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Yan-Yeung Luk[a]; Sean F. Campbell[b]; Nicholas L. Abbott Corresponding author[a]; Christopher J. Murphy Corresponding author[b]
[a] Department of Chemical & Biological Engineering, University of Wisconsin-Madison WI 53706, USA
[b] Department of Surgical Sciences-School of Veterinary Medicine, University of Wisconsin-Madison WI 53706, USA

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Non-toxic thermotropic liquid crystals for use with mammalian cells

YAN-YEUNG LUK, SEAN F. CAMPBELL†, NICHOLAS L. ABBOTT* and CHRISTOPHER J. MURPHY†*

Department of Chemical & Biological Engineering, University of Wisconsin-Madison WI 53706, USA

†Department of Surgical Sciences–School of Veterinary Medicine, University of Wisconsin-Madison WI 53706, USA

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This paper reports the results of a study that aimed to identify thermotropic liquid crystals that are not toxic to mammalian cells. Mesogenic compounds were mixed to create eight liquid crystalline phases, each with a unique set of functional groups. We investigated the toxicity of each liquid crystalline phase using two mammalian cell lines—3T3 fibroblast and SV-40 transformed human corneal epithelial (HCEC) cells. Using dual fluorescent staining assays based on calcein acetoxymethylester (Calcein-AM) and ethidium homodimer, we measured correspondingly the number of viable and dead cells following immersion of the cells in the liquid crystals. It was found that most of the liquid crystals, such as commonly used 5CB and E7, caused cell death after contact with cells for four hours. However, we identified a class of liquid crystals containing fluorophenyl groups that possess minimal or no toxicity (as indicated by results of assays based on Calcein-AM and ethidium homodimer) to cells. Following immersion in fluorophenyl liquid crystals for four hours, the cells were observed to proliferate in culture medium at rates similar to control cells exposed to phosphate-buffered saline (PBS) for four hours. In contrast, treatment of cells for 24 hours with either PBS or liquid crystals (media containing no nutrients and growth factors) resulted in cell death.

1. Introduction

Several recent studies have reported on the use of liquid crystals (LCs) as the basis of novel tools for the physical and life sciences [1–4]. For example, nematic LCs have been used to amplify protein binding events on receptor-decorated gold films that possess a topography on the nanometer-scale [2]. In addition, by using buffed films of biotinylated bovine serum albumin (BSA) covalently immobilized on glass substrates, LCs have been exploited to detect the binding of antibody to surface-bound antigens [3]. Finally, by using surfaces that present metal ions that bind mesogens, real-time detection of parts per billion (by volume) concentrations of simulants of chemical warfare agents has been demonstrated [4].

Similar to the studies reported above, this paper reports an investigation that seeks new applications of LCs. Many broadly applicable tools in the biological sciences interface with cells in culture. Examples include tools for basic research in cell physiology [5], high throughput drug screening [6], and the development of sensors using cells patterned on a chip [7]. These studies and others have established that cells respond to a range of chemical and mechanical cues to regulate cell behaviours such as adhesion to surfaces, differentiation and proliferation [8]. For example, Chen and co-workers have used surfaces presenting elastomeric posts to study mechanical force exerted by cells [9], while others have applied soft materials such as hydrogels or silicon elastomers to direct cell behavior [10]. Given the ubiquitous presence of the liquid crystalline state in biological systems [11] and the technological utility of LCs, it is surprising that few examples of the use of LC technologies involving whole mammalian cells have been reported [12]. One could envisage, for example, the use of LCs to image the expression and organization of receptors either expressed on the surfaces of cells, or secreted into the liquid crystal, in response to biochemical or biophysical cues. Alternatively, LCs might be exploited to deliver chemical or mechanical stimuli to cells and thereby guide their behaviour.

To enable the development of technologies that interface cells with liquid crystalline environments, LCs that are not toxic to cells are required. We report here

*Authors for correspondence; e-mail: abbott@engr.wisc.edu; murphyc@svm.vetmed.wisc.edu

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the results of an initial search for LCs that are *not* toxic to mammalian cells. In this work, we carried out experiments using living cells immersed under eight thermotropic LC mixtures to screen for chemical functionalities in LCs that maintain the viability of cells. Each LC mixture was composed of mesogens that contained a unique set of functional groups.

Reports on the toxicity of LCs to cells are very limited. Toxicity tests performed by industry are aimed at safeguarding human health and have generally focused on whole animal toxicity testing rather than toxicity to isolated cells [13, 14]. For example, the so-called LD_{50} test, which characterizes the dose of a compound ($mg\,kg^{-1}$ of each animal) that is lethal to 50% of the animals tested, is widely used by industry. These indices are phenomenological indicators. In contrast, in order to develop research tools for the life sciences and biotechnology, knowledge of toxicity to individual cells is required. In this work, we investigated a panel of eight thermotropic LCs that covered a broad range of physical properties and chemical functionalities. We used two mammalian cell lines—3T3 fibroblast and SV-40 transformed human corneal epithelial cells (HCECs)—to study the toxic effects of these eight LCs. These cell lines are representative of connective tissue (3T3 fibroblast) and epithelial cells (HCEC), cell types that are widely studied in the biological sciences and commonly used for *in vitro* toxicity testing.

Because the pure mesogens used in this study are solids at ambient temperature, mesogens with common sets of functional groups were mixed to create liquid crystalline phases at ambient temperatures. By using liquid crystalline phases composed of different but unique sets of functional groups, we aimed to correlate the toxic effects of the liquid crystalline phases with their chemical functional groups.

