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Rapid paper diagnostic for plasma fibrinogen concentration†

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Fibrinogen is a blood protein that is essential for clotting. It is converted into the polymer fibrin by the blood enzymes thrombin and factor XIIIa. Fibrinogen is one of the first proteins to be depleted in heavily bleeding patients. Patients with early hypofibrinogenemia need urgent fibrinogen replenishment to prevent the onset of haemorrhage and death. However, currently there is no rapid, sensitive, cheap and easy-to-use fibrinogen assay that can detect fibrinogen concentrations. In this study, we have developed a new paper-based diagnostic to quantify the fibrinogen concentration in blood at room temperature. This diagnostic is a 2-step process: first, plasma is added onto thrombin-treated paper strips where fibrinogen is converted to fibrin; then the strips are placed into an aqueous dye bath where elution occurs. The test operates by measuring the change in hydrophobicity, which increases with fibrinogen concentration under otherwise constant conditions. The diagnostic can precisely measure fibrinogen concentration within the range of 0–2 g L^{-1}, which is ideal for the clinical diagnosis of hypofibrinogenemia. Furthermore, testing needs only 12 µL of plasma, 60 mU of thrombin and 7.5 minutes of testing. This diagnostic has the potential to revolutionise point of care testing and save many lives.

Introduction

This study aims to develop a rapid and easy to use paper-based diagnostic to measure fibrinogen concentration in human blood. Martinez *et al.* in 2007 first demonstrated that patterned paper was an inexpensive medium that could wick fluids through internal capillary actions and facilitate colorimetric biochemical reactions.[1] This has influenced the emergence of many paper-based diagnostics for detecting and quantifying analytes in blood, urine and saliva[2] and for blood typing.[3] However, there has been no development of paper-based fibrinogen concentration diagnostics to date.

Fibrinogen is essential in the process of hemostasis. It is a plasma protein that usually circulates between 2–4 g L^{-1} in the human body.[4] When vessel breakage occurs, the coagulation cascade is activated that results in the activation of two key blood enzymes: thrombin and factor XIIIa (FXIIIa).[5] Thrombin converts fibrinogen into fibrin monomers that self-polymerise.[6] FXIIIa then covalently links the neighbouring fibrin monomers to each other, forming a hard clot network.[7] This hard clot network can adhere to the site of breakage where it is capable of entrapping red blood cells and platelets. The entrapment of cells allows complete blockage of the damaged site and hence prevents further blood loss until the site has healed.[4a]

Low fibrinogen concentrations are critical in the onset of major haemorrhage. In 1995, Hiippala *et al.* reported that fibrinogen is usually the first plasma protein that becomes depleted in haemorrhagic patients.[8] Patients that have early hypofibrinogenemia are at a significantly greater risk of mortality.[9] The implementation of fibrinogen concentrate (FC) into clinical major haemorrhage protocols is gaining strong interest. This is because the ability to diagnose early hypofibrinogenemia and treat it rapidly with reconstituted FC strongly increases survival in haemorrhagic patients.[10] European Guidelines states that FC therapy should be implemented when patients' plasma fibrinogen concentrations measure below 1.5–2 g L^{-1}.[11] However, there is no early hypofibrinogenemia assay currently available in clinics that is sufficiently rapid, sensitive, cheap or easy to use, particularly in cases of critically bleeding patients.

Traditionally, plasma fibrinogen concentrations have been determined through the use of standard laboratory techniques (SLTs).[12] However, SLTs are all problematic as they involve complex instrumentation for plasma analysis. Additionally, they require large volumes of plasma (*i.e.* >200 µL) to test with.[13] Finally, most SLTs need serial dilutions of known fibrinogen concentrations to be simultaneously measured with patient plasma for calibration.[14] Depending on the assay, this can require significant time and labour to perform in emergency situations.

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Given the life-threatening nature of major haemorrhage, there has been minimal evidence that the use of SLTs provide any survival advantage in hypofibrinogenemic patients.[15] Hence, clinics have avoided implementing a fibrinogen concentration diagnostic step in their major haemorrhage protocols. Instead, they have replenished fibrinogen (and all other coagulation proteins) by transfusing donated fresh frozen plasma (FFP) and cryoprecipitate into patients.[16] However, as these products are unstandardised and unpasteurised, they have been criticised as therapeutics due to their iatrogenic effects.[17]

The recent advancement of Viscoelastic Haemostatic Assays (VHAs) has allowed clinics to measure hypofibrinogenemia more easily than SLTs.[18] However, VHAs still poses clinical problems. First, assaying times of 10–15 minutes are required before a result is achieved.[19] Second, the efficacy of the required platelet inhibitors are imperfect and may result in overestimation of plasma fibrinogen concentrations.[20] Finally, the devices used to perform VHAs (*i.e.* TEG® or ROTEM®) require very significant outlay in terms of initial infrastructure requirements and costs.[21]

Likewise, the dry hematology method (DRIHEMATO®) is another emerging method.[22] It has shown nearly identical fibrinogen measurements to a Clauss Assay – the most widely used SLT for diagnosing hypofibrinogenemia in clinics. Furthermore, it can acquire results in a minute. However, it still requires the use of electricity to operate and complicated infrastructure to acquire fibrinogen readings.

