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Role of ligand-receptor interactions in visual detection of HepG2 cells using a liquid crystal microdroplet-based biosensor

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Abstract

Liquid crystal (LC) microdroplets for visual detection of HepG2 cells have been prepared using 4-cyano-4'-pentyl biphenyl molecules in the presence of sodium dodecyl sulfate as a mediator and β-galactose-conjugated poly(styrene-b-acrylic acid) block copolymer (PS-b-PA-G) as a modifier of LC-water interfaces. To clarify the effect of β-galactose-containing ligands on the orientational transitions of LC microdroplets, maltotriose as a ligand simulant was conjugated to poly(styrene-b-acrylic acid) and used as a LC modifier. Interaction of HepG2 cells with the β-galactose-conjugated block copolymer was effective in causing orientational transitions, from radial to bipolar, in LC microdroplets, whereas interactions of HepG2 cells with maltotriose-conjugated block copolymers were ineffective in inducing orientational transitions in LC microdroplets. To confirm the necessity of the PS segment of block copolymer for transmitting the ligand-receptor interaction forces from the interface to the core of the LC microdroplets, β-galactose-conjugated block copolymers (PS-b-PA-G) and homopolymers (PVLA) were

synthesized and used to prepare LC microdroplets. The LC microdroplets containing β-galactose-conjugated homopolymer did not show orientational transitions upon contact with HepG2 cells. However, LC microdroplets containing β-galactose-conjugated block copolymer showed orientational transitions from radial to bipolar, indicating that the polystyrene segment in the amphiphilic block copolymer is essential for effective transmission of ligand-receptor interactions to the core of LC microdroplets. β-Galactose anchored LC microdroplets were able to detect 1.0 ± 0.1 HepG2 cell/μm^2 of the test cell and shown significantly high reproducibility ($p < 0.05$, $n = 3$). The configurational transition in LC microdroplets that was dependent on ligand-receptor interactions was used to develop a LC microdroplet-based biosensor for the detection of HepG2 cells in biological fluids.

1. Introduction

Liquid crystals (LCs) are excellent sensing materials that transduce interfacial biomolecular interactions and biological events,[1-2] into visible optical signals.[3] The liquid crystal-based biosensor is a new research area in sensor technology that integrates modern biotechnology and advanced electronic sensing technology. LCs have become useful tools in biosensor development due to their high sensitivity to minute changes that occur at surfaces, and their ability to produce amplified optical responses, up to 100 μm into the surrounding bulk phase within tens of milliseconds, that are seen easily by the naked eye.[4-7] The orientational transitions that occur in liquid crystals are very sensitive to variations in the surface interaction energies that are caused by slight changes in the surrounding chemical environment; hence, LC molecules have been used in the fabrication of optical probes for the detection of chemicals[8-10] and of biophysical

reactions[1-3,11-12] that occur at liquid crystal-aqueous interfaces. Recently, surface-driven orientational transitions in liquid crystal molecules have been employed for the detection of toxic chemical vapors, such as thiols,[13] glutaraldehyde,[14] chemical warfare agents,[15] and chemical warfare simulants.[16] In these studies, the liquid crystal molecules on planar solid surfaces were shown to undergo orientational transitions upon interaction with vapors of chemicals and warfare agents. In comparison to liquid crystal molecules on planar solid surfaces, the application of liquid crystal molecules as microdroplets was found to be more useful in the fabrication of biosensors as they have relatively larger surface areas and orientation sensitivity. The restricted orientation states of LC molecules in microdroplets are able to change in proportional response to variations in interfacial energy[17-18] that result from interactions with chemicals/biomolecules. The optical properties of liquid crystal microdroplets depend on the types of surfactants and polymers they contain; hence, the interface of the liquid crystal microdroplets with water has been tailored using different surfactants and polymers.[19-21] The equilibrium state of the ordered LC molecules within the microdroplets is also influenced by the surfactants or polymer-mediated interactions of chemicals or biological molecules, and thereby provides a means for sensing chemicals,[13-16] biomolecules, and viruses.[22-23] Recently, polyelectrolyte multilayer (PEM)-coated LC microdroplets have been fabricated[17,24] and used for the detection of charged macromolecules, such as nucleic acids, proteins, carbohydrates, and abundantly present viruses and bacteria found in the nature. The transitional sensitivity of LC molecules in the microdroplets can be regulated by controlling the interface conditions of LC microdroplets using various functional polymers.[25-27] Although these studies have provided sufficient knowledge for the design of stimulus-response sensing systems using LC microdroplets, there is no report describing *in vitro* detection of HepG2 cancer cells in biological fluids using such a system, despite their predicted simplicity

and ease of fabrication. Various traditional methods, such as polymerase chain reaction,[28] immunohistochemistry,[29] and flow cytometry[30] are reported to be sensitive and specific in detection of various cancer cells. These methods, however, are times consuming and expensive in comparison to optical detection of cancer cells using LC microdroplets, as we have recently reported for the sensitive detection of KB cancer cells.[31] Antibody based cancer cell detection systems[32] can lose their cancer cell recognition capacity due to damage to the tertiary structure of the antibodies. To overcome this problem, aptamer-based electrochemical detection systems were introduced over the last two decades[33-35] for detection of whole cancer cells.[36] The cell-specific aptamers are thermally stable,[37] and aptamer-based biosensors have been developed for the detection of a variety of cancer cells[38-39]. As the selectivity of aptamer based biosensors to HepG2 cancer cells and other cell lines is based on an iterative *in vitro* evolution procedure, it is, however, more difficult to fabricate aptamer-based biosensors than LC microdroplet-based biosensors. The β-galactose moiety is a known ligand for asialoglycoprotein (ASGP-R) receptors[40] that are over-expressed (ASGP-R+) on hepatic cancer cells (HepG2) in comparison to normal hepatic cells (HEK293). In this study, β-galactose-conjugated block copolymers and maltotriose-conjugated block copolymers were synthesized and used in the fabrication of a LC microdroplet-based sensing system using 5CB. The ligand-receptor interactions between β-galactose-anchored LC microdroplets and HepG2 cells were found to be capable of causing configurational transitions in the LC microdroplets. To verify the necessity of PS segments in the amphiphilic block copolymers for transmission of ligand-receptor interactions to the core of LC microdroplets, a β-galactose-conjugated homopolymer (PVLA-G) was also prepared and used

the in fabrication of LC microdroplets for the detection of HepG2 cells. No configurational transitions in the LC microdroplets containing the homopolymer were detected.

