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Poloxamer 188 as a Supplement to Barium Cross-Linked Ultra-High Viscosity Alginate for Immunoisolation of Transplanted Islet Cells

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Abstract

Transplantation of Langerhans islets is a potential cure for diabetes mellitus. The main problem for routine clinical use remains the prevention of rejection without drastic side effects. Immuno-isolation is an experimental strategy to prevent graft rejection by separating the transplanted cells from the host immune system using a barrier device. The aim of the current study was to improve the physical features of encapsulated islets in a barium cross-linked ultra-high viscosity alginate by adding Poloxamer 188 (P188). Empty alginate capsules, and especially encapsulated islets, could be easily generated using UHV-P188 alginate because of its anti-foaming properties. Diabetic mice were used for evaluation of biocompatibility and graft function. Biocompatibility testing with empty capsules showed no inflammatory reaction or fibrotic overgrowth. The capsules remained intact in the intraperitoneal and intramuscular implant sites over a period of 4 weeks. Transplantation of encapsulated islets, however, led to a strong systemic inflammatory response with fibrotic overgrowth of the islet-containing capsules but no graft failure. This finding likely reflected the complement-activating property of P188. Our results clearly showed that the complex interaction of additives, xenogeneic tissue, and alginate with the host immune system could not be predicted by the behavior of the individual components. Furthermore, the mouse model described herein was an excellent tool to evaluate the physico-chemical properties and the in vivo biocompatibility and functionality of various additives. Our results will improve the biomaterials used for alginate microbeads in a clinical setting in the future.

Keywords: Microencapsulation; Islets of langerhans; Diabetes mellitus; Xenogeneic transplantation; Alginate; Islet transplantation; Poloxamer 188

Abbreviations: IEq: Islet Equivalents; P188: Poloxamer 188; GSIS: Glucose-Stimulated Insulin Secretion; Stz: Streptozotocin; M: D-Mannuronic Acid; G: L-Guluronic Acid; UHV: Ultra High Viscosity Alginates

Introduction

We have been investigating rat pancreatic xenografts as a potential source of functional islet tissue for transplantation using a mouse model. The goal of this work was to develop a therapeutic strategy to overcome the shortage of donor organs for the treatment of diabetes mellitus. To prevent strong humoral and cellular immune responses to the xenografts, we tested alginate microcapsules as an immunoisolation device. Immunoisolation technology is based on the principle that the artificial membrane protects foreign cells from the host immune system while simultaneously allowing unopposed transfer of nutrients, oxygen, and therapeutic factors [1].

According to our previously published results [2], the use of ultra-high viscosity (UHV) alginate for microencapsulation provided long-term functional rat and human islets after intraperitoneal xenotransplantation into immunocompetent diabetic mice, without the need for immunosuppression.

The composition and sequential structure of the alginate are of great importance when used as an encapsulating material. Alginate is usually isolated from marine brown algae, and its composition varies widely depending on the subspecies and growth location [3]. Depending on the nature of the cross-linking cation, the length of the polymeric chains, the mannuronic acid:guluronic acid (M:G) ratio, and the percentage of block structures, hydrogels of varying mechanical strength, elasticity, and swelling characteristics can be produced [4]. Alginates with a high content of guluronic acid block polymers produce more solid and less elastic gels with high permeability compared to alginates with a high content of mannuronic acid [5]. Therefore, the M:G ratio, length of polymeric chains, and ratio of homologous to heterologous chains must be carefully tuned to optimize the microcapsules [6].

A lack of standardization and information from manufacturers about the composition of the alginate (e.g., molecular weight, content, and ratio of D-mannuronic acid and L-guluronic acid) is a major problem for encapsulation experiments; this knowledge is of enormous importance for understanding the physico-chemical characteristics and hence biocompatibility of the alginate. Ba^{2+}-gelled capsules comprised of 1:1 mixtures of UHV alginates from the closely related species *Lessonia nigrescens* and *Lessonia trabeculata* meet the demands of high stability and flexibility (UHV: 35% M and 65% G) [7].