The structure, composition, and physical properties of the LCs used in this work are listed in tables 1 and 2. The 'E' series comprises the two olefins, 1-(4-ethylcyclohexyl)-4-[2-(4-pentylcyclohexyl)ethyl]benzene (65 mol%) and 1-ethyl-4-(4-pentylcyclohexyl)benzene (35 mol%). Because the phenyl and cyclohexyl groups are common in LCs, with notable exceptions being cholesteric and lyotropic LCs, we labelled this series of LCs as having no functional groups. The 'A' series comprises a mixture of 1-ethoxy-4-(4-propylcyclohexyl) benzene (55 mol%) and 1-ethoxy-4-(4-pentylcyclohexyl) benzene (45 mol%), both of which contain the ether functional group. The 'B' series comprises 4-ethylcyclohexanecarboxylic acid 4-ethoxyphenyl ester (50 mol%) and 4-pentylcyclohexanecarboxylic acid 4-methoxyphenyl ester (50 mol%), both of which contain the ether and ester functional groups. We included 4-cyano-4′-pentylbiphenyl (5CB) in our survey because it is a widely studied LC. The mixture E7 (a mixture of four cyanobiphenyls with different aliphatic chain lengths) was also included. The composition of E7 has been analysed by chromatography and disclosed by Cognard, from which we conclude that E7 and 5CB share the same cyano functional group [15]. The 'C' series comprises two fluoro-substituted olefins, 4′-(3,4-difluorophenyl)-4-propylbicyclohexane (50 mol%) and 4′-(3,4-difluorophenyl)-4-pentylbicyclohexane (50 mol%), both of which contain fluorophenyl groups. TL205 is a mixture of mesogens containing cyclohexane-fluorinated biphenyls and fluorinated terphenyls with aliphatic chains containing 2–5 carbon atoms, although the exact composition is proprietary (E Merck Co). However, when considering the chemical functionality present in the mesogens, TL205 is similar to the 'C' series. The cholesteric series consists of four cholesteric molecules—cholesteryl chloride, cholesteryl oleyl carbonate, cholesteryl 2,4-dichlorobezoate and cholesteryl nonanoate.

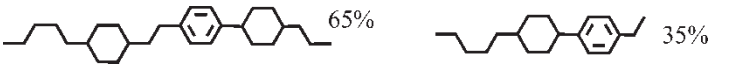
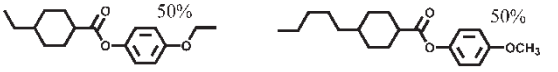
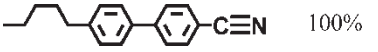
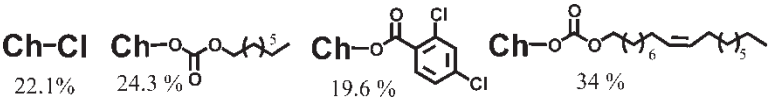
2. Experimental

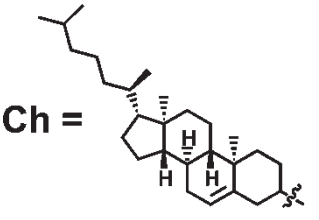
2.1. *Materials*

Foetal bovine serum, dimethyl sulfoxide (DMSO), gentamycin, ethylenediaminetetraacetic acid (EDTA) and saponin were purchased from Sigma Aldrich, St.Louis, MO. Phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4), Dulbecco's modified eagle medium (DMEM) and Ham's F-12 were purchased from BioWhittaker, Walkersville, MD; 75-cm^2 culture flasks and 96-well plates were purchased from Fisher Scientific, Pittsburgh, PA. Calcein-AM and ethidium homodimer were purchased from Molecular Probes, Eugene, OR. Porcine trypsin was purchased from JRH Biosciences, Lenexa, KS. The Cytofluor 4000 TC automated fluorescent plate reader was purchased from PE Biosystems, Foster City, CA. 3T3 fibroblasts were purchased from ATCC, Rockville, MD. SV-40 transformed human corneal epithelial cells (SV-40 HCEC) were provided as a gift from Dr Kaora Araki-Sasaki of National Kinki Central Hospital, Osaka, Japan.

Liquid crystals TL205, 5CB and E7 were purchased from EM Industries (Merck), NY. Components for the cholesteric series were purchased from Pressure Chemical Company, PA. Components for the 'A', 'B', 'C' and 'E' series were purchased from Phentex Corporation, TX.

Table 1. Structure and composition of mixtures of mesogens surveyed for toxicity to mammalian cells.

Liquid Crystal	Structure and composition (mol ratio)
'E' series	
'A' series	
'B' series	
5CB	
E7	
'C' series	
TL205	Mixture of cyclohexane-fluorinated biphenyls & fluorinated terphenyls. See text for details.
Cholesteric	



2.2. *Preparation of optical cells for imaging of liquid crystals*

The glass microscope slides were Fisher's Finest, premium grade obtained from Fisher Scientific (Pittsburgh, PA). The optical cells used to record the textures of the liquid crystals were fabricated by one of two methods. 5CB, 'E', 'B' or 'A' series LCs were heated above their clearing temperatures, and then introduced between

Table 2. Physical properties of liquid crystals used in mammalian cell toxicity tests.

