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Beneficial Effects of Coating Alginate Microcapsules with Macromolecular Heparin Conjugates–*In Vitro* and *In Vivo* Study

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Pericapsular fibrotic overgrowth (PFO) is associated with poor survival of encapsulated pancreatic islets. Modification of the microcapsule membrane aimed at preventing PFO should improve graft survival. This study investigated the effect of macromolecular Corline Heparin Conjugate (CHC) binding on intrinsic properties of alginate microcapsules and assessed the anti-fibrotic potential of this strategy both *in vitro* and *in vivo*. CHC was bound to alginate microcapsules using a layer-by-layer approach incorporating avidin. CHC binding to alginate microcapsule was visualized by confocal microscopy. Effects of CHC binding on microcapsule size, strength, and permeability were assessed, and the anti-clotting activity of bound CHC was determined by coagulation assay. Effect of CHC binding on the viability of encapsulated human islets was assessed *in vitro*, and their ability to function was assessed both *in vitro* and *in vivo* in diabetic immunodeficient mice. The potential of bound CHC to reduce PFO was assessed *in vivo* in different rat transplantation models. Confocal microscopy demonstrated a uniform coating of CHC onto the surface of microcapsules. CHC binding affected neither size nor permeability but significantly increased the tensile strength of alginate microcapsules by ~1.3-fold. The bound CHC molecules were stable and retained their anti-clotting activity for 3 weeks in culture. CHC binding affected neither viability nor function of the encapsulated human islets *in vitro. In vivo* CHC binding did not compromise islet function, and diabetes was reversed in all recipients with mice exhibiting lower blood glucose levels similar to controls in oral glucose tolerance tests. CHC binding was beneficial and significantly reduced PFO in both syngeneic and allogeneic rat transplantation models by ~65% and ~43%, respectively. In conclusion, our results show a new method to successfully coat CHC on alginate microcapsules and demonstrate its beneficial effect in increasing capsule strength and reduce PFO. This strategy has the potential to improve graft survival of encapsulated human islets.

Introduction

Microencapsulating pancreatic islets is a strategy being explored as a treatment for type 1 diabetes that may overcome the immune-mediated rejection of the graft without toxic immunosuppression. Microencapsulated islets have been shown to normalize glucose levels for extended periods in both chemically induced and spontaneously diabetic animal models of allo- and xeno- transplantation.[1] However, in all these studies, graft survival was limited and varied considerably, ranging from months to a few years. Phase 1 clinical trials using microencapsulated human islets showed that allografting of microencapsulated human islets was safe but provided only a minor clinical benefit.[2–4] Laparoscopic retrieval of the microcapsules from a human recipient at 1.3 years post-transplantation showed the presence of dense pericapsular fibrotic overgrowth (PFO) surrounding the microcapsules with no viable islets.[3] The major factor responsible for the poor graft survival may be attributed to PFO that starts in the early post-transplantation period,[5–8] as

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either a foreign body reaction against the microcapsules and/or an immunological response to small-molecular-weight antigens shed by the encapsulated islets.[9–11] This fibrotic overgrowth, which consists mainly of macrophages and fibroblasts,[12,13] interferes with the transport of nutrients and oxygen, consequently leading to hypoxia, islet cell starvation, and apoptosis.[14] Further, the immune cells in the pericapsular infiltrate can release deleterious cytokines/chemokines that are capable of permeating through the capsule pores and damaging the islets.[14] Thus, modification of the capsule membrane aimed at preventing PFO should enhance islet survival and improve transplant outcomes.

Many strategies such as dual costimulatory blockade,[15] co-encapsulation with sertoli cells, and the use of steroids[16,17] and antifibrotic agents such as HOE 077[18] have been used to try and prevent PFO in a preclinical setting. None have yet been tried in humans. Coating of heparin on biomaterials has been shown to improve their biocompatibility by inhibiting fibrotic overgrowth.[19] In this study, we coated the alginate microcapsules with macromolecular Corline Heparin Conjugates (CHC), a strategy that has been successfully used on nonencapsulated islets[20,21] and is being prepared for clinical trial. However, to our knowledge, there are no studies investigating the binding of CHC on alginate microcapsules. So, in this study, we investigated whether CHC could be bound efficiently to alginate microcapsules without adversely affecting the function of the encapsulated cells or the intrinsic properties of the microcapsules. The effect of CHC binding on encapsulated islet function was assessed both *in vitro* and *in vivo*, and its anti-fibrotic potential was investigated in both syngeneic and allogeneic transplants.

Materials and Methods

Alginate microcapsules

Barium alginate microcapsule formation was carried out by extruding highly purified 2.2% alginate (60:40 glucuronic acid: mannuronic acid, UPMVG PRONOVA; FMC Biopolymer) solution through a stainless steel air-driven droplet generator (Steinau) and collecting the droplets in a barium chloride (20 mM) precipitation bath as previously described.[22]

CHC binding to microcapsules

Alginate microcapsules were washed twice in sterile 0.9% saline and incubated with avidin (0.5 mg/mL; Corline Systems AB) in a volumetric ratio of 1:1 for 15 min at room temperature, followed by washings and incubation with CHC (1 mg/mL; Corline Systems AB) in a volumetric ratio of 2:3 for 60 min at room temperature with gentle mixing. Final washing in 0.9% saline was done twice to remove any unbound CHC. Alginate microcapsules without CHC served as controls. Hereafter, the CHC-coated alginate microcapsules are referred to as "heparinized microcapsules," and the nonheparin-coated microcapsules are referred to as "non-heparinized microcapsules," respectively.

Confocal microscopy

CHC bound to alginate microcapsules was visualized by confocal microscopy after incubating empty microcapsules for 15 min with avidin-Texas red (0.01 mg/mL) (Molecular Probes Europe BV) followed by incubation with CHC (0.02 mg/mL) labeled with Alexa 488 (Molecular Probes Europe BV) for 60 min at room temperature. Images were acquired with a Nikon confocal microscope A1 (Nikon Corporation) equipped with a Nikon Eclipse inverted Ti microscope stand using the 487 and 561 nm laser line, 500–550 and 570–620 band pass filters for Alexa 488 and Texas Red, respectively. Three-dimensional projections of the acquired Z-stacks were analyzed using NIS-Elements software (Nikon Corporation).

