Analytical Biochemistry 558 (2018) 1–11

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

In vitro detection of allergen sensitized basophils by HSA-DNP antigen-anchored liquid crystal microdroplets

Hanbyeol Shin[a], So Jung Park[a], Kailash Chandra Gupta[a,b,**], Dong Yun Lee[a], Soo-Young Park[a], Inn-Kyu Kang[a,*]

[a] Department of Polymer Science and Engineering, Kyungpook National University, 80, Daehak-ro, Buk-gu, Daegu 41566, South Korea
[b] Polymer Research Laboratory, Department of Chemistry, I.I.T. Roorkee, Roorkee -247667, India

ARTICLE INFO

Keywords:
Basophils
FcεRI receptor
HSA-DNP-Antigen
Allergen
LC microdroplets

ABSTRACT

A simple and easy to handle biosensing technique for *in vitro* detection of HSA-DNP antigen induced allergen reactions in patients has been developed through the detection of sensitized basophils expressed with anti-IgE receptor (FcεRI) by using human serum albumin-dinitrophenol (HSA-DNP) antigen-anchored liquid crystal (LC) microdroplets emulsion. The radial to bipolar transition in nematic 4-cyano-4′-pentyl biphenyl liquid crystal molecules (5CB) confined in HSA-DNP antigen anchored LC microdroplets (8.5 pg HSA-DNP/LC microdroplet) is found to be sensitive in PBS solution in detection of allergen sensitized basophils expressed with a minimum amount of anti HSA-DNP (anti-IgE) receptor (≥ 4.5 pg/basophil). The detection of allergen sensitized basophils was possible within a contact time of 30 min in presence of control cells and with 10% solution of human blood plasma. The HSA-DNP antigen anchored LC microdroplets in presence of macrophages or non-sensitized basophils did not show radial to bipolar transition in 5CB molecules in PBS or solution with 10 wt% human blood plasma. Thus HSA-DNP antigen anchored LC microdroplets biosensor may be used for *in vivo* detection of stage I allergen reaction basophils in blood samples.

Introduction

The allergen induced reactions in patients are mostly diagnosed by clinical history, skin testing, and by detecting the presence of allergen specific antigen (IgG) in biological fluids. But in patients without clinical history, the sensitive detection of antigen sensitized basophils is only a method for the diagnosis of allergy in them [1]. Currently, the accepted test for detection of sensitized basophils is based on detection of anti-IgE receptor (FcεRI) or CD63 marker, which are over expressed on antigen sensitized basophils in allergic patients [2]. Since the life of CD63 marker is found to be very short (~20 min); hence the detection of sensitized basophils through high affinity anti-IgG receptor (FcεRI) is found to be more reliable in patients with allergic reactions. The sensitized basophils have high binding affinity to IgG antigen [3] and on binding cross-linking reactions with IgG antigen they start releasing the stored or newly formed mediators or effectors molecules such as cytokines (IL-4, IL-6 and IL-13), histamine [4], and chemokines [5]. The release of these mediators in blood by sensitized basophils on binding with IgG antigen is considered responsible in showing anaphylaxis in humans [6,7]. Since basophils are not tissue dwelling cells, hence mostly they remain in blood or spleen and also able to infiltrate into inflamed tissue in skin dermatitis or in patient's airways with respiratory allergies [8,9]. The sensitized basophils are always found in patients suffering with autoimmune diseases such as asthma, rhinitis, eczema, urticaria, dermatitis, or autoinflammatory disease and in patients with parasitic infections by helminthes and tapeworms [10]. Clinically, the allergies are diagnosed by skin prick or sera test methods by detecting the presence of allergen specific antibodies expressed on sensitized basophils. Though skin prick testing method is considered a primary option for the detection of allergen-specific IgG antigen in clinics [11] but serum immunoassaying of IgG antigen is found to be more sensitive and without side effect to the patients. Similarly, the antibodies labeling methods such as radio allergosorbent test (RAST) and enzyme linked immunoassaying (ELISA) test are found to be simple in immunoassaying of IgG antigen in sera but sensitivity of these methods is found to be poor in comparison to skin prick testing methods [11]. The ELISA test based on IgG antigen is also used in detection of anti-IgG receptor antibody in patients suffering from atopic, non-atopic dermatitis [12], and chronic urticarial [13]. The sensitivity of ELISA test in detection of anti-IgG receptor antibody in sera of schistosomiasis

