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Quantifying the Ultrasonic Properties of Cells During Apoptosis using Time Resolved Acoustic Microscopy

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***Abstract*—Time resolved acoustic microscopy was used to determine the thickness, sound velocity, acoustic impedance, density, bulk modulus and attenuation of cells before and after apoptosis. Measurements of the cellular properties at 375 MHz before and after the onset of structural changes associated with apoptosis showed an increase in the thickness from 13.6 ± 3.1 μm to 17.3 ± 1.6 μm, and the attenuation from 1.08 ± 0.21 dB/cm/MHz to 1.74 ± 0.36 dB/cm/MHz. The other parameters were similar within experimental error. Acoustic images at 1.2 GHz indicated a clear delineation between the nucleus and cytoplasm for apoptotic cells, a feature not observed at lower frequencies. Rapid temporal variations were observed within apoptotic cells as indicated by changes in the spatial distribution of regions of increased backscatter within the cell. These results indicate structural changes that alter the cellular ultrasound backscatter are occurring during apoptosis on time scales on less than a minute.**

***Index Terms*—Acoustic microscopy, apoptosis, mechanical cell properties, attenuation**

I. Introduction

Apoptosis describes a controlled method of cell death, a sequence of events designed to safely dispose of toxic intracellular contents [1]. It is thought to be an integral part in the response of tumors to chemotherapeutic treatment [2]. Morphological features that are observed during apoptosis include membrane blebbing and rounding to a spherical shape due to the breakdown of structural proteins, a hallmark feature of apoptosis.

The study of apoptosis is critical to understanding cancer growth and formulating treatments for this disease. How cellular properties change during apoptosis, such as the thickness, density, speed of sound, acoustic impedance, bulk modulus, and attenuation is not well understood [3] yet may provide important information for the potential use of ultrasound for treatment monitoring [4].

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Various techniques have been used to measure the viscoelastic properties of cells, including embedded particle tracking, laser tracking microrheology, magnetic twisting cytometry, micropipette aspiration, microneedles, optical and magnetic tweezers, and atomic force microscopy [5]. These techniques are invasive, requiring staining and/or a cellular response to an external stimulus and cannot be used to measure cells during rapid processes. Ultrasound has distinct advantages over other measurement methods; it is non-invasive and can be localized to specific regions of interest within the cell.

Previous studies using high frequency ultrasound (20-60 MHz) observed an increase in the ultrasound backscatter intensity of acute myeloid leukemia (AML) cells when treated with a chemotherapeutic agent to induce apoptosis [6-8]. It was hypothesized the increase was due to structural changes within the cell, however at the frequencies used, individual cells could not be resolved.

Acoustic microscopy (100+ MHz) has resolutions approaching 1 μm at 1 GHz, which allows for resolving of cellular features such as the nucleus and some organelles [9]. The scattered ultrasound from cellular microstructures would provide valuable information as to the origin of the increased backscatter, and help understand previous studies at high frequencies.

This research aims to measure the properties of cells during apoptosis. These results will be useful in cellular modeling, molecular imaging and other ultrasound scattering simulations currently in progress [10].

II. Theory

A. Acoustic Microscopy

Acoustic microscopy uses highly focused ultrasound pulses to resolve echoes from a cell. A typical acoustic setup showing the echoes from the cell are in fig. 1. The quantitative methods require arrival time of the echoes that are received from the top of the cell, t_1, the substrate-cell interface, t_2, and a reference measurement from the substrate, t_0 (usually measured beside the cell). In addition, the maximum amplitude A from each interface must be obtained using a $V(z)$ measurement, where the transducer is focused at a series of different axial locations z to maximize the backscatter signal

from interfaces of interest [9].

