Electronic Supplementary Information

Multilayer nanocoatings incorporating superparamagnetic nanoparticles for tracking of pancreatic islet transplants with magnetic resonance imaging

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Experimental methods

Materials and reagents

Tissue culture reagents, including minimum essential medium Eagle (MEME), RPMI-1640 medium, penicillin, streptomycin, newborn calf serum (NCS) and fetal bovine serum (FBS); chitosan (low molecular weight, 50−190 kDa, 75% deacetylation), Histopaque-1077, collagenase (type XI), poly-L-lysine, fluorescein isothiocyanate (FITC) and streptozotocin were from Sigma-Aldrich (Poole, UK). Heparin sodium salt was provided by AppliChem (Darmstadt, Germany). Hanks' buffered salt solution (HBSS) was from PAA Laboratories (Pasching, Austria). Rabbit polyclonal antibody to insulin was purchased from Gene Tex (Irvine, CA, USA). Goat anti-rabbit IgG (H+L)-HRP conjugate was provided by Alpha Diagnostic International (San Antonio, TX, USA). Dako Liquid DAB + substrate chromogen system was purchased from Dako (Glostrup, Denmark).

FITC-poly-L-lysine was prepared according to the previously described procedure (T. Bhaiji, Z.L. Zhi & J.C. Pickup. *J Biomed Mater Res A*. 2012, 100, 1628-36).

Heparin-poly-aldehyde was prepared by oxidation of heparin with sodium meta-periodate using the procedure described in *Bioconjugate Technique* (*pp* 114-116), G. T. Hermanson, (Academic Press, San Diego, USA). The degree of oxidation (10%) was controlled by varying amounts of sodium meta-periodate. The mixture solution was dialysed against water using Slide-A-Lyzer 3.5 k dialysis cassettes (Pierce, Rockford, USA) for 24 h, and then freeze-dried.

Phosphorlycholine (PC)-conjugated chitosan was prepared according to the procedures described previously (Z.L. Zhi, B. Liu, P.M. Jones, J.C. Pickup, *Biomacromolecules*, 2010, 8, 610).

Synthesis of FITC-chitosan-PC

FITC was dissolved in dimethylsulfoxide (1 mg/mL). Chitosan-PC (200 μL or 2 mg) taken from a 1% (W/V) stock solution (dissolved in 1% acetic acid) was diluted to 1 mL with 50 mM HEPES, pH 8.1. FITC solution (0.1 mL) was then added to the chitosan solution and mixed overnight at 4 °C. To remove excess dye, the solution was dialysed against water using Slide-A-Lyzer 3.5 k dialysis cassettes (Pierce, Rockford, USA) for 16 h, and then 50 mM HEPES buffer for 4 h. This solution, which was frozen at -20 °C during storage, was used for encapsulating islets as a fluorescence labelled polycation layer.

Experimental animals

Eight-week aged male C57 BL/6 mice (20 – 25 g, Charles River, Margate, UK) were used as donors and recipients of pancreatic islet grafts for syngeneic transplantation. The mice were made diabetic by injection of a single *i.p*. streptozotocin (180mg/kg) 5 days before transplantation, and only the mice with a blood glucose concentration (> 20 mmol/L) in non-fasting conditions were chosen as the recipients of islets transplants. All animal procedures were approved by our institution's Ethics Committee and carried out under license, in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986.

Islet isolation

Islets were isolated from mice by injecting collagenase (1 mg/mL, 3 mL) into the pancreas via the common bile duct, followed by enzymatic digestion for 10 min at 37°C in a water bath. The islets were purified by centrifugation in a density gradient of 10 mL Histopaque-1077 (3,500 rpm, 25 min; Universal 320R, Hettich Zentrifugen, Tuttingen, Germany). The purified islets were incubated for 16 h in RPMI-1640 medium with 10% FBS, and 0.1% penicillin and streptomycin before use.

Deposition of nanocoating layers on islets

Multiple types of polyelectrolytes were used in this study and were all made to a working solution of 1 or 2 mg/mL by dissolving the solids in HBSS (supplemented with 2 mmol/L Ca^{2+}, to stabilize the cell to cell interactions). Chitosan-PC was dissolved in HBSS after overnight stirring. Heparin was also dissolved into HBSS.

