A method to measure cellular adhesion utilizing a polymer micro-cantilever

Angelo Gaitas,^{1,2,a)} Ricky Malhotra,¹ and Kenneth Pienta³

¹PicoCal, Inc., 333 Parkland Plaza, Ann Arbor, Michigan 48103, USA

²Electronic Instrumentation Laboratory, TU Delft, Mekelweg 4, 2628CD Delft, The Netherlands

³Department of Urology, Johns Hopkins University School of Medicine, Marburg 121, 600 N. Wolfe Street, Baltimore, Maryland 21287-2101, USA

(Received 24 June 2013; accepted 6 September 2013; published online 19 September 2013)

In the present study we engineered a micro-machined polyimide cantilever with an embedded sensing element to investigate cellular adhesion, in terms of its relative ability to stick to a cross-linker, 3,3'-dithiobis[sulfosuccinimidylpropionate], coated on the cantilever surface. To achieve this objective, we investigated adhesive properties of three human prostate cancer cell lines, namely, a bone metastasis derived human prostate cancer cell line (PC3), a brain metastasis derived human prostate cancer cell line (DU145), and a subclone of PC3 (PC3-EMT14). We found that PC3-EMT14, which displays a mesenchymal phenotype, has the least adhesion compared to PC3 and DU145, which exhibit an epithelial phenotype. © 2013 AIP Publishing LLC.

[<http://dx.doi.org/10.1063/1.4821946>]

Micro-cantilevers have been used for cellular adhesion measurements in the past as part of atomic force microscopy systems (AFM).^{1,2} Typically a cantilever is coated with a protein that binds to a cell's membrane, and then it is retracted to measure adhesion.³ In some other instances, cells have been attached to a cantilever and brought in contact with other cells for cell-cell adhesion measurements.² Micropipettes have also been used to attach cells for adhesion measurements.⁴ Other techniques include centrifugation assays,⁵ shear flow,⁶ and cell poking.⁷

For this preliminary study we have developed specialized cantilevers made from polyimide offering very high compliance, sensitivity, robustness, and durability compared to conventional silicon or silicon nitride cantilevers. The cantilever has the advantage to operate in transparent and opaque liquid environments. A sensing element is embedded in the cantilever eliminating the need for the laser feedback used in AFMs (Fig. 1(a)). These cantilevers are used in a custom made scanning system capable of spanning 80- μ m distance in the z-axis enabling the measurement of large adhesive events.

In the present study we compared three human prostate cancer cell lines, DU145, PC3, and a subclone of PC3, PC3-EMT14. The first two, DU145 and PC3, show a typical epithelial pattern, while PC3-EMT14 shows a more mesenchymal morphology. The rationale of using these three phenotypically distinct cell lines was to identify differences in adhesion forces, if any, using our micro-cantilevers. This short communication is a concerted attempt to define an approach for evaluating cellular mechanobiology, as well as physical and adhesive properties by the inherent ability of cells to bind to artificially coated cantilever surfaces, namely, DTSSP, as described below. Our results demonstrate that the three cell lines are quite distinct in their property to bind to the DTSSP-coated cantilever. The results hold promise for

this cell biology technique to evaluate extracellular adhesion events utilizing a specialized sensor.

The dimensions of the cantilever play a critical role in the accuracy of the measurement as well as in determining the spring constant, which affects the sensitivity. While a lower spring constant offers higher sensitivity, extremely high compliance is not desired. The width of the cantilever is important in determining the number of cells that can be contacted during a measurement. The polyimide cantilevers are 40 μ m wide, 150 μ m long, and 2 μ m thick (Fig. 1). The first polyimide layer is 1.5 μ m thick. The sensing element is a thin film of Cr/Au with 2 nm/10 nm thickness. Then 0.5 μ m of polyimide is deposited to form the top layer. Cr/Au with 5/25 nm is deposited to form an absorption layer. A $3 \times 1.4 \times 0.5$ mm³ chip serves as the base of the cantilever. The cantilever has a spring constant k of ~ 0.059 N/m and a typical resistance between 160–170 Ω . The polyimide cantilevers were wired to establish electrical contact, and then they were insulated with polydimethylsiloxane (PDMS) to insulate the electrical contacts. The cantilevers are very stable in liquids.

