**Integrin- Mediated Platelet Adhesion as a Mechanism for Hemostatic Activity of a Keratin Biomaterial.**

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**Statement of Purpose:** Keratin based hydrogels demonstrate novel hemostatic characteristics in several animal models (Aboushwareb et al. 2009). The major constituents of this biomaterial are alpha and gamma keratin protein subtypes isolated from human hair. Although records of human hair being utilized to prevent blood loss date back to the 1500s; little is known about the mechanism by which these proteins aid in blood clotting. Analysis of the amino acid composition of known keratins indicates the presence of several binding domains including Arg-Gly-Asp (RGD) and Leu-Asp-Val (LDV). These binding domains are involved in integrin mediated adhesion of platelets to the extracellular matrix proteins in the clotting cascade. It is hypothesized that the keratin hydrogels contain specific binding motifs that facilitate rapid clotting by activating and binding platelets, an essential step in the blood clotting cascade. The purpose of this work is to determine which integrins are involved in platelet adhesion to the keratin hydrogel surface aswell as identify the keratin sub-types to which platelets demonstrate the strongest binding affinity.

**Methods:** Keratin Hydrogel Preparation: Untreated human hair obtained from a commercial vendor was cut into small fibers and reduced with thioglycolic acid to initiate isolation of the keratin proteins. Further extraction and separation of the alpha and gamma keratin sub-types was accomplished via denaturation and isoelectric point precipitation, respectively. The separated alpha and gamma proteins were then dialyzed for further purification. Hydrogels were prepared by allowing the disulfide linkages to re-form by exposure to oxygen during an overnight incubation at $37^0$C.

*Colorimetric Assay*: A colorimetric assay relating absorbance to platelet adhesion was utilized to quantify platelets bound to the surface of keratin hydrogels with various alpha/gamma combinations. Keratin, fibrin and collagen hydrogels were incubated in microtiter plates at $37^0$C to allow for proper gelation. Platelet-rich-plasma (PRP) was obtained from consenting donors and isolated using differential centrifugation. Platelets were pre-incubated with anti- integrin antibodies before incubation on the hydrogels. After incubation, non adherent platelets were gently washed off the gels followed by addition of a para-nitrophenylphosphate (pNPP) solution. Alkaline phosphatases on the surface of platelets react with the pNPP substrate resulting in a color change after quenching with 2N sodium hydroxide. An end point absorbance reading was taken at 405nm.

*Scanning Electron Microscopy*: SEM was used to observe morphological changes in the platelets after adhesion to the keratin biomaterial. Keratin and fibrin hydrogels were prepared in chambered slides and allowed to gel at $37^0$C.Platelets were then layered on to the chambers and incubated for 10 minutes followed by removal of unbound platelets. Samples were then fixed, dehydrated and sputter coated with gold-palladium prior to examination in the microscope.

**Results:** Scanning electron microscopy experiments have shown activated platelet morphology on the surface of keratin hydrogels (Figure 1) resembling platelets on the surface of ECM proteins. Platelet adhesion to the surface of keratin appears to significantly ( $p<0.01$) decrease with blocking of the β1 and β3 integrin subunits (Figure2); while on collagen, platelet adhesion was reduced when β1 was blocked, consistent with published results. The alpha/gamma proportions do appear to effect biological function of the keratin coatings, (Figure 3), with the highest platelet adhesion being a 98% alpha, 2% gamma mixture of keratin proteins.

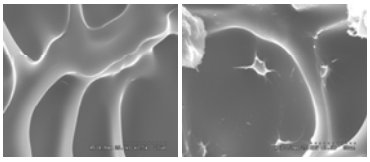

**Figure 1**.(A) SEM image of lyophilized keratin hydrogel. (B) SEM image of keratin hydrogel with activated platelets.(1500 X)

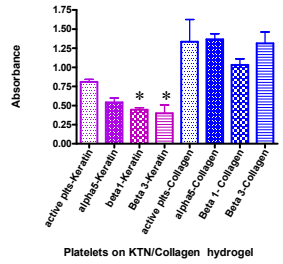


**Figure 2**. Platelet adhesion on the surface of keratin and collagen in the presence and absence of antibodies against α5, β1 and β3 integrin subunits. (*$p<0.01$)

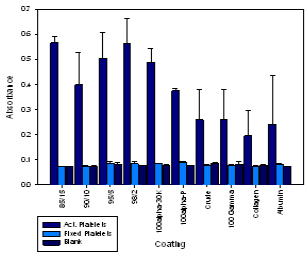


**Figure 3**. Platelet adhesion on the surface of keratin coatings with varying alpha/gamma keratin combinations.

**Conclusions:** Keratin based hydrogels appear to be pro-adhesive with functional similarities to fibrin and collagen. Optimizing this novel material can only occur with an understanding of the biochemical mechanisms involved in keratin's unprecedented hemostatic abilities aswell as the functional differences between keratin subtypes. This project has shown evidence for integrin-mediated adhesion of platelets and the variation of efficacy by altering the keratin hydrogel's protein composition.

**References:** Aboushwareb T. J Biomed Mater Res Part B: Appl Biomater. 2009;90B:45-54.