Fibrin Glue Based Clotting Factor Cocktail for High Throughput in vitro Thrombogenicity Screening

by

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George Truskey

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Brenton Hoffman

Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the Department of Biomedical Engineering in the Graduate School of Duke University

2013
ABSTRACT

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Abstract

About one in four deaths in the United States are currently due to some form of heart disease (Roger, Go et al. 2012). Patients with cardiovascular disease usually require some form of perfusion-based surgical intervention at some point in their lives. To this end, biomaterials used in these applications elicit thrombus formation at some point after implantation. To date, accurately predicting the thrombogenicity of biomaterials remains difficult, and current testing methods are not comprehensive (Ratner 2013). This is due to the fact that although blood clotting has been a topic of research for many decades, the interplay between the myriad of biochemical and physical reactions that occur in this process is not yet fully understood. One problem is the unpredictability of working with whole blood, as its precise contents vary from person to person, and its activity varies with handling methods (Ratner 2013). Therefore, it would be interesting to use a bottom-up approach and build an in vitro assay with minimal and specific clotting components to determine differences in thrombogenicity between different blood-contacting surfaces that may be used on an implantable biomaterial.

By combining clotting factors Va, Xa, II, and fibrinogen and focusing on anti-thrombotic modulators antithrombin III and heparin, samples were tested to show a dose response in clotting times and fibrin clot formation. This was done by measuring turbidity in a plate reader, and by manually assessing the time at which the first fibrin
strand could be pulled up with a pipette tip. R squared values of greater than 0.96 for all correlation plots demonstrated a strong correlation between manual and plate reading times in all cases. Clotting factor cocktails with thrombin and prothrombinase complex factors showed comparable clotting times at 1U, 0.5U, 0.25U, and 0.125U/mL of equivalent thrombin activity in plasma. A significant dose response to heparin sodium salt with 0.01U/mL antithrombin III and 0.1U/mL prothrombinase complex was demonstrated at 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 μg/mL of heparin sodium salt. These results show that it will be possible to construct specific microplate-based thrombogenicity assays with purified components of clotting.
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1. Introduction

1.1 Coagulation Cascade and Thrombosis

As of 2010, 34.3% of all deaths in the United States are due to cardiovascular disease (Pankajakshan and Agrawal 2010). By 2030, about two in five of Americans are predicted to have some form of cardiovascular disease (CVD) (Heidenreich, Trogdon et al. 2011). CVD patients will require some form of perfusion-based surgical intervention at some point in their lives. The improvement of cardiovascular devices by promoting desirable blood-biomaterial interactions is of paramount importance for the care of this large population of current and future patients with CVD. There are a few options for vascular occlusive diseases, which include stenting, angioplasty, endarterectomy, and bypass graft surgery (de Mel, Cousins et al. 2012). The devices involved in these surgical interventions include vascular stents, bypass grafts, and heart valves (de Mel, Cousins et al. 2012). In many cases, the occluded vessel cannot be bypassed by an autologous source because the possible autologous vessel sources are either diseased or of inadequate caliber, in which case a synthetic graft must be used (Sarkar, Sales et al. 2007). In the case of small diameter grafts (less than six millimeters in diameter), one of the leading causes of graft failure is thrombosis (Pankajakshan and Agrawal 2010). This happens when the native tissue responds to the graft material by eliciting the foreign body response, which triggers the intrinsic coagulation pathway. Namely, proteins
adsorb at the surface, then platelets attach to the foreign surface via adherent proteins, which activate neighboring platelets, and fibrin stabilizes the thrombi in a local network structure (Jung, Braune et al. 2012).

In the case of vascular grafts, the vessel becomes newly occluded by thrombus formation caused by this response. Because every patient has unique pathology and reactions to different drugs, it is sometimes difficult for the attending physician to determine the best course of action for a patient with cardiovascular disease with occluded blood vessels. Therefore, it would be useful to answer specific questions about the thrombogenicity of such biomedical products. In this way, doctors could make better informed decisions to treat their patients. Currently, there is an incomplete understanding of the relationship between thrombogenic potential and surface properties of the medical device (Sarkar, Sales et al. 2007).

Although blood clotting has been a topic of research for many decades, the interplay between the myriad of biochemical and physical reactions that occur in this process is not yet fully understood. To date, accurately predicting the thrombogenicity of biomaterials remains difficult, and current testing methods are not comprehensive (Ratner 2013). One problem is the unpredictability of working with whole blood, as its precise contents vary from person to person, and its activity varies with handling methods (Ratner 2013). Therefore, it would be interesting to use a bottom-up approach
and build an assay with minimal and specific clotting components to determine specific differences in thrombogenicity between different blood-contacting surfaces that may be used in an implantable biomaterial. In this way, this method could provide useful information in determining the clottability of biomedical materials treated with a variety of anti-thrombotic modalities.

