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Investigating the Kinetics of Blood Coagulation using Ultrasound

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***Abstract*—The study of blood coagulation can provide provide useful prognostic and diagnostic information for a range of diseases. Coagulation of whole mouse blood was monitored via a high-frequency ultrasound system with a central frequency of 80 MHz. Simultaneous acquisition of M-mode images and of reflected frequency spectra from signals propagated through the sample provided information on the motion of red blood cells and acoustic attenuation, respectively.**

This work aimed (i) to show the benefit of simultaneous acquisition of reflection spectra and M-mode data as blood coagulations, and (ii) to highlight the advantages of using high frequencies ($>$ 60 MHz) in identifying features associated with blood coagulation which aren't visible at lower frequencies.

***Index Terms*—high-frequency ultrasound, blood coagulation, clotting kinetics, frequency spectrum, M-mode imaging**

I. Introduction

Hemostasis is the set of biological mechanisms that work to maintain healthy blood flow through the regulation of clot formation and breakdown. A crucial component of hemostasis is the localized coagulant response at sites of injury. This response involves the initiation of a protein activation cascade that ultimately results in the formation of a protein 'mesh' composed of fibrin polymers [1]. These fibrin meshes, which constitute the underlying structure of blood clots, act to minimize blood loss. However, their inherent ability to inhibit blood flow makes clots themselves a risk to healthy circulation. For example, if they are not broken down sufficiently following the closing of the injury site, they may become lodged within the vasculature. When hemostasis is disrupted by medication, injury, or disease, the excessive bleeding or undesirable presence of clots that result may manifest in morbidity or mortality [1]. Monitoring hemostasis, therefore, is of significant interest in medicine, as it can be used to provide both prognostic and diagnostic information for a range of diseases [2].

A number of coagulation tests are used routinely in patient care to probe the coagulant process. The most common of these are the prothrombin time (PT) and the activated thromboplastin time (aPTT) tests, both of which were developed primarily to monitor patients on anti-coagulant medications. Performed in plasma (the liquid component of blood that remains when blood cells are removed), these tests measure the time required for in vitro clot formation following activation with specific clotting agents [2]. However, with major advancements in our understanding of the coagulant process, the use of a single metric (clotting time) to assess such a complex biochemical process has been brought into question [3]. Moreover, routine coagulation tests have been shown to provide little predictive value for assessing the risk of either bleeding or clotting [2].

Increasing interest has been directed towards tests that examine a more global perspective of coagulation by acquiring kinetic information throughout the hemostatic process [2]. An array of tests have been developed in this area that have shown promise in predicting the risk of excessive bleeding [2], although there remains a lack of standardization in the field and only limited translation to the hospital has been achieved. Consequently, global coagulation tests remain an active area of research.

Ultrasound (US) has been proposed as a method of monitoring coagulation due to its ability to assess optically non-transparent samples such as whole blood (rather than plasma), as well as its sensitivity to structural changes that occur in the blood during the coagulation process [4], [5]. Indeed, previous work has shown that clot formation causes changes in the acoustic velocity, attenuation, and backscatter [6]. These studies have all been performed at frequencies lower than 45 MHz, and, although results have shown promise for the use of US as a hemostatic monitoring agent, difficulty in correlating the stages of clot formation with ultrasonic features has led to mis-interpretations of data. For example, increases in the acoustic velocity (on the order of 30 m/s) were initially attributed to blood clot formation [5]. However, later examination demonstrated that the increase in velocity actually corresponded to a phenomenon known as clot retraction, whereby the clot contracts and pulls away from the vessel wall resulting in discrete regions of dense clot (fibrin mesh entrapping blood cells) and of serum (plasma with lower quantities of coagulant proteins due to the formation of a clot) [6].

Building on these studies, we developed a system to probe changes in the acoustic properties of blood during coagulation using higher frequencies (central frequency 80 MHz) than those previously reported. The improved spatial resolution afforded by this higher frequency allowed for the acquisition

of high-resolution M-mode images. The attenuation variations that occurred at these higher frequencies also allowed for novel insights into clot formation by correlating the temporal changes in attenuation to features detected in the M-mode data. Experiments were performed using whole blood from mice with no known bleeding or clotting disorders.

