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COMPARISON OF TWO DIFFERENT INDENTATION TECHNIQUES IN STUDYING THE IN-SITU VISCOELASTICITY BEHAVIOR OF LIQUID CRYSTALS

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ABSTRACT

Liquid crystal is a new emerging biomaterial. The physical property of liquid crystal plays a role in supporting the adhesion of cells. Nano and microball indentation techniques were applied to determine the elastic modulus or viscoelasticity of the cholesteryl ester liquid crystals in the culture media. Nano-indentation results (108 ± 19.78 kPa, N = 20) agreed well with the microball indentation (110 ± 19.95 kPa, N = 60) for the liquid crystal samples incubated for 24 hours at 37°C, respectively. However, nanoindentation could not measure the modulus of the liquid crystal (LC) incubated more than 24 hours. This is due to the decreased viscosity of the liquid crystal after immersion in the cell culture media for more than 24 hours. Alternatively, microball indentation was used and the elastic modulus of the LC immersed for 48 hours was found to decrease to 55 ± 9.99 kPa (N = 60). The microball indentation indicated that the LC did not creep after 40 seconds of indentation. However, the elastic modulus of the LC was no longer measurable after 72 hours of incubation due to the lost of elasticity. Microball indentation seemed to be a reliable technique in determining the elastic moduli of the cholesteryl ester liquid crystals.

Keywords: nanoindentation, microball indentation, liquid crystal, in-situ, elastic modulus, viscoelasticity.

INTRODUCTION

Liquid crystals was discovered with applications in biosensing (Lockwood *et al*., 2006; Mashooq *et al*., 2014; Chin Fhong Soon *et al*., 2015) and it is a biomaterial that could interface with cells (Hwang *et al*., 2002). To extend the exploration of liquid crystals (LC) as a new class of biomaterials, it is essential to determine their physical properties for further applications in cell and tissue engineering. However, for the cell and tissue engineering application, the stability of the amphiphilic molecules of the liquid crystals were subjected to the interaction with cell culture media (C. F. Soon *et al*., 2011). Past studies (Fairhurst *et al*., 1998; Helfrich, 1994; Porcar *et al*., 2005) indicated that the introduction of water molecules to the LC could cause the hydrophobic tails and hydrophilic heads of the LC molecules to re-orientate and self-assembled into multi-tier lyotropic system. However, the report on the time-dependent viscoelasticity properties of the liquid crystal is scarce. In addition, the major technique used for the study of viscoelastic behaviour of the liquid crystals is rheometry which is not able to provide information on the in-situ viscoelastic behaviour of the liquid crystal in the cell culture media.

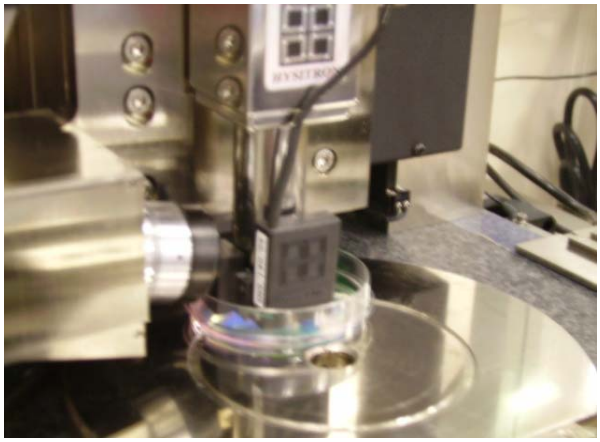
Therefore, this paper aims at examining the viscoelasticity properties of the cholesteryl ester liquid crystals after exposure to aqueous solution over a period of time by using the nanoindentation and microball indentation techniques. The data acquired also enabled the computation of the elastic modulus of the liquid crystals. Studying the physical properties of the liquid crystals in-situ is fundamental and essential in understanding the physical strength of the cholesteryl ester liquid crystals in a fluidic environment.