To review, the classic clotting cascade represents an overview of the series of protein interactions that propagate chemical signals which ultimately result in the control of fibrin clot formation. Broadly, this cascade has two branches- the extrinsic, or tissue factor pathway, and intrinsic or contact activation pathway- that converge at the common pathway from which a stable, cross-linked fibrin clot ensues (Fig. 1) (Wolberg 2007; Versteeg, Heemskerk et al. 2013).
Figure 1. Simplified overview of clotting cascade.
In general, the extrinsic pathway is activated by injury to the tissue, while the intrinsic pathway is activated by contact with a charged surface (Gailani and Renné 2007). The common pathway begins with activated clotting factor X, which mediates the conversion of prothrombin to thrombin in the presence of its cofactor, activated factor V (Walker and Royston 2002). Together, factors Xa, II, and Va, in the presence of calcium ions, form the prothrombinase complex (Fig. 2) (Swords and Mann 1993).

Figure 2. Simplified view of the prothrombinase complex. Briefly, activated factor V acts as a cofactor to serine protease activated factor X in the presence of calcium ions to cleave prothrombin and reveal its active site, thus forming thrombin.

From there, thrombin cleaves fibrinogen into fibrin, which is ultimately cross-linked by factor XIII (Walker and Royston 2002). It is important to note that the current model of coagulation is a closer depiction of the in vivo dynamics of clotting, showing it as a three step process by which the intrinsic and extrinsic pathways both play a part. This is the cell based model of coagulation introduced by Hoffman et al. (Hoffman and Monroe 2001), which shows how the clotting factors interact with platelets to go through
initiation, propagation, and amplification phases in the stabilization of a fibrin plug.

However, this project focuses on the direct inputs of fibrin clotting; namely, the factors in the common pathway and its anticoagulants.

One well-studied anticoagulant pathway is the antithrombin III/heparin pathway. Heparin acts as a cofactor to antithrombin III, increasing its activity by as much as three orders of magnitude in vivo (Olson and Chuang 2002). Together, they act to inhibit clotting by inactivating factor Xa and thrombin (Fig.3).

![Antithrombin Anticoagulation Pathway Diagram]

**Figure 3.** A schematic to show the antithrombin anticoagulation pathway. Heparin acts as a cofactor to antithrombin III by inactivating Xa and sequestering thrombin.

Thus, by combining clotting factors Va, Xa, II, and fibrinogen and focusing on anti-thrombotic modulators, Antithrombin III and heparin, samples can be tested for
elongated clotting times and differences in fibrin clot formation. This is possible because fibrin clot structure has been shown to vary with ionic strength, local pH, and concentrations of fibrinogen, dextran, and thrombin, as well as other clotting factors (Wolberg 2007).

Thus, this project describes the development of a high throughput microplate based *in vitro* assay showing a dosage response to the common anticoagulant heparin.
2. Materials and Methods

2.1 Materials

Activated factor V, activated factor X, prothrombin, as well as protein C, activated protein C, protein S, rabbit thrombomodulin, and antithrombin III were purchased through Haematologic Technologies Inc. (Essex Junction, VT). Heparin sodium salt from porcine intestine, fibrinogen type 1-S from bovine plasma, bovine thrombin, and bovine serum albumin were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco’s phosphate-buffered saline, with calcium and magnesium (DPBS, pH 7.4) was purchased from Gibco®. All clotting tests were performed in tissue culture treated polystyrene 96-well plates from Beckton Dickinson and Company (Franklin Lakes, NJ). Absorbances were monitored with a μQuant microplate reader (BioTek, Winooski, VT).

2.2 “Fibrin Glue” Fabrication Methods

All methods were performed at room temperature (approximately 20˚C). The solutions were prepared to test manual and plate reader clotting times in parallel on the same day.

Fibrin glue solutions were prepared by combining solutions of 12 mg/mL bovine fibrinogen and final concentrations equivalent to 1, 0.5, 0.25, and 0.125, U/mL of thrombin in plasma were obtained through serial dilution, each in Dulbecco’s phosphate-buffered saline (DPBS, pH 7.4, Gibco) supplemented with 1% (w/v) bovine
serum albumin (Sigma). This resulted in a series of solutions with 6mg/mL fibrinogen, and 0.5, 0.25, 0.125, and 0.0625 U/mL thrombin final concentrations.

