Spectrum analysis for assessing red blood cell aggregation using high-frequency ultrasound array transducer

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Abstract The purpose of this study is to investigate a spectrum analysis technique for detecting and monitoring red blood cell (RBC) aggregation using a high-frequency array transducer. To assess the feasibility of this approach, the backscattered radio-frequency signal from non-aggregated and aggregated RBC samples with two hematocrit levels were acquired by using a 30-MHz linear array transducer and analyzed in frequency domain. Three parameters such as spectral slope, midband fit and *Y* intercept were extracted in a static condition. Fresh porcine blood was used and degrees of aggregation were changed by diluting plasma concentration. From the experiments, it was demonstrated that the spectral slope related to a size of scatterer progressively declined as the level of aggregation increased; its mean values at hematocrit of 40% were 1.10 and -0.22 dB/MHz for RBCs suspended in isotonic phosphate buffered saline and solution with 70% plasma concentrations, respectively. For the midband fit and *Y* intercept, the mean values were increased by 9.1 and 46.4 dB, respectively. These results indicated that the spectrum analysis technique is useful for monitoring RBC aggregation and can be potentially developed for assessing aggregation in clinical applications.

Keywords Spectrum analysis · High-frequency ultrasound · Red blood cell aggregation · Array transducer

1 Introduction

The aggregation of red blood cell (RBC), which is a normal and reversible phenomenon in the circulation of mammalian species, is of interest in the field of pathophysiology [1]. The exact mechanism of RBC aggregation (referred to as rouleaux) is still obscure and is being explored but general agreement exists that enhanced RBC aggregation is correlated with elevated levels of macromolecules, e.g., fibrinogen and plasma protein. Enhancement of RBC aggregation has been observed in many clinical conditions such as circulatory disorder, type 2 diabetes, malaria and sickle cell disease [2–4]. Thus, development of techniques for detection and analysis of RBC aggregation is important for understanding the mechanism and the techniques can potentially be used in clinical diagnosis.

The properties of RBC aggregation have been studied with various methods. A photometric measurement method has been used to monitor the degrees of aggregation by measuring backscattered or transmitted light from RBC suspension [5]. Size and contrast of speckle patterns in X-ray phase contrast imaging was also investigated with varying degrees of RBC aggregation and hematocrit levels [6]. Photoacoustic imaging was used to simultaneously measure aggregation and oxygenation levels [7].

Ultrasound based techniques has been widely utilized to study the properties of blood due to its cost-effectiveness and real-time capability. In addition, it has the potential for assessing RBC aggregation in animal and human vessels noninvasively, which would be challenging for optical based techniques due to its high optical scattering in biological tissues. Previously, an aggregation effect in a whole blood sample was investigated based on ultrasound echogenicity [8]. In this method, the echogenicity profiles were measured by averaging B-mode images. Ultrasound

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backscattering has been extensively explored to assess RBC aggregation [9–11]. In addition, ultrasound propagation velocity and attenuation have been measured in a static condition [12, 13]. Doppler ultrasound was also used to assess blood velocity and backscatter intensity simultaneously [14]. Analysis of statistical distribution of backscattered signal and integrated backscattering were also performed under steady flow [15]. The shape parameters of speckle intensity such as skewness and kurtosis were explored in a whole blood sample [16]. Although these methods showed potential of assessing the degrees of RBC aggregation, the methods mainly rely on the power of backscattered signal that is affected by levels of hematocrit, thus it would be insufficient for characterizing intrinsic properties.

Spectrum analysis techniques of ultrasound backscattered signal have been developed to provide additional information related to tissue pathologic states [17, 18]. The spectrum analysis method can reveal microstructure of tissue constituents such as effective scatter size, acoustic impedance and effective concentration. It has been extensively investigated and has shown promising results in prostate and breast cancer diagnosis, cell apoptosis monitoring, osteoporosis characterization, ophthalmology and single cell analysis [19–25].