MATERIALS AND METHODS

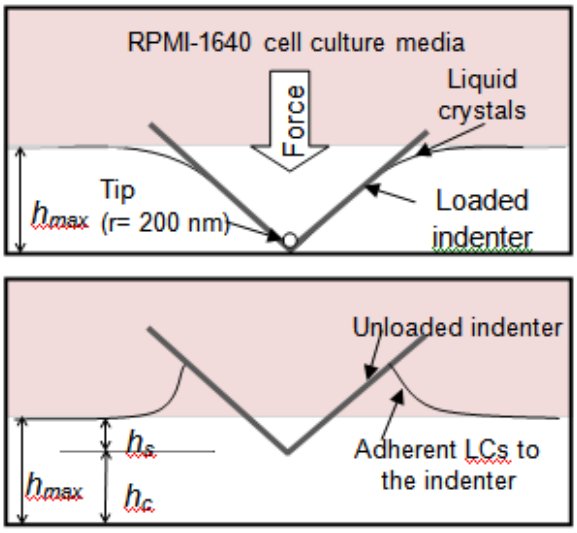
Nano-indentation techniques

The study is aim to determine the elastic modulus (*E*) of the LC substrates in culture media using the nano-indentation technique. First, cholesteryl ester liquid crystal was prepared and the gel with a thickness of approximately 100 µm was coated in a petri dish as described in (Chin Fhong Soon *et al*., 2014). Next, 6 ml of RPMI-1640 cell culture media was added to the petri dish coated with LC coating and the LC sample was incubated at 37°C for 24 hours. After incubation, the sample containing the media was placed in a Hysitron Triboscope nano-mechanical testing system (Figure-1a). For the nano-indentation, a Berkovich pyramidal indenter was loaded to the test sample at rate of 750µN/s and paused for 3 seconds before it was unloaded to complete the measurement (Figure-1b). The hold time was compensation to any creep of material. In the Hysitron Triboscope 3.5 software linked to the system, the load-displacement curve for the LC sample was obtained. Similar measurements were repeated to three similar LC samples at twenty different indentation sites. For the nano-indentation, the loading hold time, fluid buoyancy, and number of indentation cycles were handled with great care

because the measurements were highly sensitive to any changes to these factors.



(a)



(b)

Figure-1. (a) In-situ nano-indentation of the cholesteryl ester liquid crystals in the culture media. (b) Graphical diagrams illustrating the loading and unloading of an indenter and the associated displacement depths. The parameter, r denotes the radius of a tip.

Statistical Package for Social Sciences (SPSS) software was used to plot the data points of load-displacement obtained from the Hysitron Triboscope 3.5 software. Based on the unloading curve of the load-displacement graph, the contact stiffness, *S*, of the LC samples was then computed using the power law fitting (Doerner *et al.*, 1986; Oliver *et al.*, 1992; Pharr, 1998),

$$S = \frac{dp}{dh}(h = h_{\max}) = Am \cdot \left(h_{\max} - h_f\right)^{m-1} \quad (1)$$

where p is the loading force in μN and h is the displacement. The parameters, h_{max} and h_f are the maximum displacement and final displacement in nm, respectively. A and m are the curve fitting parameters were computed by the Triboscope software.

Based on the contact depth and load-displacement curve data, h_c was calculated (Oliver and Pharr, 1992) as follows:-

$$h_c = h_{\max} - h_s \quad (2)$$

$$h_s = \varepsilon \frac{P_{\max}}{S} \quad (3)$$

where, P_{max} is the maximum loading force and h_s is the perimeter contact depth. The indenter geometric constant, ε = 0.75.

With the parameter h_c computed, the contact area for an ideal Berkovich indenter can be established using the following equation (Oliver and Pharr, 1992),

$$Area = 24.5 h_c^2 \quad (4)$$

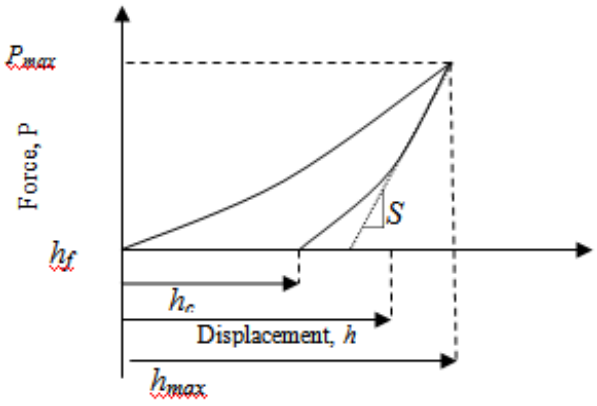


Figure-2. A graph of load-displacement curve.

