more than 20 units of insulin daily, we implanted two devices in each of ten animals in the next series of experiments. In two instances the implant had little effect on fasting blood glucose concentrations and the devices were surgically removed after 19 and 88 days for gross and microscopic evaluation. Each of these devices contained a high percentage (>50%) of nonviable islets, but the underlying cause for this loss of viability was not apparent. The vascular connections to the devices were free of any occlusion, and there was no evidence of any immune reaction. In one of these experiments the in vitro control device also secreted very low levels of insulin (≤4 units per day), suggesting that the lack of function was unrelated to implantation. A third animal died shortly after surgery because of a rupture of the suture line at one of the anastomoses.

Implantation of two devices completely supplanted exogenous insulin therapy in six of the remaining seven animals (Table 1). This includes four animals in which the insulin requirement was 30 or more units per day. The average fasting glucose values ranged from 107 to 168 mg/dl for these six implants. In the case of the seventh animal (PS35), the implant reduced the insulin required to maintain the fasting glucose levels in the 200 to 250 mg/dl range from 30 to 14 units per day, but it was not sufficient to replace the entire insulin dose. This experiment was terminated and it was determined that one of the implanted devices had clotted. Termination of experiments PS28 and PS30 was due to health complications rather than loss of device patency or function. Each of these animals manifested clinical signs of infection including fever, respiratory problems, and weight loss that persisted despite antibiotic treatment. Postmortem studies revealed systemic bacterial (cocci) infection that appeared to have originated at the site of the anastomosis.

A profile of fasting glucose levels from one of the six dogs that no longer required insulin after device implantation (PS22) is shown in Fig. 3. These data illustrate the ability of the implanted devices seeded with allogeneic islets to maintain an average fasting glucose level of 107 ± 3 mg/dl (mean ± SEM) for 5 months in the absence of exogenous insulin. During the implant period, the fasting glucose levels for this animal averaged 107 ± 3 mg/dl (mean ± SEM).

Despite good control of fasting glucose concentrations, additional studies indicated that glycemic control in response to a meal or an intravenous glucose tolerance test remained abnormal (*14*). The rate of glucose disappearance from the circulation (K rate) measured after device implantation was less than 1.0% per minute in each of these animals, whereas the rate in normal dogs is 4.1 ± 0.2% per minute (mean ± SEM, n = 17). This is consistent with the observation that there was no significant peak in circulating insulin levels during the 2-hour period after intravenous glucose administration. In contrast, normal dogs exhibit a 4.9 ± 1.1-fold (mean ± SEM, n = 6) increase over basal circulating insulin concentrations within 10 min. In vitro data (Fig. 2B) suggest that islets seeded in these devices should respond to sharp rises in glucose

Table 1. Exogenous insulin requirements and fasting blood glucose (FBG) concentrations before and after device implantation. Evaluation of the in vivo function of biohybrid artificial pancreas devices was done with adult female dogs (16.5 ± 0.7 kg) purchased from Biomedical Associates Inc. and housed in compliance with USDA Regulations Part III (Animal Welfare Act) at the Animal Resources Center facility of the Harvard Medical School. To establish a diabetic model, a pancreatectomy that removed at least 95% of the pancreas was performed. Before surgery, animals were kept without food overnight but had free access to water. Atropine (0.01 mg/kg) and, if required, acepromazine maleate (0.5 mg/kg) were administered intramuscularly 30 min before surgery. Anesthesia was induced with an intravenous injection of 4% Bio-tal (thiamylal sodium, 0.5 ml/kg) and maintained with 1 to 2% halothane in 100% oxygen given by an endotracheal tube. Lactated Ringer solution (50 ml/kg) was given intravenously during surgery. After surgery, acepromazine maleate, or Talwin (pentazocine lactate), or both, were given for pain relief if necessary. Antibiotic treatment (500 mg of amoxicillin) was given for 3 days postoperatively. Pancreatic enzymes were replaced by administration of Viokase tablets mixed with multivitamins in the food. Fasting blood samples were taken two to three times per week, and glucose levels were determined by means of an Accucheck II blood glucose monitor (Boehringer Mannheim, Indianapolis, Indiana). A daily injection of porcine Lente insulin (NovoLabs, U-100) was administered with the dose adjusted for maintenance of circulating fasting glucose levels below 250 mg/dl. A second surgical procedure was performed 2 to 3 weeks after pancreatectomy in which the devices were implanted by end-to-side anastomosis to the external iliac artery and vein by means of standard vascular surgical techniques. On completion of the anastomoses, the grafted devices were placed between the omentum and anterior abdominal wall and sutured to the abdominal wall to prevent migration and kinking of the graft. Pre- and postoperative care was carried out as described for pancreatectomy except that aspirin (75 mg) was also administered daily. We determined blood flow through the grafted device noninvasively by listening for the sound of flow (bruit) over the implant. In each experiment in which devices were seeded for implantation, a control device was seeded at the same time for in vitro perfusion culture. Thus, the effect of implantation on islet function could be assessed and the potential variability between islet preparations could be controlled. Each of the devices was seeded with 160 ± 8 × 10^3 canine islets per device, and each dog received two devices. Values shown represent the means of all determinations made during the course of the experiment.

Animal	Before implant		After implant		Duration of implant (days)	Status
	Injected insulin (units/d)	FBG (mg/dl)	Injected insulin (units/d)	FBG (mg/dl)		
PS22	18	246	0	107	157	Ongoing
PS23	30	279	0	158	11	Ongoing
PS26	30	323	0	165	100	Ongoing
PS28	24	295	0	168	54	Terminated*
PS30	18	248	0	130	27	Terminated*
PS35	30	222	15	248	38	Terminated*
PS36	30	218	0	142	22	Ongoing

*See text for details.

