Mechanical Heterogeneity of Cancer Cells as Revealed by Using an AFM-Based Asymptotic Analysis

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The Hertz model has been widely utilized to determine the elastic moduli of cells from force curves obtained using atomic force microscopy (AFM). Because this model assumes a small indentation over an infinite hemisphere, previous studies investigated only regions close to the cell cortex. The mechanical heterogeneity of cells observed under high stress is often ignored despite its biological importance. In this study, we performed an asymptotic analysis in order to determine the elastic moduli of deeper cytoplasmic regions. We also demonstrated how to determine the tip-to-sample contact point without ambiguity. We confirmed the validity of the asymptotic analysis by comparing values from the asymptotic analysis with those from the Hertz model in the low-stress regime. In conclusion, we found that cytoplasmic regions beyond the cell’s cortex were mechanically less deformable than the cell’s cortex, indicating the mechanical heterogeneous nature of breast cancer cells.

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I. INTRODUCTION

Over the past decades, the enhanced deformability has been considered as a hallmark of cancer cells. From the diagnostic point of view, the mechanical deformability, i.e., elastic modulus, has been noticed as a promising marker, which can be generally applied to various types of cancer. Among many mechano-biological techniques, the atomic force microscopy (AFM) – based indentation experiments have been most widely utilized to determine elastic moduli of cancer cells [1, 2]. Experimentally, a simple indentation yields a force-distance \((f - d)\) curve on the precisely determined nano-domain over a single cell. Elastic moduli can be determined by analyzing \(f - d\) curves using the Hertz model, the most widely utilized model [3–5]. Because the Hertz model assumes small indentations over an infinite hemisphere, previous experiments were carefully performed by precisely controlling the stress regime where a cell can be considered as homogeneous material [6–8].

The cytoskeleton governing mechanical rigidity and structural stability of cells is known to be altered in cancer cells. The cytoskeleton is composed of filamentous proteins including actin, microtubules, and intermediate filaments with accessory proteins [8]. The cell cortex is composed of actin filaments-rich layers. The dissected views of the confocal microscopic images showed that cancer cells have short and scattered actin filaments [9]. This alteration in actin organization was considered to be responsible for the increase in mechanical deformability [10]. However, recent studies in cancer biology reported the significant rearrangements of other cytoskeletal components during the cancer progression [12, 13]. The fact that other cytoskeletal components exist in cytoplasmic regions under the cell cortex imposes difficulties in evaluating their roles in mechanical deformability [13]. In addition, microtubules are thicker and show 10³ times higher bending stiffness than actin filaments in vitro [8]. The bending stiffness of intermediate filaments in vitro was intensified at high strains while actin filaments were fluidized at high strain [8]. The elastic response at low...
stress might be dominated by an actin-rich cortex. However, a considerable amount of stress has to be applied in order to deform other components found in cytoplasm beyond the cell cortex [13]. Consequently, there is a need for a new mathematical model which resolves the violation of the Hertz assumption due to the high stress.

Some efforts were made to resolve this issue. By expanding the total pressure distribution on a sample as a sum over a series of partial pressure distributions, elastic moduli were successfully determined from a single thin film on hard substrates [3,15]. Polymeric thin films and thin regions of cells, i.e., lamellipodia, were investigated in previous studies [3,16]. Furthermore, we demonstrated how to determine elastic moduli from mechanical multi-layers by adapting a semi-empirical model modified from the Hertz model [9,14,16]. We depicted a cell as a double-layered elastic structure composed of a thin actin-rich cortex and a cytoplasmic layer containing other cytoskeletal components and applied high stress to induce the considerable deformation of both layers. In this model, the force-distance curves were analyzed by hyperbolic fits by considering the asymptotic behavior of each layer. Nevertheless, there is a lack of evidence whether this asymptotic analysis will allow us to reveal the mechanically heterogeneous nature of biological cells, which was ignored in the previous AFM studies due to the limitation in mathematical models.