With the contact area estimated, the reduced modulus, E_r can be calculated based on,

$$E_r = \frac{S\sqrt{\pi}}{2\sqrt{Area}} \quad (5)$$

where the reduced modulus, E_r is a measurement determined by the contact stiffness of the LC sample and the indenter parameters. Subsequently, the following equation (Doerner and Nix, 1986) was used to compute the elastic modulus of the liquid crystal:-

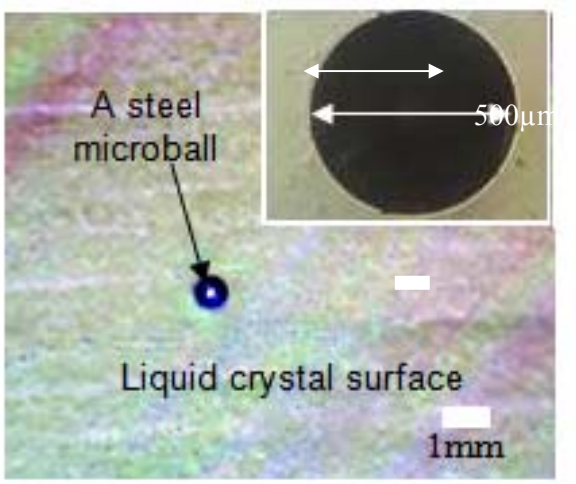
$$\frac{1}{E_r}=\frac{1-v^2}{E}+\frac{1-v_i^2}{E_i} \quad (6)$$

where E and v are the elastic modulus and Poisson's ratio of the test samples. E_i (1141 GPa) and v_i (0.07) are the same parameters defined for the diamond Berkovich pyramidal indenter (Pharr, 1998). The Poisson's ratio for liquid crystals was estimated at 0.5, similar to the viscoelastic rubber. The equation indicated that the physical properties of the test sample and indenter play a role together in the overall measurement. The specifications of the indenter can be obtained from the datasheet provided by the manufacturer of the nano-mechanical system.

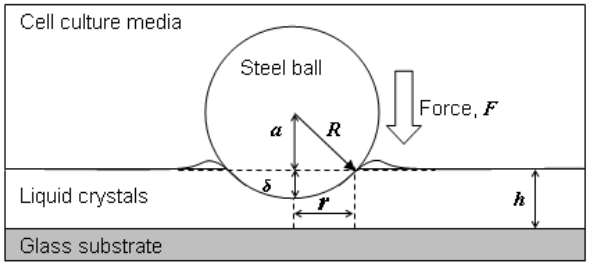
Microball indentation technique

Microball or spherical indentation was previously applied in studying the modulus of soft permeable and semi-permeable membrane based on the bi-axial deformation principle. (Ahearne *et al*., 2005; Dimitriadis *et al*., 2002; Munevar *et al*., 2001). A Digital camera system was used to capture the lateral deformation induced by a sphere load to a suspended membrane in air or fluid. Based on the theory of Hertz's contact mechanics, the elastic modulus of a membrane can be determined. This is the first use of similar concept in determining the elastic modulus of the liquid crystals coated to a petri dish in the presence of culture media. Three similar LC coatings with approximate thickness (h) of 100 µm were prepared in petri dish filled with 6 ml of cell culture media. These samples were incubated at 37ºC for 24, 48, and 72 hours, respectively.

After the designated period of incubation, the sample was placed on a heating pad with heating temperature regulated at 37ºC. At a constant temperature, the chrome steel ball (simple bearings co., UK) having a diameter of 500 µm and density of 7.849 g/cm^3 (Figure-3a) was placed gently using a pair of tweezers to the LC surface. Based on the datasheet provided by the manufacturer, the chrome ball has a mass and weight of 0.5 mg or 5 µN, respectively. However, the weight of the steel ball was corrected at ~ 4.6 µN due the effect of fluid buoyancy.