Liquid Crystal	Mesophase[a]	Functional groups	Transition temperature[b]/°C	$\varepsilon_{\parallel}$, $\varepsilon_{\perp}$[c]	n_e, n_o[d]	ρ[e]/g cm^{-3}
'E' series	Nematic	None	Cr < 19m53 I	—	—	—
'A' series	Nematic/smectic	–O–	Cr < 19m38 I	—	—	—
'B' series	Smectic	–O–, –COO–	Cr < 19m55 I	—	—	—
5CB	Nematic	–CN	Cr22.5m35 I	19.7, 6.7	1.7360, 1.5442	1.0065
E7	Nematic	–CN	m58I	19.0, 5.2	1.7464, 1.5211	—
'C' series	Nematic	Fluoro-phenyl	Cr < 19m108I	—	—	> 1
TL205	Nematic	Fluoro-phenyl	m87.4I	9.1, 4.1	1.7445, 1.5270	> 1
Cholesteric series	Cholesteric	–Cl, –O(CO)O–	N*73–75I	—	—	—

[a]The mesophases at 22°C.
[b]The transition temperature between Cr (crystalline), m (mesophase), N* (chiral nematic) and I (isotropic) phases.
[c]$\varepsilon_{\parallel}$ and $\varepsilon_{\perp}$ are the dielectric permittivities parallel and perpendicular to the director of the liquid crystal, respectively.
[d]n_e and n_o are the two principal refractive indices corresponding, respectively, to the extraordinary and ordinary ray of refracted light.
[e]ρ is the density of the liquid crystal.

two sandwiched glass substrates spaced by a 13 μm thick Saran wrap. The isotropic phases filled the optical cells by capillary action. For the 'C' series, TL205, E7 and cholesterics, a drop of liquid crystal was placed onto one of the two glass substrates with the Saran wrap spacer (~13 μm thick) in place, and then compressed by the other glass substrate to create an optical cell.

2.3. *X-ray diffractometry*

Polycrystalline (powder) X-ray diffraction [16] patterns for the liquid crystals 5CB, 8CB, 'E', 'A', 'B', 'C' and 'D' series were collected on a Siemens analytical X-ray instrument. The LCs were injected into capillary tubes (2 mm in diameter, Hampton Research, CA). Data were collected with Cu K_α radiation using an incident beam monochromator ($\lambda = 1.54$ Å) on an area detector.

2.4. *Cell culture*

3T3 fibroblasts were cultured in DMEM (supplemented with 10% foetal bovine serum plus 40 μg ml^{-1} gentamycin) and grown in a humidified incubator at 37°C and 5% CO_2 SV-40 HCECs were cultured in supplemented hormonal epithelial medium (SHEM)—a basal medium for epithelial cell growth—with 10% foetal bovine serum (FBS). SHEM is a mixture of DMEM and Ham's F-12 (50/50) plus 0.5% DMSO and 40 μg ml^{-1} gentamycin. Hormones were not added in these experiments. The culture medium was changed every other day for both cell types, until the cells reached a confluency of 90–95% at which time they were passaged using trypsin/EDTA (~0.25%/25 mM).

2.5. *Viability/cytotoxicity assays*

Both cell lines (3T3 fibroblasts and SV-40 HCEC) were plated at a concentration of 10,000 cells/well on a 96-well plate and allowed to attach and proliferate overnight under the corresponding medium of each cell line. The medium was then removed, 25 μl/well of either liquid crystal, cell culture medium or PBS (pH 7.4) were added to six wells for each solution (total of 120 wells); PBS was used as a control. Saponin (0.1% w/v) was added to one row of cells that had not been exposed to liquid crystal as a control for complete cell death. The cells were incubated at 37°C for 4 or 24 h under these solutions. The solutions (liquid crystal, cell culture medium and PBS) were then removed. The cells were rinsed three times with PBS; 50 μl of ethidium-homodimer (4 μM) or 50 μl of Calcein-AM (0.6 μM) were added to each well and the plate was incubated for an additional 2 h. Fluorescence was measured using a Cytofluor 4000TC automated fluorescent plate reader. For ethidium homodimer, the fluorescence was measured using 530 nm (excitation) and 620 nm (emission). For Calcein-AM, fluorescence was measured using 485 nm (emission) and 530 nm (excitation).

In one set of experiments, we correlated the Calcein-AM fluorescent signal with actual cell number. After 4 h of incubation in the presence of TL205 or PBS, cells were labelled with Calcein AM and fluorescence quantified using the plate reader. Cells were then detached from wells using trypsin/EDTA and manually counted using a hemocytometer.

2.6. *Proliferation assay*

SV-40 HCECs were plated at a concentration of 5,000 cells/well on a 96-well plate, and allowed to attach and proliferate overnight. Medium was removed and 25 μl/well of either the 'C' series liquid crystal,

SHEM cell culture medium or pure PBS were added to six wells for each solution (total of 18 wells). The SV-40 HCECs were incubated for 4 h under these three solutions; the three solutions were then removed, and the cells rinsed with SHEM medium. Following incubation of the cells in SHEM medium with 10% FBS at 37°C for 5 days, cell number was estimated using Calcein-AM (see §2.5). The magnitude of the Calcein-AM fluorescent signal correlates directly to the number of viable cells present.

3. Results and discussion

3.1. *Mesophases of liquid crystal materials*

The mesogens comprising the 'E', 'A', 'B' and 'C' series are solid or isotropic at room temperature. Our first task was to determine the compositions of mixtures of the mesogens that would form mesophases at room temperature. Mesophases formed by preferred mixtures are shown in figure 1. By combining the optical textures of the liquid crystals in figure 1 with X-ray diffraction, we have identified each mesophase. The X-ray diffraction patterns of the 'A' and 'B' series show two sets of arc-shaped reflections perpendicular to each other—a pair of outer diffuse crescents corresponding to the intermolecular spacing and a pair of inner crescents corresponding to the molecular layer spacing in the smectic phase [17]. The layer spacings of the smectic 'A' and 'B' series are smaller than that of smectic 4′-octyl-4-biphenylcarbonitrile (8CB). However, the diffractograms of the 'E' and 'C' series possess only the diffuse outer crescents that correspond to the translational period for the nematic phase.