Firstly, islets were picked up from the Petri dishes into 2 mL transparent Eppendorf tubes. The medium was removed as much as possible from the tubes and the islets were washed twice with 400 μL of HBSS in the tubes by allowing the cells to settle to the bottom and removing the supernatants. The native charge on the surface of islets is negative, and thus chitosan-PC was deposited as the first layer. 200 μL chitosan-PC was added the suspended cells for 10 min and mixed gently by a pipette manually two or three times for effective adsorption. The coating solution was removed and the islets were washed three times with 400 μL HBSS washing buffer to eliminate the remaining chitosan-PC before adding the next coating solution, heparin. After coating 2 layers on the surface of islets, the negative-charged SPIOs (100 μL, 0.1 mg/mL) were deposited as the third layer. After removing the remaining SPIOs, chitosan-PC was deposited again. The layer-by-layer steps were repeated until reaching the desired number of nanolayers. Finally, a chitosan-PC layer and a heparin-poly-aldehyde layer were deposited in a same manner. It took about 2 h to complete the whole coating process.

In vitro cell viability/apoptosis (caspase 3/7 activity) tests

The islets were counted and placed into a 96-well plate (5 islets/well in triplicates). The total volume of the medium taken with the cells was 100 μL. For viability measurements, 20 μL of CellTiter-Blue® cell viability assay reagents (Promega, Madison WI, USA) was added to each well and incubated at 37°C for 3 h. Meanwhile, Apo-ONE® homogeneous caspase-3/7 assay regents from Promega were used to determine the level of apoptosis in the cultured islets according to the manufacturer's instructions. The contents were mixed by gently shaking for 30 seconds and then placed in a 37°C incubator for 2-4 h. A Spectra Max Gemini EM microwell plate-reader (Molecular Devices, Wokingham, UK) was used to measure the fluorescence intensity.

Insulin secretion of the encapsulated islets

Islets were incubated for 16 hours in a 37°C incubator before they were coated and tested for insulin secretory function. The rate and patterns of in vitro insulin secretion from the coated and control islets were assessed using a temperature-controlled (37°C) multi-channel perifusion system, as described previously (Hauge-Evans AC, King AJ,

Carmignac D et al, *Diabetes* 2009, 58, 403–11). Briefly, 40 islets were loaded onto nylon filters in Swinnex filter holders (Millipore, Cork, Ireland) and perifused with a bicarbonate-buffered physiological salt solution (Gey & Gey buffer, pH 7.2, made in house) supplemented by 2 mmol/L $CaCl_2$, 0.5 mg/mL bovine serum albumin and a concentration of glucose as indicated below. Fractions were collected every 2 min during (i) a 10 min perifusion period with buffer containing 2 mmol/L glucose, (ii) a 20 min perifusion with 20 mmol/L glucose and (iii) an additional 20 min perifusion with 2 mmol/L glucose. Insulin content was assessed by radioimmunoassay, using an in house-generated antiserum against bovine insulin raised in a guinea pig (100% cross-reactivity to mouse insulin) and ^{125}I-labelled insulin in a homogeneous competitive format. Bovine ^{125}I-insulin was prepared by the iodogen method, and purified by gel filtration. Purified rat insulin was used as the standard (Jones PM, Salmon D M W, Howell S L. *Biochem J.* 1988, 254, 397-403).