Paper-based diagnostics present a great opportunity to address the many shortcomings associated with current early hypofibrinogenemia assays.

In this study, we demonstrate a novel concept for measuring fibrinogen concentrations on paper for diagnostic application. Previously, Li *et al.*[23] and Guan *et al.*[24] had developed diagnostics that could determine clotting time and blood type respectively through lateral flow wicking. This concept works similarly. By adding fibrinogen and thrombin solutions to paper strips, allowing thrombosis to occur, placing the strips into an aqueous dye bath and allowing elution to proceed, we show that the distance the dyed fluid elutes up each strip correlates precisely to the fibrinogen concentration of the solution. Therefore, this study aims to quantify the effect that varying the controlling parameters has on the sensitivity of the test. The parameters that are analyzed in this study include: the type of paper, the strip width, the volume of fibrinogen solution added, the individual concentrations of thrombin and FXIIIa used as well as the reaction and elution times. Additionally, this study also measures the effect of non-specific blood proteins on the test to emulate plasma-like conditions.

Experimental methods

Materials

Low density (tissue) and high density (filter) papers were selected to create the paper strips. The filter paper used was Whatman 41, whereas the tissue papers were produced in the Kimberly Clark Experimental Forming Unit (EFU), Neenah (WI), USA. Tissues consisted of eucalyptus fibres formed with a 3 layer headbox. 10 kg per T PAE wet strength agent was added to each layer. Tissues were moulded and thoroughly air-dried. Paper properties are listed in Table SIII.† A 1 × 1 mm^2 square steel bar was cut 35 mm long to hold the paper strips over the elution bath. Two elution tanks (13.1 cm tall) were used as mounts to position the steel bar over the elution bath. The elution bath consisted of a Petri dish (15.5 cm in diameter and 2.7 cm tall) filled with an aqueous dye. MilliQ water was used as the bulk fluid of the elution bath. Brilliant Blue FCF (from Queen Blue Food Colouring) was used as the aqueous dye to colour the elution bath. Ponceau 6R (from Queen Red Food Colouring) was used as the aqueous dye to stain cellulose and fibrin fibres for microscopic imaging. Thrombin vials and fibrinogen powder were sourced from Sigma Aldrich. FXIIIa was purchased from Zedira GmbH, Darmstadt, Germany. Plasma (anticoagulated with EDTA) and serum was sourced from the Australian Red Cross. Bovine Serum Albumin (BSA) concentrate was bought from CSL Behring. Glycerol was sourced from Astral Scientific.

For hydrophobicity testing, Menzel Gläser 22 × 22 mm coverslips were used as the substrate. Analytical grade sodium methoxide solution and cellulose acetate was sourced from Sigma Aldrich. Acetone was purchased from Ajax Finechem. Methanol was sourced from Merck. All reagents and solvents were used as supplied without further purification.

Methodology

Paper cutting. Paper strip cut-outs of different strip widths were prepared on CorelDRAW X6 with the dimensions shown in Fig. S1.† Paper was cut on a Laser Cutter (60 Watt Epilog Helix) using the cut-outs prepared on CorelDRAW X6. The print settings used were 50% speed, 4% power and 500 Hz frequency.

Fibrinogen solution preparation. A 4 g L^{-1} fibrinogen solution was prepared by dissolving 90 mg fibrinogen powder into 10 mL PBS at 37 °C. As the powder contained an inconsistent mixture of fibrinogen and other salts, its concentration was more precisely determined on a UV-Vis spectrometer (Agilent Cary 60). The fibrinogen concentration was determined using Beer's law at 280 nm with a 1 cm cuvette. An extinction coefficient of 1.51 was used to convert absorbance units to g L^{-1} fibrinogen. The fibrinogen solution was adjusted to exactly 4 g L^{-1} by measuring its concentration on the UV-Vis spectrometer and diluting it with the appropriate amount of PBS. From there serial dilutions of 2 g L^{-1}, 1 g L^{-1}, 0.5 g L^{-1} and 0.25 g L^{-1} fibrinogen solution were also prepared. All of these solutions were made into small aliquots, snap frozen in liquid nitrogen and stored at −80 °C until used.

Test setup. The elution bath was placed in between the 2 mounts. The elution bath was prepared by pouring 250 mL solution of 0.1% Brilliant Blue FCF in MilliQ water into the Petri dish. A schematic of this is provided in Fig. 1a.