The 'E' series is a class of apolar liquid crystals that usually exhibit lower viscosities than their polar counterparts [18]. While the pure component 1-(4-ethylcyclohexyl)-4-[2-(4-pentylcyclohexyl)ethyl]benzene in the 'E' series exhibits a smectic phase [19], its mixture with 1-ethyl-4-(4-pentylcyclohexyl)benzene appears to be nematic at ambient temperatures based on both the X-ray diffraction pattern and the liquid crystal texture (figure 1). Based on the X-ray diffraction pattern, the liquid crystal 'C' series appears to be nematic at ambient temperatures. The 'C' series exhibits broad mesophase range(s) with a high clearing temperature, reflecting the general trend that both the presence of cyclohexyl rings and fluorine substitution on the aromatic rings increase the clearing temperature of the mesophase [20].

3.2. *Cell attachment and spreading under liquid crystals*

We next investigated the behaviour of cells immersed under the LCs shown in figure 1. The viability of a living cell is reflected in a range of different dynamic processes, from its morphological appearance to the molecular interactions that occur within distinct intracellular compartments. These processes include cell attachment and spreading on a surface, formation of focal adhesions, activation of protein signalling pathways, differentiation and proliferation. Of these phenomena, attachment has been shown to reflect the viability of cells [21], and it is of prime importance for cell-based biotechnology [22].

For adherent cells [23], the most basic requirement for viability is attachment and adhesion to a surface [24]. We first investigated the spreading of attached

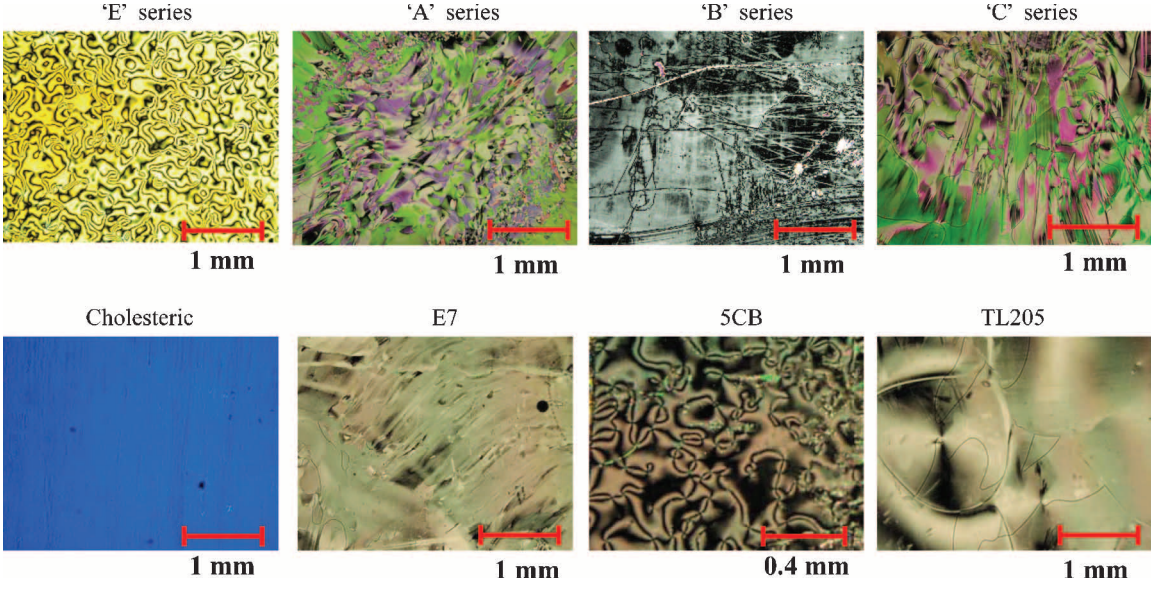


Figure 1. Optical images (crossed polarizers) of the eight liquid crystals used in this study.

cells—both 3T3 fibroblast and SV-40 HCEC cells—immersed under LCs and immersed in normal culture medium (figure 2). After plating the cells overnight in culture medium (see § 2), the medium was replaced with an LC layer (25 µl) having a thickness of about 1 mm. The cells immersed under the LCs were then further incubated for 4 h. Figure 2 compares the optical images (without polarizers) of the morphology of the plated 3T3 fibroblast cells under 5CB (A), under the 'C' series liquid crystal (B), after the 'C' series LC was removed from the plated cells (C), and cells cultured with normal culture medium (D).

Inspection of figure 2 shows that cells immersed under 5CB bulge up or 'round up' whereas cells under the 'C' series spread to an extent that is indistinguishable from cells under normal culture medium. The low resolution of the images of the cells under the LCs was caused by LC birefringence. Following the removal of the 'C' series LCs from contact with the cells, details of the morphology of the spread cells such as lamellipodia and the presence of the nucleus are indistinguishable from a normal healthy living cell. In contrast, upon removal of 5CB, the 3T3 fibroblasts rounded up, and many were displaced from the surface by rinsing. One major factor that causes the rounding-up of a cell from a spread morphology is cell death. From this experiment, it is clear that cells respond differently to treatment with LCs with different functional groups. In experiments described below, we report the results of fluorescent assays for cell viability and death that were performed after treatment of the cells with LCs.

We also incubated 3T3 fibroblasts and SV-40 HCECs for 24 h under all eight liquid crystals as well as PBS buffer (pH7.4). After 24 h of immersion in the LCs, all cells rounded up or detached from the surface of the well indicating that the cells were dead. We note that incubation of cells in PBS buffer for 24 h also resulted in cell death. Although cells are deprived of nutrients when incubated in both PBS and LCs, we do not conclude from these experiments that the causes of cell death are the same in both cases.

3.3. *Fluorescent imaging of the viability of cells treated with liquid crystals*

We carried out fluorescent assays (figure 3) to confirm the viability and death of cells treated with LCs. In one experiment, calcein acetoxymethylester (CAL-AM) was used to assess the number of living cells. In a second experiment, a fluorophore, phenanthridinium, 5,5′-[1,2-ethanediylbis(imino-3,1-propanediyl)]-bis(3,8-diamino-6-phenyl)-, dichloride, dihydrochloride (Ethidium homodimer), was used to assess the number of dead cells.