2. Experimental

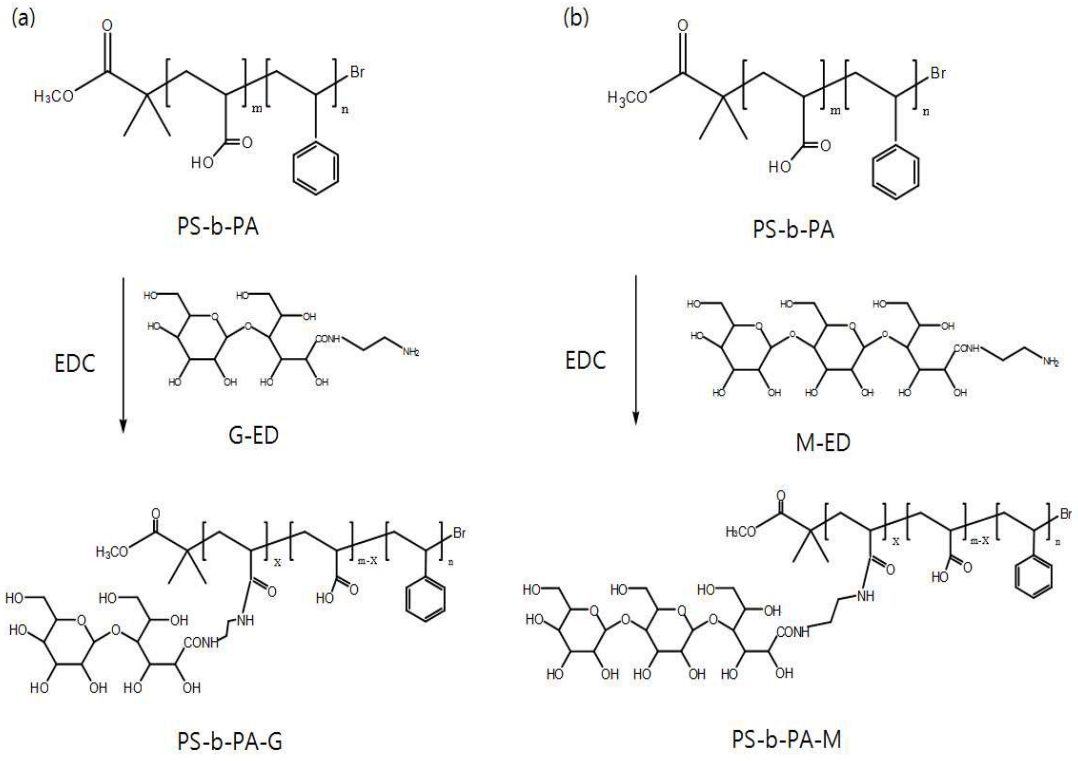
2.1 Materials

Poly(styrene-b-acrylic acid) (PS-b-PA, Mw: 7246 g mol^{-1}), 4-cyano-4'-pentyl biphenyl (5CB, Mw: 249.15 g mol^{-1}, m.p.: 24 °C), di-tert-butyldicarbonate, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC), lactobionic acid (LA) and maltotriose were purchased from Sigma-Aldrich Chemical Company (USA) and used as received. The HepG2 (liver hepatocellular cells), KB cancer cells (oral squamous cells) and fibroblast cells were purchased from the Korea cell bank. The HepG2 cells were cultured in minimum essential Dulbecco's Modified Eagle Medium (DMEM), pH 7.4, supplemented with 10% fetal bovine serum (FBS), streptomycin (100μg/mL) and penicillin (50 units/mL). Phosphate-buffered saline solution (PBS), pH 7.4, was used for washing and resuspension of LC microdroplets.

2.2 Synthesis of β-galactose and maltotriose-conjugated poly(styrene-b-acrylic acid)

Ethylene diamine (9.1 mL, 136.5 mmol) was dissolved in dichloromethane (40 mL) and added drop wise to a round-bottom flask containing di-tert-butyldicarbonate (5.55 mL, 24.15 mmol) and dichloromethane (10 mL) under nitrogen atmosphere at 20 °C for 20 h to produce N-tertiarybutyloxycarbonyl-ethylamine (Boc-ED). Dichloromethane was removed by rotary evaporation and the reaction product was extracted with ethylacetate (30 mL). The extract was purified by petroleum ether:ethylacetate (1:9) column chromatography. The Boc-ED was obtained by removing the solvent using a rotary evaporator. Lactobionic acid (25 mg, 70 mmol)

was dissolved in dichloromethane (20 mL) and mixed with EDC to activate the carboxyl group of lactobionic acid (LA). The activated LA was mixed with Boc-ED (11.08 mL, 70 mmol) in dichloromethane and stirred continuously for 1 d. The reaction product was then hydrolyzed with a mixed solution of trifluoroacetic acid and methyl alcohol (1:10) to produce lactobionic acid-ethylenediamine (G-ED). To activate the carboxyl groups of block copolymer, 100 mg (25.8 mmol) of block copolymer were dissolved in dimethylformamide (10 mL) and mixed with EDC (200 mg, 1.28 mmol). Subsequently, 10 mg (24.98 mmol) of G-ED was added to the carboxyl group-activated block copolymer and stirred for 24 h at 20 °C (Scheme 1a).



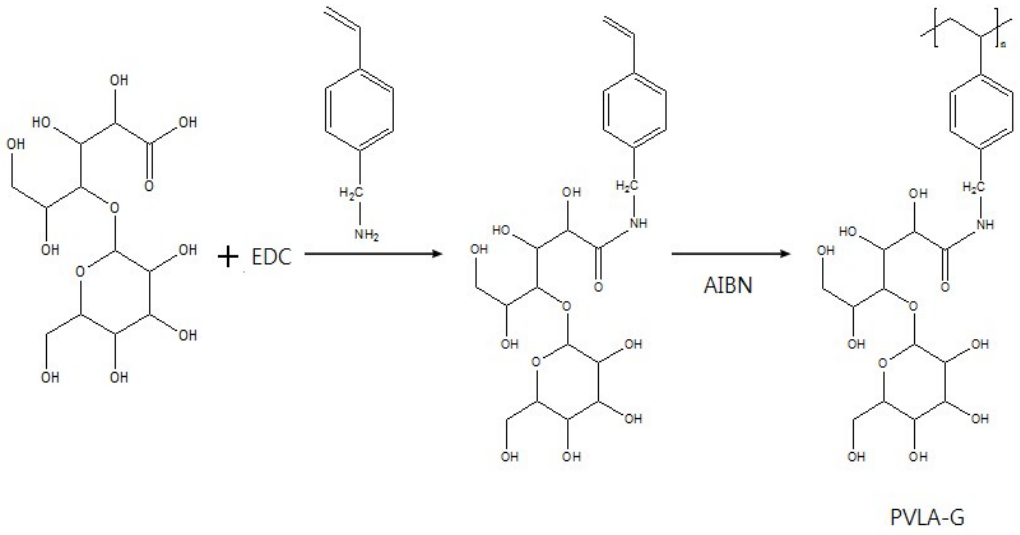
Scheme 1 (a) Synthesis of β-galactose-conjugated (PS-b-PA-G) and (b) maltotriose-conjugated (PS-b-PA-M) block copolymers

After removing the solvent by rotary evaporation, the reaction product was dialyzed with a regenerated cellulose membrane (MWCO: 2,000) using double-distilled water. Finally the reaction solution was lyophilized, yielding a powdery β-galactose-conjugated block copolymer (PS-b-PA-G). To synthesize maltotrionic acid, maltotriose (5.9 g, 11.7 mmol) was dissolved in a 4% (w/v) KOH in methanol solution and stirred for 2 h. The resulting potassium maltotrionate was then recrystallized with a mixed solution of methanol and water (1:1). The crystallized potassium maltotrionate was dissolved in 1N hydrochloric acid and stirred for 24 h. The reaction product was filtered and dried under vacuum to produce maltotrionic acid. Maltotrionic acid-ethylenediamine (M-ED) and maltotriose-conjugated block copolymer (PS-b-PA-M) were prepared by following the procedure (Scheme 1b) described for the synthesis of β-galactose-conjugated block copolymer (PS-b-PA-G). X-ray photoelectron spectra (ESCA, ESCA LAB VIG Microtech, East Grinstead, UK, using Mg Kα radiation) and FT-IR spectra (Jasco, FT-IR 300E spectrophotometer) of the materials spotted on KBr pellets were recorded to confirm the presence of β-galactose and maltotriose conjugates in the block copolymers.