The thrombin solution was prepared by reconstituting a vial of thrombin with 300 μL of MilliQ water. This made a vial of 30 NIH units per mL thrombin (these units are simplified to

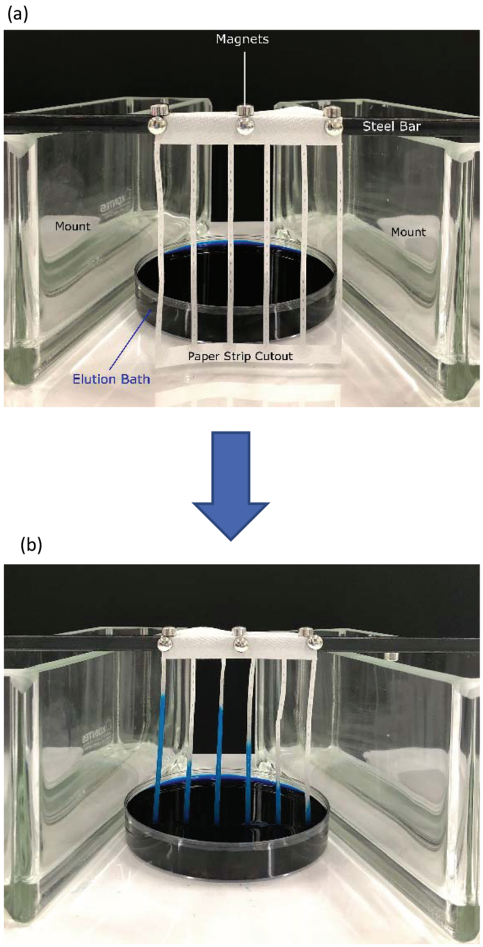

Fig. 1 Set up of paper strips. Thrombin solution is applied to the strips followed by fibrinogen solution (a). The strips are placed in the elution bath and allowed to elute. Different fibrinogen concentrations causes the dye to elute at different rates (b).

U mL^{-1} in this paper). However, different thrombin concentrations could be made by reconstituting each vial with different volumes of MilliQ water.

The thrombin solution was made differently when FXIIIa was included. First, a 60 U mL^{-1} solution of FXIIIa was prepared by dissolving 150 μL of MilliQ water into a vial of FXIIIa. Then, serial dilutions of 30 U mL^{-1}, 15 U ml^{-1}, 8 U mL^{-1} FXIIIa were prepared. Afterwards, a 60 U mL^{-1} vial of thrombin was prepared. Finally, equal volumes of thrombin and FXIIIa solutions were mixed into separate Eppendorf tubes. This created thrombin solutions of different FXIIIa concentration.

Test procedure. A paper strip cut-out was attached perpendicularly to the steel bar with magnets. The steel bar's bottom and top edges were positioned 12.5 and 13.5 cm from the bottom of the cut-out respectively. The slits 13.5 cm from the bottom of the cut-out (as shown in Fig. S1†) helped align the steel bar. The steel bar was placed on the mounts (but not directly over the elution bath) with the cut-out vertically orientated. This prevented the cut-out from making contact with the table.

Thrombin solution was pipetted near the bottom of each strip (see Fig. S4† for exact positionings). The fibrinogen solution was then pipetted 5 mm above each wetting of thrombin solution. As the fibrinogen solution wicked in both directions, it meant that only half of it could mix with the enzymes. However, it ensured that: (1) The fibrinogen and thrombin mixed properly with each other, (2) The fibrin barrier formed at the bottom of each strip. The reaction between fibrinogen and thrombin occurred for 30, 60 or 90 seconds. Afterwards, the paper strip cut-out was moved into the elution bath. The 0 mm marking of the paper strips sat level with the dye surface of the elution bath. The fibrin barrier formed at the bottom of each strip caused elution to be perturbed immediately. The paper strips were eluted for 1, 3, 5, 7 or 10 minutes.

Finally, the cut out was removed from the elution bath and dried on paper towel. The elution height of each strip was measured by finding the mid-point of each elution front and rounding to the nearest millimetre. Images of the cut-outs were taken by photocopier (Epson V370 Photo Scanner). A summary of the procedure is given in Fig. 2.

Coverslip preparation. Cellulose acetate solution was made by dissolving 0.25% w/v cellulose acetate in acetone. Coverslips were plasma cleaned (Harrick Plasma Cleaner PDC-002-HP) for 3 minutes on one side. Cellulose acetate was spin-coated (Laurell WS-650-23 Spin Coater) on the plasma cleaned sides for 20 seconds at 2000 rpm. Sodium methoxide solution in methanol was prepared at 0.5% w/v. The cellulose acetate coated slides were immersed into the sodium methoxide bath for 10 hours. This converted the cellulose acetate into a cellulose film. Afterwards, the coverslips were washed with methanol followed by MilliQ water and were left to dry.

