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Cell Membrane Deformation Induced by a Fibronectin-Coated Polystyrene Microbead in a 200-MHz Acoustic Trap

Jae Youn Hwang,

Department of Information and Communication Engineering, Daegu Gyeongbuk Institute of Science and Technology, Daegu, Republic of Korea

Changyang Lee,

National Institutes of Health (NIH) Resource Center for Medical Ultrasonic Transducer Technology, Department of Biomedical Engineering, University of Southern California, Los Angeles, CA

Kwok Ho Lam,

Department of Electrical Engineering, The Hong Kong Polytechnic University, Hung Hom, Kowloon, China

Hyung Ham Kim,

National Institutes of Health (NIH) Resource Center for Medical Ultrasonic Transducer Technology, Department of Biomedical Engineering, University of Southern California, Los Angeles, CA

Jungwoo Lee, and

Department of Electronic Engineering, Kwangwoon University, Seoul, Republic of Korea (jwlee@kw.ac.kr)

K. Kirk Shung

National Institutes of Health (NIH) Resource Center for Medical Ultrasonic Transducer Technology, Department of Biomedical Engineering, University of Southern California, Los Angeles, CA

Abstract

The measurement of cell mechanics is crucial for a better understanding of cellular responses during the progression of certain diseases and for the identification of the cell's nature. Many techniques using optical tweezers, atomic force microscopy, and micro-pipettes have been developed to probe and manipulate cells in the spatial domain. In particular, we recently proposed a two-dimensional acoustic trapping method as an alternative technique for small particle manipulation. Although the proposed method may have advantages over optical tweezers, its applications to cellular mechanics have not yet been vigorously investigated. This study represents an initial attempt to use acoustic tweezers as a tool in the field of cellular mechanics in which cancer cell membrane deformability is studied. A press-focused 193-MHz single-element lithium niobate (LiNbO_3) transducer was designed and fabricated to trap a 5- μm polystyrene microbead near the ultrasound beam focus. The microbeads were coated with fibronectin, and trapped before being attached to the surface of a human breast cancer cell (MCF-7). The cell membrane was then stretched by remotely pulling a cell-attached microbead with the acoustic trap. The maximum cell membrane stretched lengths were measured to be 0.15, 0.54, and 1.41 μm at input voltages to the

transducer of 6.3, 9.5, and 12.6 V_{pp}, respectively. The stretched length was found to increase nonlinearly as a function of the voltage input. No significant cytotoxicity was observed to result from the bead or the trapping force on the cell during or after the deformation procedure. Hence, the results convincingly demonstrated the possible application of the acoustic trapping technique as a tool for cell manipulation.

I. Introduction

The mechanics of a cell play a key role in cellular morphological changes, sensing, and reaction to mechanical environments [1]. In particular, the elastic and viscoelastic properties of the membrane of a living cell are affected by structural and molecular alterations induced from the progression of diseases and the invasion of foreign organisms [2]. Therefore, the measurement of mechanical properties of cells would be critical to develop a complete knowledge of the developmental processes associated with disease progression.

A variety of biophysical techniques, including atomic force microscopy [3], optical tweezers [4], and micro-pipette aspiration [5], have been developed to spatially manipulate micro-particles or cells with precision. Among these, optical tweezers have been well-established and therefore widely utilized as a noninvasive tool to manipulate small particles such as fibronectin-coated microbeads [6], [7]. By trapping two microspheres attached to the opposite sides of a red blood cell, the cell was deformed to estimate its shear modulus [8]. In addition, mechanical stress was locally exerted on the cells and the linkage between fibronectin-integrin-cytoskeleton was elucidated by trapping the microbeads attached to specific locations on fibroblast cells [9]. Such cell-conjugated microbeads were also used to stretch actin fibers in human umbilical vein endothelial cells (HUVECs) by displacing fibronectin-coated beads tethered to the apical surface of the cells [10]. Despite their excellent precision and versatility, however, optical tweezers have a few practical limitations: 1) their use is primarily limited to optically transparent objects; 2) the trapping force in the piconewton range is so weak that this optical method is effective only for handling small biological specimens e.g., bacteria, DNA, organelles, etc.; and 3) the high energy generated by a focused light beam may also inflict damage on trapped samples [11], [12].