A discussion of the theory behind time resolved acoustic measurements and a verification of the methodology was presented in [11, 12]. The thickness d, sound velocity c, acoustic impedance Z, density ρ, bulk modulus K and attenuation α can be calculated using

$$d = \frac{c_0}{2}(t_0 - t_1), \quad (1)$$

$$c = c_0 \frac{t_0 - t_1}{t_2 - t_1}, \quad (2)$$

$$Z = Z_0 \frac{A_0 + A_1}{A_0 - A_1}, \quad (3)$$

$$\rho = \frac{Z}{c}, \quad (4)$$

$$K = cZ = \rho c^2, \quad (5)$$

$$\alpha = \alpha_c + \frac{1}{2d} \ln\left[\frac{A_0}{A_2} \frac{Z_s - Z}{Z_s + Z} \frac{4ZZ_0}{(Z + Z_0)^2} \frac{Z_s + Z_0}{Z_s - Z_0}\right], \quad (6)$$

where c_0 is the sound velocity in the coupling fluid, A_0, A_1 and A_2 are the maximum amplitudes from the substrate, cell surface and cell-substrate interface measured from $V(z)$ curves, and Z_s and Z_o are the acoustic impedances of the substrate and coupling fluid, respectively. The attenuation coefficient α_0 can be calculated using

$$\alpha = \alpha_0 f^n, \quad (7)$$

where a linear frequency dependence was assumed with n=1, a reasonable estimate for tissue [13].

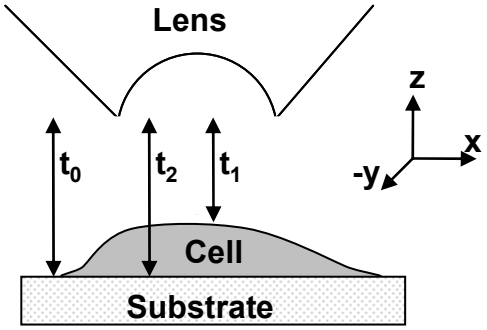

Fig. 1. Experimental setup. The acoustic lens is positioned a specific distance z above the cell. The times t_0, t_1 and t_2 are the time of the ultrasound echoes from the substrate, surface of the cell and cell-substrate interface, respectively.

III. Materials and Methods

A. Cells

MCF-7 breast cancer cells (ATCC, VA, USA) were grown in cell culture flasks using Dulbecco's modified eagle's medium cell culture medium (ATCC) with 10% fetal bovine serum (FBS) and 0.1% insulin. Cells were incubated at 37ºC with 5% CO_2 and were passed every 3 days to maintain exponential growth. 72 hours prior to experimentation, cells were dissociated using trypsin and transferred to Lab-Tek II chambers (Nunc, Germany). After 48 hours, the medium was replaced with a solution consisting of the DMEM cell culture medium (without FBS or insulin), 3 mg/mL caffeine and 20 ng/mL paclitaxel to induce apoptosis [14], then the cells were incubated overnight (approximately 15 hours) prior to experimentation at 37ºC with 5% CO_2. The entire microscope was enclosed in a climate controlled box to maintain a constant temperature of 36ºC with 5% CO_2 during experimentation.

B. Acoustic Microscope

The SASAM 1000 acoustic microscope (Kibero GmbH, Saarbrücken, Germany) consists of an Olympus IX81 inverted optical microscope with an acoustic module positioned above the sample, enabling simultaneous optical and acoustic observation.

Movement of the transducer within the acoustic module is controlled via a piezoelectric (Piezosystem Jena GmbH, Jena, Germany). The piezo controller is capable of movement in the *x, y, z* direction with a resolution of 0.1 µm. The transducer is scanned over the surface of the sample with step sizes from 0.1 to 2 µm. The RF signal recorded at each position is amplified by a 40 dB amplifier and digitized at a rate of 8 GHz. The electronics contain two monocycle pulse generators, one at 300 MHz and the other at 1 GHz each with a 100% bandwidth with a 10 Vpp amplitude. The pulse repetition rate was 500 KHz.

Measurements were made with two transducers. A 375 MHz transducer with a semi-aperture angle of 30º and a -6 dB bandwidth of 42% was used for the quantitative analysis, while a 1.2 GHz transducer with a semi-aperture angle of 50˚ and a -6 dB bandwidth of 27% was used for high resolution imaging.

To reduce noise, 400 a-lines were averaged at 375 MHz and 1000 a-lines averaged at 1.2 GHz. A bandstop filter was used to remove FM-radio frequency noise. Post processing was used to remove a constant background reference signal due to electronic and thermal noise to increase the signal to noise ratio (SNR).