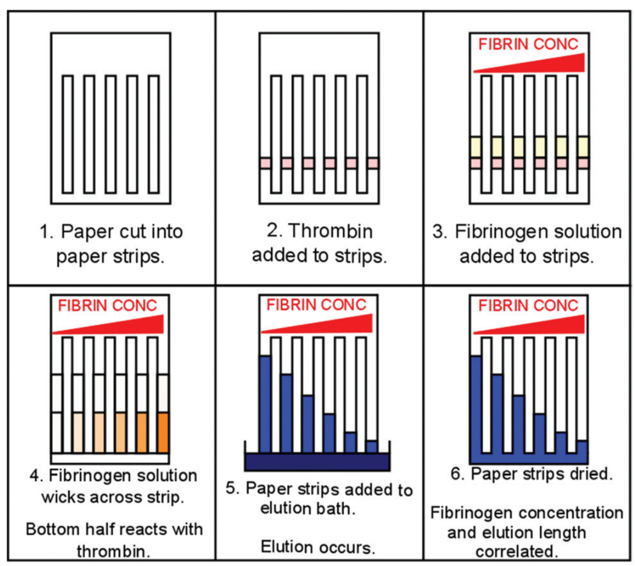

Fig. 2 Summary of test procedure. Paper strips are wetted with the thrombin solution. Then the fibrinogen solution is added where it wicks across the strips and half of it reacts with the thrombin. Finally, the cut-out is placed into the elution bath where elution occurs. The elution height up each strip can be correlated to the fibrinogen concentration. Dimensions of the paper strip cut-out used can be found in Fig. S1.†

Thrombin solution was made to 10 NIH units per mL in MilliQ water and a layer was spin-coated on top of the cellulose film for 20 seconds at 2000 rpm. Likewise, fibrinogen solution was made to 2 g L^{-1} in PBS and a layer was spin-coated on top of the thrombin layer for 20 seconds at 2000 rpm. This converted the fibrinogen into fibrin.

Hydrophobicity testing. Contact angle (Dataphysics OCA35) was used to measure the hydrophobicity of the spin-coated coverslips. Growing water droplets were created by dripping MilliQ water at a rate of 2 μL per second on top of each coverslip surface. The dispensing needle was in contact with the growing droplet throughout each recording. The advancing contact angle of the droplet was used to quantify hydrophobicity, with the general observation that larger angles corresponded to a more hydrophobic surface.

Viscosity testing. Glycerol : water mixtures of composition 0 : 100%, 85 : 15% and 100 : 0% were made. Their viscosities were measured in Cannon-Fenske viscometers of size 200 (~0.1 cSt s^{-1}), size 450 (~2.5 cSt s^{-1}) and converted to centipoise. The glycerol : water mixtures were then added to the paper strips and eluted as per the test procedure. Full details can be found in Table SI.†

Fig. 3 Elution of serum *vs.* plasma. Both blood products were collected from the Australian Red Cross. Test conditions can be found in Table SIX.† Each test was performed in triplicate.

Results

Fig. 3 demonstrated that the elution of plasma samples (which contains fibrinogen) eluted much less than the serum samples (which does not contain fibrinogen). Therefore, this phenomenon was explored in further detail.

Thrombin and Factor XIIIa (FXIIIa) were deposited onto paper strips. Then fibrinogen solution was added where it reacted with the enzyme to form fibrin. Finally, the strips were placed vertically in a blue dye bath, eluted for a set period of time and dried afterwards. There was a clear and reproducible correlation between the height that the blue dye eluted up each strip and the concentration of the fibrinogen solution. Higher fibrinogen concentrations resulted in lower elution heights. This discovery formed the concept of a sensitive, rapid and reproducible paper diagnostic for quantifying fibrinogen concentrations in blood.

The variables estimated to be important were tested individually and their impact on the diagnostic's sensitivity were quantified. The variables investigated included: paper structure, strip width, fibrinogen solution volume, thrombin concentration, FXIIIa concentration, reaction time and elution time, and the results of this testing were discussed in detail below.

This analysis elucidates the controlling mechanisms and optimises the conditions for developing a robust blood based diagnostic.

Conceptual mechanism

The effect of fibrinogen, thrombin and fibrin coating on cellulose film hydrophobicity was quantified (Fig. 4a). The film becomes significantly more hydrophobic when it is coated with fibrin. This is the concept behind the paper diagnostic. The coating of un-polymerised fibrinogen or thrombin on the cellulose film does not contribute significantly to the hydrophobicity. The effect of viscosity was also quantified using glycerol–water mixtures as model solutions (Fig. 4b). Viscosities above 100 cP do perturb elution. However, its exact contribution towards perturbation after fibrin polymerisation is unknown. Additionally, the potential effect of paper pore blockage (Fig. S9 and S10†) was investigated by microscopy and surface topography. Paper pore and surface structure is slightly and not affected by fibrin coating, respectively. The fibrin coating effect on the diagnostic is minimal. Therefore, the diagnostic is driven predominantly by the increase in hydrophobicity that results from the polymerisation of fibrinogen.

Effect of paper structure elution rate

The effect of fibrinogen concentration on elution height for four papers differing in structure was investigated (Fig. 5). Table SIII† reports the structural properties of these papers. There are three observations of interest. First, elution height strongly decreases with fibrinogen concentration, with the steepest decrease in the desired 0–2 g L^{-1} concentration range, and these results are very reproducible. Second, the sensitivity is a strong function of paper density, with the tissue papers showing much higher sensitivity than the filter papers (see Fig. S2†). Third, the sensitivity increases with basis weight for the tissue papers. This can be correlated to Table SIII† which shows that higher basis weighted papers are thicker.