II. Materials and Methods

A. Sample holder and preparation

Disposable polystyrene 96-well microtitre plates were used to mimic earlier optical transmittance studies of coagulation in plasma that utilized spectrophotometric plate readers [7]. The plate was blocked for two hours prior to experiments using a 1% solution of Tween-20 in HEPES-Buffered Saline (HBS; Boston BioProducts), which contains 25 mM HEPES and 150 mM Sodium Chloride. It was thoroughly rinsed using ultrapure water (Milli-Q®, Millipore Ltd.) and allowed to dry prior to use. The samples were prepared by pipetting a 2 μL droplet of 750 mM calcium chloride (CaCl) into the centre of a well (to act as a clot initiator) followed by the addition of 98 μL of citrated mouse blood (blood collection was performed using a 3.2% citrate BD Vacutainer). Following the addition of blood to the well, approximately half of the volume was drawn in and out of the pipette 5 times. Circular mixing ($\sim$4 times) of the sample was also performed using the pipette tip as a stir stick. The final sample volume was 100 μL and had a working CaCl concentration of 15 mM, which was comparable to concentrations previously reported to initiate clot formation in whole blood samples [6]. Control samples, which don't undergo coagulation, the 2 μL of CaCl was replaced by a solution of 0.01% Tween-20 in HBS.

B. System

A single-element focused transducer with a central frequency of 80 MHz was used to generate US pulses. RF data were acquired by averaging fifty RF lines acquired with a 24.24 KHz pulse repetition frequency and a 1 GHz sampling rate. The transducer was connected to a custom stand that allowed for manual adjustment of the transducer's height. The transducer was coupled to the bottom of a well of the microtitre plate using a droplet of water as shown in Fig. 1. Fig. 1 also shows an acrylic well cap. This cap was designed with a 2 mm diameter column which was immersed into the sample and provided a surface from which the acoustic wave could be reflected. A representative RF line obtained with this configuration is shown in Fig. 2.

C. Data acquisition and processing

The microtitre plate was positioned on a mechanical stage, which allowed for the preliminary alignment of the well with the transducer as well as the acquisition of cross-sectional scans. Cross-sectional scans were performed by shifting the plate laterally with respect to the transducer and acquiring RF lines at 50 locations across a 7 mm span (in 140 μm steps) to ensure complete coverage of the $\sim$6.5 mm inner diameter of the well. These scans were repeated every 30 seconds for the duration of the experiment, which typically lasted between 30 minutes and 1 hour. Analysis was performed on the 1.7–2 mm region (12-14 RF lines) which contained a reflection from the well cap.

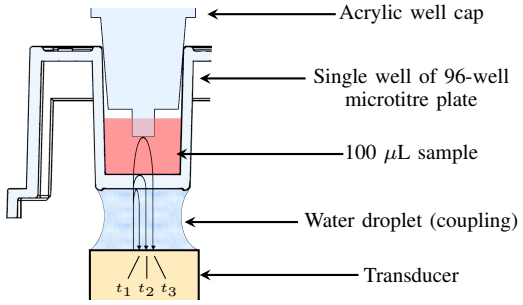

Fig. 1. A labelled schematic of the system setup. The three main acoustic reflections are labelled as t_1, t_2 and t_3.

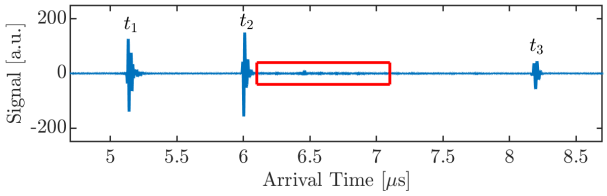

Fig. 2. A representative RF line. The acoustic reflections are labelled as t_1, t_2 and t_3, and correspond to the interfaces indicated in Fig. 1. The red box indicates the region used to construct M-mode images.

1) M-mode imaging: Changes in the backscatter signal were recorded by constructing M-mode images for each of the 12-14 RF lines examined in a sample. The region selected for the M-mode image corresponded to a 1 μs section of the RF line located directly above the bottom surface of the well (the region indicated by the red box in Fig. 2). Spatially, this corresponded to the backscatter signal from the bottom $\sim$1.5 mm of the blood sample. However, data was still reported in terms of the signal arrival time due to potential variations in the acoustic velocity. This region was chosen because of its close proximity to the transducer and the fact that the focus of the transducer fell within it ($\sim$0.5 mm from the bottom of the well); it contained the strongest scattered signals from the blood.

2) Reflected frequency spectra: Acoustic wave propagation through the medium was assessed by examining the frequency spectrum of the signal reflected from the well cap (reflected spectrum). In this way, information was obtained on the cumulative attenuation as the acoustic waves were transmitted through the sample. Variability in the axial location of the cap between experiments prevented the calculation of the attenuation coefficient.

The data are presented as a normalized power spectrum (S), which was calculated as:

$$S = 20\log_{10}\left(\frac{|F|}{|F_{\text{ref}}|}\right) , \quad (1)$$

where F is the raw spectral data and F_{ref} is a normalization factor. Due to the significant attenuation in blood at 80 MHz, a non-conventional approach to the normalization was taken. In order to better distinguish changes occurring across all frequencies, a frequency dependant normalization was implemented. The normalization factor was chosen to be the maximum value that was recorded for each frequency component of one experiment considering the entire time course of the experiment.

