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Effect of prolonged gelling time on the intrinsic properties of barium alginate microcapsules and its biocompatibility

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Abstract

Pericapsular fibrotic overgrowth (PFO) may be attributed to an immune response against microcapsules themselves or to antigen shedding through microcapsule pores from encapsulated islet tissue. Modification of microcapsules aimed at reducing pore size should prevent PFO and improve graft survival. This study investigated the effect of increased gelling time (20 vs. 2 min) in barium chloride on intrinsic properties of alginate microcapsules and tested their biocompatibility *in vivo*. Prolonged gelling time affected neither permeability nor size of the microcapsules. However, prolonged gelling time for 20 min produced brittle microcapsules compared to 2 min during compression test. Encapsulation of human islets in both types of microcapsules affected neither islet viability nor function. The presence of PFO when transplanted into a large animal model such as baboon and its absence in small animal models such as rodents suggest that the host immune response towards alginate microcapsules is species rather than alginate specific.

Keywords: islets, microencapsulation, transplantation

Introduction

Microencapsulation is a strategy that should prevent rejection of the grafted tissue without the need for anti-rejection drugs. In the context of diabetes, the principle of microencapsulation involves coating of pancreatic islets within a semi-permeable membrane without compromising islet function (Chang, 1964; Calafiore et al., 1995). The microcapsules used for encapsulation studies are mostly produced from polymers known as hydrogels which are derived either naturally or through synthetic routes. In islet transplantation, the naturally obtained alginate hydrogels are widely used as they can be gelled under physiological conditions without the use of toxic chemicals and without affecting islet function (Ménard et al., 2010). Alginates microcapsules cross-linked with Ca^{2+} or Ba^{2+} are used successfully in both allograft and xenograft settings without the need for anti-rejection drugs, at least in rodents, with recipients becoming normoglycaemic (Silva et al., 2006). Previously, we have demonstrated successful encapsulation of human and foetal pig islets into simple barium alginate microcapsules and established their function *in vivo* (Foster et al., 2007; Vaithilingam et al., 2010). Recently, a phase 1 clinical trial by the Sydney group using barium alginate microcapsules showed that allografting of microencapsulated human islets is safe although only a minor clinical benefit was observed. Laparoscopic retrieval of the microcapsules showed the presence of dense fibrotic overgrowth around microcapsules with no viable islets (Tuch et al., 2009). Usually, this pericapsular fibrotic overgrowth (PFO) is either seen as a foreign body reaction against the microcapsules themselves (Clayton et al., 1991; Soon-Shiong et al., 1991; Fritschy et al., 1994) or immunological rejection to islet tissue within the

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microcapsules, which can be attributed to the shedding of antigens or chemokines leaking through the pores of the microcapsules (de Groot et al., 2004). Thus, the two intrinsic properties, namely biocompatibility and permeability are the major factors determining the functionality of alginate microcapsules particularly used for islet transplantation studies.

The PFO consisting mainly of macrophages and fibroblasts interferes with the diffusive transport of nutrient and oxygen consequently leading to hypoxia, islet cell starvation and apoptosis (Vandenbossche et al., 1993a, b; Strand et al., 2001). Further, the deposited immune cells in pericapsular infiltrate (PCI) can release deleterious cytokines/chemokines which can permeate through the capsule pores and damage the islets (de Vos et al., 1999). Though the entry of immune cells through the capsule membrane can be easily prevented, it is impossible to prevent the entry of these low molecular weight chemokines/cytokines secreted by immune cells. The challenge of keeping these molecules outside the capsule membrane is more serious, since many of these components are smaller or similar in size to essential cell nutrients or products (Silva et al., 2006). However, at least certain molecules of the immune system, especially immunoglobulins should ideally be completely prevented from entering the capsule membrane to avoid or minimize the host reaction to the implant. Thus, strategies aimed at reducing the permeability of these microcapsules should prevent PFO and improve biocompatibility.

It has been shown previously that the divalent cation Ba^{2+} formed stronger gels with reduced permeability because of their greater affinity to alginate compared to the traditional Ca^{2+} ions (Mørch et al., 2006). The authors speculated that increasing the gelling time of alginate microcapsules with barium chloride might further reduce the molecular weight cut-off (MWCO)/pore size and increase mechanical strength without affecting the functionality of alginate microcapsules. Therefore, in this study, we intended to characterize our barium alginate microcapsules and determined the effect of increased gelling time on the intrinsic properties of the microcapsules. We also studied the biocompatibility of these barium alginate microcapsules using different animal models to determine whether the host immune response is alginate or species specific.

Methods

Preparation of empty barium alginate microcapsules

The encapsulation procedure was carried out with highly purified 2.2% alginate (60:40 glucuronic acid: mannuronic acid, UPMVG PRONOVA, FMC Biopolymer) using a stainless steel air-driven droplet generator connected to a syringe driver (Torsten Steinau Verfahrenstechnik, Berlin, Germany) as described previously (Foster et al., 2007). The syringe (1 mL) was drawn with 0.8 mL of alginate suspension which was then attached to the driver and the air gas cylinder turned on to allow a flow rate of 8 L/min at 100 kPa. Turning on the power to the syringe driver resulted in the plunger pushing the syringe handle downwards, thereby forcing the alginate-islet suspension through the droplet generator to produce microcapsules. The alginate droplets/beads were collected in a 145-mm petri dish, containing 30 mL $BaCl_2$ gelling solution, placed 10 cm from the tip of the nozzle of the generator. The alginate-islet beads were incubated in this solution for 2 or 20 min and then washed three times with 0.9% NaCl to remove excess $BaCl_2$. The microcapsules were allowed to settle between washings, and the supernatant discarded. Microcapsules were then placed in saline and maintained at 37°C in air and 5% CO_2 until further use.

