In Vivo Selection of Biocompatible Alginates for Islet Encapsulation and Subcutaneous Transplantation

Sophie Vériter, Ph.D. (cand.),[1] Julien Mergen, M.D. (cand.),[1] Rose-Marie Goebbels, B.Sc.,[1]
Najima Aouassar, B.Sc.,[1] Charles Grégoire, M.D. (cand.),[1] Bénédicte Jordan, Ph.D.,[2]
Philippe Levêque, Ph.D.,[2] Bernard Gallez, Ph.D.,[2] Pierre Gianello, M.D., Ph.D.,[1] and Denis Dufrane, M.D., Ph.D.[1]

Islet encapsulation requires several properties including (1) biocompatibility, (2) immunoprotection, and (3) oxygen diffusion for islet survival and diabetes correction. New chemical alginates were tested *in vivo* and compared with traditional high-mannuronate and -guluronate alginates. New alginates with coupled peptide sequence (sterile lyophilized high mannuronate [SLM]-RGD3% and sterile lyophilized high guluronate [SLG]-RGD3%), to improve encapsulated cell adherence in the matrix, and alginates with a very low viscosity (VLDM7% and VLDG7%), to reduce implant size by loading a higher number of islets per volume of polymer, were implanted subcutaneously in 70 Wistar rats for comparison with alginates of high viscosity and high content of mannuronic (SLM3%) or guluronic acids (SLG3%). Permeability of alginates to 36-, 75-, and 150-kDa lectins coupled to fluorescein isothiocynate was quantified before implantation and at 2, 4, and 12 weeks after implantation. Biocompatibility (fibrosis, graft stability, immunologic infiltration by CD3/CD68 cells, and neovascularization) was assessed at each explantation time. Permeability to small molecules was found for all alginates. Impermeability to 150-kDa molecules, such as IgG, was observed only for SLM3% before implantation and was maintained up to 12 weeks after implantation. SLM3% and SLG3% demonstrated better graft stability with lower CD3/CD68 recruitment and fibrosis than the other alginates. SLM3% induced a significantly higher angiogenesis and maintained oxygen pressure at ∼40 mm Hg for up to 4 weeks after implantation as measured by *in vivo* electronic paramagnetic resonance oximetry. SLM-encapsulated pig islets implanted subcutaneously in rats demonstrated no inflammatory/immunologic reactions and islets functioned for up to 60 days without immunosuppression. A traditional alginate made of high mannuronic content (SLM3%) is an adapted material to immunoprotect islets in subcutaneous tissue. No improvement was found with lower viscosity and use of GRGDSP-peptide sequence.

Introduction

ISLET TRANSPLANTATION IMPROVES the quality of life and prognosis for selected patients with type 1 diabetes mellitus (T1DM).[1] However, despite increasing efficacy rates (44–66% insulin independence at 1 year),[2–4] islet transplantation remains limited by side effects related to continuous immunosuppression (tacrolimus, sirolimus, and daclizumab), such as nephrotoxicity, hypertension, carcinogenicity, and hypersensitivity to infections, among others.[1,5,6] An attractive alternative to immunosuppressive drugs is cell immunoisolation by encapsulation in a semipermeable matrix to protect transplanted tissues against immune cells from the recipient as well as against antibodies (autoimmunity of T1DM, ABO/human leukocyte antigen incompatibility, and preformed antibodies against α-Gal and other antigens in xenotransplantation).

Macroencapsulation and microencapsulation systems have been proposed for cell immunoisolation.[7–13] A lack of biocompatibility, with a nonspecific reaction of fibrosis resulting in hypoxia and necrosis of encapsulated cells, is often designated as the major cause of graft failure.[7,8,14–18] Nonselective permeability (cytokines and antibodies), implant degradation, and limitation of nutrient diffusion are also reported as major causes of encapsulated islet dysfunction.[19] Although several materials have been assessed (agarose, chitosan, copolymers of acetonitrile, AN69, poly(2-hydroxyethyl methacrylate), polyurethane, monomethoxy poly(ethylene glycol), and Biodritin),[20–26] alginate is currently the main material used in the field of islet transplantation to provide immunoisolation to encapsulated cells.[27–31] This material, extracted from brown alga, is a polysaccharide composed of subunits of mannuronic (M) and guluronic (G)

---

[1]Laboratory of Experimental Surgery and [2]Biomedical Magnetic Resonance Unit, Université Catholique de Louvain, Faculté de Médecine, Brussels, Belgium.

acids. The M/G ratio directly affects physical and biocompatible properties of implants.

High-G alginates are more stable and therefore more resistant to mechanical stresses than high-M alginates after implantation.[7] In contrast, a smaller pore size, found in high-M alginates,[32] can promote selective permeability for small molecules, avoiding immunoglobulins and immune cells.

Alginates of high viscosity and high content of mannuronic (sterile lyophilized high-mannuronate [SLM]) or guluronic acids (sterile lyophilized high guluronate [SLG]) are most commonly reported in the literature. In this study, new alginates with coupled peptide sequence (glycine-arginine-glycine-aspartic acid-serine-proline [GRGDSP] [Novatach M RGD/Novatach G RGD]) were assessed to improve encapsulated cell adherence in the matrix.[33] Alginates with a very low viscosity (VLDM and VLDG) were also tested to reduce implant size by loading a higher number of islets per volume of polymer. The content in M and G acids as well as alginate viscosity and the use of peptide sequences[34] may influence biocompatibility.[8,35]

Therefore, there is a need to select an encapsulation material that possesses ideal biocompatible properties for islet encapsulation such as (1) stability during the graft process, (2) immunologic protection (impermeability to molecules >150 kDa such as IgG), (3) permeability to molecules of low molecular weight such as insulin, glucose, nutrients, and metabolites (<75 kDa), and (4) promotion of angiogenesis to allow (5) a sufficient oxygen pressure (pO_2) to ensure encapsulated tissue survival and function. To avoid nonspecific immune response against alginates, each material is characterized by a low level of endotoxin content (<100 EU/g).

In this study, we used the subcutaneous site for transplantation of alginate capsules. We previously demonstrated in the same Wistar rat model that the biocompatibility of encapsulated pig islets was improved by transplantation in the kidney subcapsular space and subcutaneous tissue in comparison with the intraperitoneal site.[36]

This study investigated, *in vivo*, biocompatible properties of different chemical alginates and their potential use for islet encapsulation and subcutaneous transplantation.[27,37]

Materials and Methods

Testing materials and transplantation

Alginates composed of either high mannuronic or high guluronic content were tested (Table 1). Three subtypes in each group were used: (1) high viscosity (SLM vs. SLG), (2) very low viscosity (VLDM vs. VLDG), and (3) peptide (RGD)-coupled alginate (SLM-RGD vs. SLG-RGD) (NovaMatrix, Sandvika, Norway). Freeze-dried alginates were reconstituted as previously described[27] at a concentration of 3% (wt/vol) for all alginates except 7% (wt/vol) for VLDM and VLDG. Approximately 500 μL aliquots of alginates were placed into the wells of a 24-well multiplate and crosslinked by 7 min of incubation with 1×3-(N-morpholino)propanesulfonic acid (MOPS; Sigma-Aldrich, St. Louis, MO) containing 100 mM $CaCl_2$ (Merck, Darmstadt, Germany). After rinsing in 1×MOPS for 5 min, alginate beads were stabilized by overnight incubation at 37°C, 5% CO_2 in CMRL 1066 (Mediatech, Manassas, VA) serum-free medium with 1.8 mM $CaCl_2$.[27] For the positive control (Ctrl+), in an effort to induce fibrosis, inflammation, and graft destruction, SLM3% (wt/vol) was supplemented with 10% (vol/vol) human serum (Cambrex, Walkersville, MD) and, after crosslinking, immersed in 1% (wt/vol) poly-L-lysine hydrobromide (Sigma-Aldrich)/1×MOPS over 5 min.

Alginate implants of disc-like shape of about 1–1.5 cm² and a thickness of 3–6 mm were subcutaneously implanted in the paravertebral space of Wistar rats.

The procedures were approved by the local ethics committee for animal care of the Université Catholique de Louvain (Brussels, Belgium).