Various acoustic techniques have also been developed to overcome the aforementioned shortcomings of optical tweezers [13]–[18]. In particular, acoustic trapping methods using standing surface acoustic waves (SSAWs) were developed to manipulate microparticles, cells, and entire organisms [19]–[21]. Using the SAW-based technique, a single bovine red blood cell and *Caenorhabditis elegans* were trapped and manipulated in a microchip that consisted of polydimethylsiloxane (PDMS) channels and two orthogonal pairs of chirped interdigital transducers [19]. In addition, cells and microparticles moving in microfluidic channels were patterned by the acoustic trap derived from SAWs. Fluorescent polystyrene beads with the diameter of 1.9 μm , red blood cells, and *Escherichia coli* were successfully patterned in one- or two-dimensional domains via the acoustic trapping system integrated with microchannels. The results showed that such noninvasive SAW trapping systems might further be developed as miniaturized devices appropriate for flow cytometry studies [20].

More recently, a standing wave trapping chamber including a 16-element ultrasound array was developed to manipulate microparticles. Within the chamber, the movement of the Bessel-function pressure field was achieved by changing the phase of the sinusoidal signals applied to the array elements, thus resulting in microparticle transportation [21].

In contrast, we developed a two-dimensional transverse trapping method to precisely manipulate cells or microparticles on the focal plane [22], [23]. A 26-MHz high-frequency linear phased array was shown to be capable of trapping and moving polystyrene microbeads to target positions without mechanical scanning of the transducer [22]. In addition, by using a single-element focused transducer, our proposed approaches had simpler instrumentation than the aforementioned standing wave techniques. A 30-MHz LiNbO_3 single-element transducer was built to laterally trap and direct lipid droplets with the size of over 100 μm toward the trapping beam's focus within the range of hundreds of micrometers [23]. More recently, we experimentally applied the same transverse technique to trapping individual leukemia cells with a 200-MHz ZnO focused ultrasound transducer [24]. The trapping force in the nanonewton range enabled objects of tens to hundreds of micrometers to be spatially manipulated with high precision [25].

In this paper, we report experimental results that demonstrate the potential of this trapping method applicable to forcing local membrane deformation for studying membrane elastic properties. A 193-MHz single-element lithium niobate (LiNbO_3) transducer was fabricated to produce membrane deformation of human breast cancer cells. Fibronectin-coated polystyrene microbeads, in which the fibronectin binding to integrins expressed in cancer cells is relevant to activation of intracellular signaling molecules that induce tension-dependent deformation changes in the cells [26], were attached to the cell membrane. In our experiment, a fibronectin-coated microbead was first trapped by the focused beam produced from the transducer, before being tagged to the membrane. The trapped microbead was then translated in the perpendicular direction to the beam axis by mechanically scanning the trap center (or the transducer's focus) toward the cell, until it was attached to the membrane. As the trapped bead tethered to the cell body was subsequently displaced, both deformed membrane and bead motion were recorded in video files. The deformation was quantitatively analyzed with commercial software by measuring the stretched length of the membrane, which depended on the given input voltage to the transducer. The results convincingly demonstrate the capability of acoustic tweezers as a cell manipulation tool.