The aims of the current study were 1) to improve the physical features of barium cross-linked UHV alginate by the addition of Poloxamer 188 (P188) as a detergent, and 2) to establish the muscle as a novel transplantation site.

Poloxamer is comprised of ABA-type block polymers that consist of a central, hydrophobic block of polypropylene oxide that is edged by two hydrophilic blocks of polyethylene oxide. Poloxamer 188 (P188),

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also called Luterol® F68, has an average molecular weight of ~ 8600 Da. The polyoxyethylene units represent approximately 81% of the compound [8]. P188 has diverse applications in various biomedical fields ranging from drug delivery, gene transfer, tissue engineering [9], and vaccine adjuvanting [10] to medical imaging for the management of vascular diseases and disorders, and cancer therapy. We chose P188 as a supplement for the alginate because of its lack of toxicity and its non-thrombogenic properties [11,12].

Many transplantation sites for islets in animal models have been reported (e.g., kidney [13], peritoneum [2], omentum [14], muscle [15], testis [16], and bone marrow [17]); however, in humans, the liver remains the only transplantation site that is used in a clinical setting. Due to the comparatively large size of alginate-encapsulated islets, which significantly increases the risk for portal thrombosis, the liver is not a suitable transplantation site for microencapsulated islet cells. As an alternative site, the muscle is a preferred location because it is highly vascularized and easily accessible if graft removal becomes necessary [18]. Successful auto-transplantation of parathyroid glands [19] and islets [20] into the forearm has been reported. However, for xenotransplantation of encapsulated islets, the requirements for a mechanically stable and highly biocompatible encapsulation material are significantly higher for implantation into muscle compared to subcutaneous tissue or the peritoneum due to problems with mechanical sheer forces. Therefore, we investigated the muscle as a transplantation site for UHV-P188 alginate-encapsulated islets to increase the stability and biocompatibility of the capsules.

Materials and Method

Islet cell isolation and viability assessment

Islets of Langerhans were isolated from healthy Wistar rats by a collagenase digestion method. In brief, 10 ml Collagenase solution containing 20 PZ-U NB1, 1U NP and 1 mg AEBSF (Serva, Germany) were slowly injected into the common bile duct after occlusion of the distal end just proximal to the duodenum. The distended pancreas was excised and the digestion was performed in a waterbath at 37°C for 20 min. The tissues were incubated with UW solution for 30 min. Islet purification was achieved using a three-step, discontinuous density gradient (Biochrom, Germany). Islets were collected from the interface between the 1•077 and 1•038 g/ml layer. All animal procedures were approved by our institution's Ethics Committee (Landesuntersuchungsamt Rheinland-Pfalz) and carried out under license (no. G 12-1-068), in accordance with the Ethical Committee for Animal Research at the University Medical Center, Mainz, Germany. After purification, islets were recovered overnight at 37°C and 5% CO_2 in TCM 199 (Biochrom) supplemented with 1% Penicillin /Streptomycin (Invitrogen) and 10% FCS (Seromed Biochrom KG). Single cells and small cell aggregates were removed from the islet suspension by filtration through a 40µm Nylon Cell Strainer (BD Falcon). The volume of isolated islet was expressed as the number of islet equivalents (IEq), defined as an islet 150 µm in diameter. The Ricordi algorithm was used to convert islet number into IEq [21].

Viability of islets was assessed using fluorescein diacetate (FDA) (Sigma-Aldrich, St. Louis, MO, USA) and PI (Sigma-Aldrich) staining. Briefly, islets in the inserts were transferred in PBS. 8 µl FDA (5 mg/ml in acetone) and 50 µl PI (0.5 mg/ml in PBS) stock solutions were added to the sample. After 30 s of adding the stain islets were placed on the fluorescent microscope, visualized and photographed using the filter FITC for fluorescein (em ~ 530 nm) and texas red (em >615 nm). Dead cells were stained red and viable cells were stained green. For computer-based viability determination of multicellular systems we used a method based on colour intensity of the live-dead stain as previously proposed and used for pancreatic islets [17]. The software used was ImageJ 1.34 s (NIH, USA). The pictures were split into three colour channels: red, green and blue. The islets were manually marked in both, the green and the red channel, and the mean intensity (of each channel) of the islet area was automatically given. Viability was calculated as followed: Viability [%] =living cells/ (living+dead cells) * 100%

