Microencapsulation of Pancreatic Islets

A Non-Vascularised Transplantation Model

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Abstract

Transplantation of pancreatic islets is a potential treatment of type 1 diabetes that aims to restore normal blood glucose control. By encapsulating the islets in alginate, they can be protected from rejection. The aim of this thesis was to study the biology of encapsulated islets and to use the technique of microencapsulation to study the effect of transplantation in a system that is separated from direct contact with the vascular system and the host tissue at the transplantation site.

Encapsulated islets can effectively reverse hyperglycaemia after transplantation into the peritoneal cavity of diabetic mice. A period of culture before encapsulation and transplantation did not affect their insulin release or curative capability. Pre-culture with exendin-4 improved insulin secretion, but not to the extent that the long term outcome in our transplantation model was improved. Despite being able to reach and retain normoglycaemia, microencapsulated islets transplanted intraperitoneally decreased in size. More specifically the number of beta cells in each individual islet was decreased. However, in contrast to previous studies using non-encapsulated islets, the alpha cell number was maintained, and thus the capsule seems to protect these peripherally located and otherwise exposed cells. As the capsule also prevents revascularisation of the islets, the model was used to study the importance of vascular supply for islet amyloid formation. Islet amyloid is a possible reason for the long-term failure of transplanted islets. It is likely that their low vascular density causes a disturbed local clearance of IAPP and insulin that starts the aggregation of IAPP. Indeed, encapsulated islets had an accelerated amyloid formation compared to normal islets, and might serve as a model for further studies of this process.

In conclusion, although revascularisation is not a prerequisite for islet graft function, it plays an important role for islet transplantation outcome.

Keywords: Microencapsulation, Islet transplantation, revascularisation, alpha-cell, exendin-4, islet amyloid

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"Har man tagit Fan i båten får man hitta på resten själv."

*Family saying*
List of Papers

This thesis is based on the following papers, referred to in the text by their Roman numerals:


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<table>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>G</td>
<td>Guluronic acid</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon like peptide-1</td>
</tr>
<tr>
<td>IAPP</td>
<td>Islet Amyloid Poly Peptide</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1 beta</td>
</tr>
<tr>
<td>M</td>
<td>Mannuronic acid</td>
</tr>
<tr>
<td>NOD</td>
<td>Non-obese diabetic</td>
</tr>
<tr>
<td>PLL</td>
<td>Poly-L-lysine (PLL)</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
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Introduction

Diabetes

Diabetes is a chronic disease characterised by hyperglycaemia. There are two major forms of diabetes; type 1 and type 2. In the year 2000, the worldwide prevalence of diabetes was estimated to 171 million, of these approximately 18 million people are diagnosed with type 1 diabetes. A worldwide rise in incidence has occurred both in low and high incidence populations, especially in children under the age of five years. The increase rate in incidence is 3-4% per year.

The most common form, type 2 diabetes, is characterised by peripheral insulin resistance combined with impaired insulin production. In most of these patients deposits of amyloid are seen in the pancreatic islets. The amyloid deposits are associated with reduced beta cell mass and function. However, since it is also formed in the pancreas of some non-diabetic elderly persons its contribution to the pathogenesis of diabetes is not clear.

Type 1 diabetes is an autoimmune disease, in which the immune system attacks the insulin producing beta cells. Inflammatory cells infiltrate the islets and produce beta cell toxic cytokines. This results in a total insulin deficiency leading to hyperglycaemia and development of ketoacidosis, if not treated with exogenous insulin. By the time of debut of hyperglycaemia generally 70-80% of the beta cells have been destroyed.

In the long term, diabetes increases the risk for nephropathy, neuropathy and retinopathy, but intensive insulin treatment in type 1 diabetes has been shown to delay the onset, and slow the progression of these complications. In type 2 diabetes, life-style changes in terms of diet and physical exercise may counteract the insulin resistance.

The islet of Langerhans

The human pancreas contains 1-2 million islets of Langerhans, which together constitute 1-2% of the total weight of the pancreas. The majority of cells in the pancreatic islet is insulin producing beta cells. Insulin promotes glucose uptake in muscles and fat, stimulates glucose storage in the form of glycogen in the liver and suppresses gluconeogenesis. The beta cell also produces Islet Amyloid Poly Peptide (IAPP) also known as amylin, which is
the main component of islet amyloid. The second most common cell type in the islet of Langerhans is the alpha cell that produces glucagon which mainly stimulates glycogenolysis and gluconeogenesis in the liver. In rodent islets the alpha cells are positioned peripherally, whereas they are more evenly distributed in the human islet. Other endocrine cells in the islet produce somatostatin, pancreatic polypeptide and ghrelin. The islet also contains endothelial cells, nerves, fibroblasts and dendritic cells. The pancreatic islets have a rich glomerular-like vascular system in order to supply oxygen and nutrients, but also to transport secreted hormones to target organs.7,8