Iron oxide nanoparticle characterization and SQUID analysis

Iron oxide nanoparticles were prepared in house by Dr Thanh's research group according to a modified procedure (C. Blanco-Andujar, D. Ortega, Q. A. Pankhurst, N. T. K. Thanh, *J. Mat. Chem.* 2012, 22, 12498). The morphology, particle size and size distribution of iron oxide nanoparticles were examined with a JEOL JEM 1200-EX transmission electron microscope (TEM) (JEOL, Welwyn Garden City, UK) operated at an acceleration voltage of 120 kV. Samples were prepared by dropping the aqueous dispersions onto a carbon-coated copper grid and allowed to air-dry. Particle z-potential was measured with a Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, UK). Magnetization curves of the iron oxide loaded islets were carried out in a Quantum Design (San Diego, USA) hybrid Superconducting Quantum Interference Device-Vibrating Sample Magnetometer (SQUID-VSM) at 300K, with fields up to 7 T. Fixed samples containing 200 islets each were transferred into a polycarbonate capsule containing a small amount of cotton to limit sample dispersion and allowed to air-dry. The obtained capsules were filled with cotton to obtain background homogeneity and transferred into a gel capsule. The final samples were mounted on a straw to minimise noise and allow for small magnetic moment measurements.

Transplantation of islets in diabetic mice

C57 BL/6 Mice were anaesthetized by inhalation of isoflurane, and 300 islets (coated and controls) were transplanted into mice under the kidney capsule, according to a procedure reported previously [Z.L.Zhi, A. Kerby, A.J. King, P.M. Jones, J.C. Pickup. *Diabetologia* 2012, 55, 1081] (We are thankful to Drs A Kerby and A King for the assistance with the islet transplantation). Briefly, a lumbar incision was made, the kidney exposed and an incision made in the capsule. Coated and control islets that had been centrifuged into pellets in PE50 polyethylene tubing (Becton Dickinson, Franklin Lakes, NJ, USA) were placed underneath the kidney capsule using a Hamilton syringe (Fisher, Two Rivers, WI, USA). All islets were transplanted with a delay of no more than 2 h after encapsulation. After transplantation, the blood glucose concentration and body weight of the recipient mice were monitored every day for 7 days. After 7 days, the islet graft-bearing kidneys were removed and fixed in 4% paraformaldehyde (PFA) for 2 days and then placed in phosphate-buffered saline (PBS) prior to magnetic resonance imaging (MRI).

Ex vivo MR image of the kidney samples

Paraformaldehyde-fixed kidneys with and without transplanted islets were embedded in 1% agarose for MRI. The tube was then positioned in a quadrature volume coil (33 mm inner diameter, RAPID Biomedical GmbH, Rimpar, Germany) tuned to 400 MHz ^{1}H frequency for MRI. MRI was performed on a 7T Agilent MRI scanner (Agilent Technology Inc. Santa Clara, USA). Following acquisition of scout images, MRI of the kidneys was performed using a T2-weighted spin-echo sequence: repetition time (TR), 2000 ms; echo time (TE), 20 ms; 1 scan; field of view, 30 x 30 mm; matrix size, 128 x 128 and 13 consecutive, longitudinal-axis, 1 mm thick slices were recorded.

Hypointense regions were observed within the kidney capsule with the transplanted labelled islets. These areas of low signal intensity observed by T_2-weighted MRI are consistent with the sites of transplanted iron oxide nanoparticle-labelled islets.

