Uncontrolled hemorrhage is the leading cause of death on the battlefield and second leading cause of death in civilian trauma. Recent animal testing using a lethal arterial injury model compared a variety of woven and non-woven products with granular products, and found only one product (WoundStat) gave consistent animal survival (J Trauma. 2007;63:276-284). This product is a clay mineral substance which seals the hemorrhaging wound in 30 seconds. However, the United States Army Institute for Surgical Research has put forth the challenge to develop an intact dressing with comparable hemostatic properties. Thus we have undertaken to study this substance with cotton derivatives that will confer structural integrity, sealing properties, and be compatible with clotting and wound physiology. Product prototypes were tested in a lethal vascular injury model followed by in vitro assessment of the clotting properties of the cotton composites. The challenge in developing this type of cotton product include putting together the material properties compatible with battlefield administration, and understanding the effect on clotting properties that synergize with the sealant properties to give the materials their unique hemostatic properties.

Table 1. Dressings approved for hemorrhage control and their basic material composition.

<table>
<thead>
<tr>
<th>Dressing</th>
<th>Material Composition</th>
</tr>
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<tbody>
<tr>
<td>Quickclot</td>
<td>Zeolite</td>
</tr>
<tr>
<td>HemCon</td>
<td>Chitosan</td>
</tr>
<tr>
<td>Fibrin Sealant</td>
<td>Thrombin &amp; Fibrin</td>
</tr>
<tr>
<td>Army Field Dressing</td>
<td>Cotton</td>
</tr>
<tr>
<td>Quickclot Combat Gauze</td>
<td>Kaolin-Impregnated Gauze</td>
</tr>
<tr>
<td>Woundstat</td>
<td>Bentonite</td>
</tr>
</tbody>
</table>
Most of the dressings listed in Table 1 have been deployed by the armed forces most recently in Iraq or Afghanistan. The prohibitive price of Fibrin Sealant which consists of fibrinogen and thrombin ($500 – $1000 per dressing) prevents widespread deployment of this type of dressing. The dressings evaluated by the USISR were the Army Field Dressing (a cotton product of long-standing use), Quickclot, HemCon, and Fibrin Sealant. The Army Field Dressing, which is the standard field dressing used by the military consists of two layers of gauze that wrap densely packed cotton. It absorbs a large volume of blood, and the cotton strands stimulate platelet aggregation. Quickclot is a granular mineral zeolite that rapidly absorbs water in an exothermic reaction (2). Some improvements on zeolite in the form of kaolin-impregnated gauze (Quickclot Combat Gauze) and bentonite (Woundstat) have been made recently. Kaolin and bentonite are also clay minerals which act as sealants. HemCon (chitosan) as previously described has strong tissue adhesive properties that seal the wound and stop bleeding through promotion of platelet aggregation. Previously we described some of the properties of chitosan-treated cotton gauze (3). Recent work by Ward et al. (4) has demonstrated the relative efficacy of products like Quickclot, HemCon, and Woundstat. The Woundstat, which is principally bentonite gave 100 percent survival in the swine femoral artery model. The substance acts to rapidly seal the hemorrhaging artery, and aggregated red blood cells and the formation of fibrin matrix were noted 1 minute following application. The authors note that Woundstat adheres well, without becoming inextricably linked to tissue, and there is no exothermic reaction.

This paper examines the relative effects of cotton and bentonite on blood clotting. Little is known about the actual effect of these materials or their combination on clotting. Since clotting is a function of the biochemical cascade of zymogen serine proteases being activated in succession as clotting factors with both an extrinsic component and an intrinsic one, the central role of thrombin has been well documented. Thrombin is a serine protease that, as shown in Figure 1 converts fibrinogen to fibrin. Here we show how a fluorogenic assay based on thrombin release can be adopted to assess the effect of bentonite and cotton on blood clotting. The benefits of a fluorogenic assay is that the fluorescence signal is only minimally effected by adsorption due to the blood or the appearance due to a clot and turbidity, fibrinogen does not need to be removed, and platelet aggregation does not effect it.

![Figure 1. Thrombin converts fibrinogen to fibrin.](image)

**Materials and Methods**

An assay for monitoring blood clotting via thrombin release and hydrolysis of a fluorogenic substrate was developed. The assay adopted from TECHNOTHROMBIN TGA is based on monitoring the fluorescence generated by the cleavage of a peptide fluorogenic substrate (Carbobenzoxy-Gly-Gly-Arg-AMC) by thrombin during the clotting process. From the changes in fluorescence over time, the concentration of thrombin (nM) in the sample can be calculated using the respective thrombin calibration curve. The increase in thrombin concentration with time then allows to calculate generation of thrombin in the sample and to plot such thrombin values over time for the whole coagulation process. This results in the visualization of the different phases of clot formation. The assay procedure was followed as outlined in ‘TECHNOTHROMBIN TGA; for research only’ obtainable at
The assay was adapted to a microtitr plate reader containing a fluorescence detector. Samples of bentonite and cotton were pre-weighed and added to the plates prior to adding the reagents. The assay was completed as prescribed and evaluation software (www.technoclone.com) was used to calculate thrombin generation in the samples over time and the results were given in nM thrombin generated in the samples for each point of time during the whole coagulation process. Upon initiation of coagulation in the samples by addition of CaCl2 and the phospholipid/tissue factor mixture, generation of thrombin is initiated after a lag period; thereafter thrombin generation per minute increases, reaching a maximum of thrombin generated and decreases thereafter.

Figure 2. Plots of thrombin-monitored coagulation.

Results and Discussion

The thrombin-monitored coagulation to evaluate the relative effects of bentonite and aminized cotton on clotting is shown in Figure 2. The thrombin parameters that underscore the onset and process of coagulation are lag phase, slope, peak thrombin, and inactivation phase. The lag phase which is the clotting time or time from the addition of reagents until the first burst of thrombin was shorter for bentonite than for aminized cotton. The relative slopes which are a measure of the steepest rate of thrombin formation were about the same. The peak thrombin concentration for aminized cotton was five-fold higher for aminized cotton compared with bentonite. In a similar vein the area under the curve for aminized-based coagulation appears to be considerably more robust than with the bentonite. This measurement of thrombin under the curve is indicative of the amount of work that can potentially be done by thrombin, or how much and how long it is active. Finally the inactivation phase for the aminized cotton was more long-lived that for the bentonite.

Summary

Previously we have observed that both aminized cotton and bentonite show promise in serving as hemostatic agents and in lethal arterial hemorrhage control respectively. Although the sealant properties of bentonite are well documented, and the potential to use aminized cotton based on its ability to promote aggregation of negatively charged platelets has been observed, little is known about the effects of both of these materials on blood clotting. We have shown here that the relative effects of both of these materials have similarities and differences. The nearly similar lag phase which accounts for clotting time is interesting. On the other hand thrombin formation and potential seem more pronounced with the aminized cotton. Further studies will be needed to understand the full meaning and potential of these findings. However it is clear that this result shows the promise for use of these agents in hemostatic control.

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Reference