Islet transplantation

Transplantation of islets of Langerhans could provide a good alternative to insulin treatment for type 1 diabetes patients. It has recently been shown that islet transplantation can improve blood glucose homeostasis quite efficiently on a regular basis in a large series of patients.9 The most common site for clinical islet transplantation is percutaneous injection into the portal vein, where islets are spread and embolised throughout the liver, by a surgical procedure that is relatively small compared to whole pancreas transplantation.10

Due to the requirement of large numbers of islets to cure a diabetic individual, (2-3 pancreata per recipient9) the limitation of islet transplantation is the shortage of donor material. Much effort has been devoted to find alternative sources of insulin producing cells such as stem cells and xenogeneic sources, but also to find means to improve islet function and increase beta cell mass prior to and after transplantation. This is essential, as it has been estimated that in diabetic rodents approximately 50% of the grafted islets do not survive the first few days of transplantation,11-13 especially if a suboptimal number of islets is transplanted.14 In humans it is harder to assess the survival of islets after transplantation, but based on functional studies it has been estimated that only 20% of the islets successfully engraft.15 However it is not evident from these studies whether whole islets die or whether a proportion of beta cells within the islets die. Another problem that has become evident with islet transplantation is a blunted glucagon response or loss of alpha cells from the grafted islets, which has been demonstrated in patients16,17 as well as in several animal models.18-22 It has recently been suggested that alpha cell loss is associated with close contact of the alpha cells with the implantation organ, which seems to be particularly problematic in the liver.23

Islet amyloid formation has not only been described in type 2 diabetes. It has also been described in transplanted human islets, and is a possible reason for their long-term failure.24 Recently the first report of islet amyloid formation in human islets transplanted into the liver of a type 1 diabetic
The reason for aggregation of IAPP in transplanted islets is still unknown. Disturbances in the ratio insulin/IAPP in the secretory granules might initiate the amyloid formation. Moreover, since oversecretion or increased intracellular degradation of IAPP is not sufficient for amyloid formation, another factor, possibly disturbances in the local clearance of IAPP in the islets caused by their low vascular density, must be involved.26,27

Immunosuppression

The success of clinical islet transplantation has been dependent on the use of life long immunosuppression in order to prevent rejection of the graft and autoimmune recurrence of diabetes.9 Unfortunately, immunosuppressive drugs are associated with side effects such as increased susceptibility to infections, increased risk for the development of malignancies, and toxic effects in general.28 Side-effects seen in the Edmonton protocol include kidney toxicity in patients who already have nephropathy and high triglycerides in patients who already have increased risk of cardiovascular problems, effects that are particularly dangerous for people with diabetes.24 Since these risks often are considered worse than insulin treatment, islet transplantation is restricted to a small group of patients that have a low quality of life due to frequent and severe hypoglycaemic episodes as well as being at great risk to develop diabetic complications, i.e. patients with highly variable blood glucose concentrations and hypoglycaemia unawareness, and/or for patients who have already received a transplanted organ (usually a kidney).29 It would therefore be ideal to be able to transplant islets in the absence of immunosuppression and thus make it available to a wider group of patients.

Microencapsulation

Microencapsulation in simple alginate beads is a technique that could enable islet transplantation in the absence of immunosuppression.30 It is based on physical separation of the islets from the immune system. The large immune cells are excluded whereas small molecules such as nutrients, oxygen, glucose and insulin easily can pass through the alginate gel barrier (Figure 1).31 Microencapsulation was first described by Chang in 1964,32 and in 1980 Lim and Sun applied the technique for encapsulation and transplantation of pancreatic islets.31 Sodium alginate, a polysaccharide composed of linked guluronic acid (G) and mannuronic acid (M), has been the most commonly used polymer for cell encapsulation. Alginate is a non-toxic polymer present in brown algae and is also produced by some types of bacteria.33 Because of its gelling properties it is widely used in
food and medical industry. The capsule properties depend on the alginate composition. High G sequences are required for the alginate to form a stable and strong gel, but also make the gel more permeable than high M gels. The alginate chains are cross-linked with divalent positive ions, usually Ca$^{2+}$, but also Ba$^{2+}$ that makes the capsules more stable. Traditionally, a poly-L-lysine (PLL) layer is added to the alginate core to improve the stability and reduce the permeability of the capsules. However, the positively charged PLL-layer reduces the biocompatibility of implanted capsules by provoking an immune response and inducing a cellular reaction around the capsule.$^{34,35}$ Cellular overgrowth causes a physical and metabolic barrier to nutrients and insulin, and contains macrophages that produce islet suppressing cytokines, such as IL-1$\beta$ and TNF-$\alpha$. $^{36,37}$ Although most previous studies have been carried out on capsules with PLL, some encouraging results using non-PLL capsules with barium as cross-linker to provide better stability have been published.$^{30,38}$

Due to their large volume, microencapsulated islets are usually transplanted into the peritoneal cavity. It is easily accessible and thus only a small surgical operation is needed. However, this site is rich in macrophages and is poorly vascularised resulting in a low oxygen tension.$^{39}$ Results from experiments with transplantation of microencapsulated islets to the peritoneal cavity have not been encouraging, with low reproducibility and with large numbers of islets being required to reverse hyperglycaemia.$^{30,40,41}$ It would therefore be of great interest if the outcome could be improved by for example treatment of the encapsulated islets prior to transplantation.