C. Measurements

Optical time lapse images were made of an area containing approximately 200 cells. Cells undergoing apoptosis (responsive cells) were identified by observing morphological features of apoptosis such as membrane blebbing. Cells that had not yet showed any signs of apoptosis (unresponsive cells) were also identified.

Seven unresponsive cells and five responsive cells were measured using the 375 MHz transducer. A preliminary c-scan image was made with the focus approximately 8 µm above the substrate, then V(z) scans were recorded over the center of the cell with a step size of 1 µm in the z-direction. The properties of the cells were then calculated using equations 1-7.

IV. Results

Acoustic attenuation and backscatter c-scan images were compared to optical images for responsive and unresponsive

cells (fig. 2). The morphological differences between cells in both states is apparent.

Using equations 1 to 7, the thickness, sound velocity, acoustic impedance, density, bulk modulus and attenuation were calculated for cells in each state. The average and standard deviation for cells in both states are shown in table I.

TABLE I
MCF7 CALCULATION RESULTS

Property	Untreated	Treated
d (μm)	13.6 ± 3.1	17.3 ± 1.6
c (m/s)	1582 ± 19	1574 ± 19
Z (MRayls)	1.56 ± 0.01	1.55 ± 0.02
ρ (kg/m^3)	987 ± 14	984 ± 20
K (GPa)	2.47 ± 0.03	2.44 ± 0.03
α (dB/cm/MHz)	1.08 ± 0.21	1.74 ± 0.36

The properties (thickness, sound velocity, acoustic impedance, density, bulk modulus, attenuation coefficient) of treated and untreated MCF-7 cells.

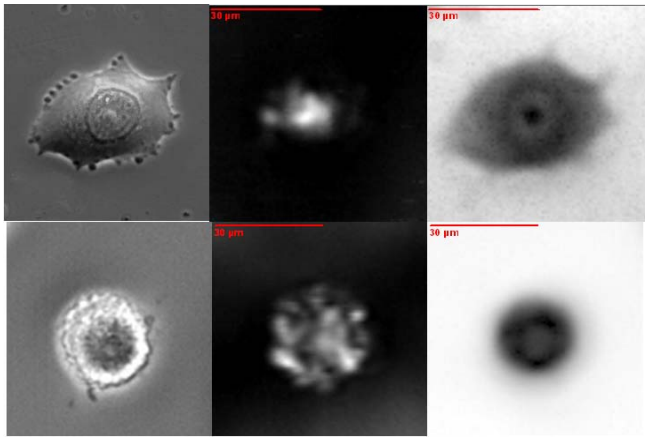

Fig. 2. A comparison of the optical and acoustic images of a treated cell showing the first signs of membrane blebbing, an early sign of apoptosis (top) and a responsive cell undergoing the full structural changes associated with apoptotis (bottom). From left to right: Optical phase contrast image, acoustic backscatter image, acoustic attenuation image. The images shown in this figure are from two different cells, however the scales are similar allowing direct comparison of the acoustical properties.

The thickness of responsive cells was 17.3 ± 1.6 μm, compared to 13.6 ± 3.1 μm for unresponsive cells. The attenuation for responsive cells was 1.08 ± 0.21 dB/cm/MHz compared to 1.74 ± 0.36 dB/cm/MHz for unresponsive cells. The other mechanical properties, including the sound velocity (1582 ± 19 to 1574 ± 19 m/s), acoustic impedance (1.56 ± 0.01 to 1.55 ± 0.02 MRayls), density (987 ± 14 kg/m^3 to 984 ± 20 kg/m^3) and bulk modulus (2.47 ± 0.03 to 2.44 ±0.03 GPa), were similar within measurement error.

Acoustic attenuation images of the same MCF-7 cell were made at 1.2 GHz and 375 MHz (fig. 3). A quantitative analysis was not possible using the backscatter from the 1.2 GHz transducer as the backscatter could not be reliably detected due to the poor SNR.

Apoptosis is an active process where morphological variations are clearly visible in real time. Optical and acoustic attenuation and backscatters images from an apoptotic cell were measured one minute apart with the focus at the substrate to determine how the acoustic signal varies with time (fig. 4). The intensity scales are the same for the each type of ultrasound images for direct comparison.