Research design and experimental groups

In vivo biocompatibility testing. Biocompatibility studies were performed on Wistar rats weighing 100–200 g (obtained from the local animal facility of the Université Catholique de Louvain) to assess the stability, inflammatory reactions, and graft permeability.

Graft stability and inflammatory reactions: Seven experimental groups of seven rats ($n = 49$) were created: one group per alginate type (SLM, SLG, SLM-RGD, SLG-RGD, VLDM, VLDG) and one positive control group. Each animal from the seven experimental groups received two implants, which were placed in small subcutaneous pockets located on each side of the dorsal column. In each group, three rats were killed after 2 weeks (21 rats/42 implants) and two additional rats were euthanized at 4 weeks (14 rats/28 implants) after implantation. After 12 weeks, the last four implants were explanted from the two remaining rats in each group (14 rats/28 implants).

Alginate implants were weighed before and after implantation to assess weight recovery and then the percentage of graft recovery. Surrounding tissues and implants (structured and destructured) were taken for investigations. Implant weight is an indication of graft course, which is as-

TABLE 1. PROPERTIES OF TESTED ALGINATES

Alginate	% (wt/vol)	% M	% G	Coupled peptide	Viscosity (mPa·s)	Endotoxin content (EU/g)
SLM	3	>50		No	>100	<100
SLG	3		>60	No	>100	<100
SLM RGD	3	>50		Yes	>100	<100
SLM RGD	3		>60	Yes	>100	<100
VLDM	7	>50		No	<20	<100
VLDG	7		>60	No	<20	<100

SLM, sterile lyophilized high mannuronate; SLG, sterile lyophilized high guluronate; VLDM, very-low-density mannuronate; VLDG, very-low-density guluronate.

sociated with fibrosis and inflammatory characterization (see below).

After overnight fixation in formol at room temperature, explants were embedded in paraffin. Sections were thereafter routinely colored with silver methenamine and Masson's trichrome to assess, respectively, the degree of fibrosis and angiogenesis.

Lymphocyte (CD3) and macrophage (CD68) infiltrations were assessed by immunohistochemistry as previously described.[27]

The numbers of macrophages, lymphocytes, and vessels were quantified histomorphologically (in 10 nonoverlapping areas per slide; original magnification×25, with a 400× $400 \mu m^2$ grid).

Graft permeability: For characterization of the permeability of different alginates, before and after implantation, implants of each alginate were incubated with FITC-coupled lectins of different molecular weights: 36 kDa (FITC-Triticum vulgare WGA [EY Lab, San Mateo, CA]), 75 kDa (FITC-Maackia Amurensis MAL-I [Vector Laboratories, Burlingame, CA]), or 150 kDa (FITC-Sambuca nigra SNA [EY Lab]) at a concentration of 200 μg/mL for WGA and 1 mg/mL for MAL-I and SNA.[38]

After a 48 h incubation at 4°C on a mechanical rocker, beads were briefly rinsed in Hank's balanced salt solution (Invitrogen, Merelbeke, Belgium), embedded in Tissue Tek (Sakura, Zoeterwoude, The Netherlands), and frozen in a bath of 2-methylbutane placed into liquid nitrogen. Sections of 40 μm thickness were cut, embedded with Vectashield mounting medium (Vector Laboratories), and examined microscopically with an Axioskop 40 coupled with a FluoArc (Zeiss, Gottingen, Germany). Fluoroscopic videos of beads were thereafter captured by an Infinity X camera (Deltapix, Ottawa, Ontario, Canada; original magnification×10). Image analysis was performed with Scion Image, Beta 4.02, acquisition and analysis software (Scion, Frederick, MD). The number of fluorescent lectins having penetrated in alginate beads were counted in four nonoverlapping areas per slide and reported to an area of 100,000 pixels.[2]

Oxygenation study. One implant per rat was used to assess the pO_2. Twenty-one additional rats were followed for 1 month: two animals were transplanted with a device made of SLG, VLDM, or VLDG alginate or positive control; three recipients were implanted with a device made of SLM-RGD or SLG-RGD alginate; and seven animals were transplanted with SLM alginate. At 1, 2, 3, and 4 weeks after implantation, pO_2 inside the graft was monitored (see below) for each animal. Rats were killed at 4 weeks after implantation.

Electronic paramagnetic resonance (EPR) oximetry was used to assess evolution in pO_2 inside grafts *in vivo* up to 4 weeks and to evaluate *in vitro* a possible gradient of pO_2 inside the SLM3% grafts. The measurement is based on the oxygen-dependent broadening of the EPR spectrum of a paramagnetic oxygen sensor.[39]

In vivo pO_2 *inside grafts:* The pO_2 inside the alginate implants placed subcutaneously in rats ($n = 17$) was studied up to 4 weeks after transplantation with an EPR spectrometer (Magnettech, Berlin, Germany) equipped with a low-frequency microwave bridge operating at 1.2 GHz and an extended loop resonator (1 cm depth sensitivity).[40] Paramagnetic carbon

(Codex® Depurato; Carlo Erba Reactifs, Val de Reuil, France) was used as the oxygen-sensitive probe.

Five-hundred microliters of a suspension of carbon at 200 mg/mL in MOPS was mixed with 1 mL alginate 4.5% or 10.5% (for VLD). After Ca^{2+} crosslinking and overnight stabilization as previously described,[27] implants were transplanted subcutaneously in Wistar rats weighing 100 g. Adding carbon exclusively to alginate implants ensures the graft specificity of the signal measured.

At 1, 2, 3, and 4 weeks after implantation, pO_2 inside the grafts was monitored. Rats were anesthetized and placed in the EPR spectrometer, with the region of graft implantation under the surface coil. EPR spectra were recorded with a modulation amplitude less than one third of the peak-to-peak line width.

pO_2 gradient in SLM grafts in vitro*:* For assessment of the pO_2 gradient in the thickness of the graft, SLM3% alginate with crystals of lithium phthalocyanine,[40] as an oxygen-sensitive probe, was placed inside Teflon tubes up to a height of about 3 cm and thereafter crosslinked in a bath of $1 \times MOPS + 100 mM Ca^{2+}$. Tubes were kept in CMRL 1066 until measurements were taken. The pO_2 inside the alginate was evaluated in a two-dimensional spectral mode on an X-Band Bruker Elexsys E540 Imager under normoxic conditions at room temperature.

Efficacy of SLM alginate in the subcutaneous tissues of diabetic recipients. Streptozotocin-induced diabetic (STZ; 55 mg/kg) rats were given transplants of encapsulated pig islets in either subcutaneous tissue ($n = 8$; SC) or intraperitoneal tissue ($n = 4$; IP). As controls, empty alginate capsules ($n = 6$; Sham) or free pig islets ($n = 4$; Ctrl+) were implanted subcutaneously.

Freshly isolated pig islets were encapsulated as previously described[41] in an SLM alginate matrix (Batch 110064; FMC BioPolymer, Drammen, Norway) containing a high concentration of mannuronic acid (high-M; 56%)[27] and a low endotoxin level (endotoxin < 25 EU/g). Diabetic rats received 8000 macroencapsulated islets equivalent, empty alginate capsules, or free pig islets (see earlier). Nonfasting blood glucose was assessed weekly and survival of islets was assessed by dithyzone and insulin staining.

Statistics

Values are presented as means ± standard error of the mean (except when otherwise specified). The one-sample Kolmogorov Smirnov test was used to assess the normal distribution of values. The statistical significance of differences between experimental groups was tested by one-way analysis of variance with a Bonferroni *post hoc* test. The statistical tests were performed with Systat version 8.0. Differences were considered significant at $p < 0.05$.

Results

In vivo *biocompatibility testing*

In vivo biocompatibility was characterized by evaluation of graft stability, neoangiogenesis in periphery of implants, recruitment of lymphocytes and macrophages, and assessment of graft permeability to small molecules and to the immune system of the receiver.