Intrinsic properties of microcapsules

Size. The size of heparinized and non-heparinized microcapsule was estimated using optical microscopy as previously described.[23] The diameter of randomly selected microcapsules was measured, and the mean ± SEM was calculated.

Strength. The mechanical properties of heparinized and non-heparinized microcapsules were tested on a Texture Analyzer TA-2Xi (Stable Micro Systems) that was equipped with a force transducer of resolution 1 mN as previously described.[23]

Permeability. Penetration of IgG labeled with Alexa Fluor 488 (Molecular Probes-Invitrogen) into heparinized and non-heparinized microcapsules was measured by confocal laser scanning microscopy using a microscope Zeiss LSM 510 (Carl Zeiss) on Axiovert 200 with objective 20 × LD Achroplan and pinhole 1.8 airy unit. Briefly, 3–5 microcapsules were incubated for 2 and 24 h, respectively, in 10^{-7} M IgG solution in CMRL medium containing 0.1 wt% sodium azide. The excitation wavelength of 488 nm and emission wavelength of 505 nm were used. The percentage of mean fluorescence intensity of penetrated IgG was calculated as

$$I_{mean} = \frac{I_{inside}}{I_{outside}}.100,$$

where I_{inside} and I_{outside} are the intensities measured inside and outside (in the IgG solution) of microcapsules, respectively.

Activated partial thromboplastin time assay

The anticoagulant activity of bound CHC on empty alginate microcapsules was determined by activated partial thromboplastin time (APTT) assay. Briefly, the heparinized and non-heparinized microcapsules (100 μL) were added into an eppendorf tube containing 500 μL of fresh human plasma. Homogenization of the microcapsules was carried out using a glass rod, and the resulting plasma supernatant was collected. The plasma supernatant was then incubated with 0.1 mL of APTT reagent (TriniCLOT™ aPTT S; Tcoag Ireland Limited) for 2 min at 37°C. After incubation, 0.1 mL of 0.02 M calcium chloride was added, and the time was recorded from this point until the fibrin clot was formed. Both positive and negative controls, namely plasma with heparin conjugate and plasma alone, were used.

Islet isolation, encapsulation, and CHC binding

Rat islets. Male Lewis rats (Harlan Industries), weighing 175–200 g, were used as pancreas donors for islets. A

laparotomy was performed followed by incision into the thoracic cavity and section of the heart for euthanasia by exsanguination. Collagenase type XI (Sigma) 2.2 mg/mL was injected via the bile duct into the main pancreatic duct to distend the pancreas. After excision, each pancreas was suspended in 7.5 mL of its respective perfusion solution, incubated at 37°C for 16 min followed by washing in ice-cold RPMI media. Islets were then separated from exocrine tissue by discontinuous Ficoll density gradients (Mediatech, Inc.) and cultured in supplemented RPMI media for 24 h before encapsulation. The islets were then encapsulated in 2.2% alginate solution (1:8) and heparinized as described earlier.

Human islets. Human islets were isolated from pancreases of brain dead cadaveric multiorgan donors ($n=3$) at the Cell Isolation Laboratory of the University of Illinois at Chicago, USA, as previously described[24] before being shipped to Sydney. Viability of these islets has been previously demonstrated.[25,26] The islets were then encapsulated in 2.2% alginate solution (1:8) and heparinized as described earlier.

FAO-ins culture, encapsulation, and CHC binding

FAO-ins is an insulin synthesizing rat liver cell line (FAO) that is derived from the Reuber H35 rat hepatoma belonging to the inbred AxC rat strain.[27] The cells were grown to confluence in high glucose supplemented DMEM media as previously described.[28] Cells were trypsinised to form a single-cell suspension for cell counting and encapsulated in 2.2% alginate solution (1:8) and heparinized as described earlier.

Viability

Viability of cultured heparinized and non-heparinized encapsulated human islets or FAO-ins cells was assessed 24 h postencapsulation using live/dead staining with the fluorescent dyes 6-carboxyfluorescein diacetate (6-CFDA; Sigma) and propidium iodide (PI; Sigma) as previously described.[26]

Insulin secretion and content of encapsulated human islets

Insulin secretion and content were determined as previously described.[26] Briefly, aliquots of heparinized and non-heparinized encapsulated human islets were exposed to either 2.8 mM glucose (basal) or 20 mM glucose (stimulus) for 1 h at 37°C, and the supernatant was collected for insulin measurement by radioimmunoassay (RIA; Linco). The remaining pellet was lysed with cold acid ethanol, kept at 4°C overnight, and the supernatant was analyzed for insulin content by RIA.

Transplantation of encapsulated human islets into immunodeficient mice

Encapsulated human islets were injected into the peritoneal cavity of diabetic male NOD/SCID mice (6–8 weeks) using a 20-gauge catheter as previously described.[26] Mice were transplanted with 2000 IEQs of either heparinized or non-heparinized encapsulated human islets. An oral glucose tolerance test (OGTT) was carried out by fasting the animals overnight followed by oral gavage (3 mg/g of 300 mg/mL glucose solution), and BGLs were measured at 0, 20, 40, 60, and 120 min after glucose administration. Blood samples (200 μL) were collected both before and 20 min after glucose administration, and human C-peptide was measured by RIA (Linco Research).

Transplantation of encapsulated islets or FAO-ins cells into immunocompetent rats

Syngeneic transplantation. Male Lewis rats (10 weeks) were transplanted intraperitoneally with non-heparinized or heparinized encapsulated syngeneic rat islets using a 20-gauge catheter. Each rat received 1000 IEQ encapsulated islets in 3 mL of 0.9% saline.

Allogeneic transplantation. Female Wistar rats (4–5 weeks) were transplanted intraperitoneally with both heparinized and non-heparinized encapsulated FAO-ins cells (30×10^6 cells per animal). Wistar rats express the major histocompatibility locus RT1u, while the FAO-ins cell line that was derived from the inbred AxC rat strain,[27] a substrain of ACI rats which express the major histocompatibility locus RT1a. As such, the transplantation of FAO-ins cells into Wistar rats represents an allogeneic transplant.

Transplantation of empty microcapsules. As controls, non-heparinized empty barium alginate microcapsules were transplanted into the peritoneal cavity of both Lewis and Wistar rats.