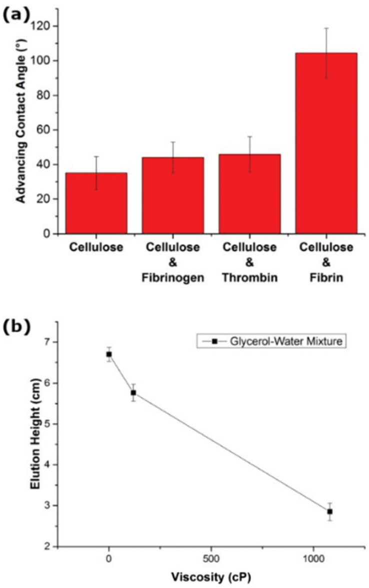

Fig. 4 Effect of hydrophobicity on the different cellulose surfaces (a). The contact angle of water droplets deposited on the spin-coated coverslips was measured. Effect of glycerol viscosity of elution height (b). Effect of solution viscosity on elution height. Glycerol–water mixtures were made to different viscosities and tested. Test conditions can be found in Table SI.† Each test was performed in quadruplet and the average and standard deviations are reported as the bar and error bar respectively.

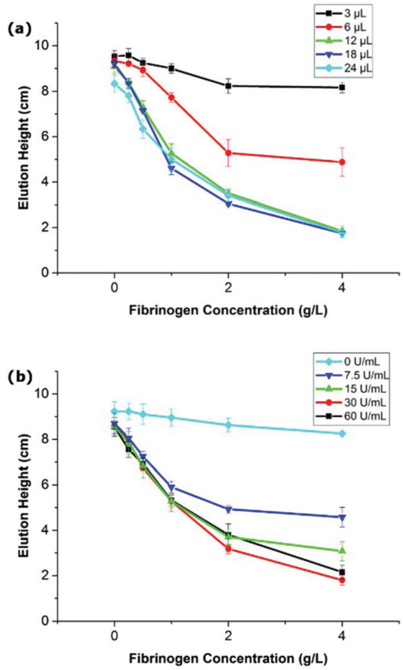

Fig. 6 Effect of fibrinogen solution volume and fibrinogen concentration on elution height (a). Effect of thrombin concentration and fibrinogen concentration on elution height (b). Test conditions can be found in Tables SII, SV and SVI.† Each test was performed in quadruplet and the average and standard deviations are reported.

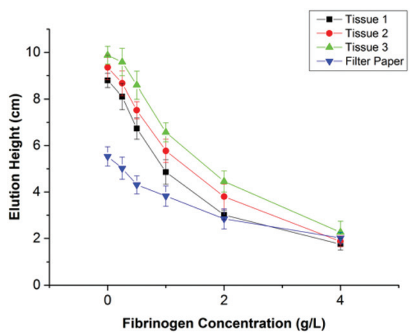

Fig. 5 Effect of paper structure and fibrinogen concentration on elution height. Test conditions can be found in Tables SII and SIV.† Each test was performed in quadruplet and the average and standard deviations are reported.

Effect of fibrinogen solution volume and thrombin concentration

The effect of thrombin concentration on elution height for different fibrinogen concentrations was investigated (Fig. 6b). Higher thrombin concentrations significantly increase sensitivity (see Fig. S4†). With no thrombin, the fibrinogen concentration–elution height relationship is virtually horizontal. However, at higher thrombin concentrations, sensitivity drastically increases and the elution height decreases with fibrinogen concentration. Insignificant changes are observed with thrombin concentrations of 30 U mL^{-1} (60 mU total enzyme) or higher.

The effect of fibrinogen solution volumes on elution height for different fibrinogen concentrations was also analysed (Fig. 6a). A very similar effect is observed. At 3 μL volume, the fibrinogen concentration–elution height relationship is flat and insensitive, as denoted by the low slope. At higher fibrinogen volumes, sensitivity drastically increases and the elution height decreases with fibrinogen concentration. Insignificant changes are observed with volumes of 12 μL and higher.

Effect of elution and reaction time

The effect of reaction time on elution height for different fibrinogen concentrations was investigated (Fig. 7a). The reaction time is the time allowed for the fibrinogen solution to react with the thrombin before it is placed in the elution bath. Longer reaction times increase sensitivity. However, once the reaction time goes above 30 seconds, insignificant changes occur.

The effect of elution time on elution height for different fibrinogen concentrations was also investigated (Fig. 7b). Longer elution times cause a remarkable increase in sensitivity (see Fig. S5†). By 1 minute, the fibrinogen concentration–elution height relationship is flat and sensitivity is only present between 0–1 g L^{-1} fibrinogen. By 7 minutes, sensitivity is present between 0–2 g L^{-1} fibrinogen. Finally, by 10 minutes, clear sensitivity can be seen across 0–4 g L^{-1} fibrinogen.

