A New Method for Incorporating Functional Heparin onto the Surface of Islets of Langerhans

SANJA CABRIC, Ph.D.,¹ TORSTEN EICH, M.D.,¹ JAVIER SANCHEZ, Ph.D.,¹
BO NILSSON, M.D., Ph.D.,¹ OLLE KORSGREN, M.D., Ph.D.,¹ and ROLF LARSSON, Ph.D.^{1,2}

ABSTRACT

A novel technique is described to conjugate macromolecular heparin complexes to cell surfaces. The method is based on the dual properties of avidin-expressing binding sites for both biotin and a macromolecular complex of heparin. A quartz crystal microbalance with dissipation monitoring (QCM-D) revealed sequential binding of biotin, avidin, and heparin complexes. Large particle flow cytometry confirmed functional integrity. Confocal microscopy of the heparinized islets showed evenly distributed fluorescence. An *in vitro* Chandler loop model demonstrated that the biocompatibility of the new method is comparable to the previous method used on artificial materials with regard to coagulation and anti-thrombin uptake. The technique presented allows human islets of Langerhans to successfully be covered with functional heparin as a means to reduce instant blood-mediated inflammatory reactions induced by the innate immune system.

INTRODUCTION

SIGNIFICANT ADVANCES IN THE FIELD of pancreatic islet transplantation have been made in recent years and have contributed to making the procedure a viable alternative for patients with brittle type 1 diabetes.^{1–4} However, the limited number of pancreata available for transplantation and the present need for multiple donors to achieve normoglycemia still greatly limit the application of this approach. A number of features, including coagulation activation and subsequent insulin dumping, were consistently found after clinical islet transplantation in patients receiving islets by infusion into the portal vein. These observations describe the instant blood-mediated inflammatory reaction (IBMIR),^{5–9} which is a likely cause of both the loss of transplanted tissue and the intraportal thrombosis associated with clinical islet transplantation. Strategies to modulate the islets prior to the transplantation would offer a valuable improvement in

addition to systemic anticoagulant treatments, which have obvious limitations because of their potential adverse effects on hemostasis in the recipient.³

Surface-immobilized functional heparin on artificial surfaces, the Corline Heparin Surface (CHS), is associated with high blood compatibility, inhibited coagulation, and complement activation and reduced platelet adhesion and activation.^{10–16} The main component of the CHS is a macromolecular conjugate composed of an aliphatic carrier chain onto which approximately 70 heparin molecules have been covalently attached, as has been described elsewhere.¹⁷ The chemical approach to attach heparin has been carefully chosen not to interfere with the antithrombin (AT)–binding site of heparin. Numerous reports have demonstrated excellent blood compatibility for CHS applied to artificial materials both *in vitro* and in clinical studies.¹⁸ Due to the fact that CHS heparin conjugate is prepared separately and is carefully purified to remove all cross-linking agents prior to

¹The Rudbeck Laboratory, Division of Clinical Immunology, Department of Oncology, Radiology, and Clinical Immunology, Uppsala University, Uppsala, Sweden.

²Corline System AB, Uppsala, Sweden.

use, this unique molecule can be applied onto a wide range of substrate surfaces, including biological surfaces such as the superficial surface of, for example, living cells, at physiological conditions. A number of strategies to heparinize artificial surfaces have been developed¹⁸; however, to our knowledge there are no other heparin-coating techniques that can be applied to living cells.

In this communication, we describe the development of a method suitable for applying the CHS to islets of Langerhans as a means to mimic the protective anticoagulant activity normally occurring at the endothelial lining of the vascular wall and thereby reducing the tendency of these cells to elicit an injurious inflammatory response at the time of transplantation,^{5,6,8} ultimately leading to the improved islet graft survival and engraftment.

The new protocol, CHS-CT (CHS on cells and tissue), differs from the previous CHS by a different conditioning layer onto which the heparin conjugates are attached, but the final layer facing blood is the Corline Heparin Conjugate, as will be described in more detail below.