2.3 Synthesis of β-galactose-conjugated poly(vinyl benzyl-galactopyranosyl-gluconamide)

The β-galactose-containing poly(vinyl benzyl-galactopyranosyl-gluconamide) homopolymer (PVLA-G) was prepared according to reported methods[31]. Briefly, lactonolactone (15.3 g, 45 mmol) was dissolved in methanol (140 mL) and then added to a solution of methanol containing *p*-vinyl benzylamine (6.0 g, 45 mmol) to produce vinyl benzyl-galactopyranosyl-gluconamide monomer (VLA). The VLA monomer (2.37 g, 5 mmol) and azobisisobutyronitrile (AIBN, 4 mg) were dissolved in dimethyl sulfoxide (5 mL). After degassing with nitrogen, the monomer solution was poured into a polymerization ampoule and heated at 60 °C for ~8 h. Finally, the

reaction product was precipitated in methanol and the precipitate was lyophilized, yielding a white solid β-galactose-conjugated homopolymer (PVLA-G) (Scheme 2). The conjugation of β-galactose and maltotriose onto the block copolymer was confirmed by recording FT-IR spectra of β-galactose (PS-b-PA-G) and maltotriose (PS-b-PA-M) conjugated block copolymers spotted on KBr pellets using a Jasco FT-IR 300E spectrophotometer, with a resolution of 4 cm^{-1}.



Scheme 2 Synthesis of β-galactose-conjugated poly(vinyl benzyl-galactopyranosyl gluconamide) homopolymer (PVLA-G).[40]

2.4 Preparation of LC microdroplets

To prepare ligand-anchored LC microdroplets, 10 mg (4.7×10^{-5} mmol) of β-galactose-conjugated block copolymer (PS-b-PA-G) was added to a 100 mL round-bottom flask containing 10 mL PBS and 50 mg (0.174 mmol) of sodium dodecyl sulfate (SDS). The mixture was stirred for 30 min at 800 rpm to properly mix the PS-b-PA-G and SDS. Upon obtaining a homogeneous

mixture of PS-b-PA-G and SDS, the LC microdroplets were prepared by drop wise addition of 50 mg (0.2 mmol) of 4-cyano-4'-pentyl biphenyl (5CB) into the homogeneous solution of PS-b-PA-G and SDS. The resulting mixture was stirred continuously for 24h to produce homogeneously dispersed microdroplets of 5CB. The dispersed LC microdroplets were centrifuged at 800 rpm in the presence of PBS solution to separate LC microdroplets from unused 5CB, SDS and PS-b-PA-G. After removing the supernatant, the centrifuged LC microdroplets were dispersed in PBS solution and centrifuged again to collect size-selected samples of LC microdroplets. Finally, the LC microdroplets were stored in PBS solution, pH 7.4, until needed for analysis of cell interaction. In addition, LC microdroplets containing ligand-conjugated block copolymer (PS-b-PA-M) or ligand-conjugated homopolymer (PVLA-G) were prepared by adding 5CB drop wise into separately prepared homogeneous mixtures of maltotriose (PS-b-PA-M) and SDS, or β-galactose-conjugated homopolymers (PVLA-G) and SDS. The subsequent steps followed the same procedure used for the synthesis of LC microdroplets containing β-galactose-conjugated block copolymer.

2.5 Characterization of LC microdroplets

Samples of LC microdroplets with a narrow size distribution were obtained by centrifuging the purified samples of LC microdroplets and separately collecting the LC microdroplets samples from the supernatant and the bottom of the centrifuge tube. The size distribution of the microdroplets was examined using particle size analyzer (Beckman coulter, N5/LS-13320, USA). The orientation state of the liquid crystal molecules in the microdroplets was determined by taking 1.0 mL of LC microdroplets into a 1.0 cm diameter optical cell and recording polarized-light micrographs of the LC microdroplets. The presence of block copolymer in the LC microdroplets was confirmed by recording confocal laser scanning micrographs (Zeiss LSM410,

Zeiss, Oberkoshen, Germany) of LC microdroplets containing FITC- and β-galactose-conjugated block copolymers (PS-b-PA-G).

2.6 *In vitro* interactions of LC microdroplets with target and control cells

To study the interactions of cells with LC microdroplets, target (HepG2) and control cells (KB cancer cells and fibroblasts) were cultured in tissue culture dishes using cell-appropriate media. HepG2 (human liver cancer cells) were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% FBS, penicillin (50 units/mL), and streptomycin (100 μg/mL) under a 5% CO_2 in humidified chamber at 37 °C. After culturing for 72h, the cells were collected and separated from the medium by centrifugation at 1200 rpm for 3 min. The cells were rinsed with sterile Dulbecco's phosphate-buffered saline (DPBS), pH 7.4, containing calcium chloride (10 mM) and magnesium chloride (5 mM). The cell pellet was redispersed in DPBS to obtain a homogeneous cell suspension and the cell density was measured using a hemocytometer. A calculated volume of HepG2 cells was used to maintain a cell density of 8000 HepG2 cells/mL during incubation with LC microdroplets (1 mL) for 3 h in 10% FBS solution at 30 °C. The KB cancer cells were cultured in tissue culture dishes in the presence of 5% CO_2 at 30 °C using Dulbecco's modified eagle medium (DMEM, GIBCO) containing 10% FBS (GIBCO), penicillin-streptomycin (50 units/mL and 50 μg/mL, respectively; GIBCO) and glutamine (1 mM; GIBCO). To maintain a fixed density of 8000 KB cancer cells/mL with the LC microdroplets, a calculated volume of the medium containing KB cancer cells (5×10^4 cells/mL) was added to the culture dish containing LC microdroplets (1 mL) and allowed to incubate for 3 h in 10% FBS solution at 30 °C. The normal fibroblast cells were also cultured in complete medium in tissue culture dishes, and harvested fibroblast cells with a cell density of 5×10^4

cells/mL were used to maintain a cell density of 8000 cells/mL when studying their interactions with LC microdroplets. To confirm the interactions of LC microdroplets with HepG2 cancer cells, KB cancer cells and fibroblast cells, optical and polarized-light micrographs were recorded after the 3 h incubation periods. The HepG2 cancer cells at a cell density of 8000 cells/mL were able to induce orientational transitions in LC microdroplets after 3h, but the control cells did not induce orientational transitions in LC microdroplets, even after 24h of incubation. Therefore, subsequent analyses of LC microdroplet and cell interactions were recorded after incubating LC microdroplets with selected cells for a maximum time period of 3h. To differentiate the interactions of KB cancer cells, fibroblasts and HepG2 cancer cells with LC microdroplets, the HepG2 cells were stained with methylene blue before incubation with LC microdroplets. The unstained KB cells and fibroblast cells were incubated with LC microdroplets in the presence and absence of stained HepG2 cancer cells in 10% FBS solution. Optical and polarized light microscopic images of LC microdroplets were recorded using an Olympus IX 71 inverted fluorescence microscope working in both cross-polarization and transmission modes. To test the reproducibility and accuracy in detection of HepG2 cells, the polarized microscopic images were recorded in triplicates at three different places in the test solution for an area of ~1.8 μm^2. A freshly prepared FITC solution in anhydrous DMSO (1 mg/mL) was used to conjugate FITC to the block copolymer (PS-b-PA-F). The reaction of FITC was carried out in the dark at 5 °C for ~8h. A confocal laser scanning microscope (Zeiss LSM410, Zeiss, Oberkoshen Germany) was used for detection of FITC-conjugated block copolymer (λ_{Ex} = 495 nm, λ_{Em} = 519 nm) in LC microdroplets at a brightness of 100 Cd/mm^2.