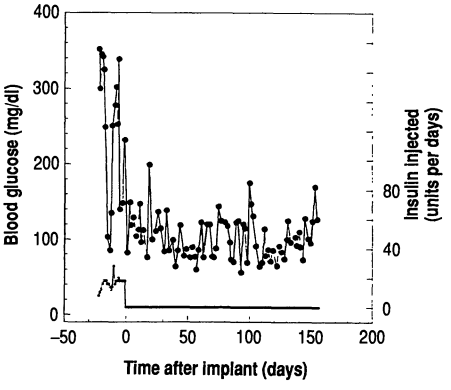

Fig. 3. Exogenous insulin requirements and fasting glucose concentrations before and after device implantation. These data were obtained for animal PS22, which received two devices containing a total of 4 × 10^5 islets. As discussed in the text, this pancreatectomized dog had required 18 units per day of insulin to maintain an average fasting glucose of 246 mg/dl. After implantation of the device, the fasting glucose levels averaged 107 mg/dl in the absence of any exogenous insulin. (·, insulin; ●, glucose.)

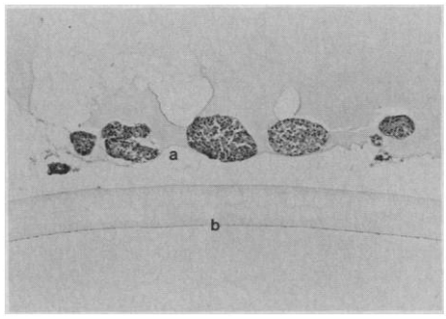

Fig. 4. Histological evaluation of the biohybrid pancreas after removal. In this experiment, a biohybrid pancreas device seeded with allogeneic islets was implanted for 78 days. After device removal, specimens from the compartment and membrane were fixed in 10% buffered formalin, embedded in paraffin, and 6-μm sections were prepared for light microscopy. Sections were stained with hematoxylin and eosin; immunohistochemical staining (not shown) was used for evaluation of the level of stored insulin within the β cells (*17*). On the cell chamber side of the membrane (a) is a group of islets made up of cells with well-defined nuclei and some cytoplasmic vacuolization; immunoperoxidase staining revealed granulated β cells. The luminal surface of the membrane (b) shows a small accumulation of fibrin but no adherence or infiltration of immunocytes or inflammatory cells (× 100).

concentration. These intravenous glucose tolerance data can be explained if maintenance of fasting glucose concentrations in vivo requires the maximal secretory capacity of the implant. Clearly, attainment of normal circulating glucose concentrations after glycemic stress represents an important feature of biohybrid artificial pancreas function that must be achieved. Our efforts are now focused on approaches to optimize insulin output and provide improved responses to glycemic stress.

The data presented here provide evidence for the potential of the biohybrid artificial pancreas as a therapy for diabetes. These results clearly demonstrate that islet function and viability can be supported in vivo by the device. This suggests that the exchange of nutrients and metabolites across the selectively permeable membrane is at least sufficient to maintain islet function and provide regulation of fasting glucose levels. Furthermore, data from both in vivo and in vitro experiments indicate insulin output can continue for at least 6 months.

In addition to the functional data, histological evaluation of 20 devices that were removed indicates that the membrane does not appear to promote fibrotic or thrombogenic responses. A micrograph of a section of membrane from a device that had been implanted for 78 days illustrates this point (Fig. 4). The luminal surface of the membrane, which is in contact with the circulating blood, has very little fibrin accumulation. This observation has been characteristic of all of the devices that have been removed. In the cell chamber on the other side of the membrane, viable islets with well-maintained morphology can be seen, confirming that the membrane provides a protective barrier against immune rejection of the allografts. No evidence of lymphocytes or inflammatory cell infiltration into the islet cell chamber or accumulation on the luminal surface of the membrane has been observed in any device seeded with canine islet allografts. In contrast, in two experiments in which canine islet allografts were placed inside sealed chambers fabricated from tubular membranes and implanted into the peritoneal cavity of pancreatectomized dogs, breakage of the membranes was associated with islet necrosis. An additional issue is whether immune isolation can also prevent the autoimmune β-cell damage associated with Type I spontaneous diabetes. In this regard, a recent report (*15*) indicates that microencapsulation can protect allografted islets from autoimmune destruction in spontaneously diabetic BB rats.

A device containing bovine islets implanted in a pancreatectomized dog for 80 days maintained an average fasting glucose level of 124 ± 12 mg/dl in the absence of exogenous insulin. Before the device was implanted, this animal required 14 units per day of insulin to maintain an average fasting glucose level of 200 ± 33 mg/dl. The results from this xenograft support the feasibility of using more readily available animal islet preparations in human implants.

The results presented here from seeded devices also demonstrate long-term patency of the device itself. In a related set of experiments, unseeded devices have been implanted in normal dogs so that the biocompatibility of the device as a vascular shunt can be evaluated. Nine unseeded devices are ongoing after periods ranging from 9 to 13 months (*13*). As discussed previously, the insulin output from isolated islets decreases over time in culture. Although the data in Table 1 suggest that devices can control fasting blood glucose concentrations for as long as 5 months, their average duration of function in vivo has not yet been precisely established. These devices are therefore designed to be reseedable through their silicone rubber ports (Fig. 1) in the event that the islets must be replaced periodically.

Clearly, the ultimate therapy for diabetes should provide glycemic regulation similar to that normally provided by pancreatic islets. These data suggest that, with improvements in device design, the biohybrid artificial pancreas should approach this goal. In addition, this same technology could lead to the development of other biohybrid organs for treatment of human diseases.

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