Supplemental Information

Assembly of bioactive multilayered nanocoatings on pancreatic islet cells: incorporation of α 1-antitrypsin into the coating

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1. Materials and methods

Reagents

Tissue culture reagents, including minimum essential medium Eagle (MEME), RPMI-1640 medium, penicillin, streptomycin, newborn calf serum (NCS) and fetal bovine serum (FBS) were from Sigma-Aldrich (Poole, UK). α 1-antitrypsin (AAT), glycol chitosan (GCh), propidium iodide, sodium periodate, Histopaque-1077, collagenase and fluorescein isothiocyanate (FITC) were also provided by Sigma-Aldrich (Poole, UK). Hanks' buffered salt solution (HBSS) was a PAA Laboratories (Pasching, Austria) product. Heparin sodium salt (MW, 8k–25k) was purchased from AppliChem, Darmstadt, Germany.

Heparin polyaldehyde (Hep-de) was prepared by oxidation of heparin with sodium meta-periodate using the procedure described previously (G. T. Hermanson, Bioconjugate Techniques, Academic Press, San Diego, USA, 1996, pp. 114–6). The degree of oxidation (2.5%, 5.0% and 7.5%) was controlled by varying amounts of sodium meta-periodate. For example, 200 mg heparin was added 1.573 mg sodium meta-periodate in 1 mL water for 4 hours at room temperature to give a resulting aldehyde density of 2.5% on heparin. The mixture solution was dialysed against water using Slide-A-Lyzer 3.5k dialysis cassettes (Pierce, Rockford, USA) for 24 h at 4 °C, and then freeze-dried.

Synthesis of FITC-glyco chitosan

FITC was dissolved in dimethylsulfoxide (1 mg/mL). Glyco Chitosan (200 μ L or 2 mg) taken from a 1% (W/V) stock solution (dissolved in water) was diluted to 1 mL with 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (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 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 coating islets as a fluorescence labelled polycation layer.

Synthesis of α 1-antitrypsin- FITC

FITC was dissolved in dimethylsulfoxide (10 mg/mL). AAT (MW 52K Da) (10 mg) was dissolved in 1 mL with 50 mM HEPES, pH 8.1. FITC solution (75 μ L) was then added to the AAT solution and mixed overnight at 4 °C. To remove excess dye, the solution was dialysed against PBS using Slide-A-Lyzer 3.5k dialysis cassettes for 16 h. This solution, which was freeze-dried during storage, was used for coating islets as a fluorescence labelled AAT.

Islet isolation

Islets were isolated from eight-week aged male ICR mouse (25–30 g; Harlan Laboratories, Oxfordshire, UK) pancreata by ductal collagenase injection. Approximately 2.5 mL of collagenase (1 mg/mL) was injected via the common bile duct into the pancreas. The pancreas was removed and digested at 37°C for 10 min. The islets were washed thrice in 25 mL MEME (+10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin), with the supernatant removed upon centrifugation. The tissue was subsequently suspended at the interface between Histopaque (15 mL) and MEME (10 mL), centrifuged for 24 min (3,500 rpm, 24 min; Universal 320R, Hettich Zentrifugen, Tuttingen, Germany). The islets were collected at the interface and washed thrice more in 50 ml MEME. Islets were transferred to RPMI-1640 culture medium (+10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin) and incubated at 37°C under 5% CO₂ for 16 h.

Nanocoating of islets

100-500 Islets (note the number of islets that can be coated in each batch is unlimited) were handpicked and transferred to 2 mL Eppendorf tubes. The medium was removed and replaced with 200 μ L HBSS (supplemented with 2 mmol/L Ca²⁺). The buffer was removed on settling of the islets and the islets were subsequently treated with 200 μ L Hep-de (2.5 mg/mL), GCh (3 mg/mL) or AAT (3 mg/mL) in suspension, depending on the specific layering scheme required, for 10 min for each deposition step. Hep-de was deposited as the first layer. 200 μ L Hep-de was added to the suspended cells for 10 min and mixed gently by a pipette manually three times for effective adsorption. The coating solution was then removed and the islets were washed two times with 400 μ L HBSS washing buffer to eliminate the remaining Hep-de before adding the next coating solution, GCh. The coating process was repeated n times per batch of islets according to the following layering order: islet/(Hep-de/GCh)_n/Hep-de, where n represents the number of Hep-de/GCh bilayers. To incorporate AAT in the coating, islets were first coated with 4 layers of Hep-de and GCh. The negative-charged AAT (100 μ L, 5 mg/mL) was deposited as the 5th layer. After removing the remaining AAT, GCh was deposited again. The layer-by-layer steps were repeated until reaching the desired number of nanolayers. It took about 2 h to complete the whole coating process.

Dead cell staining assay

Cell death in islets after coating was evaluated by fluorescence staining using propidium iodide (PI) (Sigma). The culturing medium was removed and working solutions of PI were added (0.6 pg of PI in 0.2 mL HBSS) and incubated for 3 min at room temperature. The staining solution was removed and cells were washed thrice with 0.5 mL HBSS. The cells were viewed under a fluorescence microscope with 530–560 nm excitation filters and 573–648 nm emission filters, to allow imaging of red (dead) fluorescing cells.