Optical microscopy

Microcapsule size was estimated using optical microscopy. It consisted of optical microscope (Kapa 2000, Kvant, Bratislava, Slovakia) equipped with a colour charge-coupled device camera (Mintron CC-63KW1P, Mintron, Malaysia) operated with Prover Image Forge v1.1 software (Prover s.r.o, Bratislava, Slovakia). The polydispersity was calculated by measuring the diameter of 100 microcapsules produced by 2 and 20 min gelation in barium chloride, respectively.

Permeability using inverse size exclusion chromatography

Inverse size exclusion chromatography (SEC) with pullulan standards (Gearing Scientific, Ashwell, Herts, UK), which are a type of polysaccharide polymer, of molecular weight range 0.7–7.88 kDa and saccharose (0.3 kDa) was used for the determination of the MWCO of microcapsules (Brissová et al., 1996). The equipment consisted of a glass column 10 × 250 mm (Omnifit, Cambridge, UK), containing the microcapsules, fitted with an adjustable plunger attached to a Waters SEC set-up consisting of Waters 515 pump, Rheodyne Injector 7725i with 100 μL loop and differential refractive index detector Waters 2410. Supplemented CMRL media was passed over the microcapsules in the column at a flow rate of 0.2 mL/min for 12 h, to allow equilibration to occur. Thereafter, pullulan standards of concentration ~3 mg/mL were injected. A calibration curve was constructed from the elution volumes corresponding to 50% of the area of each pullulan standard, and the partition coefficient K_{SEC} calculated. This was used for the determination of the MWCO, using WinGPC®7.2 software (PSS, Mainz, Germany). The elution volumes obtained at 50% of the area of each pullulan standard were used to create the calibration curve providing the MWCO as its exclusion volume. Finally, the differential distribution referring to the pore size distribution is obtained as the first derivative of the Boltzmann fit (expressed in the molecular weight values which can be converted to the size).

Mechanical properties

The mechanical properties of microcapsules were tested on a Texture Analyzer TA-2Xi (Stable Micro Systems, Godalming, UK) equipped with a force transducer of resolution 1 mN. Texture Expert software version 1.16 was used for determination and evaluation. The equipment consisted of a mobile probe moving vertically at a constant velocity. The mechanical stability of microcapsules was measured by compressing individual microcapsules. A microcapsule was placed on a plate in the shipping solution and the probe was moved with a constant speed of 0.5 mm/s towards the microcapsule until the travelled distance had reached 98% of the initial distance between plate and probe. The pre-test and post-test speeds were 0.5 and 2.0 mm/s, respectively. The force displacement data were recorded with a frequency of 100 Hz. The force (expressed in grams) exerted by the probe on the microcapsule was recorded as a function of the displacement (compression distance).

Human islet isolation and shipment

Human islets were isolated from pancreases of deceased multiorgan donors ($n = 3$) at the Cell Isolation Laboratory of the University of Illinois at Chicago, IL, USA. Briefly, the human islets were isolated by digestion with Serva Collagenase NB 1 (Nordmark, Germany) using the method described previously (Qi et al., 2008). The isolated human islets were then cultured in supplemented CMRL-1066 media (Mediatech, Herndon, VA) containing 1.5% human albumin for 1–2 days before being shipped to Sydney using a commercial courier service. The islets were then cultured for a day before being encapsulated. All the procedures regarding obtaining human islets were approved by the Institution's Human Research Ethics Committee.

Microencapsulation

The cultured human islets were pooled together and washed in 0.9% NaCl. The human islets were then suspended in highly purified 2.2% alginate (60:40 glucuronic acid: mannuronic acid, UPMVG PRONOVA, FMC Biopolymer) solution in 1:8 ratio. The microcapsule formation was carried out in an air-driven droplet generator as described above. Microencapsulated human islets were cultured for a day in CMRL-1066 media prior to use.

Viability

Viability of microencapsulated human islets was assessed using the fluorescent dyes 6-carboxyfluorescein diacetate (Sigma, St. Louis, MO) and propidium iodide (PI, Sigma). The percentage of green (live cells) to red (dead cells) fluorescence was assessed to evaluate the viability ($n = 100$, for each time point and preparation). Samples were analysed under a Zeiss-Axioskop 2 microscope using Axiovision LE software.

Static stimulation and insulin content

Aliquots of encapsulated human islets were exposed to either 2.8 mM glucose (basal, $n = 3$) or 20 mM glucose (stimulus, $n = 3$) for 1 h at 37°C. The supernatant was then collected and human insulin measured by radioimmunoassay (RIA, Linco, St. Charles, Missouri, USA). The remaining pellet was washed in Hanks Balanced Salt solution (HBSS) solution followed by addition of cold acid ethanol and vortexed vigorously to enhance cell lysis. The cell extract was kept at 4°C overnight and the supernatant collected the following day and analysed for insulin content by RIA.