Table 2. Implant Weight Recovery

	2 Weeks (%)	4 Weeks (%)	12 Weeks (%)
SLM	98.59 ± 27.19	85.64 ± 39.23	72.89 ± 22.84
SLG	86.18 ± 3.85	64.26 ± 12.35[a]	83.42 ± 10.35
SLM RGD	185.5 ± 103.09	65.90 ± 28.55	41.94 ± 59.31
SLG RGD	63.73 ± 7.94	95.56 ± 3.63	144.03 ± 69.23
VLDM	30.66 ± 22.65	63.98 ± 122.06	27.88 ± 9.96
VLDG	167.84 ± 45.24	47.59 ± 51.43	49.92 ± 41.54[b]
Ctrl+	259.38 ± 276.82	0.00 ± 0.00[a]	0.00 ± 0.00[b]

Implant stability, during the complete graft course, was only observed for SLM and SLG. More than 100% of weight recovery indicated a serious fibrosis surrounding the implant. Most of alginates were degraded at 12 weeks (<50% of initial implant). Note that SLM-RGD, VLGD, and Ctrl+ alginates induced early fibrosis (2 weeks) before serious implant degradation.
[a]$p<0.05$, SLG and Ctrl+ for 4 versus 2 weeks, respectively.
[b]$p < 0.05$, VLGD and Ctrl+ for 12 versus 2/4 weeks, respectively.

Graft stability and inflammatory reaction.

Graft stability: Implants were weighed before and after each explantation time to calculate the percentage of weight recovered after implantation (Table 2). Control material was totally degraded at 4 weeks after implantation. The percentage of weight recovery > 100% indicated serious fibrosis surrounding Ctrl+ (after 2 weeks), SLM-RGD (Fig. 4B), and VLDG and SLG-RGD (after 12 weeks; Table 2). Serious implant degradation was also observed for SLM-RGD at 12 weeks (−58% of graft weight), VLDM from 2 weeks after implantation (−70%), and VLDG (−52%; Table 2). Suitable implant stability, up to 12 weeks after implantation, was observed only for SLM (−27%; Fig. 4A) and SLG (−16%). The weight of the SLG implant, however, decreased significantly from 2 to 4 weeks, whereas the weight recovery of the SLM implant was stable during the complete graft course without a serious fibrosis process. Although the weight of implant (after explantation) is a semiquantitative parameter, it was significantly related to inflammatory (CD3, CD68) and fibrosis responses (Table 3).

Angiogenesis: This process is required to allow oxygenation of transplanted tissues. Therefore, angiogenesis was quantified by histomorphologic analysis of tissues surrounding alginate implants (number of vessels/0.16 mm^2) at each explantation time (Fig. 1). Angiogenesis surrounding the alginate material was significantly higher in SLM (Fig. 4C) than in other alginates at 2 weeks (Fig. 4D, SLM-RGD) and 4 weeks after implantation. Although SLG and Ctrl+ demonstrated a transient angiogenesis at 2 weeks, it was not maintained at 4 and 12 weeks after implantation (Fig. 1).

Inflammatory reaction: Low lymphocyte infiltration (<35 lymphocytes/0.16 mm^2) was observed for all experimental alginates at each explantation time (data not shown). However, a higher degree of lymphocyte infiltration was found at 2 weeks after implantation for SLM-RGD (Fig. 4F) and Ctrl+ (22.45 ± 5.85 and 34.55 ± 5.30, respectively, vs. a mean of 5.18 ± 0.61 cells/0.16 mm^2 for other alginates). At 4 and 12 weeks after implantation, VLDM and VLDG, respectively, demonstrated the highest lymphocyte recruitment (16.70 ± 1.46 and 10.10 ± 2.20, respectively, vs. a mean of 6.46 ± 0.68 cells/0.16 mm^2 for other alginates). In contrast, a lower recruitment of CD3$^+$ cells was observed for SLM (Fig. 4E) and SLG at each explantation time (a mean of 4.98 ± 1.09 and 2.42 ± 0.48 cells/0.16 mm^2, respectively).

Macrophage recruitment during the graft process is presented in Figure 2. Two weeks after implantation, SLM-RGD (Fig. 4H), VDLM, VDLG, and Ctrl+ were characterized by significantly higher macrophage infiltration than that in SLM (Fig. 4G), SLG, and SLG-RGD. After 4 weeks, CD68$^+$ cell infiltration persisted at a higher level for VLDM and VLDG than other alginates. At 12 weeks after implantation, VLDG and even SLG-RGD demonstrated a significantly higher infiltration of macrophages than that in SLM.

Throughout the whole graft process, SLM showed a constantly low level of macrophage infiltration similar to that in SLG and even SLM-RGD at 4 and 12 weeks.

Table 3. Alginate Characteristics Required for Subcutaneous Transplantation of Encapsulated Islets

		SLM	SLG	SLM RGD	SLG RGD	VLDM	VLDG	Ctrl+
Permeability to molecules of 150 kDa	Prior implantation	No	Yes	Yes	Yes	Yes	Yes	Yes
	At each explantation time	No	Yes	nd	Yes	nd	No	nd
Degradation		No	No	Yes	Yes	Yes	Yes	Yes
Fibrosis		No	Yes	Yes	Yes	Yes	Yes	Yes
Lymphocyte recruitment		+	+	+++	++	++	+++	++++
Macrophage recruitment		+	+	+++	++	+++	+++	++
Angiogenesis		+++	++	+	++	+	+	++
Ideal pO$_2$	pO$_2$>10 mm Hg	Yes	Yes	No	Yes	No	No	No
	pO$_2$>39 mm Hg	Yes	No	No	No	No	No	No

nd, nondetermined because of graft degradation.
pO$_2$, oxygen pressure.
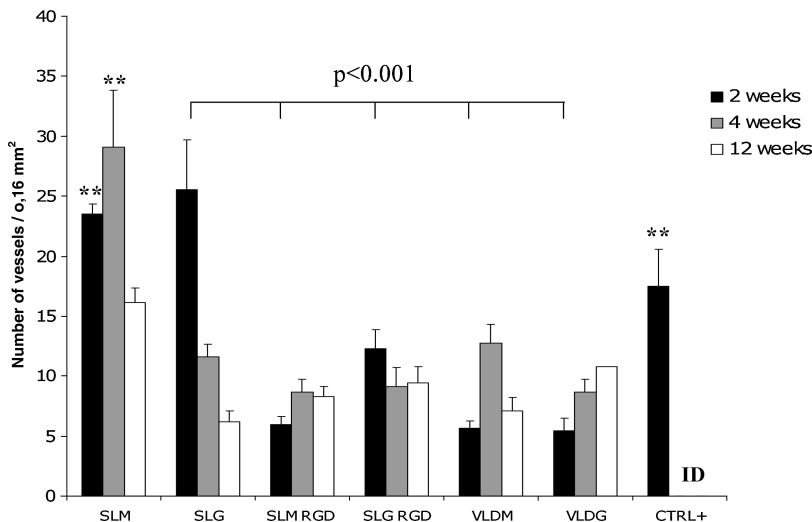
FIG. 1. Angiogenesis around alginate beads. A higher degree of angiogenesis was observed for SLM at 2 and 4 weeks after implantation (**$p < 0.005$ vs. other alginates). Although SLG and Ctrl+ demonstrated a transient angiogenesis at 2 weeks (**$p < 0.005$), it was not confirmed at 1 month after implantation because of implant degradation (ID). Three and two independent experiments were performed at 2 and 4/12 weeks after implantation, respectively (10 replicates per experiment). SLM, sterile lyophilized high mannuronate; SLG, sterile lyophilized high guluronate.

Graft permeability:

Permeability before implant transplantation—The permeability of the six alginates and control material to lectins of 36, 75, and 150 kDa was tested *in vitro* before implantation. The six alginates and the control material were permeable to small-molecular-weight molecules (36 and 75 kDa; 18.88–149.00 lectins/pixel^2). In contrast, lectins of 150 kDa could not penetrate SLM alginate, whereas similar lectins penetrated all other tested materials (0.35 ± 0.35 lectins/pixel^2 for SLM vs. 11.80 ± 6.99 lectins/pixel^2 for other alginates). Three independent experiments were performed, each in 24 replicates.

Permeability after implant in vivo *explantation*—All alginate devices implanted in rats were explanted after 2, 4, and 12 weeks for permeability testing. As the permeability assay for lectins requires well-structured alginates, permeability characterization was not performed on SLM-RGD, VLDM, and Ctrl+ materials at 4 and 12 weeks because they lost their structure after 2 weeks.