(b)



(b)

Figure-3. (a) A chrome ball of 250 µm in radius on the surface of the LC surface in the presence of culture media (Scale bar: 1 mm); Inset: The microcopy image of a steel ball. (b) A representation illustrating the microball indentation to the surface of the liquid crystal substrate in-situ and the parameters associated with the indentation.

Measurements of creep were performed to determine a suitable loading time to the LC surface. The measurement to creep was carried out by loading the steel ball to the LC surface at a time interval of 10 seconds and increased to 60 seconds. After the time interval, the ball was removed and a series of images at an time interval of 5 seconds were recorded using a digital camera fitted to a Nikon TS-100 inverted microscope.

For every time interval, the data of the deformation on the LC surface was captured and geometrical data of the steel ball were used to calculate the elastic modulus of the LC. The diameter of the contact impression formed on the LC surface was measured using ImageJ software after the steel microball was removed. The steel microball has a radius of R when in contact with the LC surface caused the formation of a circular impression area with a radius of r (Figure-3b). The displacement (δ) of the LC surface moved downwards at the centre of the steel microball (Figure-3b) was calculated by using $\delta = R - a$ where $a = \sqrt{R^2 - r^2}$.

The elastic modulus, E, of the liquid crystals was computed based on the Hertz contact equation as provided in equation (7),

$$E=\frac{3(1-\nu^2)F}{4R^{1/2}\delta^{3/2}} \quad (7)$$

where F is the force equivalent to the weight of a microball. δ is the vertical displacement due to the indentation of the microball. R is the radius of the microball and ν is the Poisson's ratio of the LC being indented. Due to the rich water content in the lyotropic liquid crystals formed after immersion in the culture media, the Poisson's ratio was set at 0.5 as that of the impressible fluid. For three independent samples of LC, similar experiments were repeated at twenty different indentation sites.

RESULTS AND DISCUSSIONS

The load-displacement of LC measured using nano-mechanical system

A nano-mechanical testing system was applied to determine the elastic modulus of the LC surfaces after being exposed to culture media for 24, 48 and 72 hours. However, the measurement for the LC after exposure to media for 48 and 72 hours using the nano-indentation technique was not successful. It was very difficult to obtain even a load-displacement curve for the LC samples incubated for long period of time. This is closely associated with the reduced viscosity of the liquid crystal as a result of water molecules infusion to the lyotropic liquid crystals (C. F. Soon *et al.*, 2011). Moreover, such a system was originally designed to indent solid materials and the sensitivity of the system may not be sufficient to provide readouts for low modulus gel such as the liquid crystals. However, this technique was viable to obtain load-displacement curve for the LC samples incubated for 24 hours or with higher viscoelastisicity.

A load-displacement curve for this LC sample is as shown in Figure-4. This figure shows the graphical description of the locations of the indenter during loading, hold and unloading phases. During the loading phase, the indenter breaks into the LC sample from air. The load to the measured force is attributed to the cohesion forces between the LC molecules. The rise of the loading force is linear after 3000 nm of displacement indicating the viscoelastic behavior of the liquid crystal. The 3 seconds hold time at peak load caused some data points existed around the peak force due to the rheological flow of the liquid crystals. After the hold time, the indenter was unloaded and attempted to break the lateral adhesion forces of derived from the liquid crystal. However, the load is lower in comparison with the load of the loading phase leading to the hysteresis of load-displacement curve. The curve during unloading was used to compute the elastic modulus of the liquid crystals using equation (1)-(6).