Transplantation and retrieval of empty microcapsules from rodents

To test the bioreactivity of empty microcapsules, they were transplanted into the peritoneal cavity of (a) immunodeficient mice – non-obese diabetic/severe combined immunodeficient (NOD/SCID) (female, 6–8 weeks); (b) immunocompetent mice – C57/BL6 (male, 8 weeks) and Balb/c (male, 8 weeks); (c) immunodeficient rats – Nude (male, 4–5 weeks) and (d) immunocompetent rats – Wistar (female, 4–5 weeks). Briefly, the mice were anaesthetized by an intraperitoneal injection of pentobarbitone 65 mg/kg and the rats with ketamine 90 mg/kg and xylazine 10 mg/kg. The empty microcapsules were then injected into the peritoneal cavity through a 20-gauge catheter. The incision site was sutured and stapled. All the groups of mice and rats were transplanted with 300 μL empty microcapsules, a volume that would contain 3000 islet equivalents of human islets. All the procedures were approved by the Institutions' Animal Care and Ethics Committee (ACEC).

Transplantation and retrieval of empty microcapsules from a baboon

All the procedures and protocols relating to the experiments were approved by the ACEC of University of Illinois at Chicago, USA. Recipient animal was fasted for 12 h prior to surgery. On the day of the surgery, the recipient animal was sedated with ketamine [10 mg/kg, intramuscular (im)], induced with propofol [3–5 mg/kg, intravenous (iv)] and anaesthetized using continuous isoflurane gas infusion. Additionally, buprenorphine (0.01–0.03 mg/kg, im), cefazolin (25 mg/kg, im) and bupivacaine (1 mg/kg) were given preoperatively. The equipment used for implanting the microcapsules into the baboon and visualizing this was an Olympus 10 mm videolaparoscope with Olympus Visera digital video processor,

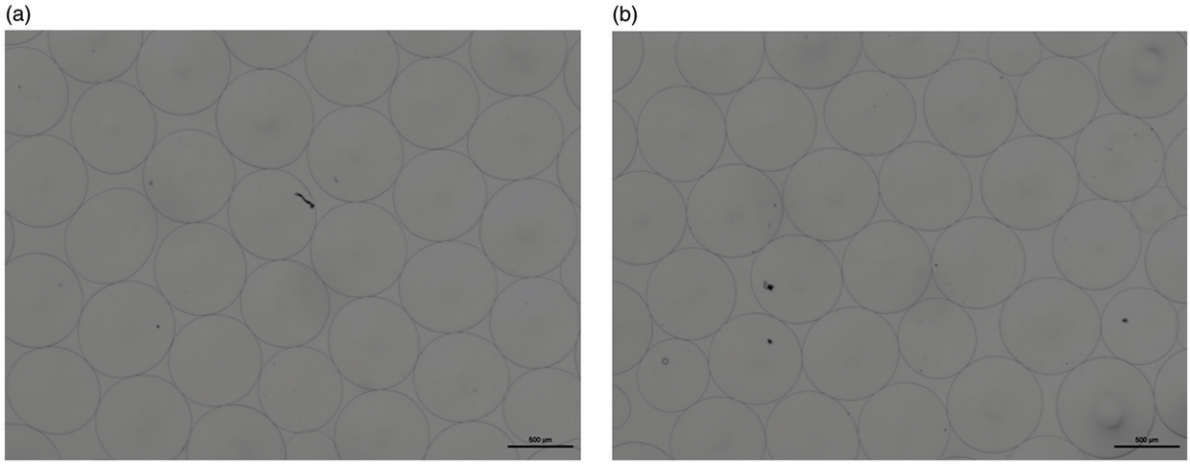

Figure 1. Prolonged gelling time did not affect bead size. Optical images of (a) 2 min and (b) 20 min barium alginate microcapsules in saline. Bar is equal to 0.5 mm. Representative images of 2 and 20 min barium alginate microcapsules after 60 min storing in fresh CMRL medium and after permeability measurement.

OTV-S7; light source, CLV-S40; high-flow insufflations unit, UHI-3 and a colour video monitor, OEV 203. The operation was carried out as follows. The baboon was placed in the supine position and a small (2 cm) supraumbilical incision was made and a 5 mm trocar (Endopath Xcel F4N315, Ethicon Endo-surgery, USA) was inserted into the peritoneal cavity. A pneumoperitoneum was created by insufflating CO_2 gas with the pressure kept at 12 mm Hg. The videolaparoscope was warmed by 70°C saline and inserted into peritoneal cavity through the 5 mm trocar. Another small incision (1 cm) was created and a 5 mm trocar (Endopath Xcel F4N315) was inserted into the peritoneal cavity under the view of the videolaparoscope. A Microcapsule Delivery Device (MDD) consisting of a 1 mL-pipette and one 60 mL syringe (Becton Dickinson, NJ, USA) connected by a 15-inch long sterile silicon tube (Masterflex, Cole-Parmer, IL, USA) was inserted through this second incision. Approximately, 80 000 empty microcapsules (approximate 30 mL in volume) supplemented with 200 mL HBSS were infused into the peritoneal cavity at the rate of 30 mL/min. Even distribution of the microcapsules throughout the abdominal cavity was achieved by rotating the 1 mL-pipette of the MDD around the liver, behind the stomach, around the spleen, in the omentum and behind the small bowel. The implantation procedure was filmed using a video recorder (Panasonic VHS, PV-9400). The peritoneal cavity was viewed for complications and unusual events during the implantation procedure.