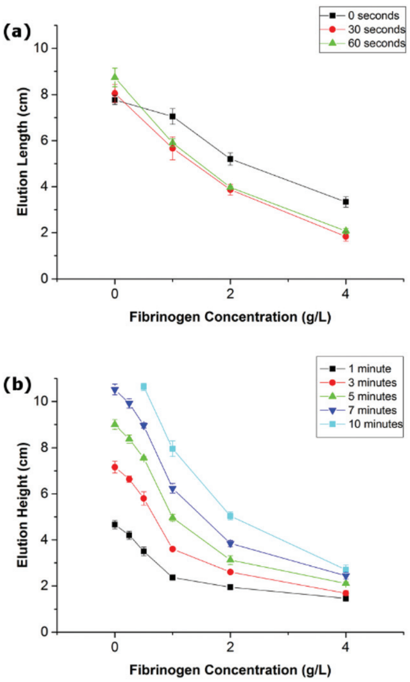

Fig. 7 Effect of reaction time and fibrinogen concentration on elution height (a). Effect of elution time and fibrinogen concentration on elution height (b). Test conditions can be found in Tables SII and SVI.† Each test was performed in quadruplet and the average and standard deviations are reported.

Effect of other plasma proteins

The effect of BSA concentration on elution height for different fibrinogen concentrations was investigated (Fig. 8). Albumin is the most abundant protein in plasma constituting up to 70% of total plasma protein.[25] Therefore, fibrinogen solutions in 80 g L^{-1} BSA were prepared to emulate plasma-like conditions in human blood. The effect of BSA is moderate. At low fibrinogen concentrations, the solution with BSA does elute less than the solution without. However, at high fibrinogen concentrations, this effect is nullified. The paper thickness also plays a critical role in the visibility of the elution front with the plasma-like samples (see Fig. S6†). Tissue 2 produces a fading blue gradient at the elution front. This makes it difficult to interpret. However, tissue 3 produces a much clearer elution front.

Optimal conditions

Optimal conditions were decided based on parameters that could best distinguish fibrinogen concentrations between 0–2 g L^{-1} in plasma-like conditions. Parameters are shown in Table SVIII.†

Discussion

Concept

In this study, we have developed a new paper-based diagnostic to quantify the fibrinogen concentration in blood. The diagnostic concept relies on fibrin forming a hydrophobic barrier to fluid flow in a porous and wettable medium, such as paper. While not linear, the sensitivity of the test in the concentration range of 0–2 g L^{-1} is highest, which perfectly fits clinical needs for diagnosing early hypofibrinogenemia. The total duration needed for the test is less than 8 minutes (Fig. 9).

Unlike other assays that require laborious preparations or specialist training,[21a,26] using the paper diagnostic is only a two-step process. First blood plasma is added onto thrombin-treated paper strips where the fibrinogen present is converted to fibrin. This is an enzyme triggered polymerization process operating at room temperature. Then the paper strips are eluted with a standard aqueous dye. We discovered that as fibrinogen is converted into fibrin upon interaction with thrombin, the elution barrier it induces on paper drastically increases. Therefore, the maximum elution height of the dye drastically decreases.

The test follows Washburn kinetics of imbibition through a porous medium (Fig. S11†). Despite the elution barrier effect that fibrin exerts on the test, it does not cause it to deviate away from Washburn kinetics.

The hydrophobicity increases remarkably when fibrinogen is converted into fibrin. When 2 g L^{-1} fibrinogen and throm-

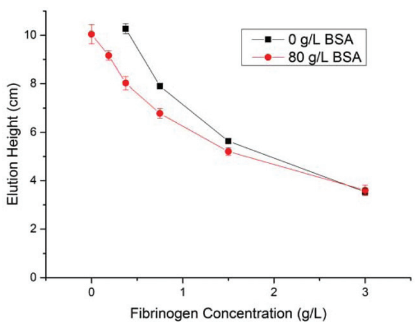

Fig. 8 Effect of BSA concentration and fibrinogen concentration on elution height. Test conditions can be found in Tables SII and SVII.† Each test was performed in quadruplet and the average and standard deviations are reported.

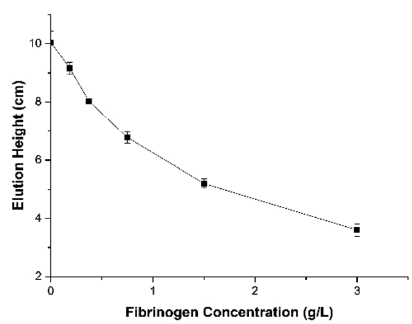

Fig. 9 Optimal test conditions. Fibrinogen in 80 g L^{-1} BSA was used as the model fluid to emulate plasma-like conditions. Test conditions can be found in SVIII.† Each test was performed in quintuplet and the average and standard deviations are reported.

bin reacts, it increases the water–surface contact angle by over 60° (Fig. 4a). Paper elution is governed by the capillary pressure:

$$P_C = \frac{2\gamma}{r}\cos(\theta) \quad (1)$$

where γ is the air–water surface tension, r is the capillary radius, θ is the water-capillary surface contact angle and P_C is the capillary pressure.[27] The higher P_C is, the greater the extent of elution. As the polymerisation of fibrinogen into fibrin causes such a large increase in θ, it induces a major decrease in P_C. Therefore, the test is predominantly driven by hydrophobicity. Although the roughness and fibre structure of paper would certainly result in additional complexities when compared to the hydrophobicity measurements undertaken on idealised smooth cellulose films, the basic controlling concept remains the same.

