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Mapping Single Cell-Substrate Interactions by Surface Plasmon Resonance Microscopy

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Abstract

We report on imaging of cell-substrate adhesion of a single cell with sub-cellular spatial resolution. Osmotic pressure was introduced to provide a controllable mechanical stimulation to the cell attached to a substrate, and high-resolution surface plasmon resonance microscopy was used to map the response of the cell, from which local cell-substrate adhesion was determined. In addition to high spatial resolution, the approach is non-invasive and fast, and allows for continuously mapping of cell-substrate interactions and single cell movements.

Keywords

Single cell imaging; Surface Plasmon Resonance imaging; osmotic effect; cell adhesion

1. Introduction

Studying cell-substrate interactions at the single cell level is critical to the understanding of many cellular behaviors, ranging from cell adhesion, growth and detachment¹ to the cell migration induced by various stimuli.^{2, 3} It is now well accepted that cell interactions with extracellular matrices (ECM) are mediated by integrin via the formation of ECM-integrin-cytoskeleton linkages.^{4, 5} The interactions allow a cell to adhere to a substrate, which provides mechanical support for the cell, a process essential for the biological functions of the cell. Studies have further indicated that cell-substrate adhesion strength exhibits high spatial and temporal variations to regulate different cellular functions.⁵ A technique that can resolve the local adhesion strength between a single cell and substrate is thus highly desired in order to understand the cell-substrate interactions and related cellular movements on surfaces.

The most developed technique to access the adhesion force information is single cell force spectroscopy, which analyzes the response of a cell by applying various mechanical stimuli, such as a direct contact force with a solid probe and a fluidic shear force by flowing solution across the cell.^{6–10} Although powerful, the lack of spatial resolution makes these techniques difficult to resolve the local distribution of the adhesion force, which is necessary for

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Supporting Information

Details regarding the location of regions of interests, calibration on SPR bulk effect, and seven-layer SPR model can be found in the supporting information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

understanding cellular processes, such as cell migration, growth and detachment. In order to obtain local adhesion information, two approaches have been proposed, one is to scan a mechanical probe over the cell surface,^{11, 12} and the second is to use an array of probes to measure the local adhesion force.^{13, 14} However, the spatial resolution is still limited and direct mechanical stimulations by the probes may damage the cell or affect the cellular behaviors, leading to results that may not directly reflect the cell properties in their natural states. Furthermore, these approaches cannot easily analyze multiple cells simultaneously, which is needed for statistical analysis.

Optical microscopy is an alternative technique to investigate cell-substrate interaction with high spatial resolution such as the total internal reflection fluorescence microscopy,¹⁵ fluorescence interference contrast,¹⁶ interference reflection microscopy^{17, 18} and surface plasmon resonance (SPR) imaging.^{19–23} SPR measures the local refractive index distribution, which is particularly suitable for real time and label-free investigation of cell-substrate interactions. Two approaches have been proposed for SPR imaging of single cells, prism-based and optical microscope objective-based systems. The former has been used to study single cell-substrate distance¹⁹ and the local mass distribution.²¹ Although successful, the spatial resolution is limited and the images suffer from distortion due to the prism.²⁰ The latter, developed recently, uses a high numerical aperture objective to achieve the spatial resolution up to the optical diffraction limit of the incident light.^{23–25} For example, using 60x to 100x objectives, sub-micron-resolution and distortion-free images have been obtained.^{26, 27} By taking advantage of the high spatial resolution, we have reported SPR imaging of single viruses,²⁶ and subcellular features of single adherent cells.²⁷

Here we demonstrate a non-invasive study of local cell-substrate adhesion strength. We achieved this task by controlling extracellular osmotic pressure and analyzing the associated local movement of cell membrane vertical to the substrate with surface plasmon resonance microscopy (SPRM)²⁸ (Scheme 1). Surface plasmon resonance (SPR) is known to be sensitive to the local refractive index distribution near a metal surface and has been applied to image cells,^{19–21, 27} but it has not yet been used to evaluate single cell adhesion strength. Previous SPR studies focused on the mass re-distribution of individual cells or a confluent monolayer of cells during certain chemical, mechanical or biological stimulations.^{22, 29, 30} To the best of our knowledge, the present work is the first attempt to investigate the cell-substrate adhesion strength by monitoring the vertical displacement of individual cells when changing the extracellular osmotic pressure. The extracellular osmotic pressure was increased by introducing a hypertonic buffer surrounding a target cell, which caused the cell to shrink and drove the bottom of the cell to detach from the substrate. The driving force competed with the cell-substrate adhesion force provided by the integrin-ECM linkage, which is usually not uniformly distributed across the cell bottom surface.^{4, 5} By monitoring the local detachment of the cell associated the hypertonic stimulation with SPRM, we obtained local information of the cell-substrate interactions.