II. Materials and Methods

A. Transducer Characteristics

A press-focused 193-MHz single-element lithium niobate (LiNbO_3) transducer (bandwidth = 114%) was constructed via a procedure previously described [11] to generate a highly focused ultrasound beam for trapping a microbead [Fig. 1(a)]. The transducer's aperture diameter was 0.65 mm and its focal length was 0.75 mm (f -number = 1.1). Note that there has been no established method for calibrating absolute pressure levels at the high-frequency range used in this work. Previous reports showed that the wire-target technique is a useful alternative to a hydrophone method, which has been well known as a standardized procedure in the determination of absolute acoustic pressure, in characterization of acoustic radiation

patterns from ultrasonic transducers [27], [28]. Therefore, both lateral and axial beam profiles [Figs. 1(b) and 1(c)] of the transducer were obtained by receiving echo-signals from a tungsten wire target with the diameter of $2.5\text{ }\mu\text{m}$ while the tungsten wire is scanned in both the axial and lateral directions. The pulse intensity integral (PII) was calculated to determine the beam size in each direction, being defined as the time integral of the intensity of a received echo taken over the time when the acoustic pressure is nonzero [28]. A spatial point at which the PII was reduced by 6 dB from its maximum value was then detected from the received RF signals reflected back from the wire target. The resultant lateral beam width and axial depth of focus were 9.0 and $65.9\text{ }\mu\text{m}$, respectively. In particular, the beam width was in approximate agreement with the predicted value of $8.47\text{ }\mu\text{m}$ ($= \text{f-number} \times \text{wavelength}$).

B. Microbead Coating With Fibronectin

Human breast cancer cell lines (MCF-7, American Type Culture Collection, Manassas, VA) were used to show the capability of our trapping method for local cell membrane deformation, because they express integrin receptors on their membrane. Hence, specific types of integrins that are associated with cancer cells have been widely considered as appropriate candidates for a targeted therapy during cancer mechanotransduction process [26], [29].

Polystyrene microbeads with the diameter of $5\text{ }\mu\text{m}$ (Polysciences Inc., Warrington, PA) were coated with fibronectin (Sigma-Aldrich Corp., St. Louis, MO) for specific binding to integrins expressed in MCF-7 cells according to the following procedure: 1) The microbeads were collected from $500\text{ }\mu\text{L}$ of 10% bead solution diluted with phosphate buffered saline (PBS; Invitrogen Corp., Carlsbad, CA) after being centrifuged and washed with PBS three times. 2) The collected microbeads were mixed with $50\text{ }\mu\text{g/mL}$ fibronectin solution, and then incubated at 4°C for 1 h. 3) After mixture centrifuge and supernatant aspiration, the microbeads were once again incubated in 10 mg/mL bovine serum albumin (BSA, Invitrogen Corp., Carlsbad, CA) at 4°C for 3 h. 4). After the BSA removal, the fibronectin-coated microbeads were finally diluted with PBS and stored at 4°C for experiment.

C. Procedure for Microbead Acoustic Trapping

An experimental setup for our transverse acoustic trapping system and a photograph are shown in Figs. 2(a) and 2(b), respectively. A sample chamber containing fibronectin-coated microbeads was placed on top of a microstage in an inverted microscope (IX71, Olympus America Inc., Center Valley, PA). A 200-MHz sinusoidal burst waveform with the peak-to-peak voltage amplitude of 6.3 V_{pp} was used to drive the transducer to generate the trap. The duty factor of the burst was 1% and the pulse repetition frequency (PRF) was set to 1 kHz. Prior to the trapping experiment, a pulse-echo test was performed to ensure that the microbeads were located near the ultrasound beam. Fig. 3 illustrates a simplified diagram for cell attachment and deformation procedure by a microbead. A fibronectin-coated microbead was randomly selected within the field of view of the microscope, and subsequently trapped by the focused beam [Fig. 3(a)]. The trapped microbead was brought to a neighboring cell by transversely scanning the transducer [Fig. 3(b)]. When the microbead was in contact with the cell, the transducer was immediately switched off to avoid further mechanical disturbances during the microbead-cell binding process. After 10 min, which allowed a

strong fibronectin-binding to the cell membrane, the transducer was turned on once again. Note that the binding time varied for individual cells. It was then checked whether the trapped microbead stretched the attached cell in the same direction as the transducer was laterally moved. This procedure was repeated until the microbead–cell attachment was visually confirmed through the microscope.