* Corresponding author.
** Corresponding author. Polymer Research Laboratory, Department of Chemistry, I.I.T. Roorkee, Roorkee -247667, India.
E-mail addresses: kcgptfcy@iitr.ac.in (K.C. Gupta), ikkang@knu.ac.kr (I.-K. Kang).

https://doi.org/10.1016/j.ab.2018.07.025
Received 5 June 2018; Received in revised form 14 July 2018; Accepted 30 July 2018
Available online 01 August 2018
0003-2697/

patients was improved by 62% on removing the interference of IgG antibodies with protein G immobilized Sepharose beads [14]. The signal amplification power of immuno-polymerase chain reaction (IPCR) in combination with ELISA has also been used in detection of IgG antigen in the sera for a concentration range from 187.5 pg/mL to 12 ng/mL with a lower detection limit of 5.7 pg/mL or sometime 1 fg/mL [15]. In comparison to other methods such as ELISA, RAST, and community acquired pneumonia (CAP) systems for the detection of IgG antigen, the IPCR immunoassaying needs a small volume (0.3 μl) of human sera for detection of IgG antigen. In addition to ELISA, the flow cytometry alone [16–19] or in combination with mass spectrometry [20] has also been used for the detection of sensitized basophils in whole blood sample without their separation. The real time surface plasmon resonance (SPR) sensors also found useful in detection of sensitized basophils in school going children diagnosed with food allergies [21–24] and in patients with allergic reactions [25]. The sensitivity of fluorogenic [26,27] or enzymatic amplification methods [28–30] in detection of IgG antigen in serum is found to be in between 0.5 and 1 ng/mL. Thus allergic reactions in patients have been monitored either by detection of allergen specific polyvalent IgG antigen in sera or by detection of anti-IgE receptor (FcεRI) antibody on sensitized basophils by using ELISA, flow cytometry, surface plasmon resonance, and fluorogenic methods. But these methods are found to be either time consuming or costly in recording their response to sensitized basophils. At the same times many of these methods are not used for the detection of HSA-DNP antigen induced basophils expressed with anti-HSA-DNP (IgE) receptor. Recently in various studies, the long range orientation order of liquid crystal molecules has been used successfully to develop biosensors for label free detection of proteins and cancer-specific antigens using interfacial events at solids [31] or water–liquid crystal interfaces [32]. In comparison to layered liquid crystal molecules at interface, the confined liquid crystal molecules as microdroplets have shown high sensitivity in detection of interfacial events involving interactions with chemicals [33,34] or viruses [35]. The fabrication and handling of liquid crystal based biosensor is very easy and economical in comparison to other methods of detection of protein and metal ions. The LC microdroplets emulsion is able to generate optical signal by the orientation transition from bipolar to radial or vice versa in 5CB molecules on interactions with antigen in solution, which can be detected by using simple polarized light microscope. Thus considering the optical properties of 5CB liquid crystal molecules, we have developed biosensors for naked eye detection of cancer cells [36] and antigens [37]. The literature survey has indicated that so far no report is available for *in vitro* detection of HSA-DNP antigen sensitized basophils by using LC microdroplets emulsion anchored with HSA-DNP antigen. Therefore, in these studies an effort has been made to develop a biosensor based on optical properties of LC microdroplets as a tool for *in vitro* detection of HSA-DNP antigen sensitized basophils in PBS solution and in presence of human blood plasma without any interference from blood IgG antibodies.