Several studies have previously demonstrated successful modifications of islet surface by using different microencapsulation techniques¹⁹ or gene therapy.²⁰ The heparinization approach is distinctly different from coatings, such as microencapsulation, that create not only a barrier for molecules and cells but also a significant dead space, leading to diffusion barriers for nutrients and secreted products. Moreover, unlike other pretreatment procedures such as gene therapy, the heparinization of islet surface should not be associated with an increased risk of inducing inflammatory or adaptive immune responses.²¹ Also, islet functionality was not affected by the heparinization procedure as may be the case by using adenoviral vectors.^{22,23}

The study includes investigations using quartz crystal microbalance with dissipation monitoring (QCM-D), large particle flow cytometry, confocal microscopy, and evaluation in a whole blood test model *in vitro*.

Preclinical evaluation *in vitro* and *in vivo* of heparin-modified islets using CHS has recently been reported separately.²²

MATERIALS AND METHODS

Islet isolation

Islets from human cadaver donors were isolated according to a protocol approved by regional research ethics committees of Uppsala University and performed in accordance with local institutional and Swedish national rules and regulations.

Islets were isolated using a Liberase perfusion followed by continuous-density Ficoll gradient purification in a refrigerated COBE 2991 centrifuge (COBE Blood Component Technology, Lakewood, CO) as previously described.^{24–26}

After the isolation and purification, the islet preparations were placed in untreated single-transfer packs for platelets

1300 (Baxter Medical, Deerfield, IL) and kept at 37°C in an atmosphere of 5% CO₂ in humidified air in culture medium, CMRL 1066 (Invitrogen, Carlsbad, CA) supplemented with 10 mM nicotinamide (Sigma-Aldrich, St. Louis, MO), 10 mM HEPES buffer (Invitrogen AB), 0.25 µg/mL Fungizone (Invitrogen AB), 50 µg/mL gentamicin (Invitrogen AB), 2 mM L-glutamine (Invitrogen AB), 10 µg/mL Ciprofloxacin (Bayer AG, Leverkusen, Germany), and 10% (v/v) heat-inactivated human serum (Uppsala blood bank).

Islet purity was determined by staining with diphenylthiocarbazone. The purity of the islet preparations used varied from 70% to 95%.

Modification of cell surfaces with immobilized heparin

Human islets were biotinylated by incubating the islets for 60 min at 37°C in culture medium containing EZ-Link™ Sulfo-NHS-LC-Biotin (SNL biotin) (Pierce Biotechnology, Rockford, IL) at 1 mg/mL. The islets were then rinsed by changing the culture medium three times. In the next step, the islets were incubated for 30 min at 37°C in culture medium supplemented with 1 mg/mL of avidin (Pierce Biotechnology), and again rinsed by changing the culture medium three times. Finally, 1 mg/mL macromolecular conjugates of heparin, ~70 heparin molecules covalently linked to an inert carrier chain (Corline Systems AB, Uppsala, Sweden), in culture medium, were allowed to bind to the biotin/avidin coating for 60 min at 37°C. The islets were finally rinsed by changing the culture medium three times. This preparation will henceforth be referred to as CHS-CT.

Quartz crystal microbalance with dissipation monitoring

To monitor the various steps in the heparinization procedure, a QCM-D technique (Q-Sense AB, Gothenburg, Sweden) was used. This technique relies on the fact that a mass adsorbed onto the sensor surface of a shear-mode oscillating quartz crystal causes a proportional change in its resonance frequency (f). Changes in f reflect the amount of mass deposited onto the surface of the crystal.¹³ The corresponding dissipation change (ΔD) indicates frictional (viscous) losses induced by deposited materials. Sensor crystals (5 MHz), sputtered with stainless steel SIS2343, were used. All measurements were carried out at 25°C. Since islets cannot be used in QCM, the sensor crystals were modified with human albumin to serve as a substitute for the islet surface. The crystals were incubated in 2% human albumin (Baxter AG, Vienna, Austria) for 30 min at room temperature.

Confocal microscopy

The degree of heparinization of the islets was visualized by confocal microscopy after incubating the islets at room temperature for 15 min with avidin Texas Red (0.01 mg/mL)

(Molecular Probes Europe BV, Leiden, The Netherlands). DAPI (10 $\mu\text{g/mL}$) (Sigma-Aldrich) was used to detect cell nuclei in the islets. Images were acquired with a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss, Jena, Germany) equipped with an Axiovert 200 microscope stand. Z-stacks of the islet surfaces were acquired using the 543 nm laser line, a 10 $\times$ objective, and a 560–615 BP filter. Three-dimensional projections of the acquired Z-stacks were analyzed using Imaris software (Bitplane, Zurich, Switzerland).