The effect of viscosity was investigated using a series of glycerol–water solutions (Fig. 4b). As glycerol is a hydrophilic substance, it cannot act as a hydrophobic barrier to fluid flow. Therefore, it can only restrict elution purely on viscosity. Elution is impeded at viscosities over 100 cP and can be significant once viscosities rise over 1000 cP. However, the extent to which this applies to fibrin polymerisation is unknown. This is because the viscosity of fibrin is affected by the shear rate exerted on it.[28] However, viscosity effects are expected to play a secondary role in the diagnostic. This is because the viscosity of the dye solution is unaffected by the formation of fibrin.

The effects of physical pore blockage due to fibrin formation was also deciphered (Fig. S9 and S10†). Images from Fig. S9† show that the paper pore size can exceed 100 μm and that the fibrin formed cannot fill the pores. Profilometry data from Fig. S10† shows that the surface roughness does not decrease when fibrin is formed on paper. Therefore, physical pore blockage does not occur.

In essence, the test predominantly consists of measuring the change in hydrophobicity, which increases with fibrinogen concentration under otherwise constant conditions. Various methods to measure paper hydrophobicity already exist. These include the contact angle test and water droplet adsorption time.[29] However, this elution based test was chosen due to its superior efficacy and easy-to-read result to the naked eye.

Variables affecting sensitivity

Paper structure, fibrinogen volume, thrombin concentration, reaction time and elution time are the main variables affecting the sensitivity of the test. Strip width and FXIIIa, however, show no impact. Additionally, BSA concentration has a small but non-detrimental effect.

The sensitivity of the test correlates strongly with the elution rate of the paper (Fig. 5). The sensitivity is significantly better for the tissue papers than for the filter paper, indicating that low density papers are preferred. The elution height difference for the tissues between 0–2 g L^{-1} fibrinogen are all around 6 cm. This is easy to visualise and hence these papers enable determination of the fibrinogen concentration using only the naked eye. The sensitivity is also much higher than the diagnostic reported by Li *et al.* which can only distinguish clotted from non-clotted blood samples in the range of 4 mm.[23a] Whereas, filter paper only shows 2.5 cm difference between this range and hence requires more subjective interpretation of results. Low elution rate papers thereby provide lower sensitivity than high elution rate papers. Table SIII† highlights the effect of paper structure properties and how they influence the elution rate. A clear difference is demonstrated in basis weight and density between the tissue and filter papers. As the tissue papers have a lower density than the filter paper, they are able to elute a lot faster. In 2012, Su *et al.* investigated the paper structure needed to separate agglutinated from non-agglutinated blood cells and reported a similar finding. Thin and porous papers were reported to provide greater sensitivity than thick and dense papers.[30] Paper of low density and high porosity is needed for such fibrinogen diagnostics.

The effect of fibrinogen solution volume provides the basis for the amount of blood plasma required (Fig. 6a). Likewise, the effect of thrombin concentration determines the amount of thrombin to be used per test (Fig. 6b). The trend is the same for both relationships. Increased fibrinogen solution volume and thrombin concentration both increase the sensitivity of the test. The sensitivity of the test becomes optimised once the volume and concentration reaches certain threshold values: 12 μL and 30 U mL^{-1}, respectively. This means that the reagents needed to perform such a test are significantly lower in quantity than any other currently available fibrinogen assay. For instance, Clauss Assays require 100 μL thrombin solutions (of up to 200 U mL^{-1}) and 200 μL of diluted plasma in order to provide reliable results.[31] Likewise, FIBTEM tests require 300 μL of blood and 40 μL of extra reagents.[32] As our test needs only 60 mU of thrombin and a small drop of plasma (~12 μL), it can save significant reagent usage from the clinic and the heavily bleeding patient.

The effect of reaction and elution time combined define the testing time (Fig. 7). The reaction time shows optimal sensitivity at only 30 seconds. In 1953, Biggs *et al.* stated that normal plasma could be clotted at 37 °C in 10 seconds.[33] The fact that our study was performed at room temperature (instead of 37 °C) indicates that temperature has little impact on the rapidity of the test. Likewise, the sensitivity improves with larger elution times. By 7 minutes, concentrations between 0–2 g L^{-1} are very easy to distinguish. Therefore, this was selected as the optimal time needed for the test. It is quicker than FIBTEM which can require up to 11 minutes to get a reading.[19] This paper test can give clear results in half the time of a FIBTEM.

We hypothesised that including FXIIIa would improve the sensitivity of the test. This is because FXIIIa forms covalent cross-links between fibrin monomers that strengthens the fibrin. Therefore, we thought that this would increase the hydrophobicity of the elution barrier and impede further elution. However, FXIIIa has no clear impact on the sensitivity of our test (Fig. S7†).

