Engineering a simple lateral flow device for animal blood coagulation monitoring

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Increasing numbers of animals are diagnosed with thromboembolism, requiring anticoagulation treatment to prevent thrombotic events. Frequent and periodic coagulation monitoring is critical to ensure treatment effectiveness and patient safety by limiting blood coagulation ability within the desired therapeutic range. Point-of-care diagnostics is an ideal candidate for frequent coagulation monitoring due to rapid test results and no need for laboratory setting. This article reports the first utilization of *no-reaction* lateral flow assay (nrLFA) device for simple and low-cost animal blood coagulation monitoring in resource-limited setting. The nrLFA device consists of sample pad, analytical membrane and wicking pad, without conjugate pad, reagent printing or membrane drying. Citrated and heparinized animal blood were utilized to mimic different blood coagulation abilities *in vitro* by adding reversal agents CaCl_2 and protamine sulfate. The travel distance of red blood cells (RBCs) on the nrLFA after a pre-determined test time serves as endpoint marker. Upon adding 500 mM CaCl_2 solution to citrated bovine, canine, rabbit and equine blood, the average travel distance decreases from 10.9 to 9.4 mm, 8.8 to 5.7 mm, 12.6 to 9 mm, and 15.3 to 11.3 mm, respectively. For heparinized bovine and rabbit blood, the average distance decreases from 14.5 to 11.4 mm and from 9.8 to 7.2 mm, respectively, when adding 300 mg/l protamine sulfate solution. The effect of hematocrit on RBC travel distance in the nrLFA was also investigated. The nrLFA device will potentially improve treatment efficiency, patient safety, quality of life, and satisfaction for both animal patients and their owners. *Published by AIP Publishing.*
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I. INTRODUCTION

With thrombosis being the leading cause of morbidity and mortality worldwide for human,¹ in veterinary medicine thrombophilia is becoming more frequently recognized and treated among animal patients.² Thrombosis is the localized coagulation or clotting of the blood inside of the vascular system, resulting in an aggregation of platelets and fibrin called thrombus.³ Thrombus can be formed in both large and small veins and arteries, and can possibly break down and travel along the bloodstream as an embolus to lodge in a vessel, causing embolism.³ Venous thromboembolism (TE) is caused by the thrombi formed in veins under low blood flow condition and primarily consist of fibrin and red blood cells (RBCs). Arterial TE is caused by the thrombi formed in arteries under high blood flow condition and primarily consist of platelets. Unlike venous TE which tends to be non-life-threatening, arterial TE often causes acute and devastating consequences. Venous thrombi are more likely to be developed in horses and cattle, whereas arterial thrombi tend to be more clinically significant in dogs and cats.³

Acute and chronic anticoagulation treatment is an essential part of the treatment for arterial thrombosis and embolism among animal patients.^{3–6} Heparin is the mainstay of acute

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anticoagulation treatment. It is typically administered in combination with thrombolytic agents because those agents do not prevent the formation of new clots and anticoagulants such as heparin or warfarin do not break down the existing clots.⁵ After achieving the desired level of anticoagulation by heparin injection, oral warfarin therapy is typically administered as chronic anticoagulation treatment.⁵ Routine coagulation testing should be performed^{3–6} during both acute and chronic anticoagulation treatment to ensure therapy efficiency and patient safety. The baseline of the activated partial thromboplastin time (aPTT) should be maintained at 1.5–3.0 times the pre-heparin value, and the baseline of the prothrombin time (PT) should be maintained at 1.5–1.7 times the pre-warfarin value, or between 2.0 and 3.0 of the international normalized ratio [INR = (patient PT)/(control PT)].^{3–6} The major risk of using anticoagulants is hemorrhage, which can be fatal to patient's life. Therefore, frequent and periodic monitoring of blood coagulation by performing coagulation tests is critical for dogs and cats undergoing anticoagulation treatment.

Blood coagulation is a critical step of hemostasis. The coagulation cascade^{7–9} is a complicated process, during which blood coagulation is achieved through extrinsic pathway (begins with trauma to vascular wall and surrounding tissues), intrinsic pathway (begins in the blood), and common pathway (merged pathway) for clot formation. The process is directly regulated by more than a dozen blood-clotting factors^{7,9} including twelve factors designated by Roman numerals, platelets, prekallikrein (Fletcher factor) and high-molecular-weight kininogen (Fitzgerald factor). Figure 1 shows a simplified common pathway of the coagulation cascade. The common pathway starts when Factor X is activated from extrinsic and/or intrinsic pathways. At the end of extrinsic pathway, with the presence of Ca^{2+} ions (Factor IV), tissue factor (Factor III) complexes with Factor VIIa and subsequently acts as enzyme to convert Factor X to Factor Xa (activated form of Factor X). Factor Xa can also be formed at the end of intrinsic pathway by Factor IXa acting in combination with Factor VIIIa and tissue factor to activate Factor X. Immediately after Factor Xa is formed, with the presence of Ca^{2+} ions, it complexes with Factor V and phospholipids and subsequently forms a complex called prothrombin activator. The prothrombin activator converts prothrombin (Factor II) into thrombin (Factor IIa) with the presence of sufficient Ca^{2+} ions. The thrombin triggers the polymerization of fibrinogen (Factor I) molecules into fibrin monomers (Factor Ia), which subsequently form cross-linked insoluble fibrin fibers with the presence of Ca^{2+} ions and Factor XIIIa (fibrin stabilizing factor).