Alginat droplet generation and islet encapsulation

Production of UHV-alginates was performed as described previously [22]. UHV alginates of clinical grade were used for all islet encapsulations (viscosity of a 0.1% wt/vol solution in distilled water was 20–30 mPa·s). 0.017% Poloxamer (BASF) was added to generate UHV-P188 alginate. For microcapsule formation, the alginate was dissolved in sterile, endotoxin-free 0.9% NaCl solution at a concentration of 0.65% (wt/vol). The islets were mixed carefully with the alginate in a concentration of 8000 IEq/ml alginate solution. Alginate droplets were produced by an air-jet droplet generator and cross-linked using the crystal gun method with external and internal Ba^{2+} as described previously by Zimmermann et al. [18]. Briefly, air pressure was used to drive the dry, sterile $BaCl_2$ crystals into the alginate droplets before they reached the cross-linking solution, leading to internal gelling. External gelling occurred when the droplets came into contact with a 20 mM $BaCl_2$ solution. The osmolality of the cross-linking solution was adjusted to 290 mOsm using appropriate amounts of NaCl. After 20 minutes, the alginate/islet capsules were washed three times with 0.9% NaCl solution.

In vivo biocompatibility of microcapsules

To assess the biocompatibility of empty alginate microcapsules, the droplets were implanted by intraperitoneal or intramuscular injection into healthy BALB/c mice or NOD mice. Microcapsules were counted, measured by size and by volume before transplanted under sterile conditions. Control animals received the same volume of 0.9% NaCl solution. Grafts were retrieved 4 weeks after transplantation.

Intraperitoneal grafts were explanted by repeated flushes with warm 0.9% NaCl solution containing 20 mM $BaCl_2$. Intramuscular grafts were surgically removed. The microcapsules were removed and the percentage of recovered capsules (defined as the ratio to the volume of capsules before transplantation to that after removal) and the degree of capsular overgrowth, defined as the number of capsules presenting adherent cells on their surface per 100 capsules, were determined. Images were made with a digital camera and stored as jpg files.

In vivo test of graft function

Transplantation experiments were carried out with healthy, 5-6-week-old BALB/c mice or spontaneously diabetic NOD mice. BALB/c mice were rendered diabetic with intraperitoneal injections of streptozotocin (stz 250 mg/kg body wt; Sigma, St. Louis, MO, USA) freshly dissolved in citrate buffer 2 weeks before islet transplantation. Only animals exhibiting blood glucose higher than 350 mg/dl in two consecutive measurements were considered diabetic and were used as recipients. German guidelines for the care and use of experimental animals were strictly followed throughout the study. Each mouse received 2000 microencapsulated IEq. Transplants were made in a class 100 biological safety cabinet under sterile conditions. Islets were injected into the peritoneal cavity with a 20-gauge needle (Vasofix; Braun, Melsungen, Germany). Non fasting blood glucose level of transplanted mice was monitored every 3 days, later weekly. FreeStyle

mini (Abbott, Germany) was used for blood glucose determination. Grafts were removed after graft failure or poor general condition of the animals. Stz control mice received every 3-4 days subcutaneous administration of insulin (Novo Nordisk Levemir®, 3-4 international units/injektion).

Explanted microcapsules were counted, measured by size and by volume as well as evaluated for fibrotic overgrowth using an IX70 microscope (Olympus). Explanted encapsulated islets were fixed in paraffin blocks.