The second round of testing replaced thrombin with its prothrombinase complex equivalent of 0.5, 0.25, 0.125, and 0.0625 U/mL of thrombin in plasma. Here, we define the prothrombinase complex as the combination of activated factor V, activated factor X, and prothrombin.

The third test was to demonstrate the anticoagulation capability of antithrombin III with heparin. Briefly, 0.01 U/mL of antithrombin III and 0.1U/mL prothrombinase complex were added in with 0.2, 0.3, 0.4, 0.5, 0.6 and 0.8 μg/mL of heparin sodium salt.

2.3 Qualitative Analysis via UV-Vis Spectrophotometer

“Fibrin glue” solutions were prepared as described above to have a final fibrinogen concentration of 6 mg/mL and final concentrations equivalent to 0.5, 0.25, 0.125, and 0.0625 U/mL of thrombin in plasma. Each clotting factor solution was mixed into the fibrinogen solution in triplicate in tissue culture polystyrene 96-well plate wells, and immediately placed in a μQuant microplate reader to monitor absorbances over time at 405 nm with a reference wavelength of 620nm. Absorbance data were baseline-corrected and the time axis was offset to correct for differences between experimental runs in the initiation of clotting and the beginning of data acquisition. The clotting time was calculated to be the time at which the rate of absorption was at a maximum. This
was done by graphing the first derivative of the raw optical density data in Excel and
taking the time point at the peak of the curve. Curve fittings were also performed, fitting
all kinetic curves to cubic or quadratic polynomials and taking the time at which it took
to reach a 0.1 OD threshold.

It is important to note that the thrombin or prothrombinase complex solution
was added as the last step immediately before reading in all cases.

2.4 Semi-Qualitative Manual Fibrin Clotting Time Analysis

“Fibrin glue” solutions were prepared as described above to have a final
fibrinogen concentration of 6 mg/mL and final concentrations equivalent to 1, 0.5, 0.25,
and 0.125 U/mL of thrombin in plasma for the first tests, and 0.1U/mL of thrombin in
anticoagulation tests. The clotting time test began as soon as the thrombin or
prothrombinase complex solution was mixed into the fibrinogen solution in a
polystyrene 96-well plate well, and was performed in triplicate per concentration. The
clotting time was defined as the time at which a fibrin strand could be drawn from the
solution with a pipette tip.
2.5 Scanning Electron Microscopy

Fibrin clots were prepared for the SEM using a standard biological prep protocol. First, they were fixed in 4% paraformaldehyde in DPBS overnight at 4°C. Fixed clots were then removed from the wells and placed into Eppendorf tubes where they were washed twice with 1x DPBS, then dehydrated with a graded ethanol series (35%, 50%, 75%, 90%, 100% (twice) each for 5 minutes). Then, they were washed with HMDS three times for five minutes and stored in a desiccator. Clots were then sputter-coated with gold foil, and viewed on an FEI XL30 SEM-FEG. Images were taken at 15000x, 35000x, and 100000x, with a 10-11 mm working distance and a 10kV accelerating voltage.

2.5 Statistics

All tests were done in triplicate, and figures reflect N=3 data. Error bars were calculated using standard deviations. Differences between experimental conditions were determined using multivariate analysis of covariance (ANCOVA). Significance was assumed at p < 0.05. R squared values of greater than 0.95 were considered to be a good fit.
3. Results

3.1 Baseline with Traditional Fibrin Glue Formulation

To begin constructing this assay, it was first necessary to calculate manual and quantitative clotting times with a well-defined fibrin glue formulation. Namely, different concentrations of thrombin (0.125, 0.25, 0.5, and 1U/mL) were added to a final concentration of 6mg/mL of fibrinogen. To calculate quantitative clotting times, kinetic curves over the course of ten minutes were taken by a microplate reader, and the clotting time was calculated as the point where the rate of absorption was at a maximum. Manual clotting times were taken as the time at which the first fibrin strand could be pulled up from the sample by a pipette tip. Table 1 describes N = 3 data, where the average of three separate experiments performed in triplicate on different days with all fresh solutions. Figure 4 shows the kinetic curves plotting time in seconds versus absorbance in optical density, and demonstrates an increased lag time with a decrease in concentration. Figure 5 shows a strong correlation between manual and quantitative clotting times with an $R^2$ value of 0.9662.
Figure 4. Kinetic curve plotting time in seconds versus absorption in optical density of fibrin assembly with final concentrations: 6 mg/mL fibrinogen, 1U/mL, 0.5U/mL, 0.25U/mL, and 0.125U/mL thrombin, in DPBS, pH 7.4 with calcium and magnesium supplemented with 1% BSA. N=3, all done in triplicate. Error bars reflect standard deviations between N values.
Figure 5. Correlation between plate reader and manual clotting times for prothrombinase complex and thrombin plus fibrinogen including trend lines. R squared values of 0.9968 and 0.9662 respectively.