![Diagram of Alginate Encapsulated Pancreatic Islet](image)

*Figure 1. Alginate encapsulated pancreatic islet. The large immune cells are excluded whereas small molecules such as nutrients, oxygen, glucose and insulin easily can pass through the alginate capsule.*
Microencapsulation as an experimental model

Microencapsulation is not only a way to avoid rejection; it also has advantages in experimental studies, as it is possible to retrieve the encapsulated islets after transplantation to perform *ex vivo* studies of individual islets. It is also a technique to create an islet graft that can not become revascularised, although preliminary results indicate that endothelial cells remain in transplanted encapsulated islets for at least one month. Moreover, in the encapsulated islets, the most peripheral cells are protected from contact with cells at the transplantation site, which has previously been reported to be detrimental for alpha cells transplanted into the liver.23

Islet transplantation improvement

As mentioned above, the poor survival of transplanted islets and the shortage of human islets is a huge problem. It is therefore important that transplantation efficiency is improved, as that will reduce the number of islets needed to successfully reduce blood glucose levels and make the treatment available to more people.

Although culture of islets may be desirable in human islet transplantation,42-45 it has been suggested that the use of freshly isolated islets could improve transplantation outcome. For example the success of the Edmonton protocol has partly been attributed to the use of freshly isolated islets.9 This in turn could be explained by the fact that fresh islets are more appropriately revascularised after transplantation than cultured islets.46 Since encapsulated islets have no possibility to become revascularised the importance of revascularisation for the outcome of islet transplantation can be studied.

Pre-treatment of islets prior to transplantation could be an effective way to improve transplantation outcome. One advantage of pre-treatment of islets in culture is that the patient does not have to be given the drug systemically and thus it is a safer option. A variety of compounds have been used in islet culture in the hope of improving transplantation outcome including nicotinamide,47 nerve growth factor,48 caspase inhibitors,49 and interleukin 6.50 Exendin-4, a glucagon like peptide-1 receptor agonist, is an ideal compound, as it has a wide variety of positive effects on beta cells.51-56 Previous studies using non-encapsulated islets indicate that pre-culture of the islet graft with this peptide improves the outcome in islet transplantation.57-59 With regard to islet transplantation, the most interesting effects of exendin-4 may be its stimulation of beta cell proliferation and its anti-apoptotic properties.51-53
Aims of thesis

The overall aim of this work was to study the biology of encapsulated islets and to use the technique of microencapsulation to study the effect of transplantation in a system that is separated from direct contact with the vascular system and the host tissue at the transplantation site.

More specific aims were:

- To study the efficacy of encapsulated islets in reversing hyperglycaemia in chemically induced as well as spontaneous models of diabetes
- To examine whether conventional culture prior to encapsulation of islets affects the outcome of transplantation
- To investigate the effect of long term implantation on the morphology and cellular composition of transplanted encapsulated islets
- To study acute and chronic effects of treatment with exendin-4, on \textit{in vitro} and \textit{in vivo} function of microencapsulated islets
- To look for the presence of amyloid deposits in microencapsulated human and hIAPP+/+ transgenic mouse islets
Study Design and Methods

An overview of the methods used is described below. All methods as well as manufactures of the equipment and chemicals are described in more detail in the individual papers.

Study Design

Paper I
Islets collected from each collagenase digested pancreas were randomly assigned to either culture (three days) followed by microencapsulation, or to immediate microencapsulation (<4 hours after collagenase injection). Directly after encapsulation, 350 or 700 microencapsulated islets were syngeneically transplanted into alloxan-diabetic mice. In addition, 350 or 700 freshly isolated non-encapsulated islets were transplanted in the same way. The efficacy of the transplantation was determined by blood glucose concentration measurements and glucose tolerance tests. Retrieved encapsulated islets were examined by measurements of glucose oxidation rates and insulin release. To assess the quality of islets prior to transplantation, glucose oxidation and insulin release rates were measured in vitro in encapsulated cultured or non-cultured islets and in non-encapsulated islets.

Paper II
Alloxan-diabetic mice received either a graft of 700 syngeneic (C57BL/6J) non-encapsulated islets, 700 syngeneic microencapsulated islets or 700 allogeneic (BALB/c) microencapsulated islets. Spontaneously diabetic NOD mice were implanted with grafts of either 700 microencapsulated C57BL/6J mouse islets or 700 BALB/c mouse islets. As a control, diabetic NOD mice were grafted with 700 empty microcapsules. In addition, normoglycaemic C57BL/6J mice received a syngeneic (C57BL/6J) graft of 300 encapsulated islets. The blood glucose concentrations were monitored for 12 weeks. Thereafter capsules were retrieved, sectioned and stained for insulin and glucagon. Some encapsulated syngeneic islets were retrieved after four
weeks and insulin release and insulin, glucagon and DNA contents were measured.

