Silica Sol-Gel Encapsulation of Pancreatic Islets

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Abstract. **We developed a biocomposite material containing silica ceramic. The sol-gel technology in which ceramic materials are polymerized from liquid solutions at room temperature and physiologic pH can be used to produce ceramics that have a determined pore size and that contain living organisms or cells. Capsules were stable to extreme acid and base conditions as well as to trypsin *in vitro* for 6 months. We used insulin-secreting murine islet cells as the first mammalian material for encapsulation. Two approaches to generating successful encapsulation of islets were used: drop-tower sphere generation and emulsion. Sphere diameters of less than 1 mm were associated with positive insulin secretory capacity as documented by a static batch incubation technique. Average pore sizes were 161 Å for drop-tower spheres and 105 Å for emulsion spheres. Capsules allowed the passage of insulin and cytokines but not the passage of antibody. Implantation of encapsulated islets did not result in fibrosis of the capsule *in vivo,* and retrieval of capsules after 1 month *in vivo* documented continued insulin secretory capacity. Further *in vivo* experiments documented increased survival of transplant recipients despite failure to achieve normoglycemia in all but a few cases. Silica sol-gel encapsulation provides a potentially useful alternative for encapsulation of cells for transplantation or drug delivery, and further work is warranted to develop this potentially useful approach for the treatment of diabetes mellitus.** [P.S.E.B.M. 1998 Vol 218]

Presently, the long-term outlook for most persons with type 1 diabetes mellitus includes multiple daily injections and a high probability of complications resulting from the disease (1). Transplantation of insulin secreting cells has been viewed as a potential solution for persons with type I diabetes (2, 3). The development of biocompatible encapsulation for insulin secreting cells would avoid the risk of immune rejection that is the hallmark of the disease. We have developed a biocomposite material containing silica ceramic (4–6). The ceramic forming sol-gel technology in which ceramic materials are polymerized from liquid solutions at room temperature and physiologic pH can be used to produce ceramics that have a given pore size (7) and that contain living organisms or cells (8, 9). We studied insulin-secreting islet cells as the first mammalian material for encapsulation and herein demonstrate developments in capsule generation, pore size measurements, permeability of pores to insulin but not antibodies, *in vitro* capsule stability, insulin secretion in response to a stimulus, capsule viability *in vivo,* and increased survival of transplant recipients.

Research Design and Methods

Mice. All protocols were approved by appropriate institutional review. Non-obese diabetic (NOD) mice or C57 BL6 mice were raised in the animal facility of Sansum Medical Research Foundation or obtained from Jackson Laboratories (Bar Harbor, ME). All mice were maintained at 23°C in a relative humidity of 50% and under a normal light/dark cycle. All animals were administered untreated tap water and Purina Mouse Chow (5015) *ad libitum.*

Sol-Gel Preparation. Silica gels were prepared (9) based on the two-step method of Brinker (10) in which hydrolysis of the silicon alkoxide precursor is achieved under acidic conditions, and gelation is induced through the

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addition of base. Tetraethoxysilane (TEOS, 42 ml), purchased from Alfa/Aesar (Ward Hill, MA) was vigorously mixed with 10.0 ml 0.1 *M* HCl solution. After 30 min of stirring, the initially turbid mixture became a clear solution due to the hydrolysis of TEOS. The solution was then chilled in an ice bath to reduce the rate of polycondensation under the addition of base, and ammonium hydroxide solution (15.0 ml, 0.1 *M*) was added rapidly.

Islet tissue in 1-ml Hanks balanced salt solution (HBSS) was added rapidly to the sol-gel mixture and stirred. This mixture was added dropwise to a beaker filled with vegetable oil for the emulsion method; or the mixture was added dropwise to the top of a 6-foot tower containing vegetable oil for the drop-tower method. For the emulsion method, the slurry was stirred on a stir plate until the microspheres had formed (approximately 30 sec). Using the drop-tower method, gelled spheres were collected in a flask at the bottom of the drop tower. The time of transit from top to bottom of the tower was approximately 2 min. The spheres were retrieved and washed thoroughly with HBSS and placed into culture medium (RPMI 1640).

Determination of Pore Size. The porosity of the encapsulant material from both the drop tower and emulsion methods was determined using the Brunauer Emmett Teller (BET) method at Quantachrome (San Juan Capistrano, CA). Surface area, pore volume, average pore diameter, and peak or most common pore diameter within the sample were measured, based upon the quantity of gas adsorbed at various relative pressures. Pore size distributions were made by measuring the volume of gas adsorbed or desorbed over a wide range of pressures at constant temperature.

