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Photoacoustic response of suspended and hemolyzed red blood cells

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The effect of confinement of hemoglobin molecules on photoacoustic (PA) signal is studied experimentally. The PA amplitudes for samples with suspended red blood cells (SRBCs) and hemolyzed red blood cells (HRBCs) were found to be comparable at each hematocrit for 532 nm illumination. The difference between the corresponding amplitudes increased with increasing hematocrit for 1064 nm irradiation. For example, the PA amplitude for the SRBCs was about 260% higher than that of the HRBCs at 40% hematocrit. This observation may help to develop a PA method detecting hemolysis noninvasively. © *2013 AIP Publishing LLC*. [http://dx.doi.org/10.1063/1.4816245]

The photoacoustic (PA) technique has evolved as a potential biomedical imaging modality over the last few years.[1–3] It is a nonionizing and hybrid imaging modality. It provides a spatial map of absorbed photons detected through ultrasonic means. A short-pulsed laser is used to irradiate a sample. The illuminated region of the sample gets heated due to absorption of light and undergoes thermoelastic expansion. The rapid expansion induces wideband ultrasonic waves. The PA technique can provide an image with high contrast because only endogenous chromophores contribute to the generation of PA signals and non-absorbing tissue constituents present no background.[1] Moreover, it is possible to image depths beyond one optical transport mean free path ($\sim$1 mm).[2] This is because scattering of pressure waves is two to three orders of magnitude less than that of light allowing to detect pressure waves from deep tissue and thus image formation.

Tissue functional state can also be assessed by sensing and analyzing the PA signals.[1–3] For example, blood oxygen saturation can be determined by measuring the PA signals at two optical wavelengths followed by a spectroscopic analysis.[4,5] In this context, a theoretical model has been presented to study the generation of PA signal from blood.[5] It essentially considers blood as a homogeneous medium containing hemoglobin (Hb) molecules as the dominant chromophores and thus ignores the fact that the Hb molecules are enclosed within red blood cells (RBCs). Another theoretical model has recently been developed by exploiting the cellular aspect of blood.[6] The PA field from a single RBC (approximated as a fluid sphere) suspended in a fluid medium has been calculated by employing a frequency domain approach.[7] The PA field from blood has been obtained by summing the fields emitted by the individual RBCs as they are the dominant light absorbing particles in blood.[6] This model assumes that the light absorption takes place in the molecular level but acoustic emission takes place in the cellular level. Further, in this picture, Hb molecules are bounded within the RBCs and their concentration (inside a cell) and oxygen saturation states define the amplitude of the PA field emitted by a cell. This theoretical model provides a framework to study how spatial organization of RBCs[6] and oxygenation of individual erythrocytes affect PA signals.[8] Recent experimental results demonstrate good qualitative agreement with the theoretical results.[9]

Intuitively, the continuum picture would be suitable to describe the PA fields generated by samples comprising of hemolyzed RBCs (HRBCs) because for such samples Hb molecules are unconfined and distributed homogeneously within the medium. On the other hand, the particle picture might provide a good framework to study the PAs for samples containing suspended RBCs (SRBCs) for which Hb molecules are confined within the cells. A fundamental question is how these two types of samples would differ from the PA point of view. In other words, what would be the effect of confinement of hemoglobin molecules on PA signal. In this letter, an attempt has been made to address this issue experimentally. Therefore, the PA experiments were conducted with the SRBCs and the HRBCs over a wide range of hematocrits (i.e., volume fractions occupied by the RBCs) examining the PA response of each system.

Fresh porcine whole blood was collected from a local slaughterhouse and mixed with ethylene diamine tetra acetic acid (EDTA) at 3 g/l to prevent coagulation. It was centrifuged for 30 min at 3000 rpm at $\approx$4 °C and subsequently, the plasma and the buffy coat were removed gently. The concentrated RBCs were then washed twice with the isotonic phosphate buffered saline (PBS).[10] A centrifugation at 3000 rpm for 15 min was applied at each round of washing. Five different blood samples were prepared by suspending the packed RBCs in PBS to achieve hematocrits of about 10%, 20%, 30%, 40%, and 50%. Another set of blood samples was prepared by mixing the concentrated RBCs and hypotonic solution[11] so as to attain nearly the same hematocrit levels. This batch was kept for 1 h allowing erythrocytes to imbibe water due to osmotic pressure difference and then centrifuged at 14 000 rpm for 1 h to prepare samples with the HRBCs.

