Effects of an Enzyme, Depolymerization and Polymerization Drugs to Cells Adhesion and Contraction on Lyotropic Liquid Crystals

Chin Fhong Soon, *Member, IAENG,* Mansour Youseffi, Nick Blagden and Morgan Denyer.

***Abstract*— A novel cell force transducing assay based on liquid crystals has been developed. Human keratinocytes (HaCaTs) attached to and formed localized deformation on the surface of highly flexible cholesteryl ester liquid crystals. Cells have shown affinity to the lyotropic phase of the cholesteric liquid crystals which was immersed in culture media. In studying the nature of the attachment, 30µM cytochalasin-B and 0.25% Trypsin-EDTA were applied in independent experiments to qualitatively evaluate the force transmitted from the cytoskeletons and adhesion proteins to the liquid crystals substrate. 2% formaldehyde was used to fix the cells and to interrogate the mechanical creep effects in the liquid crystals. Cytochalasin-B reduced the forces exerted by the cells on the liquid crystals indicating that the liquid crystal surface could be used to sense forces generated internally by actin filaments. This study was supported by an additional experiment in which cells attachment was inhibited by the trypsin indicating the forces induced on the liquid crystals by the actin filaments were transmitted to the surface via protein couplings, i.e., focal contacts. Cells morphologies were also distinctly different in both treatments. The study on the creep effects at micro scale showed that a constant stress on the material imposed a regular strain on the material. Liquid crystals has shown stability in response to a constant and long term stress over a period of three days. The experiments demonstrated that the cholesteric liquid crystals could provide a flexible substrate to which cells readily attached, whilst enabling stable transduction of forces generated internally and transmitted to the liquid crystals film via cell surface receptors over a period of several days.**

***Index Terms*— Cell adhesion, Cell contraction, Human keratinocytes, Lyotropic Liquid Crystals.**

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Chin Fhong Soon, Morgan Denyer, Nick Blagden are with the Institute of Pharmaceutical Innovation, University of Bradford, BD7 1DP, United Kingdom. (Phone: 01274 234747; Fax: 01274 236060; e-mail: s.c.fhong@bradford.ac.uk).

Mansour Youseffi is with the School of Design, Engineering and Technology-Medical Engineering, University of Bradford, BD7 1DP, United Kingdom. (e-mail: m.youseffi@bradford.ac.uk).

I. Introduction

The ability of a cell to adhere and contract informs on the structural integrity of a cell and the cytoskeletal expressions. Numerous techniques based on silicone rubber, polyacrylamide with fluorescence markers, quartz microbalance and patterned PDMS have been developed to measure cell traction forces [1]. Soft substrate techniques showed promise in sensing contractile responses in high resolution but these techniques required a coating of extracellular matrix (ECM) proteins (e.g. collagen, fibronectin, matrigel, etc.) to promote cells adhesion. Hence, it is controversial whether the cells responses to these substrates were mediated by ECM proteins or the substrate stiffness [2]. To simplify the system, we aimed to use Lyotropic liquid crystals (LC) to measure cell forces without functionalization with ECM proteins. Lyotropic liquid crystal will function more effectively for the following two reasons:

a) Cell membranes are constructed from amphiphile molecules of lyotropic liquid crystals. Hence, liquid crystal might form a good adhesion layer.
b) Shear sensitive liquid crystals might be elastic, flexible and sensitive to external perturbations.

One of the goals of this study is to determine how cells attached to such liquid crystals.