3.3.1. *Calcein acetoxymethylester—viability assay*

Live cells are distinguished from dead cells by the presence of intracellular esterase activity and plasma membrane integrity. In order to quantify the viability of cells treated with LCs, we carried out a fluorescent assay based on intracellular esterase activity [25]. This viability assay uses the fluorescent precursor calcein acetoxymethylester (CAL-AM), which is permeable to the membrane of cells. Upon entering the cells, CAL-AM is a substrate for the endogenous esterase that can hydrolyse CAL-AM to afford a green fluorescent product (about 530 nm)—Calcein (CAL)—in the cytosol (figure 3 (*b*)). Hence, the presence of green fluorescence from CAL in cells is evidence of esterase activity as well as an intact membrane that retains the esterase products in the cells, both of which are indicators of a living cell.

By measuring the level of intensity of CAL fluorescence in cells treated with different LCs relative to that measured without LC treatment (in culture medium), we quantified the effect of eight LC materials on the viability of 3T3 fibroblast and SV-40 HCEC cells. A high relative fluorescence indicates that the LC treatment has a minimal effect on the viability of cells whereas a low relative fluorescence indicates a high death rate of cells due to LC exposure.

Figure 4 shows the relative fluorescence of CAL in cells treated with LCs for 4 h. Before discussing the effects of the LC on fluorescence of CAL in cells, we

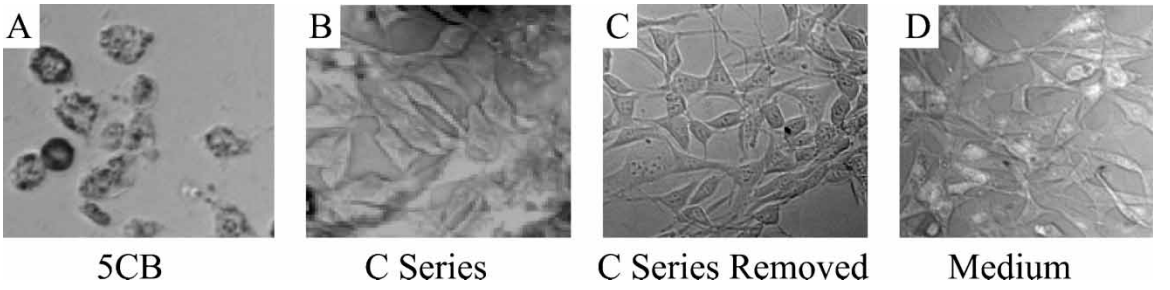


Figure 2. Optical micrographs of 3T3 fibroblasts immersed under either culture medium or one of two liquid crystals for 4 hours: (A) 5CB, (B) 'C' Series, (C) after 'C' series is removed from the 3T3 fibroblasts, (D) under normal DEME culture medium.

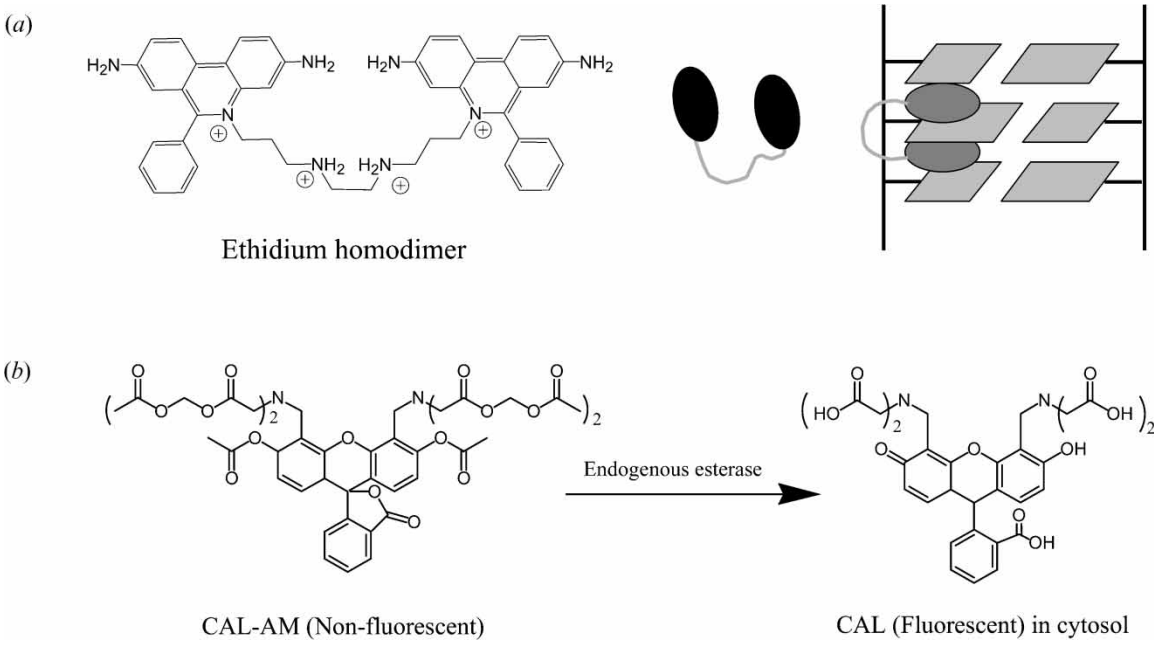


Figure 3. (*a*) Ethidium homodimer binds to nucleic acids and undergoes a 40-fold enhancement of fluorescence. (*b*) Hydrolysis of the non-fluorescent CAL-AM by esterase in cytosol affords the green fluorescent CAL in cells.