2. Materials and Methods

2.1 Materials

The isotonic extracellular fluid (ECF) buffer contained NaCl (120 mM), KCl (3mM), CaCl₂ (2mM), MgCl₂ (2mM), D-Glucose (25 mM), HEPES (15 mM). A proper amount of NaOH was added to bring the buffer pH to 7.4. The hypertonic ECF was prepared by adding different amounts of mannitol to the isotonic ECF, and the hypotonic ECF was obtained by diluting isotonic ECF by 0.8 fold. All the reagents were analytical grade, purchased from Sigma, except otherwise stated. Deionized water was used to prepare all the buffers.

2.2 Cell culture

SH-EP1 cells (kindly provided by Dr. Ronald J. Lukas, Barrow Institute, Phoenix, AZ) were cultured in a humidified atmosphere at 37 °C with 5% CO₂ and 70% relative humidity. SH-EP1 cells are human epithelial cell line and they may share neuroepithelial origins with neurons.^{31, 32} Cells were grown in Dubelco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA) with 10% FBS (Invitrogen) with 100 units/ml penicillin and 100 µg/ml streptomycin (BioWhittaker, Basel, Switzerland). Cells were passaged with 0.05% Trypsin and 0.02% EDTA in Hank's balanced salt solution (HBSS; Sigma-Aldrich, St. Louis, MO) when they reached a confluency of approximately 75%. The average size of adherent SH-EP1 cells on gold chips was found to be around 50 µm.

2.3 Surface plasmon resonance microscopy (SPRM) recording

The imaging setup was a surface plasmon resonance microscopy system introduced by Zare et al.²⁴ The optical system comprises of a fiber coupled 680 nm super-LED light source (Qphotonics LLC, Ann Arbor, MI), an inverted microscope (Olympus X81) with total internal reflection fluorescence (TIRF) imaging attachment, and a charge-coupled device (CCD) camera (Pike F-032). The sensor chips were BK7 glass coverslips coated with ~2 nm chromium followed by ~47 nm gold. Each chip was washed with water and ethanol followed by hydrogen flame annealing to remove surface contamination before each experiment. A Flexi-Perm silicon chamber (Greiner Bio-One) was placed on top of the gold chip to serve as a cell culture well and electrochemical cell. 80 µL of 50 µg/mL collagen solution (prepared in 1% HAc solution) was added in the chamber and kept in an incubator overnight to achieve the surface modification prior to cells seeding. The thickness of collagen layer was estimated to be ~ 1.1 nm by an SPR experiment.

~5,000 SH-EP1 cells in 300-µL growth medium were added on a chip with a FlexiPerm well attached. After overnight incubation to allow the cells to attach and grow, the growth medium was replaced by isotonic ECF solution before each experiment.

2.4 Drug perfusion system

A gravity-based multi-channel drug perfusion system (SF-77B, Warner Instruments, CT) was used to control the local solution surrounding the target cell. The typical transition time between different solutions was less than 1 second.

3. Results and discussion

3.1 Hypertonic stimulation

We first investigated the cell responses to the hypertonic solution, which led to an increase in the extracellular osmotic pressure. Fig. 1(a) shows the SPRM image of a single cell adhered on a collagen-modified gold film in 300 mOsm isotonic solution before the hypertonic stimulation. The high quality SPRM images of single cells were captured with an objective-based system.^{24, 27} At t=53 s, we replaced the buffer with 350 mOsm (300 mOsm isotonic buffer + 50 mM mannitol), creating a hypertonic condition, and the change in the cell-substrate interactions was monitored continuously by recording the SPRM image over time (see Movie S1 in Supporting Material to view the video). The hypertonic buffer was introduced with a drug-perfusion system, which was able to change the buffer around the target cell with a transition time less than 1 second. Figs. 1b–d display three snapshots of the cell recorded at t=80, 160 and 325 s after the buffer change, respectively. Upon the change in the osmotic pressure, some regions of the cell decrease in intensity and appear as “holes” in the image, which last for a few minutes and gradually recover after ~4 minutes. These variations in intensity reflect the local movement of the cell membrane in contact with the substrate via the integrin-ECM linkage, and the SPRM video provides detail temporal and