Graft retrieval and evaluation of pericapsular fibrosis

The rats transplanted with encapsulated rat islets, FAO-ins cells, and empty microcapsules were culled at 3 weeks post-transplantation and microcapsules were recovered by peritoneal lavage as previously described.[29] Microapsules were assessed for (1) retrieval rate, (2) state of retrieved microcapsules, (3) evidence of breakage, (%) and (4) fibrotic overgrowth.

(1) Graft retrieval rate was calculated using the equation, Graft recovery (%) = (PCV post-transplant/PCV pre-transplant) ×100, where PCV is the packed capsule volume.
(2) State of the retrieved microcapsules was determined by manual observation as to whether they were freely floating in the peritoneal cavity, clumped, or adherent to abdominal organs.
(3) Number of broken microcapsules was calculated using the equation,

Intact microcapsules (%) = (No. of broken microcapsules/Tot) ×100, where Tot is the total number of microcapsules analyzed for that sample.

(4) A random aliquot of retrieved microcapsules was taken from each rat, and the degree of fibrotic overgrowth was assessed in a blinded fashion using the scoring system as described elsewhere[29]: Score 0 = no overgrowth, Score 1+ = < 25% overgrowth, Score 2+ = 25–50% overgrowth, Score 3+ = 50–75% overgrowth, and Score 4+ = > 75% overgrowth. The cell adhesion score was calculated as (0 x% Score 0) + (2 x% Score 1+) + (4 x% Score 2+) + (8 x% Score 3+) + (16 x%Score 4+).

Histological analysis

Retrieved microcapsules were fixed in 10% neutral buffered formalin, embedded in paraffin blocks, sectioned at

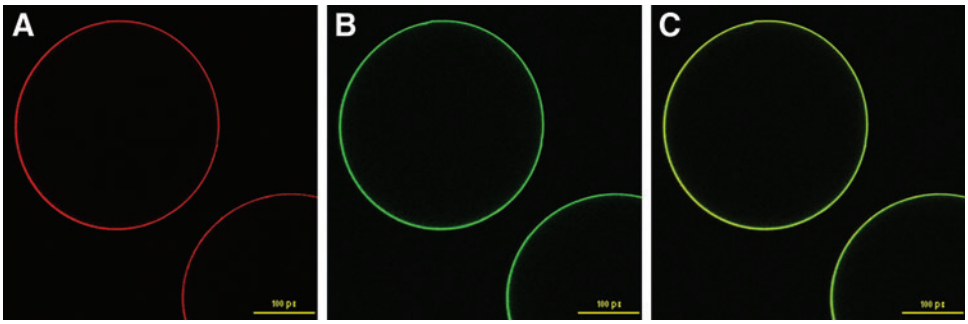

FIG. 1. Corline Heparin Conjugates (CHC) bound to surface of alginate microcapsules. Confocal microscopy images of CHC bound to the surface of barium alginate microcapsules showing the adsorption of **(A)** avidin (labeled with Texas Red, red) and **(B)** CHC (labeled with Alexa 488, green) and **(C)** merged images of avidin and CHC on the surface of microcapsules. Color images available online at www.liebertpub.com/tea

5 μm intervals, and stained with haematoxylin-eosin to assess the degree of PFO.

Ethics approval

All procedures relating to mice and rats were approved by the appropriate Institution's Animal Ethics Committee. Approval to human islets was obtained from the Institution's Human Research Ethics Committee.

Statistical analysis

All data are presented as mean ± standard error of mean (SEM). Differences between two groups were analyzed by the two-tailed Student's *t*-test and of more than two groups by one-way ANOVA with *post-hoc* Duncan's Multiple-Comparison test using NCSS 2004 (NCSS). Significant differences among data groups were assigned when $p < 0.05$.

Results

CHC binding increased capsule strength without altering permeability

Confocal microscopy images demonstrated that a uniform coating of CHC was established on the surface of the alginate microcapsules using the simple two-step procedure incorporating avidin as the cationic linker (Fig. 1A–C). CHC binding did not alter the microcapsule size but significantly enhanced the mechanical strength by ~1.3-fold compared with non-heparinized capsules (Table 1). CHC binding did not alter permeability, and both types of microcapsules were permeable to IgG, with mean fluorescent intensity similar at ~25% (Fig. 2).

Table 1. Effect of Corline Heparin Conjugates Binding on Size and Strength of Microcapsules

Microcapsule properties	*Nonheparinized microcapsules*	*Heparinized microcapsules*
Size (μm)	575 ± 0.011	593 ± 0.007
Mechanical strength–compression resistance at 65% deformation (g/microcapsule)	10.1 ± 0.58	12.9 ± 0.57[a]

CHC binding did not alter the size but increased the strength of alginate microcapsules. Values = mean ± SEM.; $p = 0.185$ for size ($n = 120$ capsules for each group).

[a]$p < 0.001$ for strength ($n = 25$ capsules for each group) between nonheparinized and heparinized empty alginate microcapsules (Student's *t*-test).

CHC, Corline Heparin Conjugates.

Bound CHC retained its anti-clotting activity

The clotting time for empty heparinized microcapsules was significantly higher at all the time points measured compared with the non-heparinized and normal plasma controls (Table 2). However, the clotting time of heparinized microcapsules diminished with time, being 58.1 ± 0.63 s at day 21 compared with 170 s on day 1 post-heparinization.

CHC binding did not affect cell viability

CHC immobilization did not alter the viability of encapsulated FAO-ins cells (heparinized vs. non-heparinized: 76.6% ± 1.43% vs. 74.7% ± 1.44%; $p = 0.356$; $n = 100$ for each group) or human islets (heparinized vs. non-heparinized:

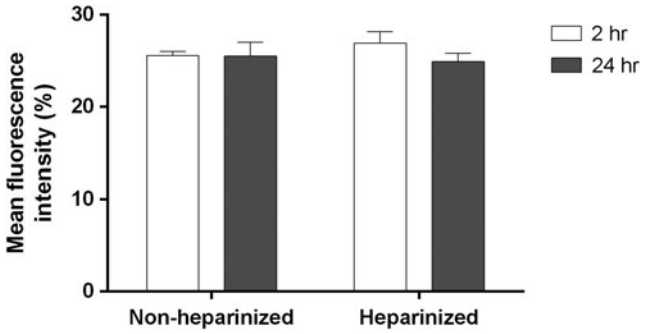

FIG. 2. Effect of CHC binding on permeability of alginate microcapsules. Quantification of permeated-labeled IgG as a percentage of mean fluorescence intensity measured inside the capsule compared with the intensity detected outside. Values are corrected for background before rationing. Values = mean ± SEM ($n = 5$); $p = 0.306$ and $p = 0.732$ for mean fluorescence intensity between non-heparinized and heparinised microcapsules for 2 and 24 h respectively (Student's *t*-test).