Table I summarizes the physiological range of important blood parameters in various animals and human, including hematocrit (Hct), hemoglobin (Hgb), blood total and ionized

FIG. 1. Simplified common pathway of coagulation cascade.

TABLE I. Physiological range of various blood parameters in rabbit, porcine, bovine, equine, canine and human.

Species	Hct (%)	Hgb (g/dl)	Plasma protein (g/dl)	Total Ca (mM)	Ionized Ca (mM) (Factor IV)	Plasma fibrinogen (mg/dl) (Factor I)	Plasma prothrombin (U/ml) (Factor II)	Factor X (U/ml)
Rabbit	33–50 ¹⁰	10–17 ¹⁰	5.4–8.3 ¹⁰	3.24–3.40 ¹³	1.35–1.39 ¹³	169–223 ¹⁸	2.37–2.43 ¹⁸	1.75–1.85 ¹⁸
Porcine	36–43 ¹⁰	10–16 ¹⁰	6.0–8.0 ¹⁰	1.77–2.89 ¹⁴	0.87–1.45 ¹⁴	270–426 ¹⁸	0.38–0.98 ¹⁸	1.64–3.44 ¹⁸
Bovine	24–46 ¹⁰	8–15 ¹⁰	6.0–8.0 ¹⁰	2.42–3.09 ¹⁴	1.20–1.55 ¹⁴	160–200 ¹⁸	0.32–0.52 ¹⁸	0.79–1.39 ¹⁸
Equine	27–43 ¹⁰	10–16 ¹⁰	6.0–8.5 ¹⁰	2.99–3.69 ¹⁵	1.61–1.89 ¹⁷	240–306 ¹⁸	1.25–1.43 ¹⁸	2.08–2.12 ¹⁸
Canine	35–57 ¹⁰	12–19 ¹⁰	6.0–7.5 ¹⁰	2.37–2.82 ¹⁶	1.30–1.46 ¹⁶	165–265 ¹⁸	1.13–1.73 ¹⁸	1.78–2.58 ¹⁸
Human	36–54 ¹¹	12–18 ¹¹	6.5–7.9 ¹²	2.28–2.34 ¹³	1.03–1.05 ¹³	150–450 ¹⁸	0.70–1.30 ¹⁸	0.75–1.25 ¹⁸

calcium, plasma fibrinogen, plasma prothrombin and plasma clotting Factor X. Hematocrit (Hct) is the packed volume percentage of RBCs in whole blood. Canine and human, and bovine and equine have similar Hct values, which are 35%–57% and 24%–46%, respectively.^{10,11} Canine and human, and rabbit, porcine and equine have very similar Hgb values, which is 12–19 g/dl and 10–17 g/dl, respectively.^{10,11} All animal species including human have similar plasma protein concentrations ranging from ~6 to ~8 g/dl.^{10,12}

A noticeable difference in blood calcium concentrations (both total and ionized) can be observed in Table I among various species. The same is also true for plasma fibrinogen, prothrombin and Factor X. Rabbit and equine have the highest concentrations of total calcium (3.24–3.40 mM and 2.99–3.69 mM, respectively), and porcine and human the lowest (1.77–2.89 mM and 2.28–2.34 mM, respectively).^{13–16} For ionized calcium (Ca^{2+}) and clotting Factor IV in coagulation cascade, equine has the highest concentration of 1.61–1.89 mM, and porcine and human the lowest (0.87–1.45 mM and 1.03–1.05 mM, respectively).^{13,14,16,17} Concentration of ionized calcium is ~50% of total calcium for most mentioned species except for rabbit, where it is close to ~40%–43%. This results in a higher ratio of citrate to fresh rabbit blood for effective clotting prevention.¹³ For plasma fibrinogen and clotting Factor I, bovine, rabbit and canine have the lowest concentrations of approximately 160–265 mg/dl, and porcine the highest (270–426 mg/dl).¹⁸ For plasma prothrombin and clotting Factor II, bovine and porcine have the lowest concentrations (0.32–0.52 U/ml and 0.38–0.98 U/ml, respectively), and rabbit the highest (2.37–2.43 U/ml).¹⁸ For Factor X, human and bovine have the lowest concentrations (0.75–1.25 U/ml and 0.79–1.39 U/ml, respectively), and equine the highest (2.08–2.12 U/ml).¹⁸ It is noted that equine has the highest concentrations in various coagulation factors among the mentioned species, which generally results in faster induced coagulation from anticoagulated equine blood than in other species.