For carrying out the cell deformation test, the transducer was displaced from the cell-attached microbead by a certain distance D while it was turned off [Fig. 3(c)]. Input voltages of 6.3, 9.5, and 12.6 V_{pp} were then applied to the transducer to stretch the cell by the acoustically trapped microbead [Fig. 3(d)]. At each given voltage, the attractive motion of the trapped microbead was captured in image files using a CCD camera (ORCA -Flash2.8, Hamamatsu Photonics K.K., Hamamatsu, Japan) installed in the microscope. Both microbead displacement and cell stretched length were analyzed from the stored images with a position tracking software program (Metamorph Advanced, Olympus Corp., Tokyo, Japan).

D. Cell Viability Test

Cell viability after carrying out the cell deformation test was examined with a viability dye, Calcein AM (Invitrogen Corp., Grand Island, NY). In live cells, the dye is converted to a green fluorescent calcein, and thus its fluorescence intensity represents cells' viability [30]. For loading of the calcein dye to MCF-7 cells incubated in complete Dulbecco's modified Eagle's medium for 24 h, 1 μ M calcein solution (final concentration) was added in the sample chamber containing the cells. In 30 min, the cells were thoroughly washed with PBS, followed by fluorescence imaging of the cells before and after the trapping force application, as shown in the Section II-C. For the fluorescence imaging, the same microscope was utilized (excitation: 488 nm and emission: 532 nm). Fluorescence images were here acquired both before attaching a bead to cell membrane and after the force application to compare changes in cell viability resulting from the trapping force application. The calcein fluorescence in cells after trapping force application was then normalized by their own fluorescence before the trapping force application. For the statistical analysis, the mean of normalized calcein fluorescence was compared between the cells with and without (control) the force application. All data were expressed as mean $\pm$ standard deviation of indicated sample sizes ($n = 10$), and were analyzed by a two-tailed paired t -test, with the level of significance set at p -value < 0.01 . Additionally, the viability was compared between the cells that were exposed to the trapping force and treated with 1% bleach for 20 min.

III. Results and Discussion

Fig. 4 illustrates sequential images of the motion of a trapped microbead as the trap center is moved before cell-microbead attachment (S1). The microbead was moved from an arbitrary point [Fig. 4(a)] to the target position [Fig. 4(b)] along a designated path. The trapped bead was displaced upward by 13 μ m as shown by Figs. 4(a) and 4(b). Subsequently, the bead was moved to the left by 10 μ m [Fig. 4(c)], upward by 5 μ m [Fig. 4(d)], and to the right by 10 μ m [Fig. 4(e)], before it finally reached the cell surface [Fig. 4(f)]. The arrow here indicates the bead's displaced direction. After the attachment was set, the trap center was

repositioned at $D = 3 \mu\text{m}$ from the microbead. Note that the bead's contact position with the cell was adjusted to precisely attach the fibronectin-coated microbead to the cell.