Large particle flow cytometry

A BioSorter 1000 large particle flow cytometer from Union Biometrica (Holliston, MA) that can detect and sort large (40–1500 μm) particles in a continuously flowing stream at a rate of 10–50 objects per second was used.²⁷ For these experiments, the instrument was equipped with a three-laser configuration (325 nm UV, 488–514 multiline argon, and 670 nm diode lasers). Particles were distinguished by size, optical density, and intensity of fluorescence markers.

Islets were heparinized using the methods described above. Heparinized islets were detected using AT (1 U/mL) labeled with the fluorochrome Alexa Fluor 488[®] (Molecular Probes, Eugene, OR) or avidin Alexa Fluor 488 (0.01 mg/mL) (Molecular Probes Europe BV). In addition, islets from each step of the heparinization process were analyzed for background fluorescence. Biotinylated islets were also stained with fluorescein isothiocyanate (FITC)-streptavidin, and the background fluorescence of the heparinized islets was evaluated using unlabeled AT alone (data not shown).

In addition, heparinized islets marked with fluorochrome-labeled AT or avidin were incubated with coagulation factor Xa (0.667 nKcat/mL) for 15 min at room temperature. After incubation, the islets were rinsed three times with islet culture medium, and thereafter analyzed.

The Chandler loop model

For the evaluation of biomaterials in circulating blood, Chandler loop model was used as previously described.^{11,13,28} Fresh whole blood was drawn from healthy volunteers, who had received no medication for 10 days, using surface heparinized equipment only, without soluble anticoagulants. Pieces of polyvinyl chloride (PVC) tubing (length 200 mm, inner diameter 4 mm) furnished with immobilized CHS or CHS-CT heparin or left untreated were used. The CHS-CT was applied to tubings that had been preadsorbed with 2% human albumin (Baxter AG). Each tubing was filled with 2 mL of fresh whole blood and turned into closed circuits using surface heparinized connectors (CHS) of thin-walled steel (length 20 mm). The tubing loops were rotated vertically at 33 rpm in 37°C water bath for 1 h. After incubation, 1 mL blood samples were collected in EDTA (4.2 nM, final concentration) and centrifuged at 3500 rpm, 4°C, for 20 min.

Platelet counts were assessed using a Coulter AcT diff analyzer (Beckman Coulter, Fullerton, CA). Plasma concentrations of thrombin–antithrombin (TAT) complexes were quantified using a commercially available ELISA kit (Enzygnost TAT; Dade Behring, Marburg, Germany). AT binding capacity was assessed as previously described.^{13,29}

Statistics

To avoid the effect of individual variations, platelet loss was calculated as percentage of the values of the samples taken before the experiment. All other parameters were given as absolute values. Data are presented as mean $\pm$ SEM.

RESULTS

Optimization of the heparinization procedure using QCM-D

To screen a variety of agents and strategies for coating surfaces with heparin using biotin and/or avidin as an intermediate layer, a QCM-D device was used. The most promising combinations are shown in Figure 1. The results demonstrated sequential binding of biotin, avidin, and heparin complexes, where a mass adsorbed onto the oscillating

FIG. 1. Use of QCM-D to monitor the stepwise build-up of a heparin coat with biotin/avidin. The polystyrene surface was initially coated with albumin in order to create a matrix to which biotin could bind. For each step in which an increased mass is added to the sensor, a corresponding dampening of the frequency is obtained (f , blue line). The corresponding dissipation change, ΔD , is indicated by the pink line. This binding was followed by exposure to avidin and finally to the heparin conjugate. The biological function of the coat was then tested by allowing AT to bind to the surface-linked heparin.

sensor surface causes a proportional change in its resonance frequency, f (blue line). The corresponding dissipation change, ΔD , is indicated by the pink line. These changes in frequency reflect the amount of mass deposited onto the sensor surface. Due to a low molecular weight, binding of biotin resulted in a low frequency shift, following a rapid frequency increase due to the buffer exchange. Binding of avidin to biotin was shown to occur with change in Δf , but no change in ΔD , indicating a rigid binding, whereas binding of the heparin conjugates to avidin resulted in a distinct change in both ΔD and Δf , indicating a more loose and flexible structure. Binding of AT to the final heparin surface was shown to occur by a distinct change in both Δf and ΔD .