After explantation, each tested alginate maintained its permeability to molecules of low molecular weight (36 kDa)

at each explantation time (Fig. 3A). Permeability to lectins of 36 kDa was significantly higher for SLG-RDG than for other alginates (except SLM) at each explantation time (Fig. 3A). Only SLM and SLG maintained their permeability to molecules of 75 kDa during the entire graft process (Fig. 3B). SLM preserved the level of selective permeability to 150 kDa up to 12 weeks after implantation (Fig. 3C), whereas a significantly higher degree of permeability to such molecular weight molecules was evidenced for SLG and SLG-RGD (Fig. 3C).

Oxygenation study.

In vivo *pO_2 inside grafts:* Because the major cause of encapsulated cell death is probably hypoxia, pO_2 was assessed *in vivo* inside alginate implants at 1, 2, 3, and 4 weeks after implantation in 21 rats. Only SLM, SLG (Fig. 5A–C), and SLG-RGD alginates showed a $pO_2 > 10$ mm Hg during the 4 weeks of follow-up. SLG alginate showed an increasing pO_2 from 10 mm Hg after 1 week up to 25 mm Hg after 4 weeks. pO_2 with SLG-RGD varied during the 4 weeks, but the mean pO_2 was about 20 mm Hg. In this context, SLM clearly demonstrated a constant and much higher oxygenation
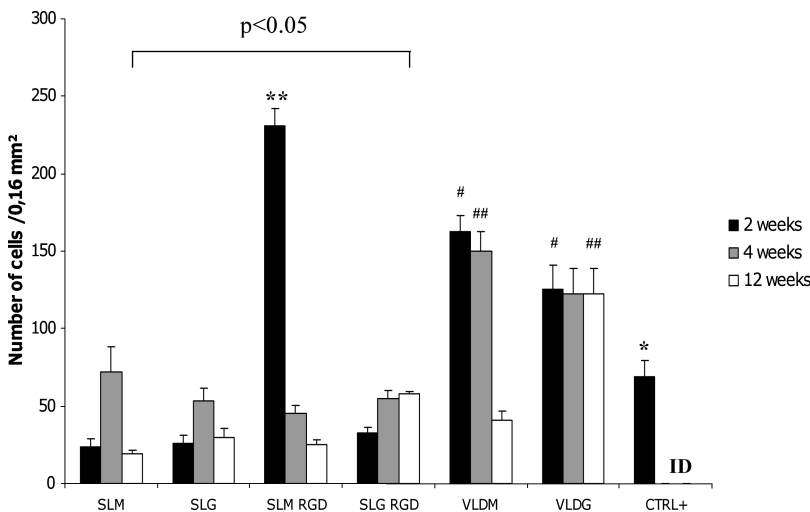
FIG. 2. Macrophage infiltration post-implantation. Before implant degradation, Ctrl+ was characterized by a higher infiltration of macrophages than SLM, SLG, and SLG-RGD at 2 weeks after implantation (*$p < 0.05$). Implants made of SLM-RGD demonstrated a higher degree of CD68 cell infiltration at 2 weeks in comparison to other groups (**$p < 0.005$). VLDM and VLDG were characterized by a higher recruitment of CD68$^+$ cells at 2, 4, and 12 weeks after implantation (#$p < 0.05$; ##$p < 0.005$ vs. other alginates). At 12 weeks after implantation, SLG-RGD was more infiltrated by macrophages than SLM ($p < 0.05$). Three and two independent experiments were performed at 2 and 4/12 weeks after implantation, respectively (10 replicates per experiment). VLDM, very-low-density mannuronate; VLDG, very-low-density guluronate.

A

36 kDa

B
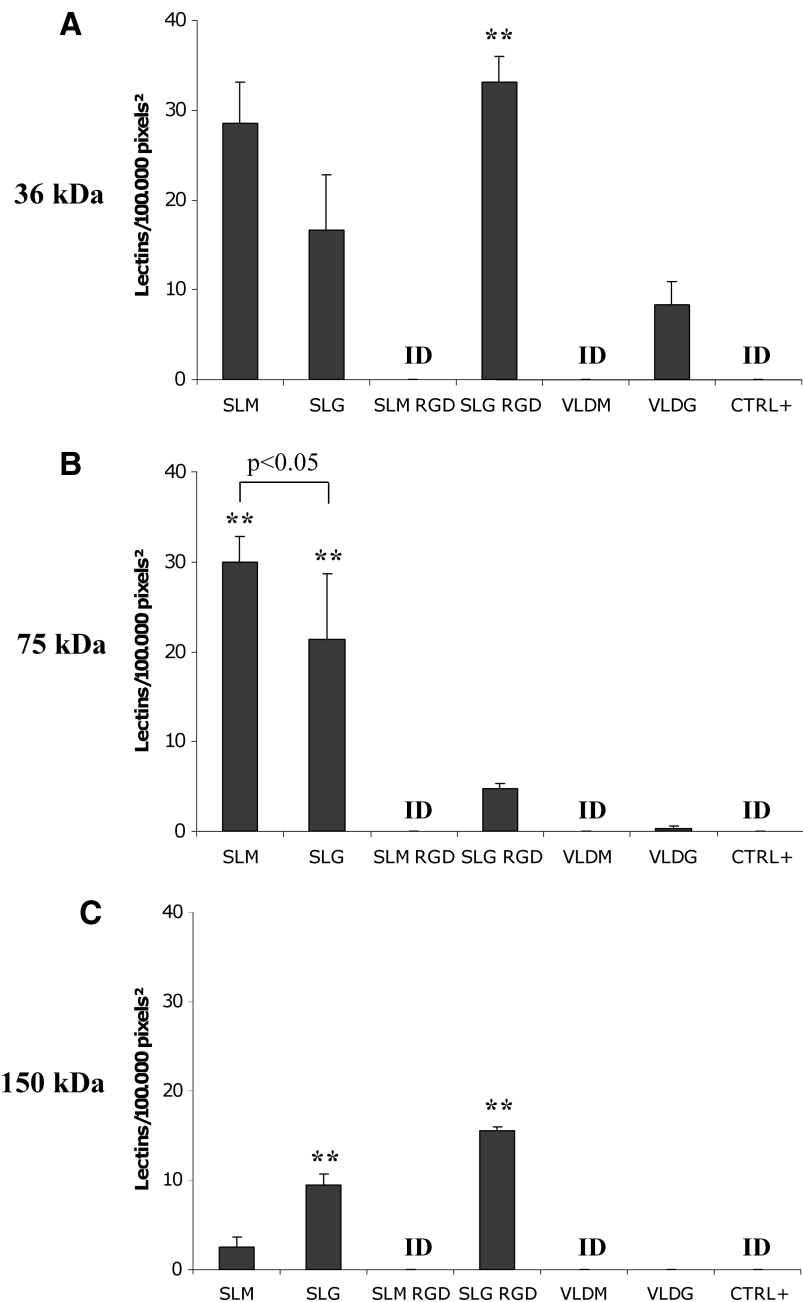
75 kDa

FIG. 3. Alginate permeability at 12 weeks after implantation. All alginates showed permeability to lectins of small molecular weight (36–75 kDa) at 2, 4, and 12 weeks after implantation (**A**, ******$p < 0.005$ for SLG-RGD vs. other alginates, except for SLM; **B**, ******$p < 0.005$ for SLM and SLG vs. other alginates). SLM maintained a constant capacity of low diffusion of 150 kDa lectins up to 12 weeks after implantation (**C**, ******$p < 0.005$) in comparison to other experimental alginates (SLG, SLG-RGD). Three and two independent experiments were performed at 2 and 4/12 weeks after implantation, respectively (24 replicates per experiment). Permeability was not assessed for implant degraded (ID).

C

150 kDa

($\sim 40\,mm\,Hg$) during the entire 4-week follow-up. Destructured alginates (such as VLDG) induced a significantly lower pO_2, below $10\,mm\,Hg$ (Fig. 5A, D).

pO_2 gradient in SLM grafts in vitro: To ensure the oxygenation was optimal everywhere in the implant, pO_2 in the thickness of the SLM implant was also measured *in vitro*. Results showed no pO_2 gradient (reflecting gas diffusion) in the total depth of the implant. A pO_2 of $\sim 160\,mm\,Hg$ was found at three different depths (2.4 vs. 2.6 vs. $3.4\,cm$; Fig. 5E).