In this study, we investigated whether the AFM indentation studies could reflect the mechanical heterogeneous nature of breast cancer cells. To do this, we determined elastic moduli of both the cell cortex and body by varying the applied stress. We also verified the validity of the asymptotic analysis by confirming the consistency between elastic moduli determined from the Hertz model with low stress and those from the asymptotic analysis.

II. EXPERIMENTS

1. Cell culture

The breast cancer cells (MD-MB-231) were cultured with DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 2 mM L-Glutamine at 37 °C, 5% CO₂. Approximately 1 x 10⁴ cells were seeded on pre-cleaned glass slides two days before the experiments.

Fig. 1. (Color online) A representative f – d curve obtained from a breast cancer cell. The contact point between a tip and a sample was determined from the secondary derivative of the sigmoidal function derived from the experimental data. Note that the 1st and 2nd derivative functions are enlarged by 10³ and 10⁵ times in the plot. We determined the contact point as the distance at which the secondary derivative meets one twentieth of its maximum value. The arrow indicates the contact point.

2. AFM indentation experiments

A commercial AFM (MFP3D®, Asylum Research) mounted on an inverted optical microscope (Olympus IX-81) was used to indent the localized regions of cells to obtain force-distance (f – d) curves. V-shaped silicon nitride cantilevers (Microlever®, Bruker) modified with spherical beads (bead radius = 4 μm) were used. Prior to measurements, the cantilever force constant (k = 0.03 – 0.05 N/m) was accurately calibrated by the thermal noise fluctuation method. Indentation points on the cell body were located by looking through an inverted optical microscope. We varied the maximum applied forces from 125 pN to 20 nN with the indentation speed of 30 - 200 nm/s. Using the customized scripts of IGOR®, asymptotic analysis based on the Hertz model were applied to calculate the elastic moduli from the f – d curves.

III. RESULTS AND DISCUSSION

Unlike the mechanically stiff sample, it is not trivial to detect the contact between a tip and a sample from the force curve because there is no abrupt change in the force due to the tip-sample contact. Special attention was paid to determine the contact point between a tip
and a sample. We utilized the customized IGOR® script to determine the point where the slope of the force curve initially deviated from zero force. As shown in Fig. 1, we derived a sigmoidal function from a $f - \delta$ curve. The rate of change right after a tip made contact is more exaggerated in the second derivative of the sigmoidal function than the first derivative or the sigmoidal function itself. Thus, we determined a point where the second derivative of the sigmoidal function becomes non-zero. For the practical purpose, we found the distance at which the second derivative function becomes one twentieth of its maximum value.

Once the contact point is determined, we calculated the indentations ($\delta$) by subtracting the cantilever deflections from the scanner displacements in order to convert $f - d$ curves to $f - \delta$ curves. The conventional Hertz model shown in Eq. (1) was applied to calculate elastic modulus, i.e., Young’s modulus $(E)$ from force-indentation $(f - \delta)$ curves [4].

$$f = \frac{4}{3} \frac{E}{1 - \nu^2} \sqrt{R \delta^3}$$  \hspace{1cm} (1)

Here, $R$ is the radius of the probe. We assumed that the Poisson ratio ($\nu$) is 0.3.

A representative set of $f - \delta$ curves obtained from a breast cancer cell with varying the maximum applied forces $(f_t = 0.125 \sim 20 \text{nN})$ was shown in Fig. 2. Regardless of the applied stress, the regions where the indentations are less than 1 $\mu$m coincide with the prediction from the Hertz model. The significant deviation from the Hertz model was observed from the $f - \delta$ curves taken with the higher stress regime $(f_t > 3 \text{nN})$. Deviations were further exacerbated in the higher stress regime $(f_t \geq 10 \text{nN})$. This deviation from the Hertz model implies that deeper cytoplasmic regions of breast cancer cells were mechanically different from the cell cortex. Cells display a homogeneous elastic behavior when the strain is confined to the actin-rich cortex. However, the compositional variation in the cytoskeleton beyond the cell cortex might contribute for the deviation of the $f - d$ curves obtained with high stresses from the Hertz prediction. Despite of much higher complexity in molecular compositions of cancer cells, we simplified a cell as a double-layered elastic structure in order to extract the empirical features of mechanical heterogeneity.

