Development of Synthetic Platelets for Hemostatic Applications

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Statement of Purpose: Uncontrolled bleeding following traumatic injury is the leading cause of death among trauma patients.[1] Thirty-four percent of these deaths occur in the prehospital setting, highlighting the need for better hemostatic agents for emergency medicine applications. Clot formation is critical to the cessation of bleeding and involves the formation of a platelet plug embedded within a fibrin mesh, however, clot formation is impaired during hemorrhaging due to massive dilution of platelets and other critical clotting factors. During clot formation, platelets bind multiple fibrin fibers thereby cross linking and stabilizing the developing clot. We have designed synthetic platelets (SPs) that mimic this feature of natural platelets by interacting with fibrin with high affinity and specificity at the site of injury. To maximize interactions with fibrin networks, we utilize highly deformable, ultra-low crosslinked pNIPAm microgels with multiple sites for chemoligation as the base material for our hemostatic material. To impart fibrin specificity to the microgels, humanized synthetic single chain variable fragment (scFv) antibodies with high affinity for fibrin were identified through molecular evolutionary techniques and then coupled to the microgels. Like platelets, our SPs will specifically target the site of injury and prevent nonspecific clotting in the bloodstream.

Methods: ScFv antibodies with high affinity for fibrin and low affinity for fibrinogen were identified using two phagemid libraries, Tomlinson I and the domain antibody library, in biopanning assays against fibrin. Ten clones were identified per library, and the specificity and binding affinity of each clone to fibrin were evaluated with surface plasmon resonance (SPR). To create SPs, the clone found to have the highest affinity for fibrin, clone H6, was conjugated to the microgels using EDC/NHS coupling. We utilized fibrinogen and fibrin-based ELISAs, along with the Fortébio BLITZ system, to characterize SP binding to fibrin. We then characterized the effect of SPs on fibrin network structure using confocal microscopy. Finally, to characterize the effect of SPs on clotting *in vitro*, we utilized an endothelialized microfluidics device that accurately recapitulates the cellular, physical, and hemodynamic environment of microcirculation.[2] Clotting of platelet poor plasma (PPP) in the absence or presence of SPs was analyzed in real time using confocal microsopy. As a control, clotting of platelet rich plasma (PRP) or PPP in the presence of non-fibrin binding microgels was also investigated.

Results: Using fibrinogen and fibrin ELISAs, we observed binding of SPs to fibrin but not fibrinogen coated surfaces, demonstrating the specificity of the SPs for fibrin. Binding of SPs to fibrin surfaces was further confirmed in a label free system utilizing the Fortébio BLITZ. Confocal microscopy analyzing the effect of SPs on fibrin network formation demonstrated that SPs interact extensively with fibrin networks compared to control microgels; furthermore, increasing concentrations of SPs during fibrin polymerization result in a denser fibrin network compared to control gels. Finally, *in vitro* clotting experiments in an endothelialized microfluidic device demonstrate robust clotting of PPP in the presence of SPs, similar to that observed with PRP, while PPP alone or in the presence of non-fibrin binding microgels resulted in minimal clotting (Figure 1). Furthermore, clotting was also observed upon addition of SPs to PPP diluted 1:1 with buffer, conditions which simulate the dilution of clotting factors observed during hemorrhage.

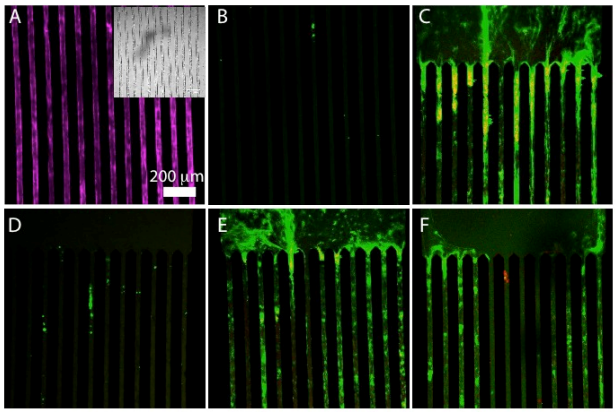

Figure 1. *In vitro* clotting assay. Endothelial cells prior to plasma injection stained with cell mask deep red (A) and phase (inset). Clotting of PPP alone (B), PRP (C), PPP + non-fibrin binding microgels (D), PPP + SPs (E) and diluted PPP + SPs (F). Fibrin=green; microgels/platelets=red

Conclusions: These studies combine innovative biopanning techniques with highly deformable microgels to develop SPs that augment the natural clotting cascade. Like natural platelets, our SPs specifically target fibrin rather than fibrinogen and interactive extensively with fibrin networks, successfully fulfilling the critical design criteria for our hemostatic agents. Finally, the addition of SPs to PPP results in robust clot formation in an *in vitro* clotting assay, demonstrating the success of our design. The utility of SPs in augmenting clotting will be further verified in future studies *in vivo* utilizing bleeding time assays in a rat femoral artery injury model.

References:

1. Rossaint, R, *et al*.Crit Care. 2010;14(2):R52.
2. Myers, DR, *et al*. J Vis Exp. 2012; (64).