The efficacy of SLM alginate in the subcutaneous tissues of diabetic recipients. Diabetic rats given transplants of free pig islets or empty capsules revealed rapid graft destruction and alginate biocompatibility, respectively, without diabetes correction. After transplantation of encapsulated pig islets

into the peritoneum, a transient blood glucose control was obtained up to 10 days but rapid decline of function followed. The diabetes was totally controlled, during 2 months after transplantation, with encapsulated islets in subcutaneous tissue (Fig. 6A, B). After graft explantation, viable insulin-staining islets without graft fibrosis were found (Fig. 6C).

Discussion

Islet immunoisolation for T1DM treatment requires an ideal encapsulation material with specific properties: (1) permeability to small molecules to ensure sufficient diffusion of nutrients, glucose, insulin, metabolites, and oxygen; (2) immunologic protection against immune cells and natural/elicited antibodies; (3) stability of implant after transplantation; (4) no

SLM   SLM-RGD

Fibrosis

Angiogenesis

CD3
recruitment
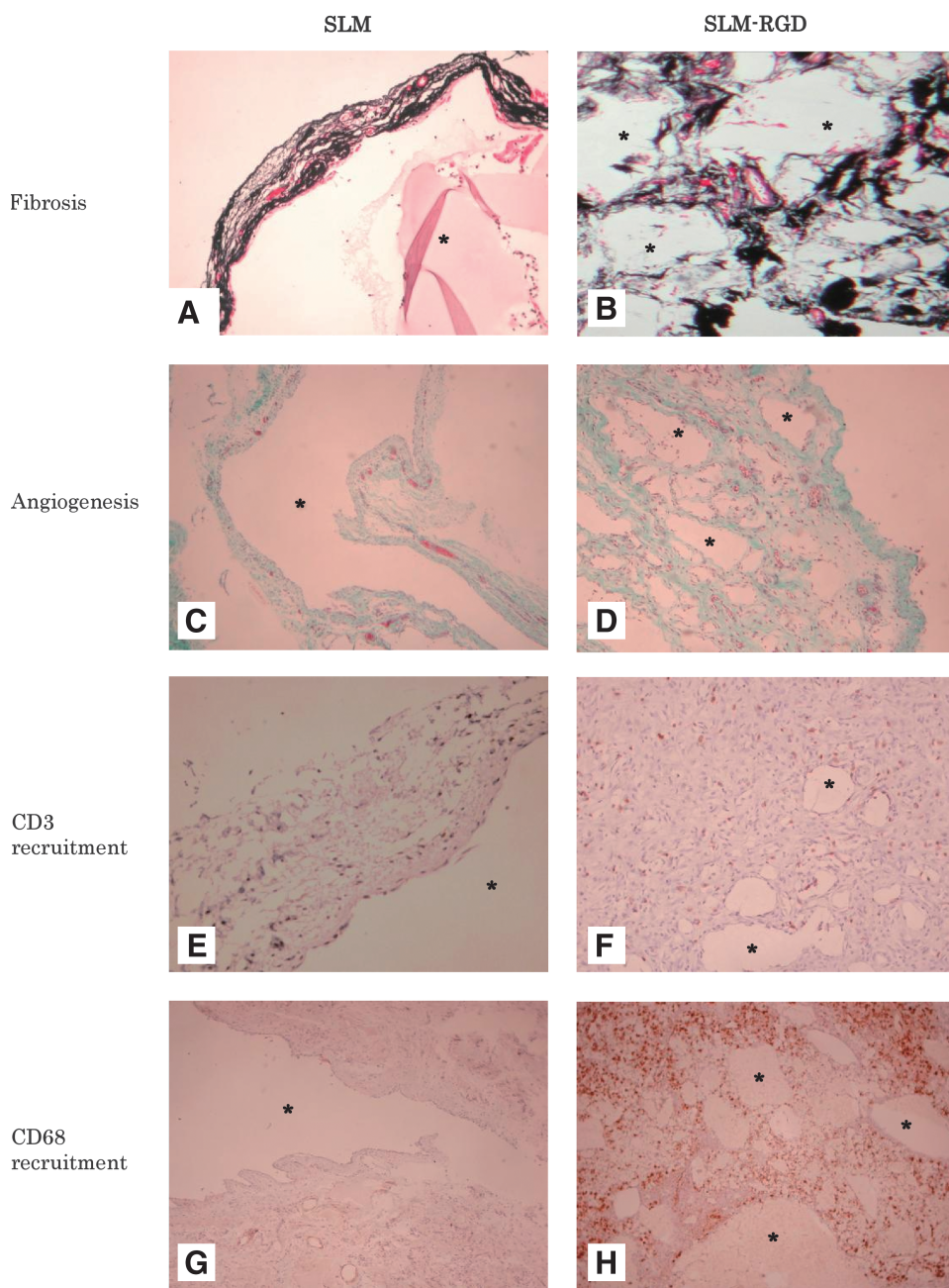
CD68
recruitment

FIG. 4. Biocompatible properties of SLM versus SLM-RGD. Note a significantly lower implant fibrosis (**A, B**) and CD3 (**E, F**) and CD68 recruitment (**G, H**) for SLM alginates in comparison to SLM-RGD. In contrast, a significantly higher angiogenesis in close contact to the implant was observed for SLM in contrast to SLM-RGD (**C, D**). Original magnification×5. Alginates are indicated by asterisks (∗).

induction of inflammation; (5) promotion of neoangiogenesis; and (6) sufficient pO_2 delivery to encapsulated tissues.

Material for encapsulation must possess selective permeability for nutrients while preventing the passage of immune cells and antibodies associated with islet allotransplantation and xenotransplantation for T1DM. The cutoff for the immunoglobulin molecular weight is 150 kDa for IgG, 500 kDa for activated complements, and 800 kDa for IgM. Reported studies used a multilayer encapsulation system to obtain a selective permeability.[13,31,42] Polylysine, a polyamino acid polycationic, is currently added to alginate to confer semipermeable properties to alginate beads.[43,44] But this latest technique remains associated with a nonspecific inflammation reaction after implantation.[45–47] Double alginate membranes have also been tested for islet immunoprotection, but these assessments were

performed in experimental *in vivo* models without the presence of antibodies against encapsulated pig islets.[42] In our study, among several compositions of tested alginates, a high-M alginate with a high viscosity obtained a selective permeability for molecules under 150 kDa without an overlayer membrane. We demonstrated, for the first time, that SLM alginate can promote selective properties without an additional overlayer. Although alginate properties are well established *in vitro*, selective permeability could be modified by the transplantation in correlation with inflammation and graft stability.