Table 1. Raw data showing manual and plate reader clotting times for thrombin and prothrombinase solutions.

<table>
<thead>
<tr>
<th>Units</th>
<th>Thrombin Fibrin Pulling (sec)</th>
<th>Turbidity (sec)</th>
<th>Units</th>
<th>Prothrombinase Fibrin Pulling (sec)</th>
<th>Turbidity (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00</td>
<td>56 ± 24</td>
<td>64 ± 12</td>
<td>1.00</td>
<td>33 ± 6</td>
<td>65 ± 18</td>
</tr>
<tr>
<td>0.500</td>
<td>97 ± 29</td>
<td>106 ± 6</td>
<td>0.500</td>
<td>71 ± 9</td>
<td>86 ± 33</td>
</tr>
<tr>
<td>0.250</td>
<td>139 ± 17</td>
<td>169 ± 23</td>
<td>0.250</td>
<td>136 ± 11</td>
<td>143 ± 37</td>
</tr>
<tr>
<td>0.125</td>
<td>276 ± 45</td>
<td>312 ± 86</td>
<td>0.125</td>
<td>244 ± 14</td>
<td>312 ± 15</td>
</tr>
</tbody>
</table>
3.2 Substituting Prothrombinase Complex for Thrombin

The next step in the process was to replace thrombin with the prothrombinase complex (activated factor V, activated factor X, and prothrombin) in order to create a bioactive clotting platform that can be manipulated. Here, concentrations equivalent to those previously used of thrombin (0.125, 0.25, 0.5, and 1U/mL) were added to a final concentration of 6mg/mL of fibrinogen. To calculate quantitative clotting times, kinetic curves over the course of ten minutes were taken by a microplate reader, and the clotting time was calculated as the point where the rate of absorption was at a maximum. Manual clotting times were taken as the time at which the first fibrin strand could be pulled up from the sample by a pipette tip. Table 1 describes the average of three separate experiments performed in triplicate on different days with all fresh solutions. Figure 6 shows the kinetic curves plotting time in seconds versus absorption in optical density, and demonstrates an increased lag time with a decrease in concentration similar to that of the fibrin glue baseline. Figure 6 shows a strong correlation between manual and quantitative clotting times with an R² value of 0.9668. Figure 7 shows a strong correlation in clotting times between prothrombinase and thrombin formulations for both manual and plate reader derived calculations with R² values of 0.9832 and 0.9853 respectively.
Figure 6. Kinetic curve plotting time in seconds versus absorption in optical density of fibrin assembly with final concentrations: 6 mg/mL fibrinogen, 1U/mL, 0.5U/mL, 0.25U/mL, and 0.125U/mL thrombin equivalents of prothrombinase complex, in DPBS, pH 7.4 with calcium and magnesium supplemented with 1% BSA. N= 3, all done in triplicate. Error bars reflect standard deviations between N values.
3.3 Anticoagulation Effect of Antithrombin and Heparin

Having obtained a clotting time baseline with the prothrombinase complex, it is now possible to manipulate the clotting times using common anticoagulative proteins. Because heparin is such a common component of vascular graft biomaterials (Gorbet
and Sefton 2004), a dosage response to heparin was determined. Clotting times were calculated as described above. Using a constant 12mg/ml of fibrinogen, 0.1U/mL of prothrombinase complex and 0.01U/mL of antithrombin III per well, a dosage response to heparin sodium salt was shown with 0.3, 0.4, 0.5, 0.6, and 0.8 μg/mL (Figure 8 and Table 2). The negative control of prothrombinase complex with antithrombin III-only behaved as expected, showing now significant difference to the prothrombinase complex-only negative control within the time scale of the experiment. 0.2 μg/mL of heparin sodium salt did not show a statistically significant difference to the negative controls. Thus 0.3μg/mL was taken to be the lower detection limit for heparin sensitivity.

Table 2 describes the average manual and quantitative clotting times of three separate experiments performed in triplicate on different days with all fresh solutions. Figure 9 shows a strong correlation between manual and quantitative clotting times with an R² value of 0.9769.
Figure 8. Correlation between plate reader and manual clotting times for antithrombin III-heparin sodium salt-prothrombinase plus fibrinogen including trend lines. R squared value of 0.9769.

Table 2. Raw data showing manual and plate reader clotting times for increasing concentration of heparin sodium salt.