In the presented study, a feasibility of spectrum analysis using high-frequency ultrasound for detecting and measuring RBC aggregation was investigated using a porcine blood samples in a static condition. In the experiment, ultrasonic backscattered radio-frequency (RF) data from varying degrees of RBC aggregation with two hematocrit levels (20 and 40%) were acquired using a 30-MHz linear array transducer. Three parameters (i.e., spectral slope, midband fit and Y intercept) that describe physical properties were extracted. The experiment results demonstrated that the spectral slope, midband fit and Y intercept are useful parameters to investigate and monitor the changes in RBC aggregation.

2 Materials and methods

2.1 Spectrum analysis technique

The detail theoretical framework of spectrum analysis can be found elsewhere [17]. The spectrum analysis is first performed by selecting a region of interest (ROI) from a B-mode image. The ROI is typically located at a transmit focal depth to avoid diffraction effect. Digitized backscattered RF echo signals are then acquired at the ROI and a window function (e.g., Hamming window) is multiplied to minimize spectral leakage (i.e., sidelobes) coming from the discontinuity at the endpoints of the data.

Spectrums of acquired RF echo signal are computed using a fast Fourier transform (FFT) algorithm. A calibrated power spectrum is calculated by dividing the RF echo power spectrum by a reference power spectrum obtained from a planar target, e.g., glass or quartz. By doing this, system dependencies (i.e., transfer function of electronics in transmitter/receiver and transducer) can be removed, thus only backscattered RF echo signal from tissue composites is used in the spectrum analysis. After calibrating the power spectrum, a linear fit calculated by linear regression analysis is computed over effective transducer bandwidth, e.g., -6 -dB bandwidth.

Figure 1 shows spectrums of RF echo from tissue (RBC) and quartz plate along with a calibrated spectrum and linear fit, respectively. As shown in Fig. 1a, the frequency response of reference signal at the effective transducer bandwidth (i.e., -6 -dB bandwidth) produce smooth curve with high magnitude, thus the calibrated power spectrum always exhibits stable response. From the linear regression of calibrated power spectrum, three parameters (i.e., spectral slope, midband fit and Y intercept) can be extracted. The spectral slope (dB/MHz) is associated with an

Fig. 1 **a** Spectrums of RF signal from red blood cells and quartz plate and **b** calibrated spectrum along with a corresponding linear fitted line

Fig. 2 Microscopic images of **a** non-aggregated RBCs suspended in PBS and **b** aggregated RBCs in plasma₇₀, respectively. The scale bar denotes 10 μm

effective scatterer size; the slope decreases as scatterer size increases. The midband fit is defined as the value at the midpoint of the linear fitted line within the effective bandwidth, which is related to acoustic impedance and concentration. The Y intercept is the value obtained by extrapolation of linear fit to zero frequency. Statistical

procedures such as cluster analysis and neural networks can be used to identify disease in conjunction with database classifiers that delineates the spectral features of particular disease.

2.2 Blood sample preparation

All experiments were performed with fresh porcine blood (Sierra Medical Inc., Whittier, CA). The blood was centrifuged at $3000 \times g$ for 10 min at room temperature to separate plasma and buffy coat. Isotonic phosphate buffered saline (PBS) was used to wash the RBCs. Collected plasma was diluted with PBS to obtain 30% (plasma₃₀) and 70% (plasma₇₀) plasma solutions. The diluted plasma results in reduced RBC aggregation due to the low concentration of plasma protein, thus varying degrees of aggregation can be achieved [5]. The packed RBCs were then suspended in PBS, plasma₃₀ and plasma₇₀, and thoroughly mixed to achieve homogeneity. The presence of aggregation was assessed using an inverted microscope (Olympus IX-71, Center Valley, PA) and representative images of non-aggregated and aggregated RBCs are shown in Fig. 2. Two hematocrit levels (20 and 40%) with different plasma concentrations (i.e., PBS, plasma₀ and plasma₇₀) were examined and all experiments were performed within 2 h of blood sample preparation at room temperature.

