Effects of Trypsin and Cytochalasin-B Treatments to Cell Traction Forces

A study on the cell traction forces of keratinocytes treated with trypsin and cytochalasin-B using liquid crystal-cell traction force transducer

Chin Fhong Soon[1, 2], Nafarizal Nayan[1]
[1]Microelectronic and Nanotechnology-Shamsuddin Research Center (MiNT-SRC)-Biosensor and Bioengineering Laboratory,
Faculty of Electrical and Electronic Engineering,
Universiti Tun Hussein Onn Malaysia
83000 Batu Pahat, Johor, Malaysia
soon@uthm.edu.my

Mansour Youseffi[3], Nick Blagden[2], Morgan Clive Thomas Denyer[2]
[2]School of Life Sciences [3]School of Engineering, Design and Technology-Medical Engineering,
University of Bradford
Bradford BD7 1DP, United Kingdom
m.denyer@bradford.ac.uk

Abstract— **This study aimed at characterizing the responses and cell traction forces of keratinocytes after treated with trypsin and cytochalasin-B using a liquid crystal based cell traction force transducer. The physical restructuring of the cell and the deformation lines induced in the liquid crystal surface were profiled using phase contrast microscopy. The liquid crystal based cell traction force transducer allowed experiments involving the dissolution of the cytoskeleton via cytochalasin-B and trypsinization to identify clear differences in cell responses to the two treatments indicated that the attachment of the cells was mediated by integrins and enforced by actin filaments. This work indicates that the new liquid crystal based sensor can be used to interrogate the kinetic and cytoskeletal responses of cells to the infusion of compounds known to interact with the actin cytoskeleton and adhesion proteins.**

Keywords- Liquid crystals; cytochalasin-B; trypsin; keratinocyte; cell traction force

I. INTRODUCTION

The ability of a cell to adhere and contract is closely associated with the structural integrity of a cell. In wound healing, keratinocyte migration and proliferation involve the formation of stress fibres as a result of actin filament polymerization [1]. Actomyosin in epithelial cells may not be used for driving cell motility alone, but may function to provide static and coordinated contraction in closing a gap in the epithelium [2]. Monitoring the force generated in a single cell would provide a more complete picture of the contractile functionality of a cell, which in turn, could be useful in pharmacological applications.

In search for a material that is suitable to function as a cell traction force transducer based on force-deformation measurement, we propose the use of cholesteric liquid crystals (LC). In the context of biocompatibility, cholesteryl ester liquid crystals have been shown to be non-toxic, provide affinity for cells attachment and to be thermally stable between 20 ^{o}C to 50 ^{o}C [3]. In many respects, liquid crystals are the basic structure of the phospholipid bilayer [4]. In addition, cholesterol moieties have been positively described with several attractive advantages in enhancing the physical properties of cells during attachment and proliferations [5]. Liquid crystals are highly flexible and have linear viscoelastic responses to cell derived traction forces [6].

Cell traction forces are the forces exert by a cell tangentially on a surface. These forces are associated with the forces generated within the cell and transmitted via the focal adhesion to the underlying surface. Any disruption to the cell biological system would disturb the transmission of forces to that surface. In this paper, the nature of the adhesion and contraction of HaCaT cells on the liquid crystals coated substrate was examined by disrupting the cytoskeletons using cytochalasin-B and cleaving cell membrane adhesion proteins by using crude trypsin.

II. MATERIALS AND METHODS

A. Preparation of lyotropic liquid crystal substrate

Ester based cholesteryl liquid crystal [7, 8] compounds were mixed to synthesize a shear sensitive cholesteryl liquid crystal using the procedures reported in [6]. The physical mixtures contained in a glass vial were heated up to an isotropic phase at melting temperatures of 74-77 ^{o}C. When all the solids were melted, 5 µl of the fluid mixtures were deposited on a glass cover slip and immediately transferred to a centrifuge and spin coated at 1000 rpm for a minute. The thickness of the liquid crystals coating was controlled using thin gold grids (Agar Scientific, UK) with a thickness of about 100 µm. The glass cover slip coated with cholesteryl ester liquid crystals were immersed in a Petri dish containing 6 ml of RPMI-1640 culture media (Aldrich Sigma, UK).