The implanted microcapsules were retrieved at 1, 2 and 4 weeks after infusion. The surgical set-up for microcapsule retrieval was similar to the implantation procedure. In brief, after anaesthesia, pneumoperitoneum was created and two trocars were inserted in peritoneal cavity of the recipient baboon. Before harvesting the implanted microcapsules, the peritoneal cavity was viewed and detected for microcapsule clumping, inflammatory adhesion and organ abnormality. Pictures were taken using the camera attached to laparoscopy. In order to harvest the microcapsules, a 60-mL of HBSS was injected through MDD to gently flush the microcapsules. The floating microcapsules were aspirated back into the 60 mL syringe and collected for histological analysis.

Statistical analysis

All values were expressed as mean ± SEM, and the statistical analysis package Number Cruncher Statistical Software (NCSS97©) (Utah, USA) was used to perform the statistical analysis. A two-sample *t*-test was used to compare the data between two groups. A one-way analysis of variance (ANOVA) and Duncan's multiple comparison tests were used to compare data from more than two groups. Differences among groups were considered significant if $p < 0.05$.

Results

Prolonged gelling time did not affect bead size

The optical microscope images of 2 and 20 min barium alginate microcapsules are shown in Figure 1(a) and (b). Both batches of microcapsules were washed and their sizes measured 60 min after being stored in fresh CMRL medium and after permeability measurements, respectively. Microcapsules did not exhibit a significant difference in their size either after wash with fresh CMRL medium or after permeability determination (Table 1). In addition, these data show that the droplet size is much better controlled and the polydispersity in the microcapsule diameter is close to the range typically seen for microcapsules prepared by the air-stripping technique.

Table 1. Evaluation of the bead size of 2 and 20 min barium alginate microcapsules before and after permeability measurements by optical microscopy.

No.	Microcapsules	Capsule size measured by optical microscopy (mm)
1	2 min barium alginate microcapsules in saline	0.58 ± 0.11
2	20 min barium alginate microcapsules in saline	0.56 ± 0.04
3	No. 1 washed and stored in fresh CMRL medium after 60 min	0.58 ± 0.05
4	No. 2 washed and stored in fresh CMRL medium after 60 min	0.56 ± 0.08
5	No. 1 after permeability measurement in CMRL medium	0.60 ± 0.03
6	No. 2 after permeability measurement in CMRL medium	0.60 ± 0.05

Note: There was no significant difference in the microcapsule diameters of both 2 and 20 min barium alginate beads at the different time points studied.

Prolonged gelling time did not affect bead permeability

Both types of barium alginate microcapsules, namely 2 and 20 min were analysed using inverse SEC. A typical evaluation of the inverse SEC raw data (elution curves) is shown in Figure 2(a). Pullulan standards of 788 000 and 109 400 Da and dextran standards of 70 000 and 60 000 Da do not permeate the microcapsule pores. Pullulan standards of 47 300, 22 800, 11 800 and 5900 Da permeate partially and 667 and 342 Da standards fully permeate the column (microcapsule) pores. Resulting calibration curves with Boltzmann function fitting are shown in Figure 2(b). A slight shift the fit for 20 min barium alginate microcapsules was considered as negligible and we concluded that microcapsules are characterized by the similar MWCO value being ~50 kDa towards polysaccharide standards, which corresponds to ~250 kDa for proteins. This suggested that both the types of barium alginate microcapsules are permeable to IgG. The similarity in the MWCO of 2 and 20 min gelled barium alginate microcapsules points out that even the gelling for times >2 min may result in a slightly different product the ''outer'' gel layer responsible for the MWCO value does not vary significantly with time in the used range. There is no major difference in the pore size distribution for the all microcapsules.

Prolonged gelling time reduced bead strength

Profiles of compression tests for both batches of microcapsules before and after permeability measurement are shown in Figure 3, with the level of mechanical resistance for each capsule being calculated at 70% of deformation. The mechanical strength of 2 min barium alginate microcapsules of 16 g/microcapsule was greater than that for 20 min barium alginate microcapsules, which had a