3. Results and Discussion

The response of liquid crystal systems to the presence of chemicals or biomolecules depends on the extent of interaction of the liquid crystals with the molecules. The magnitude of the interactions depends on the type of liquid crystal system used and the nanostructures produced at the interface of the amphiphilic polymers and the surfactants. Amphiphilic block copolymers and surfactants used in fabrication of LC microdroplets can form self-assembled nanosized structures with an interface that can transduce and amplify responses of the LC microdroplets upon their interaction with HepG2 cells and other cell lines. Certain polysaccharides that show selective and strong affinities for cancer cells have been used in targeted drug-delivery methods. To evaluate the cancer cell-targeting properties of carbohydrates for detection of HepG2 cells, and to clarify the role of amphiphilic block copolymers (PS-b-PA) and homopolymer (PVLA) in transduction of interaction forces from the interface to the core of LC microdroplets, β-galactose (PS-b-PA-G) and ligand simulant (PS-b-PA-M) conjugated block copolymers were prepared and used to fabricate LC microdroplets. The properties of the fabricated LC microdroplets, and their cell-type specificities, are discussed below.

3.1 Characterization of ligand containing block copolymers and LC microdroplets

Comparisons of photoelectron spectra of block copolymer (PS-b-PA), β-galactose (PS-b-PA-G) and maltotriose (PS-b-PA-M)-conjugated block copolymers, clearly indicated the conjugation of β-galactose and maltotriose moieties onto the block copolymer (PS-b-PA) through the ethylene diamine spacer. The photoionization peak for nitrogen was only visible in the ligand and ligand simulant-conjugated block copolymers (Fig. 1b,c). The percentage of atomic nitrogen in the maltotriose-conjugated block copolymer (2.5%) was lower than that in the β-galactose-conjugated block copolymer (4.3%) (Table1). This variation in nitrogen percent is due to the

difference in molecular weight of the conjugated β-galactose and ligand simulant (maltotriose) on the block copolymer.

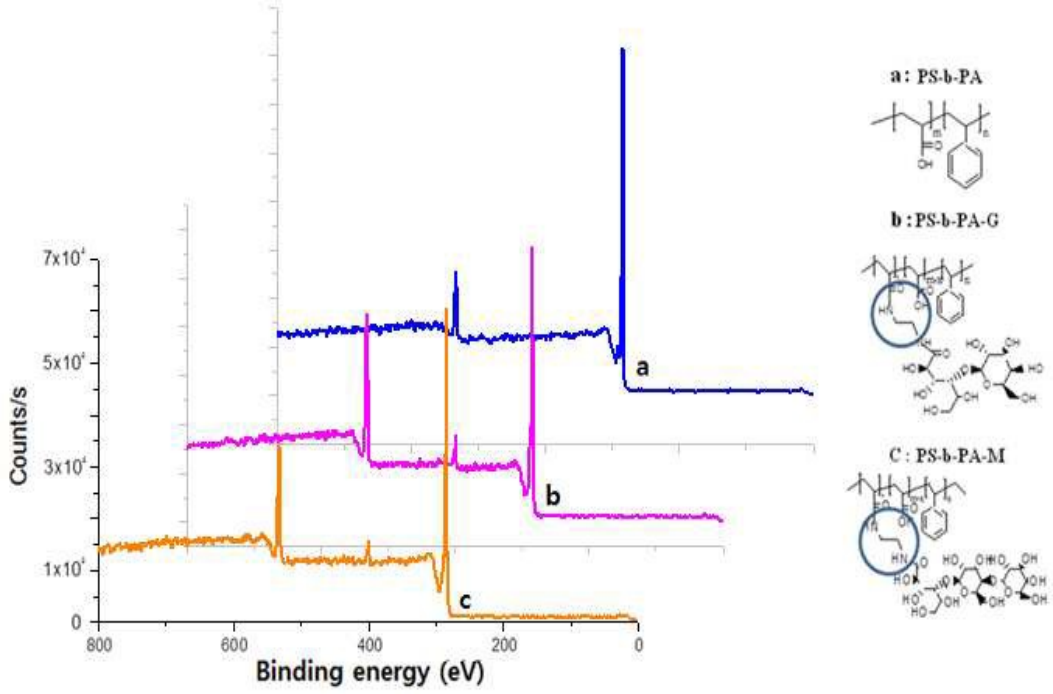


Fig. 1 X-ray photoelectron spectra of (a) block copolymer, (b) β-galactose- and (c) maltotriose-conjugated block copolymer.

The comparison of the photoelectron spectra (Fig. 1 & Table 1) and atomic percentages of nitrogen of block copolymer (PS-b-PA) with ligand (PS-b-PA-G) and simulant (PS-b-PA-M) conjugated block copolymers confirmed the successful conjugation of β-galactose and maltotriose moieties onto the block copolymer (PS-b-PA).

Table 1 Atomic percentage* of elements present in block copolymers of LC microdroplets

Atomic %	PS-b-PA	PS-b-PA-G	PS-b-PA-M
C	93.6	84.1	88.4
O	6.4	11.6	9.1
N	-	4.3	2.5

* The percentage is calculated from the XPS survey scan spectra.

The conjugation of β-galactose and maltotriose moieties onto the block copolymer was further confirmed by comparing the FT-IR spectrum of the block copolymer with those of the β-galactose- and maltotriose-conjugated block copolymers (Fig. 2).