<table>
<thead>
<tr>
<th>[Heparin] (μg/mL)</th>
<th>Fibrin Pulling (sec)</th>
<th>Turbidity (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prothrombinase Only</td>
<td>199 ± 29</td>
<td>232 ± 28</td>
</tr>
<tr>
<td>Prothrombinase and ATIII Only</td>
<td>200 ± 30</td>
<td>246 ± 31</td>
</tr>
<tr>
<td>0.2</td>
<td>220 ± 23</td>
<td>248 ± 31</td>
</tr>
<tr>
<td>0.3</td>
<td>237 ± 12</td>
<td>286 ± 23</td>
</tr>
<tr>
<td>0.4</td>
<td>281 ± 19</td>
<td>322 ± 16</td>
</tr>
<tr>
<td>0.5</td>
<td>328 ± 10</td>
<td>350 ± 17</td>
</tr>
<tr>
<td>0.6</td>
<td>409 ± 15</td>
<td>408 ± 78</td>
</tr>
</tbody>
</table>
Figure 9. Kinetic curve plotting time in seconds versus absorption in optical density of fibrin assembly with final concentrations: 6 mg/mL fibrinogen, 0.1U/mL thrombin equivalent of prothrombinase complex, 0.01U/mL antithrombin III, and increasing concentrations of heparin sodium salt (0.2, 0.3, 0.4, 0.5, 0.6, and 0.8 μg/mL) in DPBS, pH 7.4 with calcium and magnesium supplemented with 1% BSA. ‘P only’ refers to the negative control with prothrombinase complex added only, and ‘P and ATIII’ only refers to the second negative control of prothrombinase and antithrombin III only. No significant difference between ‘P only’, ‘P and ATIII’ only, and 0.2 μg/mL heparin. P values less than 0.05 when comparing concentrations of 0.3, 0.4, 0.5, 0.6, and 0.8 μg/mL heparin to each other and to controls and 0.2 μg/mL heparin. N= 3, all done in triplicate. Error bars reflect standard deviations between N values.

### 3.4 Preliminary Scanning Electron Microscopy Imaging Data

SEM images of fibrin clots were taken to show differences in clot architecture. Namely, solutions with final concentrations of 0.1U/mL of thrombin, 0.1U/mL of...
prothrombinase complex, and 0.5U/mL of prothrombinase complex all in 6mg/mL of fibrinogen were compared.

Figure 10. The availability of thrombin affects the cleavage of fibrinogen and polymerization of fibrin. Final concentrations: 6 mg/mL fibrinogen, 0.1U/mL thrombin (first row), 0.1U/mL equivalents of prothrombinase complex (second row), and 0.5U/mL equivalents of prothrombinase complex (third row) in DPBS, pH 7.4 with calcium and magnesium supplemented with 1% BSA. The scale bars indicate 2μm (first and second column), and 500nm (third column).
4. Discussion

Error bars in figure 4 are most likely due to bubbles noted in some of the wells during the experiment. Although the correlation plots show good correlation between turbidity and manual clotting tests, it is important to note that it may not be accurate to conclude that the clotting times correspond to the same stage of fibrin polymerization. Also, it is possible that the same activity units of thrombin in the prothrombinase complex created more available thrombin, which would explain the higher final optical densities in the prothrombinase complex kinetic curves (figure 5) versus the thrombin complex kinetic curves (figure 4). Indeed, preliminary SEM images seem to indicate that more available thrombin leads to thinner fibers (figure 10). More rigorous SEM studies are necessary to make any conclusions.

The immediate next step will be to show that this system can assess differences in clotting time with different concentrations of heparin adsorbed onto surfaces. In the future, it will be interesting to develop a similar assay using the thrombomodulin, protein C pathway. It will also be useful to see if endothelial and endothelial progenitor cells overexpressing and underexpressing thrombomodulin can be differentiated using this assay. It is also possible to build platforms to test procoagulants such as tissue factor and snake venoms if prothrombinase complex concentrations are low enough to increase the time scale of the negative controls. The specificity of this assay can be considered a limitation in the case of wanting to test more general clottability.
In conclusion, this project describes a platform which can be manipulated to isolate specific questions about thrombogenicity of surfaces which can be found in biomaterials used in biomedical devices. This was done using a combination of previously developed methods of fibrin glue formulations (Brennan 1991), and turbidity measurements of fibrin clot formation (Carr Jr and Hermans 1978; Wolberg 2007; O’Leary and Isbister 2010; Tilley, Levit et al. 2011). Being able to isolate the clotting factors from the heterogeneous environment of blood allows us to build a fully controllable and easily manipulated system by which we can directly measure differences in fibrin assembly and fibrin clot architecture in response to biomaterial surfaces.
References