Experimental section

Materials and methods

Poly(styrene-b-acrylic acid) (PS-b-PA) (Mw, 8000 g mol^{-1}), 98% nematic 4-cyano-4′-pentyl biphenyl liquid crystal (5CB) (Mw, 249.15 $gmol^{-1}$, m.p. 24 °C), 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC) (Mw, 155.25 g mol^{-1}), N-hydroxysuccinimide (NHS) (Mw, 115.09 g mol^{-1}), sodium dodecyl sulfate (SDS) (Mw, 288.38 g mol^{-1}), folic acid (FA) (Mw, 441.40 g mol^{-1}), human serum albumin dinitrophenol (HSA-DNP) antigen (Mw, 66 kDa), and o-phthalaldehyde were purchased from Sigma-Aldrich Chemical Company, USA and used as received. The macrophages (Oral squamous cells) and basophils were purchased from Korean cells bank. The α-minimum essential medium (α-MEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 1.0% penicillin G-streptomycin was used to culture macrophages at 37 °C under 5% CO_2 atmosphere. Basophils were cultured for 24 h in complete RPMI-1640 culture medium containing 2 mM L-glutamine and 25 mM Hepes (Biochrom), 10% heat-inactivated fetal calf serum (FCS) (Seromed) and antibiotics (100 IU/mL penicillin, 100 μg/mL streptomycin) and cells were used after 4–8 passages throughout the study. One day before the experiment for sensitization, the basophils were harvested using trypsin solution and then sensitized with HSA-DNP antigen to produce anti-IgE receptors at their surfaces [25]. To record optical micrographs, the glass bottom dish cultured basophils were stimulated by adding 50 ng/mL HSA-DNP antigen and fixed with 4% paraformaldehyde for 15 min. Following two times washes, the cells were treated with phosphate-buffered saline (PBS) solution containing 0.1% Triton X-100 and 5% bovine serum albumin for 30 min. All measurements were carried out in PBS solution (0.01 M phosphate buffer, 0.0027 M potassium chloride, and 0.137 M sodium chloride) at pH 7.4. The UV–vis spectra were recorded using double beam spectrophotometer (Jasco-650, UV–vis spectrophotometer, USA). The size distribution of LC microdroplets was determined using optical microscope (Nikon Eclipse TS100, Japan) and particle size analyzer (Beckman coulter, N/LS-1332, USA). The optical and polarized light micrographs of LC microdroplets were recorded using Olympus IX 71 inverted fluorescence microscope in cross-polarization and transmission modes. The upright fluorescence microscope (Olympus BX61, Olympus America Inc., USA) with fluorescent filter tube above the objective lenses coupled with digital camera was used to record FITC fluorescence ($\lambda_{Ex,}$ 519 nm) images of LC microdroplets to confirm the conjugation of HSA-DNP antigen on LC microdroplets. The ultrapure water was prepared using Milli-Q system and used in experimental work. The HSA-DNP antigen and HSA-DNP-FITC conjugate anchored LC microdroplets were synthesized by anchoring HSA-DNP antigen and HSA-DNP-FITC conjugate on LC microdroplets after using EDC and NHS as activators.