Mouse Islet Isolation. The abdomen of the mouse was shaved, and the anesthetized animal (chloral hydrate, 0.08 mg/g body weight; and pentobarbital sodium, 0.03 mg/g body weight given intraperitoneally) was laid on its back. A horizontal nick was made just through the fur in the lower abdomen followed by a vertical incision up to the sternum. At both the top and bottom of the vertical incision, horizontal cuts were made extending from midline out to both sides. Forceps were used to peel back the fur before replicating incisions through the peritoneum.

The exposed intestines were pushed aside gently, and the median lobes of the liver were retracted to expose the pancreas. Forceps were used to fan out the pancreas from the gall bladder to the duodenum. HBSS was then injected into the pancreas. All pinkish tissue interconnected by clear membrane was excised from the first anterior loop of the small intestine, the back of the stomach, and the large intestine and spleen. The pancreas was placed in HBSS in a sterile petri dish and stored on ice until all required pancreata were excised.

The tissue was minced with iris scissors, and the pieces were transferred to a sterile conical tube. The mixture was centrifuged gently (1 min), resuspended into 8 ml fresh HBSS, and centrifuged again. The HBSS was aspirated, and collagenase was added to each tube (collagenase XI; 4 ml/pancreas; 2.5 mg/ml) and transferred to a sterile culture flask. The mixture was agitated in a controlled temperature water bath, 140 oscillations/min, at 37°C for 10 min.

The digested material was transferred to a fresh sterile conical tube and rinsed with 3–4 ml fresh HBSS. Three washes were performed with 8 ml HBSS at each rinse, and the pieces were resuspended after each centrifugation. After the final wash the pieces were resuspended in 8 ml HBSS and stored on ice for 45–60 min.

To perform islet picking, a small amount of tissue was added to a black-bottomed 100-mm petri dish containing enough HBSS to cover the bottom of the dish. Islets were picked under a dissection microscope and moved to a second black-bottomed 100-mm petri dish with a 9-in. Pasteur pipette. The picked islets were then placed in a fresh petri dish containing filtered complete growth medium.

Islet Viability. Islet viability was tested using the static batch incubation method described below. Islets for testing were transferred to fresh sterile conical tubes and centrifuged for 1 min to pellet the tissue. With care not to agitate islets, 1 ml of room temperature Krebs Ringer Buffer (KRB) was added to each tube with a micropipettor. Tubes were placed in a water bath for 1 hr with oxygen flow over them at 6 liters/min. After 60 min, each tube was aspirated under the dissecting microscope and stored immediately in labeled tubes on ice. The labeled tubes were collected and assayed as described below.

Islet Fractional Stimulatory Ratio. The fractional stimulatory ratio was performed as described previously (11). Briefly, islets or capsules were transferred to plastic tubes and incubated in 1 ml Krebs-Ringer buffer (KRB), pH 7.40 containing 25 m*M* HEPES, 2 m*M* glucose and 0.3% BSA at 37°C, in an atmosphere of 95%/25% CO_2. After 1 hr or longer, buffer was removed, and fresh KRB identical to the above but containing sufficient glucose to bring the total concentration to 25 m*M* was introduced, and islets were reincubated for an equal period. After each incubation, samples of buffer were removed for radioimmunossay of insulin (Diagnostic Products Corporation, Los Angeles, CA) release using rat insulin as a reference standard. The fractional insulin secretion rate during the first hour (F_1) of incubation was the amount of insulin secreted as a percentage of total insulin (sum of insulin secreted during the first and second hours plus the nonsecreted insulin). The fractional secretion rate during the second hour of incubation (F_2) was the amount of insulin secreted as a percentage of total remaining insulin (sum of insulin secreted during the second hour plus the nonsecreted insulin). The fractional stimulation ratio (FSR) was defined as F_2/F_1 and expresses the insulin secretory capacity. The FSR for nonencapsulated islets ranged from 1.4–3.6.

Capsule Implantation. Cultured islets from donor mice (C57 BL6 *ob/ob* or -/- males) were encapsulated for the *in vivo* experiments. Islets were pooled and divided into aliquots for encapsulation. A small portion of the pooled islets was set aside for insulin production assay (FSR) using

the static batch method described above. Pooling of the islets was done to prevent the possibility of giving poorer performing islets to some recipients and superior ones to others. Thus we could compare the performance of the two encapsulation methods performed with a given batch of islets. Encapsulation was performed 4 days prior to implantation. Encapsulated islets were maintained in culture (RPM1 1640, 37°C, 5% CO_2) until implantation.