In order to fabricate the micro-cantilever, first, a silicon wafer is oxidized, and then 1.5 μ m of polyimide is coated and patterned. Then the sensing element made of chrome/gold (Cr/Au) with thickness 2 nm/10 nm is deposited and patterned. Then the pads are formed by depositing titanium/gold/titanium (Ti/Au/Ti) 20 nm/500 nm/2 nm. A 0.5 μ m layer of polyimide is deposited and patterned to form the top layer. Cr/Au with 5 nm/25 nm is deposited to form the absorption layer. Finally the silicon wafer is etched, while the front side and the handles are protected, to form the cantilever. These sequential steps are schematically shown in Fig. 1(c).

Thin insulated wires were stripped at the ends and attached with conductive epoxy on each of the two pads. The chips were attached to a glass slide using PDMS. PDMS was used to insulate the exposed pads and wire on the chip. The cantilevers were placed in Coplin jars and immersed for 10 min in methanol, acetone, isopropanol-2, and distilled water. Then the cantilever was functionalized with the

^{a)} Author to whom correspondence should be addressed. Electronic mail: angelo@picocal.com

FIG. 1. (a) Scanning electron micrograph of the cantilever depicting the location of the embedded sensing element (in dashed lines), sandwiched between two polyimide layers and the gold absorption layer. (b) Light photomicrograph illustrating the cantilever in contact with PC3 cells. Magnification 100 $\times$. (c) Cantilever fabrication steps.

water-soluble cross-linker DTSSP (Thermo Scientific). DTSSP was dissolved in 5 mM sodium citrate buffer (pH = 5.0) at a concentration of 1.5 mM before use as DTSSP is moisture sensitive and degrades easily. The cantilevers were immersed in 1.5 mM DTSSP for 2 h at room temperature resulting in adherence of DTSSP to the gold surface by disulfide linkage. Following derivatizing with DTSSP the devices were immersed in phosphate buffered saline (PBS) for 5 min before the start of measurements.

The three human prostate cancer cell lines, DU145, PC3, and PC3-EMT14 were maintained in the Pienta laboratory at the University of Michigan Medical Center, Ann Arbor, MI. PC3-EMT14 was generated in Dr. Pienta's laboratory (unpublished observations). These cell lines were propagated in cell culture flasks in complete media consisting of RPMI-1640 GlutaMax supplemented with 10% Fetal Bovine Serum and an antibiotic antimycotic-mixture and placed in a humidified CO₂ incubator at 37°C in an atmosphere of 5% CO₂ and 95% air. The medium was changed every three days. The cultures were passaged every 7 days using 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA). For the purpose of measurements utilizing our cantilevers the cells were finally trypsinized and plated on sterile round glass coverslips placed inside 12 well cell culture plates. On the day of measurements a completely confluent layer of cells on the coverslips were first rinsed in PBS to remove all traces of media and placed on a glass slide and maintained under a warm layer of PBS.

A confluent layer of cells growing on a round coverslip was placed on a clean glass slide and the cells soaked in 20 μ l of warm PBS to prevent drying. All measurements were completed within a 30-min time window to maximize cell viability. The slide was placed on a microscope stage equipped with a motorized stage and a piezo-electric stage. The change in resistance of the deflection-sensing element of the micro-cantilever (with in-plane displacement) was directly measured using a micro-Ohm meter (Agilent Technologies, HP-34420A) and the data acquired with a LabVIEW program. A piezoelectric XYZ stage with 100- μ m

range and nanometer resolution on each axis (PiezoJena, Tritor 100) was used to finely position the sample directly underneath the microcantilever. Resistance vs. displacement curves are produced using the microcantilever and a sample on the XYZ piezoelectric stage. The piezoelectric XYZ stage used here allows us to monitor and measure interactions at ranges larger than 70 μ m.