The transducer was then turned on again at several input voltages. This allowed the displaced microbead to stretch and deform the cell surface. At the input voltage of $6.3 V_{pp}$, no noticeable microbead displacement and cell stretching was observed [Fig. 5(a), left column] (S2), whereas at the voltages of $9.5 V_{pp}$ [Fig. 5(a), middle-left column] and $12.6 V_{pp}$ [Fig. 5(a), middle-right column] (S3 and S4), the microbead was directed toward the trap center, and the cell membrane was deformed by the bead displacement. In contrast, without the microbead attachment to the cell membrane, membrane deformation was not observed as a result of the acoustic trapping force [Fig. 5(a), right-column], indicating that the cell deformation was made by pulling the microbead attached to cell membrane. Fig. 5(c) shows the change in displacement over time at each input voltage, suggesting that a higher voltage input to the transducer produces a larger displacement. After the acoustic trap was off, it was found that the microbead would return approximately to the original position where it was located before the trapping force application [Fig. 5(a), bottom row]. The stretched lengths of the cell membrane were measured to be 0.15 , 0.54 , and $1.41 \mu\text{m}$ at 6.3 , 9.5 , and $12.6 V_{pp}$, respectively [Fig. 5(d)], demonstrating that the stretched length was nonlinearly related to the voltage input to the transducer. Previous works showed that cells' mechanical response at low forces is largely determined by the actin cortex, whereas the cell response for more than a few hundred nanometers of deformation is increasingly determined by viscous effects [31]. Thus, the mechanical response of cells is expected to exhibit nonlinear characteristics at different levels of force. Our results [Fig. 5(d)] also showed the nonlinear deformation as function of the trapping force, mainly caused by the cells' viscoelastic behavior. The cell was then visually inspected, immediately after the transducer was turned off to examine whether the ultrasound beam caused any cell damage. Serious morphological damage on the cell e.g., noticeable membrane blebbing, which is an irregular bulge in the cell membrane, or fragmentation was not observed during or after the deformation procedure. Total ultrasound exposure time was less than 5 min throughout the experiment. Additionally, cell viability after trapping force applications was examined to confirm the effects of acoustic traps on the cells. The cell viability was somewhat decreased by ~ 0.92 due to the acoustic traps compared with the viability of the control cells (without acoustic trapping) (~ 0.96) [Fig. 6(a)], but it was not significant (p -value: $0.65 > 0.01$). In this experiment, we observed that most cells exhibited no significant viability changes due to the acoustic traps. Interestingly, a few cells' viability decreased by ~ 0.7 as a result of the acoustic traps, but the cells were still viable. Furthermore, we compared the viability between the cells receiving trapping force and those treated with 1% bleach for 20 min. The cells receiving the trapping force still emitted strong fluorescence at 20 min, indicating no significant viability changes [Fig. 6(b), lower], whereas the cells treated with 1% bleach lost their viability at 20 min after the treatment [Fig. 6(b), upper]. Taken together, these results demonstrated that the trapping force at $12.6 V_{pp}$ had no significant effects on viability of the cells under the experimental condition.

In Fig. 5(a), we observed that the deformation was reversible within a short period (less than a few seconds) after the initial deformation of cell membrane was made by an acoustic trap

at the indicated voltages. Interestingly, several excitations of trapping force on the bead were required to achieve the initial membrane deformation. Previous studies showed that cells receiving mechanical stress for a long period exhibited cell remodeling such as changes in focal adhesion composition, contractility and cell stiffness, implying that the mechanical properties of cells vary as a function of time because of mechanical stress [32], [33]. Therefore, the duration of trapping force application to cells may have to be minimized for probing the mechanical properties of cells in a reversible manner.

For the purpose of demonstration, the trapping force at 9.5 V_{pp} was calibrated using drag force measurement described in a previous report (see the supplementary figure) [25]. The force at 9.5 V_{pp} was found to be ~25.47 pN at the distance of 2.45 μm from the beam center. The membrane stretched length was ~0.55 μm when such trapping force was exerted on the microbead [Fig. 5(d)]. In the previous study, deformation of 0.2 μm in mouse fibroblasts was made at forces of 30 pN [31]. In contrast, our results showed that the lower trapping force led to more deformation, perhaps the result of using different types of cells.

Cell membranes are viscoelastic. Therefore, the selection of the measurement techniques depends on which mechanical property in cells is probed. In previous works, using AFM, the local viscoelastic properties of a single cell can be probed with large indentations (>~1 μm). However, the lowest force exerted by AFM is limited by the thermal noise of the AFM cantilever in liquid (~20 pN). In contrast, the forces below the 20 pN limit of AFM can be achieved with magnetic twisting cytometry, optical stretchers, and optical traps. In particular, the optical traps were used to probe the cells' membrane tension with small scale deformation (less than 0.2 μm) by trapping the bead attached to the membrane [31], [32]. Our study showed that the proposed method was capable of probing both the small- and large-scale membrane deformation (0.15 to 1.41 μm), see Fig. 5(d), suggesting its potential to measure viscous (large deformation) and elastic (small deformation) properties of the cells separately.