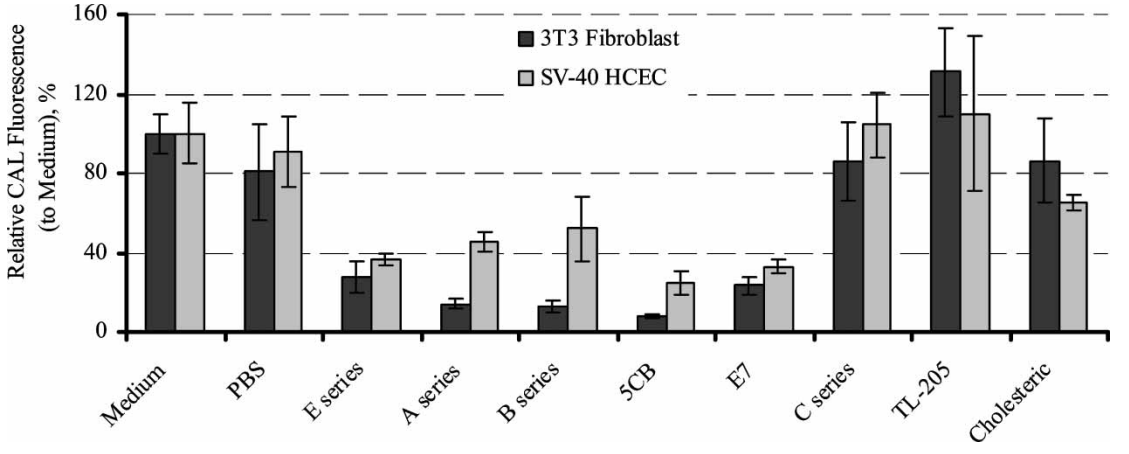


Figure 4. Relative intensities of the fluorescence from CAL-AM added to 3T3 fibroblasts (black bars) and SV-40 HCEC (grey bars) after treatment with each liquid crystal (4 h, 10,000 cells/well, six wells/treatment).

note that a control experiment in which LC was replaced with pure PBS buffer for 4 h caused no significant decrease of the relative fluorescence of CAL in cells. This result is important because it shows that the short-term starvation (4 h) due to removal of the nutrients does not affect the viability of cells in this experiment. Hence, any effect of the LCs on the cells can be largely attributed to their chemical/toxic effect. Figure 4 reveals that, in general, the 3T3 fibroblasts and SV-40 HCECs respond in a similar manner to each LC. In particular, the 'E', 'A', 'B' series of LCs, as well as 5CB and E7, are toxic to both 3T3 fibroblasts and SV-40 HCECs. The treatment of cells with these five LCs caused the CAL fluorescence to decrease to less than 30% for 3T3 fibroblasts, and to less than 50% for SV-40 HCECs. In addition, it is evident that the CAL fluorescence reveals the cholesteric series of LCs to be toxic to SV-40 HCECs but not 3T3 fibroblasts. However, treatment of both cell lines with the 'C' series and TL205 led to levels of CAL fluorescence that were similar to the CAL fluorescence measured with cells not treated with LCs (both culture medium and

pure PBS buffer). We believe these two LCs are not toxic to 3T3 fibroblasts and SV-40 HCECs during 4-h incubations.

We also note that the CAL fluorescence in both cell lines (3T3 fibroblast and SV-40 HCECs) treated with fluorine-substituted liquid crystals ('C' series and TL205) for 4 h tends to be higher than for cells treated with PBS solution (pH 7.4). This trend was consistently observed in several repeat experiments (data not shown). In a separate experiment we manually counted, using microscopy, the number of SV-40 HCECs adherent to the well bottoms after the 4 h treatment with TL205 and the PBS solution. Figure 5 shows the number of cells in comparison with the CAL fluorescence from SV-40 HCECs after treatment of PBS and TL205 for 4 h. Figure 5 demonstrates that a greater number of cells tend to be present after treatment with TL205 than after exposure to PBS solution for an identical period of time. In another control experiment, we first labelled SV-40 HCECs with CAL-AM for 2 h and then treated the cells with either PBS or TL205 for 4 h. We observed no difference in the CAL fluorescence from these two treatements. Both of these results confirm that the increase in CAL fluorescence after treatment of cells with fluorine-substituted LCs ('C' series and TL205) is not due to an increase in intracellular esterase activity. A possible interpretation of the increased CAL fluorescence in cells treated with fluorine-substituted LCs is that cell death is mildly inhibited in the presence of fluorine-substituted LCs as compared with PBS.

3.3.2. *Ethidium homodimer—cytotoxicity assay*

Dead cells have damaged membranes that permit the permeation of the fluorophore ethidium homodimer into the nucleus of the cells. Ethidium homodimer is a bis-intercalating dye that has a high affinity for nucleic acids. Upon intercalation between base pairs of nucleic acids, it undergoes a 40-fold enhancement of fluorescence intensity (figure 3 (*b*)) [26]. Thus, ethidium homodimer produces a bright red fluorescence (>600 nm) in the presence of dead or damaged cells by intercalating with nucleic acids in those cells with damaged cell membranes.

Because the experiments depicted in figure 4 reveal a decrease in viability signal (CAL-AM) after contact with 5CB, we next examined the fluorescence of ethidium homodimer in cells treated with 5CB for 4 h and cells not treated with LCs (cells incubated in ether medium or PBS). In a control experiment, cells were incubated with medium containing 0.1% w/v of saponin. Saponins are highly membrane-active amphiphiles that consist of a lipophilic fused six-member ring and hydrophilic side chains of sugar residues [27]. Because saponins lyse cells and cause maximum exposure of nucleic acids [28], fluorescence of ethidium homodimer from cells treated with saponins indicates completely dead cells.