The resistance of a typical sensing element is between 160 and 170 Ω . Additional resistances from the cabling and the wiring are less than 1 Ω and therefore do not have a significant contribution to the overall resistance. A schematic representation of the experimental setup is depicted in Fig. 2(a) showing the sample on the piezoelectric stage and the motorized stage, which is initially brought in close proximity to the microcantilever. The cantilever is maintained at a constant angle and the piezoelectric stage is moved while the cantilever's resistance is simultaneously measured. A representation of change in resistance vs. distance traveled is shown in Fig. 2(c). The electrical signal of the cantilever is on the y-axis in $\mu\Omega$ and the movement of stage in μ m on the x-axis. The stage is programmed to move by 70 μ m, and subsequently the resistance is recorded for every 1 μ m step movement of the stage. These fine incremental movements coupled with exquisite sensitivity of the probe enabled us to record min changes in resistance. We monitored simultaneously by real-time video recording the actual time of contact of the cantilever with the surface of the cells as shown by time-lapse pictures in Fig. 2(b).

In a typical graph of contact and re-traction of cantilever shown in Fig. 2(c) the numbers (1) to (6) denote events as follows and correlate with images shown in Fig. 2(b). From points (1)–(2) the electrical resistance is constant because the cantilever is not in contact with the surface of the cells while the piezoelectric stage is moved up vertically in micrometer incremental steps. At point (2) the cantilever makes initial contact with the cells and from (2)–(3) the cantilever makes further contact with the surface of the cells leading to a rise in the resistance. From (3)–(4) the cantilever is programmed to remain in contact with the cell surface for

FIG. 2. (a) Schematic representation of the cantilever in contact with the surface of cells in PBS. (b) Images of the light reflected from cantilever at different phases of its movement. A detailed description of these events is described in the text. (c) A typical graph of contact and re-traction of cantilever on a cell. The electrical signal of cantilever is on the y-axis in Ω and the movement of stage in μm on the x-axis.

15 min to enable strong binding/cross-linking of the DTSSP coated cantilever tip with the cell surface proteins and receptors. An increase in resistance of the cantilever seen in the graph between (3)–(4) is attributable to small changes in surface tension due to evaporation of PBS covering the cells. The time-scale of the actual adhesion measurement is much smaller ($<3\text{--}4\text{ s}$) compared to the time-scale where this type of drift becomes significant (over several min), and therefore the net change in resistance is negligible. From (4)–(5) the cantilever and the cells on the piezoelectric stage are programmed to move away from each other until the contact finally breaks off at (5). Note the images in (1)–(2) and (5)–(6) look similar, which is indicative of no contact of cantilever with the cell surface (however the cantilever is still immersed in PBS). The distances traveled from point (2) to point (3) and subsequently from point (4) to point (5) are indicative of relative adhesion forces, which in our observations vary between various cell types. Hypothetically, if there was no adhesion, the length of segment (2)–(3) and segment (4)–(5) would be the same. However, in the schematic shown in Fig. 2(c) the segment (4)–(5) is longer indicating that the cantilever has to move a greater distance before it breaks off contact with the cell. The force value of the difference in (2)–(3) and (4)–(5) corresponds to force necessary to break the adhesion from the surface. Finally the segment (5)–(6) is indicative of no contact between cantilever and cells and therefore the resistance remains constant. It should be noted that the contact area between the cantilever and the cells may be as large as $40\text{ }\mu\text{m} \times 30\text{ }\mu\text{m}$, therefore the cantilever may make contact with more than one cell simultaneously. This observation is further validated by the photomicrograph shown in Fig. 1(b) showing the tip of the cantilever in contact with the surface of monolayer of cells.

We first used a cantilever for measurement with cells placed on a coverslip. Then we performed baseline measurements by allowing the cantilever to come in contact with a glass slide with a few drops of PBS without any cells as a control. After completion of these sets of measurements, a different cantilever is used for the next cell line.