Assessment of insulin secretion

The static incubation assay was used to assess the insulin secretion response of islets after a glucose challenge, as previously described [23]. 50 IEq were transferred into a culture insert (membrane pore diameter 12 µm; Millicell PCF, Millipore, France) and put into a well of a 24-well culture-plate (Falcon Multiwell; Becton, Dickinson). Six wells were prepared for each experimental group. Basal insulin secretion was measured after 60 min of incubation at 37°C in Medium199 +10% FCS. The inserts with islets were transferred into hyperglycemic culture medium (Medium199 + 10% FCS + 200 mg/dl D-glucose) for additional 60 minutes. The supernatant was frozen at -21° until determining the insulin concentration using sandwich ELISA. ELISA-plates (high binding; Greiner) were coated with 1µg/ml mouse mAb to insulin (abcam, Cambridgeshire, UK) diluted in 0.1 M bicarbonate buffer, pH 9.6. Plates were incubated overnight at 4°C. After incubation, plates were washed four times with PBS containing 0.05% Tween 20 and blocked for 2 h at 37°C by adding 50 µl of blocking solution (Candor, Germany) to each well. Thawed supernatants and insulin standards (Sigma, St. Louis, MO, USA) were added at a volume of 35 µl/well (1:3,5 in PBS). Plates were incubated overnight at 4°C then washed. 0.15 µg/ml of secondary HRP conjugated mouse mAb to insulin (abcam, Cambridgeshire, UK) diluted in low cross binding buffer (Candor, Germany) was added at 25 µl/well. Plates were incubated for 2 h at room temperature. After washing, plates were developed by adding 25 µl/well of TMB (Sigma, St. Louis, MO, USA). The reaction was stopped by adding 25 µl/well of 1 N H_2SO_4.The extinction was measured in a ELX800G (Bio-Tek Instruments, Winooski, USA) equipped with KC4 software using a reference wavelength of 630 nm and a test wavelength of 450 nm.

Immunhistochemistry

In brief, serial sections (5 µm) were cut from 5% formaldehyde-fixed paraffin-embedded pancreas or retrieved graft tissue. Serial sections (5 µm) were deparaffinized, rehydrated, and subjected to antigen unmasking with citrate buffer (pH 6.0).

Sections were treated with 1 % bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 60 min to block nonspecific antibody binding. The primary antibody (chicken polyclonal anti-insulin antibody (1:100; abcam, Cambridgeshire, UK) was diluted with PBS containing 1% BSA and applied to the sections. Rabbit anti chicken HRP (abcam, Cambridgeshire, UK) was used as secondary antibody, diluted 1:200 in PBS and incubated for 60 min. Insulin was identified with 3,3′ diaminobenzidine tetrachloride (H-2200, DAB, Daco, Germany) and sections were counterstained with Mayer hematoxylin. Digital images of the stained slices were obtained using Olympus IX70 microscope.

Statistics

All experiments were repeated a minimum of three times. Data are reported as means ± S.D. All statistical analyses were performed using SPSS 12.0 (SPSS Inc., Chicago, IL, USA). Significant differences were identified using Levene´s Test for equality of variances and independent samples t-test. In all tests, the significance level was set at 5% ($P<0.05$).

Results

In vitro evaluation of UHV-P188 alginate-microencapsulated islets

Before transplantation of the encapsulated islets, their insulin secretory capacity was determined after 1 day of culture. As shown in Figure 1, encapsulated rat islets responded well to a high-glucose stimulus. There was no significant difference in insulin secretion and release into the medium between the non-encapsulated islets and the microencapsulated islets after glucose challenge.

Biocompatibility of empty alginate capsules

The biocompatibility of pure UHV-P188 alginate droplets was tested by intramuscular (im) and intraperitoneal (ip) transplantation into healthy BALB/c and diabetic NOD mice. Five weeks after transplantation, the retrieved empty alginate droplets did not exhibit any cellular overgrowth on their surfaces (Figure 2).

Graft function *in vivo*

Graft function was tested in diabetic mice. Specifically, diabetes was induced chemically (streptozotocin, stz) in BALB/c mice; diabetes developed spontaneously in NOD mice. Non-fasting blood glucose levels in the transplanted stz diabetic BALB/c mice and spontaneously diabetic NOD mice were monitored. The mice were transplanted ip or im with microencapsulated islets. Normoglycemic blood glucose levels were generally reached within 24 h of transplantation and lasted up to 15 days in the NOD mice and >70 days in the BALB/c mice (Figure 3 a and 3b).