**Paper III**

Encapsulated or non-encapsulated islets were cultured with 1 nM exendin-4 added to half the number of the culture dishes. After three days of culture, insulin accumulation in the culture media was measured, and insulin release and glucose oxidation rates of the islets were determined. During both assays, one half of the islets of each group was exposed to exendin-4 (Figure 2).

Alloxan-diabetic mice were transplanted with a suboptimal number of 350 encapsulated islets, which had been cultured with or without exendin-4. Some of the animals receiving exendin-4 cultured islets were intraperitoneally injected with exendin-4 immediately after the surgery and then daily for a period of 14 days. Control mice were injected in the same way but with saline. The efficacy of the transplantation was determined by blood glucose concentration measurements.

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**Figure 2.** Study design for *in vitro* experiments with exendin-4.

**Paper IV**

Transgenic hIAPP+/+ mouse islets were either microencapsulated or kept non-encapsulated under normal culture conditions for one or three weeks. Islets were then fixed for electron microscopy and stained immunohistochemically for IAPP. Encapsulated and non-encapsulated islets cultured for one week were also examined by measurements of stimulated
insulin and IAPP release. Moreover, 50 encapsulated or 150 non-encapsulated human or transgenic mouse islets were transplanted beneath the capsule of the left and right kidney of nude mice. The islet grafts were retrieved after four weeks, prepared for light microscopy and sections were stained with Congo red.

Animals (Papers I-IV)

Inbred male C57BL/6J and BALB/c mice were used as islet donors, whereas male C57BL/6J and spontaneously diabetic female NOD mice, weighing 25-30 g, served as recipients. Recipient C57BL/6J mice were made diabetic by an intravenous alloxan injection (75 mg/kg body weight). Mice with a non-fasting blood glucose concentration higher than 16.7 mmol/l were considered diabetic and used as transplant recipients. For amyloid studies transgenic male and female mIAPP-/-hIAPP+/+ mice were used as islet donors, and normoglycaemic male C57BL/6J nu/nu mice served as recipients.

During the experiments, the animals had free access to tap water and pelleted food. Prior to the glucose tolerance tests, the animals were fasted overnight. The animal experiments were approved by the local animal ethics committee for Uppsala University.

Human islets (Paper IV)

Human islets from eight heart-beating donors were isolated at the Human Islet Isolation Core Facility for the Nordic Countries at Uppsala University and kindly provided by Professor Olle Korsgren.

Islet isolation and culture (Papers I-IV)

Pancreatic islets were isolated using collagenase digestion and density gradient purification. In terminally anaesthetised mice, 2-3 ml cold collagenase solution were injected into the pancreas via the common bile duct. After removal of the pancreas, enzymatic digestion was carried out at 37°C. Density gradient separation was then performed, where pelleted digested tissue was resuspended in Histopaque-1077 solution, and RPMI 1640 was carefully added to form a sharp interface. After centrifugation islets were collected from the interface.

Islets were cultured free-floating in groups of 150 for three days, at 37°C (95% air and 5% CO₂), in culture medium RPMI 1640 supplemented with 1% L-glutamine (200 mM), benzylpenicillin (100 U/ml), streptomycin
(100 μg/ml), and 10% (vol/vol) foetal calf serum and 11 or 5.5 mM D-glucose for mouse and human islets, respectively. The culture medium was changed every second day.

**Microencapsulation (Papers I-IV)**

Non-PLL high G-alginate capsules of approximately 700 μm were formed using an electrostatic bead generator (previously described by A. King et al\textsuperscript{36}) (Figure 3). Islets were mixed with 1.8% (wt/vol) alginate solution containing 67 or 73% guluronic acid and 33 or 27% mannnuronic acid respectively in a syringe and then pushed through a narrow needle (0.35 mm in diameter) by a rate of 4-10 ml/h, into a beaker containing a solution of 50 mM CaCl\textsubscript{2}, 1 mM BaCl\textsubscript{2} and 0.15 M mannitol as an osmolyte. In order to avoid formation of large droplets at the end of the needle, the alginate beads were pulled down by an electrostatic potential between the needle and the solution. The positive electrode was also connected to a vacuum pump that sucked out excessive liquid, to compensate for the increased volume in the lower beaker as the droplets fell, thus keeping the distance between the surface and the needle constant. The positive divalent ions in the lower beaker cross-link the alginate chains, so that a gel network is formed. Cross-linkage was allowed for 5 min before the capsules were washed 4 times in 0.9% NaCl. Approximately 1.5 ml alginate solution was used for each encapsulation (up to 700 islets) in these experiments. Capsules containing islets were hand-picked prior to transplantation.