Blood sample was loaded in a transparent glass cuvette (Optiglass, UK; 45- $\times$ 12.5- $\times$ 32.5-mm) which was positioned centrally within a water tank. The cuvette was cleaned with distilled water and dried before loading of each sample during the experiment. Further, prior to loading, the sample was gently but thoroughly shaken to ensure homogeneity. A Q-switched pulsed Nd:YAG laser (Brilliant,

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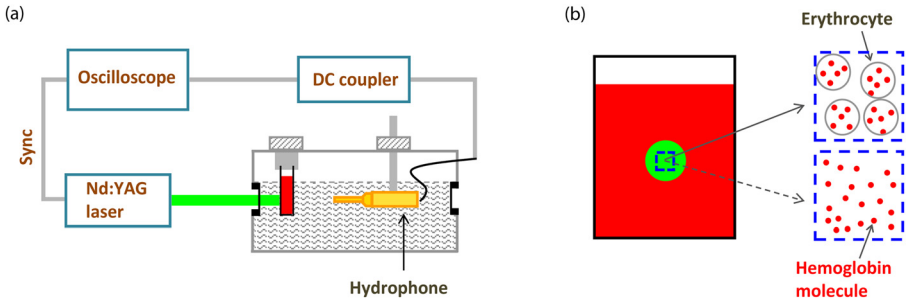

FIG. 1. (a) An embodiment of the experimental arrangement used for the PA measurements. (b) An illustration of the illuminated region containing either the SRBCs or the HRBCs.

Quantel, France; 20 Hz pulse repetition frequency, 5 ns pulse width, 6 mm beam diameter) was used to irradiate the samples. The PA measurements were conducted at 532 and 1064 nm illuminations. The laser fluences were set nearly at 18 and 353 mJ/pulse/cm^2 at those wavelengths, respectively. A needle hydrophone (Precision Acoustics, UK; 1 mm diameter) was placed in the forward direction to detect acoustic signals. The signals were displayed in a digital oscilloscope (TDS5034B, Tektronix Inc., USA) and 100 such signals for each sample were recorded via MATLAB 2009b. All the measurements were performed at the room temperature and within 12 h from blood collection. The experimental setup is shown in Fig. 1(a). Fig. 1(b) illustrates the illuminated region for a sample with the SRBCs or the HRBCs.

The recorded radio frequency (RF) lines were filtered using a linear phase FIR lowpass filter (based on the Parks-McClellan algorithm[12] with cutoff frequencies at 50 and 60 MHz and an attenuation of 100 dB) to increase the signal to noise ratio. The envelope of the signal was obtained using the filtered signal and its Hilbert transform.[12] The peak signal amplitude corresponding to the illuminated sample surface was noted. Its mean and standard deviation were calculated from 100 captured lines for each sample. The variation of the peak amplitude as a function of hematocrit was studied for both the blood samples.

A typical PA waveform from a blood sample with the SRBCs at 40% hematocrit for 532 nm incident optical radiation is shown in Fig. 2(a). The signal within the time window (denoted by two vertical lines) has been generated by the irradiated surface of the sample. A very strong PA pulse has been produced by this surface. The pulses arising due to multiple reflections of the first pulse by the glass surfaces of the cuvette wall appear outside the gated region successively with decreasing amplitudes. The filtered signal corresponding to the gated region is further shown in the inset. Fig. 2(b) displays a RF line detected for the same input laser from a blood sample composed of the HRBCs at the same hematocrit level. The RF line looks similar to that of Fig. 2(a).