spatial information of the local movement. The observation that the cellular response varies differently from location to location, demonstrates the need of a spatially resolved technique, and the value of the present approach. Note that the SPRM images of cells completely recovered after the removal of osmotic stimulation in terms of both morphology and intensity, which ensured that the experiment did not permanently damage the cell, at least in terms of SPRM images. Similar recovery was also reported in the recent publications,^{29, 30} in which the average mass density of cells did not significantly change before and after changes in osmotic pressure over 10 minutes.

To analyze the local information quantitatively, 12 regions of interest (ROI) were selected as shown in Fig. 1b. Region *R* is from the collagen-coated gold area without cell coverage, which serves as a reference, regions *S1–S5* are from cellular areas that display a small SPR intensity increase, and regions *W1–W6* are selected from the areas that exhibit large SPR intensity decrease, *i.e.*, the “hole” regions. The SPR intensity vs. time in each ROI is shown in Figs. 1e–f. Note that the bulk index of refraction change due to the introduction of 50 mM mannitol was corrected by taking the SPR signal from surrounding regions without cell coverage as a reference signal (see Section 2 in Supporting Material for details). *S* regions display a small and fast initial decrease in the SPR intensity, which is followed by a modest amount of increase and then reach a plateau within ~1 minute (Fig. 1e). In contrast, *W* regions show a large intensity decrease within the first 10 seconds and followed by a slow increase, reaching the same intensity level as the *S* regions after ~4 minutes. We denote the fast initial SPR intensity decrease as phase 1 and the later slow intensity increase process as phase 2.

SPR intensity is known to be extremely sensitive to the distance between the bottom membrane of the cell and the substrate,³³ and the large intensity decrease during the initial process of the osmotic modulation reflects local detachment of the cell bottom membrane from the substrate. We point out that although local buffer or ionic concentration changes can also change SPR intensity, the observed SPR intensity would correspond to ~0.5 M concentration increase inside the cell (count as NaCl solution) when stimulated by 150 mM mannitol. Such a concentration change within cells is too high to occur. Furthermore, the observed intensity change is highly localized, and diffusion of ions over the time period (several minutes) would have washed out any local concentration variations. Moreover, the observed signal was believed to reflect the vertical movement of cell membrane rather than local mass redistribution because of the following considerations. Firstly, the SPR intensity changes immediately after the introduction of osmotic pressure stimulation. It is too fast to be mass redistribution across the cell membrane, which usually takes place within minutes or even hours. Secondly, the change in SPR intensity was greatly relevant to the mannitol concentration, indicating the observed signal was a response to mechanical stimulation, such as the membrane movement. In addition, the SPR intensity could recover after the removal of the stimulation, further supporting the hypothesis that cell membrane movement was responsible for the SPR responses.

The driving force for the detachment comes from the sudden change in the osmotic pressure. The regions showing larger intensity decrease (*W* regions) have weaker cell-substrate adhesion strength than those showing smaller intensity changes (*S* regions). Using a seven-layer SPR model (see Section 3 in Supporting Material for details), we were able to establish a relationship between the SPRM intensity and cell-substrate distance, from which we determined the local cell-substrate distance from the SPRM intensity. Similar strategy has long-time been applied to measure the cell-substrate distance in many optical techniques including SPR¹⁹ and reflection interference contrast microscopy³⁴. The obtained results are in good agreement with the cell-substrate distance measured by fluorescence interference contrast microscopy, demonstrating the feasibility of such multi-layer optical model.³⁵ Fig.

