Supplementary Material for

Correcting the Effect of Hematocrit in Blood Coagulation Analysis on Paper-Based Lateral Flow Device

Hua Li[1], Daewoo Han[1], Giovanni M. Pauletti[2], Michael A. Hegener[2] and Andrew J. Steckl[1*]

[1] *Nanoelectronics Laboratory, Department of Electrical Engineering and Computer Science, University of Cincinnati, Cincinnati, 45221, USA*

[2]*Winkle College of Pharmacy, University of Cincinnati, Cincinnati, 45267, USA*

1. Materials and Methods for nrLFA Device Fabrication

The nrLFA device is based on a conventional lateral flow immunoassay (LFIA) test strip and utilizes a plastic cassette as strip holder which has a cut-out window for flow monitoring within the strip. The nrLFA test strip consists of a fiber glass sample pad (Grade 8950, Ahlstrom-Munksjö, Helsinki, Finland), a nitrocellulose lateral flow membrane (Hi-Flow™ Plus HF075, MilliporeSigma, Billerica, MA), a cellulose wicking pad (Grade 470, Whatman, Maidstone, United Kingdom), and a self-adhesive plastic backing card (MIBA-020, Diagnostic Consulting Network, Carlsbad, CA). Unlike conventional LFIA, no conjugate pad or pre-stored reagent is needed in the nrLFA strip. The overall strip dimension is 4 mm × 53 mm, and the dimension of each component is 4 mm × 13 mm for sample pad, 4 mm × 30 mm for analytical membrane, and 4 mm × 20 mm for wicking pad. The overlaps of the cellulose membrane with the sample pad and with the wicking pad are 6 and 4 mm, respectively. Long sheets of the three components are stacked and assembled on a self-adhesive plastic backing card and then cut into narrow strips of 4 mm width using a guillotine cuter (CM4000, BioDot, Irvine, CA). The completed nrLFA strip is then placed within a plastic cassette (MICA-125, Diagnostic Consulting Network, Carlsbad, CA) that consists of two plastic pieces that snap together (top and bottom pieces). The cassette has an oval-shape sample reservoir and a 16.5 mm-long observation window. The observation window was covered using transparent adhesive tape to prevent the sample evaporation during testing. No reagent printing or membrane drying process is needed in the fabrication process.

The material selection and dimension design of the nrLFA strip components (sample pad, analytical membrane and wicking pad) are carefully performed to accommodate the physical properties of the blood as well as the desired sample volume and test time. Fiber glass was selected to be the material of sample pad due to its very low bed volume (~1-2 μL/cm^2)[1] and excellent release properties. We also selected the thinnest fiber glass on the market (Grade 8950 from Ahlstrom-Munksjö) to ensure the bed volume is the lowest. Millipore HF075 nitrocellulose membrane was selected to be the analytical membrane for blood tests due to its highly-reproducible lateral flow properties, very short capillary rise time (77±2 sec/4 cm according to our measurements), very large pore size (14.5±4.7 μm according to our measurements), as well as a clear separation between RBCs and plasma when using blood samples. Because of the relatively high viscosity of blood (compared to other body fluids) and the size of RBCs (~6-8 μm in diameter)[2], short capillary rise time and large pore size ensure fast, uniform and reproducible flow of blood samples within the membrane without clogging the capillaries. This results in a relatively short test time, such as 4 min for blood coagulation testing using the nrLFA device. Nitrocellulose does have relatively large bed volume (~10 μL/cm^2)[3] which can potentially be reduced by casting thinner membranes by the manufacturers. Cellulose was selected to be the material of wicking pad due its high absorbency/bed volume (>25 μL/cm^2)[1] that provides continues capillary drive and prevents fluid back flow in the test duration. The dimension of the strip as well as those of each strip components were selected to properly match the existing plastic cassette with the lowest bed volume. The current sample volume for blood coagulation testing using nrLFA is 30 μL. This sample volume can be further reduced if the bed volume of the nrLFA strip can be reduced by fabricating narrower strips or thinner nitrocellulose membrane.