Recipient mice were given streptozotocin (165 mg/kg body weight; dissolved in sterile saline) 4 days prior to surgery. Diabetes onset was documented by glucosuria of 2%. Anesthesia for surgery consisted of pentobarbital (10 μl injected IP; diluted with 4 parts sterile saline to 1 part pentobarbital) and chloral hydrate (10 μl injected IP; 1 g in 30 cc sterile saline). Animals were kept warm both before and after surgery using a warming platform at 37°C. All surgery was performed in a sterile Class 100 biological materials hood, inside a Class 1000 clean room.

Midline abdominal incisions were made of a sufficient size (c. 1.0 cm) into which capsules were placed intraperitoneally. Wound closure was performed with a two-layer procedure. A 6-0 synthetic absorbable suture was used for the internal closure, followed by methylacrylate closure of the skin. Each recipient received approximately 60 to 65 islet clusters or approximately 500 islets/kg.

Lymphocyte Isolation and Encapsulation. Human lymphocytes were obtained from fresh whole blood using a Ficoll-Paque gradient (Pharmacia) as described previously (12). The cells were resuspended in Hanks' buffered salt solution (HBSS) and centrifuged at 60–100*g* for 10 min at 18–20°C. The last step was repeated, before resuspension of cells in either HBSS or culture medium.

To determine whether cytokines could traverse the capsules, the resuspended cells were placed in complete growth medium with concanavalin A and added to the sol-gel mixture as described above and either encapsulated by the drop-tower method (Group A) or placed in complete growth medium with concanavalin A (Group B). Another batch of cells was placed in another petri dish and received no concanavalin A (Group C). The retrieved capsules were washed several times with phosphate-buffered saline (PBS) and placed in complete growth medium. The dishes were kept in the incubator for 48 hr, and the supernatants were collected. Presence of cytokines IL-2 and IL-4 were measured using sandwich ELISA assays (Endogen, Cambridge, MA).

Antibody. To determine if antibody could cross the capsules, FITC-conjugated anti-CD4 human IgG1 kappa, MW 59 kDa (DAKO, Glostrup, Denmark) was used according to the manufacturer's instructions. The cells were washed twice with PBS and resuspended in either HBSS for encapsulation or complete growth medium for nonencapsulated controls. Fluorescence measurements were performed with a Perkin-Elmer Applied Biosystems 7200 Fluorescence Sequence Detector (with attached 96-well plate reader), at an emission wavelength of 525 nm.

Statistics. Results are given as means ± SEM. Significance levels (P) were determined using a paired or unpaired t test as appropriate (Statview 4.01; Abacus Concepts, Berkeley, CA).

Results

To investigate the stability of the encapsulant *in vitro,* spheres were placed in a variety of incubation media. Acid stability was tested by placing several spheres in a 15-cc conical vial containing 1 *M* HCl. Base stability was tested by placing several spheres in a conical vial containing 1 *M* NH_4OH. Other spheres were placed in PBS containing 5 mg trypsin. After 6 months, all spheres were examined for macroscopic changes. No change was observed for any of the spheres under any of the above conditions, indicating *in vitro* stability under a wide variety of conditions.

Sol-gel porosity measurements showed that the two encapsulation procedures resulted in slightly different pore size despite the same gel formulation (Table I). The emulsion method spheres had greater surface area and smaller pore density.

Pore size was also estimated biologically by two experiments documenting that cytokines could traverse the capsules whereas antibodies could not. In response to concanavalin A (conA) stimulation, nonencapsulated control human peripheral blood lymphocytes (PBL) secreted 46.0 (0.05) pg/ml IL-2 and 2.52 (1.50) pg/ml IL-4; the lymphocytes encapsulated with conA secreted 40.0 (31.9) pg/ml IL-2 and 3.31 (1.27) pg/ml IL-4; and the nonencapsulated controls with no concanavalin A stimulation secreted 6.73 (0.31) pg/ml IL-2 and 0 (4.78) pg/ml IL-4. Each cytokine has an M_r of approximately 20,000 Da. Lymphocytes did not escape the capsule nor were they found under microscopic examination to be immersed in the pores of the sol-gel.