By definition, lyotropic liquid crystals mean liquid crystals forming in the presence of a solvent [3]. They are found in many living systems such as cell membrane and serum lipoproteins. Lyotropic liquid crystals encompass the gel phases and lamellar liquid crystalline phases. Once exposed to a solvent, the mesogens would re-orientate the polar and non-polar part of the molecules to adapt in the water. Non-polar molecules do not dissolve at an lipid-water interface and are insoluble in bulk. One of the examples of amphiphilic molecules is the cholesteryl esters that could form stable water insoluble monolayer [4]. The insoluble membrane could function as a flexible culture substrate that may allow the forces generated to be monitored. In this study, the forces generated will be studied by using cytochalasin-B and EDTA-Trypsin because they can disrupt cytoskeleton and cell attachment. Cytochalasin-B extracted from the fungus, Drechslera dematioideum inhibits cell division by inhibiting formation of contractile microfilaments [5],[6], depolymerized cells microfilament [7] and prohibited keratinocyte migration [8]. EDTA-Trypsin is a common solution used in cell culture which is applied to digest integrins on the surface of the cell membrane hence preventing cells from attaching to any surface. The liquid

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crystals used in this study is a viscoelastic material [10] and may undergo accommodating creep in response to the long term forces associated with cells attachment. Fixing the cells with Formaldehyde would stabilize the forces on liquid crystals [11], hence, changes on the liquid crystals deformation over time could relate to the degree of creep under constant stress. The aim of this work was to study the flexibility and stability of the liquid crystals in response to stress exerted due to the adhesion and contraction of the human keratinocytes cell line (HaCaTs) in response to an enzyme, polymerization and depolymerization drugs.

II. Experimental

A. Lyotropic liquid crystal coating and cell culture

Lyotropic liquid crystals were formed by immersing a glass cover slip coated with cholesteryl ester liquid crystals in RPMI-1640 culture medium prior to cell plating. Cholesteryl ester liquid crystal has a chemical structure of a cholesterol that consists of a hydrophilic head except that the hydrophilic tail is an ester bond instead of a hydroxyl group (-OH). The preparation of shear sensitive cholesteryl liquid crystals and coating were as described previously [12]. After the cover slip was coated, cell suspensions were re-harvested using a standard culture procedure [10] and ready to be used for the next experiment. In the study of thermo-analytical method, these liquid crystals are stable over the room and incubation temperature 37^0C and biocompatible to HaCaTs cell line [12].

B. Treatment with Cytochalasin B, EDA-Trypsin and Formaldehyde

Cytochalasin B (Sigma Aldrich) were diluted in 0.042% (v/v) ethanol in distilled water (35mg/ml). Before the application of cytochalasin B, HaCaT cells were cultured on cholesteryl ester liquid crystal in RPMI-1640 culture media at a density of 2 x 10^4cells/ml. The RPMI-1640 media was supplemented with Fetal Calf Serum, L-glutamine, Fungizone, Penicillin and Streptomycin for cell culture. Two flask of similar cultures were prepared for the control and Cytochalasin-B treatments. Subsequently, they were incubated at 37^0C for 24 hours. After incubation, the cultured cells were treated with 5µl of 30µM Cytochalasin B for one hour at 37^0C. As a control, 0.042 (v/v)% of Ethanol in the distilled water was applied to the cells under the same culture conditions. These experiments were repeated three times.

Under similar cell culture conditions and treatment time, independent experiments were conducted with the application of 0.25% Trypsin-EDTA solution to a substrate cultured with HaCaT cells. The old media were discarded before 3ml of Trypsin-EDTA solution was added.

Following the two studies, we identified the source of stress on the liquid crystals as being due to the physiological contraction by cells and not due to natural film deformation. A constant stress needed to be applied in order to investigate if more localized deformation lines would be formed (creep) within 96 hours of treatment time. After discarding the old media, 3ml of 2% formaldehyde was applied to the cells cultured on liquid crystals. Subsequently, measurements of deformation length were taken to examine if any slow deformations occurred when the cells were fixed at 37^0C.