We converted the change in resistance reading to force by calibrating using the control measurement described above on a hard surface. We determined using these baseline measurements that a $1\text{ }\mu\text{m}$ movement represents approximately a $0.016\text{ }\Omega$ change in resistance in the cantilever. The

changes in resistance $\Delta R_{(4)-(5)}$ and $\Delta R_{(2)-(3)}$ (illustrated in Fig. 2(c)) were converted to changes in force $\Delta F_{(4)-(5)}$ and $\Delta F_{(2)-(3)}$ for both, the control (empty slide) and cellular contact measurement by multiplying the change in distance with the spring constant of the cantilever. The difference in the force between the cantilever retracting from the cells' surface and the cantilever pressing against the cell membrane is an indicator of the "stickiness" or adhesion of the sample. The difference between the actual cellular contact measurement and the control measurement gives us a fairly accurate idea of the changes in adhesive force and the ratio of these two measurements aids in comparing different cell lines by "normalizing" for each cantilever in an attempt to eliminate any cantilever-to-cantilever variations.

In order to demonstrate our method we compared the adhesive forces between the three human prostate cancer cell lines, PC3, DU145, and the PC3-EMT14 as well as the ratio of the cellular contact measurement to the control measurement with no cells. We also obtained measurements on a standard fibroblast cell line (3T3) during our standardization phase (data not shown). The data obtained from four independent experiments on each cell line is shown in Fig. 3. These initial results demonstrate that both PC3 and DU145 cells, which have a predominant epithelial phenotype, have stronger adhesive forces to DTSSP, while PC3-EMT14 has the least "stickiness" to the DTSSP coated cantilever. The average adhesion forces ($\Delta F_{\text{adhesion-cell}} - \Delta F_{\text{adhesion-control}}$) were $0.113\text{ }\mu\text{N}$ for PC3-EMT14, $0.46\text{ }\mu\text{N}$ for DU145, and

FIG. 3. Adhesion values (on y-axis in arbitrary units) as measured by change in resistance and normalized to respective control (no-cells) measurements are shown in PC3, DU145, and HR14 cells. $**P < 0.001$ vs. PC3 and DU145 using analysis of variance (ANOVA) and student's t test. $N = 4$.

0.72 μN for PC3 (average values from 4 separate experiments).

One of the confounding phenomena in cancer and cancer related deaths is metastasis resulting in uncontrolled spread of cancer cells from the primary tumor through the blood to new organ sites. It is now widely believed that aberrant activation of an epithelial-mesenchymal transition (EMT) plays a predominant role in the metastatic process.^{8–12} During EMT E-cadherin and other adhesion proteins are repressed and cell motility is increased.^{13–15} Epithelial cells exhibit greater adhesive strength compared to mesenchymal cells.^{10,14} We utilized DTSSP as a vehicle or tool to bind or cross-link the gold-coated cantilever surface with the proteins on the cell membrane. DTSSP binds in a non-specific manner directly to primary amine groups ($-\text{NH}_2$) on proteins, particularly lysines and the N-terminal amine.¹⁶ It is conceivable that the extracellular proteins and receptors present on the surface of these cell lines are distinctly different in their free amino groups that are available for binding to the DTSSP-coated cantilever. The differential ability of DTSSP to cross-link the receptors on the cell membranes of the three cell lines may explain their relative “stickiness” to the cantilever surface. It was recently demonstrated¹⁷ that receptor cross-linking Con A receptors with DTSSP could greatly increase cell adhesion utilizing Con A coupled to an AFM tip in NIH3T3 fibroblast cells. During the acquisition of EMT characteristics, cells are also known to rev up the expression of mesenchymal markers and also increased activity of matrix metalloproteinases.^{18,19} Thus PC-3EMT14 may present a completely different extracellular protein milieu to the DTSSP-coated cantilever that results in a diminished binding to the cantilever surface.