Table 2. Anti-clotting Activity of Bound Corline Heparin Conjugates Measured Over 21 Days Postheparinization

Groups	*Day 1 postheparinization (s)*	*Day 7 postheparinization (s)*	*Day 14 postheparinization (s)*	*Day 21 postheparinization (s)*
Plasma alone	35.7 ± 0.58	35.2 ± 0.91	34.7 ± 0.64	36.4 ± 0.35
Plasma + non-heparinized microcapsules	47.4 ± 0.83	47.6 ± 1.02	46.2 ± 1.04	48.3 ± 1.03
Plasma + heparinized microcapsules	170 ± 0.0[a]	84.3 ± 3.07[a]	74.9 ± 2.37[a]	58.1 ± 0.63[a]
Plasma + heparin	170	170	170	170

APTT test was carried to determine the long-term bioactivity of CHC coated onto empty alginate microcapsules. Values = mean ± SEM ($n = 3$ for each time point and group).

[a]$p < 0.001$ clotting times (in second) for heparinized microcapsules at days 1, 7, 14, and 21 compared with non-heparinized microcapsules and plasma alone at each time point (one-way ANOVA with *posthoc* Duncan's Multiple-Comparison test). Upper limit of the assay is 170 s.

87.7% ± 0.66% vs. 86.3% ± 0.52%; $p = 0.119$; $n = 100$ for each group) as assessed by live/dead staining.

CHC binding did not affect human islet function in vitro

Heparinization of alginate microcapsules also did not affect the function of human islets, as assessed by secretion of insulin by static exposure to 20 mM glucose. Heparinized encapsulated islets secreted insulin in a similar manner to non-heparinized controls, with a stimulation index of 1.9 versus 2.4 respectively (Fig. 3).

CHC binding preserved human islet function in vivo

All the mice receiving heparinized encapsulated human islets achieved euglycemia by 3.3 ± 0.88 days (range 2–5 days), and the BGLs declined from 23.1 ± 1.41 to 4.6 ± 0.31 mM, similar to non-heparinized controls. All these animals remained euglycemic till the end of the study at 60 days post-transplantation (Fig. 4A). To assess graft function further, an OGTT was carried out at 30 days post-transplantation. All the mice receiving heparinized and non-heparinized encapsulated human islets handled glucose normally and exhibited lower blood glucose levels

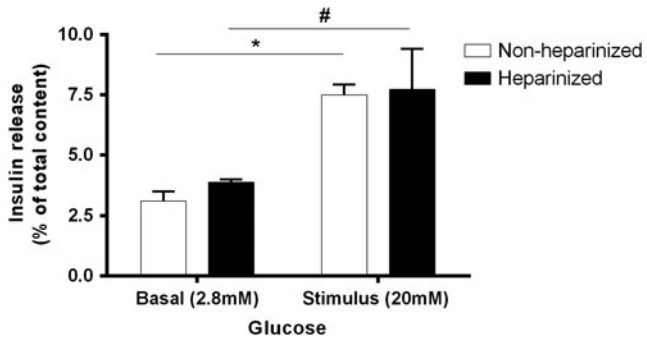

FIG. 3. Effect of CHC binding on islet function *in vitro*. Effect of heparin binding on encapsulated human islet function after 24 h: basal (2.8 mM glucose) and stimulated (20 mM glucose) insulin secretion as measured by RIA. Values are mean ± SEM ($n = 3$); *$p < 0.001$ and #$p = 0.041$ for basal versus stimulated insulin secretion for non-heparinized and heparinized encapsulated human islets, respectively (Student's *t*-test).

than the diabetic controls but similar to what was observed with nondiabetic controls (Fig. 4B). Further, both groups of mice responded with a significant rise in the plasma human C-peptide levels 20 min after oral glucose administration (Fig. 4C).

CHC binding reduced PFO in both a syngeneic and allogeneic transplantation setting

Syngeneic transplantation. All microcapsules retrieved at 3 weeks post-transplantation were intact and free floating without any clumping or adherence to abdominal organs (Table 3 and Fig. 5A, B). Retrieval rates in both non-heparinized and heparinized groups were similar to controls transplanted with empty microcapsules. The majority of microcapsules had no PFO, and the percentage of microcapsules with a score 0 was similar in both heparinized (87.3% ± 6.17%) and non-heparinized (85.7% ± 6.55%) groups (Fig. 6A). On the contrary, microcapsules with a fibrotic score ≥ 3 + were seen only in the non-heparinized groups (ranging from 0.7–2.0 ± 0.67–0.58%). The extent of PFO as measured by the cell adhesion score was significantly reduced by ~2.7-fold in the heparinized compared with the non-heparinized group (0.26 ± 0.13 vs. 0.75 ± 0.06) (Fig. 6B).