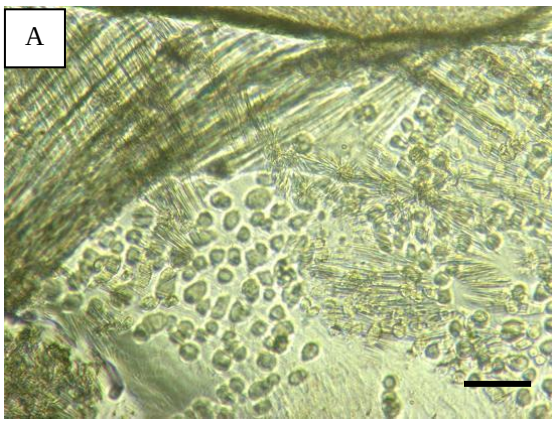
For the cytochalasin-B and EDTA-trypsin treatment, the petri dishes were placed on a hot stage maintaining at 37^0C while 5-minutes time lapsed images were captured using a phase contrast microscope for one hour. The treatment with formaldehyde was kept in the incubator at 37^0C and imaged using a phase contrast microscope every 24 hours up to 96 hours. Identification of the lyotropic phase was performed with a crossed polarising microscope under the condition where the cover slip coated with liquid crystals was removed from the culture media.

III. Result and Discussion

A. Identification of lyotropic phase of liquid crystals

When the bulk cholesteryl ester liquid crystals were immersed in a solvent, the amphiphilic lipids molecules re-orientated with the hydrophilic head pointing towards the water and hydrophobic carbon chain towards the bulk liquid crystals. Such a self-assembly characteristic has literally transformed the cholesteric liquid crystals into the uni-lamellar or multi lamellar layer interlaced by water layers of lyotropic liquid crystals. The ternary system (water-lipid interface) has been described as a translucent layer, basically showing fluid like properties [13] as has been observed in Fig. 1A. Under the illumination of phase contrast microscopy, the phase changes could not be seen when the liquid crystals membrane ripped off the bulk lyotropic liquid crystals due to hydrodynamic pressure (Fig. 1A). The unique optical structures of lyotropic lamellar phase of liquid crystals are well documented and they were usually studied using crossed polarizing microscopy [14]-[19]. When the sheared liquid crystals were examined in a crossed polarising microscope, defects of the layer were projected with focal conic textures that have been identified as lyotropic Smectic or lyotropic lamellar mesophase of the liquid crystals (Fig. 1B).

However, under equilibrium condition, the lyotropic lamellar layer is essentially a transparent layer where cells are attached. Under phase contrast microscopy, no major phase changes were observed when cells contracted on the thin membrane except dark/bright deformation lines (Fig. 1A).



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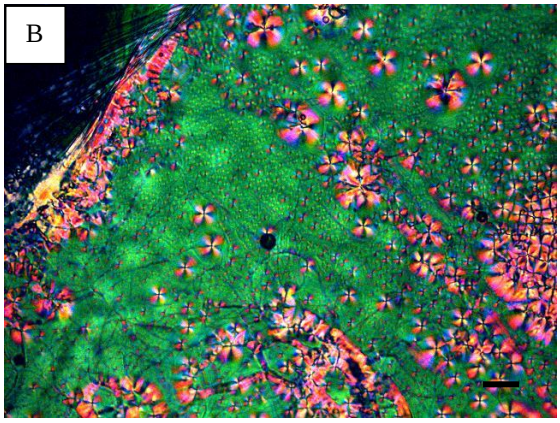

Fig. 1. Lyotpropic liquid crystals ripping of the bulk lyotropic liquid crystals imaged with (A) phase contrast and (B) crossed polarising microscopy. Phase contrast microscopy shows the cells attached on a liquid crystal film ripping off the bulk liquid crystals while the crossed polarising microscopy shows the focal conic texture of a typical lyotropic lamellar phase (Scale bar: 25µm)

B. Effects of Cytochalasin-B and EDTA-Trypsin to cells attached to liquid crystals

The objective of this experiment was to validate if actin filament is the main cytoskeleton involved in supporting HaCaTs attachment on LC and consequently causing the liquid crystal deformation lines. After 24 hours culture of HaCaTs on the liquid crystals, cells were attached on the LC and the attachment may made intense deformation lines (dark/bright groove lines) on the surface of the LC in the test and control cultures. For control and two test culture experiments, three cells of similar size were chosen. Under the phase contrast microscopy, HaCaTs attached to LC were not fully spread but ruffles could be observed at the cell periphery at 0 minute (Fig. 2A, B and C). When ethanol was added to the control through in 60 minute, no major changes of cell activity and deformation lines of the liquid crystal were observed (Fig. 2A).

