

Identification of red blood cell rouleaux formation using photoacoustic ultrasound spectroscopy

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ABSTRACT

Red blood cell (RBC) rouleaux formation is a reversible phenomenon that occurs during low blood flow and small shearing forces in circulation. Certain pathological conditions can alter the molecular constituents of blood and properties of the RBCs leading to enhanced rouleaux formation, which results in impaired perfusion and tissue oxygenation. In this study rouleaux were artificially generated using Dextran-70 and examined using a photoacoustic (PA) microscope. Individual rouleau were irradiated with a 532 nm pulsed laser focused to a 10 μm spot size, and the resulting PA signals recorded with a 200 MHz transducer. The laser and transducer were co-aligned, with the sample positioned between them. The frequency-domain PA ultrasound spectra were calculated for rouleaux with lengths ranging from 10 to 20 μm . For the rouleaux, a single spectral minimum at 269 ± 4 MHz was observed. The spectral minima were in good agreement with a theoretical thermoelastic expansion model using an infinite length cylindrical absorber, bearing a diameter equivalent to an average human RBC (7.8 μm). These results suggest that PA ultrasound spectroscopy can be potentially used as a tool for monitoring blood samples for the presence of rouleaux.

Keywords: Photoacoustics, red blood cell aggregation, spectral analysis

1. INTRODUCTION

1.1 Red blood cell aggregation

Red blood cells (RBCs) can adhere together forming stacks of coin like structures called rouleaux. RBC aggregation, a phenomenon normally present in normal physiology, is an influential factor in determining hemorheological properties. During low shear rates typically present in the post capillary venules, RBCs aggregate and increase blood viscosity. However the forces causing aggregation are weak and the aggregates disperse under high shearing conditions in blood flow. This change in blood viscosity under high shearing rate is known as the shear thinning effect of blood¹.

RBC aggregation is mainly known to occur in athletic species and is almost nonexistent in sedentary species¹. For athletic species aggregation can be beneficial in maintaining homeostasis since the change in blood viscosity under low and high shearing rates can regulate resistance to flow in microcirculation. During periods of high oxygen demand the blood flow to certain organs can increase by a significant amount. Hence the increased flow velocity (and increased shearing rate) disaggregate the RBCs and reduce blood viscosity in postcapillary venules. Lower blood viscosity, decreases the resistance to flow and increases the volume of blood flowing away from organs. So aggregation, in conjunction with regulation of vessel diameter, acts as an auto regulatory mechanism of maintaining blood pressure at the micro circulatory level².

The formation of aggregates depends on both the RBC membrane surface properties (charge, elasticity) and suspending medium properties¹. The widely accepted model for RBC aggregation is the depletion model³⁻⁵. The presence of macromolecules like fibrinogen and $\alpha 2$ macroglobulin can enhance red blood cell aggregation in autologous plasma. Rouleaux formation can also be induced by suspending RBCs in physiological solutions of macromolecules like dextran (DEX). The strength of the aggregation depends both on the molecular weight of the dextran and its concentration in solution¹ (figure 1).

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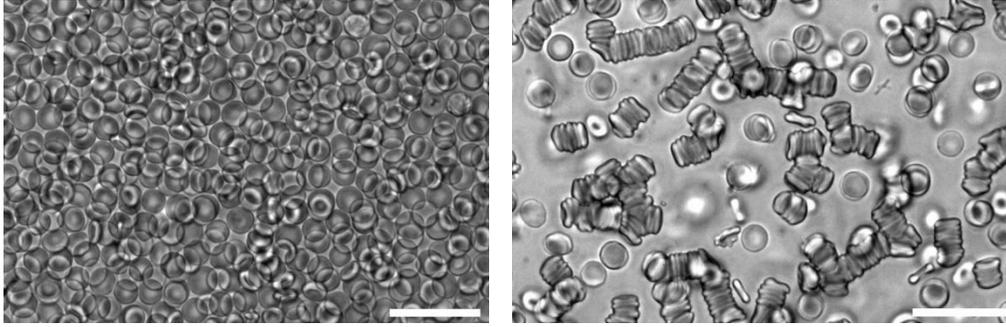


Figure 1: Optical images of (Left) non aggregated human RBCs in autologous plasma, (right) aggregated RBCs with 3% dextran-70 kDa PBS added to whole blood (scale bar 20 μm).