Allogeneic transplantation. All microcapsules retrieved at 3 weeks post-transplantation were intact, and the retrieval rates were similar in both non-heparinized and heparinized groups (Table 3 and Fig. 5C, D). In the group receiving non-heparinized encapsulated FAO-ins cells, most microcapsules had a fibrotic score of 3 + (25.8% ± 1.68%) or 4 + (65.6% ± 1.81%) and were found clumped and adherent to abdominal organs (Fig. 6C). On the other hand, in rats that received heparinized encapsulated FAO-ins, microcapsules were freely floating, with no clumping, and very few capsules adherent to abdominal organs. The majority of retrieved microcapsules in the heparinized group had a fibrotic score of 2 + (28.8% ± 4.42%) or 3 + (42.2% ± 1.68%), and the percentage of microcapsules with a score of 4 + (16.8% ± 1.36%) was less than that of the non-heparinized group (Fig. 6C). The extent of PFO as measured by the cell adhesion score was also significantly reduced by ~1.8-fold in the heparinized compared with the non-heparinized group (7.3 ± 0.22 vs. 12.9 ± 0.27) (Fig. 6D). Histological analysis showed the presence of dense fibrotic overgrowth

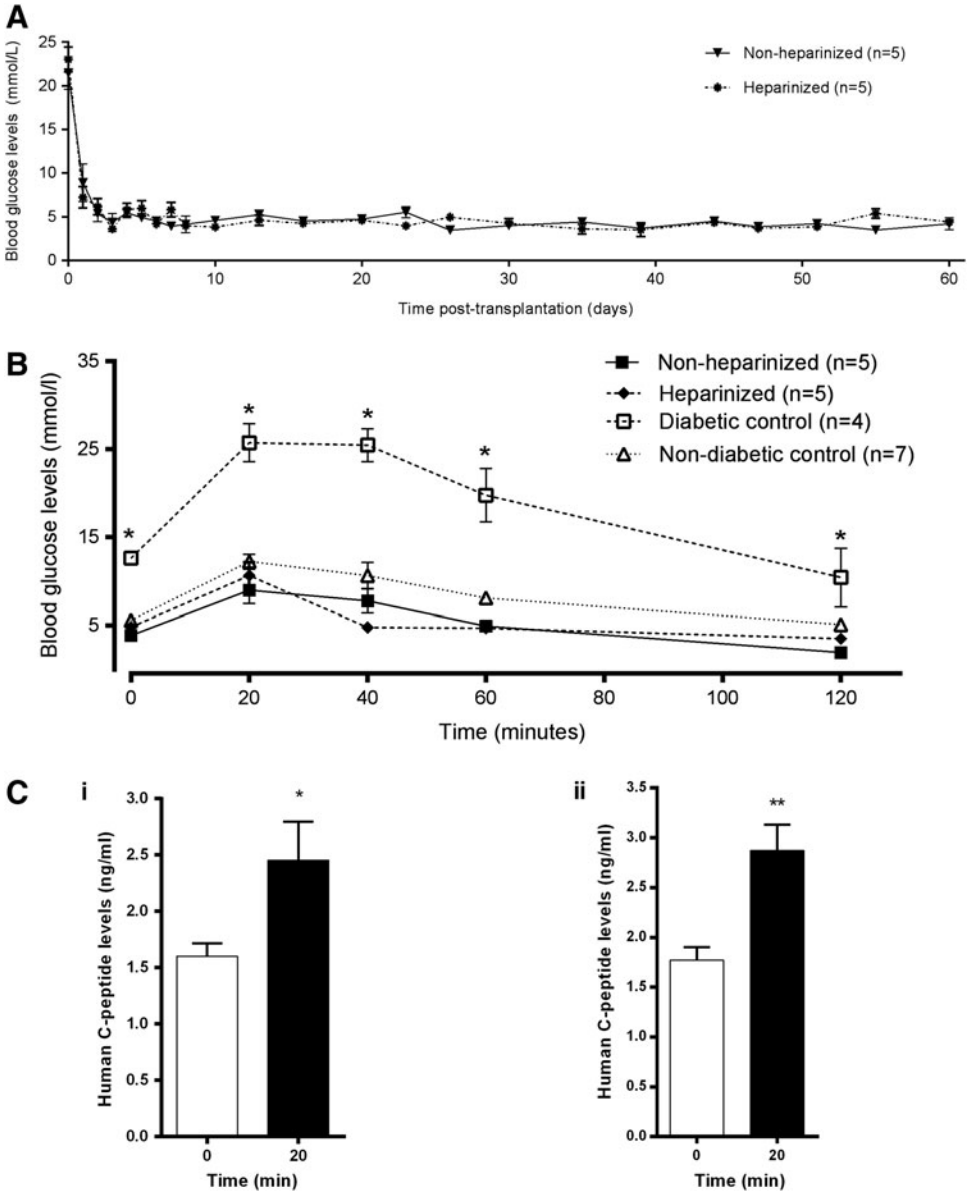

FIG. 4. Effect of CHC binding on islet function *in vivo*. 2000 IEQs of heparinized or non-heparinized encapsulated human islets were transplanted into the peritoneal cavity of diabetic NOD/SCID mice and monitored for approximately 2 months post-transplantation. **(A)** Normoglycemia was achieved in 100% mice receiving both non-heparinized and heparinized encapsulated human islets by 3.3 ± 0.88 days post-transplantation. Values = mean ± SEM. **(B)** OGTT done at day 30 post-transplantation after normalization of BGL. Values = mean ± SEM; $*p < 0.001$ at 0, 20, 40, 60, and 120 min; at all time points, diabetic control > nondiabetic, heparinized and nonheparinized groups (One-way ANOVA with *posthoc* Duncan's Multiple-Comparison test). **(C)** Human C-peptide levels at 0 and 20 min after oral glucose administration in mice transplanted with (i) non-heparinized and (ii) heparinized encapsulated human islets, respectively. Values = mean ± SEM ($n = 5$); $*p = 0.029$ and $**p = 0.0019$ at 0 and 20 min for mice receiving non-heparinized and heparinized encapsulated human islets, respectively (Student's *t*-test).

surrounding a single or group of microcapsules in the non-heparinized (Fig. 7A, B) compared with the heparinized microcapsules (Fig. 7C, D).

In the control Lewis and Wistar rats, that received non-heparinized empty alginate microcapsules, there was no fibrotic overgrowth in the majority of the microcapsules (Table 3 and Fig. 5E, F). Almost all (≥95%) were free floating and had a fibrotic score of 0, with < 5% having a fibrotic score of 1 + (Fig. 6A, C), with a cell adhesion score of 0.08–0.09 ± 0.014–0.006 (Fig. 6B, D). Histological analysis confirmed the absence of PFO on the surface of empty microcapsules (Fig. 7E, F).