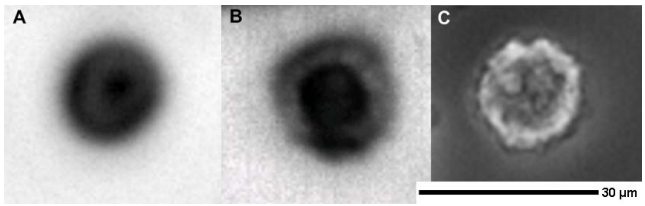

Fig. 3. A comparison of an MCF-7 cell. Acoustic attenuation images at 400 MHz (A), 1.2 GHz (B) and an optical phase contrast image (C).

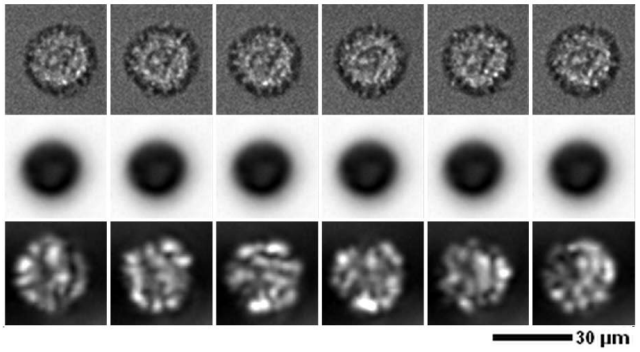

Fig. 4. Optical and acoustic images of a MCF-7 cell measured 1 minute apart. Optical images (top row), acoustic attenuation images (middle row), acoustic backscatter images (bottom row). The attenuation images remained similar, however, the optical and acoustic backscatter images show variations over time.

V. DISCUSSION

Treated cells were measured using a 375 MHz transducer before and after morphological changes of apoptosis were observed (fig. 2). The attenuation images clearly show some cellular features such as the cell and nuclear membrane when compared to the optical images. Significant backscatter was observed around the center of the cell before and after apoptosis.

The backscatter intensity from each scattering region within the cell before and after apoptosis were similar (transducer voltage readings between 5 and 25 mV). However the total integrated intensity from the entire cell was larger after the onset of apoptosis. This is due to the increase in the number of localized regions of scattering from within the cell as shown in fig. 2 and fig. 4.

Apoptotic cells were also imaged using the 1.2 GHz transducer and compared to optical imaging (fig. 3). The 1.2 GHz transducer showed detail not resolved at lower frequencies. The nuclear and cytoplasm regions are clearly delineated, and the attenuation in the nucleus appears to be higher than the cytoplasm region. Additionally, protrusions from the nucleus into the cytoplasm, and occasionally outside the cell not visible at lower frequencies were observed.

Extensive activity within the cell was observed optically and acoustically during apoptosis. Six measurements were made on cells one minute apart to show temporal variations within the cell (fig. 4). The acoustic attenuation images were relatively unchanged throughout the time period, indicating the attenuation is not changing during the time period of the

extensive intracellular structural variations. The backscatter intensity variations and changes indicate extensive activity occurring within the cell during apoptosis. No such variations were observed for non-responding cells. This correlates to optical images taken during the acoustic measurements, where membrane blebbing and minor morphological changes were visible. The rapid variations in cell backscatter and but not attenuation is expected as in general ultrasound backscatter is related to structural features of the cell (which as know to undergo rapid changes during apoptosis) but attenuation is related to cell bulk composition which does not undergo such rapid variations (even though the attenuation does increase when comparison a cell before and after the apoptotic response).

Optical images of the cell show that morphological variations occur in apoptotic cells over a time period of seconds. Acoustic measurements were made one minute apart, which is not fast enough to observe how the backscatter is changing with the same frame rate as the optical microscopy. Larger step sizes or lower scan areas could improve the scan time, but at a cost of image resolution.

