Injury-targeted Enzyme-responsive Direct Delivery of Thrombin for Hemostatic Treatment of Coagulopathy

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Statement of Purpose: The term 'hemostasis' refers to the body's natural ability of blood coagulation at a vascular injury site, to stanch bleeding. This process involves: 1) platelet adhesion and aggregation at the site, 2) tissue factor-mediated and platelet mediated amplification of thrombin at the site, and 3) thrombin-induced conversion of fibrinogen to fibrin which deposits as a biopolymeric mesh on the aggregated platelets to render a stable blood clot that 'seals' the injury. Dysfunction and defects in any of these cellular (e.g. platelets) and molecular (e.g. coagulation factors) components results in coagulopathy and suboptimal hemostasis, that can lead to acute, chronic and inherited bleeding disorders (e.g. in trauma, surgery, hemophilia, vWF disease, Glanzmann's thrombasthenia, Bernard-Soulier syndrome etc.). We hypothesized that injury-targeted direct delivery of thrombin can circumvent these cellular and molecular defects, and locally amplify fibrin generation to rescue and augment hemostasis. For this, we utilized a liposome-templated nanoparticle platform that can encapsulate thrombin, bind specifically to the injury site via interaction with exposed collagen, locally deposited vWF, locally aggregated active platelets or locally formed fibrin, and release the thrombin triggered by the action of injury site-specific enzymes like phospholipase A_2 (PLA_2). Here we present our results on injury site-relevant targeting, thrombin release and hemostatic augmentation by this technology.

Methods: Cholesterol, Distearoyl phosphatidylcholine (DSPC), Polyethylene glycol-modified distearoyl phosphatidylethanolamine (DSPE-PEG) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Platelet-targeting, fibrin-targeting peptides were obtained from Genscript (Piscataway, NJ, USA). PLA2 was obtained from Sigma-Aldrich (St. Louis, MO, USA). Thrombin was obtained from Haematologic Technologies (Essex Jn., VT, USA). Lipid-tethered Rhodamine B fluorescent probe was obtained from Setareh Biotech (Eugene, OR, USA). Rotational thromboelastometry (ROTEM) instrument and reagents were from Instrumentation Lab (Bedford, MA, USA). Human whole blood was obtained from donors using CWRU IRB-approved protocol. A variety of coagulation and anticoagulation agents obtained from Haematologic Technologies (Essex Jn., VT, USA). For the proposed technology, targeting peptides were conjugated to DSPE-PEG, and the DSPE-PEG-peptides, DSPC and cholesterol were self-assembled into nanovesicles (~200 nm diameter) loaded with thrombin using the thin film rehydration and extrusion technique. Targeting of these nanovesicles to injury site-relevant human platelets and to fibrin were characterized by immunofluorescence confocal microscopy. PLA2-triggered thrombin release from the vesicles was characterized by UV-Vis spectrometry. Effect on blood coagulation was assessed by ROTEM and microfluidics. Hemostatic efficacy was evaluated in a rat model of liver hemorrhage.

Results: The nanovesicles could effectively anchor onto activated platelets and fibrin, thus demonstrating feasibility to anchor onto injury site relevant components. PLA_2 was able to trigger the enhanced release of thrombin from the vesicles. Released thrombin was able to rescue/amplify coagulation parameters (e.g. clotting time, clot firmness and stability) as evident from ROTEM studies. In presence of simulated platelet dysfunctions (e.g. thrombocytopenia) and coagulation dysfunctions (e.g. anticoagulant effects), the thrombin-releasing vesicles were able to rescue clot kinetics and coagulation outputs (e.g. fibrin formation and clot stability). In vivo studies in rat model demonstrated efficacy in augmenting hemostasis and reducing blood loss. **Figure 1** shows some representative results.

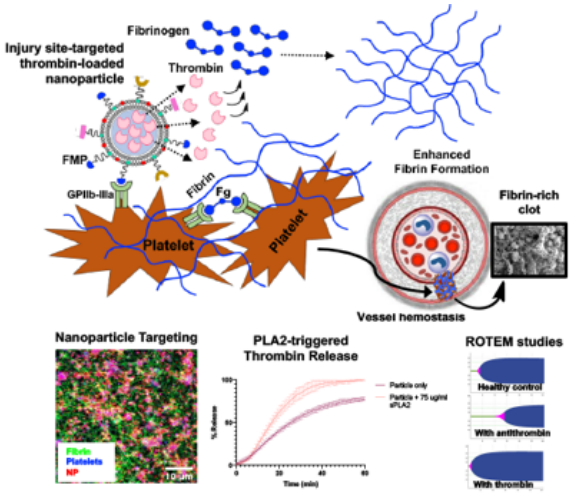

Figure 1. Schematic of targeted thrombin delivery, leading to enhanced fibrin generation for augmenting hemostasis; Representative data show NP targeting, PLA_2-triggered enhanced thrombin release, and ROTEM data for thrombin rescue of clot defect in antithrombin-treated blood.

Conclusions: Irrespective of dysfunction in the cellular and/or molecular components of blood coagulation and hemostasis mechanisms, a central issue in many coagulopathies is the attenuated generation of thrombin at the injury site, which then results in reduced fibrin generation. We rationalize that such issues can be treated by directly delivering thrombin in a targeted manner at the injury site. We tested this possibility by utilizing an injury site-targeted liposome-templated enzyme-responsive nanoparticle system as a carrier platform for thrombin. Our results demonstrate the feasibility of this approach to deliver thrombin for localized generation of fibrin for hemostatic augmentation. This strategy can be potentially used to treat bleeding complications in prophylactic as well as emergency settings. Future studies will be directed at testing this strategy for treatment of several inherited and acquired clinically important hemostatic dysfunctions.

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