The LC immersed in the cell culture media was loaded with a peak force of P_{max} = 4.84 µN at a maximum indentation depth of h_{max} = 4910 nm (Figure-4). The final displacement depth of unloaded indenter, h_f, was found from the unloading data at 2713 nm. Withdrawal of the indenter was ceased at a displacement depth of h_s = 1068 nm. As obtained from the Triboscope software and using least square fitting methods (Oliver and Pharr, 1992), the power law fitting data to the unloading curve was 1.1 and 1.957 for A and m, respectively. The mean stiffness of the liquid crystals computed from equation (1) was 0.0017 ± 0.00067µN/nm (mean ± SD) and the elastic modulus derived from equation (6) was 108 ± 20 kPa (mean ± SD). Out of the three independent liquid crystal samples incubated for 24 hours, one sample could produce 20 repeatable and measureable results.

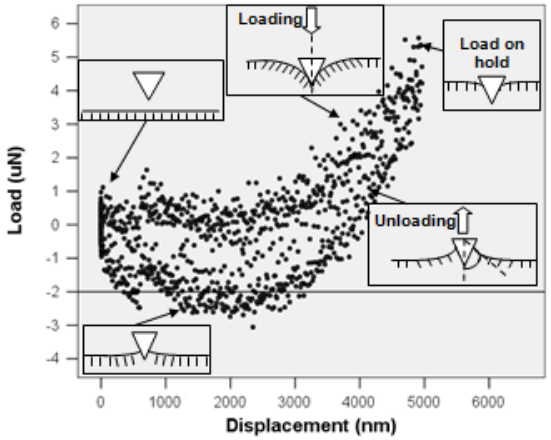


Figure-4. The load-displacement curve of a liquid crystal sample and the depiction on the different positions of the Berkovich indenter.

The elastic modulus of the liquid crystals determined using microball indentation

The less repeatable experiments of the nano-indentation to produce stable data had led to the application of microball indentation in determining the elastic modulus of the liquid crystals. The microball indentation experiments were able to produce repeatable measurement of the displacement formed in the liquid crystal surfaces.

The birefringence of the liquid crystal surface after indented with a steel microball is as shown in Figure-5a. These images were captured soon upon removal of the microball indenter from the liquid crystal substrate with a thickness, h of 100 µm. At the indented site, the weight of a steel ball loaded to the liquid crystal surface induced a dark circular region indicating the dispersion of the liquid crystal molecules into pseudo-isotropic phase. Focal conic texture was found at the periphery of the circular dark region due to the deformation in bi-axial direction (Figure-

5a, 0 second). When the microball was being unloaded from the surface of the liquid crystal, the dislocated liquid crystal molecules were seen rapidly moved and returned to the centre region of the contact area. After 15 seconds, the indented area was found closed. The defective texture found in a small area at the centre region was probably due to the dislodgment of the LC gel, which was adhered to the microball during. These LC gel was also found on the Berkovich indenter of the nano-indentation system.

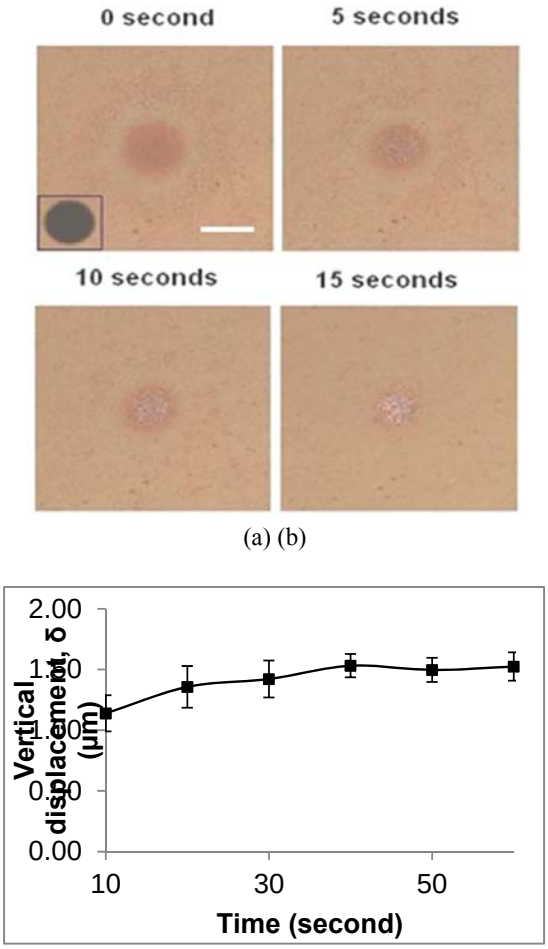


Figure-5. (a) Cross-polarising micrographs indicating the resurgence of the deformed LC surface (0 - 15 seconds) after the microball was removed. The inset in (a) is the microball used with a radius of 250 μm. (Scale bar: 50μm) (b) A relationship of the vertical displacement (δ) to the loading time (N = 3) of the LC.