In our study, the subcutaneous site was used as a simple clinical procedure for transplantation of encapsulated islets. We previously demonstrated in the same Wistar rat model that the biocompatibility of encapsulated pig islets was improved by transplantation in the kidney subcapsular space
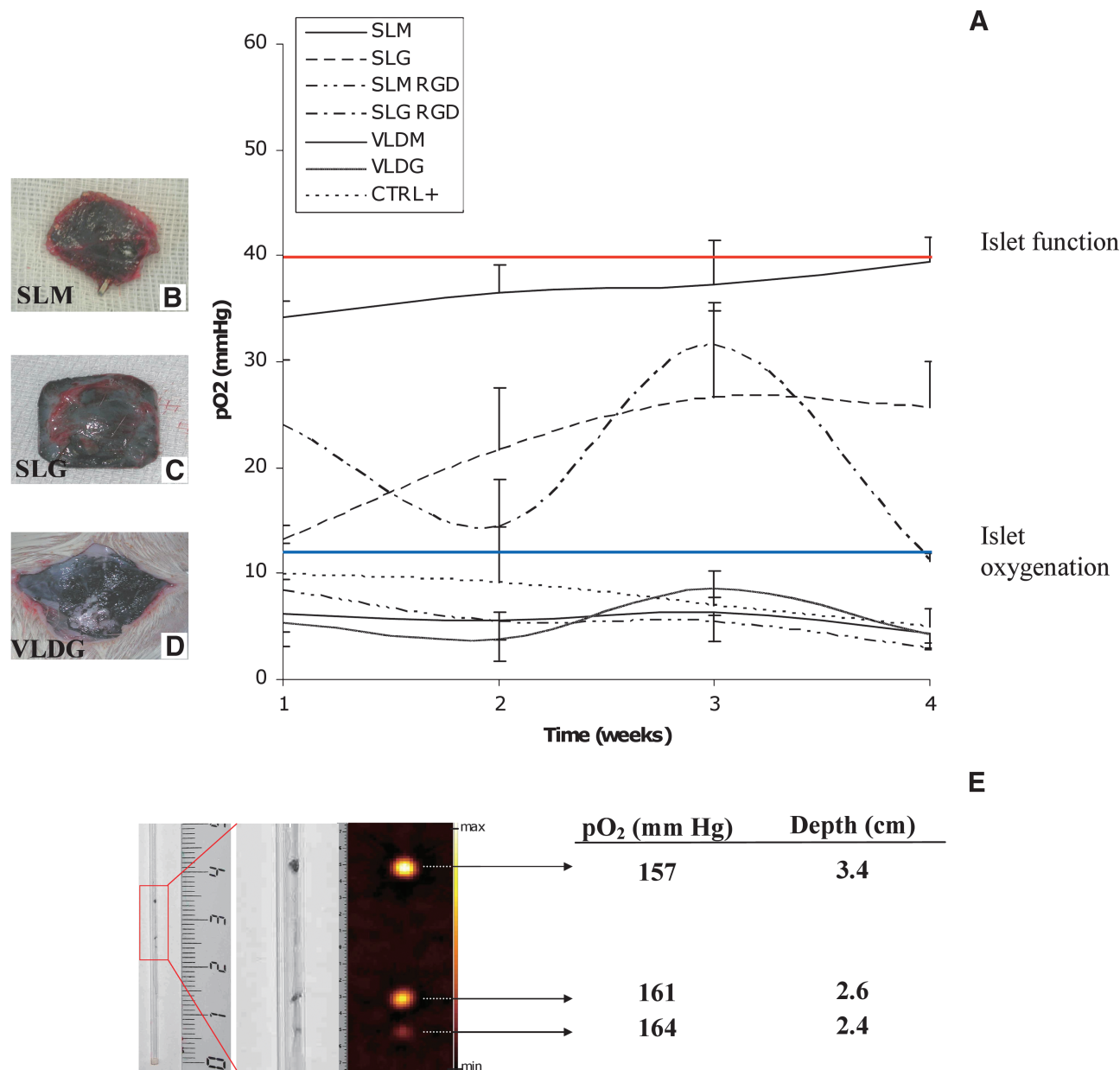
FIG. 5. Oxygenation inside alginate implants. Oxygen pressure (pO_2) inside implants, up to 4 weeks, was directly affected by graft outcome. Note that alginates completely degraded (as VLDM, VLDG [**D**], Ctrl+, SLM RGD) were characterized by a very low pO_2 (<10 mm Hg). (**A**) In contrast, SLM (**B**) was the only alginate with a sufficient oxygenation ($\sim$40 mm Hg) required for islet survival and function. SLM demonstrated significantly higher pO_2 than other alginates ($p < 0.05$) at each tested time, except SLG and SLG-RGD at 3 weeks after implantation. SLG (**C**) and SLG-RGD, partially degraded and infiltrated by fibrosis, presented intermediate pO_2 (10–40 mm Hg). Setup used for oxygen measurement in grafts: left and center: lithium crystals in 3% SLM placed in a Teflon tube; right: electronic paramagnetic resonance image. The spectral analysis was interpreted in terms of local pO_2. Note that an homogeneous diffusion of O_2 was found in the total depth of SLM (**E**). SLM, $n = 7$; SLG, $n = 2$; SLM-RGD, $n = 3$; SLG-RGD, $n = 3$; VLDM, $n = 2$; VLDG, $n = 2$; Ctrl+, $n = 2$.

and subcutaneous tissue in comparison with the intraperitoneal site.[36] Our current results confirmed that an alginate of SLM composition does not recruit lymphocytes and macrophages and avoids fibrosis. This absence of inflammatory response is correlated with implant stability in the subcutaneous tissue, thus maintaining the selective permeability of SLM alginate up to 3 months after transplantation. These results are relevant for a potential clinical application as previously demonstrated by translational studies rat to primate.[27,36]

Although several studies reported that high-G alginates induce a stronger inflammatory response than high-M alginates,[48,49] in our study no significant difference between the two types of alginates was found in terms of graft stability and inflammatory response, which can be explained by a similar endotoxin content between alginate batches. Variation of alginate biocompatibility was essentially observed for VLD and coupled peptide alginates investigated to reduce graft size and to improve islet survival, respectively. A serious inflam-
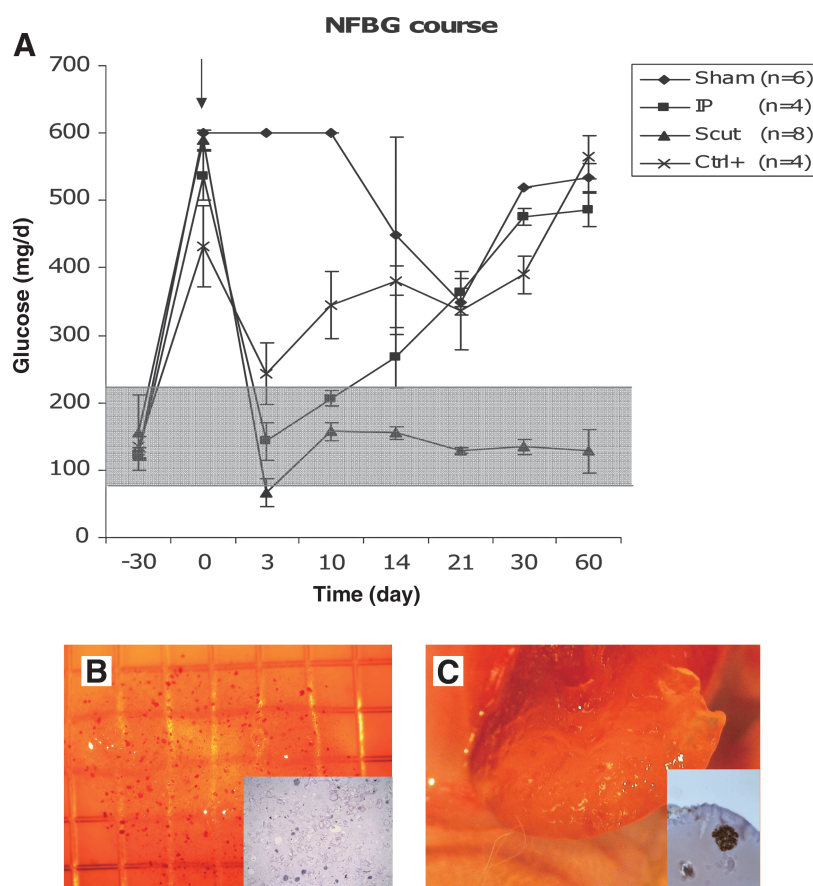
FIG. 6. Proof of concept of encapsulated islets in the subcutaneous tissue. Nonfasting blood glucose (NFBG) of streptozocin-induced diabetic rats (**A**) transplanted with free pig islets (Ctrl+), empty SLM capsules (Sham), and encapsulated pig islets (**B**, islets stained in red by dithyzone) implanted intraperitoneally (IP) or subcutaneously (Scut). Note that diabetes was totally controlled for up to 60 days for rats transplanted with encapsulated islets in the subcutaneous space without any fibrosis and with islet survival (**C**, insulin-staining islet). Gray rectangle in (**A**) indicates range of normal NFBG for rats; arrow indicates transplantation.

matory response, fibrosis, and consequently, implant degradation were found in both types of alginates without specification for high-M and high-G acid content. The VLD alginates did not resist mechanical friction and irritation in the subcutaneous tissue compared with the high-viscosity alginates. A rapid degradation was found with a strong inflammatory response.

Alginates coupled with peptide sequences to improve cell spreading and growth after encapsulation were unfortunately associated with a high degree of degradation and fibrosis in contrast to SLM/SLG without peptide. Additional GRGDSP sequences induced a severe inflammatory response. The major difference between uncoupled and coupled GRGDSP alginates was found in SLM because the major properties of biocompatibility were affected by GRGDSP sequences. Selective permeability was also affected by the addition of an GRGDSP sequence, with permeability to molecules >150 kDa found for SLM-RGD alginate. If SLM appears to be the ideal material for islet immunoprotection and subcutaneous transplantation, this latest technique must be adapted for angiogenesis to provide sufficient oxygenation for islet encapsulation.