1g shows the cell-substrate distance or displacement of the bottom membrane from the substrate as a function of time in phase 1. The displacement of *S* regions increases by a few nm over 1–2 s. In contrast, the displacement of *W* regions is as large as several tens of nm and takes much longer time. Note that the large variations in the displacement values are due to the intrinsic spatial variation of the cell-substrate interactions rather than errors in the measurement. Note also that in this model, cell-substrate distance was determined by the absolute SPR angle, which can be measured for each ROI. In our experiment, the average SPR angle in all 11 ROIs before hypertonic stimulation is 73.6 ± 0.3 degrees, which gives us an average cell-substrate distance (d_0) of 66 ± 12 nm. These results are consistent with previous reports on cells attached to a protein modified substrate.¹⁹

Following the initial SPR intensity decrease was a much slower intensity increase. It is known that when cells are exposed to a hypertonic solution, water flows out of cells, cytoplasm concentration increases, and the cells shrink. This process continues until a new equilibrium is established between the intra- and extracellular osmotic pressures. Consequently, cytoplasm refractive index (RI) increases, which is responsible for the intensity increase in all the regions of the cell. This process is ~10 times slower than the initial cellular detachment process, reflecting relative slow diffusion of water across the cellular membrane surface. In two recent papers, Robelek and Wegener studied the SPR responses of a monolayer of cells to the changes in osmotic pressure.^{29, 30} However, this work did not reveal the different response in different subcellular locations because it was basically lack of spatial resolution and therefore could only obtain the average response of a full monolayer of cells. Moreover, the present work distinguished the contribution from cell-substrate distance and that from cell swelling by virtue of a quantitative multi-layer optical model.

Since osmotic pressure was the driving force of the cell detachment, the vertical displacement of the cell bottom surface with respect to the substrate should depend on the extracellular osmolarity, which was controlled by mannitol concentration. Fig. 2a shows the linear relationship between the relative displacement ($\Delta d/d_0$) in each ROI and the concentration of mannitol (C_{mannitol}) in a series of hypertonic experiments by increasing the mannitol concentration. If we take the change in osmotic pressure ($\Delta \Pi$) as the driving force and take the relative displacement as the response, the following equation could be used to evaluate the Young's modulus of the local adhesion strength:

$$E = \frac{\Delta \Pi}{\Delta d/d_0} = \frac{RTC_{\text{mannitol}}}{\Delta d/d_0} \quad (1)$$

where E is the Young's modulus of the adhesion strength $\Delta \Pi$ is the change in extracellular osmotic pressure, $\Delta d/d_0$ is the relative displacement in phase 1, R is the gas constant, and T is the absolute temperature. Using equation 1, the average Young's moduli in *W* and *S* regions were estimated to be 0.16 ± 0.08 MPa and 1.00 ± 0.35 Mpa, respectively. The Young's modulus of cell-substrate adhesion measured in the present work is in good agreement with the previous report on mammalian cells, in which the cells were pressed by AFM tips to measure the mechanical response.^{36, 37} The average Young's modulus in *S* regions is 5 times larger than that in *W* regions. The local Young's moduli in all the ROIs can vary by up to 30 folds, which underscores the importance of imaging local adhesion strengths.

We noted earlier that the slow SPR intensity increase in phase 2, following the rapid intensity decrease in phase 1, was due to the increase in cytoplasm RI associated with the cell shrinkage. This conclusion is further supported by quantitative analysis described below. Studies have indicated that cells act as ideal osmometers,³⁸ where the equilibrium cell volume is inversely proportional to the extracellular osmotic pressure according to the

van't Hoff relation. Since the cytoplasm RI decreases inversely with the cell volume, and the SPR angle increases with RI, one expects a linear increase in the final SPR angular shift in phase 2 with mannitol concentration. Indeed we observed such a linear relationship between the SPR angular shift and mannitol concentration (red triangles in Fig. 2b). Furthermore, since the cell-substrate distance was known, we calculated SPR angular shift vs. mannitol concentration based on the RI of cytoplasm at different concentrations, and plotted the results as black squares in Fig. 2b. It is clear that the measured and calculated SPR angular shifts are in quantitative agreement, which further supports the interpretation of the SPR intensity increase in phase 2.