An increased level of aggregation has detrimental effects on micro perfusion⁶ and oxygenation of tissue^{7, 8}. Abnormal increase in aggregation levels can arise from any surgical procedure requiring extracorporeal circulation of blood or from any pathophysiological conditions that induce inflammation, affect circulatory or metabolic functions. Since the elevated level of RBC aggregation is an indicator of a disease or adverse effects of a clinical procedure⁹, the development of real time, non-invasive techniques for aggregation detection is an ongoing field of investigation.

1.3 Detection of RBC aggregation

Several methods are used for RBC aggregation detection; most of them are invasive or technologically complex. Examples of some invasive methods include erythrocyte sedimentation rate (ESR), intravital microscopy, low shear viscometry and light transmission in RBC suspensions¹⁰. As a non-invasive technique ultrasound backscattered signal analysis has been developed for detection of RBC aggregation^{9, 11}. However the backscattered ultrasound signal strength has a non-linear relationship with the blood hematocrit level¹², which presents difficulty in monitoring aggregation in the presence of various hematocrit levels.

Hemoglobin, present in RBCs, is an endogenous contrast agent for photoacoustic (PA) imaging which makes blood an excellent candidate for PA. While PA has been used extensively to image blood vessels and estimate their oxygenation states, there are only a limited number of studies on its use to examine RBC aggregation. Simulations of PA signal¹³ as a function of frequencies ranging from 0-1000 MHz and experimental results¹⁴ at 5 MHz from our group have demonstrated the potential of using PA to investigate RBC aggregation. Detection at ultra-high frequencies (UHF, over 100 MHz)¹⁴ has also been used in our group to show that the size and shape of the probed RBC produces unique spectral features. RBC rouleaux formation represents an increase in effective absorber size and change in shape, thus this study was aimed at investigating the effect of RBC aggregation on the PA signal and its spectral features at UHFs.

1.4 Thermoelastic expansion model photoacoustic signal from an infinite cylinder

If an infinitely long optically thin cylinder with sound speed c_c and density ρ_c surrounded by a transparent fluid with sound speed c_f and density ρ_f is irradiated with an optical light with intensity I_0 modulated by a frequency ω , the heat deposited in the cylinder per unit volume and time can be written as

$$H = \mu_a I_0 e^{-i\omega t} \quad (1)$$

where μ_a is the optical absorption coefficient of the cylinder and t is the time. Ignoring the effects of viscosity, energy relaxation and assuming no heat conductivity, the wave equation for the PA pressure generated can be represented by

$$\left(\nabla^2 - \frac{1}{c^2} \frac{\partial^2}{\partial t^2} \right) p = -\frac{\beta}{C_p} \frac{\partial H}{\partial t} \quad (2)$$

where β is the thermal expansion coefficient, C_p is the heat capacity per mass, and c the sound speed of the absorbing structure. For a heating function described by equation (1) the solution to the partial derivative for PA pressure (equation 2) can be found using the boundary conditions of linear acoustics; the pressure and acceleration across the cylinder-fluid boundary must be continuous. The PA pressure in the fluid can be written as

$$p_f(\hat{q}) = \frac{i\mu_a \beta I_0 c_c a}{C_p} \left[\frac{J_1(\hat{q}) H_0^{(1)}(\hat{c}\hat{q}\hat{r})/\hat{q}}{J_1(\hat{q}) H_0^{(1)}(\hat{c}\hat{q}) - \hat{\rho}\hat{c} J_0(\hat{q}) H_1^{(1)}(\hat{c}\hat{q})} \right] e^{-i\hat{q}\hat{t}} \quad (3)$$

where a is the radius of the cylinder, $\hat{\rho} = \rho_c/\rho_f$ is the dimensionless density ratio, $\hat{c} = c_c/c_f$ is the dimensionless speed of sound ratio, \hat{r} is the radial coordinate, $J_0(x)$ is the zeroth order Bessel function, $H_0^{(1)}(x)$ is the zeroth order Henkel function of the first kind, $\hat{q} = \omega a/c_c$ is the dimensionless frequency and $\hat{t} = c_c t/a$ is the dimensionless time. The far field solution for equation (3) at large radial distances as a function of frequencies produces the analytical PA signal (thermoelastic expansion model)¹⁵ for an infinite cylinder.

