Correlation between microbubble-induced acoustic cavitation and hemolysis in vitro*

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Microbubbles promise to enhance the efficiency of ultrasound-mediated drug delivery and gene therapy by taking advantage of artificial cavitation nuclei. The purpose of this study is to examine the ultrasound-induced hemolysis in the application of drug delivery in the presence of microbubbles. To achieve this goal, human red blood cells mixed with microbubbles were exposed to 1-MHz pulsed ultrasound. The hemolysis level was measured by a flow cytometry, and the cavitation dose was detected by a passive cavitation detecting system. The results demonstrate that larger cavitation dose would be generated with the increase of acoustic pressure, which might give rise to the enhancement of hemolysis. Besides the experimental observations, the acoustic pressure dependence of the radial oscillation of microbubble was theoretically estimated. The comparison between the experimental and calculation results indicates that the hemolysis should be highly correlated to the acoustic cavitation.

Keywords: acoustic cavitation, passive cavitation detection, microbubbles, hemolysis

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1. Introduction

Ultrasound contrast agents (UCAs) are usually composed of suspensions of gas-filled microbubbles with diameters in micrometer encapsulated by a thin stabilization coating (e.g., lipid, albumin, or polymer).[1] UCA was first introduced clinically to improve echocardiographic image contrast since microbubbles in fluids could create an acoustic impedance mismatch between the host fluids (e.g., blood) and the surrounding tissues. Besides increasing reflection of ultrasound, the presence of UCA is also believed to help increasing the absorption of sonic energy and then enhance ultrasound-induced bioeffects. In the past decades, the potential therapeutic applications of UCAs have also been investigated, both in vitro and *in vivo*.[2−5] Ultrasound-mediated gene/drug delivery, one of the most promising ultrasound therapeutic applications, is claimed to be significantly improved by UCA-assisted cavitation activities.[6−8] As reported, ultrasound cavitation assisted by UCAs could enhance the cell membrane permeability, which could make cell membrane temporarily "open" to facilitate the flux of foreign gene/drugs into cells (sonoporation).[9,10] It has been hypothesized that ultrasound-induced inertial cavitation (IC) activities should play an important role at sonoporation process[10,11] and the added UCA microbubbles could facilitate the acoustic energy absorption and lower the cavitation threshold by acting as cavitation nuclei.[12] However, hemolysis may also occur due to irreversible membrane damage. As we know, extensive hemolysis could significantly increase morbidity and mortality. Knowledge of these physical mechanisms is therefore of vital importance and would provide a framework wherein drug and gene techniques can avoid clinically extensive hemolysis.

This study aims to examine the hemolysis induced by the moderate-intensity ultrasound in the presence of UCAs for the application of drug delivery. To achieve this purpose, the human red blood cells mixed

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with a kind of home-made encapsulated microbubbles are exposed to 1-MHz pulsed ultrasound with different acoustic pressure amplitudes. The hemolysis is evaluated with flow cytometry, and the occurrence of cavitation during ultrasound exposure is examined by using a passive cavitation detection (PCD) system. Besides the experimental studies, the mechanism of hemolysis is discussed relative to the radial oscillation of the encapsulated microbubbles.

2. Materials and methods

2.1. Microbubble and cell preparations

The UCA used here is a kind of self-developed encapsulated microbubbles with a mean radius of $\sim$1.0 μm, whose concentration is about 3×10^8 microbubbles/ml. Anticoagulated human blood was obtained from a Blood Transfusion Center and stored at 4 °C. Red blood cells were collected by centrifugation at 1000 rpm for 10 min, washed by saline (0.9%) three times and the leukocyte layer carefully removed. For each ultrasound exposure, samples were added into two plastic culture tubes with 10 mm in diameter and 50 mm in length, which were kept on ice just before use in the measurement. Microbubble solution (about 1×10^6 microbubbles/ml) was then added into one of the test tubes with a microbubbles/cells ratio of 20:1. These two samples were respectively ultrasound-exposed (sham), and ultrasound-exposed in the presence of UCAs.