3.2 Sub-cellular mapping to cell-substrate interaction

By taking advantage of the high spatial resolution of SPRM, a map of cell-substrate distance could be created by performing similar analysis to each pixel in the image of the cell. Fig. 3 shows the calculated cell-substrate distance (or displacement of the cell bottom surface) image in phase 1, which was obtained by subtracting the cell-substrate distance at $t=50$ s from that at $t=60$ s. It is clear that different locations within the cell show quite different displacements under the same hypertonic stimulation. The weakly adhered regions, *i.e.*, “holes” in Fig. 1b show much larger displacement. Therefore, this displacement image could be considered as a map of the cell-substrate adhesion strength distribution, in which red locations have weaker adhesion strength. Similar results have been observed for about 50 cells (see Movie S2 in Supporting Material).

3.3 Hypotonic stimulation

The case of hypotonic stimulus was also investigated on the same cell by decreasing the extracellular osmolarity from 300 to 240 mOsm. The SPR intensities in *S* and *R* regions and *W* regions are shown in Figs. 4a and 4b, respectively. SPR intensities in all ROIs show a two-phase response, *i.e.*, faster increase followed by a slower decrease, which is opposite to that in the hypertonic experiment. More specifically, when the extracellular osmotic pressure decreased, two effects took place: 1) the bottom of the cell bottom was pushed towards the substrate quickly, resulting in the faster SPR increase in phase 1, and 2) water moved into the cell slowly to reduce the cytoplasm RI, which was responsible for the SPR intensity decrease in phase 2. The SPR profiles in phase 1 were also location-dependent. For example, *W* regions showed a much larger SPR increase in phase 1 than *S* regions did.

A quantitative analysis was carried out to obtain the displacement curves in each ROI in phase 1 (Fig. 4c). The cell swelling in phase 1 led to the movement of the cell bottom membrane towards the substrate by 8 ± 2 nm and 21 ± 5 nm in *S* and *W* regions, respectively, demonstrating the weaker cell-substrate interactions in *W* regions than those in *S* regions. Furthermore, the single pixel-based analysis was performed to obtain a cell displacement image (Fig. 4d), in which larger displacement (blue color) means weaker adhesion strength. The adhesion strength map (Fig. 4d) is in good agreement with that in hypertonic experiment (see Fig. 3), indicating that the cell-substrate adhesion strength can be evaluated by either hypertonically or hypotonically modulating the osmotic pressure surrounding the individual cells.

4. Conclusions

In conclusion, spatial mapping of local cell-substrate adhesion strength was achieved by monitoring the cell responses to a well-defined mechanical stimulus introduced by extracellular osmotic pressure with high resolution SPRM. The entire dynamic process of the cellular response to extracellular osmotic pressure was followed and studied to provide a detailed picture of cellular response under hypertonic or hypotonic conditions. This optical,

label-free and non-invasive technique measured the cell-substrate adhesion strength under natural conditions, which minimized the possible damage of external forces to cell behaviors. We believe that approach will provide a new tool for continuous monitoring of dynamic cell-substrate interactions in different cell movements, such as its adhesion, migration and detachment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. SPRM images of a single cell adhered to a substrate captured at $t = 40$ (a), 80 (b), 200 (c) and 325 s (d) in a hypertonic experiment. Red numbers, 1–6, mark the locations of “holes” (W regions), blue numbers, 1–5, mark the locations of adjacent regions (S regions), and purple “R” marks a reference region on gold in (b). The detailed ROI are described in the Section 1 in Supporting Material. SPR intensity changes in R and S (e) and W (f) regions. (g) The displacements of S and W regions vs. time in phase 1.

Figure 2.

(a) Relative displacements ($\Delta d/d_0$) in each ROI in phase 1 under the stimulation of a series of different concentrations of mannitol. (b) SPR angle shifts in phase 2 ($\blacktriangle$: measured, $\blacksquare$: calculated) induced by hypertonic solutions containing different concentrations of mannitol.

Figure 3. Mapping local cell-substrate adhesion strength, where larger displacement reflects weaker adhesion strength.

Figure 4. SPR intensity changes in *R*, *S* (a) and *W* regions (b) in a hypotonic experiment. (c) Local displacements vs. time in *S* and *W* regions in phase 1. (d) Mapping local cell-substrate adhesion strength, where larger displacement reflects weaker adhesion strength. Note that the negative displacement values reflect that the cell bottom surface moves closer to the substrate.

Scheme 1.

Schematic illustration of mapping cell-substrate interactions with SPRM. Osmotic pressure is introduced to move the bottom surface of the cell towards or away from the substrate, and the local displacement is imaged to create a map of adhesion strength.