978-1-4673-1666-8/12/$31.00 ©2012 IEEE

B. *Cell culture on the liquid crystals and treatment with cytochalasin B and EDTA-Trypsin*

Human keratinocyte cell line was prepared [3] and plated at a density of 500 cells/cm^2 and maintained in RPMI-1640 media supplemented with fetal calf serum, L-glutamine, fungizone, penicillin and streptomycin. The cell suspensions were added into three petri dishes containing the liquid crystal coated cover slips in culture media and each Petri dish was incubated at 37 ^{o}C for 24 hours. After incubation, the cells cultured on the liquid crystals were treated with 5 µl of 30µM cytochalasin B for one hour at 37 ^{o}C. Cytochalasin B (35 mg/ml, Sigma Aldrich) was solubilized in 0.042 % (v/v) ethanol (in distilled water). As a control, 0.042 % (v/v) of ethanol was applied to the cells in an identical cell culture.

Under similar conditions of cell culture and treatment time, independent experiments were conducted with the application of 0.25 % Trypsin-EDTA solution to a liquid crystal substrate cultured with HaCaT cells in which, cell bath was replaced with EDTA-trypsin solution. These experiments were conducted to identify the source of the forces exerted by the cells on the liquid crystal substrate. For the three treatments, the Petri dishes were placed on a hot stage maintaining at 37 ^{o}C while time lapsed images were captured via a GDX-2 phase contrast microscope bundled with a GT VisionCX digital camera every 5 minutes over an hour. These experiments were performed in triplicates.

C. *Measurement and Mapping of Cell Traction Forces*

The measurement and mapping of the cell traction forces in a two-dimensional force map were performed based on our method published in [9]. Briefly, the Poisson's ratio of single deformation line was characterized using actin cytoskeleton relaxation technique. In terms of the stress points to the liquid crystal, the focal contact area was estimated using immunofluorescence staining of the vinculins. With the physical parameters of the liquid crystal deformation determined, force-deformation relation was established using Hooke's in considering the linear viscoelasticity of the cholesteryl ester liquid crystals at low shear rate [6].

III. RESULTS AND DISCUSSION

After culturing HaCaT cells on the liquid crystal substrate for 24 hours, cells were found attaching to the liquid crystal surface and the attachment caused intense deformation lines (dark/bright groove lines) on the surface of the liquid crystals in both test and control cultures as seen using a phase contrast microscopy (Figure 1a-c, 0 minute). The physical interactions of cells and liquid crystals were studied using cytochalasin-B and trypsin. In this experiment, cytochalasin-B disrupted the functionality of the actin filaments and crude trypsin digested the integrins receptors that mediate cells attachment to a substrate. For the control and two test culture experiments, three cells of similar size were chosen for the treatment and analysis.