![Figure 3](image-url)

*Figure 3. Electrostatic bead generator. Islets mixed with alginate solution are pushed through a narrow needle into a solution of positive divalent ions that cross-link the alginate chains into a gel network. The alginate droplets are pulled down by an electrostatic potential between the needle and the solution in the lower beaker. A vacuum pump sucks up excessive liquid to keep the distance between the surface and the needle constant.*
Intraperitoneal transplantation (Papers I-III)

Microencapsulated or non-encapsulated islets were syngeneically or allogeneically transplanted to the peritoneal cavity of isoflurane anaesthetised mice. The inhalation gas contained 2.2-2.3% isoflurane in a mixture of 40% oxygen and 60% nitrogen, and was continuously administered by an isoflurane pump (Univentor 400 Anaesthesia Unit). A small incision was made in the skin and then in the linea alba. The islets or capsules were delivered into the peritoneal cavity using a 1 ml-pipette (Figure 4).

At the end of the experiments the animals were killed by cervical dislocation, the peritoneal cavity injected with 5-6 ml 0.9% NaCl, and the capsules washed out. In general, 60-70% of the transplanted capsules were retrieved.

*Figure 4. Intraperitoneal transplantation of microencapsulated islets.*
Subcapsular transplantation (Paper IV)

For transplantation under the kidney capsule animals were anesthetised by an intraperitoneal injection of 0.02 ml/g body weight avertin (2.5% (vol/vol) solution consisting of 10 g 97% 2.2.2-tribromoethanol in 10 ml 2-methyl-2-butanol. Thereafter, 50 encapsulated or 150 non-encapsulated islets were packed in a braking pipette and implanted beneath the capsule of the left and right kidney, respectively (Figure 5). Grafts were retrieved by removal of the entire kidney after the mouse was killed by cervical dislocation.

Figure 5. Encapsulated (left) and non-encapsulated (right) islets transplanted under the kidney capsule.

In vivo functional tests

Blood glucose measurements (Papers I-III)

Non-fasting blood glucose concentrations of transplanted diabetic mice were measured using a glucose meter (FreeStyle Mini™; Abbot Scandinavia AB) with blood obtained from the snipped tail on day 0, 1, 3, 7 and then once a week throughout the experiments. Mice with blood glucose levels lower than 11.1 mM were considered normoglycaemic. Intraperitoneal and intravenous glucose tolerance tests were carried out on day 28 and 35, respectively (Paper I). The animals were injected with a glucose solution (2 g/kg body weight), and blood glucose concentrations were subsequently measured at 15-30 minutes interval for two hours.
In vitro functional tests

Hormone measurements (Papers I-IV)
Long-term insulin accumulation in the culture media was measured after approximately 40 hours culture (Paper III), whereas short-term rates of baseline and stimulated insulin and IAPP secretion per hour were measured at 1.7 and 16.7 mM glucose. In paper IV a third hour of stimulated insulin and IAPP release at 16.7 mM glucose plus 5 mM arginine was added. After the short-term incubations, insulin and glucagon were extracted from the islets by means of acid ethanol.61 Insulin concentrations in incubation media and homogenates were determined by ELISA (Mercodia). Glucagon concentrations of the islet homogenates were determined by radio-immunoassay (Linco Research, Millipore) (Paper II). IAPP in the incubation media was measured by ELISA (Linco Research, Millipore) (Paper IV).

Glucose oxidation (Papers I and III)
To evaluate islet metabolism, glucose oxidation rates were measured according to a previously described method.61 The radioactivity of the $^{14}$CO$_2$ entrapped in hyamine was measured by liquid scintillation counting. Non-radioactive glucose was added to give final glucose concentrations of 1.7 and 16.7 mM. A higher oxidation rate in high glucose indicates good islet cell viability.

DNA measurements (Paper II)
Islet homogenates were diluted and DNA contents were determined by fluorophotometry (PicoGreen dsDNA Quantitaion Kit; Molecular Probe).

Electron microscopy (Paper II)
Islets were fixed for electron microscopy in a solution of 265 mM glutaraldehyde in a buffer (pH 7.2) containing 36 mM disodium hydrogen phosphate and 14 mM potassium dihydrogen phosphate, and then post-fixed in a buffer containing 40 mM osmium tetroxide.62 The islets were then dehydrated and subsequently embedded in Epon812®. Ultrathin sections were contrasted with uranyl acetate and lead citrate. Electron microscopy was carried out using a H-7100 transmission electron microscope (Hitachi).
Electron microscopy (Paper IV)

Electron microscopical investigations in Paper IV were performed at Linköping University. Islets were fixed for electron microscopy in a solution of 4% paraformaldehyde and 0.5% glutaraldehyde in a buffer (pH 7.4) containing 0.1 M sodium cacodylate and 0.1 M sucrose. Excised islet grafts were then dehydrated and embedded in Epon812®. Sections were immunolabelled with rabbit antiserum to IAPP (A110) visualised with 10 nm protein A-gold labelled secondary antibodies, and contrasted with uranyl acetate and lead citrate. Electron microscopy was carried out using a JEOL 1200 electron microscope.