2.3 Experiment setup and data acquisition

The experiment setup is shown in Fig. 3. To assess a feasibility of this approach, a high-frequency linear array that has 50 μm azimuth pitch (i.e., λ in water) and 2 mm elevation length focused at 7.3 mm was utilized [26]. The center frequency was 28 MHz and -6-dB fractional bandwidth was 40%. The transducer was driven by a custom built 64-channel imaging system. A transmit focal depth was set to 6.4 mm and an excitation voltage was 40 V_{pp}. The porcine blood samples were loaded in cylindrical

Fig. 3 Schematic diagram of high-frequency array ultrasound system. A white dotted box in a B-mode image represent a region of interest located at a transmit focal point

Fig. 4 B-mode images of RBCs suspended at hematocrit of 40% in **a** PBS ($\text{RBC}_{\text{H40,P00}}$), **b** plasma₃₀ ($\text{RBC}_{\text{H40,P30}}$) and **c** plasma₇₀ (and $\text{RBC}_{\text{H40,P70}}$), respectively. All images were logarithmically compressed with a dynamic range of 40 dB

polystyrene containers. Pre-beamformed RF data from a ROI (0.5 mm $\times$ 0.5 mm) were acquired and transferred to a personal computer (PC) and off-line processing was

Fig. 5 Representative calibrated spectrums and corresponding linear fit for **a** non-aggregated ($\text{RBC}_{\text{H40,P00}}$), **b** and **c** aggregated RBCs ($\text{RBC}_{\text{H40,P30}}$ and $\text{RBC}_{\text{H40,P70}}$) at 40% hematocrit

performed using the Matlab (The Math works Inc., Natick, Massachusetts). A reference signal for calibration was obtained from a quartz plate at the transmit focal point. The surface of plate was perpendicular to the beam axis. A Hamming window was used and 512-point FFT was conducted in the spectrum analysis. Note that the spectrum analysis is performed at effective transducer bandwidth (i.e., -6 -dB bandwidth, 22.4–33.6 MHz in this experiments) that provide high signal-to-noise ratio (SNR), thus noise compensation is not typically conducted in the spectrum analysis method. A t test was performed to compare the spectral parameters (i.e., spectral slope, mid-band fit and Y intercept) from non-aggregated and aggregated RBCs.

3 Results and discussion

Figure 4 shows B-mode images of RBCs (hematocrit level of 40%) suspended in PBS ($\text{RBC}_{\text{H40,P00}}$), plasma₃₀ ($\text{RBC}_{\text{H40,P30}}$) and plasma₇₀ ($\text{RBC}_{\text{H40,P70}}$), respectively. All images were logarithmically compressed with a dynamic range of 40 dB. From visual assessment, higher echogenicity can be observed in the B-mode image of aggregated RBCs (Fig. 4b, c) than that from non-aggregated RBCs (Fig. 4a). These results were consistent with the previous studies [16].

Representative calibrated spectrums and corresponding linear fit for $\text{RBC}_{\text{H40,P00}}$, $\text{RBC}_{\text{H40,P30}}$ and $\text{RBC}_{\text{H40,P70}}$ are, respectively, plotted in Fig. 5. As can be seen in Fig. 5, the spectral slope decreased as the degrees of aggregation increased. The values of spectral slope for these samples were 1.12, 0.36, -0.45 dB/MHz for $\text{RBC}_{\text{H40,P00}}$, $\text{RBC}_{\text{H40,P30}}$ and $\text{RBC}_{\text{H40,P70}}$, respectively. Since the spectral slope is directly correlated with a size of scatterers (the slope decreases as scatterer size increases), it can be readily recognized that a size of rouleaux is changed; the size of rouleaux increased with higher concentration of plasma protein. Note that the spectral slope is only associated with a size of scatterer and not influenced by acoustic impedance and concentration [18]. In the case of midband fit, the value increased with increasing plasma concentration; those were -40.9 , -33.6 and -28.3 dB for $\text{RBC}_{\text{H40,P00}}$, $\text{RBC}_{\text{H40,P30}}$ and $\text{RBC}_{\text{H40,P70}}$, respectively. The midband fit is related to acoustic impedance that is product of density and acoustic propagation velocity. Since the Y intercept value take both scatterer size (spectral slope) and acoustic impedance (midband fit) into account, differences

Fig. 6 Means and standard deviations of **a** midband fit, **b** spectral slope and **c** Y intercept obtained from non-aggregated and aggregated RBCs with different hematocrit levels (20 and 40%), respectively. The error bars represent the standard deviation of ten ultrasound signals

according to aggregation levels are more distinct; -71.4 , -43.4 and -16.2 dB for $\text{RBC}_{\text{H40,P00}}$, $\text{RBC}_{\text{H40,P30}}$ and $\text{RBC}_{\text{H40,P70}}$, respectively. The advantage of Y intercept is that it does not affected by attenuation so that it has the potential for clinical applications [18].

