Sequential Immobilization of Thrombomodulin and Endothelial Protein C Receptor on Polyurethane.

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Statement of Purpose: Polyurethane is often used in biomedical devices because of its mechanical properties and natural biocompatibility. However, polyurethane has been reported to be thrombogenic. To overcome this property, studies have been conducted on the surface immobilization of proteins or biomolecules such as heparin, cholesterol and albumin.

Thrombomodulin (TM) is a glycoprotein which is found on the surface of endothelial cells. In vivo, TM binds to thrombin inactivating the thrombin and forming a complex which activates Protein C (PC) to form Activated Protein C (aPC) a powerful anticoagulant which prevents the activation of thrombin from thrombus. On endothelial cells, TM is found in close proximity to Endothelial Protein C Receptor (EPCR) which has a high affinity for PC which can then be activated by the TM / thrombin complex. In vivo, EPCR has been shown to have a 3-4 fold increase in the activation of PC.

Because of the cooperative nature of the two proteins, a patterned co-immobilization was desired to sequentially immobilize the two proteins. Current methods of patterning such as photo mask immobilization have been resolved for cell scale levels, 100 nm, but are not precise enough for that of enzymes. To overcome this limitation a chemical process was used to create a bi-dentate functional group extension from the polyurethane surface. This bridge (Figure 1) allows one protein to be immobilized through a coupling reaction while the other protein is immobilized through a nucleophilic reaction while tethering the two proteins to a single polyurethane monomer.

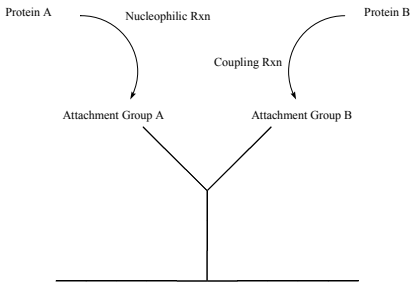

Figure 1

The objective of this study is to determine a method to covalently immobilize 2 proteins, TM and EPCR in a sequential method and to then test whether those proteins can activate the anticoagulant aPC.

Methods: Extruded samples of ChronoFlex AR medical grade polyurethane (PU) was generously provided CardioTech International, Inc. The PU was cut into small working samples and the surfaces activated on the secondary nitrogen of the carbamate subunit. Analysis of the modifications to the PU was done by FTIR, XPS and titration assays. The mechanical analysis of the polymer, pre and post modification, were conducted by DMA. Biocompatability of the modified polymer were compared to control unmodified samples using MTT assays with cells grown both on the polymer samples and in contacted media for leachables. The thrombogenic properties of the modified polymer were analyzed using a thromboelastograph (TEG).

Recombinant cytosolic EPCR was produced in Picia Pastoris and the protein purified and analyzed for size and activity prior to immobilization.

Protein C activation was measured on samples immobilized with TM, EPCR, both or TM and RCR2, an antibody which binds to the carboxy terminus of the EPCR. Samples were incubated with thrombin, then incubated with PC and the reaction stopped using anti-thrombin. aPC was quantitated using spectrozyme.

Results: The chemical modification of polyurethane was able to produce bi-dentate bridge and these results were confirmed by FTIR and XPS analysis. Protein immobilization experiments using hIgG showed approximately 1.5 μg / cm^2. Co-immobilization of the surface was verified by using fluorescently labeled antibodies.

The biocompatibility of the modified surfaces showed no statistical increase in the toxicity of the material over that of the medical grade PU starting material. Samples analyzed by TEG showed no significant change in the clotting characteristics including the clotting time and the clot strength.

aPC assays showed that samples with EPCR had no activation of PC. Samples with EPCR co-immobilized with TM had a significant increase in the activation of PC to aPC, Figure 2.

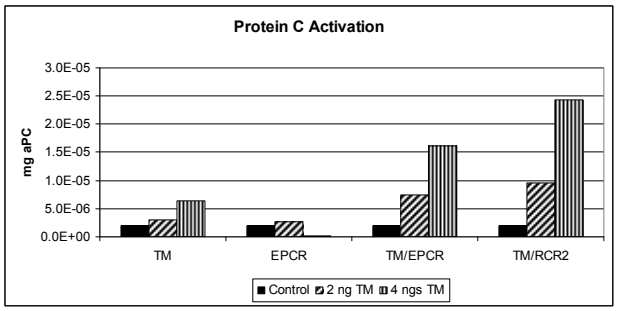

Figure 2

Conclusions: We have shown that the modification of polyurethane can produce a surface capable of sequential immobilization of biomolecules or proteins on an enzymatic scale. We have also shown that the co-immobilization of EPCR with TM increases the activation of PC to the anticoagulant aPC, similar to the native endothelium.

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