Although the results described in this report in terms of cellular adhesion are preliminary in nature, they reinforce earlier reports on tumor progression and the role of EMT to decrease cell adhesion and increase cell motility and invasion.^{8,10,13–15} Our technique utilizing a DTSSP-coated polymer cantilever with a deflection-sensing element represents an experimental cell biological tool and a method of interpreting the data for both mechanobiology and nanotechnology scientists to further explore cellular adhesive

properties. Further in-depth studies are warranted to expand the scope of this study to demonstrate the reproducibility of our micro-cantilever device and its utility in measuring adhesive forces as they relate to cancer cell biology.

The authors thank Dr. T. Li and Dr. W. Zhu for help with the set-up, the micro-cantilever fabrication, and preparation, Dr. H. Roca and Dr. S. Sud in Prof. Pienta's laboratory for help with cell culture and materials, Dr. S. Akiyama for his valuable feedback. The micro-cantilevers were fabricated at the Lurie Nanofabrication Facility of the University of Michigan, Ann Arbor. The work of was partially supported by the National Institutes of Health (Grant No. GM084520).

¹M. Benoit, D. Gabriel, G. Gerisch, and H. E. Gaub, *Nat. Cell Biol.* **2**(6), 313–317 (2000).

²M. Benoit and H. E. Gaub, *Cells Tissues Organs* **172**, 174–189 (2002).

³U. Dammer, O. Popescu, P. Wagner, D. Anselmetti, H. Guntherodt, and G. Misevic, *Science* **267**(5201), 1173–1175 (1995).

⁴E. Evans, R. Merkel, K. Ritchie, S. Tha, and A. Zilker, in *Methods for Studying Cell Adhesion*, edited by P. Bongrand and A. Curtis (Springer, Berlin, 1994).

⁵N. John, M. Linke, and H.-W. Denker, *In Vitro Cell Dev. Biol.* **29A**, 461–468 (1993).

⁶R. Alon, D. A. Hammer, and T. A. Springer, *Nature* **374**, 539–542 (1995).

⁷G. I. Zahalak, W. B. McConnaughey, and E. L. Elson, *J. Biomech. Eng.* **112**, 283–294 (1990).

⁸J. P. Thiery, *Nat. Rev. Cancer* **2**, 442–454 (2002).

⁹S. E. Moody, *Cancer Cell* **8**, 197–209 (2005).

¹⁰J. M. Lee, S. Dedhar, R. Kalluri, and E. W. Thompson, *J. Cell Biol.* **172**(7), 973–981 (2006).

¹¹R. Kalluri and R. A. Weinberg, *J. Clin. Invest.* **119**(6), 1420–1428 (2009).

¹²M. Yu, A. Bardia, B. Wittner, S. Stott, M. Smas, D. Ting, S. Isakoff, J. Ciciliano, M. Wells, A. Shah *et al.*, *Science* **339**, 580–584 (2013).

¹³B. M. Gumbiner, *Nat. Rev. Mol. Cell Biol.* **6**, 622–634 (2005).

¹⁴X.-S. Ke, Y. Qu, N. Goldfinger, K. Rostad, R. Hovland, L. A. Akslen, V. Rotter, A. M. Øyan, and K. H. Kalland, *PLoS ONE* **3**(10), e3368 (2008).

¹⁵K. Lee and C. M. Nelson, *International Review of Cell and Molecular Biology* (Elsevier, Amsterdam, 2012), Vol. 294.

¹⁶A. Lodge, Thermo Fisher Scientific, private communication (2013).

¹⁷A. Chen and V. T. Moy, *Biophys. J.* **78**(6), 2814–2820 (2000).

¹⁸Z. Wang, Y. Li, D. Kong, and F. H. Sarkar, *Curr. Drug Targets* **11**(6), 745–751 (2010).

¹⁹C. Min, S. F. Eddy, D. H. Sherr, and G. E. Sonenshein, *J. Cell. Biochem.* **104**(3), 733–744 (2008).