Light microscopy (Paper II and IV)

Islet grafts were fixed in 4 or 10% formaldehyde, rinsed in graded concentrations of ethanol followed by xylene and subsequently embedded in paraffin and sectioned at 5 μm. For immunohistological staining (Paper II), sections were incubated with primary antibodies against insulin and glucagon overnight in a moist chamber at 4°C. The slides were thereafter incubated for 30 min with a secondary swine anti-rabbit antibody. Rabbit peroxidase anti-peroxidase antibody, was applied to the slides for 30 min, and then developed with 3,3’-diaminobenzidintetrahydrochloride. Islet sections were evaluated in a light microscope by one observer unaware of the origin of the samples. The number of alpha and beta cells in each islet section was counted.

For visualisation of islet amyloid (Paper IV), the grafts were first stained with haematoxylin and then incubated with Congo red solution A (NaCl-saturated 80% ethanol with 0.01% NaOH) for 20 min and thereafter in Congo red solution B (solution A with Congo red) and incubated for 20 min. Islet sections were photographed in a fluorescence microscope at 20x or 40x magnification, and Congo red positive areas were calculated as percentage of total graft area using Image analysis software (ImageJ 1.41, the National Institutes of Health, http://rsb.info.nih.gov/ij/).
Results and Discussion

Effects of islet microencapsulation (Paper I, III and IV)

C57BL/6J mouse islets
The glucose oxidation rate of C57BL/6J mouse islets was not affected by the microencapsulation procedure (Paper III). In Paper I and III microencapsulated islets cultured for three days had slightly lower insulin content when compared to non-encapsulated islets, however the difference was only statistically significant in Paper III. It should be mentioned that the lower insulin content could be due to technical difficulties of extracting all the insulin out of the encapsulated islets. Glucose stimulated insulin release of encapsulated islets after three days of culture was comparable to that of non-encapsulated islets (Paper III).

mIAPP-/-/hIAPP+/+ mouse islets
In Paper IV, where transgenic hIAPP islets were studied, the insulin content of encapsulated islets was about 50% of that of non-encapsulated islets after seven days of culture. Both glucose and arginine stimulated insulin release was lower from the encapsulated islets than from the non-encapsulated islets. In this case it should be mentioned that these transgenic islets contained amyloid deposits that most likely affect the islet function. However, glucose stimulated insulin release as well as insulin content of encapsulated and especially of non-encapsulated transgenic hIAPP islets were higher than observed for C57BL/6J mouse islets.

Intraperitoneal transplantation of encapsulated islets (Paper I and II)
Intraperitoneal transplantation of 700 encapsulated islets effectively lowered blood glucose levels in both syngeneic and allogeneic alloxan-diabetic C57BL/6J mice (Figure 6) as well as spontaneously diabetic NOD mice. All animals were normoglycaemic (<11.1 mM glucose) on day one, and the majority of animals, 70-80%, remained completely normoglycaemic.
throughout the 6 or 12 week study periods in Paper I and Paper II, respectively.

In contrast, intraperitoneal transplantation of non-encapsulated islets had no effect on the serum glucose concentrations (Figure 6). This indicates that the intraperitoneal site is not a very favourable site for islet transplantation.\textsuperscript{41,64}

In the liver or in the renal subcapsular site 200-300 islets are needed to restore normoglycaemia in an alloxan-diabetic mouse,\textsuperscript{46,65} and our group has previously shown that in the peritoneal cavity 1000 non-encapsulated islets are needed to reverse hyperglycaemia.\textsuperscript{41} The reason why so many more islets are needed in the intraperitoneal site is not known. Other groups have also shown better success with encapsulated islets than non-encapsulated islets in this site.\textsuperscript{66} It may, among other things, be attributed to less efficient engraftment in this particular site. Nevertheless, the fact that encapsulated islets do not revascularise at all should be taken to indicate that there are other explanations to why the non-encapsulated islets are so ineffective in this site. The capsule itself may serve as a protection against shear stresses. Moreover, the islets may survive better in the sense that the capsule acts as a supporting matrix, whereas non-encapsulated islets are unsupported in the peritoneal cavity.\textsuperscript{67}

Both intraperitoneal and intravenous glucose tolerance tests of mice grafted with encapsulated islets were as good as for non-diabetic mice and better than for mice transplanted with non-encapsulated islets. This shows that encapsulated islets located in the peritoneal cavity can be reached by and respond to glucose administered directly to the site as well as systemically via intravenous injections.

Figure 6. Blood glucose concentrations of alloxan-diabetic mice transplanted with 700 microencapsulated syngeneic (filled circles), allogeneic (open circles) or non-encapsulated syngeneic islets (filled squares).
Encapsulated islets retrieved after transplantation (Paper I and II)

Encapsulated islets retrieved six weeks after implantation had decreased glucose oxidation rates compared with non-transplanted encapsulated islets. Encapsulated islets harvested from animals transplanted with a lower number of encapsulated islets tended to oxidise even less glucose.