Based on the birefringence changes, the impression of the circular area provided information associated with the contact radius formed by the microball. The contact radius, r and vertical displacement into the liquid crystal, δL for the three LC samples (N = 20 per sample) were approximately 26 ± 1.6 μm and 1.33 ± 0.16 μm, respectively.

The vertical displacements (δ) over time in Figure-5b indicates saturation of LC displacement after 40 seconds of microball loading in which the microball was stably loaded to the LC surface. This result suggested the suitable loading time for determining the elastic modulus of the liquid crystals. The increment in the vertical displacement from 1.14 ± 0.15 μm to 1.50 ± 0.10 μm indicated the creep and viscoelastic behavior of the liquid crystals. When the liquid crystal was loaded with microball, the vertical force exerted was distributed omni directional to the liquid crystal causing the liquid crystal molecules to move laterally and vertically around the contact area. The displaced positions of the LC molecules re-polarised incident lights and hence, the birefringence changes could be observed clearly. If the loading force is low, some of the liquid crystal molecules were able to revive to their original position when the microball when unloaded.

Based on Hertz's contact equation, the elastic modulus (E) of the three liquid crystal samples after 24 hours of incubation in cell culture media was found to be 108 ± 22.31 kPa, 111 ± 17.97 kPa, and 109 ± 21.33 kPa, respectively. The average value of the elastic modulus (E) was 110 ± 21.33 kPa (N = 60). The confidence interval at 95% for the mean elastic modulus was $104.87 < E < 115.18$ kPa. The results show similar elastic moduli determined by using microball indentation for the liquid crystal substrates after incubation for 24 hours at 37°C. Compared to the nano-indentation technique, the measurements using microball indentation technique were reproducible and highly repeatable.

The results obtained from microball indentation also indicated that the LC surface presented a viscoelastic behavior at a shear strain of ~25% (r = 25 μm, h = 100μm) and a small axial strain of $\delta/h < 2\%$ (δ = 1.33 μm, h = 100 μm). Based on our previous rheological results (C. F. Soon *et al.*, 2011), the strains are well within the linear viscoelastic region of the liquid crystals. This explained the possible elastic recovery of the indented area as found in Figure-5(a).

Table-1. Comparison of elastic modulus of cholesteryl ester based lyotropic liquid crystals (after 24 hours of incubation) determined by using nano-indentation and microball indentation.

	Elastic modulus (kPa)	
	Mean ± SD	**Mean ± SD**
Nano-indentation (N = 20 for a LC sample)	108 ± 19.78	-
Microball indentation (N = 20 for each of the three LC samples)	111 ± 17.97	110 ± 19.95 for N = 60
	108 ± 22.31	
	109 ± 21.33	

The comparison of using two different techniques in studying the elastic modulus of the liquid crystals after 24 hours incubation in culture media is as presented in Table-1. The elastic modulus of the liquid crystal studied using nano-indentation and microball indentation were calculated at 108 ± 19.78 kPa and 110 ± 19.95 kPa, respectively. Both measurement techniques similarly corresponded to 20% of standard deviation.