Table II in shows the most commonly used anticoagulants for patient interventions and laboratory applications. Commonly used medical anticoagulants include warfarin, heparin and novel oral anticoagulation drugs (NOACs—apixaban, edoxaban, rivaroxaban and dabigatran). Warfarin is an oral anticoagulant that prevents or treats venous thrombosis as well as other thromboembolic complications.¹⁹ Its anticoagulation capacity is achieved by reducing the amount of active vitamin K in the tissues and thus suppressing the liver's production of clotting factors including Factors II, VII, IX and X.^{9,19} Heparin (sodium heparin or calcium heparin) is another common anticoagulant that is typically administered by intravenous injection in high concentrations to prevent intravascular clotting.^{9,20} Although by itself heparin has little or no anticoagulation property,⁵ when combined with antithrombin III, the resulting complex increases the effectiveness of thrombin removal by a hundred to a thousand times,⁷ thus serving as anticoagulant. The complex of heparin and antithrombin III also removes other clotting factors including Factors XIIa, XIa, Xa and IXa.^{7,20} Unlike warfarin or heparin which inhibits several clotting factors, NOACs are a group of new anticoagulants that selectively inhibit one single coagulation factor—either thrombin or Factor Xa.²¹ Also, no reversal agent for NOACs has received market approval yet,²² whereas anticoagulation effect from warfarin or heparin can be reversed by vitamin K (for warfarin) or protamine sulfate (for heparin). Three of the

TABLE II. Conventionally used anticoagulants for patient interventions and laboratory applications.

Anticoagulants	Mechanism	Intended use/application	Reversal agent
Apixaban, edoxaban, rivaroxaban	Factor Xa inhibition	Oral medication (NOAC)	N/A
Dabigatran	Thrombin (Factor IIa) inhibition	Oral medication (NOAC)	N/A
Warfarin	Vitamin K suppression; pro-thrombin (Factor II), Factors VII, IX and X inhibition	Oral medication	Vitamin K; fresh blood/plasma
Heparin (sodium heparin/calcium heparin)	Antithrombin III (AT III) activation; thrombin (Factor IIa) and Factor Xa inhibition	Injection medication, heart-lung and artificial kidney machines, laboratory blood tests, blood transfusion bags	Protamine sulfate; fresh blood
Citrate (sodium citrate/acid citrate dextrose)	Binds to Ca^{2+} ions (Factor IV) in blood; reversible by addition of Ca^{2+} ions	Laboratory blood tests (coagulation tests), blood transfusion bags	Ionized calcium
Oxalate	Binds to Ca^{2+} ions (Factor IV) in blood; reversible by addition of Ca^{2+} ions	Laboratory blood tests (glucose tests)	Ionized calcium
EDTA (ethylenediamine-tetraacetic acid)	Binds to Ca^{2+} ions (Factor IV) in blood strongly and irreversibly	Laboratory blood tests (whole blood tests)	None

above mentioned anticoagulants have been utilized in veterinary applications. Heparin utilizations in veterinary medicine include the management of disseminated intravascular coagulation, aortic TE and other hypercoagulability in dogs, cats and horses.^{3–6,9,23} Warfarin has been utilized for treatment of cardiac embolism including aortic TE and pulmonary TE in dogs and cats.^{3–6,9,24} Rivaroxaban has also been reported to experimentally treat thrombotic complications in dogs.^{3,25} Commonly used anticoagulants for laboratory applications include heparin, citrate, oxalate and EDTA (Table II). Detailed information on these anticoagulants can be found in [supplementary material](#).

The simplest and cheapest way to provide frequent and periodic blood coagulation monitoring for animals undergoing anticoagulation treatment is to utilize point-of-care (POC) diagnostic devices. POC diagnostics has gained increasing popularity due to its ability to achieve rapid, reasonably sensitive and accurate test results in non-laboratory settings.²⁶ Technologies of POC testing (glucose test strips, pregnancy test strips, etc.) have been significantly improved in the past two decades,²⁷ leading to the successful development of easy-to-use and low-cost devices with accurate qualitative or quantitative detection of a broad range of analytes including cells,²⁸ bacteria,²⁹ viruses,³⁰ biomarkers,³¹ pesticides³² and heavy metals.³³ By allowing at-home patient self-testing and rapid diagnostics during emergency, POC devices have transformed the healthcare delivery system to allow healthcare professionals as well as patients to perform rapid and accurate biochemical tests.^{27,34}

The benefits of POC devices extend beyond human applications with the help of biosensor technologies developed for health management of animals and livestock.³⁵ Due to their portability, rapid detection and low cost,^{26,27} veterinarians and animal specialists have been evaluating POC devices in both the clinic and in the field³⁴ for the past decade to measure blood physiological parameters such as glucose,^{36,37} lactate,^{38,39} ions⁴⁰ and gases.^{40,41} The evaluation of POC devices in assessing hemostatic functions^{42–44} in dogs was reported, as well as measuring the concentration of hemoglobin in pigs⁴⁵ and creatinine in cats.⁴⁶ Recently, POC devices specially designed for household pets have started to emerge on the market. Abaxis VetScan series include five handheld or small bench-top POC analyzers for veterinary clinics to perform blood/plasma chemistry, coagulation and fibrinogen testing, complete blood count, and vector-borne and fecal disease testing. At-home-use veterinary POC devices are currently limited to pet blood glucose meters, such as AlphaTRAK 2 (Zoetis Services LLC, Parsippany, NJ) and EverPaw (EverPawTM LLC, Brooklyn, NY) blood glucose monitoring systems for dogs and cats.