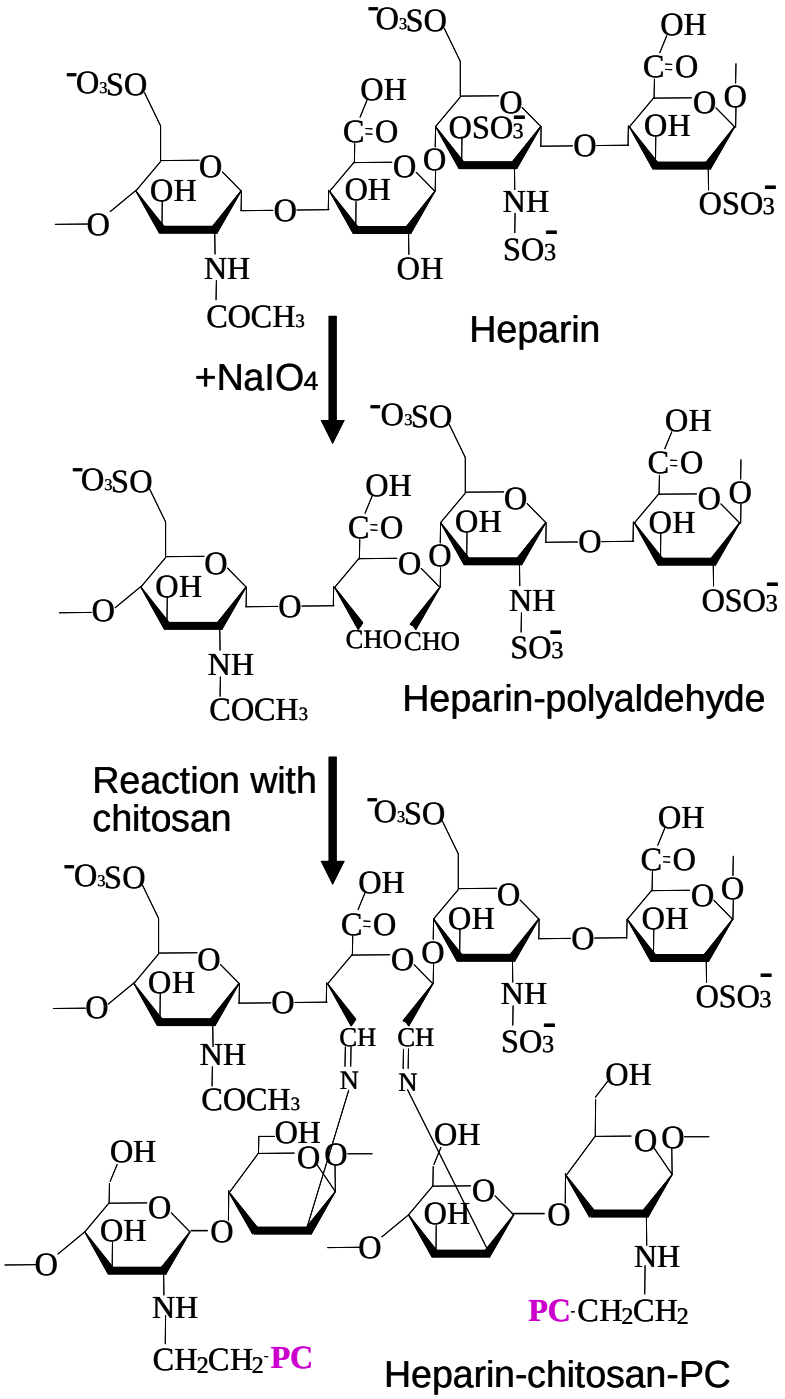
Graft morphology, histology and immunohistochemistry.

At the end of the study, the recovered graft bearing kidneys were fixed in 4% (vol/vol) neutral-buffered formalin and paraffin-embedded, and specimens were sectioned at a thickness of 5 μm. The paraffin sections were re-hydrated by sequential 5 min

incubations in xylene (twice), 100% ethanol, 95% ethanol and then 70% ethanol. The re-hydrated sections were stained with hematoxylin/eosin for routine morphology studies. The graft sections were further evaluated by immunohistchemical staining with antibodies targeting insulin. The de-waxed sections were microwave-heated in 10 mM citrate buffer (pH 6.0) with 0.05% Tween at 100 °C for 20 min to retrieve the antigen (the microwave was initially set at 700 Watts, after boiling, it was adjusted to 120 Watts to keep boiling minimal). The samples were then blocked using 1% BSA in PBS buffer for 10 min and incubated with primary antibody (diluted 200 times with the same blocking buffer) for 1 h in a humidifying chamber followed by washing in tris-bufferd saline (TBS), and addition of horseradish peroxidase-conjugated rabbit anti-rabbit IgG (H+L) secondary antibody for 1 h in the humidifying chamber. The bound peroxidase was colour-developed with the liquid DAB and substrate chromogen system containing 3,3'-diaminobenzidine. Sections were then counterstained with hematoxylin/eosin to identify the presence of cell nuclei in the section.

Statistical analysis

Differences between groups were compared by a two-way analysis of variance (ANOVA) (for curves) or t-test (for means). Differences were considered to be statistically significant when the p values were less than 0.05. Results are shown as mean ± SD unless otherwise indicated.