Preparation of LC microdroplets

To prepare LC microdroplets emulsion, 10 mg (1.4 μmol) of PS-b-PA block copolymer was added to a 100 mL round bottom flask containing 10 mL PBS solution and 10 mg (35 μmol) of sodium dodecyl sulfate (SDS). The mixture was magnetically stirred for about 30 min at 1100 rpm to obtain a homogeneous solution of SDS with amphiphilic PS-b-PA block copolymer. In homogeneous solution of PS-b-PA block copolymer and SDS surfactant, 50 mg (200 μmol) of 98% nematic 4-cyano-4′-pentyl biphenyl liquid crystal molecules (5CB) were added drop-wisely. The mixture so obtained was stirred continuously at 11,000 rpm for about 24 h to obtain a homogeneous dispersion of LC microdroplets. The resultant homogeneous dispersion of LC microdroplets was centrifuged at 800 rpm in PBS solution (pH 7.4) to separate out LC microdroplets from unused 5CB, SDS, and PS-b-PA block copolymer. After removing the supernatant having small-sized LC microdroplets (< 10 μm diameter), the centrifuged LC microdroplets were dispersed in PBS solution and centrifuged again to collect size-selected samples of LC microdroplets. These LC microdroplets were preserved in PBS solution for anchoring HSA-DNP antigen and to study their interactions with HSA-DNP antigen sensitized basophils in PBS solution. The size distribution of LC microdroplets was determined using optical microscope (Nikon Eclipse TS100, Japan) and particle size analyzer (Beckman coulter, N/LS-1332, USA). To confirm the orientational state of 5CB molecules in LC microdroplets, the polarized light micrographs of LC microdroplets were recorded using Olympus IX 71 inverted fluorescence microscope, which was able to operate in cross-polarization and transmission modes.

Anchoring of HSA-DNP antigen on LC microdroplets

To synthesize HSA-DNP antigen anchored LC microdroplets, the carboxylic groups of polyacrylic acid blocks in amphiphilic block co-polymer (PS-b-PA) in LC microdroplets were activated by taking 1.0 mL LC microdroplets emulsion (1×10^4 microdroplets/mL) having average microdroplet size of 27 ± 2 μm in a round bottom flask containing 10 mL PBS solution. To this solution, 100 mg (320 μmol) of NHS and 100 mg (870 μmol) of EDC was added and solution was kept for 1 h in dark at 5 °C. To this solution, 100 ng of HSA-DNP antigen was added under stirring and left for overnight at 5 °C. The resultant mixture was dialyzed using regenerated cellulose nitrate membranes (MWCO, 12 kDa) to remove unused EDC, NHS, HSA-DNP antigen, and other impurities. Finally, HSA-DNP antigen anchored LC microdroplets were washed with PBS till pH 7.2 and preserved in PBS solution (5.0×10^4 microdroplets/mL) for sensing experiments with sensitized basophils. The anchored amount of HSA-DNP antigen on LC microdroplets was determined by analyzing the solution for remaining amount of HSA-DNP antigen at 360 nm (Bradford method) using calibration curve, which was drawn using standard dilute solutions of HSA-DNP antigen. The anchoring of HSA-DNP antigen on LC microdroplets was found to be ~85% (8.5 pg HSA-DNP antigen/LC microdroplet).

Anchoring of HSA-DNP-FITC conjugate on LC microdroplets

To confirm the anchoring of HSA-DNP antigen on LC microdroplets, first of all HSA-DNP-FITC conjugate was prepared. The HSA-DNP-FITC conjugate was prepared by drop-wise addition of 10 mL PBS solution containing 0.1 μg (0.1 μmol) of HSA-DNP antigen to a 100 mL round bottom flask containing 100 mg each of EDC and NHS dissolved in 10 mL PBS solution to activate the carboxyl groups of HSA-DNP antigen. After 2 h, a freshly prepared 10 mL solution of FITC (0.5 μg) in dimethyl sulfoxide was added and mixture was left in dark at 5 °C. After 8 h, the reaction mixture was dialyzed using regenerated cellulose acetate membranes (MWCO, 14 kDa) and PBS solution. Finally, HSA-DNP-FITC conjugate was washed with PBS solution and lyophilized to obtain a powdery white solid. The conjugation of FITC with HSA-DNP antigen was confirmed by recording UV–vis spectra of HSA-DNP antigen, FITC, and HSA-DNP-FITC conjugate using UV–vis spectrophotometer. To anchor HSA-DNP-FITC conjugate on LC microdroplets, a 10 mL PBS solution containing 100 ng of HSA-DNP-FITC conjugate was added to a 100 mL round bottom flask containing 1.0 mL LC microdroplets (1×10^4 microdroplets/mL) and activating agents (EDC and NHS, 100 mg each). The mixture of HSA-DNP-FITC and LC microdroplets was centrifuged at 8000 rpm for 10 min. The mixture was left in dark at 5 °C and after 8 h, the supernatant was removed. The HSA-DNP-FITC conjugate anchored LC microdroplets were washed with PBS solution to remove unused HSA-DNP-FITC, EDC and NHS. The anchoring of HSA-DNP-FITC conjugate was confirmed by recording green fluorescent images of HSA-DNP-FITC conjugated LC microdroplets (λ_{EX}, 519 nm) using upright fluorescence microscope (Olympus BX61, Olympus America Inc., USA) with fluorescent filter tube above the objective lenses coupled with a digital camera. The anchoring of HSA-DNP-FITC conjugate on LC microdroplets was found to be around 72% (i.e., 7.2 pg HSA-DNP-FITC/LC microdroplet). The orientation state of 5CB molecules in HSA-DNP-FITC conjugated LC microdroplets was determined by recording polarized light micrographs of LC microdroplets using Olympus IX 71 inverted fluorescence microscope, which was able to operate in cross-polarization and transmission modes.