In contrast to native pancreatic islets, which require high vascularization, encapsulated cells are not directly vascularized after transplantation. Then, for promotion of encapsulated islet survival and function, it remains crucial to develop a vascularization process around the alginate implants. In case of proinflammatory reaction, as observed for SLG, Ctrl+, and VLD alginates, a transient angiogenesis occurs at 2 weeks after transplantation but is followed by a strong fibrotic process. For SLM alginate, progressive vascularization occurred, with a peak at 4 weeks after transplantation. However, a crucial question remains about the relationship between angiogenesis and the oxygen tension required for encapsulated islet survival and function.

The pO_2 measured in native islets and in islets of the whole pancreas graft is 40 mm Hg.[50] Dionne and colleagues showed that for isolated islets in a perfusion chamber, the first phase of insulin secretion was insensible to hypoxia but, during the second phase, the secretion diminished when pO_2 fell below 60 mm Hg.[51,52] In islets implanted into the liver, the classic site for islet transplantation, the pO_2 was around 5 mm Hg at 9–12 weeks after implantation.[53] In this study, we report the first published investigation of pO_2 inside subcutaneous implants. An ideal pO_2 of about 40 mm Hg was found for SLM up to 4 weeks after implantation. This high pO_2 found in the implant is related to the graft stability, the absence of inflammation/fibrosis, and the high level of structured angiogenesis in close contact with the SLM alginate. In contrast, destructured alginates (SLM-RGD, VLDM, VLDG, Ctrl+) demonstrated significantly lower pO_2 (<10 mm Hg). A similar pO_2 was found in native islets and SLM implants up to 4 weeks after implantation. Further, the diffusion of oxygen was assessed *in vitro* through a maximum of 3.4 cm of SLM alginate. Only a very low gradient of oxygen tension was found but this latest is not significant with respect to the thickness of alginate used *in vivo* (3–6 mm). The thickness of the graft in view to concentrate islets is therefore not an important factor to consider for the O_2 diffusion inside the implant.

Conclusion

As the function of SLM-encapsulated islets is limited to 6 months,[27] we have extensively tested new alginates with respect to SLM to improve islets survival after subcutaneous implantation. Among these six tested materials, high mannuronic with high viscosity alginate is still superior with all required criteria for an ideal bioartificial pancreas (Table 3). This SLM alginate (1) is permeable to molecules of low molecular weight and ensures impermeability to the immune system, (2) is stable during the graft process, (3) does not induce serious fibrosis (low inflammation), and (4) promotes angiogenesis for survival of the islets.

Acknowledgments

Disclosure Statement

No competing financial interests exist.

References

1. Shapiro, A.M., Lakey, J.R., Ryan, E.A., Korbutt, G.S., Toth, E., Warnock, G.L., Kneteman, N.M., and Rajotte, R.V. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 343, 230, 2000.

2. Ryan, E.A., Paty, B.W., Senior, P.A., Bigam, D., Alfadhli, E., Kneteman, N.M., Lakey, J.R., and Shapiro, A.M. Five-year follow-up after clinical islet transplantation. Diabetes 54, 2060, 2005.

3. Mineo, D., Ricordi, C., Xu, X., Pileggi, A., Garcia-Morales, R., Khan, A., Baidal, D.A., Han, D., Monroy, K., Miller, J., Pugliese, A., Froud, T., Inverardi, L., Kenyon, N.S., and Alejandro, R. Combined islet and hematopoietic stem cell allotransplantation: a clinical pilot trial to induce chimerism and graft tolerance. Am J Transplant 8, 1262, 2008.

4. Shapiro, A.M., Ricordi, C., Hering, B.J., Auchincloss, H., Lindblad, R., Robertson, R.P., Secchi, A., Brendel, M.D., Berney, T., Brennan, D.C., Cagliero, E., Alejandro, R., Ryan, E.A., DiMercurio, B., Morel, P., Polonsky, K.S., Reems, J.A., Bretzel, R.G., Bertuzzi, F., Froud, T., Kandaswamy, R., Sutherland, D.E., Eisenbarth, G., Segal, M., Preiksaitis, J., Korbutt, G.S., Barton, F.B., Viviano, L., Seyfert-Margolis, V., Bluestone, J., and Lakey, J.R. International trial of the Edmonton protocol for islet transplantation. N Engl J Med 355, 1318, 2006.

5. Hirshberg, B., Rother, K.I., Digon, B.J., III, Lee, J., Gaglia, J.L., Hines, K., Read, E.J., Chang, R., Wood, B.J., and Harlan, D.M. Benefits and risks of solitary islet transplantation for type 1 diabetes using steroid-sparing immunosuppression: the National Institutes of Health experience. Diabetes Care 26, 3288, 2003.

6. Ryan, E.A., Lakey, J.R., Paty, B.W., Imes, S., Korbutt, G.S., Kneteman, N.M., Bigam, D., Rajotte, R.V., and Shapiro, A.M. Successful islet transplantation: continued insulin reserve provides long-term glycemic control. Diabetes 51, 2148, 2002.

7. de Groot, M., Schuurs, T.A., and Van Schilfgaarde, R. Causes of limited survival of microencapsulated pancreatic islet grafts. J Surg Res 121, 141, 2004.

8. De Vos, P., Hamel, A.F., and Tatarkiewicz, K. Considerations for successful transplantation of encapsulated pancreatic islets. Diabetologia 45, 159, 2002.

9. Knazek, R.A., Gullino, P.M., Kohler, P.O., and Dedrick, R.L. Cell culture on artificial capillaries: an approach to tissue growth in vitro. Science 178, 65, 1972.

10. Chick, W.L., Like, A.A., and Lauris, V. Beta cell culture on synthetic capillaries: an artificial endocrine pancreas. Science 187, 847, 1975.

11. Scharp, D.W. Isolation and transplantation of islet tissue. World J Surg 8, 143, 1984.

12. Efrat, S. Cell replacement therapy for type 1 diabetes. Trends Mol Med 8, 334, 2002.

13. Lim, F., and Sun, A.M. Microencapsulated islets as bioartificial endocrine pancreas. Science 210, 908, 1980.

14. De Vos, P., Van Straaten, J.F., Nieuwenhuizen, A.G., de Groot, M., Ploeg, R.J., De Haan, B.J., and Van Schilfgaarde, R. Why do microencapsulated islet grafts fail in the absence of fibrotic overgrowth? Diabetes 48, 1381, 1999.

15. De Vos, P., De Haan, B.J., Wolters, G.H., Strubbe, J.H., and Van Schilfgaarde, R. Improved biocompatibility but limited graft survival after purification of alginate for microencapsulation of pancreatic islets. Diabetologia 40, 262, 1997.

16. King, A., Lau, J., Nordin, A., Sandler, S., and Andersson, A. The effect of capsule composition in the reversal of hyperglycemia in diabetic mice transplanted with microencapsulated allogeneic islets. Diabetes Technol Ther 5, 653, 2003.

17. Fritschy, W.M., De Vos, P., Groen, H., Klatter, F.A., Pasma, A., Wolters, G.H., and Van Schilfgaarde, R. The capsular overgrowth on microencapsulated pancreatic islet grafts in streptozotocin and autoimmune diabetic rats. Transpl Int 7, 264, 1994.

18. De Vos, P., De Haan, B.J., and Van Schilfgaarde, R. Upscaling the production of microencapsulated pancreatic islets. Biomaterials 18, 1085, 1997.

19. De Vos, P., Smedema, I., van Goor, H., Moes, H., van Zanten, J., Netters, S., de Leij, L.F., de Haan, A., and De Haan, B.J. Association between macrophage activation and function of micro-encapsulated rat islets. Diabetologia 46, 666, 2003.

20. Iwata, H., Kobayashi, K., Takagi, T., Oka, T., Yang, H., Amemiya, H., Tsuji, T., and Ito, F. Feasibility of agarose microbeads with xenogeneic islets as a bioartificial pancreas. J Biomed Mater Res 28, 1003, 1994.