2.2. Ultrasound exposure system and flow-cytometer

All ultrasound exposure experiments were performed in an acrylic tank filled with degassed water. As shown in Fig. 1, an arbitrary waveform generator (Agilent 33250A, CA, USA) was used to supply 1-MHz sinusoidal pulses (12 cycles, period repetition frequency (PRF) = 250 Hz). The output signals from the waveform generator were amplified through an RF power amplifier (ENI A150, Rochester, NY, USA) with a fixed gain of 50 dB, which were used to drive a 1-MHz home-made focused source transducer with a radius of 9.2-cm, and 6.6-cm focal distance. A plastic test tube of 10 mm in diameter and 50 mm in length was filled with sample suspension. The tube was capped by a rubber stopper that was used as a sound absorber, and then sealed with paraffinum to minimize undesired bacteria infection. Before the ultrasound exposure, the test tube was aligned axially with the source transducer and positioned 6.8 cm away from the surface of the transmitter, so that the centre of the suspension was situated at the focal area. The *in situ* acoustic pressure of the source transducer was calibrated using a needle hydrophone (TNU001A, NTR Systems Inc. Seattle, WA, USA) with a 30-dB preamplifier (HPA30, NTR Systems Inc. Seattle, WA, USA). The attenuation of the test tube wall was determined by measuring the ultrasound amplitude with/without placing the test tube *in situ*.

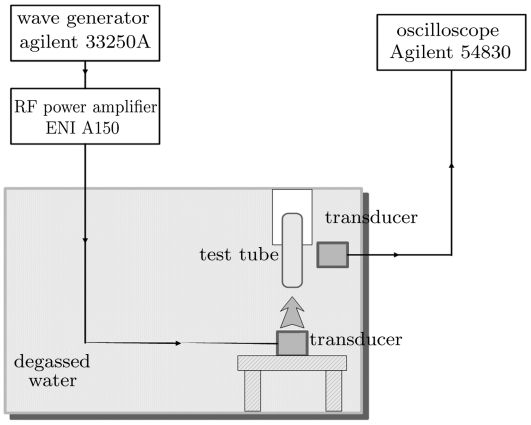


Fig. 1. Illustration of the ultrasound exposure and cavitation detection system.

A 5-MHz, 11-mm diameter home-made single-element transducer used for passive cavitation detection (PCD) measurements was located perpendicularly to the 1-MHz focused transducer so that its focus was orthogonal to the source transducer. The bubble scattering and emission signals in the focal volume were collected by the 5-MHz 'listening' transducer and digitized by an oscilloscope (Agilent 54810, Santa Clara CA, USA). The recorded PCD waveforms were stored in a personal computer (PC) for subsequent signal processing using MATLAB program (the MathWorks, Inc., Natick, WA). A LabView program (National Instruments, Austin, TX, USA) was used to control the waveform generator and the oscilloscope.

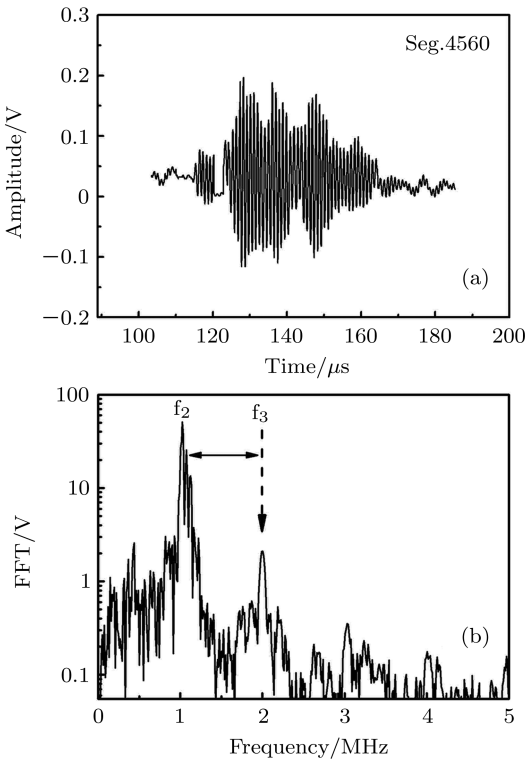
2.3. Assessment of hemolysis and cavitation dose