2. METHOD

2.1 Sample preparation

RBCs were extracted from a healthy volunteer in accordance with the guidelines of the Ryerson Ethics Board (REB #2013-016). A lancet was used to obtain a small drop of blood from the volunteer's finger and deposited in 1 mL Dulbecco's modified essential medium (DMEM) containing 10% fetal bovine serum (FBS) (Sigma Aldrich, St. Louis, MO) and 3% DEX 70 kDa (Sigma Aldrich, St. Louis, MO). The blood was allowed to aggregate in the dextran solution for 1 minute. A transfer pipette (Fisher Scientific, Waltham, MA) was used to deposit a thin coat of the aggregated blood in a 35 mm glass-bottom dish (Mattek Corporation, Ashland, MA) prefilled with 4 mL of DMEM+FBS+DEX solution and coated with a 200 μm layer of 1% agar (to prevent ultrasound backscatter from the glass surface). Physical disturbance to the aggregates was minimized during the transfer process. The sample was prepared this way to ensure a sparse distribution of the aggregates (figure 2) so that the laser spot would only illuminate one rouleau at a time.

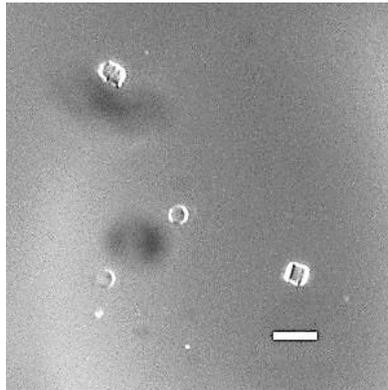


Figure 2: Optical images showing typical sizes of rouleaux and non-aggregated single cells selected for PA signal generation (scale bar 20 μm).

2.2 Photoacoustic measurements

An acoustic microscope (Kibero GmbH, Germany) was used for PA measurements. An IX81 inverted optical microscope (Olympus, Japan) was modified to include a transducer positioned above the sample holder. The laser and transducer were co-aligned, with the sample positioned between them. Aggregates containing five to ten RBCs in the rouleaux (10–20 μm length) were chosen to conduct measurements on. Each aggregate was illuminated using a 532 nm laser (Teem Photonics, France) collimated through the side port of the microscope, then focused onto the sample using a

10x optical objective. The focused laser spot size was 10 μm in diameter. Laser energy was maintained at 40 nJ. The transducer was used in receive mode to record the PA signals generated from the aggregates. Details of the experimental system can be obtained elsewhere¹⁶. All measurements were made with a transducer that had a center frequency of 200 MHz (60° aperture, 42% bandwidth) and 8 μm lateral focal spot size. The laser had a pulse width of 330 ps and pulse repetition frequency (PRF) of 4 kHz. All signals were averaged 40 times, amplified by a 40 dB amplifier (Miteq, USA) and were sampled at 8 GHz. A Hamming window was applied to the signals and then the power spectrum was calculated using the Fast Fourier transform. The power spectra from the samples were then normalized by removing the transducer response. The transducer response was obtained from the PA spectrum of a 200 nm gold film¹⁷.

3. RESULTS AND DISCUSSION

3.1 Spectral features of non-aggregated red blood cells

Figure 3 shows the PA power spectra for 10 horizontally positioned RBCs measured using the 200 MHz transducer. The PA signals from ten single horizontal RBCs are spectrally flat within the transducer bandwidth (100 to 300 MHz). This is in agreement with the results from previous studies¹⁷ that used a 375 MHz transducer to measure signal from horizontal RBCs. Another study using photoacoustic frequencies at 1.2 GHz¹⁶ showed the first spectral minima for single horizontal RBCs occur at 800 MHz. Since these minima are far above the 200 MHz transducer bandwidth (100-300 MHz) a spectrally flat signal in this frequency range is reasonable.