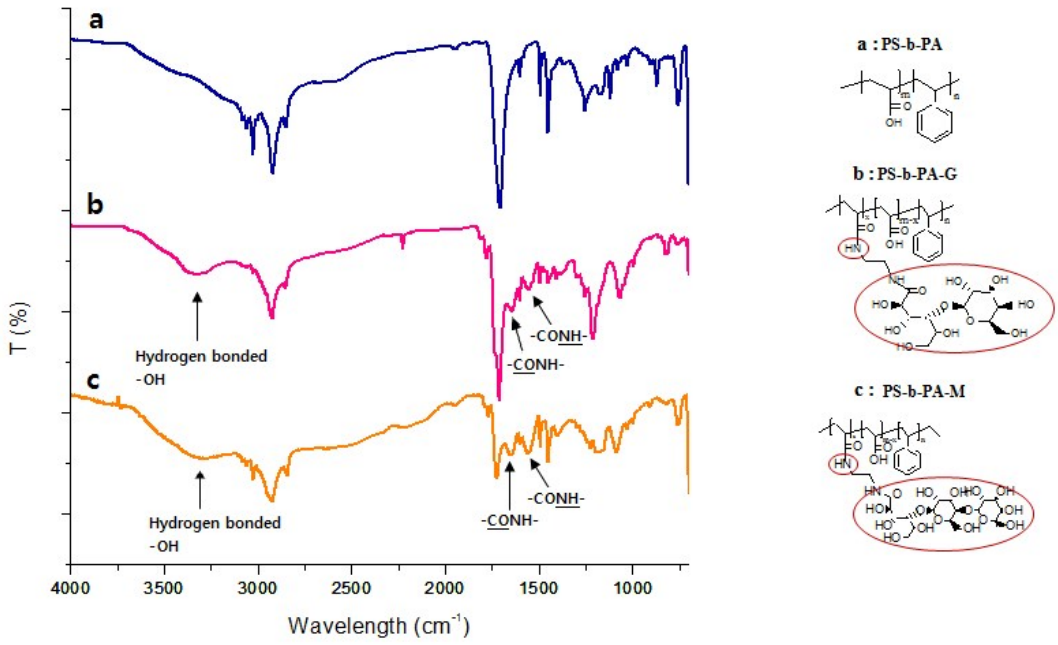


Fig. 2 FT-IR spectra of (a) block copolymer, (b) β-galactose- and, (c) maltotriose-conjugated block copolymer.

The FT-IR spectrum of the β-galactose-conjugated block copolymer showed absorption bands at 1640 cm^{-1} and 1029 cm^{-1}, corresponding to the amide I and II and ether groups of β-galactose molecules on the block copolymer. In comparison to FT-IR spectra of the block copolymer, the β-galactose- and maltotriose-conjugated block copolymers showed a broad band at 3282 cm^{-1} for the hydroxyl groups of β-galactose and maltotriose on the conjugates. The size distribution of LC microdroplets ranged from 5–25 μm in diameter, with a maximum size distribution of 12.5 μm (Fig. 3a). The LC microdroplets prepared using β-galactose- and maltotriose-conjugated block copolymers with sodium dodecyl sulfate at ambient temperature exhibited a radial orientational configuration, which was confirmed was polarized light micrographs as reported for folic acid anchored LC microdroplets.[31] The stability of liquid crystal molecules in a radial configuration might be due to the large size of the microdroplets that were formed and the presence of a sufficient amount of sodium dodecyl sulfate. These results are contrary to those reported by other workers.[41]

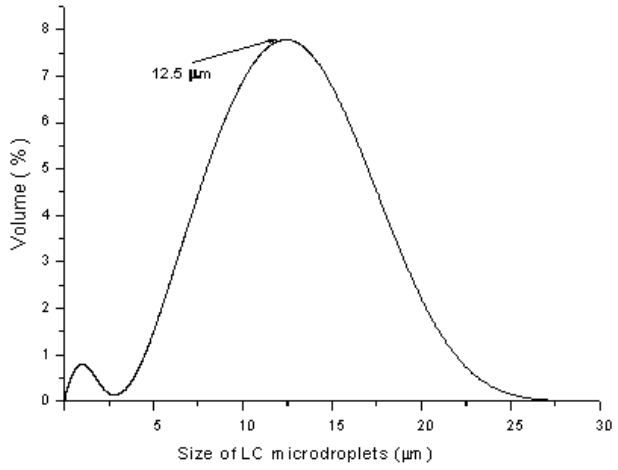


Fig. 3a The size distribution curve for LC microdropets showing size variation from 5-25 μm.

To confirm the presence of ligand-conjugated block copolymers in the LC microdroplets, LC microdroplets were also prepared using FITC- and β-galactose-conjugated block copolymer.

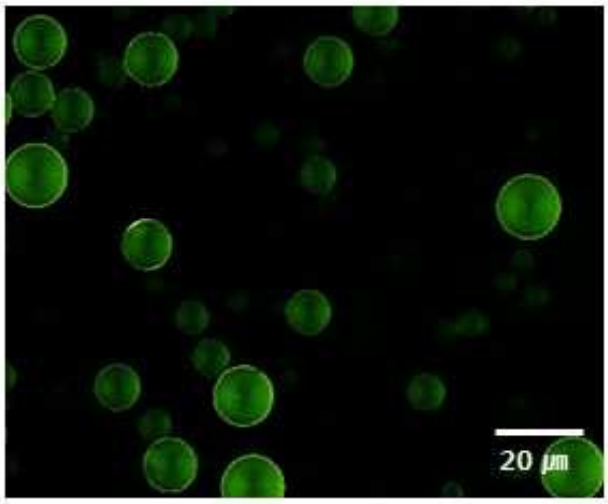
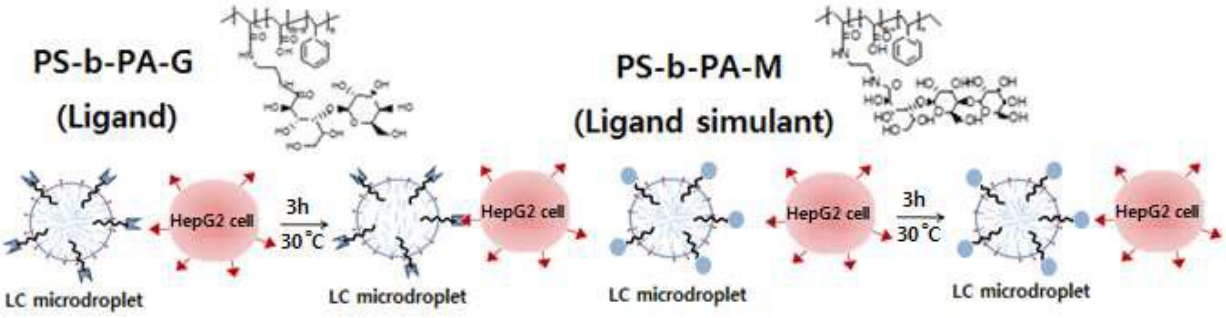


Fig. 3b Confocal laser scanning micrograph showing the presence of β-galactose- and FITC-conjugated block copolymer (PS-b-PA-G-F) in LC microdroplets.

The presence of β-galactose-conjugated block copolymers in LC microdroplets was confirmed by analysis of confocal laser scanning micrographs (CLSM) of LC microdroplets. The presence of green-colored LC microdroplets in the confocal laser scanning micrographs (λ_{Ex}= 495 nm, (λ_{Em}= 519 nm) confirmed the presence of β-galactose-conjugated block copolymers in the LC microdroplets (Fig. 3b). Recent studies have indicated that configurational transitions in LC microdroplets can be significantly influenced by variations in the amounts and types of surfactants used.[21] The present investigation on LC microdroplets was therefore carried out using a constant amount of sodium dodecyl sulfate (50 mg, 0.174 mmol). The observed radial configuration in the LC microdroplets anchored with β-galactose and maltotriose-conjugated

block copolymers also indicated the need for interaction between the crystal molecules and block copolymers. If conjugated ligands do not influence the π–π interactions between the benzene rings of the liquid crystal molecules and block copolymers, then the configuration state of the liquid crystal molecules will remain unaltered (Scheme 3). In these studies, the π–π interactions played a significant role in transmitting the ligand-receptor interaction forces to the liquid crystal molecules and influencing the ordering of liquid crystal molecules to the variable interactions between the anchored ligand and the receptor on the target cells. However, the response of liquid crystal molecules did depend on the magnitude of the interfacial interactions, as was clear from the final configuration of LC microdroplets prepared with β-galactose- versus maltotriose-conjugated block copolymers upon interaction with HepG2 cells (Scheme 3).