Addition of cytochalasin B to the cultures caused remarkable changes in both cell morphology and liquid crystal's deformation lines distribution when compared with the control. Time lapsed images taken every 30 minutes (Fig. 2B) show the effects to HaCaTs and the deformation lines correlated with the cells response. By 15 minutes of treatment, HaCaTs did not show much changes of morphology but the deformation lines started to shrink (Image not shown). After 30 minutes, the attachment area of the cell seemed expanded even though the ruffles on the cell body were greatly reduced. A marked reduction of deformation lines was identified when membrane ruffles were reduced. By the end of 60 minutes treatment, the cell remained attach, relaxed with a flattened cell body and the intense deformation lines of the liquid crystal have disappeared and turned into fine lines (Fig. 2B). Every deformation line represented the force exerted via the compression arising from a group of focal adhesions. Following the depolymerization of actin filaments, the force generated in the cells were reduced and this was implied by the reduction of deformation lines. From this experimental result, it is clearly understood that cytochalasin B has inhibited the function of actin filament in supporting HaCaTs contraction. In this case, Cytochalasin-B did not detached cells from the surface indicating that the actin polymerization which response in generating the force within the cells were not associated with anchoring the cells to the surface, hence, were mediated by the focal adhesions [20]. This attachment to the liquid crystals was probably maintained by the interaction of the focal adhesions and liquid crystals. Focal adhesions are a group of specific macromolecules assemblies such as vinculin, paxillin or talin connected to a pair of αβ integrins that directly interfaced to the ECM [21]. This is also where the mechanical force from cytoskeleton is transmitted to the extracellular matrix [22]. In contrast, we could not find this type of transducing effects when cells attached to the petri dish (stiff surface) (Fig. 3A) were treated with cytochalasin-B (Fig. 3B). Comparatively, other soft substrate force measurement method such as PDMS, polyacrylamide and collagen sheet [2], [23], [24] could not provide such a flexibility and localized deformations as presented by liquid crystals because of the rigidity which was limited by the cross linkers in the polymer.

Experiments performed with EDTA-trypsin were to confirm that cells adhesion to liquid crystals was mediated by integrins or adhesion proteins as suggested by the result in the cytochalasin-B treatment. However, the previous result was very different from the trypsinisation effects to HaCaTs on liquid crystals where a total loss of liquid crystal deformation line were observed (Fig. 2C). Crude EDTA-Trypsin cleaved the adhesion proteins that bound cells to the substrate, caused the periphery of the cell edges to detach and cell spread area were reduced. After 60 minute of trypsinisation, the cell changed into more rounded morphology, shrunk and cells had lifted off the surface of liquid crystals (Fig. 2C). This indicated that the actin filaments had restructured to sustain a more rounded morphology.