As a POC technology that features portability, ease of use, simple fabrication and low cost, lateral flow (immuno)assay (LFA) is widely utilized in biomedicine, food, agriculture and environmental science to detect tumor biomarkers,⁴⁷ microorganisms,⁴⁸ heavy metals,⁴⁹ toxins⁵⁰ and pesticides.³² LFA typically consists of cellulose sample pad, fiberglass conjugate pad, nitrocellulose analytical membrane and cellulose wicking pad.⁵¹ Lateral flow takes place in porous materials which take advantage of the capillary effect, and allows fluid samples to transport within the same material or from one material to another.⁵¹ To pursue even cheaper and simpler devices, Whitesides group in the Harvard University developed microfluidic paper-based analytical devices (μ PAD)^{52,53} that consist of laminated paper strips with impregnated non-immunological reagents for colorimetric detection. The low-cost and disposable nature of paper and membrane materials utilized in μ PAD promotes broad application and frequent usage of the device, as well as minimizes cross-contamination between tests.

In this article, we report the first utilization of a simple and low-cost *no-reaction* lateral flow assay (nrLFA) device to monitor animal blood coagulation in resource-limited settings (home, small clinics, shelters, etc.). Meyer dos Santos *et al.* reported⁵⁴ a microfluidic blood coagulation test based on Rayleigh surface-acoustic wave (SAW) and demonstrated a prolonged SAW-induced clotting time with increasing dose of various anticoagulants including heparin, as well as NOACs such as argatroban, dabigatran and rivaroxaban. Especially in the case of rivaroxaban, SAW-induced clotting time was more sensitive than activated partial thromboplastin time (aPTT) to the increasing concentration of anticoagulant. Also, Lin *et al.* reported⁵⁵ an integrated blood coagulation testing system on lab-on-disc platform and demonstrated its capability of performing both prothrombin time (PT) and aPTT tests. High correlations ($R > 0.96$) between the test results from lab-on-disc device and from a clinical coagulation analyzer were shown for both PT and aPTT tests using fifty clinically acquired blood samples. Furthermore, Yu *et al.* reported⁵⁶ a microfluidic system for continuous monitoring of real-time thrombin generation by adding fluorogenic substrates into continuously extracted thrombin using a microdialysis probe and splitting the mixture into in-line microdroplets. However, none of the above methods fit into the concept of simple and low-cost POC coagulation tests. In our previous research, we initiated the concept of using nrLFA device for blood coagulation monitoring and reported its comparable performance to a clinical instrument for clotting time measurement.⁵⁷ We also demonstrated the excellent reproducibility of the nrLFA device and various related materials in the device fabrication.⁵⁸ Moreover, we conducted clinical trial using the nrLFA device with patients on warfarin therapy and successfully demonstrated the nrLFA's capability to monitor blood coagulation in conjunction with existing POC coagulation analyzers.⁵⁹ Existing POC coagulation analyzers are not widely affordable for everyday use due to the high initial cost and the per-use cost of the test strips. Our approach fits the everyday routine monitoring using low-cost platform. Based on the conventional LFA technology, nrLFA device primarily consists of a sample dispensing pad, a porous analytical membrane, a wicking pad and a plastic housing. The device has very low material costs due to its simple structure, and is self-powered by the capillary force driving fluid flow in the porous membrane. After being applied to the sample pad, whole blood migrates onto the analytical membrane where the separation of RBCs and plasma occurs due to the filtration property of the membrane. Since whole blood viscosity changes during the coagulation process, RBC travel distance at a given time is related to the coagulation state of the blood. The RBC travel distance serves as endpoint marker due to its easy visibility to the naked eye.

II. MATERIALS AND METHODS

A. Fabrication of nrLFA device

Figure 2 shows the structure of nrLFA device utilized in this study. The device is based on a conventional LFA test strip held in a 2-piece plastic cassette, with a fluid reservoir for sample dispensing and an observation window for measuring flow distance. The length of the observation window is 16.5 mm. Unlike conventional LFA, no conjugate pad is included for nrLFA. The nrLFA test strip consists of a fiber glass sample pad (Ahlstrom, Helsinki, Finland), a nitrocellulose lateral flow membrane (MilliporeSigma, Billerica, MA), a cellulose wicking pad

FIG. 2. Structure of nrLFA device.

(Whatman, Little Chalfont, United Kingdom) and a plastic backing card (Diagnostic Consulting Network, Carlsbad, CA). The width of all the components is 4 mm, and the lengths of each component are 13 mm for sample pad, 30 mm for analytical membrane and 20 mm for wicking pad, with an overall strip dimension of 4 mm × 53 mm. The overlaps of the analytical membrane with the sample pad is 6 mm and with the wicking pad is 4 mm. Narrow sheets of the three components are stacked and glued onto a plastic backing card and then cut into 4 mm width strips with the CM4000 guillotine cutter (BioDot, Irvine, CA). The resulting nrLFA strip is then placed inside the plastic cassette (Diagnostic Consulting Network, Carlsbad, CA). The observation window is covered with transparent tape to prevent fluid evaporation during tests. No membrane drying or reagent printing procedure is used during device fabrication.