Scheme 3 Configurational state of LC microdroplets containing block copolymer conjugated with (a) β-galactose (PS-b-PA-G), and (b) ligand simulant maltotriose (PS-b-PA-M) in 10% FBS medium at 30 °C.

The ligand-receptor interactions of HepG2 cells were stronger with β-galactose-anchored LC microdroplets than with maltotriose-anchored LC microdroplets. β-galactose-anchored LC microdroplets showed a configurational transition from radial to bipolar upon interaction with HepG2 cells, but no configurational change was noticed when maltotriose-anchored LC

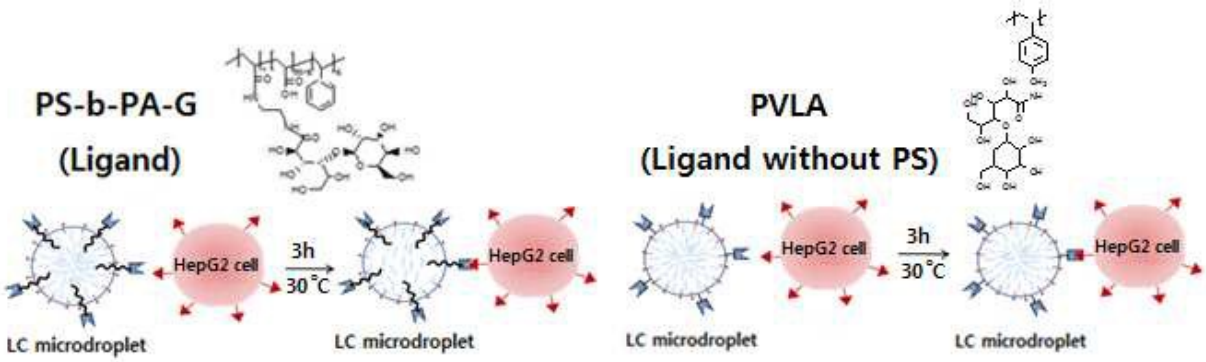
microdroplets interacted with HepG2 cells (Scheme 3). The configurational transition in liquid crystal molecules took place only when the interfacial interaction energy was sufficient to induce configurational transition of the liquid crystal molecules in the microdroplets.

To avoid an effect of the amount of amphiphilic polymer on the configurational state of liquid crystal molecules in LC microdroplets, a fixed amount of ligand-conjugated block copolymer (10 mg) was used. This ensured that the ratio of liquid crystal molecules to block copolymer remained constant, and that a uniform coating of ligand-anchored block polymer was formed on the LC microdroplets[42] to reduce variations in the interactions of the liquid crystal molecules with HepGe2 cells. The LC microdroplets reported in these studies were highly sensitive to interfacial interactions, and showed configurational transitions upon interaction with HepG2 cells. Therefore, the responses of LC microdroplets prepared here were comparable to those of earlier studies that described the application of LC microdroplets to detection of chemicals and biomolecules.[7,15, 43-45] The determination of optimal amounts of β-galactose- or maltotriose-conjugated block copolymer and SDS allowed for formation of dynamic nanostructures at the LC microdroplet-water interface. These nanostructures transduced the interfacial interactions to the core of LC microdroplets via the styrenic benzene of the block copolymer, in a manner similar to that of natural lipids, which act as transducers in biological systems. The sensitivity of the prepared LC microdroplets to interfacial interactions and configurational transitions was significantly higher than that in polymer dispersed liquid crystal systems,[46-49] which are known to be less sensitive to interfacial activity than LC microdroplets. These studies therefore demonstrated that the LC microdroplets prepared with small amounts of amphiphilic block copolymer and SDS surfactant were more sensitive in transduction of interfacial interactions to the core of LC microdroplets than LC microdroplets prepared with greater amounts of

amphiphilic block copolymer, or without SDS surfactants. In these studies, the use of amphiphilic block copolymers containing β-galactose ligand also contributed significantly to the sensitivity of the LC microdroplets to interfacial events, when compared to LC microdroplets prepared with β-galactose-conjugated homopolymer (PVLA-G) and SDS (as discussed below). These results have provided an understanding of the role of amphiphilic block copolymers in transmitting the interfacial energies to the core of the microdroplets.

3.2 LC microdroplets anchored with β-galactose-conjugated homopolymer

To validate the role of the styrenic benzene of the block copolymer as a transducer of interfacial interaction forces to the LC molecules in the microdroplets, LC microdroplets were also prepared using a homopolymer with β-galactose-conjugated benzene (PVLA-G), and the prepared LC microdroplets were allowed to contact HepG2 cells in 10% FBS solution. No configurational changes in the LC microdroplets were detected, despite strong interactions between the LC microdroplets and HepG2 cells (Scheme 4).



Scheme 4 Configurational state of LC microdroplets anchored with β−galactose -conjugated (a) block copolymer (PS-b-PA-G) and (b) homopolymer (PVLA-G) in 10% FBS at 30 °C.

In these LC microdroplets, the benzene of the homopolymer (PVLA-G) was not able to penetrate to the core of the LC microdroplets; hence it was not possible to transmit the ligand-receptor interactions to the LC molecules, and no configurational transition occurred in the LC microdroplets upon interaction with HepG2 cells (Scheme 4).

3.3 Interactions of HepG2 cells with LC microdroplets anchored with β-galactose- and maltotriose-conjugated block copolymers

The LC microdroplets prepared with β-galactose- and maltotriose-conjugated block copolymers were allowed to make contact with HepG2 cells in 10% FBS, and the effect of ligand-receptor interactions on configurational state of liquid crystal molecules was analyzed using polarized light micrographs. It is interesting to note that the ligand-receptor interactions remained constant as the amount of FBS was varied from 0–10% during the interactions of HepG2 cells and other cell lines with the LC microdroplets. These results suggested that the LC microdroplets could be used to sense the presence of HepG2 cells in biological fluids without interference from plasma proteins, and that the configurational transitions in the LC microdroplets would be totally dependent on ligand-receptor interactions. In consideration of this lack of interference from FBS, the cell-sensing experiments with the LC microdroplets were carried out in the presence of 10% FBS, and cells were allowed make contact with LC microdroplets for 3 h at 30 °C. The LC microdroplets prepared with β-galactose and maltotriose-conjugated block copolymers were allowed to make contact with HepG2 cells, and the effect of HepG2 cell interactions on the configurational state of liquid crystal molecules was analyzed using micrographs collected with a polarized light microscope. The optical images of LC microdroplets cultured with HepG2 cells (Fig. 4a,b) demonstrated the interactions of HepG2 cells with LC microdroplets. The interactions of HepG2 cells with LC microdroplets containing β-galactose-conjugated block copolymer were

effective in changing the configurational state of the LC microdroplets from radial to bipolar, as shown in polarized light micrographs of LC microdroplets (Fig. 4b). On the other hand, contact of HepG2 cells with LC microdroplets anchored with maltotriose-conjugated block copolymer (Fig. 4c,d) did not induce configurational changes in the LC microdroplets (Fig. 4d). This indicated that the HepG2 cells did not interact effectively with the LC microdroplets anchored with maltotriose-conjugated block copolymer.