Islet embedding for transmission electron microscopy (TEM)

For TEM analysis, the coated or control islet cells were fixed for 4 h in 4% (w/v) paraformaldehyde, 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and post-fixed in 1% (w/v) osmium tetroxide for 1.5 h at 4°C. Samples were then dehydrated through a graded ethanol series, equilibrated with propylene oxide before infiltration with TAAB epoxy resin and polymerised at 70°C for 24 h. Ultrathin sections (70-90 nm) were prepared using a Reichert-Jung Ultracut E ultramicrotome (Wein, Austria), mounted on 150 mesh copper grids (TAAB), contrasted using uranyl acetate and lead citrate and examined on a FEI Tecnai 12 BioTWIN transmission microscope (Oregon, USA) operated at 120 kV. Images were acquired with an AMT 16000M camera.

Islet cell apoptotic activity assay

Islets were counted and loaded into a 96-microwell plate. The coated and control islets were loaded (10 islets/well, in quadruplicates), with the total volume of RPMI-1640 fixed at 100 μ L. For apoptosis measurements, 20 μ L of Apo-ONE[®] homogeneous caspase-3/7 assay reagents (Promega, Southampton, UK) were used. The contents were incubated at 37°C incubator for 3-4 h. Fluorescence intensity was measured by Spectra Max Gemini EM microwell plate-reader (Molecular Devices, Wokingham, UK).

Dynamic perfusion for studying insulin secretion

Patterns of insulin secretion from control and coated islets were assessed in a temperaturecontrolled (37°C) multi-channel perfusion assay. Fifty islets were loaded on nylon filters in Swinnex filter holders (Millipore, Cork, Ireland), perfused with a bicarbonate-buffered physiological salt solution (Gey & Gey buffer, pH 7.2, 2 mM CaCl₂, 0.5 mg/mL bovine serum albumin and supplemented with either 2 or 20 mM glucose). Fractions were collected every 2 min during (i) a 10 min perfusion period with 2 mM glucose buffer, (ii) a 20 min perfusion with 20 mM glucose and (iii) further 22 min perfusion with 2 mM glucose. Radioimmunoassay assed insulin content, using antiserum against bovine insulin raised in a guinea pig and ¹²⁵I-labelled insulin in a homogeneous competitive format.

Activated partial thromboplastin time assay

The anti-coagulant activity of the bioactive nano-coating on islets was determined by activated partial thromboplastin time assay. The islets (coated or non-coated) (50 μ L) were added into an Eppendorf tube, 50 μ L of fresh ICR mouse plasma was added. After adding 50 μ L of 0.2 M calcium chloride, the time was recorded from this point until the fibrin clot was observed under microscope at room temperature.

Statistical analysis

Comparisons between groups were conducted using a student's T-test and a two way analysis of variance (ANOVA), for means and curves, respectively. A p-value < 0.05 was considered significant. Results were shown as mean \pm SD. All statistical calculations were conducted using IBM SPSS software.



Fig S1. Monitoring of the stepwise deposition of the Hep-de/GCh/AAT multilayers on mouse islets.
A, 1 layer of AAT, B, 2 layers of AAT, C, 3 layers of AAT, D, 4 layers of AAT. AAT was FITC labelled.
E, plot of the average fluorescence intensity against the number of AAT layers deposited.



3. Fig S2(c), (d) and (e), stability time course of the nanocoatings on islets. All islets were coated by the order of islet/(Hep-de/GCh)₂/(AAT/GCh)₂AAT (AAT was FITC labelled) and cultured at 37 °C for 1 day (c), 5 days (d) and (e) 10 days. Note due to the photobleaching of the dye during the culture process, the photos were obtained by automatic adjustment of the exposure time to get the clear images.



4. Fig S3. Effect of the nanocoating on islet cell death and activity of caspase-3 and -7 in islets (apoptosis). (a – d) PI cell staining shows the dead cells as bright red fluorescence dots. (a) control islets, (b) Hep-de(5.0)/GCh (9 layers), (c) Hep-de(7.5)/GCh (9 layers) and (d) Hep-de(5.0)/GCh/AAT (9 layers) coating schemes. (e) APO-ONE® caspase 3/7 activity assay for the various islet groups, where n = 4; error bars indicate standard error of the mean. *** represents p<0.001.</p>



4. Fig S4. Dynamic insulin production in vitro in control (non-coated), and coated (Hep-de/GCh, 9 layers; and Hep-de/GCh/AAT, 9 layers) ICR mouse islets in response to changes in extracellular glucose (from 2 mM to 20 mM, then return to 2 mM). Periods of glucose stimulation and glucose concentration were as indicated. 50 ICR mouse islets were perfused in each flow channel 2 hours after coating. Values are mean ± SD; n=4. Only half error bars are shown for each curve for clarity.