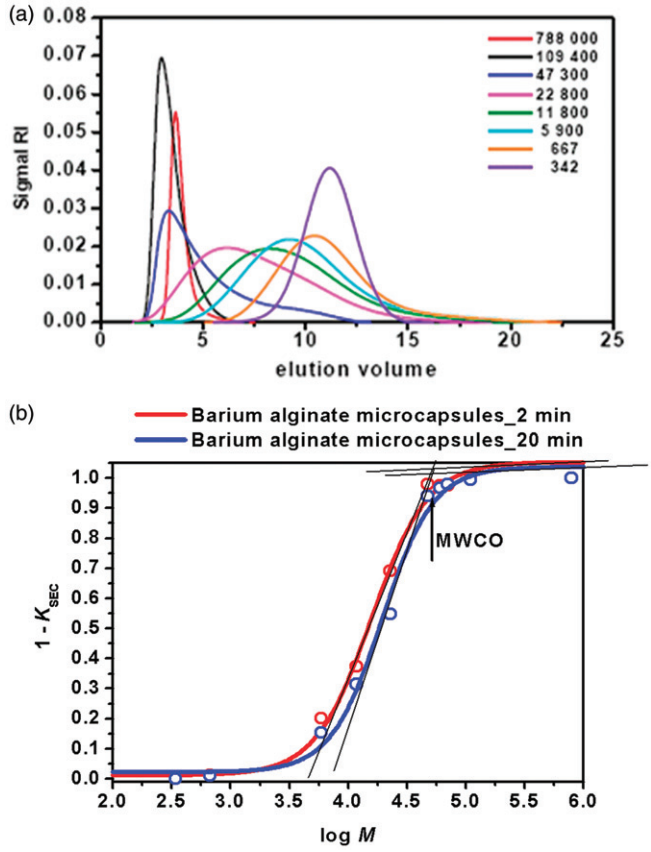

Figure 2. Prolonged gelling time did not affect bead permeability. Evaluation of MWCO for 2 and 20 min barium alginate microcapsules. (a) Representative picture of elution curves for different pullulan standards. (b) Dependence of partition coefficient on molecular weight for pullulan standards in estimating the calibration curve for the column filled with barium alginate microcapsules. Lines represent the best fits of Boltzmann function through the experimental data points. Pullulans with $(1-K_{SEC})\rightarrow 1$ are excluded from pores, pullulans with $(1-K_{SEC})\rightarrow 0$ completely permeate. The MWCO value shown by arrow is at $(1-K_{SEC})\sim 0.9$. This value correspond to ~50 kDa towards polysaccharide standards, which corresponds to ~250 kDa for proteins, suggesting that both the types of microcapsules are permeable to IgG.

mechanical resistance of 7 g/microcapsule. Thus, a longer gelling time leads to more brittle microcapsules.

Prolonged gelling time did not affect viability and insulin secretion of encapsulated human islets

To study the effect of increased gelation in $BaCl_2$ on cell survival and function, human islets were encapsulated in sodium alginate and gelled in 20 mM $BaCl_2$ for 2 and 20 min, respectively. The encapsulated human islets were cultured for a day in supplemented CMRL-1066 media containing 1.5% human albumin before being assessed for viability and glucose induced insulin secretion. Gelation in $BaCl_2$ for 2 or 20 min did not affect the viability of encapsulated human islets, 77.6% ± 1.5% versus 74.5% ± 1.6% (Figure 4(a); (i) and (ii)). Further, prolonged gelation did not alter encapsulated islet function with a stimulation

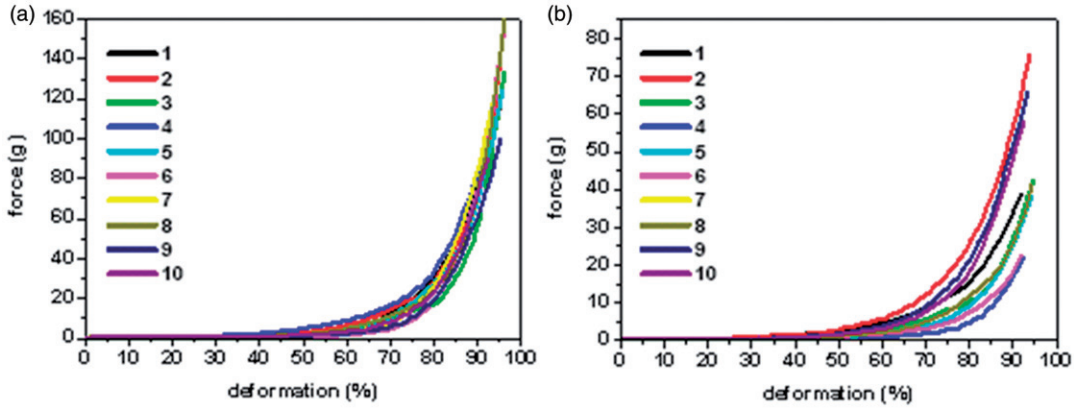

Figure 3. Prolonged gelling time reduced bead strength. Profiles for compression force vs. distance for (a) 2 min and (b) 20 min barium alginate microcapsules. Numbers refer to measurements for different microcapsules in the batch. Representative pictures of 2 and 20 min alginate microcapsules before and after permeability measurements.