In this study, we experimentally demonstrated the capability of our trapping method to deform a single cell by pulling the attached microbead in an acoustic trap. It was shown here that the reproducibility was less than 10%. Typically, the binding force between fibronectin and integrin varies even in the same type of cells [34]. Therefore, the relatively poor reproducibility may be caused by the heterogeneity of the binding force between fibronectin and integrin in individual cells. To deform the cell membrane, the binding force between fibronectin and integrin must be higher than the cell membrane tension. Therefore, to enhance the experimental reproducibility, augmentation of the binding force may be needed. It may be achieved by further refinement in fibronectin coating and the size of the microbead.

IV. Conclusion

The results demonstrated that our transverse acoustic trapping technique using a 193-MHz single element lithium niobate (LiNbO₃) ultrasonic transducer was capable of deforming and manipulating the membrane surface of cancer cells quantitatively. Using the transducer driven in a sinusoidal burst mode, a 5-μm fibronectin-coated microbead was trapped near an

ultrasound microbeam and then attached to the cell membrane. Furthermore, local membrane stretching of an individual cancer cell where the microbead was attached was made by pulling the trapped microbead using our approach. We found that the membrane's stretched length depended on voltage inputs to the transducer, suggesting that the membrane can precisely be manipulated with the proposed trapping technique by adjusting the transducer's excitation parameters. Therefore, these results experimentally verified the usefulness of this ultrasonic method as a tool for single cell manipulation.

Furthermore, the findings of this work may suggest that the use of our trapping technique can be extended to other cellular applications. For example, with calibrated trapping force, this acoustic tool may also be utilized to quantitatively probe local mechanical properties of cancer cells in the determination of their metastatic potentials. Previous studies showed that mechanical properties of cancer cells were correlated with their metastatic potentials [35]. More invasive cancer cells exhibited softer mechanical characteristics that resulted in more desirable shape change suitable for metastatic population growth. In addition, the metastatic potential measured through cancer cell invasion assay was shown to possess an inverse power-law relationship with the cell stiffness [36]. Therefore, the cell deformation study reported in this paper suggests that the proposed tool may offer a valuable alternative for the evaluation of cancer cell stiffness in determining invasion potentials of cancer cells.

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(a)

(b)

(c)

Fig. 1.

Transducer characteristics: (a) photograph of the fabricated transducer; (b) the measured lateral beam width is $9.0\ \mu\text{m}$; (c) the depth of focus is $65.9\ \mu\text{m}$ in the axial direction.

Fig. 2.

System configuration for microbead trapping experiment: (a) schematic diagram, (b) photograph of system, including micropositioners and an exposure chamber.

Fig. 3.

Illustration of experimental procedure for cell stretching: (a) initiation of microbead trapping, (b) contact of trapped bead with cell surface, (c) displacement of the transducer for cell stretching while it is turned off, (d) onset of trapping beams, and (e) completion of cell stretching. $D = 3 \mu\text{m}$.

Fig. 4. Microbead attachment to cell surface: Sequential images from (a) to (e) show that a trapped fibronectin-coated microbead is moved before it arrives at the cell surface. (f) After being successively displaced, the bead held its position at the cell surface for 10 min until it was firmly bound to the cell. Input voltage = 6.3 V_{pp}, PRF = 1 kHz, and duty factor = 1%.

Fig. 5.

Cell stretching images and analyzed data associated with the stretched length. (a) The images indicate that the stretching test is carried out at various voltage inputs to the transducer; 6.3 V_{pp} (left column), 9.5 V_{pp} (middle-left column), 12.6 V_{pp} (middle-right column), and 12.6 V_{pp} without a bead (right column). The dotted circle indicates a beam spot. (b) Contours of cell membrane and the bead before and after the trapping. (c) Temporal displacement change at each input voltage. (d) Plot of membrane stretched length versus input voltage.

Fig. 6.

Cell viability after the trapping force application. (a) Viability of cells with the force application was compared with the viability of cells without the force application (p -value: ~ 0.66). (b) Viability of cells treated with 1% bleach under the trapping force as a function of time. The red solid circle indicates a microbead.