Scheme S1 Reaction scheme for the preparation of heparin-polyaldehyde by periodate oxidation and its cross-linking with the amino groups of chitosan-PC.

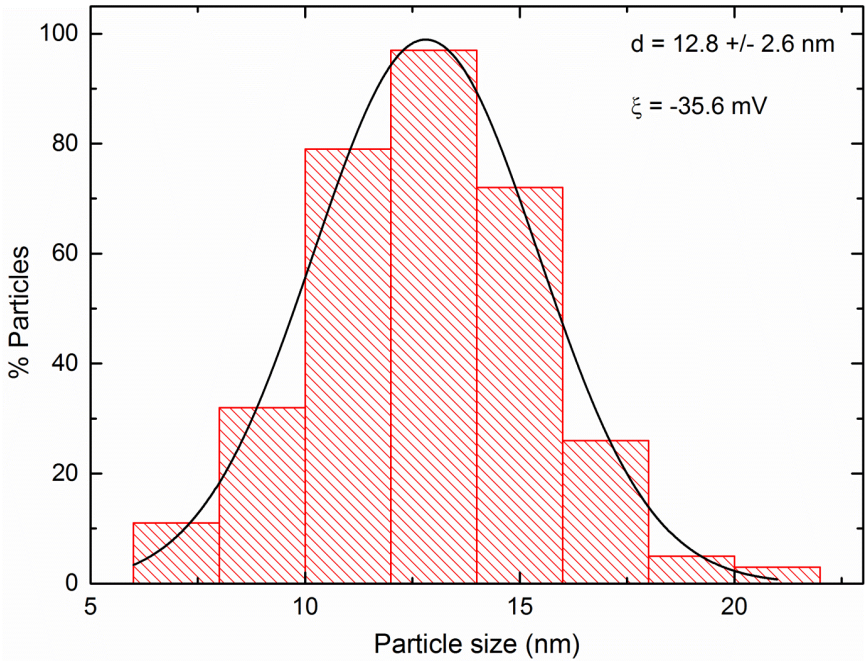


Fig. S1 Size distribution of the iron oxide nanoparticles.