Detection of basophils by HSA-DNP antigen anchored LC microdroplets

The detection of sensitized basophils has been carried out under different experimental conditions to confirm the selectivity of HSA-DNP antigen anchored LC microdroplets for allergen sensitized basophils expressed with anti-IgG-receptor.

Detection of sensitized basophils in absence of control cells

To evaluate the optical sensitivity of HSA-DNP antigen anchored LC microdroplets in detection of allergen sensitized basophils, first of all basophils were cultured for 4–6 passages and then sensitized by seeding 1.0 mL basophils (1×10^4 basophils/mL) with 100 ng HSA-DNP antigen for 2 h in incubator at 37 °C in presence of 5% CO_2. The sensitized basophils were washed with PBS solution to remove unused HSA-DNP antigen and other impurities. The analysis has indicated for 90% consumption of HSA-DNP antigen in stimulation of 1×10^4 basophils (i.e., 4.5 pg of HSA-DNP antigen was used per basophil). Thus considering the amount of HSA-DNP antigen used in stimulation of basophils, each basophil was expressed with ~4.5 pg of anti-IgE receptor to interact with HSA-DNP antigen anchored LC microdroplets. This results has also supported 1:1 mass stoichiometry between receptor and antigen. For detection of sensitized basophils, 1 mL solution of PBS containing LC microdroplets (1×10^4 microdroplets/mL) anchored with HSA-DNP antigen was added in a culture dish containing 1.0 mL sensitized basophils (1×10^4 basophils/mL). The dish was incubated for 30 min at 30 °C in presence of 5% CO_2.Parallel control experiments were also performed by adding HSA-DNP antigen anchored LC microdroplets (1×10^4 microdroplets/mL) in culture dishes having either macrophages or non-sensitized basophils and after the optimized contact time of 30 min, the optical and polarized light micrographs of incubated HSA-DNP antigen anchored LC microdroplets were recorded to visualize the contact of incubated cells with HSA-DNP antigen anchored LC microdroplets and to analyze the effect of their contacts on orientational state of 5CB molecules in HSA-DNP antigen anchored LC microdroplets.