C. Direction of HaCaT relaxation and shrinkage of CELC deformation lines

Apart from causing the cell morphology changes, we discovered that cytochalasin-B experiment not only showed the relaxation of the cells but also provided information about the force produced through the growing direction of deformation lines which is directly correlated with the cells pinching force [23]. A group of contracted cell with elongation shapes was treated with 30µM cytochalasin B in an hour on a hot stage set at 37^0C. Before treatment with cytochalasin B, contracted cells indented the surface of the LC with deformation lines growing in outward directions, perpendicular to the cell contraction force direction as seen in Fig. 4. The reaction of the LC deformation lines could be explained by Newtons' third law of action and reaction. The forces applied to the LC by the cells was equal to the repelling forces by the LC. The stronger the compression, the more intense is the deformation line as seen using a microscope. After treatment with cytochalasin-B for 30 minutes, the deformation lines of LC made by the cells were very much shortened in an inward direction perpendicular to the cell relaxation direction (Fig. 4C). From Fig. 4A and 4C, the relaxation force direction is shown opposite to the cell contraction force direction. The relaxation of HaCaTs were verified by an observation of increase in cell lengths (123µm) compared with the original cell lengths (102µm). Similar to the previous cytochalasin-B experiment, the cells remain attached on the LC with some fine deformation lines left around the cells at the end of the experiment.

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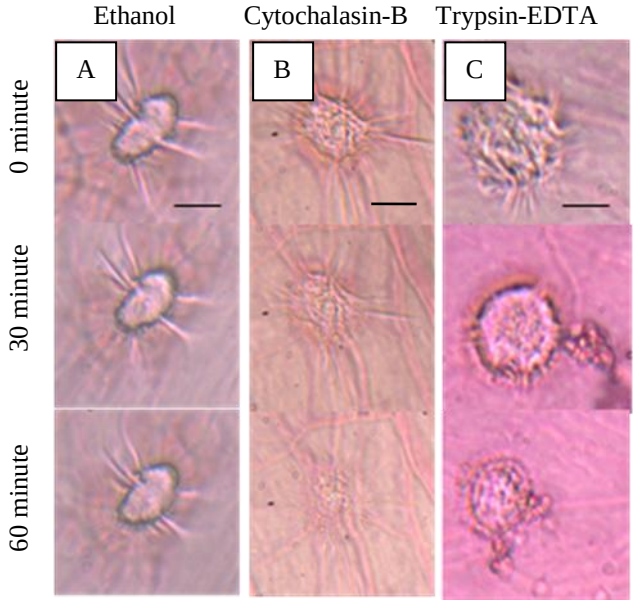

Fig. 2. Application of single dose (a) 0.042% Ethanol (Control), (b) 30µM Cytochalasin B in 0.042% Ethanol, (c) 0.25% Trypsin-EDTA solution on Cells adhered on lyotropic liquid crystals in 60minutes treatment. (Scale bar: 25µm)

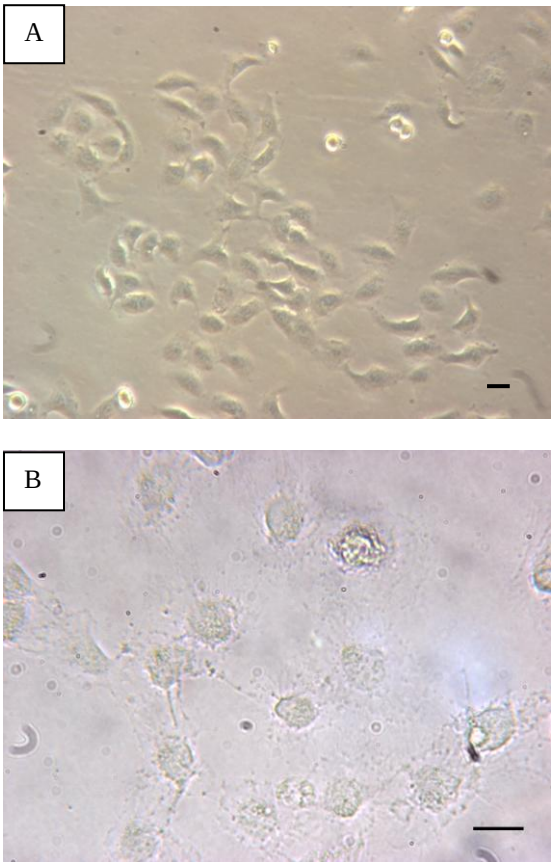

Fig. 3. HaCaTs on petri dish (A) before and (B) treated with 30µM of Cytochalasin B for 1 hour. (Scale bar: 25µm)