A representative trace of a PA signal is illustrated in Fig. 3(a) when 1064 nm laser source is used to excite a blood sample containing the SRBCs at 40% hematocrit. The signal appears like an inverted Mexican hat with multiple echoes superimposed. The gated region that corresponds to the initial part of the signal arising from the sample surface does not contain echoes. The filtered signal of this region is further elaborated in the inset. A PA signal from a sample consisting of the HRBCs at 40% hematocrit is plotted in Fig. 3(b) for the same incident laser. The signal amplitude is reduced in this case and the inverted Mexican hat appears slightly asymmetric. Note that it has been discussed theoretically in the literature that asymmetric inverted Mexican hat waveform can be generated from a PA point source due to thermal diffusion.[13]

Fig. 4(a) plots the variation of peak PA amplitude as a function of hematocrit for each type of sample when probed with 532 nm beam. It exhibits that the PA amplitude almost linearly increases with increasing hematocrit for both the cases. Additionally, the amplitude for the SRBCs is comparable to that of the HRBCs at each hematocrit. The variation of peak PA amplitude with hematocrit at 1064 nm illumination is presented in Fig. 4(b). The amplitude for the SRBCs is always higher than that of the HRBCs. Further, the

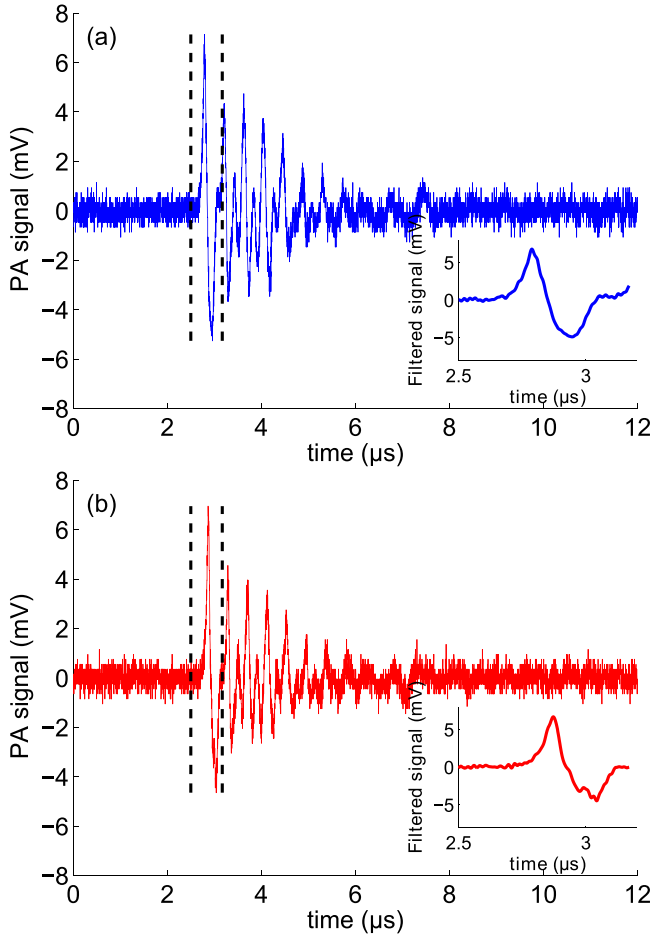

FIG. 2. (a) A representative waveform generated by the SRBCs at 40% hematocrit for 532 nm incident optical radiation. The filtered signal corresponding to the gated region (denoted by two dashed vertical lines) is presented in the inset. (b) Same as (a) but for the HRBCs at the same level of hematocrit.