Immediately after ultrasound exposure, the samples were centrifuged at 1000 rpm for 10 min, and 20-μL supernatant was drawn into an optical tube. The fraction of hemolysis cells was calculated from the optical absorption in the supernatant by using

a flow-cytometer (BD FACSCalibur, BD Biosciences, Franklin Lakes, NJ, USA). Hemolysis with 100% was calibrated by the cells lysed with pure water (osmotic lyses).

Broadband noise emissions produced by IC activities can be detected using a PCD system. Compared to other approaches that only quantify the intensities of IC activities,[13] the ICD estimation provides a "dose" measurement to assess the "amount" of IC energy over a certain ultrasound exposure duration for different treatment conditions.[14]

The scattered acoustic signals from the exposed suspension were collected with the 5-MHz "listening" PCD transducer and sampled by the oscilloscope with a sampling frequency of 25 MHz. Each waveform of all sampled time series signals was first transformed to the frequency domain using fast Fourier transfor (FFT) (Fig. 2(a)). A specific frequency window between the second and third harmonic frequency components was chosen to evaluate the amount of IC-induced broadband-noise by calculating the root mean square (RMS) amplitude of the FFT spectrum within the window (Fig. 2(b)). Then these FFT RMS amplitudes were plotted for all sampled waveforms essentially as a function of exposure "on" time (the exposure "on" time = 20 μs/pulse $\times$ PRF $\times$ treatment time) (Fig. 2(c)). The cumulated ICD was defined as the area under this curve over the entire exposure "on" time period.



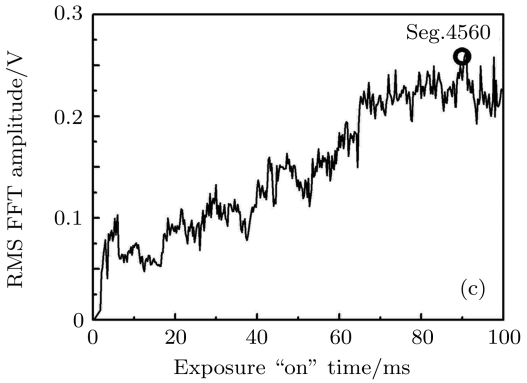


Fig. 2. Illustration of numerical calculation of IC dose.

3. Results and discussion

Figure 3 plots the amount of hemolysis as a function of incident acoustic pressure during 10-s ultrasound exposure, where the solid and open symbols are respectively measured with and without the addition of UCAs, and the error bars represent the standard deviation of each group of six identical samples. Without the addition of UCAs, cavitation production and hemolysis effects are insignificant (less than 5%) for the acoustic pressure amplitude up to 1 MPa. Lack of cavitation is likely due to the absence of cavitation nuclei in blood samples. After the addition of the microbubbles, the amount of hemolysis increases a little when the acoustic pressure is less than 0.13 MPa. As the acoustic pressure exceeds 0.38 MPa, the level of hemolysis increases significantly in the ultrasound exposure, compared with the case of no microbubbles. For example, microbubbles induce about 23% hemolysis over 3% without microbubbles at the acoustic pressure 0.58 MPa, i.e. the amount of hemolysis is enhanced by about 8 times. There is a sharp increase for hemolysis when the acoustic pressure exceeds 0.38 MPa. It suggests that the threshold pressure necessary for cavitation has been exceeded.