The retrieved encapsulated islets, independently of the number of islets grafted, responded with a five- to 30-fold increase in insulin secretion when stimulated with glucose. In Paper I, the stimulated insulin release rates were similar to those of non-transplanted encapsulated islets. However, the insulin content of islets retrieved from the low number grafted islet recipients was lower than in islets retrieved from the high number grafted islet recipients. These encapsulated islets were in a hyperglycaemic environment, and were exposed to metabolic stress. Thus, the low insulin content could at least partly be due to their extensive degranulation. One more reason might be that there is a decrease in beta cell mass in each individual islet, as both beta cell number, total number of cells per islet cross section, and islet DNA content were decreased to about 50%. This correlates well with what has previously been estimated in whole grafts of non-encapsulated islets.\textsuperscript{11-13} However, the number of beta cells was not affected by the metabolic stress imposed on the islets. Thus, in normoglycaemic mice, the number of beta cells per islet cross section was the same as in islets implanted into a diabetic mouse. In addition, whether the diabetes was spontaneous (in the NOD mouse) or chemically induced with alloxan did not influence the number of beta cells per islet after transplantation.

Surprisingly, despite a reduction in total islet mass the number of alpha cells was increased (Figure 7) and the glucagon content of individual islets remained unchanged, indicating that these cells were not detrimentally affected by the implantation. These findings contradict to some extent studies using non-encapsulated islets where alpha cell numbers diminish after islet implantation.\textsuperscript{13,18,20,21} It could be speculated that the capsule protects these peripherally located and otherwise exposed cells from the cells of the transplantation site.\textsuperscript{23} Electron microscopy revealed good alpha cell viability with well-granulated cells, in contrast to the degranulated beta cells.
Figure 7. Immunohistochemical staining of microencapsulated islets before (left) and 12 weeks after (right) transplantation into alloxan-diabetic mice. Alpha cells are visualised (brown) by staining with antibodies against glucagon.

Cultured vs. freshly isolated encapsulated islets (Paper I)

As we were able to cure more or less all animals with implantation of 700 encapsulated islets, it was unclear whether there was a difference between non-cultured and cultured islets. We therefore chose to transplant 350 encapsulated islets, in the hope that if not all animals were cured we should be able to detect differences between the groups. Indeed, 350 encapsulated islets worked well as a minimal mass model, with most mice not becoming normoglycaemic, with average blood glucose levels between 12 and 20 mM. Despite this, no differences between the cultured and non-cultured islets could be discerned. Moreover, in both the intravenous and the intraperitoneal glucose challenges, cultured and non-cultured encapsulated islets had similar effects on the blood glucose levels. In general, these mice had lower serum glucose than recipients of non-encapsulated islets. Also, in the in vitro studies performed before and after transplantation, freshly isolated and cultured encapsulated islets behaved similarly. One reason for the previously shown superiority of non-encapsulated fresh islets for transplantation outcome might be that they more easily become revascularised.\textsuperscript{9,46,57} When we in our study used the microcapsule to eliminate the advantage that fresh islets would have had through higher vascular density, they did not function better than cultured islets. These results speak in favour of the idea that indeed revascularisation may play an important role for transplantation of non-encapsulated islets. On the other hand, the fact that encapsulated islets were more effective in lowering blood glucose than non-encapsulated islets when transplanted into the peritoneal cavity, may be taken to indicate that
revascularisation in general is not of that ultimate importance for transplanted islet function as hitherto has been believed.

Exendin-4 treated encapsulated islets (Paper III)

Both in the case of encapsulated and non-encapsulated islets, insulin accumulation in the culture media was increased twofold by the presence of exendin-4. Moreover, exendin-4 increased glucose induced insulin release both acutely and long-term. The fact that this was seen to the same extent for both encapsulated and non-encapsulated islets indicates that exendin-4 can easily pass through the capsule. Despite increased insulin secretion in the exendin-4 treated islets, insulin content was unchanged.

Presence of exendin-4 in the media during the three days of culture increased the glucose oxidation rate in the encapsulated islets, but did not affect that of the non-encapsulated islets. However, no further acute effects on glucose oxidation rates were observed when exendin-4 was added to the short-term incubation media. This most probably reflects an indirect effect due to increased beta cell survival, rather than an effect on glucose oxidation per se.

Animals transplanted with a suboptimal graft of exendin-4 treated or control islets had decreased blood glucose levels initially. However, most animals were not able to maintain the normoglycaemia. Mice transplanted with exendin-4 pre-cultured encapsulated islets had lower blood glucose levels than the control mice on day three after transplantation. At all other time points tested their blood glucose levels did not differ from those of the control mice. Exendin-4 treatment of the recipients by daily intraperitoneal injections had no further beneficial effect, which is in line with a previous study. On the other hand it was envisaged that exendin-4 might have been more effective in the present study as the microencapsulated islets were located in the peritoneal cavity, the site of the exendin-4 injections.