Detection of sensitized basophils in presence of control cells

For selective detection of anti-IgE receptor bearing basophils by HSA-DNP antigen anchored LC microdroplets, the sensitized basophils were co-cultured with macrophages or non-sensitized basophils in culture dish and allowed to interact with HSA-DNP antigen anchored LC microdroplets. To carry out the selective detection of sensitized basophils, the sensitized basophils and methylene blue stained macrophages were mixed in equal cells density (5×10^3 cells/mL). The macrophages were stained using 10% methylene blue for 30 min at 37 °C and this staining of macrophages was found to be useful to make their contacts distinct with HSA-DNP antigen anchored LC microdroplets in presence of sensitized basophils. The density of sensitized basophils and methylene blue stained macrophages was determined using hemocytometer. The sensitized basophils mixed with methylene blue stained macrophages were seeded for 2 h at 37 °C in presence of 5% CO_2 in α-DMEM medium containing 10% FBS and 1% penicillin-streptomycin. After incubating sensitized basophils and methylene blue stained macrophages for 2 h, the culture dish was washed with PBS solution to wash out the residual medium and unattached cells from culture dish. To carry out the selective detection of basophils, 1.0 mL PBS solution containing HSA-DNP antigen anchored LC microdroplets (1×10^4 microdroplets/mL) was added into culture dish containing sensitized basophils, and methylene blue stained macrophages, and then HSA-DNP antigen anchored LC microdroplets were allowed to contact for 30 min. After 30 min, the optical and polarized light micrographs were recorded to visualize the contact of basophils with HSA-DNP antigen anchored LC microdroplets and to analyze the effect of interactions of anti-IgG receptor bearing basophils on orientation state of 5CB molecules in HSA-DNP antigen anchored LC microdroplets. Blank experiments were also carried out by adding 1.0 mL of HSA-DNP antigen anchored LC microdroplets (1×10^4 microdroplets/mL) in a dish seeded with macrophages alone or macrophages with non-sensitized basophils. To simulate *in vivo* detection of basophils by HSA-DNP

antigen anchored LC microdroplets in real system, the optical response of HSA-DNP antigen anchored LC microdroplets was also studied in presence of 10% blood plasma solution. The optical and polarized light micrographs of HSA-DNP antigen anchored LC microdroplets recorded in presence of basophils co-cultured with macrophages in 10% blood plasma were used to compare with micrographs of basophils co-cultured with macrophages in presence of PBS.

Results and discussion

Human basophils are important tools for the detection of allergic reactions in patients as they release a variety of mediators e.g., histamine, leukotriene on interactions with allergens. Though sensitized basophils have been detected by various methods such as; flow cytometry [16–19] and enzyme based [28–30] methods but *in vitro* detection of sensitized basophils is usually not possible with these methods. Currently, liquid crystal based methods have been used successfully in detection and assaying of various chemicals [31–37], viruses [38], and cancer cells [39–41] by recording optical signals shown by liquid crystal molecules at solids [42–45] and liquid-water interfaces [46–48]. These studies have provided a basis to develop antigen anchored LC microdroplets emulsion for detection of sensitized basophils through visibly seen radial to bipolar optical transitions in 5CB molecules in LC microdroplets (Scheme 1). The 5CB molecules in nanosized LC microdroplets are quite sensitive for detection of antigen-antibody interactions by showing radial to bipolar or vice versa transitions [46–48].

Thus considering the importance of 5CB molecules in detection of sensitized basophils in allergen reactions, the HSA-DNP antigen anchored LC microdroplets have been developed and used after characterization as a optical sensor for detection of sensitized (Scheme 1a) and non-sensitized basophils or macrophages (Scheme 1b) in PBS solution.

Characterization of LC microdroplets

The LC microdroplets, which were prepared by using a constant amount of 5CB liquid crystal molecules (50 mg, 200 μmol), PS-b-PA block copolymer (10 mg, 1.4 μmol) and SDS surfactant (10 mg, 35 μmol) in PBS solution, were found to be with radial configuration as confirmed from their polarized light micrographs (Fig. 1b). This has indicated that 5CB molecules were able to stabilize initially in radial orientations in prepared LC microdroplets having fixed size distributions (Fig. 1b).

The size distribution of LC microdroplets was determined by particle size analyzer (Beckman coulter, N5/LS-13320, USA), which indicated a maxima in size distribution curve of LC microdroplets at 27 ± 2 μm (Fig. 2). The bright field micrographs of LC microdroplets recorded with Olympus IX 71 inverted fluorescence microscope have also supported the average size distribution of LC microdroplets (Fig. 1a) as determined by particle size analyzer.