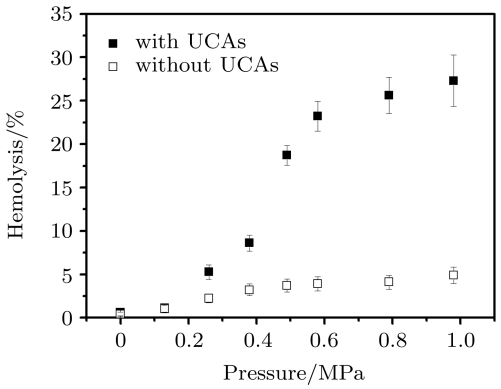


Fig. 3. Percentage hemolysis versus acoustic pressure.

In the absence of microbubbles, almost no cavitation event can be detected. After the addition of UCAs, figure 4 shows results for the cavitation dose versus acoustic pressure. Obvious cavitation events are detected as the acoustic pressure is larger than 0.2 MPa. After the acoustic pressure exceeds 0.4 MPa, the cavitation dose shows a significant increase. This result agrees well with hemolysis level in Fig. 3, which confirms that the level of hemolysis is strongly correlated with the amount of cavitation during ultrasound exposure.

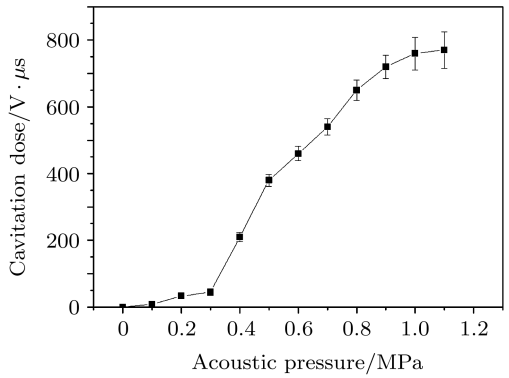


Fig. 4. Correlation of the cavitation dose with acoustic pressure.

To discuss the effect of microbubble-induced cavitation on hemolysis, we also made a numerical calculation for radial oscillation of the encapsulated microbubble surrounded by blood-like fluid. The Marmottant model[15] was employed to describe the large amplitude response of a single coated microbubble in terms of a varying surface tension. The time varying radial amplitude of the shelled microbubble is given by:

$$\begin{aligned}&\rho_{\rm L}\left(\ddot{R}R+\frac{3}{2}(\dot{R})^2\right)\\&=\left(p_0+\frac{2\sigma(R_0)}{R_0}\right)\left(\frac{R_0}{R}\right)^{3\kappa}\left(1-\frac{3\kappa}{c}\dot{R}\right)-\frac{2\sigma(R)}{R}\\&\quad-4\mu_{\rm L}\frac{\dot{R}}{R}-\frac{4\kappa_{\rm s}\dot{R}}{R^2}-p_0-p_{\rm i}(t),\end{aligned}\tag{1}$$

where R is the instantaneous radius of the bubble; $\dot{R}$ and $\ddot{R}$ are the first and second derivatives of the instantaneous radii with respect to time, respectively; R_0 is the equilibrium radius. And $\rho_{\rm L}$ and $\mu_{\rm L}$ are the density and shear viscosity of the surrounding liquid, respectively; κ is the polytropic gas exponent, and $\kappa_{\rm s}$ is the surface dilatational viscosity; p_0 is the hydrostatic pressure; $p_{\rm i}(t)$ is the time-varying incident acoustic pressure. The size-dependent surface tension $\sigma(R)$ is given by:

$$\sigma(R)=\begin{cases}0 & R\le R_{\rm buckling};\\ \chi\left(\dfrac{R^2}{R_{\rm buckling}^2}-1\right) & R_{\rm buckling}\le R\le R_{\rm breakup};\\ \sigma_{\rm water} & R\ge R_{\rm breakup}.\end{cases}\tag{2}$$