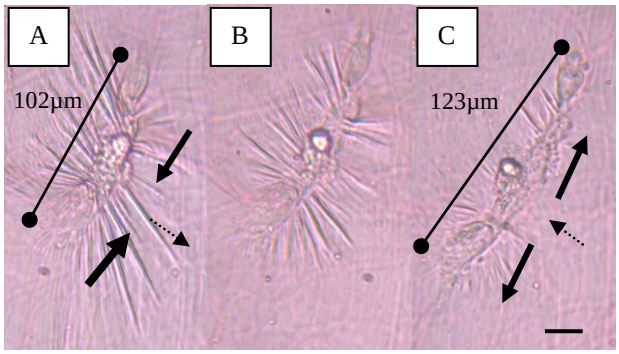

Fig. 4. Elongated HaCaTs on LLC in response to 30µM cytochalasin B and direction of CELC deformation lines shrinkage at (a) 0 minute (b) 30 minutes and (c) 60 minutes. Solid arrows show the direction of the cell contraction and relaxation after treated with cytochalasin B. Dotted arrows show the repelling force directions. (Scale bar: 25µm)

D. Effects of Formaldehyde on cells adhered to liquid crystals

A group of cells were chosen for treatment with 2% formaldehyde because they could impose higher stress on the material and would accelerate the creep effects if any. The cell fixation with 2% formaldehyde was not successful in the preliminary experiment when the treatment was followed by a wash with Hanks Balance Salt Solution (HBSS). HaCaT cells immediately relaxed after the wash with HBSS, in turn, released the deformation lines formed on LC. This is a reaction due to the difference in osmotic pressure [26]. Hence, same volume of solutions was maintained in the petri dish through out the 4 days of monitoring. Visually, HaCaTs on the liquid crystals maintained similar morphologies through out the 4 days of treatment (Fig. 5A-5E). To examine if there were any quantitative changes, measurements were taken for some indentation sites as shown in Fig. 5 (Labeled as L1-L10). After 24 hours of treatment with 2% formaldehyde, a drop of deformation length was observed on most of the deformation lines induced (Fig. 6). There were evidences [11], [25] in literature that the cells continue to shrink up to 16 hours after fixation in formaldehyde. To monitor the effect of creep under a constant stress, it is necessary to monitor the deformation lines at least 16 hours after fixation. After 16 hours, one could assume forces exerted by the cells would be constant and if there are any changes after that would be down to the re-ordering of the LC molecules.

The cells maintained the deformations through out the 24-72 hours treatment but deformation lines decreased slightly after 72 hours (Fig. 6). A decrease in deformation lines after 72 hours might be due to the deterioration of the biological material even though the cells stress was fixed or it could be due to the cells were fixed in a lower concentration of formaldehyde. However, a suggestion to keep formaldehyde fixation to the minimum (concentration and treatment time) has been proposed because part of the cytoskeleton and antigen expressions in lymphocytes were reduced after 3 days of fixation with 4% formaldehyde [25]. The experimental findings in the cells response to formaldehyde [25] seemed to have good correlations with our experimental result in which liquid crystal was innovated as a cell force transducer. This experiment has evidenced a negative creep effect on the liquid crystals up to 72 hours of

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formaldehyde treatment. The results were confirmed with three repeat of experiments. Obviously, the strain formed on the liquid crystals did not exceed the plasticity of this viscoelastic material. In addition, our rheology result on lyotropic liquid crystals indicated a decrease of viscosity when the biomaterial was immersed in culture medium for more than 3 days [27].