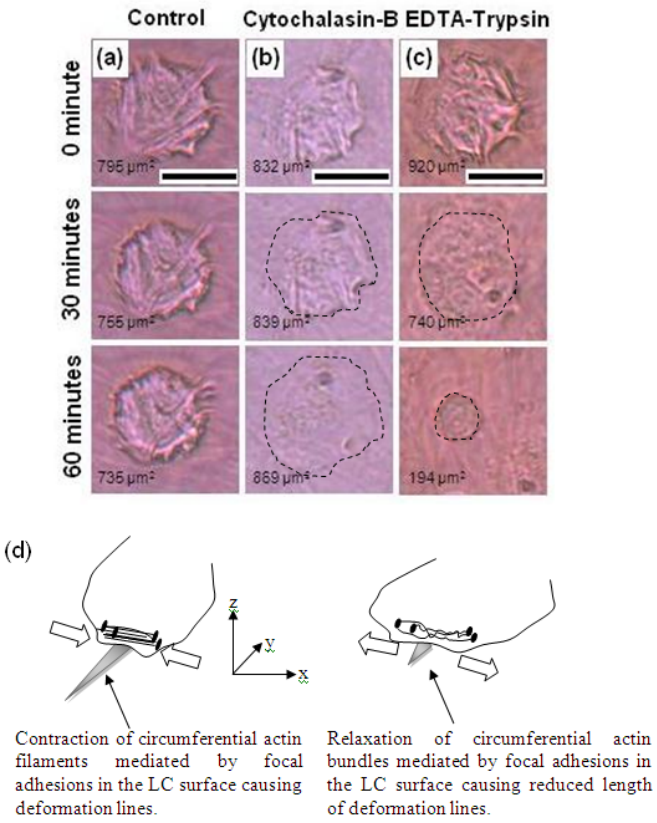

Figure 1. Phase contrast micrographs for the treatment effects of (a) 0.0042 % (v/v) ethanol (control), (b) 30 µM cytochalasin B and, (c) 0.25 % trypsin to the cells in phase contrast photomicrograph taken every 5 minutes over a period of 60 minutes. The area of cell attachment in µm^2 is as shown in each micrograph. (d) A depiction showing a correlation of the deformation line formed on the liquid crystal film with lateral shear forces induced by the cell's circumferential actin filaments polymerization anchored at the focal adhesions. Treatment with cytochalasin-B caused F-actin depolymerization, force degeneration and diminishing deformation lines of the liquid crystal film. (scale bar = 25 µm).

At 0 minute before any treatment, the adherent cells on the liquid crystals were not fully spread but wrinkles of liquid crystals could be observed at the cell periphery (Figure 1a-c). Throughout the 60 minutes treatment with ethanol as a control, no major changes of cell activity, a slight change in the projected area of attachment and deformation lines of the liquid crystals were observed (Figure 1a). Conversely, addition of cytochalasin-B to the cultures caused remarkable changes in cell morphology and liquid crystal's deformation lines distribution which correlates with the cells responses (Figure 1b). After 30 minutes with the treatment of cytochalasin-B, the deformation lines shortened and the attachment area of the cell increased slightly from 832 µm^2 to 839 µm^2 (Fig. 1b, 30 minutes). By the end of 60 minutes treatment, the cell remained attach with a flattened cell body with a surface area of 869 µm^2, and there were no intense deformation lines observed (Fig. 1b, 60 minutes). In this case, cytochalasin-B did not affect the cell surface receptors; yet, these receptors maintained their function of anchoring the cells to the liquid crystal surface even though the cell's body

The project is sponsored by Fundamental Research Grant Scheme (FRGS) Phase 1/2012, Grant No. 1050, Ministry of Higher Education Malaysia.

had collapsed. Every deformation line represented the traction forces exerted via lateral shearing is believed to arise from a group of focal adhesions that are interconnected to the circumferential F-actin and diffusely arranged at the edges of the cell membrane [9]. A focal adhesion is a group of specific macromolecules such as vinculin, paxillin or talin connected to a pair of αβ integrins that directly interface with the extracellular matrix [10].

However, the effect of trypsinization on the HaCaT cells was different from the cytochalasin-B treatment (Figure 1c). The initial attachment area of the cell was 920 μm^2. After 30 minutes of trypsinization, the deformation lines disappeared in a cell's projected area of 740 μm^2 and cells had lifted off the surface of liquid crystal towards the end of the 60 minutes treatment (Figure 1c). EDTA-Trypsin cleaved the adhesion proteins that bound cells to the substrate, caused the periphery of the cell edges to detach and cell area covered by cells to decrease remarkably to 194 μm^2. The detachment of cells from the surface allows the elasticity of the cells to pull cells into rounded morphology, leading to a decrease in cells size. The trypsinization and cytochalasin-B treatment show that the tension generated by cells on liquid crystals is dependent on the actin cytoskeleton and the relationship with the integrins coupling cells to the surface.