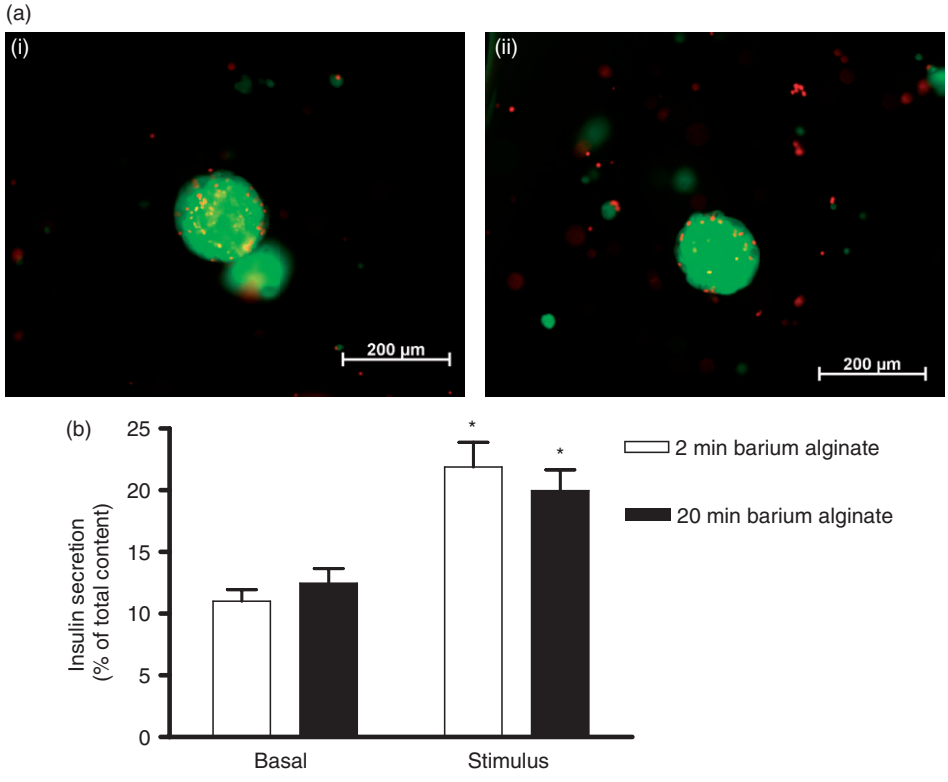

Figure 4. (a) Prolonged gelling time did not affect islet cell viability. Viability of microencapsulated human islets produced by 20 min gelation in $BaCl_2$ was similar to those produced by 2 min gelation in $BaCl_2$, respectively; $74.5 \pm 1.6\%$ vs. $77.6\% \pm 1.5\%$. Values = mean ± SEM, $p > 0.05$, ($n = 3$). (b) Prolonged gelling time did not affect islet function. Basal (2.8 mM) and glucose-stimulated (20 mM) insulin secretions of microencapsulated human islets produced by 20 min gelation in $BaCl_2$ behaved in a manner similar to those produced by 2 min gelation in $BaCl_2$, indicating that prolonged gelling time had no effect on islet function. Values = mean ± SEM. *$p < 0.05$, basal vs. stimulus, ($n = 3$).

indices 1.9 and 1.6 for 2 and 20 min $BaCl_2$ gelation, respectively (Figure 4(b)).

Biocompatibility of barium alginate microcapsules

Since capsules exposed to $BaCl_2$ for 2 min were stronger than those exposed for 20 min, and were equal in the other parameters analysed, it was decided to use them alone to test biocompatibility. For this purpose, empty capsules were transplanted into the peritoneal cavity of three different species of animals, the mice, rat and baboon, and the capsules retrieved at different time points, as mentioned in Table 2.

Mouse

After 2–3 months post-implantation, >90% of the capsules were retrieved from both immunodeficient and

Table 2. Evaluation of retrieved barium alginate microcapsules from the peritoneal cavities of different animal models.

Animal models	Days post-transplantation	Appearance of microcapsules in peritoneal cavity	Percentage of retrieved microcapsules	Percentage of intact microcapsules	Fibrosis
NOD/SCID mouse ($n=4$)	3 months	Free floating	93.5 ± 1.1	>99%	No
BALB/c mouse ($n=4$)	2 months	Free floating	91.3 ± 1.7	>99%	No
C57BL6 mouse ($n=4$)	2 months	Free floating	91.4 ± 1.2	>99%	No
Nude rat ($n=3$)	4 months	Free floating	89.1 ± 1.4	>99%	<1%
Wistar rat ($n=3$)	1 month	Free floating	90.8 ± 1.3	>99%	<1%
Baboon ($n=1$)	1 week	Free floating with some overgrowth	n/a	n/a	Moderate overgrowth
	2 weeks	Interspersed in omental tissue	n/a	n/a	Dense overgrowth
	4 weeks	Aggregated and embedded in omental tissue	n/a	n/a	Dense overgrowth

Note: In the smaller animal models such as rodents majority of the capsules were free floating and intact without any fibrotic overgrowth compared to the dense fibrotic overgrowth seen in a large animal model such as the baboon.