Large particle flow cytometry

In order to test functional capacity of bound heparin on the islet surface, large particle flow cytometry was used. Background fluorescence of control and heparinized islets is shown in Figure 2A, and B. Background fluorescence was also obtained with the coupling intermediates before binding of heparin complexes (data not shown). Bound heparin complexes on the islets surface were detected by fluorochrome-labeled AT or avidin (Fig. 2C, D). AT binds to a limited number of AT binding sites (a specific pentasaccharide sequence) in heparin and reflects the biological function of heparin, whereas Alexa Fluor 488-labeled avidin that binds to sites in the whole heparin molecule generated a markedly stronger signal.

In order to test the functional capacity of bound heparin on the islet surface, heparinized islets marked with fluorochrome-labeled AT or avidin were incubated with FXa and thereafter analyzed with respect to detection of remaining AT or avidin. Following incubation with FXa, AT preadsorbed to the heparin-coated islets were found to be removed from the islets, which then appeared to be identical to heparin-coated islets without prior exposure to AT (Fig. 2E). In contrast, heparinized islets marked with avidin remain unaffected (Fig. 2F).

Binding of heparin to human pancreatic islets as visualized by confocal microscopy

Confocal microscopy was used to verify the binding of heparin to human islets and to assess the uniformity of the binding (Fig. 3). The confocal microscopy analysis of the heparinized islets consistently revealed evenly distributed fluorescence, demonstrating that the heparin conjugates covered the whole islet surface (Fig. 3B). Cross sections of the heparinized islets showed that the heparin bound strictly to the surface of the islets (Fig. 3D). This analysis confirmed that the heparinized islets were surrounded by a coherent and smooth coating of heparin, which was predominantly confined to the surface of the islet. The coating was fixed to cultured human islets for more than 24 h. Control untreated islets are shown in Figure 3A and C.

FIG. 2. Large particle flow cytometry. Islets were analyzed in a BioSorter 1000 large particle flow cytometer. Particles were distinguished by size, optical density, and intensity of fluorescence markers. Background fluorescence of control and heparinized islets (A, B). Heparinized islets detected using fluorochrome-labeled AT or avidin (C, D). Heparinized islets labeled with AT (E) or avidin (F) after incubation with FXa. Color images available online at www.liebertpub.com/ten.

In control experiments, we used Alexa Fluor-labeled AT, which binds specifically to the AT binding sequence on the heparin molecule, and evaluated islets that were treated with heparin conjugates alone, with heparin conjugates after biotinylation (excluding the avidin step), and with heparin conjugates after treatment of the islets with avidin (excluding the biotin step). No binding of heparin conjugates or nonspecific absorption to the islet surface was detected under any of these conditions (data not shown).

The Chandler loop model

Biocompatibility of CHS-CT and CHS was compared using a modified Chandler loop model. A marked drop in platelet count and macroscopic clotting could be observed in untreated loops after 60-min incubation with whole human blood. The untreated loops also triggered a rise in the coag-

FIG. 3. Binding of heparin to human islets as visualized by confocal microscopy. Human islets were heparinized and analyzed by confocal microscopy. Bound heparin was visualized using fluorochrome-labeled avidin (red). The upper two panels show representative untreated (A) and heparinized (B) human islets. The lower two panels show representative cross sections of untreated (C) and heparinized (D) human islets. DAPI (blue) was used to detect cell nuclei in the islets. Scale bars: 200 μm .

ulation parameter TAT complex. In loops coated with either CHS or CHS-CT surface, there was no macroscopic clotting, and the drop in platelet count ($n = 3$) and generation of TAT ($n = 3$) were markedly attenuated when compared to untreated loops (Table 1). CHS-CT surface displayed slightly higher AT binding capacity compared to CHS (Table 1).

DISCUSSION

Immobilized functional heparin on artificial surfaces implies excellent blood compatibility, with inhibited coagulation and complement activation and reduced platelet adhesion and

activation.¹⁸ Surfaces of biological origin may also sometimes be thrombogenic; therefore, there is a need to be able to modify such surfaces to reduce thrombogenicity in order to avoid deleterious clotting and inflammatory reactions when biological tissue is transplanted and exposed to circulating blood. IBMIR, elicited by islets of Langerhans at the time of intraportal transplantation,^{5,6,8} is one such example that stimulated the development of the new method for applying immobilized heparin onto surfaces of biological origin as described in this paper.