These focal adhesions are anchoring points for the F-actin on the liquid crystal surface and they control the area of adhesion. F-actin polymerization induces an oblique inward force via the contact points which in turn, laterally shears the liquid crystal membrane resulting in an outward growing deformation lines (Figure 1d). As a result of applying cytochalasin-B which inhibited the polymerization of F-actin, the traction forces of cells degenerated with the depolymerization of actin filaments, which in turn, reduced the deformation lines on the liquid crystal surface (Figure 1d).

The result of the cytochalasin-B and trypsin treatments suggested that the forces generated within the cells are dependent on the cytoskeleton when they are cultured on liquid crystal coated substrate. Subsequently, these forces are transmitted to the surface via the focal adhesions. The result of the cytochalasin B and trypsin treatments clearly demonstrated that liquid crystal is sensitive and flexible enough to differentiate the integrity of F-actin or integrins. This is a degree of sensitivity and flexibility that has not been reported previously. The shear sensitivity of the liquid crystals achieved in this study was tuned by the concentration of the cholesteryl chloride incorporated in the mixtures of the compound. The level of flexibility can be explained by the properties of the uncross-linked liquid crystal molecules in comparison with polymer based measurement techniques that lack of such flexibility [11, 12].

Fig. 2 shows the time varying cell traction force map (Figure 2, right) as a result of cytochalasin-B treatment (Figure 2, left). Before cytochalasin-B treatment the forces were regularly distributed at the periphery of the cell (Figure 2a). The cell traction forces expressed by this single quiescent cell (Figure 2, 4) ranged between 68 and 90 nN. The cell traction forces expressed at four different regions (Region A-D) of the cell decreased from a maximum force of ~90 nN to ~38 nN within a treatment time of 30 minutes and diminished (0 nN) drastically thereafter (Figure 4). Consistent with other repeats of the experiment, the cells remained attach to the liquid crystal surface with a broadly spread body (Figure 1b). This result shows that 30 µM of cytochalasin-B is a drug that provides reasonably fast action in depolymerizing the actin cytoskeleton. The decreasing cell traction force rate is ~ 2 nN/min.