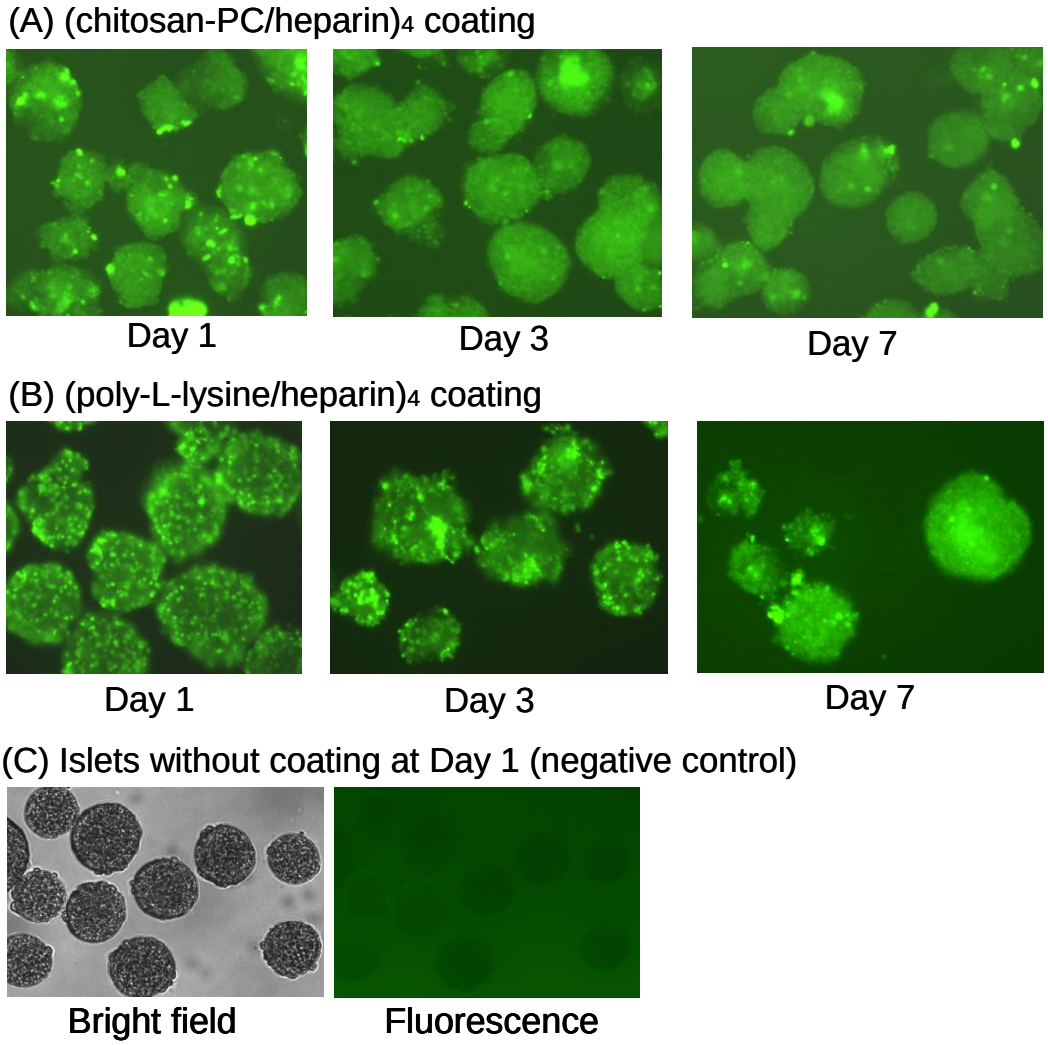


Fig. S2 Evaluation of the coating stability in culture. (A) Islets were coated with 8 layers of FITC-chitosan-PC/heparin and cultured in RPMI medium at 37°C in an incubator. (B) Islets were coated with 8 layers of FITC-poly-L-lysine-PC/heparin and cultured in the same conditions. (C) Islets without coating (as the negative controls). Fluorescence and bright field images are shown. The size of a typical islet is about 150 μm.

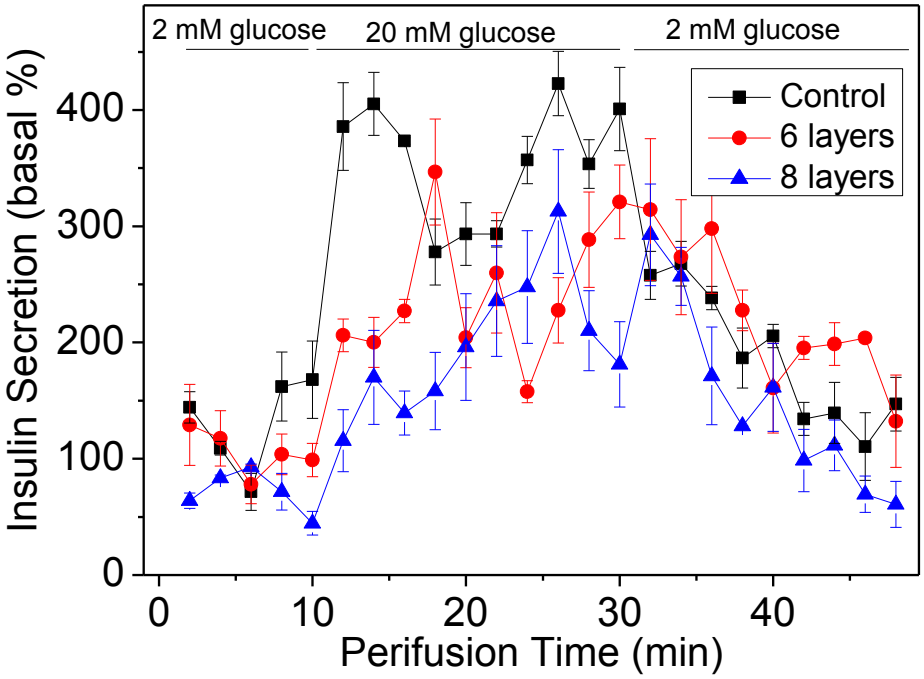


Fig. S3 Dynamic insulin secretion in vitro in the control and the nanocoated mouse islets in response to changes in glucose. Nanocoated islets including one (total 6 layers) and two (total 8 layers) layers of the nanoparticles are shown. N=4, mean ± SD. Periods of glucose stimulation and glucose concentration were as indicated.