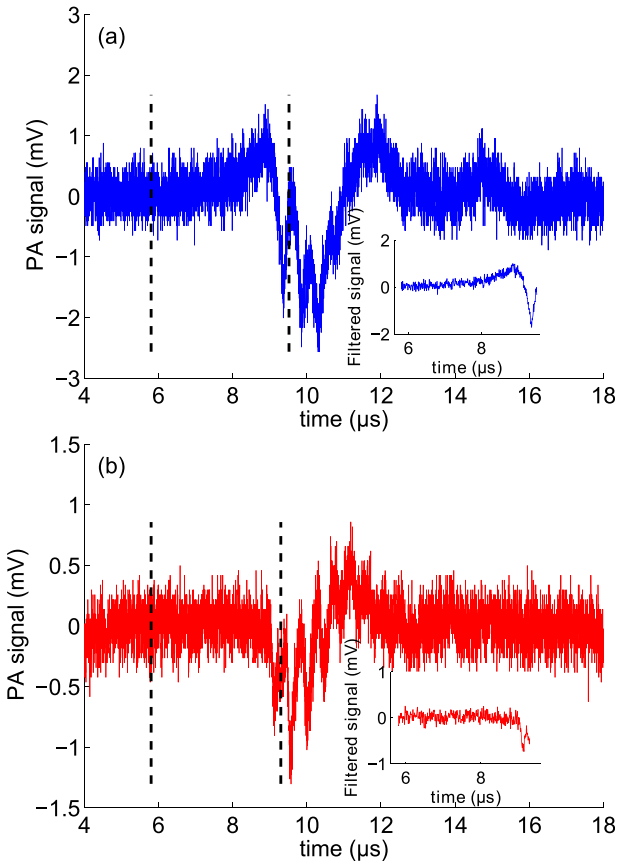

FIG. 3. (a) An illustrative trace of a PA signal emitted by the SRBCs at 40% hematocrit when excited by 1064 nm laser beam. The inset elaborates the filtered signal corresponding to the gated region (indicated by two dashed vertical lines). (b) Same as (a) but for the HRBCs at the same level of hematocrit.

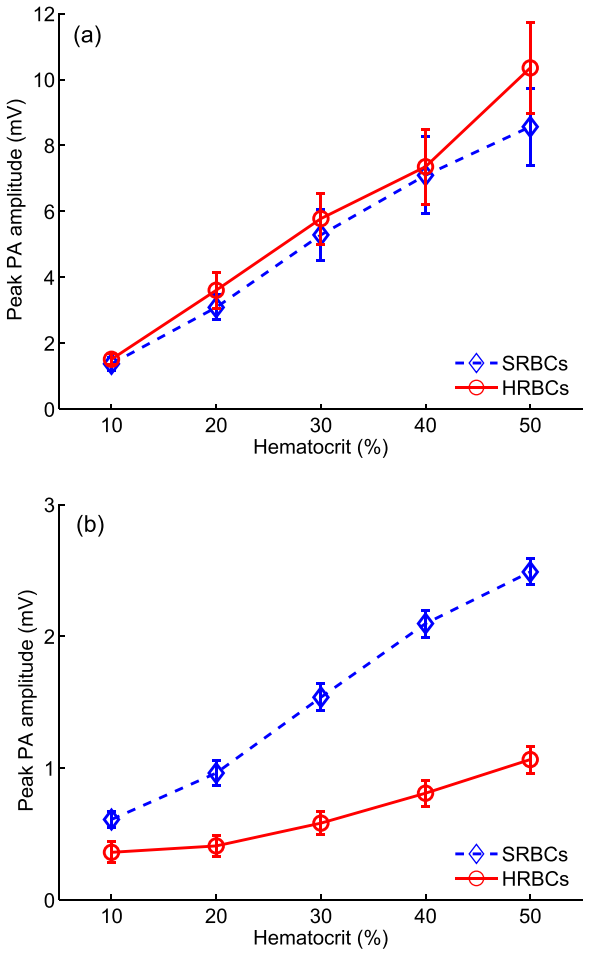

FIG. 4. (a) The variation of the peak PA amplitude (mean ± SD) with hematocrit for 532 nm input laser. (b) Same as (a) but for 1064 nm irradiating beam.

difference between the amplitudes increases as the hematocrit increases.

It seems to be an interesting observation that at 532 nm blood samples comprising the SRBCs and the HRBCs generate PA signals of nearly equal amplitudes. However, at 1064 nm completely separable lines [see Fig. 4(b)] are obtained and thus both types of samples become distinguishable from the PA point of view. In order to get some insights about the interaction of light with confined and freely suspending Hb molecules as well as to interpret the PA results, spectrophotometric measurements were made with diluted samples (Lambda 750 UV/VIS, Perkin Elmer, USA).

Fig. 5(a) displays that absorbance for the SRBCs is greater than that of the HRBCs over a wide range of wavelengths at low concentration of cells. Fig. 5(b) illustrates that for 5% concentration of cells light transmission loss is very high up to approximately 600 nm by both the samples. In this range, transmitted light intensity becomes less than the detection threshold for each sample. After 600 nm, sample with the HRBCs allows more light to transmit as compared to a similar sample with the SRBCs.