III. Results

A. Identification of clot formation

Fig. 3 shows an M-mode image and reflected spectrum for a representative control sample (no coagulation). The M-mode image (Fig. 3(a)) shows the correlated motion of red blood cell (RBC) aggregates (clusters of RBCs which form under low shear rates) through the frame. The fluctuations in the first several minutes are thought to correspond to the scattered signal prior to RBC aggregation, while the downward motion of the scatterers in the later stages of the acquisition are likely a result of sedimentation (where gravity causes settling of the aggregates at the bottom of the well). Fig. 3(c) corresponds to the normalized spectrum of the pulse reflected from the well cap and was obtained from the same location and sample as Fig. 3(a). Minor fluctuations are present and are likely attributable to variation in scatterer location and geometry as aggregates drift in and out of the frame.

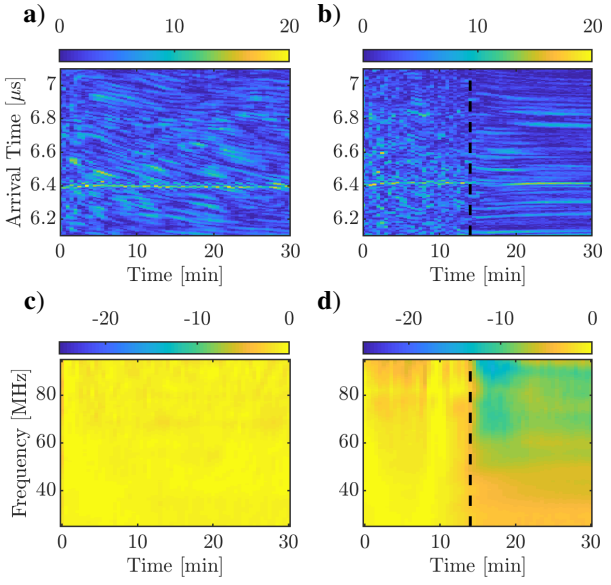

Fig. 3. Results from a control ((a), (c)) and a coagulating sample ((b), (d)). Colour scales for M-mode images ((a), (b)) are linear [a.u.], while the colour scales of the reflection spectra ((d), (e)) represent S [dB].

The M-mode image and reflected spectrum from a representative coagulating sample can be seen in Fig. 3. In the M-mode image (Fig. 3(b)), the initial time points of the experiment are characterized by fluctuations in signal along the slow-time axis (x-axis). These fluctuations are notably less correlated than the fluctuations in the control sample of Fig. 3(a). This is thought to be caused by the early stages of clot formation, in which the process of fibrin polymerization impacts the motion and aggregation of RBCs. At approximately 13 minutes (indicated by a dashed line), the motion of scatterers stabilizes, as can be seen through the high degree of correlation present in the M-mode image. This is thought to correspond to the stabilization of the fibrin mesh, whereby the RBCs become trapped and can no longer move in and out of the US frame. The stabilization of the mesh (indicated by the dashed line) results in a drop in the amplitude of the transmitted signal across all frequencies (Fig. 3(d)). This effect is more apparent in the higher frequency ranges, where changes greater than 10 dB were observed. An additional feature of interest is the increase in signal intensity (particularly visible at higher frequencies), which occurs in the minutes prior to clot stabilization. It is hypothesized that these changes are attributable to the early stages of fibrin polymerization.

B. Spatial variation of clot onset

Spatial variations in clot onset time were visible within individual samples through the stabilization of scatterers in the M-mode images. In the example in Fig. 4, three data sets from a single sample are shown, which were acquired with a lateral separation of 420 μm. Clot onset (indicated by the dashed lines) was found to vary by $\sim$5 minutes across a distance of 840 μm. While it is possible that sample preparation played a minor role in the homogeneity of the sample, protocols were obtained based on standard laboratory coagulation tests and steps were taken to ensure adequate mixing of the CaCl in the blood. Further investigation is required to determine the role of sample preparation in the homogeneity of the clot onset. However, these results indicate that care should be taken when measuring cumulative or integrated effects in coagulating samples, particularly for experiments which involve relatively large blood volumes.

C. Variability of clot microstructure

The M-mode images provided unique insight into variability in the clot's microstructure, which aided in identifying irregularities in the reflected spectra. Three examples are shown in Fig. 5.

In Fig. 5(a,d), clot retraction is shown. The contraction of the fibrin structure caused the clot to pull away from the bottom of the well, resulting in an increasingly large region free of scatterers as time increases (bottom right of Fig. 5(a)). In the reflected spectrum (Fig. 5(d)), the onset of clot retraction caused an increase in the intensity of the signal across all frequencies. This is thought to be a result of decreased attenuation in serum (as is the case in plasma) compared to blood.