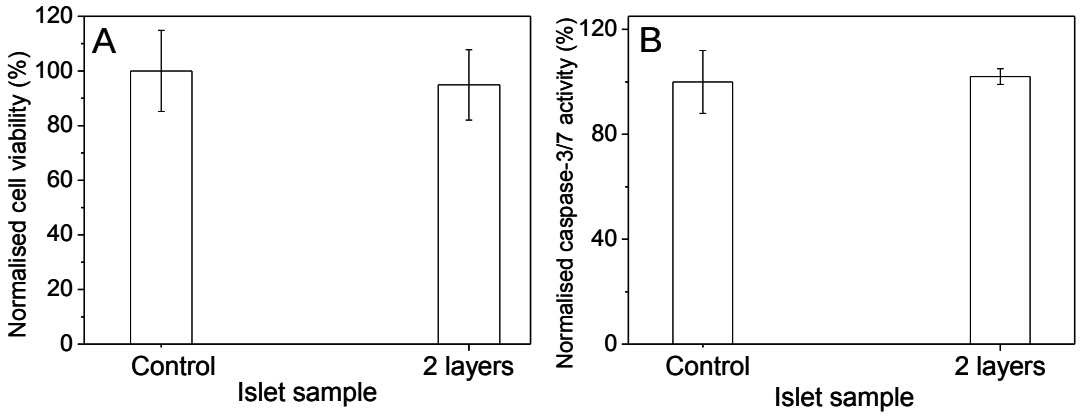


Fig. S4 Effects of the nanoparticles and polysaccharide coatings on (A) the islet cell viability ($p > 0.05$); (B) apoptosis ($p > 0.05$), in vitro. Coated islets included 2 layers of nanoparticles. N = 3, mean ± SD.

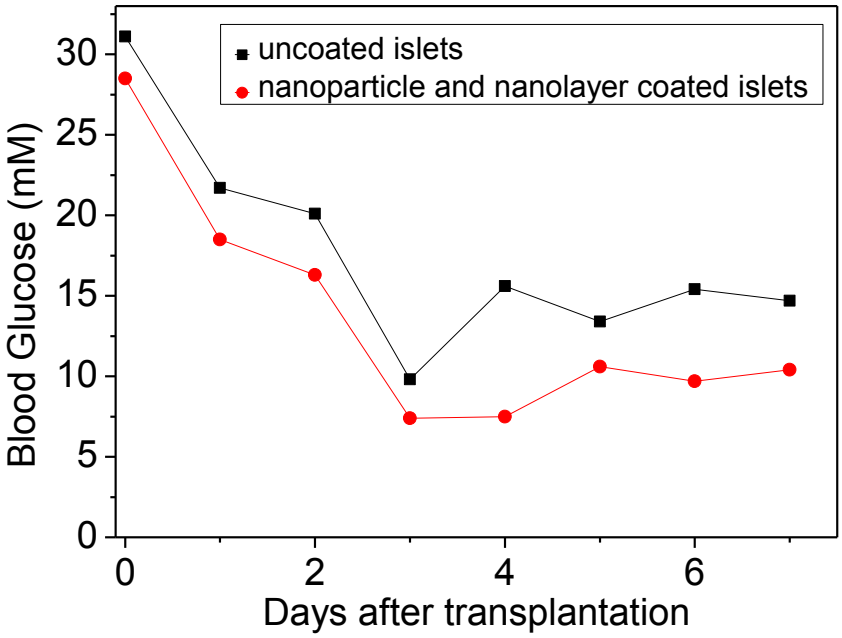


Fig. S5 Monitoring of blood glucose concentrations in the animals transplanted with 300 labelled and control islets.

