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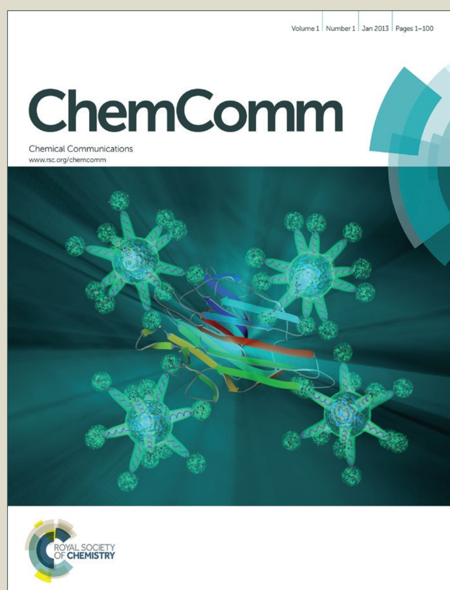

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Assembly of bioactive multilayered nanocoatings on pancreatic islet cells: incorporation of α1-antitrypsin into the coating

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A spontaneous multilayer deposition approach to presenting therapeutic proteins onto pancreatic islet surfaces, using a heparin polyaldehyde and glycol chitosan alternating layering scheme, has been developed to enable the nanoscale engineering of microenvironment for transplanted cells. The nanocoating incorporating α1-antitrypsin, an anti-inflammatory protein, exhibited effective anti-coagulant activities *in vitro*.

Despite some recent progress, the advancement of current cellular therapy from the bench to a routine clinical strategy is largely impeded by poor functional cell survival post-transplantation.[1] Cell surface engineering with bioactive nanofilm coatings is an emerging field which provides an attractive alternative to traditional genetic engineering approaches to the control of *in vivo* microenvironment for implanted therapeutic cells, enhancing their function and survival.[2] Such a cell surface modification works on the premise of generating bioactive nanocoating to resurface the molecular landscape of cells for enhancing engraftment and attenuating donor cell induced inflammatory responses for improved therapeutic benefit. Nanocoating of cells offers a scheme for the formation of a protective shield close to the cell surface with unchanged volume, preventing detrimental interactions between transplanted cells and the hostile *in vivo* surrounding environments.[3] More importantly, those biomaterials-based coatings can be used as a nanoscale matrix or delivery depot for local and sustained delivery of therapeutics at the graft site to modulate inflammatory cascades and immune responses, thereby attenuating the hostility of the *in vivo* environment.[4] The challenge with the biofunctional nanocoating is to nano-engineer effective and lasting protective layers that provide optimal cell engraftment and survival conditions, and is chemically orthogonal to cellular components while being biocompatible with the surrounding host environment.

Cell surface nanoengineering as a paradigm for enhanced cell delivery strategies is gaining increasing interest in a broad range of cellular therapies. With pancreatic islet cell transplantation for example, the development of the Edmonton protocol led to marked improvements in the success rate of clinical islet transplantation as a viable therapy for Type 1 diabetes.[5] However, a major challenge with the islet transplantation is that up to 70% of cells may be functionally impaired in the immediate post-transplantation period due to the instant blood-mediated inflammatory reaction (IBMIR), where contact of islet cells with blood results in coagulation, platelet aggregation, complement activation, and neutrophilic and monocytic islet infiltration.[6] IBMIR has therefore been a major barrier to successful islet and other therapeutic cell transplantation. To address this specific problem, a bioactive nano-coating designed to enable controlled delivery of anti-IBMIR agents to the vicinity of islets is required to provide localized anti-inflammatory protection to the transplanted islets, thereby mediating the cell dysfunction and providing a foundation for long-term islet function.

Previous effort on the development of chemical approaches to islet or other cell coatings included the use of surface-bound biotin-streptavidin chemistry,[7] exogenous insertion of lipid anchor into the cell membrane,[8] and chemoselective Staudinger ligation between azide and phosphine.[9] The principal disadvantage of these existing strategies is the requirement for significant pre-derivatisation and purification of each assembly component, complicating nanocoating fabrication. One option for nanocoating involves the deposition of multiple layers onto cells such as islets by exploiting the cell surface charge, which permits layer-by-layer deposition of electrostatically charged polymers.[10] This technique allows a predefined number of layers to be deposited, by alternating positively and negatively charged molecules, but with the added incorporation of therapeutic proteins and polymers with conjugated bioactive motifs as a molecular basis of the build-up of protective cell coatings.