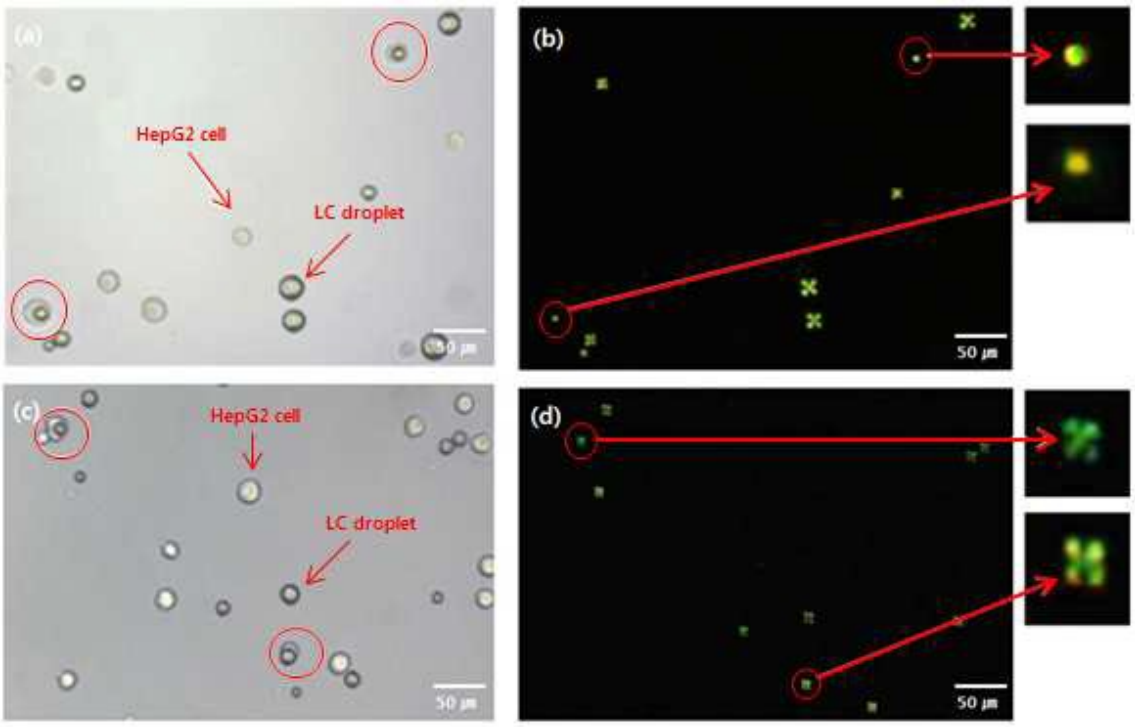


Fig. 4 Optical (a, c) and polarized light (b,d) micrographs showing interactions of HepG2 cells with LC microdroplets anchored with β-galactose- (PS-b-PA-G) (a,b), or maltotriose- (PS-b-PA-M) (c, d) conjugated block copolymer in 10% FBS at 30 °C. The microscopic images were recorded in triplicates ($p < 0.05$, $n = 3$).

This result suggested that the anchored maltotriose did not interact with the asialoglycoprotein receptor when LC droplets were allowed to contact with HepG2 cells (Fig. 4 d). In contrast, β-

galactose was able to bind selectively and strongly with asialoglycoprotein receptors, which are over expressed on HepG2 cancer cells, and the resultant ligand-receptor interaction force was able to cause a configurational transition in LC microdroplets, as was clear from the polar micrographs of those LC microdroplets in contact with HepG2 cells (Fig. 4b). Statistically, the LC microdroplets were able to detect 1.0 ± 0.01 HepG2 cells/μm^2 (Fig. 4b & d) on recording microscopic images in triplicates ($p < 0.05$, $n = 3$) for configurational transition in LC microdroplets anchored with β-galactose and maltotriose ligands (Fig. 4b & d) in a given area of ~1.8 μm^2 of optical test cell.

3.4 Interactions of HepG2 cells with LC microdroplets anchored with β-galactose-conjugated homopolymers

To validate the role of the polystyrene segment of the block copolymer in transmission of interfacial interaction energy to the core of LC microdroplets, HepG2 cells were allowed to make contact with LC microdroplets anchored with β-galactose-conjugated homopolymer (PVLA-G), and optical (Fig. 5a) and polarized light micrographs (Fig. 5b) were recorded.

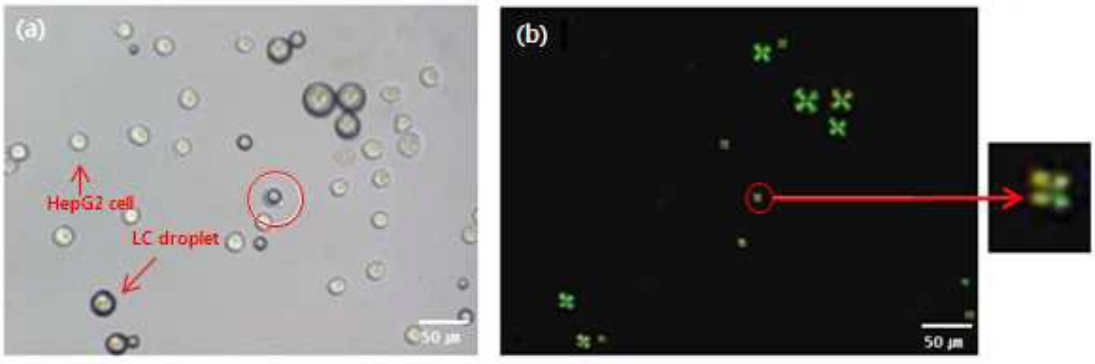


Fig. 5 Optical (a) and polarized light (b) micrographs showing interactions of HepG2 cells with LC microdroplets containing β-galactose-conjugated homopolymer (PVLA-G) in 10% FBS at 30 °C. The microscopic images were recorded in triplicates ($p < 0.05$, $n = 3$).

The polarized light micrographs of the LC microdroplets in contact with HepG2 cells did not show any configurational changes in the liquid crystal molecules. The ligand-receptor interactions took place at the interface between LC microdroplets and HepG2 cells, but the interaction force was not transmitted effectively to the core of the LC microdroplets since π–π interactions were not possible between 5CB and the β-galactose-conjugated homopolymer. The ligand-receptor interaction force was not transmitted in a way that caused a configurational transition in the liquid crystal molecules. These results indicated that configurational transitions in liquid crystal molecules are possible only if the interacting polymer contains energy transducing moieties, such as styrenic benzene, that can interact strongly with the liquid crystal molecules. These results validated the importance of the polystyrene segment component of the block copolymer when fabricating LC microdroplets for sensing applications that are based on 5CB. Statistically, the LC microdroplets containing β-galactose-conjugated homopolymer were able not able to detect any HepG2 in a given area of ~1.8 μm^2 (Fig. 5b) on recording microscopic images of LC microdrolets in triplicates ($p < 0.05$, $n = 3$).