Figure 6 compares the fluorescence of ethidium homodimer added to both cell lines (3T3 fibroblast and SV-40 HCEC) in cell culture medium, in PBS (pH 7.4), after treatment with 5CB and after incubation in cell culture medium containing 0.1% w/v of saponin. Figure 6 shows that while the fluorescence of ethidium

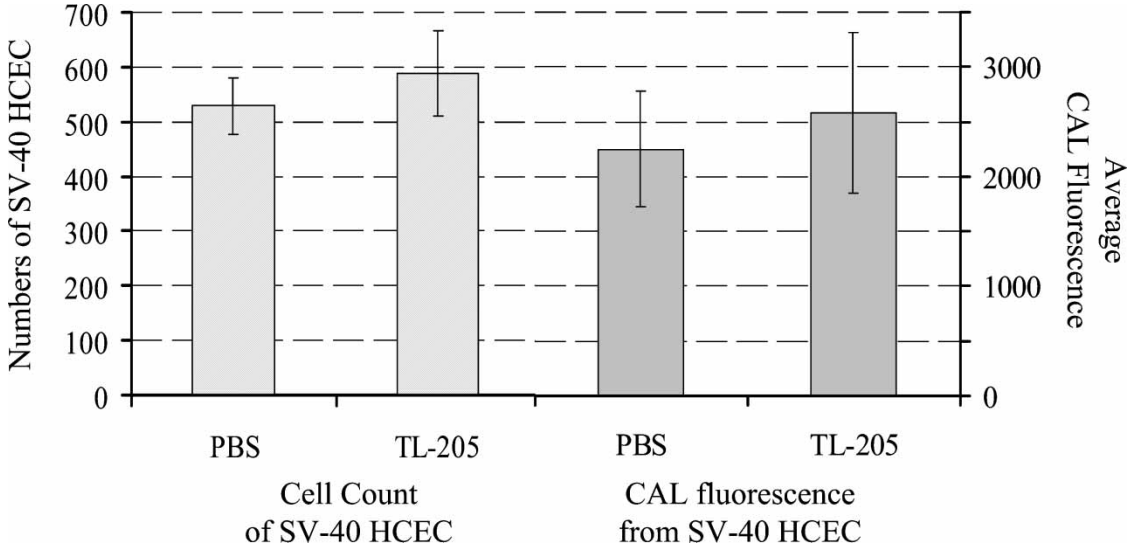


Figure 5. The number of adherent cells (shaded bars) as determined by direct microscopic counting and the average CAL fluorescence (filled bars) from SV-40 HCECs after treatment with either PBS or liquid crystal TL205 for 4 hours. Although not statistically significant, there is a trend for a greater number of cells exposed to TL205 to be adherent after rinsing procedures (reflected also by a greater fluorescent signal), compared with PBS.

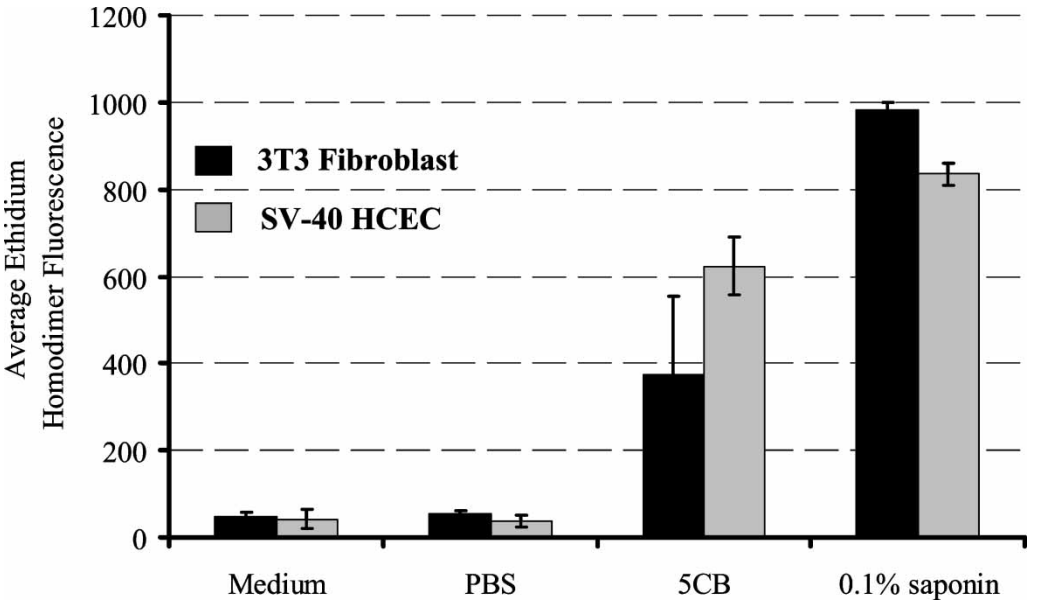


Figure 6. Average intensity of the fluorescence of ethidium homodimer added to 3T3 fibroblasts (black bars) and SV-40 HCEC (grey bars) in medium, in PBS, after treatment with 5CB and after treatment with medium containing 0.1% of saponin (4 h, 10,000 cells/well, six wells/treatment).

homodimer added to both cell lines in medium and PBS was minimal, its fluorescence when added to cells treated with 5CB and saponins was high and comparable. This result indicates that 5CB causes permeabilization of the membrane of both cell types. Combining this permeabilization with the result that cells round up and detach from the surface due to treatment with 5CB (figure 2), we conclude that 5CB is toxic to cells. We caution that it is possible for 5CB to alter the membrane permeability without causing cell death. However, other fluorescence assays that test the viability of cells have confirmed the toxicity of 5CB (see above).