Discussion

PFO is one of the major reasons for the limited graft survival of a bioartificial pancreatic device[14] due to the adsorption of fibrinogen and other proteins to the biomaterial surface. This aids in macrophage binding, thereby initiating a foreign body reaction.[30–32] Heparin-coated polymers have been shown to reduce fibrinogen adsorption compared with uncoated polymers, thus providing a strategy to reduce inflammation and prevent fibrotic overgrowth.[33–35] Thus, we hypothesized that anchoring heparin conjugates (CHC) to alginate microcapsules should improve the biocompatibility and prevent PFO in the immediate post-transplantation period. In this study, we have reported a simple method to bind CHC to the surface of the barium alginate microcapsules. Heparin conjugation has previously been shown to improve the compatibility of varied biomaterials in both experimental and clinical studies.[21] The CHC used in this study was a water-soluble macromolecule consisting of 70 heparin molecules bound to a carrier chain.[36] These heparin conjugate molecules being negatively charged can bind strongly to any cationic surface. Alginate polymers are composed of β-D-mannuronic and α-L-guluronic acids with

Table 3. Morphological Assessment of Grafts Retrieved from the Peritoneal Cavity of Immunocompetent Rats in Different Transplantation Settings

Groups	*Graft recovery (%)*	*Intact microcapsules (%)*	*State of retrieved microcapsules*
A. Transplantation of empty microcapsules			
Non-heparinized ($n=8$)	82.2 ± 3.20	100	FF
B. Syngeneic transplantation			
Non-heparinized ($n=3$)	80.3 ± 2.67	100	FF
Heparinized ($n=3$)	86.0 ± 3.00	100	FF
C. Allogeneic transplantation			
Non-heparinized ($n=5$)	71.2 ± 4.52	100	FF, A and C
Heparinized ($n=5$)	69.6 ± 4.23	100	FF and few A

FF–Freely floating in the peritoneal cavity.
A–Adherent to abdominal organs.
C–Clumping of microcapsules.

different compositions and spatial arrangements conferring a net negative charge to the capsule surface.[37] Since CHC can only bind to a cationic surface, the coating of the microcapsule with avidin renders its ionic charge positive. Avidin has a strong affinity for heparin,[38] making for the ready binding of CHC onto the microcapsule surface. By this method, we have achieved a uniform coating of CHC on the surface of alginate microcapsules.

This chemical approach to anchor CHC was chosen, as it did not interfere with the antithrombin (AT)-binding sites of heparin. Further, CHC binding altered neither size nor permeability of the alginate microcapsules but increased its tensile strength by ~28%. However, the heparin bioactivity was gradually lost during time in culture as evident from the decreased clotting times in the APTT assay. Despite this gradual loss over 3 weeks the anchored CHC achieved an

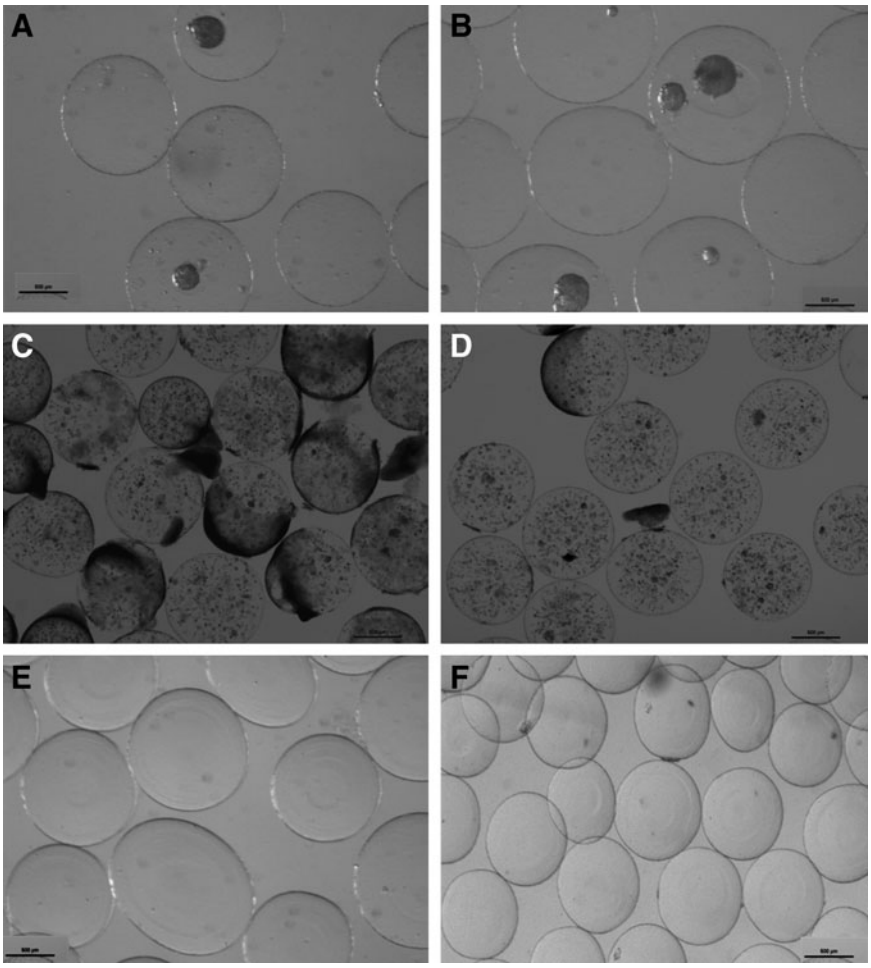

FIG. 5. Host cell adhesion to grafts in different transplant settings 3 weeks after implantation in the peritoneal cavity of immunocompetent rats. Encapsulated rat islets **(A**-non-heparinized; **B**-heparinized**)**, encapsulated FAO-ins cells **(C**-non-heparinized; **D**-heparinized**)** and non-heparinized empty microcapsules **(E**-Lewis rats and **F**-Wistar rats**)**.