2. Methods for Extracting Calibration Equation Using Citrated Rabbit Blood

In this set of experiments, citrated rabbit blood was purchased from HemoStat Laboratories (Dixon, CA). According to the vendor, fresh rabbit blood was collected in 4 wt.% trisodium citrate solution at 4:1 volume ratio (fresh blood: citrate solution) to prevent coagulation. Citrated rabbit blood with various hematocrit (Hct) values (25%, 30%, 35% and 40%) were obtained by removing freshly separated plasma from low Hct blood (19-25% for various batches from the vendor) after light centrifugation (Thermo Fisher Scientific accuSpin Micro 17, Osterode am Harz, Germany)

at 400× g for 6 min, followed by re-suspending with gentle agitation. During the calibration measurements, a camera and a camera timer were employed to document the blood travel distance in the form of digital images at a fixed time interval (2 sec). The starting point (t = 0) was selected to be the moment when red blood cells (RBCs) started to appear in the observation window, and the end point (t = 160 s) was selected to be the moment when RBCs of the blood sample with 25% Hct, which traveled the fastest among all blood samples (25%, 30%, 35% and 40% Hct), approached but not yet reached the end of the observation window (D = 16.5 mm). If RBCs reached the end of the observation window, it would be impossible to measurement the travel distance because of the limitation of the window length. For each blood sample, 10 replicates were performed (n = 10) to ensure the reliability of the test results. The number of pixels associated with RBC travel distance was measured using ImageJ and then converted into actual distance using Excel. Mean and standard deviation of RBC travel distance were calculated using Excel.

3. Clinical Trial – Study Setting, Population and Protocol

Study Setting and Population

This clinical feasibility study of the nrLFA device was conducted at two different sites, with healthy and non-anticoagulated volunteers recruited and tested at the University of Cincinnati (Cincinnati, OH), and patients on prescribed oral warfarin medication recruited and tested at the St. Elizabeth Healthcare anticoagulation clinic (Fort Thomas, KY). The recruited patients (n = 27) were established patients at the St. Elizabeth clinic who have started warfarin therapy at least 1 month ago, at least 18 years of age, and mentally competent. Exclusion criteria included diagnosed blood clotting disorders such as factor V Leiden, antiphospholipid syndrome, protein C deficiency, protein S deficiency, antithrombin deficiency, as well as pregnancy. Healthy volunteers (n = 25) were recruited from the student, faculty, and staff members in the east and west campus of the University of Cincinnati. Volunteers were eligible if at least 18 years of age and mentally competent. Exclusion criteria included prior history of any anticoagulant therapy in the past 3 months, the presence of any known blood clotting disorders, as well as pregnancy. This study received IRB approval (study ID: 2016-5324) from the University of Cincinnati and St. Elizabeth Healthcare.

Study Protocol

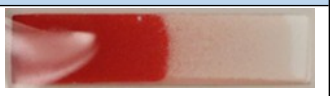
After consent form was signed by the participants, the following tests were performed using capillary whole blood sample from finger prick: (1) PT/INR test using the CoaguChek® XS POC Blood Coagulation Analyzer (Roche Diagnostics, Mannheim, Germany), (2) hematocrit (Hct) test using Zipocrit® microhematocrit centrifuge (LW Scientific, Lawrenceville, GA), and (3) RBC travel distance on the nrLFA device after 240 sec (4 min). All collections were performed by the primary investigator (physician) in the examination room where patient's blood was collected and dispensed in the nrLFA device. Then the nrLFA device was quickly transported to another room where the trial data were capture and analyzed by a blinded secondary investigator (research assistant). The sample collection and data analysis cannot be performed in the same room due to the limited size of examination room as well as being considerate to patient's privacy. During the PT/INR test, one fingertip of the participant was cleaned using an alcohol wipe before a finger prick was performed using a 1.8 mm, 23 gauge CoaguChek® Lancet (Roche, Mannheim, Germany). 8-10 μL of capillary whole blood was collected using a CoaguChek® capillary blood collection tube and applied to a commercial CoaguChek® XS test strip within 10 sec of collection. From the same finger prick site, 30 μL of capillary whole blood was subsequently collected using a calibrated Microsafe® pipette (Safe-Tec Clinical Products, Warminster, PA) and applied to the sample pad of the nrLFA device within 10 sec of collection. If the second blood sample failed to be collected within 10 sec of initial puncture, a second finger prick on a different fingertip was performed to collect the required blood sample. For Hct measurement, ~30 μL of capillary whole blood was collected from a separate finger prick performed on a different finger using a heparinized microhematocrit tube (Drummond Scientific Company, Broomall, PA). Microhematocrit tubes were placed inside of a Zipocrit® microhematocrit centrifuge (LW Scientific, Lawrenceville, GA) and spun for 5 min at 11,000 rpm, and the Hct percentage was determined visually with the aid of a standard nomogram. The RBC travel distance on the nrLFA device was measured and analyzed as described in Sec. Methods for Extracting Calibration Equation Using Citrated Rabbit Blood with but 15 sec intervals between consecutive images. Fig. S1 shows the major instruments utilized in the clinical trial.