The analytical membrane, which consists of nitrocellulose in our case, is the most important component of the nrLFA device. Nitrocellulose (NC) has been widely used as analytical material for lateral flow immunoassays due to its high protein binding capability and particularly uniform wetting in all directions. NC membranes are available in various capillary rise times (the time it takes for water to wick 4 cm vertically in a membrane strip) ranging from ~75–180 s/4 cm, giving high flexibility and versatility during assay design. We chose Millipore HF075 membrane for the nrLFA device due to its short capillary rise time and large pore size, which are essential in whole blood analysis. Since blood has a relatively high viscosity compared to other bodily fluids (plasma, urine, sweat, etc.) and contains a large quantity of cells, the membrane with short capillary rise time and large pore size will ensure a fast and uniform flow without potentially clogging the capillary passage. As reported⁵⁸ previously by our group, HF075 has a porosity of 82%, a pore size of $14.53 \pm 4.73 \mu\text{m}$ and a capillary rise time of $77 \pm 2 \text{ s/4 cm}$, which directly contributed to its excellent flow reproducibility⁵⁸ when using either anticoagulated or coagulation-activated blood.

B. Coagulation experiments using citrated animal blood

Citrated blood of five animal species were utilized for blood coagulation assessment: rabbit (HemoStat Laboratories, Dixon, CA), porcine (Innovative Research, Novi, MI), bovine (Innovative Research, Novi, MI), equine (Innovative Research, Novi, MI), and beagle (Innovative Research, Novi, MI). As illustrated in Fig. 3, 160 μl of citrated animal blood is dispensed into a 1.5 ml microcentrifuge tube (Fisher Scientific, Pittsburgh, PA), followed by adding 5 μl 0.9% NaCl (Fisher Scientific, Pittsburgh, PA) solution and then adding 15 μl CaCl_2 (Fisher Scientific, Pittsburgh, PA) solution. CaCl_2 solutions with various concentrations (up to 500 or 600 mM) were utilized as reversal agent to mimic fresh whole blood with various coagulation abilities. For the control case (no CaCl_2), a total of 20 μl 0.9% NaCl solution was added to citrated animal blood instead to maintain

FIG. 3. Experimental procedures of coagulation tests using citrated animal blood.

the hematocrit percentage constant in all blood samples, with or without CaCl_2 solution. Immediately after adding NaCl and CaCl_2 solutions, the microcentrifuge tube was submerged in a 38 or 39 $^{\circ}\text{C}$ (39 $^{\circ}\text{C}$ for rabbit, beagle and bovine, and 38 $^{\circ}\text{C}$ for porcine and equine) water bath for 2 min to fully initiate blood coagulation cascade by providing a similar temperature to the actual animal's body. Immediately after the water bath, 100 μL of blood sample mixture was dispensed into the nrLFA reservoir. As soon as the blood sample starts flowing in the analytical membrane, the slower-propagating RBC (red blood cell) front is separated from the faster-propagating plasma front due to the filtration property of the membrane. The plasma front was fairly transparent on the membrane making it difficult to observe with the naked eye, so the travel distance of RBC front was measured and subsequently considered as an indicator of blood coagulation ability.

During the tests, a digital camera with an external timer was utilized to graphically document fluid travel distance by taking high-resolution digital photographs at fixed time interval (5 s). The starting point ($t=0$) was selected when the RBC front just appeared in the observation window and the ending point was selected at $t=120$ s or 240 s, at which moment the travel distance of RBC front was measured. Each test was performed in triplicate to confirm the reproducibility of nrLFA device. The number of pixels associated with RBC travel distance was obtained using ImageJ and then converted into actual distance (mean and standard deviation) using Excel.

C. Coagulation experiments using heparinized animal blood

In this set of experiments, heparinized bovine blood (HemoStat Laboratories, Dixon, CA) and rabbit blood (Innovative Research, Novi, MI) were utilized for blood coagulation assessment. 20 μL of protamine sulfate (MilliporeSigma, Billerica, MA) solution of various concentrations (0 to 500 mg/l with an increment of 50 or 100 mg/l in 0.9% NaCl solution) was dispensed on the nrLFA and then followed by the addition of 100 μL heparinized blood when the front of protamine sulfate solution reached the end of analytical membrane. Travel distance of RBC front on nrLFA was acquired the same way as the experiments using citrated animal blood.

D. Preparation of adjusted blood samples

Citrated rabbit blood with hematocrit (Hct) values of 30% and 40% was obtained by removing freshly separated plasma from low Hct blood (24% as received from vendor) after

light centrifugation (Thermo Fisher Scientific accuSpin Micro 17, Osterode am Harz, Germany) at $400\times g$ for 6 min, and then re-suspended in the blood by gentle agitation. Citrated rabbit blood with 30% and 40% Hct were obtained by removing 200 μl and 400 μl of plasma, respectively, from 1 ml of citrated rabbit blood with 24% Hct. Citrated rabbit blood with Hct value of 20% was obtained by adding 100 μl freshly separated citrated rabbit plasma ($700\times g$ for 10 min) into 1 ml of citrated rabbit blood with 22% Hct (as received from vendor). A microhematocrit centrifuge (LW Scientific Zipocrit, Lawrenceville, GA) was utilized to measure hematocrit of blood samples from all animal species.