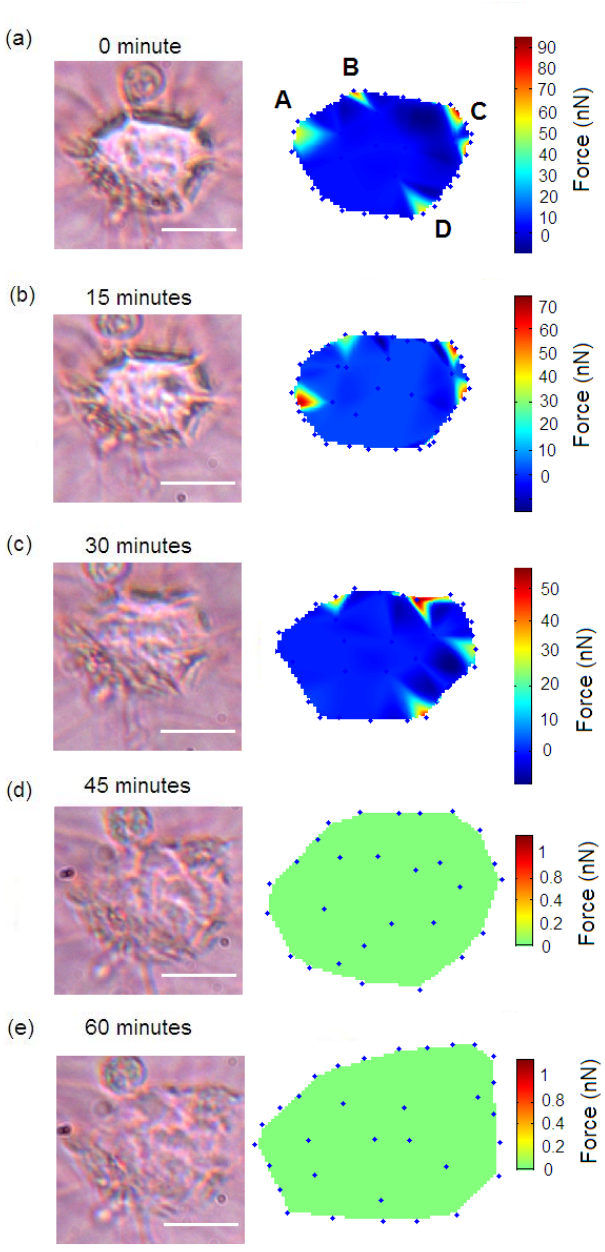

Figure 2. Effects of single dose 30 µM cytochalasin-B (left) to the cell traction forces (right) at different regions of a cell.

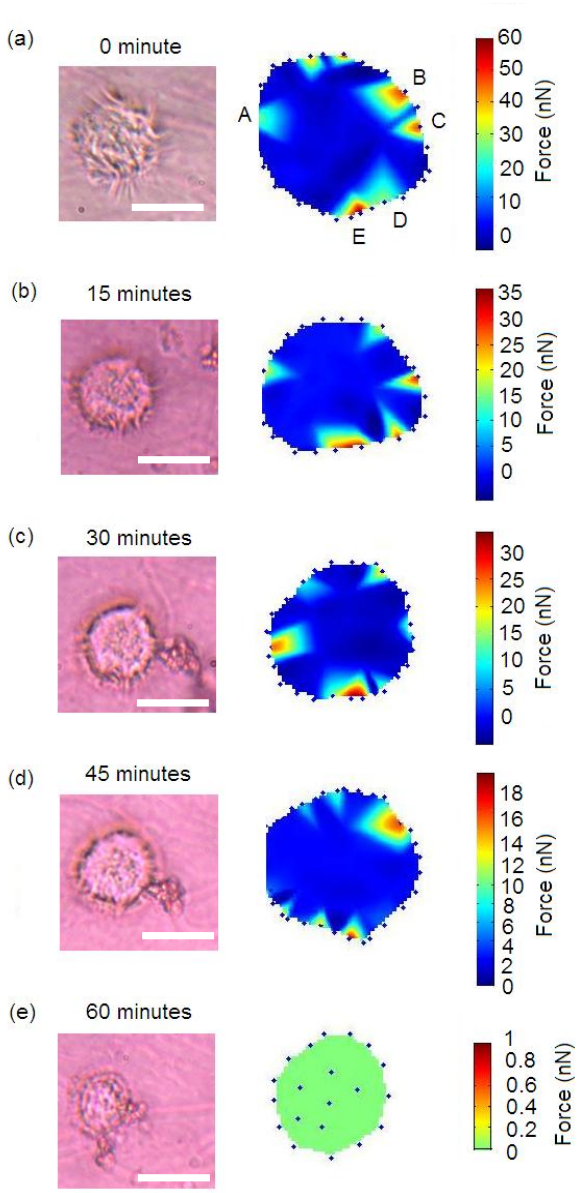

Figure 3. Phase contrast micrographs of HaCaT cells attached to the liquid crystal substrates treated with single dose trypsin at 0.25% (scale bar = 25 µm) and the associated cell traction forces at (a) 0 minute, (b) 15 minutes, (c) 30 minutes, (d) 45 minutes, and (e) 60 minutes.

For studying the effects of trypsin to cell traction forces, a cell with expressions of regular traction forces (20 - 60 nN) at the edges of the cell was selected (Figure 3). Comparatively, the cell displayed a distinctly different cell traction force response when treated with crude EDTA-trypsin. The release of the cell traction forces was much slower compared with cytochalasin-B treatment and the cell fully released the traction forces towards the end of the trypsinization process (Figure 3). At the end of the trypsinization process, this cell detached from the liquid crystal surface and formed into spherical shape as shown in Figure 3. The forces dropped drastically within first 30 minutes of treatment and decreased gradually thereafter as shown in Figure 5. In this particular example, the average cell traction force per cell decreased at a rate of ~0.4 nN/min (Figure 5). The effect of trypsinization (0.25 %) to the cell traction forces is fivefold slower than the cytochalasin-B treatment (30 µM), that were performed within the same treatment period.