However, one fundamental issue of this approach is the cytotoxicity from the polycationic components, such as poly-L-lysine and poly(allylamine hydrochloride), which pose severe limitations for this approach as a routine tool in cell surface engineering.[10] Nevertheless, a dramatic reduction in the cytoxicity can be achieved by decreasing the polycation charge density through proper grafting of polyethylene glycol chains.[11] We report here a novel nanocoating scheme permitting greater incorporation of anti-IBMIR agents with

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minimal adverse effect on the cell function, whilst providing improved *in vivo* drug characteristics and helping reduce islet cell damage in the immediate post-transplantation period.

The nanocoating on islets was constructed by alternating layering of heparin polyaldehyde (Hep-de) and glycol chitosan (GCh) in order to attain improved efficiency of the spontaneous layer deposition and reduced cytotoxicity of the formed films on islets. Although the coating of heparin on the islets has been developed previously,[7a] Hep-de is a different entity. The amine reactive Hep-de is generated by partial periodate cleavage of adjacent hydroxyl-containing carbon-carbon bonds on heparin.[12] The resulting aldehyde group permits the formation of stable Schiff bases with the endogenous amine groups of cell surface, enhancing the attachment of heparin to the cell surface. The anionic heparin increases the negativity of the cell surface thereby allowing for a stronger attraction of positive species, in this case GCh; it can also neutralize the cytoxicity of the polycationic GCh which is deposited as the counting layer. Since GCh is highly water soluble at neutral pH, compared to the insolubility of native chitosan at any pH conditions greater than 6.0, it should incorporate well into the coatings.[13] The weakly positively charged polymer chain of GCh at neutral pH means it may exert much less cytotoxicity than other strongly ionic poly-cations. Overall, the strength of this proposed electrostatic attraction is designed to improve the efficiency of the coating process and minimise the cytotoxicity of the deposited films, whilst permitting greater incorporation of anti-IBMIR agents.

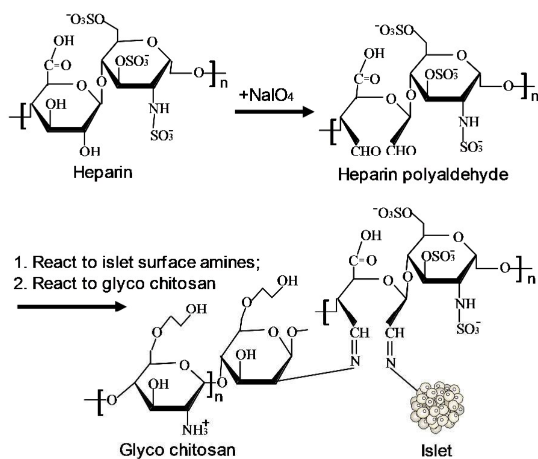

Figure 1. Scheme of the chemical reaction for generating heparin polyaldehyde (Hep-de) by periodate oxidation and its subsequent reaction with the cell surface amines and glycol chitosan (GCh) in multilayers. AAT was incorporated in the coating following the layering order of islet/$(\text{Hep-de/GCh})_2$/$(\text{AAT/GCh})_2$AAT.

Islets used for the coating experiments were isolated from 8-week old ICR (the name of the mouse stain) mice by collagenase digestion of the exocrine pancreas.[13] The conformal nano-thin films encapsulating individual islets were then constructed based on the spontaneous layer-by-layer nano-deposition technique.[13] The coating scheme (Figure 1) highlights the individual molecule layers and the position they occupy in the layering sequence, with the first molecule layer (Hep-de) immediately adjacent to the islet surface and the last molecule (Hep-de or a protein) furthest away from the islet surface. The coating process was repeated n times per batch of islets according to the following layering order islet/$(\text{Hep-de/GCh})_n$/Hep-de, where n represents the number of Hep-de/GCh bilayers.