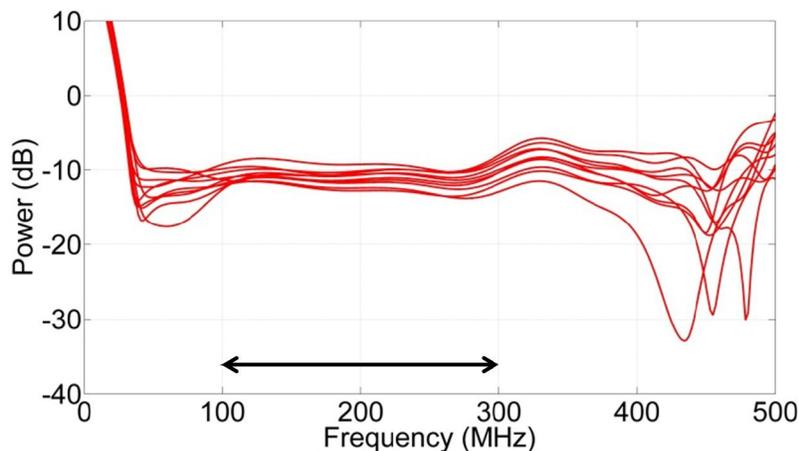


Figure 3: Power spectrum from 10 horizontal single RBCs (red), double headed arrow indicates the transducer bandwidth (100-300 MHz).

3.2 Spectral features of aggregated red blood cells

PA power spectra from 10 rouleaux are shown in figure 4 (red lines). The mean spectral minimum was observed at 269 ± 4 MHz. The black curve represents the analytical solution for the thermoelastic expansion model for PA signal from an infinite cylinder with diameter of 7.8 μm using equation (3). The theoretical curve shows a spectral minimum at 260 MHz. The theoretical and the experimental spectral minima differ by 9 MHz. This deviation may be due to the fact that during aggregation the diameters of the RBCs change due to the forces acting on them or from not applying certain corrections to the data (for example the frequency dependence of ultrasound attenuation has not been accounted for). Also the RBCs that align to form a rouleau are slightly offset from each other⁶, which is a slight deviation from a perfect cylinder.

The lengths of the rouleaux illuminated varied between 10 to 20 μm however this variation did not affect the location of the minima. This is consistent with the fact that the RBCs vertical dimension and the speed of sound are the main variables that control the locations of the minima in the PA signal for the infinite cylinder model. Irrespective of its

length (10-20 μm), each rouleau was illuminated by the exact same 10 μm laser spot size. Since the size of the rouleaux illuminated was always greater than the illumination spot size (10 μm) and the transducer focal spot size (8 μm) the assumption that the rouleaux can be modeled as infinite cylinders is likely valid. The close match between the analytical model and experimental rouleaux data confirms this assumption.

Future work includes examining rouleaux as a function of length, laser spot size and transducer center frequency. This includes obtaining spectrum from rouleaux smaller in length than the laser spot size, and comparing them to the analytical PA signal from a finite length cylinder¹⁸.

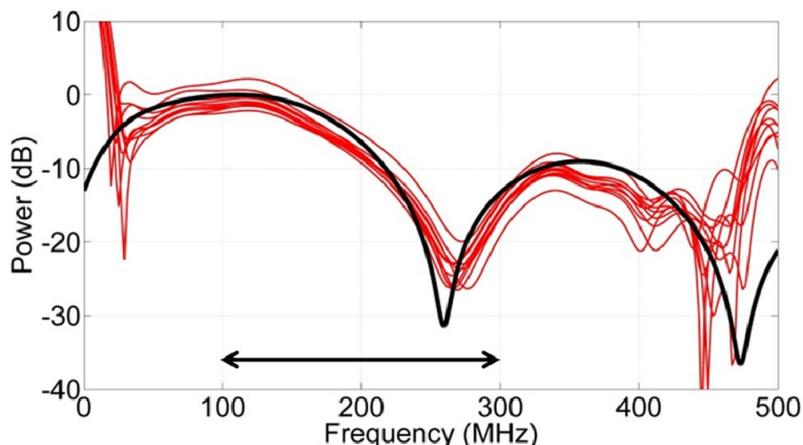


Figure 4: Power spectrum of ten different RBC rouleaux (red) and an analytical cylindrical absorber model with diameter of 7.8 μm and infinite length (black), double headed arrow indicates the transducer bandwidth (100-300 MHz).

4. CONCLUSION

This experiment shows that photoacoustic measurements using UHF produce different spectral features for aggregated and non-aggregated RBCs. This method can potentially be applied to differentiate between single RBCs and RBC rouleaux to reliably detect RBC aggregation. Understanding signal at a single cell level will help us interpret results from non-invasive lower frequency clinical studies.

5. ACKNOWLEDGEMENTS

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