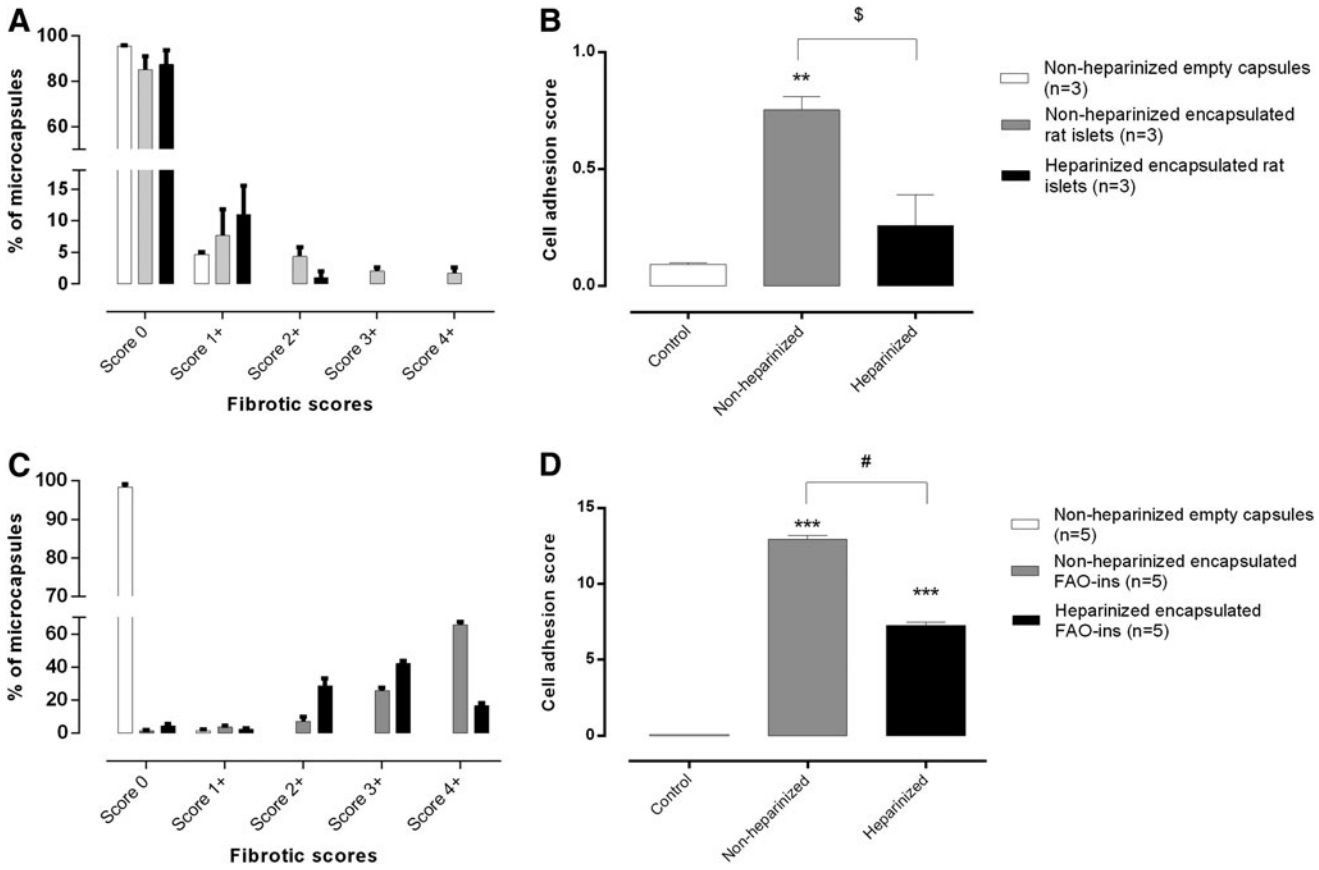

FIG. 6. Assessment of pericapsular fibrotic overgrowth. The fibrotic scores **(A, C)** and cell adhesion scores **(B, D)** on retrieved microcapsules in both syngeneic **(A, B)** and allogeneic **(C, D)** transplantation setting. Extent of host cell adhesion on retrieved grafts is represented by a cell adhesion score, on a scale of 0 (no cell adhesion) to 16 (complete host cell adhesion). Values are mean ± SEM; $^{**}p < 0.001$ and $^{***}p < 0.0001$ for cell adhesion scores of non-heparinized and heparinized microcapsules compared with non-heparinized empty capsules (control) in all transplantation settings (One-way ANOVA with *posthoc* Duncan's Multiple-Comparison test); $^{\$}p = 0.025$ and $^{\#}p < 0.001$ for cell adhesion scores between non-heparinized and heparinized groups in syngeneic and allogeneic transplantation setting, respectively (Student's *t*-test).

anticoagulation effect. A similar approach incorporating biotin, avidin, and CHC has been used to anchor heparin onto the surface of nonencapsulated islets to prevent the instant blood-mediated inflammatory reaction (IBMIR), with complete loss of the heparin coating at 4 weeks post-transplantation.[20,21] The loss of heparin bioactivity at 3–4 weeks postheparinization might not be a major concern *in vivo* considering that PFO occurs in the immediate post-transplantation period.[39] The CHC binding process employed in this study was rapid, unlike other more complex heparinization procedures that have been reported[40,41] and gentle on the encapsulated cells. The viability of heparinized and non-heparinized encapsulated cells/islets was not altered by the heparinization procedure. Further, the heparinization process did not affect the capacity of human islets to secrete insulin in response to glucose, with encapsulated islets responding in a similar manner in both heparinized and non-heparinized microcapsules when tested *in vitro*. Moreover, all the diabetic immunodeficient mice that received either heparinized or non-heparinized encapsulated human islets achieved euglycemia within 4 days post-transplantation and subsequently responded normally to an OGTT showing that CHC binding did not affect islet function *in vivo*. Similar results have been reported to occur when CHC were bound to nonencapsulated human, porcine, and mouse islets, with islet function preserved both *in vitro* and *in vivo*.[20]

Though alginate microcapsules can protect the encapsulated tissue from host immune cells and large cytotoxic antibodies such as IgM (but not IgG), they are still vulnerable to small molecules such as chemokines/cytokines that can damage the graft. The molecular weight cut-off of the barium alginate microcapsules used in this study was ~250 kDa with regard to proteins,[23] thereby making them susceptible to small cytotoxic chemokines such as interleukin-1beta (IL-1β), interferon-gamma (IFN-γ), and tissue necrosis factor-alpha (TNF-α), which can cause β-cell death.[42] Further, IL-1β can induce β-cells to secrete monocyte chemoattractant protein-1 (MCP-1) (12 kDa), which can leak through the capsule pores and attract macrophages, thereby initiating an immune cascade leading to PFO and graft failure.[9] Therefore, modifying the microcapsule surface to adsorb these small chemokines/cytokines should prevent PFO in the early post-transplantation period and enhance graft survival. It is known that heparin and polymers with heparin-like properties can adsorb cytokines and, hence, prevent their entry into the capsule.[43] In our study,