The diffusion kinetic of both cytochemical drugs and interactions with the cells is interesting to be investigated. Fig. 4 shows the changes of cell traction force induced by cytochalasin-B treatment over a treatment period of 60 minutes. At the first 15 minutes of cytochalasin-B treatment, the initial reaction of cell traction forces is low (~ 0.4 nN/min) (Figure 4). This reaction of cell traction forces is intensified in the next 45 minutes (~2.33 nN/min). For the influx of cytochalasin-B into the intracellular matrix, molecules of cytochalasin-B may take time to diffuse via the cell membrane into the cell system in order to interact with the actin cytoskeleton. Once the molecules reach the actin filaments, the depolymerization process occurred and causes the degeneration of forces within the cell at a faster reaction rate.

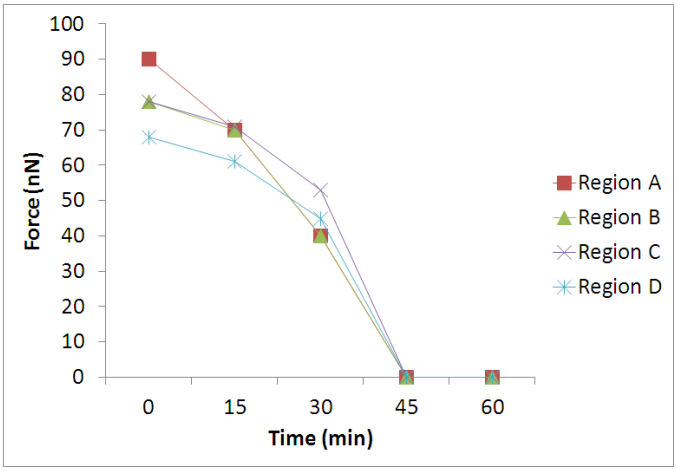

Figure 4. Changes of cell traction force induced by cytochalasin-B treatment over a treatment period of 60 minutes

Contrarily, for trypsin treatment, the reaction of cleaving the adhesion proteins is fast (~2 nN/min) in the first 15 minutes but decreased (~0.78 nN/min) in the next 45 minutes as shown in Figure 5. The initial enzymatic reaction is direct because adhesion proteins are exposed on the cell membrane compared with the actin depolymerization process that occurred within the cell. However, the enzymatic reaction decreased after 15 minutes of treatment. This may have may have been due to the serum in the media blocking the enzymatic action of the trypsin along with the tight coupling of adhesion proteins attaching the cell membrane to the extracellular matrix. This creates a large resistant for further enzymatic reaction. Hence, the kinetics of both cytochemical treatments is quite different and the effects can be investigated by monitoring the reaction of cell traction forces.

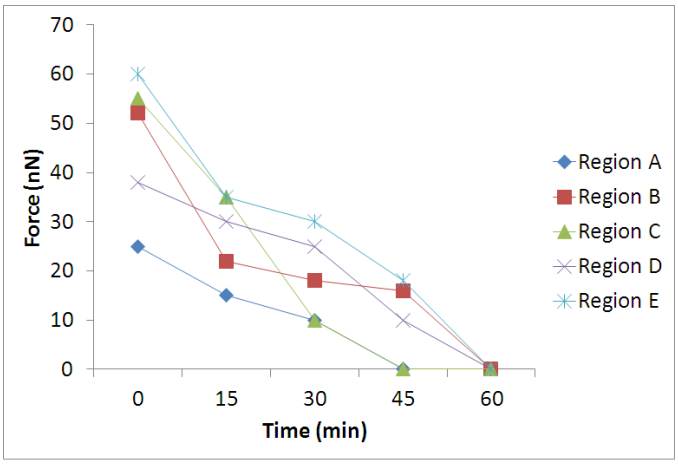

Figure 5. Effects of trypsinisation to the cell traction forces at different region of a cell over a treatment period of 60 minutes.