3.3.3. *Proliferation assay*

When cells are immersed under LCs (and PBS), they are deprived of nutrients, supplements and growth factors. Under such conditions, cells may not proliferate normally and may display abnormal physiology. Furthermore, some toxic effects may not be so acute as to cause immediate cell death, but rather affect the proliferation and/or the viability of the cells in the long term. Hence, to address this issue following treatments of cells with LCs and PBS for 4 h, we incubated the cells under optimal growth conditions—medium with serum—for 5 days to permit them to proliferate. We subsequently used the CAL-AM fluorescent assay to evaluate the extent of proliferation. Figure 7 shows the results for SV-40 HCECs; they indicate that while the average CAL fluorescence from the SV-40 HCECs treated with SHEM is almost double that of the cells treated with the 'C' series LC, the average CAL fluorescence from the SV-40 HCECs treated with the 'C' series LC is the same as that of the cells treated with PBS. This result indicates that the 4 h treatment of SV-40 HCEC with 'C' series LC and PBS stress the cells to similar extents. We speculate that the primary origin of this stress experienced during short-term incubation is deprivation of nutrients and growth factors (not toxicity).

3.4. *Chemical inertness of liquid crystals towards living cells*

The results above reveal that the 'C' series LCs and TL205 possess minimal or no toxicity to cells. Inspection of table 2 shows that the 'C' series of LCs and TL205 possess fluorophenyl functional groups, whereas 5CB and E7 both contain cyano groups. Interestingly, both the 'C' series LCs and TL205 cause no reduction of the CAL fluorescence in 3T3 fibroblast and SV-40 HCEC, whereas both 5CB and E7 are toxic to both cell lines. We further note that 5CB is an isotropic liquid whereas E7 is nematic at 37°C. These results lead us to postulate that the chemical functionality of the LC is a primary factor in determining the viability/cytotoxicity of cells. In contrast, the physical properties of LCs, such as viscosity and clearing temperature, do not appear to serve as useful indicators of cell toxicity. We also note that the 'E' series were toxic to both cell lines. Because the 'E' series is

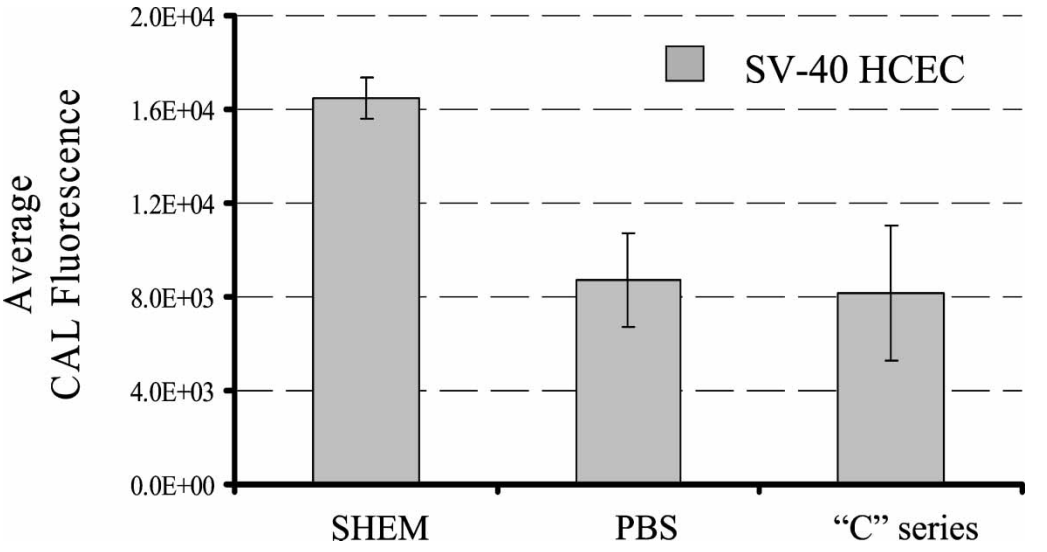


Figure 7. Proliferation assay of SV-40 HCECs after treatment with the 'C' series liquid crystal for 4 hours: average fluorescence from CAL-AM added to SV-40 HCECs after treatment for 4 h with SHEM, PBS and the 'C' series of liquid crystal. See text for details of the proliferation assay.

composed of olefins that have no special functional groups, we are led to speculate that the inertness of the 'C' series and TL205 is associated with the presence of fluorophenyl groups. Perhaps the most surprising result reported in this paper is the observation of a small *increase* in the CAL fluorescence in cells treated with fluorophenyl mesogens. We have repeated the experiments on both cell lines and confirmed this observation.

The exact mechanisms by which 'E', 'A' and 'B' series of LCs, 5CB and E7 are toxic to living cells are not yet understood. It is well known that perfluorinated alkane chains (used as blood substitutes) are largely inert to living cells, and possess exceptionally large capacities for solvating oxygen and carbon dioxide [29]. We speculate that the 'C' series and TL205 may share some of the physicochemical properties of perfluorocarbon-based blood substitutes. Furthermore, mesogens bearing fluorophenyl groups ('C' series and TL205) may also carry a sufficiently high content of oxygen/carbon dioxide to stimulate cells during the short period of time that the mesogens are in contact with the living cells.

4. Conclusions

In summary, we have investigated the effects of eight LCs with unique sets of functional groups on the viability of two mammalian cell lines immersed in them. We find that the chemical functionality of the LCs correlates closely with the toxic effect. We have identified several functional groups that, when incorporated in mesogens, are *not* toxic to cells during 4-h incubations. Treatment of cells with the LCs containing these functional groups does not affect the post-treatment proliferation of the cells as compared with PBS. The exact mechanisms by which these functional groups define liquid crystal-cell interactions and thus toxicity, in general, is not fully understood and is the subject of continuing research.

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