3.5 Selectivity of β-galactose-anchored LC microdroplets in detection of HepG2 cells

Further experiments were carried out to analyze the ability of β-galactose-conjugate block copolymer-anchored LC microdroplets to detect control cells (KB cancer cells and fibroblasts), or HepG2 cells in the presence of control cells. Incubation of KB cancer cells and fibroblast cells with β-galactose-anchored LC microdroplets separately did not induce orientational transition in the LC microdroplets, whereas incubation of HepG2 cell with β-galactose-anchored LC microdroplets did induce the transition (Fig. 4b). These results suggest that β-galactose-anchored LC microdroplets will be useful for the detection of HepG2 cells found in biological fluids, and

that other types of cells, such as KB cancer cells and fibroblasts will not create similar signals. To examine the selectivity of LC microdroplets prepared with β-galactose-conjugated block copolymer and SDS for HepG2 cells, the β-galactose anchored LC microdroplets were incubated for 3h with HepG2 cells in the presence of KB cancer cells and fibroblast cells, each at a cell density of 8000 cells/ mL, at 30 °C in 10% FBS medium. To determine the sensing selectivity of LC microdroplets for HepG2 cells in presence of KB cells and fibroblasts, HepG2 cells stained with methylene blue were mixed with unstained KB cancer cells and fibroblasts. After incubating the LC microdroplets with the cells for 3h, optical and polarized light images of the LC microdroplets were recorded, as shown in Fig. 6. The optical images indicated that stained HepG2 cells, as well as unstained control cells, were able to make contact with the LC microdroplets (Fig. 6a). The polarized light images of LC microdroplets (Fig. 6b) in contact with stained HepG2 cells and unstained control cells were then examined to ascertain the selectivity of LC microdroplets on the basis of the configurational state of the liquid crystal molecules.

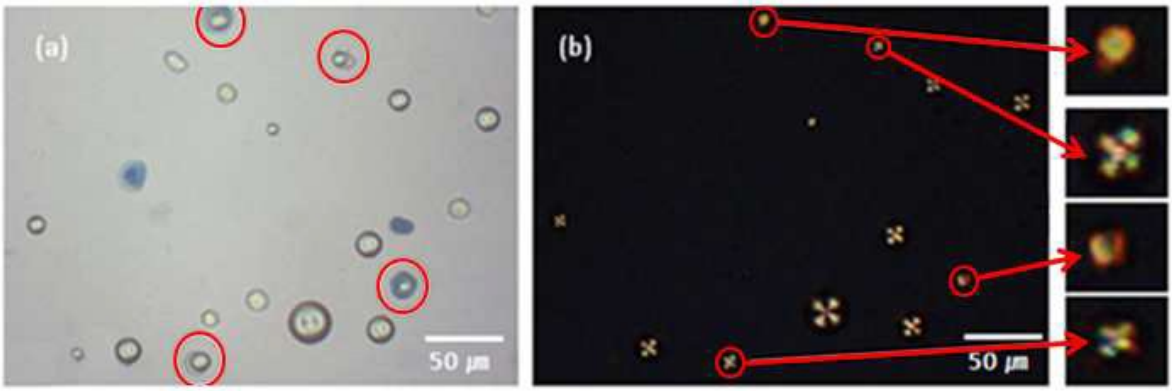


Fig. 6 Optical (a) and polarized light (b) micrographs showing interactions of methylene blue -stained HepG2 cells in the presence of control cells (KB and FB) with LC microdroplets containing β-galactose-conjugated block copolymer (PS-b-PA-G) in 10% FBS at 30 °C. The microscopic images were recorded in triplicates ($p < 0.05$, $n = 3$).

LC microdroplets in contact with two stained HepG2 cells co-cultured with control cells, (left top and right bottom circles in Fig. 6a), that have undergone configurational transition from radial to bipolar, are shown in the two upper insets in Fig. 6b by arrows. In contrast, contacts of two control cells with LC microdroplets (right top and left bottom circles in Fig. 6a) were not effective in inducing configurational transition in LC microdroplets, as is evident from the radial configuration of LC microdroplets shown in the two lower insets in Fig. 6b by arrows. These results confirmed that β-galactose-anchored LC microdroplets were able to effectively interact with HepG2 cells in a selective manner and without interference from KB cancer cells or other control cells in the coculture system. The contacts of other control cells with LC microdroplets were weaker and insufficient to cause configurational transitions of the liquid crystal molecules in the LC microdroplets. Statistically, the sensitivity of β-galactose anchored LC microdroplets in detection of HepG2 cells is found to be 1.0 ± 0.01 HepG2 cells/μm^2 on recording optical micrographs in triplicates ($p < 0.05$, $n = 3$) both in absence (Figs. 4b) and in presence of control cells (Figs. 6b) in a given area of ~1.8 μm^2 of the optical test cell.

The enhanced sensitivity of LC microdroplets in the detection of HepG2 cells was assumed to be due to the localized binding of HepG2 cells to the curved interfaces of LC microdroplets anchored with β-galactose ligand.[50] The selectivity of the prepared LC microdroplets for detection of HepG2 cells appears to be superior to aptamer-based electrochemical biosensors reported for *in vitro* detection of HepG2 cells in the presence of other cancer cells, such as human prostate, breast, and colon tumor cells.[39] The prepared LC microdroplets should have an advantage for *in vitro* detection of HepG2 cells in blood plasma, since time will not be lost in a sample preparation step that is necessary for aptamer-based electrochemical biosensors.

4. Conclusions

LC microdroplets were successfully prepared using 4-cyano-4'-pentyl biphenyl (5CB)_and β-galactose-conjugated poly(styrene-b-acrylic acid) in the presence of sodium dodecyl sulfate. The prepared LC micropdroplets were found to be highly selective for HepG2 cells in 10% FBS solution, and showed configurational transitions from radial to bipolar in liquid crystal molecules over a time interval of 3h. The LC microdroplets prepared with maltotriose-conjugated block copolymer and β-galactose-conjugated homopolymer did not show configurational transitions, either as a result of weak interactions with HepG2 cells or of not having styrenic benzene to transmit the ligand-receptor interaction force to the liquid crystal molecules in the microdroplets. The LC microdroplets showed high selectivity in the detection of HepG2 cells in presence of the control cells, such as KB cancer cells and fibroblasts. The present method found to be highly sensitive in detection of 1.0 ± 0.01 HepG2 cell/μm^2 on recording microscopic images in triplicates ($p < 0.05$, $n = 3$) in a given area of ~1.8 μm^2 of optical test cell. The interactions of LC microdroplets with HepG2 cells remained constant in the presence of different concentrations of FBS, which further indicated the applicability of LC microdroplets for detection of HepG2 cells in blood-containing samples. The presence of a polystyrene segment in the block copolymer used in fabrication of LC microdroplets was essential for sufficient transduction of ligand-receptor interaction forces to the core of LC microdroplets to allow for recording of optical images that permit visual detection of HepG2 cells in biological fluids. This approach could therefore serve as the basis of a label-free optical biosensor-based method for *in vitro* detection of HepG2 cells in biological fluids, which would not involve unnecessary loss of time for sample preparation.

Further work is in progress to fabricate LC microdroplet-based biosensors using functional polymers and surfactants for the detection of other cancer cells and biomolecules.

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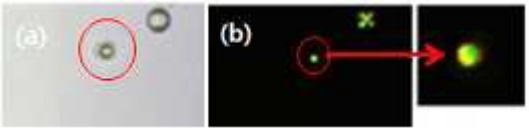
Table of content



Optical (a) and polarized (b) micrographs showing orientational transition in LC microdroplet on contacting with HepG2 cell in PBS solution.