The effect of BSA concentration provides the basis for non-specific effects on the test (Fig. 8). The reference range for total protein concentration for human plasma is 60–80 g L^{-1}.[25] Therefore, 80 g L^{-1} solutions of BSA were used to emulate human plasma-like conditions in the high concentration range. BSA does perturb the sensitivity of the test. However, this is only evident at low fibrinogen concentrations (*i.e.* below 1.5 g L^{-1}). At higher concentrations, the effects of BSA are negligible. Given that this experiment tested between an 80 g L^{-1} difference of BSA, whereas total plasma protein concentration between normal individuals varies by 20 g L^{-1}, such small effect would be nullified even further. This indicates that non-specific effects in normal plasma would be insignificant on the sensitivity of the test.

Perspectives

Non-specific effects of other plasma proteins, especially albumins and globulins, are a concern in this test. Both of these proteins have been known to adsorb to cellulose[34] and contribute to plasma viscosity.[35] Therefore, they may retard elution. Whilst we found that physiologically relevant levels of these proteins had measurable but not detrimental effects on the elution height when compared to fibrin, abnormally high levels of these proteins were not considered. Therefore, individuals with hyperviscous blood may be susceptible to giving falsely high fibrinogen concentration readings.

Non-homogeneous elution can cause the elution front to move further and hence cause a falsely low reading for fibrinogen concentration (see Fig. S3†). The main phenomenon behind inhomogeneous elution is the creation of local areas of air pockets across the width of the strip. As these air pockets are lower in pressure than the surrounding clotted plasma, the aqueous dye prefers to move through them. These air pockets are most likely formed due to variability within the paper pore structure. Fig. S8† shows that 5 mm strips produce larger variations in elution height than 3 mm strips. This is probably due to the greater chance of encountering an air pocket in a wider strip than in a narrower strip. Therefore, our test requires a high quality paper with no variability in pore structure or density.

A rapid and simple method to pre-separate blood cells from plasma must be considered before applying plasma onto our test. We found that whilst serum and plasma showed clear elution differences to each other (Fig. 3), whole blood did not perform as well (result not included). This is in contrary to Li *et al.*'s[23a] diagnostic which could distinguish whole blood samples with prolonged clotting parameters from samples with normal clotting parameters. In 2017, Kim *et al.* recently developed a high purity and high throughput pipette tip that could separate plasma and blood from each other.[36] A similar size-exclusion filtering device would perform well in the test given the minute plasma volume required.

Finally, the temperature and humidity may also have an impact on the elution height. Both of these parameters have been well known to affect elution rate in imbibition studies.[37] In this study, we only looked at the effects at room temperature and relative humidities between 30–50%. However, given that our test is intended to be a handheld, point of care (POC) diagnostic for emergency fibrinogen transfusions, it is likely to be used in both indoor and outdoor environments. In Australia, the climate can vary from hot and dry conditions in Central Australia to mild and damp conditions in Victoria and Tasmania. Therefore, such a diagnostic may have to be stored and operated under controlled temperature and humidity conditions, indicating the need for additional testing.

Conclusion

In this study, we have developed a rapid, simple, sensitive and cheap paper-based diagnostic that can quantify fibrinogen concentrations. The test is a two-step process. First, dissolved fibrinogen is added onto thrombin-treated paper strips. The thrombin converts the fibrinogen into fibrin, and then the strips are eluted with a standard elution dye. The aqueous dye will elute to a certain height, depending on the quantity of fibrin present on the strip. This is through a hydrophobicity controlled mechanism. This diagnostic is well adapted for clinical major haemorrhage protocols as it can detect within the range of 0–2 g L^{-1}, necessary for diagnosing early hypofibrinogenemia.

We found that the paper type, fibrinogen solution volume, thrombin concentration, reaction time and elution time all affected the sensitivity of the test. Whilst the test requires low density paper for rapid elution, it can achieve sensitivity with low reagent quantities (12 μL fibrinogen solution and 60 mU thrombin) and short assaying times (30 seconds reaction time and 7 minutes elution time). This is significantly quicker and cheaper than any current laboratory tests, including FIBTEM.

We also tested the non-specific effects of other blood proteins in plasma. We found that at physiologically relevant concentrations, its impact on the test was small. However, abnormally high levels were not tested and could be a limitation. Other limitations of the test include non-homogenous elution patterns (which causes the dye to elute further than it should), the requirement to pre-separate blood cells from plasma and the potential effects of temperature and humidity. Hence, further optimization is still required to develop the test into a robust POC commercial diagnostic.

The future of this test is exciting. Such a diagnostic could be used to detect early hypofibrinogenemia and save many lives. It also has the potential to revolutionise major haemorrhage protocols by introducing rapid, simple and cheap point of care diagnostics that are suitable for targeted treatment.

Ethical statement

De-identified human blood samples were provided by the Australian Red Cross Blood Service, obtained with written informed consent in accordance with the recommendations of Blood Service Human Research Ethics Committee (BSHREC).

This study was carried out with approval of and in accordance with the recommendations of the Monash University Human Research Ethics Committee (MUHREC).

Conflicts of interest

There are no conflicts to declare.

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