Fig. S1 Major instruments utilized in the clinical trial with patients on warfarin therapy.

4. Effect of Albumin on Rabbit Blood Transport in nrLFA

In order to investigate the effect of plasma protein concentration on blood transport in the nrLFA, we designed and conducted a set of preliminary experiments using bovine serum albumin (BSA) and washed rabbit RBCs (packed 80%). Five concentrations, 6 g/dL, 8 g/dL, 10 g/dL, 12 g/dL and 14 g/dL, of lyophilized BSA powder (Millipore Sigma, St. Louis, MO) in 0.9% NaCl (Fisher Scientific, Hampton, NH) solution was made and then added to 80% packed rabbit RBCs (Innovative Research, Novi, MI) at 1:1 volume ratio. The volume of both BSA in saline solution and packed rabbit RBCs are 50 µL. The resulting five blood samples have BSA concentrations of 3 g/dL, 4 g/dL, 5 g/dL, 6 g/dL and 7 g/dL respectively, and an identical Hct of 40%. During the measurement, 30 µL of the resulting blood sample is dispensed in the nrLFA device and the travel distance of RBCs were measured at t = 100 s, 150 s and 200 s. The documentation of the RBC distance is described in Sec. Methods for Extracting Calibration Equation Using Citrated Rabbit Blood but with 5 sec intervals between consecutive images. The experimental data are presented in Table S1 in the form of images. A light reflection can be seen at the left side of the observation window due to a clear plastic film placed on top of the window to prevent evaporation in the duration of experiment.

Table S1. Experimental data of the effect of albumin on blood transport in the nrLFA device.

BSA Conc. in Blood Sample	t = 100 s	t = 150 s	t = 200 s
3 g/dL			
4 g/dL			
5 g/dL			
6 g/dL			
7 g/dL			

From Table S1, we can see that RBC fronts (deep red front) of various blood samples travel approximately the same distance at t = 100 s, 150 s and 200 s despite of different concentrations of BSA in the samples. A slight decrease in the travel distance of plasma front (light red front) can be observed when BSA concentration increases. Since we only measure the transport of RBCs in all our experiments in this manuscript, we would like to conclude that different BSA concentrations do not affect the RBC travel distance of rabbit blood. No correlation can be observed between increasing albumin concentrations in blood (3-7 g/dL) and RBC travel distances in the nrLFA. Although we did not use the matching albumin and RBCs from the same animal species, we doubt the result would be different if we do use the matching albumin and RBCs.

References

1. Sigma-Aldrich: IVD Lateral Flow – Sample, Conjugate and Absorbent Pad Basics. (https://www.sigmaaldrich.com/technical-documents/articles/ivd-immunoassay/lateral-flow/pads-chemistries-selections-specifications-and-conjugates.html)
2. Red blood cells – Wikipedia. (https://en.wikipedia.org/wiki/Red_blood_cell)
3. EMD Millipore: Lateral Flow Tests Strips – Considerations for Product Development. (https://www.emdmillipore.com/Web-US-Site/en_CA/-/USD/ShowDocument-Pronet?id=201306.12550.)