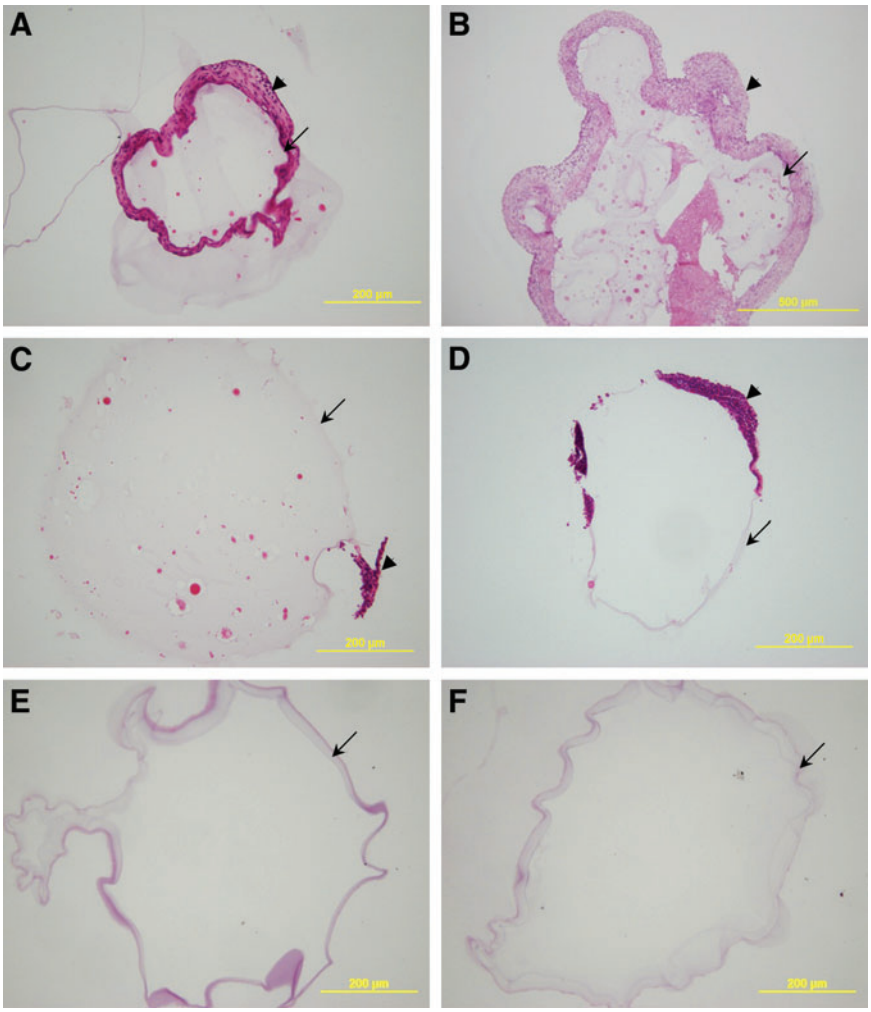

FIG. 7. Histology of retrieved grafts. Hematoxylin-eosin staining of non-heparinized **(A, B)** and heparinized **(C, D)** encapsulated FAO-ins retrieved from the peritoneal cavity of Wistar rats at 3 weeks post-transplantation. Single **(A)** and clumped microcapsules **(B)** surrounded by several layers of fibrotic tissue (arrow heads) were seen in the non-heparinized compared with heparinized groups. Arrows point to the microcapsule surface. Empty non-heparinized microcapsules retrieved from both Lewis **(E)** and Wistar **(F)** rats had no fibrotic overgrowth with a score of 0. Color images available online at www.liebertpub.com/tea

syngeneic transplantation of encapsulated rat islets resulted only in a mild PFO with a majority of microcapsules (>95%) having a fibrotic score of 0 or 1+ in both heparinized and non-heparinized groups. However, CHC binding significantly reduced the PFO by ~65%, and there were no microcapsules with fibrotic scores of ≥3+ in the heparinized groups compared with the non-heparinized controls. Similarly, allotransplantation of heparinized encapsulated FAO-ins cells did not prevent but reduced PFO by ~43% at 3 weeks post-transplantation, with the majority of microcapsules freely floating in the peritoneal cavity. In contrast, grafts retrieved from rats receiving non-heparinized microcapsules exhibited dense fibrotic overgrowth with clumping of the microcapsules. It is possible that the beneficial effect observed in both the syngeneic and allogeneic transplantation setting also may be attributed to the cytokine shielding properties of the bound heparin conjugates, in addition to their blocking adsorption of fibrin to the microcapsule. Others have shown that microcapsules made from polymers containing heparin[44] or having heparin-like properties[45] have improved biocompatibility and enhanced islet function both *in vitro* and *in vivo*. Another study investigated encapsulation of islets in heparin mimetic microcapsules; for instance, sulfated glucomannan-barium-alginate secreted less MCP-1. This resulted in reduced macrophage infiltration and subsequently, the production of IL-1β, IFN-γ and TNF-α by the host immune system when allotransplanted.[46] However, transplantation of non-heparinized empty microcapsules did not elicit an immune response and were freely floating in the peritoneal cavity with fibrotic scores ranging from 0 (97%) to 1+ (3%). This clearly suggests that the syngeneic and allogeneic immune responses seen in our study were directed against the encapsulated tissue rather than the encapsulating material, namely the alginate.

In conclusion, we have shown that macromolecular CHC can be bound successfully to barium alginate microcapsules using a simple two-step procedure via the cationic linker avidin. CHC binding did not affect islet function and was beneficial in reducing host cell adhesion in both a syngeneic and allogeneic transplantation setting. These data suggest that CHC binding may be useful to reduce the fibrotic response to various alginate microcapsules employed in designing a bioartificial pancreas for humans that utilizes human cells.

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Disclosure Statement

The authors do not have any competing financial interests in connection with this article.

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