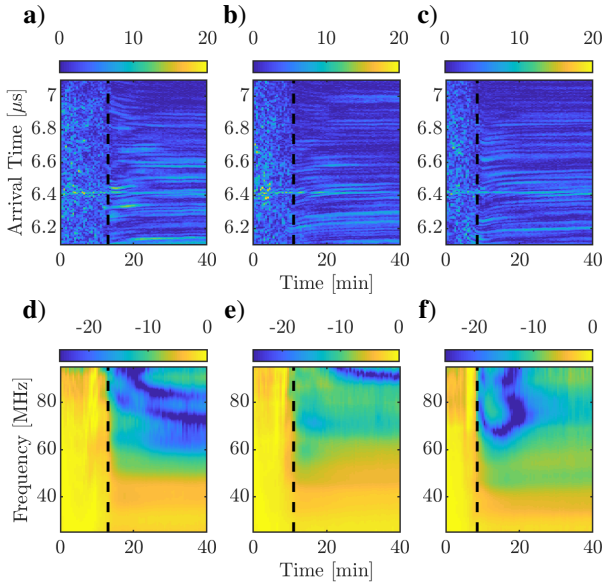

Fig. 4. Results from a coagulating samples at three locations seperated by 420 μm. Colour scales for M-mode images (a,b,c) are linear [a.u.], while the colour scales of the reflection spectrum (d,e,f) represent S [dB].

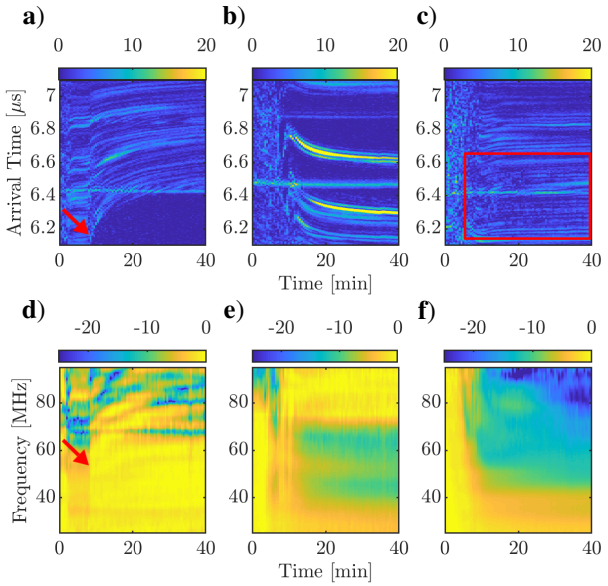

Fig. 5. Results from a coagulating samples which displayed variability in the clot microsctructure. (a) and (d) show clot retraction (red arrows indicates start time of retraction). (b) and (e) show a clot whose formation was not homogeneous. (c) and (f) show what appears to be incomplete/partial clot formation where the red box in (c) indicates a region where fluctuations in the M-mode image of the formed clot. Colour scales for M-mode images ((a), (b), (c)) are linear [a.u.], while the colour scales of the reflection spectra ((d), (e), (f)) represent S [dB].

In Fig. 5(b), the M-mode image demonstrates inhomogeneous clot formation. This effect is thought to be similar to retraction, in that the contraction of the fibrin structure causes the formation of high-density clot regions (indicated by the bright yellow lines showing regions of high scatter) and pockets lacking scatterers. This structural variation impacted the reflected spectrum (Fig. 5(e)) by causing an increase in the reflected spectral intensity at frequencies greater than 75 MHz (as opposed to the decrease observed under more homogeneous clot formation).

Finally, fluctuations in the M-mode images were present in certain samples or sample locations that seemed to indicate either incomplete clot formation or less densely-formed fibrin structures, in which the RBCs still have some freedom of motion within the mesh (Fig. 5(c)). This impacted the reflected spectrum (Fig. 5(f)) through more gradual changes at the onset of clot formation, rather than the abrupt change in intensity typically observed in more stable clots.

IV. Conclusion

This study aimed to demonstrate the advantage of using M-mode imaging to identify structural changes in the sample that occur during the complex process of blood coagulation. The information provided by these images was used to explain irregularities in the reflected spectra, as well as to identify spatial variations in the clot onset time. In the samples that coagulated without irregularities in the microstructure, stabilization of the clot resulted in a large decrease in the reflected spectral intensity, an effect that was particularly prominent at frequencies greater than 50 MHz. The higher frequencies of the spectrum also appeared to show features in the minutes prior to clot formation, which could be caused by the early stages of fibrin polymerization. In future work, these experiments will be extended to human blood.

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