We assume that within the stress applied in this study the hard nucleus composed of various proteins and nucleotides were not mechanically deformable. However, we understand that our semi-empirical model was not able to eliminate the contribution from a nucleus to the mechanical deformability.

In order to deduct elastic moduli from the $f - d$ curves obtained with high stresses $(f_t \geq 10 \text{nN})$, we adapted the mathematical model developed by Capella and coworkers [14]. The cell cortex provides a mechanical shielding for the underlying cell body and the shielding effectiveness decreases as the deformation increases. The elastic moduli extracted from the indentations near the contact point should solely describe the elastic behavior of the actin-rich layer in the cell cortex. However, the deeper indentations should reflect the mechanical behavior of the underlying cell body which might be composed of other cytoskeletal components. Indeed, both ends of a $f - \delta$ curve should independently reflect the mechanical behavior of each layer when depicting a cell as a double-layered structure. A hyperbolic fit was suitable for the plot of $\delta^{3/2}$ vs. $D$ derived from $f - \delta$ curves by considering this asymptotic behavior. Here, $D$ is the cantilever deflection. Simply, the following conditions represent mechanical behavior originated from each layer.

1. $E_C E E_B$
2. When $\delta/t \to 0$, $E \to E_C$, then $\delta/t \to \infty$, $E \to E_B$

$E_C$ and $E_B$ represent the elastic moduli of the cell cortex and the cell body, respectively. Here, $t$ is the thickness of the cell cortex. From the hyperbolic transformation,
the linear behavior of $\delta^{3/2}$ vs. $D$ expected from the Hertz model can be rewritten as [14].

$$\delta^{3/2} = \beta D + \varepsilon - \sqrt{\alpha^2 D^2 + 2\varepsilon(\beta - \gamma)D + \varepsilon^2}$$  \hspace{1cm} (2)

Here, $\alpha$ and $\beta$ reflect the elastic moduli of the cell cortex and the cell body.

$$\alpha = \frac{3k(1 - \nu^2)}{8\sqrt{R}} \left( \frac{1}{E_C} - \frac{1}{E_B} \right)$$  \hspace{1cm} (3)

$$\beta = \frac{3k(1 - \nu^2)}{8\sqrt{R}} \left( \frac{1}{E_C} + \frac{1}{E_B} \right)$$  \hspace{1cm} (4)

As a meaningful parameter, $\gamma$ is defined as the instant slope at zero load and represents the mechanical response near the contact point. $\varepsilon$ satisfies the condition of no deformation under the zero load; $\delta^{3/2}|_{D=0} = 0$.

As shown in Fig. 3, we performed a hyperbolic fitting based on Eq. (2). The experimental data were obtained with the maximum applied force of 10 nN from a breast cancer cell. When the cantilever deflection is smaller than 50 nm, $\delta^{3/2}$ vs. $D$ shows the linear behavior as expected from the conventional Hertz model. From this linear behavior, we can postulate that the hyperbolic fitting reflects the asymptotic behavior of $\delta^{3/2}$ vs. $D$ at least at the lower stress regime. Using Eqs. (2) and (4), we calculated the elastic moduli of both the cell cortex and the cell body from the fitting parameters of $\alpha$ and $\beta$.

In order to find out whether mechanical responses observed in force curves were dominated by the cell cortex or body, we used the substitution of variables $X$ and $Y$ given in Eqs. (5) and (6), rotated $\delta^{3/2}$ vs. $D$ functions about the axis of this onset point, and finally obtained the concave hyperbola as shown in Fig. 4.

$$X = D + \frac{\varepsilon}{\alpha}(\beta - \gamma)$$  \hspace{1cm} (5)

$$Y = \delta^{3/2} - \beta D - \varepsilon$$  \hspace{1cm} (6)

The solid line in Fig. 4 represents the asymptotic behavior, which is given by $Y = \pm \alpha X$. Negative $X$ represents the cell cortex – and positive $X$ represents the cell body – dominated behaviors, respectively. As expected, we found that the lower indentations of force curves obtained with the maximum applied force of 10 nN shown in Fig. 4 were mainly affected by the mechanical response of the cell cortex while others were dominated by mechanical behavior of the cell body.