The spectrophotometric data demonstrate that reduction of light energy is higher for the SRBCs in comparison to that of the HRBCs. This is quite obvious since in addition to light absorption by the Hb molecules, cells in suspension scatter light resulting in loss of photons. Note that scattering coefficient for the SRBCs is wavelength dependent and is maximum at 500 nm.[14] At 1064 nm, it is approximately half of that of 532 nm.[14] Moreover, it is known that photons after entering cells, in general, encounter multiple reflections from the cellular boundaries leading to increased optical paths.[14] The increased path length enhances effective absorption of light by the cells.

Therefore, at one hand, internal reflections of photons enhance effective optical absorption by the SRBCs. On the

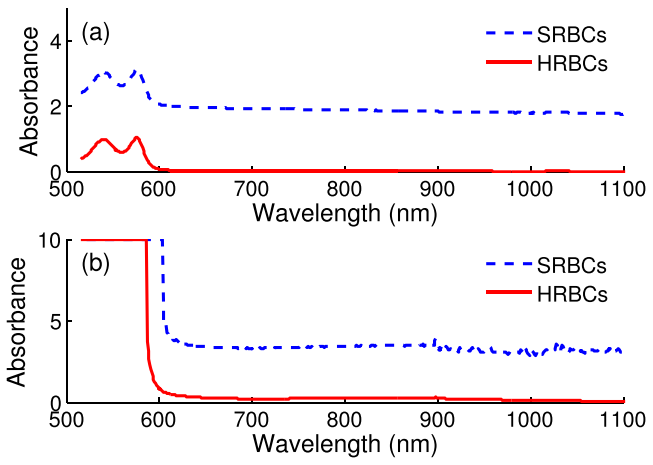

FIG. 5. The absorption spectrum for the SRBCs and the HRBCs at hematocrits of about 0.5% in (a) and 5% in (b).

other hand, cells receive less numbers of photons owing to scattering loss. It could be speculated that these two factors might have become comparable at 532 nm and canceled each other out. Hence, overall absorption becomes similar for the SRBCs and the HRBCs leading to PA emissions of equivalent strengths at this wavelength. However, the effect of internal reflections of photons is likely to dominate because scattering of light by RBCs is less at 1064 nm. It enables the SRBCs to produce stronger PA signals than the HRBCs. Although possible arguments are provided explaining the PA results in the context of the present study, other independent investigations are required to clarify the issue further.

The observation reported in this work may find an important clinical application. The intended application is the noninvasive detection of hemolysis. Hemolysis is the rupture of the erythrocyte membrane resulting in release of the Hb and other constituents into the blood serum.[15] It is associated with various medical conditions. For instance, an elevated level of hemolysis promotes circulatory disorders (i.e., disturbance in transportation of oxygen, nutrients, and metabolic wastes) which may lead to multiple organ failure. It can occur under both *in vivo* and *in vitro* conditions. *In vivo* hemolysis can take place due to bacterial and parasitic infections as well as due to some autoimmune and genetic disorders. *In vitro* hemolysis can be caused by improper technique during blood collection, processing, and transportation.[15]

Hemolysis can be detected visually. Although it is not an accurate method, it serves as a quick and easy method. The spectrophotometry based methods remain to be the best approaches to detect and quantify Hb in blood plasma.[15] The experimental results presented in this study demonstrate that the PA amplitude for the SRBCs is about 2.6 times greater than that of the HRBCs at 40% hematocrit for 1064 nm irradiation. It suggests that it may be feasible to detect hemolysis in practice using PAs. Nevertheless, further investigations are required to optimize the technique in terms of input radiation wavelength, laser fluence, acoustic receiver aperture, and bandwidth to develop it as a diagnostic tool.

In conclusion, experimental results show that the samples with confined and unconfined Hb molecules generate the PA signals of similar strengths at 532 nm illumination. However, for 1064 nm irradiation, the samples with confined Hb molecules produce much stronger PA signals as compared to the samples containing unconfined Hb molecules particularly at physiological hematocrits. Therefore, this study reveals that confinement of the Hb molecules plays a dominant role in the PA emission from blood for 1064 nm optical excitation.

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