The major component of the CHS-CT is identical to the one used in CHS, namely, a macromolecular conjugate composed of ~ 70 heparin molecules covalently bound to an aliphatic carrier chain.¹⁷ The fact that this heparin conjugate is prepared separately implies that the treatment of the cells can be carried out at physiological conditions and that no potentially harmful substances are introduced. Another important component is avidin, which, in addition to binding firmly to biotin, also expresses strong affinity for heparin.³⁰ Provided that avidin has been laid down on the cell surface, by virtue of binding to immobilized biotin at the cell surface, the heparin conjugates will then be attached by a self-assembly type of process. The conjugates will be driven to the surface by attraction to the heparin binding sites in avidin, but at the same time, the individual conjugates will also repel each other due to the high negative net charge.

The QCM-D measurements revealed sequential binding of biotin, avidin, heparin complexes, and AT to the surface. In addition, large particle flow cytometry showed that the CHS-CT surface is biologically functional as judged by the fact that the FXa was capable of removing AT preadsorbed to the immobilized heparin on the islet surface. Since AT is a natural inhibitor of FXa,³¹ the FXa-AT complex is probably formed, leading to the release of FXa-AT complex from the surface of the heparinized islets.

Confocal microscopy analysis of the heparin-coated islets revealed evenly distributed fluorescence, demonstrating that the heparin coating covers the entire islet surface. In addition, cross sections of the islets showed that the heparin is predominantly bound to the surface of the islets. The approach is fundamentally different from microencapsulation, which creates a barrier and more importantly a dead space between the encapsulated cells and their surrounding environment, leading to the diffusion barriers.¹⁹ Thus, the heparin coat gives the transplanted cells the opportunity to recover under the heparin shield and finally engraft in a process similar to nonheparinized islets. This supposition was confirmed when islets were exposed to glucose stimulation in a dynamic perfusion system, in which heparinized and untreated control islets responded similarly, indicating that insulin secretion was not affected by the heparinization procedure.²² Moreover, in a syngeneic islet transplantation model in alloxan diabetic mice, all mice that received either heparinized or untreated control islets had normalized blood glucose levels within 10 days after transplantation and subsequently

TABLE 1. THE RELATION BETWEEN THE HEPARIN SURFACES AND BLOOD COMPATIBILITY

		Platelets	
	AT (pmol/cm^2)	(% of presample) ^a	TAT (mg/L) ^a
Presample		100 $\pm$ 0.0	3.4 $\pm$ 0.5
Untreated		2.3 $\pm$ 0.9	22601.6 $\pm$ 4390.7
CHS	4.6 $\pm$ 0.2	89.8 $\pm$ 1.7	44.7 $\pm$ 20.7
CHS-CT	6.3 $\pm$ 1.5	86.7 $\pm$ 4.7	148.1 $\pm$ 35.3

^aMean $\pm$ SEM after 60 min (0 min for presample).

responded equally well to an intraperitoneal glucose tolerance test. In addition, positive insulin staining and intact morphology revealed successful engraftment and survival of both heparin and untreated control islets.²² In order to compare biocompatibility of CHS-CT and CHS at conditions as identical as possible, a modified Chandler loop assay was employed. As this assay relies on the use of tubing loops made of PVC,^{11,13,28} the CHS-CT was prepared onto a layer of adsorbed albumin that served as a substitute for a surface of biological origin. In the present study, we demonstrate that the biocompatibility of CHS-CT surface with regard to coagulation and AT uptake is nearly as effective as CHS.

It should be emphasized that the surface density and coverage of albumin on the PVC tubing may be different compared to the protein composition at the islet surface, and hence, the preparation of CHS-CT on the PVC tubing represents a compromise to comply with the model used and may not completely reflect the coating efficacy of islet of Langerhans.

CONCLUSION

Immobilization of heparin is an excellent option to render biomaterial surfaces blood compatible with regard to coagulation, complement, and platelet activation.¹⁸ Similarly, covering the islet surface with heparin offers an attractive alternative allowing for preventing the IBMIR, thereby reducing the risk of thrombosis concomitant with improving both the safety and the outcome of clinical islet transplantation.

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Address reprint requests to:
Sanja Cabric, Ph.D.
The Rudbeck Laboratory C11
Dag Hammarskölds väg 20
Uppsala SE-751 85
Sweden

E-mail: sanja.cabric@klinimm.uu.se