Although treatment of encapsulated islets in culture had positive effects on islet function in vitro, the effect after transplantation lasted less than seven days. Taken together, these results suggest that graft failure is most likely due to an inability of the encapsulated islets to compensate for the increased insulin demand. Indeed, it is possible that the islets have been stimulated too much in relation to their ability to synthesise insulin.
Islet amyloid formation in encapsulated islets
(Paper IV)

Electron microscopy of encapsulated and non-encapsulated transgenic hIAPP mouse islets revealed obvious signs of early amyloid formation after one and three weeks of culture. There were mostly intragranular fibrils and dissolved insulin cores. The encapsulated islets were most affected, and the formation was increased with increased culture time. As mentioned above, after one week of culture both insulin content and insulin release, stimulated by arginine and/or glucose, were lower in the encapsulated islets compared to the non-encapsulated islets, as were hIAPP content and release.

After transplantation under the kidney capsule both intra- and extracellular areas of amyloid were seen in microencapsulated as well as in non-encapsulated islet grafts. However, the signs of amyloid were more frequent in the encapsulated grafts (Figure 8). Moreover, the total area of amyloid relative to the total area of the islet graft was higher in the encapsulated islet grafts. This was seen in both human and transgenic mouse islets.

Since transplanted microencapsulated islets exemplify a non-vascularised transplantation model these results indicate that poor revascularisation of transplanted islets leads to impaired clearance of insulin and IAPP, which may be an important reason for the increased amyloid formation in transplanted islets. However, presently we cannot exclude the possibility that encapsulation per se is important in this respect as well.

Figure 8. Islet amyloid in a transplanted microencapsulated human islet. Beta cell with intracellular amyloid in an electron micrograph (left) and Congo red stained islet section in fluorescent light microscope (right).
Conclusions

- Encapsulated islets can effectively reverse hyperglycaemia in chemically induced as well as spontaneously diabetic mice after transplantation to the intraperitoneal cavity, in fact more efficiently than non-encapsulated islets.

- A period of culture prior to encapsulation does not affect the transplantation outcome.

- After long-term implantation, encapsulated islets have reduced beta cell mass and insulin content and, although each individual islet has a 50% reduction in total mass, alpha cells are increased in number.

- Treatment of encapsulated islets with exendin-4 in culture has got positive effects in vitro, both chronically and acutely, however pre-treatment before transplantation into diabetic mice, only has a short transient effect on blood glucose homeostasis.

- Amyloid formation is accelerated in encapsulated transplanted human and transgenic hIAPP mouse islets compared to that in revascularised non-encapsulated islet grafts.
Typ-1-diabetes är en autoimmun sjukdom som gör att kontrollen över kroppens blodsockernivå förloras på grund av att de insulinproducerande beta-cellerna förstörs. Genom transplantation av nya beta-cellsklusters, så kallade Langerhanska öar, kan kontrollen över blodsockernivån återställas. Genom att kapsla in dessa öar i alginat innan de transplanteras till patienten kan öarna skyddas från avstötning. Detta innebär att patienten kan slippa de annars nödvändiga immunosuppressiva läkemedlen och deras allvarliga biverkningar. Några av de viktigaste uppgifterna inom ötransplantationsforskningen i dag är att förbättra öarnas funktion och att förlänga deras livslångd i mottagaren.

I våra studier fann vi att transplantation av 700 enkapsulerade öar, både syngena och allogena, var tillräckligt för att bota diabetiska möss. Samma antal icke-enkapsulerade öar hade ingen effekt alls på blodsockernivåerna.

Tidigare har det visats att transplantationsresultatet vid användning av icke-enkapsulerade öar kan förbättras genom att använda färsk akeleje öar istället för odlade öar. Vi fann dock att färsk akeleje och odlade öar hade likvärdig insulinfrisättning och botande förmåga då de var enkapsulerade. Trots att dessa öar botade de diabetiska mottagarna, var insulinfrisättningen och insulininnehållet försämrat i varje enskild ö efter en tids implantation. Vi fann även att varje enkapsulerad ö förlorade ca 50 % av det totala antalet celler efter transplantationen. Den celltyp som minskade i antal var beta-cellerna medan antalet glukagonproducerande alpha-celler faktiskt ökade.

Genom att förbehandla enkapsulerade öar med exendin-4, som är en så kallad GLP-1-recepteragonist med proliferativa och anti-apoptotiska effekter på beta-cellerna, ökades insulinfrisättningen hos öarna in vitro, både akut och efter en tids odling. Efter transplantation av exendin-4-behandlade öar varade dock effekten i mindre än sju dagar.

Bildning av amyloid från Islet Amyloid Poly Peptide (IAPP) är ett fenomen som ursprungligen satts i samband med typ-2-diabetes. Nyligen har amyloid även hittats i transplanterade öar och har föreslagits vara en orsak till att dessa öar inte överlever längre än några år i mottagaren. Orsaken till amyloiddödsförekomst i de transplanterade öarna är ännu oklart. Något som skiljer transplanterade öar från endogena öar är att de har en lägre genomblödningsfrekvens. I de enkapsulerade öarna, där genomblödningsfrekvensen är ofintlig, kunde vi se en betydande ökning av amyloiddödsförekomst i jämförelse med icke-enkapsulerade
öar. En tänkbar förklaring till det är att avsaknaden av genomblödning orsakar en störd balans av IAPP- och insulinkoncentrationer lokalt inne i ön.

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References


A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)