In search of more robust and cell-biocompatible coating for islets, we first tested three variants of Hep-de, namely Hep-de(2.5), Hep-de(5.0) and Hep-de(7.5), which differed in the density of aldehyde groups generated on heparin chain. Hep-de(2.5) represents a density of 2.5% of the disaccharide units of heparin possessing adjacent hydroxyls oxidised to generate aldehydes, whereas Hep-de(5.0) and Hep-de (7.5) refer to a density of 5.0% and 7.5%, respectively, of the heparin sugar chains.

The coating efficiency of this scheme was assessed by incorporation of FITC labelled GCh (FITC-GCh) in a four-layer coating scheme (Figure 2a–e). The concentration of Hep-de was kept constant (2.5 mg/mL), regardless of the density of aldehyde groups on it. Figure 2b shows that using unmodified heparin, there is little fluorescence detected, reflecting minimal GCh localized to the islets. When the Hep-de layering was used however, a marked improvement in the coating was observed as indicated by the increase in fluorescence, indicating the aldehyde group made a clear improvement in the efficiency of GCh deposition and the layer build-up. Furthermore, GCh incorporation improved in proportion to the density of aldehyde groups generated (Figure 2c–e). Hep-de(2.5) showed relatively little islet surface fluorescence, hence limited GCh incorporation (Figure 2c). Hep-de(5.0) and Hep-de(7.5) (Figure 2d, 2e) displayed markedly more fluorescence than heparin (2b), indicating the most promising improvements in coating, as a result these were utilised in the further function studies.

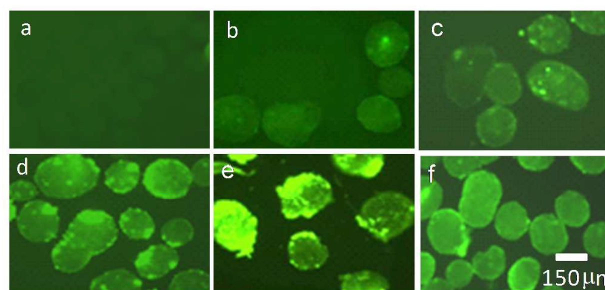

Figure 2. Increasing the density of aldehyde group on heparin chain improves the coating efficiency. FITC-GCh binding to the layered Hep-de was used to assess the efficiency of the coating schemes. (a) Control islet without coating; (b) Using normal heparin and FITC-GCh; (c) Hep-de(2.5)/FITC-GCh; (d) Hep-de(5.0)/FITC-GCh; and (e) Hep-de(7.5)/FITC-GCh; (f) AAT-FITC was used as the model therapeutic protein to be incorporated into the coating. 4 layers were deposited for (b-e), with the layering order of $(\text{Hep-de/FITC-GCh})_2$; (f) 9 layers with the layering order of $(\text{Hep-de(5.0)/GCh})_2$/$(\text{AAT/GCh})_2$/AAT, with AAT being FITC labelled.

When a model therapeutic protein is to be incorporated, in this study, α1-antitrypsin (AAT) (pI = 5.6, being negatively charged at pH 7.2), the layering order was modified to: islet/$(\text{Hep-de/GCh})_2$/$(\text{AAT/GCh})_2$AAT, with the first 2 bilayers being $(\text{Hep-de/GCh})_2$ as the seed coating, and the last layer being AAT to complete the coating process. AAT is known to be a serum protease inhibitor which possesses anti-inflammatory, anti-thrombosis, and

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immunomodulatory activities and reduces cytokine-mediated islet damage to islet cells.[14] However, AAT was usually delivered systemically into the blood stream which substantially increases the risk of undesirable side-effects such as bleeding and cancer induction; and local delivery of the protein to the islet graft has not been achieved so far. In this study, we optimised the incorporation of AAT on the islet coating using a small number of nanolayers with the goal of making nanocoating bioactive. This could help reduce blood-mediated cellular damage and ultimately improve the outcome of islet transplantation.