Based on the microball indentation, the elastic moduli determined for the three LC surfaces after 48 hours of culture were 58 ± 8.26 kPa, 55 ± 6.79 kPa and 51 ± 9.99 kPa, respectively. For the three repeats of experiments, the mean elastic modulus of the LC was 55 ± 9.99 kPa (N = 60). Comparatively, the modulus of the LC cultured for 48 hours reduced to 50% of the moduls of the LC cultured for 24 hours (Table-1). The results suggest that the viscoelasticity of the liquid crystal is a time dependent and the infusion of water molecules is an progressive effects to the liquid crystla surface as shown in Figure-6. Prolong culture in media for 72 hours reduced the elasticity of the liquid crystal yielding a highly viscous behavior of the liquid crystals. This results can be verified via the rheological study of the liquid crystals after 72 hours of incubationin culture media (C. F. Soon *et al*., 2011). The interdigitation of the water molecules weaken the cohesion force of the amphiphillic molecules leading to the lost of viscoelasticity. The viscoelasticity of the liquid crystal is intact if the liquid crystal is interacted with the culture media within 48 hours of incubation.

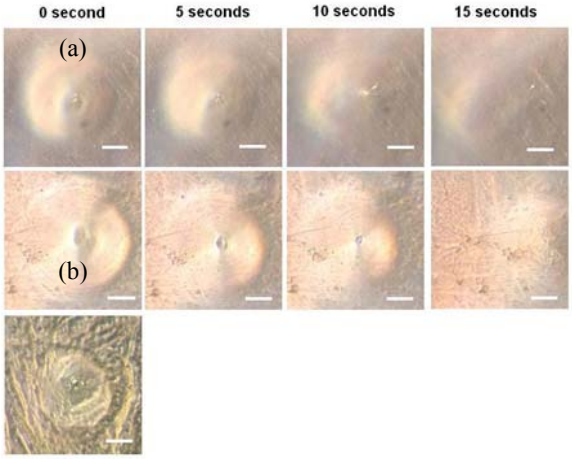


Figure-6. Phase contrast micrographs of the LC surface after microball indentation. Three indepdent samples of liquid crystal incubated in cell culture media for (a) 24, (b) 48 and 72 hours at 37°C, respectively. (Scale bar: 50 µm).

The differences of both experiments are the geometries of the indenters and complexity of system used. Nano-indentation involves with piezoresistive transducers and sensitive measurement system to sense the force loaded to the indenter. In contrast, microball indentation requires a simple steel ball to function as a load to the test material and the displacement is based on the gravity. This technique is far more reliable and repeatable to be used compared with the nano-indentation technique in determining the elastic modulus of the cholesteric liquid crystals. Liquid crystals and tissues are considered heterogeneous samples and repetitive measurements are essential (Zadpoor, 2015). The similarity between the two experiments were the use of similar peak loading force loads at approximately 5 µN and both were performed in the presence of culture media. However, both techniques produced similar measurements of elastic modulus for the liquid crystals.

The modulus obtained at around 100 kPa for the cholesteryl ester liquid crystals is considerably lower than that of the thermotropic cubic phase liquid crystals at approximately 300 kPa (Even *et al*., 2006). A lower modulus of the liquid crystal is related to the hydration factors introduce to the experiments. The range of modulus obtained indicated that it may be a suitable material to be applied in biological studies because the human epidermis layer was believed to have modulus of 82 ± 60 kPa (mean ± SD) (Hendriks *et al*., 2006; Takeo, 2007). The modulus resembling the stiffness of the soft tissues may present suitable mechanical properties for the adhesion or proliferation of cells. Suitable physical properties of a biomaterial is important to avoid identification as pathological tissue by the cells (Engler *et al*., 2004b; Levental *et al*., 2007).

CONCLUSIONS

The nanoindentation and microball indentation could be used to determine the visocelasticy behavior of the cholesteryl ester lyotropic liquid crystal in the presence of the cell culture media. Microball indentation seemed to be a better technique to reliably determine the time dependent properties of the liquid crystals in-situ. The elastic moduli as determined by both techniques showed similar measurement results for the modulus of LC samples incubated for 24 hours. However, the nano-indention technique could not measure the modulus of the LC after longer incubation period due to the change of the viscoelasticity of the LC and detection limit of the system. In comparison to the nano-indentation technique, microball indentation could provide reliable and repeatable measurement results despite of the buoyancy of fluid. It was found that the elastic modulus of the LC determined mimicks the elastic modulus of the epidermis layer reported previously. The LC may provide a suitable mechanical properties to support the proliferation of cells.

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