This model has three parameters to describe the surface tension: the buckling radius of the bubble $R_{\rm buckling}$ below which the surface buckles; an elasic modulus χ that gives the slope of the elastic regime; the third parameter $R_{\rm rupture}$ is incorporated to describe the moment of rupture. In the numerical calculation, values of the parameters for the encapsulated microbubble were as follows:[16] $R_0=1$ μm, $S_{\rm p}=2\chi=2$ N/m, $S_{\rm f}=12\pi\kappa_{\rm s}=0.27\times10^{-6}$ N, $d_{\rm s}=10$ nm, $\rho_{\rm L}=1000$ kg/m^3, $\mu_{\rm L}=0.001$ Pa$\cdot$S, $p_0=1.01\times10^5$ Pa, $\kappa=1.095$, $R_{\rm buckling}=R_0$, $R_{\rm rupture}=R_{\rm buckling}(1+\sigma_{\rm water}/\chi)^{1/2}$. The external acoustic driving pressure used is a 1-MHz tone burst signal with 12 cycles.

Figure 5 plots the percentage change in radius versus incident acoustic pressure. As the incident acoustic pressure increases, the percentage change in radius increases. When the acoustic pressure reached 0.25 MPa, the microbubble increases to 1.05 times its original size. Further increasing the acoustic pressure, the microbubble shows much greater radial oscillation. At the acoustic pressure of 0.48 MPa, the ratio of maximum radial oscillation reached a value of 3. As previously demonstrated experimentally, the fragmentation for a phospholipid-based microbubble when the relative expansion reached three times the initial radius.[17] Yeh and Su[18] found 95% definite microbubbles destruction at an mechanical index (MI) of about 0.5.[18] Therefore, it is suggested from Fig. 5 that as the acoustic pressure exceeds 0.2 MPa (MI

= 0.2), the stable cavitation may play an important role in the hemolysis; when the acoustic pressure is up to 0.4 Mpa (MI = 0.4), the transient cavitation leads to the significant increase of hemolysis. The general experimental results shown in Figs. 3 and 4 are in agreement with this hypothesis. Though only single microbubble dynamics was considered, it is reasonable because UCAs with low concentration have been used in this study. In addition, the linear summation approach could be used to estimate the results for UCAs with a size distribution.

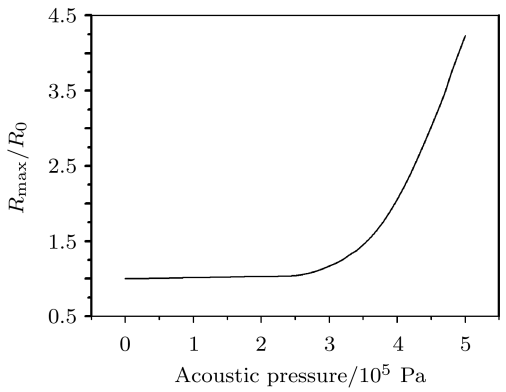


Fig. 5. Relative change in radius versus acoustic pressure.

4. Conclusion

In conclusion, the acoustic pressure dependences of hemolysis and cavitation dose have been experimentally investigated for human blood cells exposed to 1-MHz ultrasound pulses in the presence of microbubbles. The theoretical evaluation of radial oscillation of microbubble induced by ultrasound has also been carried out. Both the hemolysis level and the cavitation dose significantly increase with increasing acoustical pressure. In particular, the acoustic pressure being around 0.48 MPa driven by 1-MHz ultrasound may be enough to cause transient cavitation to neighbouring cells; the amount of hemolysis is improved by about 8 times at this applied acoustic pressure compared with ultrasonic exposure without microbubbles, but still less than 30%. This hemolysis level is insignificant in drug delivery. The comparison between the experimental and calculation results indicates that cavitation should be considered as the most important factor that affects the hemolysis process. Thus, current studies would help us to gain a better understanding of the mechanisms of ultrasound-induced hemolysis in the presence of UCAs. The long-range goal of this effort is to optimize the ultrasound-mediated gene/drug delivery.

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