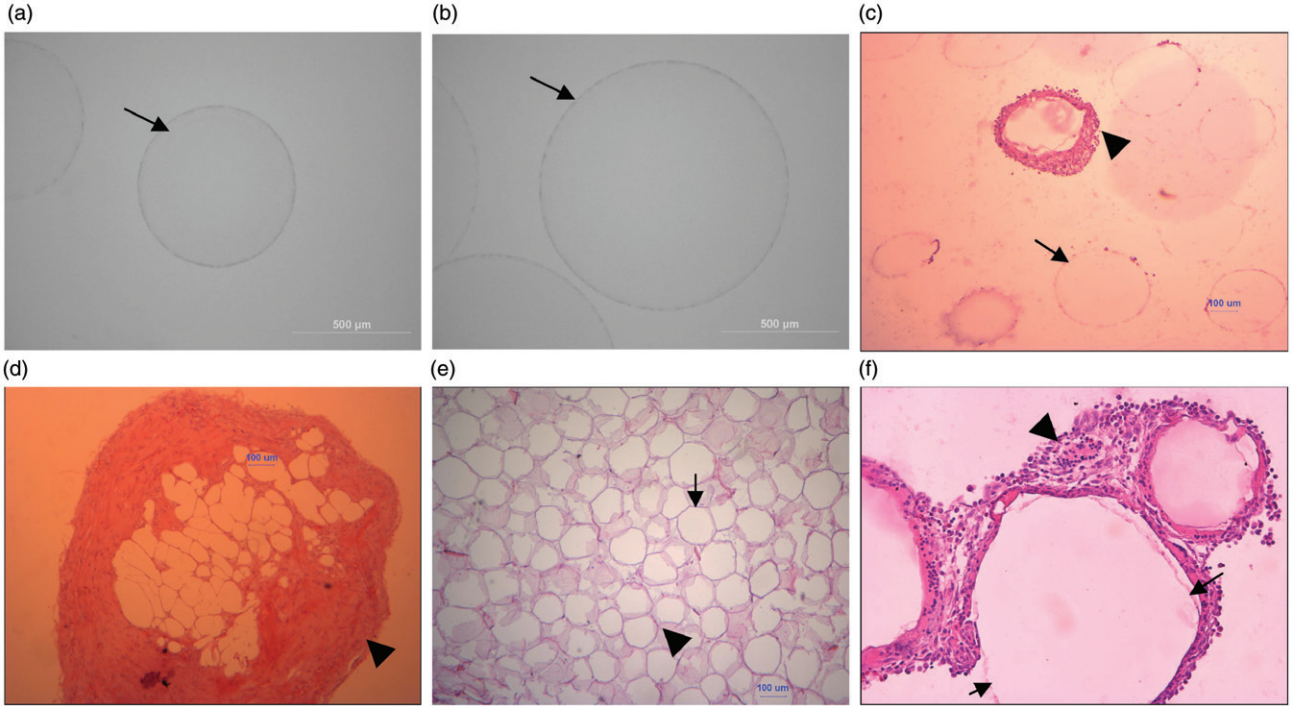

Figure 5. Transplantation of empty barium alginate microcapsules into the peritoneal cavity of different animal models. (a) Representative picture of graft retrieved from both immunodeficient ($n=4$) and immunocompetent mice ($n=8$). (b) Representative picture of graft retrieved from both nude ($n=3$) and Wistar rats ($n=3$). (c)–(e) Histology of the graft retrieved from a baboon at 1, 2 and 4 weeks, respectively. The figures show the presence of dense fibrotic overgrowth around the microcapsules with most capsules embedded within the omental tissue. (f) Higher magnification image of the graft retrieved from a baboon at 1 week post-transplantation. The arrows represent the intact capsule surface and the arrowheads the PCI.

immunocompetent mice. The retrieved microcapsules were freely floating in the peritoneal cavity and intact without any deformities on the capsule surface. The retrieved capsules were clean with no evidence of host cell adherence. This clearly demonstrates the biocompatibility of the barium alginate beads in the mouse (Figure 5(a) and Table 2).

Rat

The outcome in both nude and immunocompetent rats was similar to that observed in the mouse. Graft retrieval after 1–4 months post-implantation showed that ~99% of the microcapsules were clean with no evidence of fibrotic overgrowth and were freely floating in the peritoneal cavity. However, there were a very small number of capsules (<1%) freely floating in the peritoneal cavity but covered with adherent host cells, as seen in Figure 5(b) and Table 2.

Baboon

Contrary to the results in rodents, the outcome in the baboon was different. Transplantation of the microcapsules into the peritoneal cavity of a baboon resulted in a moderate fibrotic overgrowth even at 1 week post-implantation even though most of the capsules were freely floating in the peritoneal cavity (Figure 5(c) and Table 2). However, by 2 weeks post-implantation, most of the capsules were

not freely floating but were interspersed in the omentum surrounded by a dense relatively avascular fibrotic overgrowth (Figure 5(d)). The situation was similar at 4 weeks post-implantation (Figure 5(e) and (f)).

These studies demonstrate that the microcapsules are biocompatible in rodents, but not the baboon.

Discussion

Alginate-based microcapsules, either alginate-poly-L-lysine-alginate (APA) cross-linked with calcium or alginate cross-linked with barium are the most widely studied encapsulation systems for the generation of bioartificial pancreas (de Vos et al., 2006). Despite promising animal studies with both types of microcapsules, there has been limited success with their use in the clinic (Calafiore et al., 2006; Silva et al., 2006). Apart from hypoxia to the encapsulated islets, limited immunoprotection and bioincompatibility are believed to be factors responsible for this (de Groot et al., 2004). The limited immunoprotection may be attributed to antigen shedding or chemokines/cytokines leaking through the pores of microcapsules and initiating an immune response. Thus, strategies aimed at reducing pore size should enhance graft survival and improve outcomes both in allo- and xeno- transplantation settings. Though the presence of poly-L-lysine (PLL) decreased the porosity of APA microcapsules, the immunogenic nature of PLL makes them less preferred compared to barium alginate microcapsules (Vandenbossche et al., 1993a, b; King et al., 2001). However, even in the absence of PLL, the barium alginate microcapsules were prone to immune insult leading to PFO (Silva et al., 2006). This reduced immunoprotection of the barium alginate microcapsules may be attributed to the larger pore size allowing the leakage of immunostimulatory molecules initiating an inflammatory response resulting in PFO.