Figure 6 shows the values of mean and standard deviation of spectral slope, midband fit and Y intercept obtained from the RBCs samples of two hematocrit levels (20 and 40%) with different degrees of aggregation. The mean and standard deviation were computed with ten ultrasound backscattered data or spectra. As can be seen, the midband fit gradually increased and the value changes according to the levels of aggregation were statistically significant ($p < 0.01$) for all hematocrit levels. There was also significant difference between two hematocrit levels (20 and 40%) with the same degree of aggregation ($p < 0.01$). Note that smaller midband fit value was obtained with higher hematocrit level. This results is the same as previous study, in which back scattering coefficients of suspended RBCs in saline was peaked at between 15–20% hematocrit level [27]. For the spectral slope, the value decreased with significantly significant manner ($p < 0.01$) as the plasma concentration increased. On the other hand, the changes of spectral slope for different hematocrit levels of the same degree of aggregation were not significant ($p \geq 0.05$) except the RBCs with PBS only ($p < 0.01$). This may stem from the low back scattering coefficient, i.e., low SNR in the RBCs with 40% hematocrit ($\text{RBC}_{\text{H40,P00}}$). In this experiment, the SNR for $\text{RBC}_{\text{H40,P00}}$ was about 5 dB. This can be mitigated by using advanced imaging technique such as coded excitation method, which remains as future work. Similar results were obtained for the Y intercept for all hematocrit level ($p < 0.01$). The values between $\text{RBC}_{\text{H20,P70}}$ and $\text{RBC}_{\text{H40,P70}}$ did not show significant difference ($p = 0.18$).

From the experiments, it was learned that all three parameters are useful to determine the degrees of RBC aggregation. Compared to the previous studies that only rely on power of back scattered signal, the spectrum analysis provides better degrees of freedom in statistical analysis of RBC aggregation. For example, for the midband fit of $\text{RBC}_{\text{H20,P00}}$ and $\text{RBC}_{\text{H40,P70}}$, the mean values were -28.04 and -29.09 dB, respectively. However, its mean values of spectral slope showed clear difference; those were 0.81 and -0.22 dB/MHz for $\text{RBC}_{\text{H20,P00}}$ and $\text{RBC}_{\text{H40,P70}}$, respectively. This can be readily seen in a

Fig. 7 Scatter-plot of spectral slope and midband fit for non-aggregated and aggregated RBCs with different hematocrit levels (20 and 40%). Six clusters according to the degrees of aggregation and hematocrit are clearly distinguished

scatter plot (Fig. 7). As seen in Fig. 7, distinct clusters according to the levels of aggregation and hematocrit can be observed. This property would allow for establishing database in varying levels of aggregation and hematocrit, suggesting that the spectrum analysis has potential for detecting changes of RBC aggregation in clinical applications.

4 Conclusion

In the study, we have demonstrated the potential of spectrum analysis for detecting and monitoring changes of RBC aggregation. The advantages of spectrum analysis is that it is not affected by the system transfer function and three different parameters (i.e., spectral slope, midband fit and Y intercept) can be simultaneously obtained. The spectral slope is directly related to the scatterer size and the midband fit is correlated with the acoustic impedance ($= \rho c$, where ρ is the density and c is the acoustic propagation velocity). From the experiment results with non-aggregated and aggregated RBCs, it was shown that the method is useful for discriminating RBCs with varying levels of aggregation and hematocrit. The results suggest that it would be possible to obtain quantitative measurement of RBC aggregation, and has the potential as a noninvasive tool for detecting and monitoring RBC aggregation in superficial vessels.

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