VI. Future Work

The measurements presented in this paper can be performed on other cell lines during treatment, in particular acute myeloid leukemia (AML) cells, which have been studied extensively at high frequencies (20-60 MHz) previously in this laboratory [4, 6-8]. The methodology presented in this paper can also be used for other time sensitive biological processes, such as mitosis, locomotion and exposure of cells to other types of chemotherapeutic agents. The penetrating nature of ultrasound would give insight into the structural changes occurring within the interior of the cell during these processes.

Knowledge of the mechanical properties as a function of a cell's state will allow modeling and simulations of ultrasound scattering from cells and cell ensembles. These values are required as input to theoretical models of scattering currently being developed in our laboratory [10], [15].

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References

[1] A. H. Wyllie, J. F. R. Kerr and A. R. Currie, "Cell death: The significance of apoptosis," *Int. Rev. Cytol.,* vol. VOL. 68, pp. 251-306, 1980.

[2] G. I. Evan and K. H. Vousden, "Proliferation, cell cycle and apoptosis in cancer," *Nature,* vol. 411, pp. 342-348, 2001.

[3] G. Bao and S. Suresh, "Cell and molecular mechanics of biological materials," *Nature Materials,* vol. 2, pp. 715-725, 2003.

[4] M.C. Kolios, G. J. Czarnota, M. Lee, J. W. Hunt, M. D. Sherar, "Ultrasonic spectral parameter characterization of apoptosis," *Ultrasound in Medicine and Biology,* vol. 28, pp. 589-597, 2002.

[5] K. J. Van Vliet, G. Bao and S. Suresh, "The biomechanics toolbox: Experimental approaches for living cells and biomolecules," *Acta Materialia,* vol. 51, pp. 5881-5905, 2003.

[6] A. S. Tunis, R. E. Baddour, G. J. Czarnota, A. Giles, A. E. Worthington, M. D. Sherar and M. C. Kolios, "Using high frequency ultrasound envelope statistics to determine scatterer number density in dilute cell solutions," in *Proceedings - IEEE Ultrasonics Symposium,* 2005, pp. 878-881.

[7] M. C. Kolios, L. Taggart, R. E. Baddour, F. S. Foster, J. W. Hunt, G. J. Czarnota and M. D. Sherar, "An investigation of backscatter power spectra from cells, cell pellets and microspheres," in *Proceedings of the IEEE Ultrasonics Symposium,* 2003, pp. 752-757.

[8] G. J. Czarnota, M. C. Kolios, J. Abraham, M. Portnoy, F. P. Ottensmeyer, J. W. Hunt, M. D. Sherar, "Ultrasound imaging of apoptosis: High-resolution non-invasive monitoring of programmed cell death in vitro, in situ and in vivo," *Br. J. Cancer,* vol. 81, pp. 520-527, 1999.

[9] A. Briggs, *Acoustic Microscopy*, Oxford: Clarendon Press, 1992.

[10] O. Falou, R. E. Baddour, G. Nathanael, G. J. Czarnota, J. C. Kumaradas and M. C. Kolios, "A study of high frequency ultrasound scattering from non-nucleated biological specimens," *J. Acoust. Soc. Am.,* vol. 124, pp. E278-E283, 2008.

[11] E. M. Strohm and M. C. Kolios, "Measuring the mechanical properties of cells using acoustic microscopy," *31st Annual International Conference of the IEEE EMBS,* 2009, to be published.

[12] E. M. Strohm, "Determining the Mechanical properties of apoptotic cells using time resolved acoustic microscopy," M.Sc. dissertation, Dept. Physics, Ryerson University, Toronto, Ontario, 2009.

[13] F. A. Duck, *Physical Properties of Tissue.* London; San Diego: Academic Press, 1990.

[14] D. E. Saunders, W. D. Lawrence, C. Christensen, N. L. Wappler, H. Ruan and G. Deppe, "Paclitaxel-induced apoptosis in MCF-7 breast-cancer cells," *International Journal of Cancer,* vol. 70, pp. 214-220, 1997.

[15] R. E. Baddour, M. D. Sherar, J. W. Hunt, G. J. Czarnota, M. C. Kolios, " High-frequency ultrasound scattering from microspheres and single cells," *Journal of the Acoustical Society of America,* vol. 117, pp. 934-943, 2005.