Fig. S6 Photograph showing the site of islet transplantation under the mouse kidney capsule.

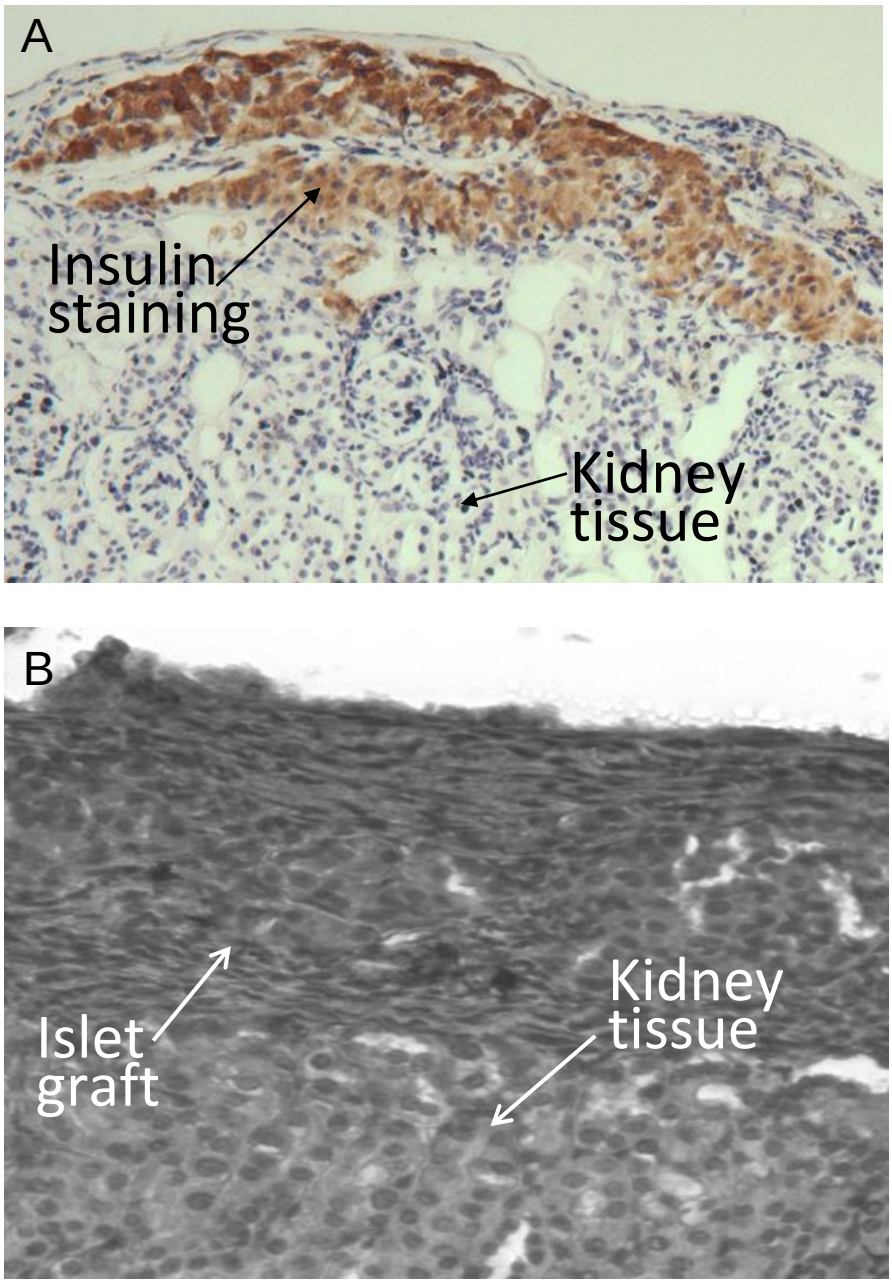


Fig. S7 Histological analysis of the labelled islet-bearing kidneys. A, insulin immuno-staining; B, hematoxylin-eosin (H&E) staining.