A detailed study by Morch et al. (2006) indicated that the gelation ion Ba^{2+} rather than Ca^{2+} yielded microcapsules of better strength and stability when used with high G alginate and not with high M alginate. Further, increasing the concentration of barium from 10 to 20 mM in the gelling bath drastically reduced the pore size and diminished the permeability to IgG. However, no study to our knowledge has demonstrated the effect of increasing the gelling time with 20 mM $BaCl_2$ on the intrinsic properties of the barium alginate microcapsules. We hypothesized that increasing the gelling time in 20 mM $BaCl_2$ should reduce the pore size and decrease the permeability. In this study, we used a commercially available high G alginate (PronovaTM UP MVG) to produce alginate beads with different gelling times of 2 and 20 min with $BaCl_2$. The results demonstrated that increasing the gelling time by 10-fold did not alter capsule permeability and both the types of microcapsules exhibited similar pore size distributions (Figure 2). Both types of the microcapsules had a MWCO of ~50 kDa for polysaccharides, which corresponds to ~250 kDa for proteins, thereby making them permeable to IgG, which has a molecular weight of 150 kDa. However, the permeability of alginate beads to IgG may not be a major issue as studies have demonstrated successful reversal of diabetes both in allo- and xenograft settings using microcapsules with high permeability such as high-M barium alginate microcapsules (Duvivier-Kali et al., 2001, 2004; Omer et al., 2003; Schneider et al., 2005). Further, increasing the gelling time reduced the mechanical strength and produced brittle microcapsules (Figure 3). This may be due to the different concentrations of alginate used in our study (2.2%, w/v) compared to the study by Mørch et al. (2006) where they used an alginate concentration of 1.8% (w/v) and a gelling time of 10 min. The authors speculate that increasing the gelling time in barium chloride from 2 to 20 min would have led to the formation of more inhomogeneous microcapsules with less alginate concentration at the surface compared to the centre thereby producing brittle microcapsules (Skjåk-Braek et al., 1989). This study raises the need to optimize the different alginate concentrations and gelling times required to produce the most favourable barium alginate beads used in islet encapsulation studies.

The other major reason for the failure of bioartificial pancreas is bioincompatibility leading to PFO. In this study, we determined the biocompatibility of the barium alginate microcapsules in both small and large animals. In the small animals, barium alginate microcapsules were biocompatible without any PFO despite being in the peritoneal cavity for up to 4 months. The capsules were found freely floating in the peritoneal cavity and were intact with a retrieval rate of ~90% (Table 2 and Figure 5). However, transplantation of empty microcapsules into the large animal resulted in fibrotic overgrowth by 1 week post-transplantation with the intensity of this reaction increasing by 2 weeks. By this time, the capsules were not freely floating but aggregated in the omentum (Table 2 and Figure 5). Similar results have been obtained with empty calcium alginate microcapsules implanted into the peritoneal cavity of the baboon (B Strand, personal communication). However, no such reaction occurs when empty alginate-polyornithine microcapsules are implanted in the peritoneal cavity of the cynomolgus monkey (Elliott et al., 2005). The reason for the fibrotic reaction to the alginate capsules in the baboon as compared to the monkey, rat and mouse, is likely to be an effect of greater adsorption of fibrinogen (Tang and Eaton, 1993) and other proteins onto the surface of the capsules. Such binding leads to adhesion of fibroblast, antigen presenting cells, phagocytes and other anchorage-dependent cells (Collier et al., 1997; Zhang et al., 2001; Castner and Ratner, 2002), further leading to fibrotic overgrowth of the capsules. It is known that the rate of formation of collagen, a key element of fibrous tissue, is greater in the baboon than in the monkey, and much less in the rat and the mouse (Collins and Jones, 1978; Mezey et al., 1983). This enhanced rate of collagen formation in the baboon will help explain why the fibrotic response to the microcapsules was magnified in this animal. Collagen formation in humans is akin to that in the baboon, suggesting that the baboon is a good animal model to study the likely outcome of implanting microcapsules clinically. Indeed, when microcapsules implanted

into humans were retrieved, they were surrounded by dense fibrous tissue (Tuch et al., 2009), although whether this was a response to the microcapsules alone or to antigens shed from the encapsulated islets is unknown.

Conclusion

In summary, this study showed that gelling of high-G alginate beads in $BaCl_2$ for 20 versus 2 min altered neither their permeability nor size, but did result in more brittle microcapsules. The fibrotic response to empty microcapsules in the baboon, but not the mouse or the rat, raises concerns about the use of rodents to predict outcomes when microcapsules are implanted into humans. This is because the rate of formation of collagen, which would be secreted by fibroblasts adhering to the capsules, is similar in the baboon and human, and 4-fold greater than that in the rodent (Collins and Jones, 1978).

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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