Despite normoglycemia, the general condition of the transplanted BALB/c mice progressively deteriorated, as shown by weight loss and dramatic leukocytosis, so that the animals had to be sacrificed on day 76. Approximately 25% of the explanted encapsulated islets showed mild to moderate cellular overgrowth (Figure 3 c and 3d). When the transplantation experiment was repeated, the same deterioration was observed and the experiment had to be stopped, despite functional islet grafts.

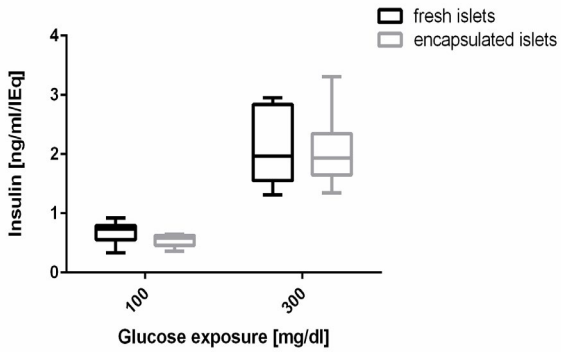

Figure1: In vitro insulin-release from fresh and microencapsulated islets
A static incubation assay was performed with fresh isolated islets and islets microencapsulated in UHV-P188 alginate. Insulin secretion was measured in supernatants using Insulin ELISA. Results are representative of three separate cell isolation and encapsulation experiments. * marks an outlier.

Recovery and examination of the explanted grafts

All microencapsulated islets retrieved from NOD mice 25 days after transplantation showed massive cellular attachment on their surfaces (Figure 3e and 3f). There was no transplantation site inflammatory reaction observed in either set of mice. Immunohistochemical staining of pancreatic tissues from stz diabetic mice confirmed the loss of beta cell mass (Figure 4a and 4b). Microencapsulated islet grafts retrieved 76 days post-transplantation were positive for insulin by immunohistochemistry, which verified the presence of viable beta cells (Figure 4c). The loss of beta cell mass within the capsule is due to the massive fibrotic cellular attachment.

Discussion

The success of alginate microbeads as an immunoisolation device depends largely on the bio-physico-chemical properties of the microbeads [24]. In the present study, we investigated P188 as an alginate supplement to improve alginate capsule biocompatibility and graft function.

We were able to demonstrate stable physico-chemical properties of the alginate capsules *in vitro*. The alginate capsules had high viability and retained regulated insulin secretory capacity and very good biocompatibility *in vivo* without any fibroblast overgrowth reaction after the transplantation of empty capsules. However, we found that the addition of P188 resulted in an unexpected immunogenic reaction when encapsulated islets were transplanted intraperitoneally or intramuscularly. These results suggested that P188 in the presence of xenogeneic tissue behaved as an immunogenic adjuvant.

A series of poloxamers has been tested for adjuvant activity using a standard oil-in-water emulsion, with egg albumin as the antigen [20].

Adjuvant activity studies of copolymers showed that antibody titers generally increased with increasing molecular weight of the hydrophobic poly(oxypropylene)amine salts (POP), along with decreasing content of hydrophilic polyoxyethylene (POE). Pure polymers of POP, however, were not as effective as adjuvants [12]. P188 does have a relatively high molecular weight (~8400 Da) and a high hydrophilic POE content of 35% [11]. Moghimi et al. [9] described the ability of P188 to activate the alternative complement pathway. Based on these results, the use of a higher molecular weight poloxamer as an alginate additive for microencapsulation of islet cells warrants further study. A promising candidate is Poloxamer 407, which has been shown to prevent nonspecific binding of IgG to the solid surface and phagocytosis by neutrophils [25].

The peritoneum is a classic site for the transplantation of microcapsules because a large amount of capsules can be easily injected. However, the peritoneum provides an ideal environment for inflammatory and immunological reactions [26]. Peritoneal mesothelial cells facilitate the induction and promotion of a strong innate immune reaction to antigen. Recently, studies of encapsulated pig islets transplanted into mice reported that the biocompatibility was improved and the survival of encapsulated pig islet transplants was prolonged by the inhibition of macrophage and lymphocyte stimulation [27]. Therefore, we attempted to evaluate a site that would be less immunoreactive for implantation of islet xenograft microcapsules, and that would also avoid the need for immunosuppression. Liver and spleen were excluded directly as graft sites because the transplantation of capsules having a diameter of ~ 500 µm greatly increases the risk for thrombosis. Therefore, intramuscular transplantation of encapsulated islets offers an attractive alternative, based on its simplicity and ease of access for noninvasive graft imaging and cell explantation.