To determine if antibodies could traverse the pores of the sol-gel capsules, we incubated freshly encapsulated human lymphocytes previously labeled with FITC-conjugated monoclonal mouse anti-human CD4 antibody (see Methods) and measured relative fluorescence of culture supernatants after 24 hr in culture. The results were as follows (fluorescence at 525 nm, relative scale): the supernatant from lymphocytes not labeled with antibody had a relative fluorescence of 18 without encapsulation and 22 if encapsulated. The supernatant from lymphocytes labeled with anti-CD4 label had a fluorescence of 31 if not encapsulated. For the encapsulated labeled lymphocytes, the supernatant relative fluorescence did not differ from unlabeled cells (relative fluorescence 21).

Table I. Sphere Pore Characteristics

Pore characteristic	Drop-tower spheres	Emulsion spheres
Surface area (m^2/g)	817	996
Pore volume (cc/g)	3.30	2.62
Average pore diameter (Å)	161	105
Peak pore diameter (Å)	89	65

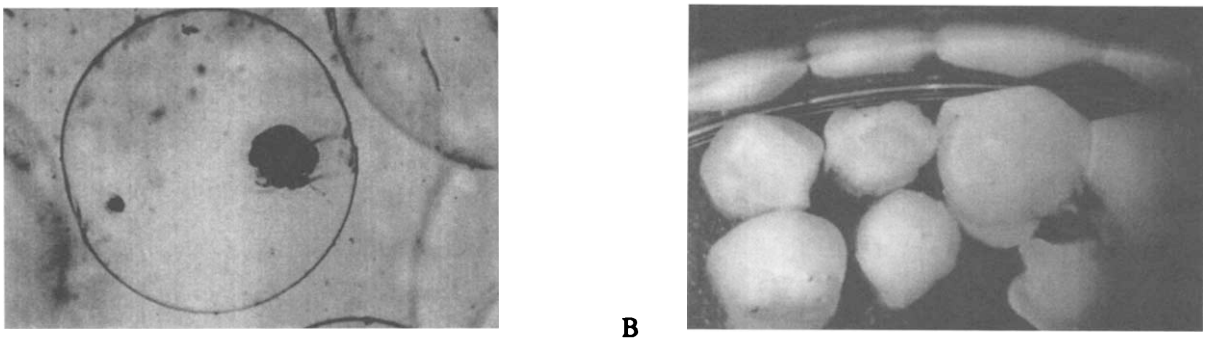

Figure 1. (A) Encapsulated islets in sol-gel ceramic. Approximate actual diameter of sphere is 3 mm. (B) Retrieved emulsion method capsules from a recipient mouse. Magnification is 10×.

The optimal sphere size and optimal encapsulation method for islet encapsulation were addressed simultaneously. We studied the effect of sphere size on islet function in capsules created using 2 encapsulation methods: drop-tower and emulsion formation. The drop-tower method created spheres in the millimeter diameter range. The emulsion method created spheres of 200–1000 μm in diameter. Capsule diameters ≤ 1mm were associated with a fractional stimulatory ratio following static batch incubation > 1.0, indicating that the tissue in these capsules secretes insulin satisfactorily in response to a glucose stimulus. Increasing capsule size above 1 mm was associated with FSR ≤ 1. For example, comparison of islet-containing capsules ≤ 100 μm (n = 12 observations) with those from 1–4 mm showed FSR values of 1.2 ± 0.2 vs 0.8 ± 0.4 respectively ($P < 0.01$).

Insulin secretory responses were found following static batch incubation in emulsion method capsules removed from a recipient mouse (Fig. 1, panel B) after 1 month *in vivo*. The high glucose (25 m*M* glucose incubation stimulus) response was 1.6 μU/ml; and the low glucose (5 m*M* glucose incubation stimulus) response was ≤ 1μU/ml, corresponding to an FSR of at least 1.6. These results show that the islet tissue inside the capsules was still capable of secreting insulin in response to a stimulus, even after 1 month *in vivo*.