IV. CONCLUSIONS

The cholesteryl ester based lyotropic liquid crystals have promoted positive cell surface interactions and they can be used as a cell adhesion substrate for human keratinocytes. This study has shown that the viscoelastic surface of the liquid crystals was very sensitive to different structural changes of the adherent cells. The kinetic of cell traction force reaction rate to cytochalasin B and trypsin can be studied by monitoring the dynamics of cell traction forces using a liquid crystal based cell traction force transducer.

ACKNOWLEDGMENT

The author would like to acknowledge Dr. Steve Britland, Dr. Peter Twigg and Dr. Samira Batista Lobo for providing technical advice and many thanks to Professor Des Tobin for his technical support. The authors are grateful to the Malaysia Ministry of Higher Education for research funding support (FRGS Phase 1, Vot No. 1050).

REFERENCES

[1] E. A. O'Toole, "Extracellular matrix and keratinocyte migration," Clinical and Experimental Dermatology, vol. 26, pp. 525-530, 2001 2001.

[2] S. Pellegrin and H. Mellor, "Actin stress fibres," Journal of Cells Science, vol. 120, pp. 3491-3499, 2007.

[3] C. F. Soon, M. Youseffi, N. Blagden, R. Berends, S. B. Lobo, F. A. Javid, and M. Denyer, "Characterization and biocompatibility study of nematic and cholesteryl liquid crystals " in Proceedings of the World Congress on Engineering vol. 2, 2009, pp. 1872-1875.

[4] D. M. Small, "Liquid crystals in living and dying systems," Journal of Colloid and Interface Science, vol. 58, pp. 581-602, 1977.

[5] J. J. Hwang, S. N. Iyer, L.-S. Li, R. Claussen, D. A. Harrington, and S. I., "Self-assembling biomaterials: Liquid crystal phases of cholesteryl oligo(l-lactic acid) and their interactions with cells " Proceedings of the National Academy Sciences of the United States of America, vol. 99, pp. 9662-9667, 13 July 2002 2002.

[6] C. F. Soon, M. Youseffi, T. Gough, N. Blagden, and M. C. T. Denyer, "Rheological characterization of the time-dependent cholesteric based liquid crystals and in-situ verification," Materials Science and Engineering C, vol. 31, pp. 1389-1397, 2011a.

[7] B. P. Nguyen, M. C. Ryan, S. G. Gil, and W. G. Carter, "Deposition of laminin 5 in epidermal wounds regulates integrin signaling and adhesion," Current Opinion in Cell Biology vol. 12, pp. 554-562, 2000.

[8] E. J. Klein and A. Margozzi, "Exploratory investigation on the measurement of skin friction by means of liquid crystals," NASA Technical Memorandum, vol. X-1774, pp. 1-11, May 1969.

[9] C. F. Soon, M. Youseffi, T. Gough, N. Blagden, and M. C. T. Denyer, "Development of a novel liquid crystal based cell traction force transducer system," Biosensors & Bioelectronics, http://dx.doi.org/10.1016/j.bios.2012.06.032, 2012., in press.

[10] B. Geiger and A. Bershadsky, "Exploring the neighborhood: adhesion-coupled cell mechanosensors," Cell Press Journal, vol. 110, pp. 139-142, 2002.

[11] T. Oliver, M. Dembo, and K. Jacobson, "Traction forces in locomoting cells," Cell Motility and the Cytoskeleton vol. 31, pp. 225-240, 1995.

[12] K. A. Beningo, "Flexible substrata for the detection of cellular traction forces," Trends in Cell Biology, vol. 12, pp. 79-84, 2002b.