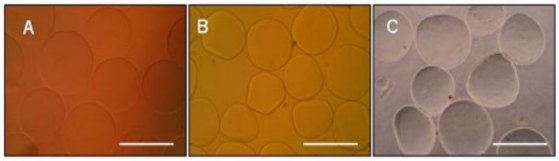

Figure2: Representative images of fresh (A) and explanted (B-C) UHV-P188 alginate capsules without encapsulated islets of Langerhans
Light microscopy of Ba2+ cross-linked UHV-P188 alginate capsules recovered 6 weeks after intraperitoneal implantation (B) or intramuscular implantation (C) into BALB/c and NOD mice. Bar scale = 500 µm.

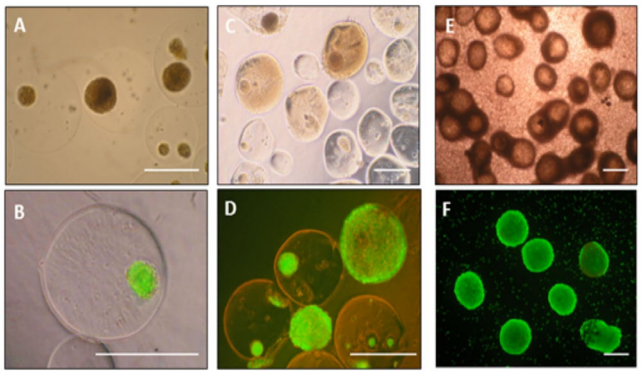

Figure 3: Representative images of fresh and retrieved microencapsulated islets
Light microscopic (A,C,E) and fluorescent microscopic images (B,D,F) of microencapsulated islets. Pictures were taken after encapsulation (A,B), at 76 days after transplantation into BALB/c mice (C,D), or at 20 days after transplantation into NOD mice (E,F). Viability staining with fluorescein diacetate (FDA; green=viable) and propidium iodide (PI; red=dead) of encapsulated islets. Bar scale- 500µm.

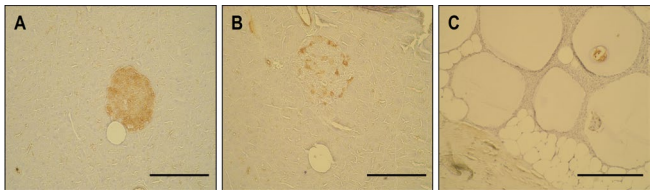

Figure 4: Representative images of immunohistochemical staining of pancreatic islets for insulin expression
(A) Pancreatic sections from a healthy BALB/c mouse. (B) Pancreatic section from an stz diabetic BALB/c mouse with a functioning graft on day 76 post-transplantation. (C) Section from an im-transplanted microencapsulated islet graft on day 76 post-transplantation.
Bar scale (A,B) 200 µm, (C) 500 µm.

Alginate microbeads have been investigated clinically for a number of therapeutic interventions, including drug delivery, cell delivery, and cell encapsulation [28]. Studies have shown promising results in these applications; however, clinical success requires the ability to control and predict solute transport in the alginate matrix, as well as possible interactions between the delivered drug/tissue, the alginate, and the host system. Our results clearly showed that the biocompatibility of such a complex system cannot be predicted by the behavior of its individual components; the complex interactions, especially in the setting of transplanted, immunoseparated xenogeneic tissue that has been encapsulated, should be tested *in vivo*. The mouse

model of xenotransplantation of islets encapsulated with our highly defined UHV alginate was excellent for the *in vivo* study of the physico-chemical properties of the alginate microbeads. The model also was ideal for testing the *in vivo* biocompatibility and functionality of the islet cell graft.

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