III. RESULTS AND DISCUSSION

A. Coagulation evaluation using citrated rabbit blood

The utilization of the nrLFA device for blood coagulation monitoring is based on the changes in whole blood viscosity during coagulation process.⁶⁰ Achieving effective hemostasis requires a complex interplay involving the vascular system, coagulation process, fibrinolytic system and platelets.⁵⁹ Upon the activation of coagulation cascade by the intrinsic or extrinsic pathway, the viscosity of whole blood gradually increases, and the blood changes its physical properties from a viscoelastic fluid to a viscoelastic solid after formation of a cross-linked fibrin clot.⁶¹ In our previous research, we investigated the relationship between fluid travel time on the nrLFA device and fluid dynamic viscosity by measuring the time required for the fluid front of various glycerin/water mixtures to reach the end of the observation window.⁵⁹ The viscosity values of selected glycerin/water mixtures were within the reported range for coagulating blood under low shear stress,⁶⁰ and a linear relationship between fluid travel time and its viscosity was observed on the nrLFA device.⁵⁹

Figure 4 shows the relationship between RBC travel distance and various CaCl_2 concentrations when using citrated rabbit blood with 20%, 30% and 40% Hct. This set of experiments is designed to investigate the effect of coagulation on RBC travel distance in nrLFA, as well as the effect of Hct on RBC travel distance when coagulation is activated. Citrated blood was utilized in this set of experiments because citrate is one of the most common anticoagulants for laboratory use and citrated blood is routinely used for coagulation studies due to the ease of reversing the coagulation effect by simply adding Ca^{2+} ions. As shown in Fig. 4, at $t = 120$ s, in all three cases of 20%, 30% and 40% Hct the RBC travel distance decreases monotonically when increasing concentrations of CaCl_2 solution are added to the citrated blood. This results in more ionized calcium in blood, which enables coagulation, and thus contributes to higher

FIG. 4. RBC travel distance vs. CaCl_2 concentration when using citrated rabbit blood with Hct 20%, 30% and 40% ($n = 3$).

blood viscosity and shorter RBC travel distance in the nrLFA membrane. When adding 600 mM CaCl_2 solution, the RBC travel distance decreases from an initial value of ~ 14 mm to 7.5 mm for the 20% Hct case, from ~ 11 mm to ~ 7 mm for the 30% Hct case, and from ~ 8 mm to ~ 6 mm for the 40% Hct case. Thus, the lowest (20%) Hct blood results in greatest decrease in RBC travel distance (~ 6.5 mm) compared to the highest (40%) Hct blood (~ 2 mm) when adding the same concentrations of CaCl_2 . In general, when Hct increases from 20% to 40%, the RBC travel distance decreases at all CaCl_2 concentrations. This effect is caused by increasing blood viscosity in the presence of an increased cellular fraction, which results in shorter RBC travel distance in the nrLFA membrane. Due to the preparation method of blood samples with various Hct (mentioned in Sec. II D), 20% Hct blood has the highest concentration of clotting factors, followed by 30% Hct blood and then 40% Hct blood. The slope of the RBC travel distance vs. CaCl_2 concentration (“resolution”) from 200 to 600 mM is 1.30 mm, 0.90 mm and 0.46 mm per 100 mM CaCl_2 for 20% Hct blood, 30% Hct blood and 40% Hct blood, respectively. This indicates that the travel slope increases with increasing effective concentration of clotting factors in citrated rabbit blood.

B. Coagulation evaluation using citrated blood from four animal species

Figure 5 shows the relationship between RBC travel distance in the nrLFA device and added CaCl_2 concentrations when using citrated blood from various animal species. Citrated animal blood was utilized in this set of experiments for the same set of reasons as indicated in Sec. III A. Experimental data using citrated porcine blood is not included. During multiple attempts the porcine blood clotted early in the microcentrifuge tube and was impossible to pipette out after even adding the lowest concentration (100 mM) of CaCl_2 solution. The Hct of citrated bovine, canine, rabbit and equine are 44%, 50%, 40% and 35%, respectively. As can be seen in Fig. 5, at $t = 240$ s, RBC travel distance of all animal species decreases monotonically with increasing concentrations of added CaCl_2 solution, as the blood sample undergoes continuous coagulation. Also, according to Fig. 5, the RBC travel distance of each species basically follows their Hct percentage. Equine blood, with the lowest Hct, results in the longest RBC distance, while canine blood, with the highest Hct, exhibits the shortest distance with added CaCl_2 concentration. This trend indicates that the rabbit blood results with high and low Hct percentage shown in Fig. 4 is fundamental and not species-dependent. As shown in Fig. 5, the average distance decreases from 10.9 to 9.4 mm, 8.8 to 5.7 mm, 12.6 to 9 mm, and 15.3 to 11.3 mm when using 0–500 mM CaCl_2 solution added to citrated bovine, canine, rabbit and

FIG. 5. RBC distance vs. CaCl_2 concentration on nrLFA device when using citrated animal blood from various species ($n = 3$).

equine blood, respectively. The resolution of the RBC travel distance on the nrLFA device when using citrated animal blood is 0.52 mm, 0.78 mm, 0.77 mm and 0.89 mm per 100 mM CaCl_2 for bovine, beagle, rabbit and equine, respectively.