21. Zielinski, B.A., and Aebischer, P. Chitosan as a matrix for mammalian cell encapsulation. Biomaterials 15, 1049, 1994.

22. Kessler, L., Legeay, G., Jesser, C., Damge, C., and Pinget, M. Influence of corona surface treatment on the properties of an artificial membrane used for Langerhans islets encapsulation: permeability and biocompatibility studies. Biomaterials 16, 185, 1995.

23. Klomp, G.F., Hashiguchi, H., Ursell, P.C., Takeda, Y., Taguchi, T., and Dobelle, W.H. Macroporous hydrogel membranes for a hybrid artificial pancreas. II. Biocompatibility. J Biomed Mater Res 17, 865, 1983.

24. Zondervan, G.J., Hoppen, H.J., Pennings, A.J., Fritschy, W., Wolters, G., and Van Schilfgaarde, R. Design of a polyurethane membrane for the encapsulation of islets of Langerhans. Biomaterials 13, 136, 1992.

25. Sawhney, A.S., and Hubbell, J.A. Poly(ethylene oxide)-graft-poly(L-lysine) copolymers to enhance the biocompati-

bility of poly(L-lysine)-alginate microcapsule membranes. Biomaterials **13,** 863, 1992.

26. Campos-Lisboa, A.C., Mares-Guia, T.R., Grazioli, G., Goldberg, A.C., and Sogayar, M.C. Biodritin microencapsulated human islets of Langerhans and their potential for type 1 diabetes mellitus therapy. Transplant Proc **40,** 433, 2008.

27. Dufrane, D., Goebbels, R.M., Saliez, A., Guiot, Y., and Gianello, P. Six-month survival of microencapsulated pig islets and alginate biocompatibility in primates: proof of concept. Transplantation **81,** 1345, 2006.

28. De Vos, P., De Haan, B.J., de Haan, A., van Zanten, J., and Faas, M.M. Factors influencing functional survival of microencapsulated islet grafts. Cell Transplant **13,** 515, 2004.

29. Schneider, S., Feilen, P.J., Brunnenmeier, F., Minnemann, T., Zimmermann, H., Zimmermann, U., and Weber, M.M. Long-term graft function of adult rat and human islets encapsulated in novel alginate-based microcapsules after transplantation in immunocompetent diabetic mice. Diabetes **54,** 687, 2005.

30. Elliott, R.B., Escobar, L., Tan, P.L., Muzina, M., Zwain, S., and Buchanan, C. Live encapsulated porcine islets from a type 1 diabetic patient 9.5 yr after xenotransplantation. Xenotransplantation **14,** 157, 2007.

31. De Vos, P., Faas, M.M., Strand, B., and Calafiore, R. Alginate-based microcapsules for immunoisolation of pancreatic islets. Biomaterials **27,** 5603, 2006.

32. Ertesvag, H., and Valla, S. Biosynthesis and applications of alginates. Polym Degrad Stability **59,** 85, 1998.

33. Burdick, J.A., and Anseth, K.S. Photoencapsulation of osteoblasts in injectable RGD-modified PEG hydrogels for bone tissue engineering. Biomaterials **23,** 4315, 2002.

34. Evangelista, M.B., Hsiong, S.X., Fernandes, R., Sampaio, P., Kong, H.J., Barrias, C.C., Salema, R., Barbosa, M.A., Mooney, D.J., and Granja, P.L. Upregulation of bone cell differentiation through immobilization within a synthetic extracellular matrix. Biomaterials **28,** 3644, 2007.

35. Orive, G., Tam, S.K., Pedraz, J.L., and Halle, J.P. Biocompatibility of alginate-poly-L-lysine microcapsules for cell therapy. Biomaterials **27,** 3691, 2006.

36. Dufrane, D., van Steenberghe, M., Goebbels, R.M., Saliez, A., Guiot, Y., and Gianello, P. The influence of implantation site on the biocompatibility and survival of alginate encapsulated pig islets in rats. Biomaterials **27,** 3201, 2006.

37. Abstracts of the Joint Meeting of the International Xenotransplantation Association (IXA), the International Pancreas and Islet Transplant Association (IPITA), and the Cell Transplant Society (CTS), Minneapolis, MN, September 15–20, 2007. Xenotransplantation **14,** 375, 2007.

38. Barnett, B.P., Kraitchman, D.L., Lauzon, C., Magee, C.A., Walczak, P., Gilson, W.D., Arepally, A., and Bulte, J.W. Radiopaque alginate microcapsules for X-ray visualization and immunoprotection of cellular therapeutics. Mol Pharm **3,** 531, 2006.

39. Gallez, B., Baudelet, C., and Jordan, B.F. Assessment of tumor oxygenation by electron paramagnetic resonance: principles and applications. NMR Biomed **17,** 240, 2004.

40. Gallez, B., Jordan, B.F., Baudelet, C., and Misson, P.D. Pharmacological modifications of the partial pressure of oxygen in murine tumors: evaluation using *in vivo* EPR oximetry. Magn Reson Med **42,** 627, 1999.

41. Dufrane, D., D'hoore, W., Goebbels, R.M., Saliez, A., Guiot, Y., and Gianello, P. Parameters favouring successful adult pig islet isolations for xenotransplantation in pig-to-primate models. Xenotransplantation **13,** 204, 2006.

42. Duvivier-Kali, V.F., Omer, A., Lopez-Avalos, M.D., O'Neil, J.J., and Weir, G.C. Survival of microencapsulated adult pig islets in mice in spite of an antibody response. Am J Transplant **4,** 1991, 2004.

43. Gugerli, R., Cantana, E., Heinzen, C., von Stockar, U., and Marison, I.W. Quantitative study of the production and properties of alginate/poly-L-lysine microcapsules. J Microencapsul **19,** 571, 2002.

44. Goosen, M.F., O'Shea, G.M., Gharapetian, H.M., Chou, S., and Sun, A.M. Optimization of microencapsulation parameters: semipermeable microcapsules as a bioartificial pancreas. Biotechnol Bioeng **27,** 146, 1985.

45. Pueyo, M.E., Darquy, S., Capron, F., and Reach, G. *In vitro* activation of human macrophages by alginate-polylysine microcapsules. J Biomater Sci Polym Ed **5,** 197, 1993.

46. Darquy, S., Pueyo, M.E., Capron, F., and Reach, G. Complement activation by alginate-polylysine microcapsules used for islet transplantation. Artif Organs **18,** 898, 1994.

47. Strand, B.L., Ryan, T.L., In't, V.P., Kulseng, B., Rokstad, A.M., Skjak-Brek, G., and Espevik, T. Poly-L-lysine induces fibrosis on alginate microcapsules via the induction of cytokines. Cell Transplant **10,** 263, 2001.

48. De Vos, P., De Haan, B., and Van Schilfgaarde, R. Effect of the alginate composition on the biocompatibility of alginate-polylysine microcapsules. Biomaterials **18,** 273, 1997.

49. Clayton, H.A., London, N.J., Colloby, P.S., Bell, P.R., and James, R.F. The effect of capsule composition on the biocompatibility of alginate-poly-L-lysine capsules. J Microencapsul **8,** 221, 1991.

50. Carlsson, P.O., and Palm, F. Oxygen tension in isolated transplanted rat islets and in islets of rat whole-pancreas transplants. Transpl Int **15,** 581, 2002.

51. Dionne, K.E., Colton, C.K., and Yarmush, M.L. Effect of oxygen on isolated pancreatic tissue. ASAIO Trans **35,** 739, 1989.

52. Dionne, K.E., Colton, C.K., and Yarmush, M.L. Effect of hypoxia on insulin secretion by isolated rat and canine islets of Langerhans. Diabetes **42,** 12, 1993.

53. Carlsson, P.O., Palm, F., Andersson, A., and Liss, P. Markedly decreased oxygen tension in transplanted rat pancreatic islets irrespective of the implantation site. Diabetes **50,** 489, 2001.

Address correspondence to:
Denis Dufrane, M.D., Ph.D.
Laboratory of Experimental Surgery
Université Catholique de Louvain
CHEX 5570
Avenue Hippocrate 55
B-1200 Brussels
Belgium

E-mail: denis.dufrane@uclouvain.be