Loading of protein into the coating was assessed using fluorescence labelled AAT (FITC-AAT). On top of a seed coating with two bilayers of Hep-de(5.0) and GCh, a total of 9 layers of the coating including 3 individual layers of AAT were deposited (Figure 2f). The localised fluorescent patches found on the islet surfaces meant that AAT was incorporated well into the coating scheme. The fluorescence intensity on the islets increased with the increase of the number of AAT layers deposited (Fig. S1, ESI). The deposition of nanolayers on islets was further confirmed by transmission electron microscopy (TEM) analysis of the islet sections (Figure 3). The nanometer-thin coating observed at the edge of the islet (Figure 3b) was likely due to the interlayer crosslinking which was further strengthened by the strong ionic interaction between heparin and GCh. The stability of the coating was studied under culture conditions and was found to be substantially stable for at least 10 days (Fig. S2, ESI).

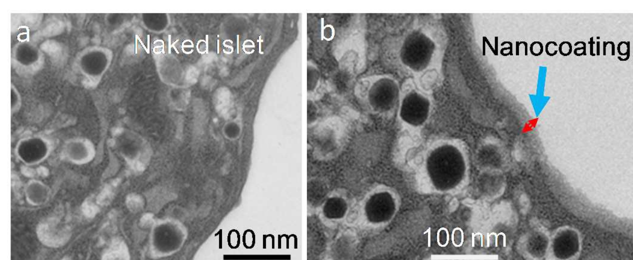

Figure 3. Transmission electron micrograph of a section of an islet. (a) Control islet; (b) Nanocoated islet. The nanocoating with the order of islet/(Hep-de/GCh)$_2$/(AAT/GCh)$_2$AAT is indicated by a blue arrow, and the thickness of the coating is about 30 nm.

The incorporation of the negatively charged protein into the coating highlights the open platform nature for therapeutic proteins and other molecules in addition to biopolymers like Hep-de and GCh (Figure 2f). In practical terms, this means that the new coating format can provide depot delivery of biomolecules to the cell grafts in a small number of layers, including therapeutic protein that could improve the cell viability and function under *in vivo* environments.

The impact of nanocoatings on islet cell survival was assessed by qualitative dead cell staining process with propidium iodide (PI), 24 hours post coating (Fig. S3, ESI), followed by assessment of apoptosis activity by measurement of caspase 3/7 activities. Fig. S3, ESI shows that the control group displayed low numbers of dead cells, with a diffusing spread of dead cells throughout the islets, as expected. Although Hep-de(5.0) and AAT coatings exhibited a few dead cells within the islets, the coated islets looked comparable to the control islets (Fig. S3, ESI). However, the Hep-de(7.5) coating displayed markedly increased numbers of dead cells (Fig. S3c, ESI), with most of the dead cells localised at the islet edges, rather than at the islet cores, indicating the death of the cells may be caused by the over-loading of Hep-de (7.5).

The APO-ONE® assay (Promega) was used to reveal caspase 3/7 activity, and hence the apoptotic activities of the islet cells (Fig. S3e, ESI). The control islets displayed low levels of caspase 3/7 activity. The Hp-de(5.0)/GCh and Hp-de(5.0)/GCh/AAT coated islets displayed similar levels of caspase 3/7 activity and were not significantly different to the control or each other ($p>0.05$). Conversely, the Hep-de(7.5)/GCh group displayed significantly increased apoptotic activity (11.5 times higher, $p<0.001$) in comparison to the other islet groups. The results of the apoptosis assay thus agreed with the dead cell PI staining data (Fig. S3, ESI).

The dead cell staining and the apoptosis assay data suggest that neither Hep-de(5.0) nor AAT coatings have any significant effect on apoptosis or viability (Fig. S3, ESI). However, the Hep-de(7.5) group showed a marked increase in dead cells and significantly raised level of caspase 3/7 activity. The increase in cell death for the Hep-de(7.5) group was consistent in the microscopic PI staining test and apoptosis assay. It is possible that there was direct toxicity of the Hep-de(7.5) coating, or potentially the cell surface negative charges was too densely engineered leading to cellular injury. This is supported by the observation that most of the dead cells were localised nearer to the islet edge, rather than at the core.