Figure 2 documents survival of diabetic mice following implantation of encapsulated islets by the drop-tower and emulsion methods. One of the mice receiving encapsulated islets using the emulsion method (n = 2) survived for up to 16 weeks post-implantation and was aglucosuric for 2 weeks. The other animal was sacrificed after 40 days to observe the capsules and document the viability of the encapsulated islets. This latter animal also had improved glucosuria for 2 weeks following implantation (P = 0.04 versus nonoperated controls). The nonoperated control animals (n = 6), all died within 12 days of streptozotocin administration. Recipients of encapsulated islets using the drop-tower encapsulation method (n = 10) survived a minimum of 4 weeks postimplantation (P = 0.0009 versus nonoperated controls; P = 0.02 versus placebo recipients). Two of the drop-tower capsule recipients experienced reduced glucosuria (0.5%) for up to 2 weeks, and a third mouse experienced the elimination of glucosuria for over 5 weeks following the transplant, after which the values returned to 2% (6). The placebo mice (n = 4; drop-tower capsules) died within 3 weeks of surgery (P = 0.02 versus drop-tower capsule recipients; P = ns versus nonoperated controls).

Discussion

In conclusion, we have provided evidence for the potential utility of silica sol-gel encapsulation of mammalian cells. The capsules are stable enough to be potentially useful as a transplant material. Capsule porosity is sufficient to allow insulin to exit the capsule, yet IgG antibodies did not traverse the sol-gel. Previously, we have shown with islets encapsulated in molds that insulin secretory capacity is

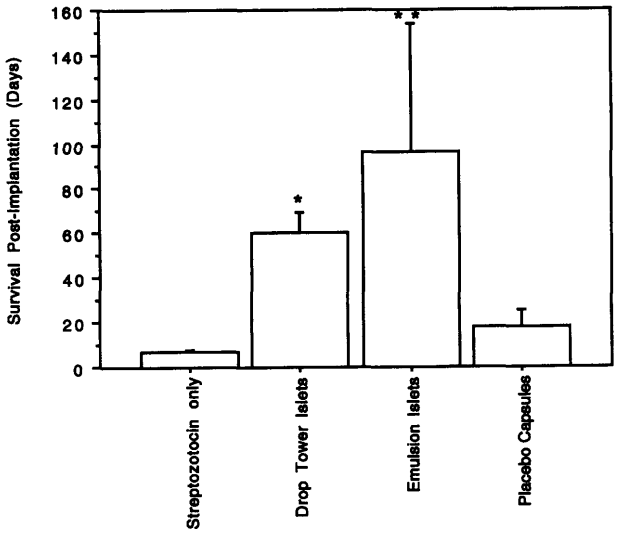

Figure 2. Comparative survival of diabetic NOD implant recipients. Animals receiving emulsion capsules survived longest (n = 2; P = ns vs. placebo; P = 0.02 vs. streptozotocin-treated animals). One animal in the emulsion group was sacrificed at 40 days to retrieve and observe the implanted capsules. Thus, the bar graph represents 40 days survival for the sacrificed animal, and 154 days for the other animal. The drop-tower recipients (n = 10) also had significantly longer survival rates compared to placebo animals (n = 4; P = 0.02) and streptozotocin-treated animals (n = 6; P = 0.0009). The placebo recipients and nonoperated controls had no statistically significant difference.

maintained *in vitro* (6). The present work documents that generation of capsules by drop-tower or emulsion may also be used to generate functioning islet capsules. In addition we have now shown that capsules retrieved after 1 month *in vivo* maintain insulin secretory capacity. To date, we have not seen a cellular response around intraperitoneal capsules that do or do not contain islets.

The approach appears nontoxic. With the dose of islets given to the recipients, we were not able to document "cure" of diabetes in this murine model. This result is not unexpected since it is generally accepted that approximately 5,000 to 10,000 islets/kg body weight are required to establish normoglycemia in mammals with complete pancreatic insufficiency (13). Nevertheless, we were able to document a biological effect in terms of glycosuria and longevity with approximately 500 islets/kg in the present study.

Thus it appears that the inorganic silica-based sol-gel encapsulation described here may provide a useful means of islet and cell encapsulation for transplant purposes in addition to the organic based encapsulation procedures that have been described and are now in commercial development (Neocrin, Irvine, CA; VivoRX, Santa Monica, CA; Encell, Greenville, NC; Islet Technologies, North Oaks, MN). The organic matrices for encapsulation of islets have included artificial membranes made of acrylic resins with (14) or without (15) physical treatments to modify permeability, alginates (16–18), or macroencapsulation matrices in permselective hollow-fiber membrane devices (19). Further work is warranted with all of these approaches to improve glycemia in the recipients, document longer-term survival of the capsules, as well as provide further evidence for lack of toxicity to both the encapsulated material as well as the transplant recipient.

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