According to the vendors, citrated animal blood of various animal species was collected using the followed protocols: bovine—10:1 volume ratio of fresh blood to 4% (w/w) sodium citrate solution; canine (beagle)—9:1 volume ratio of fresh blood to 3.8% (w/w) sodium citrate solution, rabbit—4:1 volume ratio of fresh blood to 4% (w/w) sodium citrate solution; equine—10:1 volume ratio of fresh blood to 4% (w/w) sodium citrate solution. Assuming 3.8% and 4% citrate solutions are made from anhydrous trisodium citrate powder with a molar mass of 258.06 g/mol, the original citrate concentration in citrated blood (before chelating ionized calcium) for various species is 14.1–14.7 mM for bovine, beagle and equine, and 31 mM for rabbit. As shown in Table I, average concentration of blood ionized calcium for various species is ~ 1.37 – 1.38 mM for bovine, beagle and rabbit, and 1.75 mM for equine. Assuming ionized calcium binds to trisodium citrate in 3:2 ratio and all ionized calcium is depleted in citrated animal blood, the concentration of remaining citrate in as-received citrated animal blood (after chelating ionized calcium) is 13.04–13.87 mM for bovine, beagle and equine, and 30.27 mM for rabbit. These are the final concentrations of remaining citrate in as-received animal blood. It is important to note that citrated rabbit blood with 40% Hct was obtained by extracting 400 μl of plasma from 1 ml of citrated rabbit blood originally received with 24% Hct. Therefore, the concentrations of citrate in Hct 40% citrated rabbit blood should be 18.16 mM. When adding 15 μl of CaCl_2 solution into 160 μl of as-received animal blood, the added Ca^{2+} concentration ranges from 8.33 to 50 mM for 100 to 600 mM added CaCl_2 solution. Therefore, due to the previously mentioned 3:2 binding ratio, for all animal species the blood coagulation process is fully activated after adding 200 mM CaCl_2 solution due to the complete chelation of citrate in the blood. The detailed information on the calculation of citrate and ionized calcium concentrations can be found in [supplementary material](#). After coagulation process is fully activated, adding more ionized calcium causes stronger coagulation effect, which can also be observed in Fig. 5 as the RBC travel distance continues to decrease.

It has been reported⁶² that horses have a large variation in whole blood viscosity, plasma viscosity and RBC aggregation when using standardized blood samples (Hct=40%) at low shear rate (0.7 s^{-1}), which explains the larger error bars of equine data in Fig. 5. For the case of rabbit (Figs. 4 and 5), the relatively large error bars are probably due to the centrifugation and re-suspension process associated with Hct adjustment of citrated blood sample, which damaged the shapes of some RBCs and resulted in variation in RBC aggregation in nrLFA.

C. Coagulation evaluation using heparinized animal blood

Heparin is another commonly used anticoagulant both *in vivo* and *in vitro*, and cationic protamine sulfate acts as emergency reversal agent to heparinized blood by binding to heparin at 1:1 ratio and eliminating the anticoagulation effect generated by heparin. Figure 6 shows the relationship between RBC travel distance and protamine sulfate concentration when using heparinized bovine and rabbit blood at $t = 120 \text{ s}$. As shown in Fig. 6, for heparinized bovine blood, RBC travel distance decreases as higher concentrations (50–250 mg/l) of protamine sulfate is added to heparinized blood, and reaches a floor at 250 mg/l. The same trend can be observed in heparinized rabbit blood data with a somewhat higher floor at $\sim 300 \text{ mg/ml}$. The overall resolution of the RBC travel distance on the nrLFA device when using heparinized bovine blood is 1.27 mm per 100 mg/l protamine sulfate when adding 0–250 mg/l of protamine sulfate solution. For heparinized rabbit blood, the overall resolution is 0.89 mm per 100 mg/l when adding 0–300 mg/l of protamine sulfate solution.

In Fig. 6, at very high concentrations (300–500 mg/l) of protamine sulfate, RBC travel distance stops decreasing because all heparin is neutralized by the large amount of protamine sulfate and blood coagulation process is fully activated, leaving the extra protamine sulfate of no use in promoting coagulation. According to the vendor, heparin concentration in bovine and rabbit blood is 3 IU/ml and 5 IU/ml, respectively, and the purity of protamine sulfate powder is

FIG. 6. RBC travel distance vs. protamine sulfate concentration when using heparinized bovine and rabbit blood on the nrLFA device ($n = 3$).