On confirming that the coating used in this study did not impose a cytotoxic effect on the islets, we assessed islet function by measuring glucose-stimulated insulin secretion of the islet cells with selected coating groups, including Hp-de(5.0) and AAT groups, compared to the control (unmodified) islets. Evidence from the dead cell studies already suggested that Hep-de(7.5) coating was deleterious to islets and hence this group was not investigated any further. As can be seen in Fig. S4, ESI, the three groups all showed an appropriately low level of insulin secretion for the first 10 min at 2 mM glucose concentration. At this basal level, insulin secretion was comparable between all three groups ($p>0.05$). On elevation of the glucose concentration to 20 mM for the following 20 min, there was an immediate and marked increase in insulin secretion for all three groups. A calculation of the ratio of the peak insulin concentration at high glucose against the basal insulin concentration showed the very close values of 6.91, 6.80, and 6.72, for the control, the Hep-de/GCh and the Hep-de/GCh/AAT groups, respectively, indicating the coatings did not alter the insulin secretion function.

More importantly, the insulin secretion for all groups reverted to baseline level when the glucose concentration was reduced to 2 mM for the remaining 22 min. The difference in insulin secretion between groups was not significantly different ($p>0.05$). The ability of nanocoated islets to mount an appropriate and reversible insulin secretory response to elevated glucose demonstrates that the Hep-de(5%) nanocoating does not interfere with glucose uptake into beta-cells via the Glut2 transport, nor with glycolytic metabolism, mitochondrial oxidative phosphorylation, ATP generation, β-cell depolarization and Ca^{2+} influx, all of which are essential for glucose-induced insulin secretion.[15] This is convincing evidence that the Hep-de(5%) nanocoating strategy is compatible with maintained physiological cell function.

To test the ability of the nanocoating to affect the blood-mediated response against islet cells, anti-coagulant activity of the layered

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islets were tested using the activated partial thromboplastin time assay.[16] Figure 4 shows the clotting time measured as the fibrin strand formation (an indication of the partial thromboplastin) time for islets layered with 1 layer of Hep-de(5.0) and 9 layers of a layering order of $(Hep\text{-}de/GCh)_2/(AAT/GCh)_2AAT$ compared to the islet control and the plasma control. Islets coated with 1 layer of Hep-de (5.0) showed a longer stranding time than the control islets, but this was not significant ($p>0.05$). However, islets coated with layers of $(Hep\text{-}de(7.5)/GCh)_2/(AAT/GCh)_2AAT$ caused a significant increase in stranding time compared to the control islets (Figure 4), demonstrating that the inclusion of AAT in the nanocoating could reduce the IBMIR in this *in vitro* assay. The inclusion of AAT did not completely prevent the islet-induced thrombotic response *in vitro*, most likely because the surface area of the nanocoated islets, and thus the contact area for delivery of AAT, was very small in comparison to the plasma incubation volume. Such a localised anti-coagulant property could however be more favourable over the systemic anticoagulation when used for protecting the cell transplants *in vivo*.

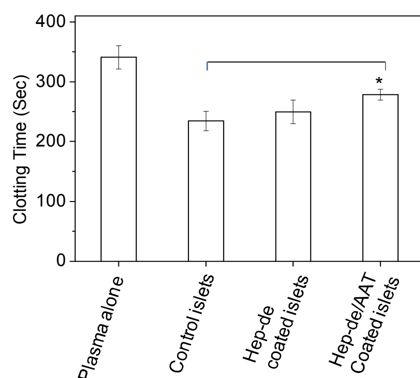

Figure 4. Anti-coagulant activity of the coated islets compared with the controls. ICR mouse islets coated with one layer of heparin-polyaldehyde and 9 layers of AAT/Hep-de(5.0)/GCh were shown. Error bars represent ± SD of the mean, n=5. * represents $p<0.05$.

In summary, our proposed layer-by-layer nanocoating technique was found to be highly efficient in depositing the biocompatible nanolayers on cell surface, whilst maintaining islet cell viability and normal insulin secretory function. The optimized coating scheme has demonstrated significantly higher anti-coagulant activities. However, the anti-inflammatory activities of such a coating and its potential for preventing cellular injury *in vivo* are yet to be tested using animal models of islet transplantation. We are also currently studying the protein payload and release behaviour by using the ^{125}I-labelled AAT and the effects of coating conditions. The data in this study highlights that this coating scheme has the potential to be used as a generally applicable strategy to reduce donor cell-induced IBMIR responses, which could be advantageous in increasing graft survival in therapeutic cell transplant applications.

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