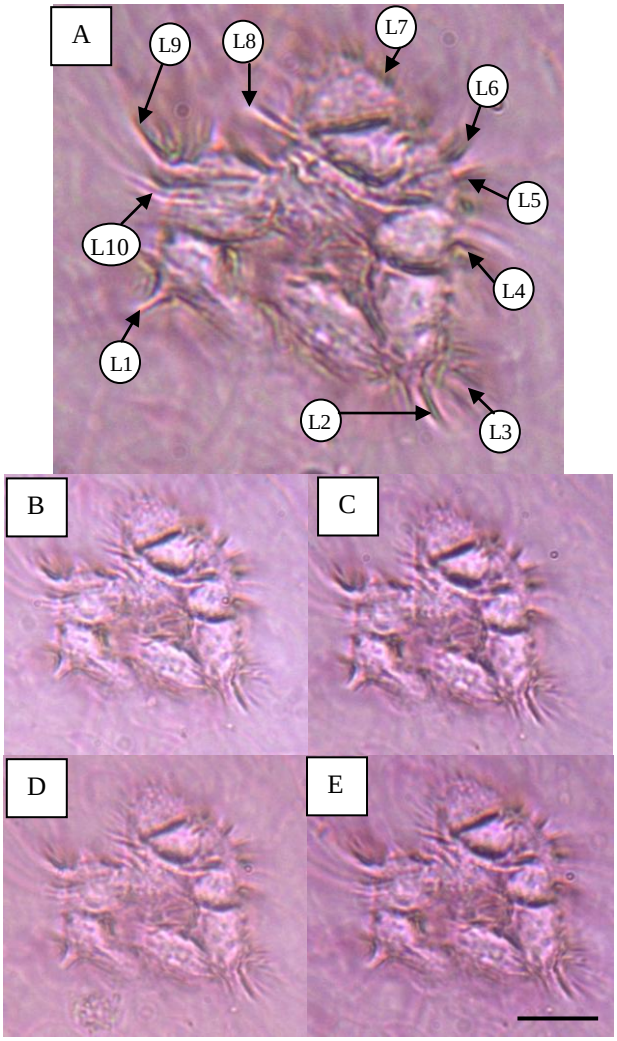

Fig. 5. (A) Pre-fixed cells and fixed cells with formaldehyde after (B) 24 hours, (C) 48hours, (D) 72hours, and (E) 96 hours (Scale bar: 25µm).

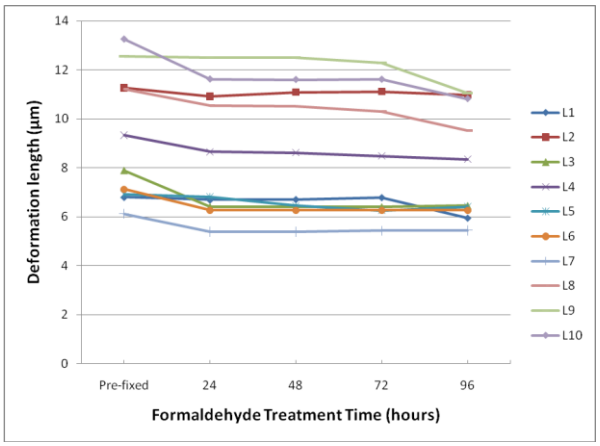

Fig. 6. Deformation length (measured for labeled L in Fig. 5A) due to the contraction of cells after treatment with 2% formaldehyde for a period of 4 days.

IV. CONCLUSIONS

This work has provided a clear indication that HaCaTs adhesion and spreading on the lyotropic liquid crystals were facilitated by adhesion proteins and forces generated by cells to the liquid crystals were associated with the forces generated by cytoskeleton. Without the formation of focal adhesions, the forces generated by the actin polymerization could not be transmitted to the liquid crystals substrate. Both focal adhesions and actin polymerizations are responsible for the force transmitted onto the extracellular matrix with a higher contribution from actin filaments. Cells contractions on the liquid crystals were characterized by pinching or lateral compression effects on liquid crystals. The slight decrease in deformations on the liquid crystals after 72 hours of treatment with formaldehyde might be due to the weakening of the gel. Future work is to determine the group of integrins and focal adhesions involved.

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