We confirmed the reliability of the hyperbolic fitting by converting the plot of $\delta^{3/2}$ vs. $D$ to the traditional $f - \delta$ curves as shown in Fig. 5. The conventional Hertz model agrees well with the obtained data only when $f < 1$ nN. The $f - \delta$ curve generated from the elastic moduli of the cell cortex calculated from the hyperbolic fitting also agrees well with the obtained data when $f < 2$ nN. Similarly, the other $f - \delta$ curve reconstructed from the elastic modulus of the cell body agrees well with the obtained data when $f > 5$ nN. This empirical observation manifestly explains that the hyperbolic fitting is suitable to deduct the elastic moduli of both the cell cortex and body. A similar approach was reported by Gus et al. [17]. They depicted a cell as a homogeneous elastic layer covered with very soft brushes such as glycocalyx. A thermodynamic equation was introduced to explain the forces generated from entropic brushes. They reported that self-consistent elastic moduli of cells were...
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In this study, we verified the validity of asymptotic analysis by determining the elastic moduli of the breast cancer cells. First, we analyzed the force curves obtained with 3 nN maximum applied force using the conventional Hertz model. Most experimental data obtained with 3 nN maximum applied agree will with the prediction from the Hertz model. The elastic moduli were calculated as 702 ± 92 Pa (n = 12 cells), which is consistent with the values from the previous AFM studies [18]. Next, we performed the indentation experiments by increasing the applied force to 10 nN on the same investigated cells. When performing the hyperbolic fitting, we found that the elastic moduli of the cell cortex and body were 738 ± 97 Pa and 997 ± 86 Pa. From the student’s t-test considering the paired-analysis, we found no difference in elastic moduli between the conventional Hertz model and the asymptotic analysis (p = 0.31345). This consistency between models indicates the reliability of the asymptotic analysis. Interestingly, the elastic moduli of the cell body determined from the asymptotic analysis turned out to be significantly higher than those of the cell cortex (p < 0.0001). This increase in elastic moduli in the deeper indentations might be resulted from the compositional complexity in cytoskeletal components. As explained earlier, the filamentous components such as microtubules and intermediate filaments showing the higher bending moduli in vitro lie under the cell cortex and thus the higher elastic moduli could be expected in the deeper regions beyond the cell cortex. We also understand that this mechanical hardening in the cell body can be originated from the existence of nuclei and the suggested asymptotic analysis may not enough to eliminate the contribution from the nuclei. Nevertheless, we believe that our experimental results pave the new way to analyze the mechanical deformability of cells and provide the new insight to utilize the mechanical deformability as a diagnostic marker.

IV. CONCLUSION

Many biomechanical studies on cancer cells were limited to investigate only the cell cortex where the deformation could be made with low stress. In this study, we have demonstrated that the asymptotic analysis could determine the elastic moduli of the deeper cytoplasmic regions as well as the cell cortex. The hyperbolic fit provided self-consistent elastic moduli of the cell cortex.
both from the conventional Hertz model and the asymptotic analysis. We also suggested a practical way without subjective ambiguity to determine the contact point between the tip and a sample, which provides a critical impact on the elastic moduli. From the asymptotic analysis, we found that the breast cancer cells were mechanically heterogeneous. A breast cancer cell can be depicted as a double-layered structure, which consists of a soft actin-rich cortex and a mechanically hardened layer in the deeper cytoplasmic region. Although we were not able to eliminate the effect from the hard nucleus, we believe that the asymptotic analysis using the hyperbolic fitting is useful to understand the mechanical heterogeneity of cancer cells. From this analysis, we expect that the cancer diagnosis based on the mechanical deformability will gain the new information about which cytoskeletal components play a major role in the cancer onset and progress.

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