96.6 IU/mg. 100 μ l of heparinized bovine blood contains 0.3 IU of heparin, and since the reaction ratio between heparin and protamine sulfate is 1:1, 0.3 IU of protamine sulfate is required for complete neutralization, which corresponds to 3.1056×10^{-3} mg and thus equals to 20 μ l of 155 mg/l protamine sulfate solution. As shown in Fig. 6, complete neutralization of heparin happens at 250 mg/l protamine sulfate data point instead of 150–200 mg/l. This is probably due to the fact that the contact between heparin and protamine sulfate molecules on nrLFA strip is not sufficient due to their sequential flow on the membrane material, and thus more protamine sulfate molecules are needed to fully neutralize heparin. The same calculation method can be applied to heparinized rabbit blood. The concentration necessary for the 20 μ l protamine sulfate solution to fully neutralize heparin is 259 mg/l, which is higher than the corresponding concentration (155 mg/l) for heparinized bovine blood. This explains why heparinized rabbit blood reaches a floor at 300 mg/l protamine sulfate data point, slightly higher than that of heparinized bovine blood.

D. Potential application in veterinary diagnostics

The proposed utilization of the nrLFA device for veterinary applications is POC blood coagulation screening device for animals undergoing anticoagulation treatment. The observation window on the nrLFA can be divided into three sections (from left to right): “too thick,” “good” and “too thin.” “Thick” and “thin” stand for strong or weak blood coagulation ability. The length of the three sections should be customized for each animal patient based on the desired therapeutic range, pre-determined test time and the patient’s Hct, as well as other blood parameters that may affect its viscosity. If the travel distance of RBC front after a pre-determined amount of time falls in section “good,” it implies that the patient’s blood coagulation ability is in the desired therapeutic range and the patient is well-protected from potential thrombotic events. If the RBC distance falls into “too thick” or “too thin” section, it indicates that the patient’s blood coagulates too fast or too slow, and further quantitative measurement is needed in a veterinarian’s office to increase or decrease drug doses under veterinarian’s instruction for effective yet safe treatment. The nrLFA device is not designed to replace traditional aPTT or PT/INR tests during anticoagulation treatment, but rather to be utilized as a qualitative tool for testing at home or in other resource-limited settings (small clinics, shelters, etc.) to determine if a follow-up visit with a veterinarian is necessary for a quantitative measurement. In clinical applications, patient self-testing of PT/INR using POC devices is already a well-established monitoring method that not only improves therapeutic range and treatment efficiency but also increases quality of life and satisfaction level when compared to traditional PT/INR monitoring.⁵⁹

It has been reported that frequent patient self-testing can significantly reduce the risk of stroke (by ~55%), major hemorrhage (~35%), and even death (~39%).⁶³ All above benefits can be readily enjoyed by animal patients if in-location coagulation monitoring is provided by POC devices such as the nrLFA. Although not being quantitative, its simple operation and low cost (<40 cents each⁵⁹) make the nrLFA an ideal candidate for local monitoring or in-clinic screening without the need of an expensive coagulation analyzer. Especially in today's connected world when remote diagnosis is becoming more common, sending POC tests results from off-site to a veterinarian followed by consulting on an urgent or regular follow-up visit will save the time and resource for both animal owners and veterinarians.

The dependence of flow properties on Hct indicates the need for periodic nrLFA calibration against known Hct concentration for the patient. However, since the Hct does not change rapidly, nrLFA tests to detect routine changes due to certain conditions and treatments can be performed as part of medical monitoring. It is recommended that the length of three sections ("too thick," "good" and "too thin") on the nrLFA device is customized for each patient, and the usage of the nrLFA devices for each patient should be performed at the same time each day and when the patient is in a "normal" condition, such as before breakfast.

IV. CONCLUSIONS

The nrLFA device has several desirable characteristics (low cost, easy to use, disposable, etc.) that make it a promising candidate in blood coagulation screening for animal patients diagnosed with TE or other hypercoagulable state. This study demonstrated that the RBC travel distance on the nrLFA device decreases as blood coagulation ability increases when using coagulation-activated anticoagulated animal blood (citratd blood from bovine, beagle, rabbit and equine, and heparinized blood from bovine and rabbit). The data indicates that the addition of different concentrations of reversal agents to anticoagulated blood causes different coagulation abilities of the blood that subsequently results in the difference in blood viscosity, and thus allow RBCs to travel at different rate on the nrLFA device. Since the hematocrit has an influence on RBC travel distance on the nrLFA, it is recommended to customize the desired range of RBC travel distance for each patient and to perform the test at the same time each day for better accuracy.

We believe the utilization of the nrLFA device in blood coagulation monitoring for animal patients undergoing anticoagulation treatment will improve not only treatment efficiency and patient safety, but also the quality of life and satisfaction level.

SUPPLEMENTARY MATERIAL

See [supplementary material](#) for conventionally used anticoagulants for patient interventions and laboratory applications (Table S1), calculated concentrations of ionized calcium and citrate in citrated animal blood during coagulation experiment (Table S2), calculated concentrations of added ionized calcium in citrated animal blood during coagulation experiment (Table S3).

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