Anchoring of HSA-DNP antigen on LC microdroplets

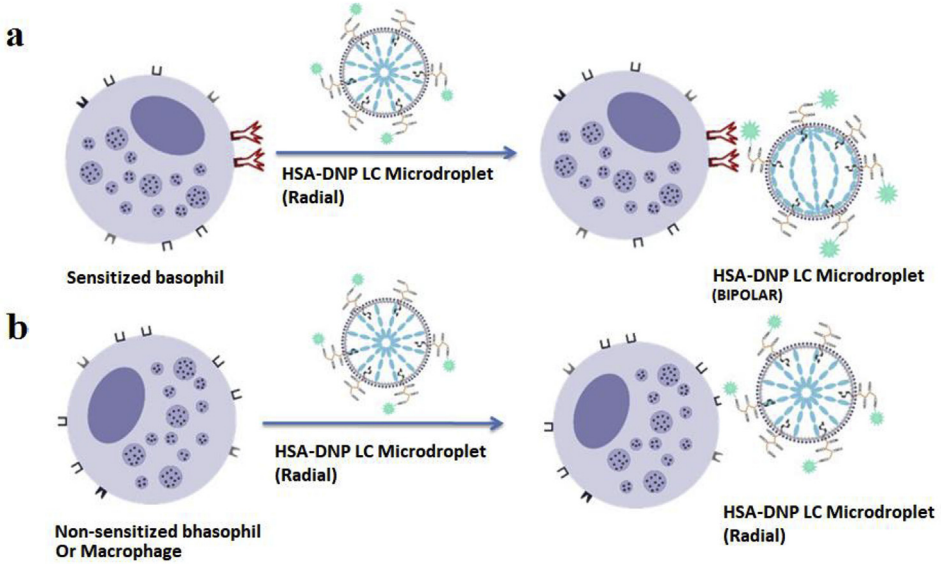
To introduce anti-IgG receptor sensitivity in LC microdroplets for the detection of sensitized basophils, first of all prepared LC microdroplets were anchored with HSA-DNP antigen. To anchor the HSA-DNP antigen on LC microdroplets, 100 ng HSA-DNP antigen was used to react with LC microdroplets (1×10^4droplets/mL) in PBS solution having EDC and NHS as activating agents to activate carboxylic acid groups of acrylic acid of PS-b-PA block copolymer in LC microdroplets. The analysis has indicated that ~85% HSA-DNP antigen was anchored on LC microdroplets. The amount of antigen anchored on LC microdroplets was determined by analyzing spectrophotometrically the remaining amount of HSA-DNP antigen in solution at 360 nm and using calibration curve, which was drawn by using diluted standard solutions of HSA-DNP antigen as per Bradford method [37]. To analyze the effect of anchoring of HSA-DNP antigen on orientational state of 5CB molecules in LC microdroplets, the polarized light micrographs of HSA-DNP anchored LC microdroplets were recorded using inverted fluorescence microscope operating both in polarization and transmission modes. To get an average view of orientation state of 5CB molecules in LC microdroplets, the micrographs of LC microdroplets were recorded at 3–4 places of culture dish.

The polarized light micrographs of HSA-DNP antigen anchored LC microdroplets have shown radial orientation of 5CB molecules (Fig. 3b) as was found in pristine LC microdroplets (Fig. 1b).

On anchoring of HSA-DNP antigen no significant size variation in LC microdroplets was observed as confirmed from optical micrographs (Figs. 1a and 3a).

Synthesis of FITC-conjugated HSA-DNP antigen

To confirm the anchoring of HSA-DNP antigen on LC microdroplets, the HSA-DNP antigen was conjugated with FITC before it was anchored on LC microdroplets. For this purpose, 100 ng of HSA-DNP antigen was conjugated with FITC (500 ng), which was activated by using 100 mg



Scheme 1. Orientational variation in HSA-DNP antigen anchored LC microdroplets on interactions with sensitized basophils (a) and non-sensitized basophils (b).

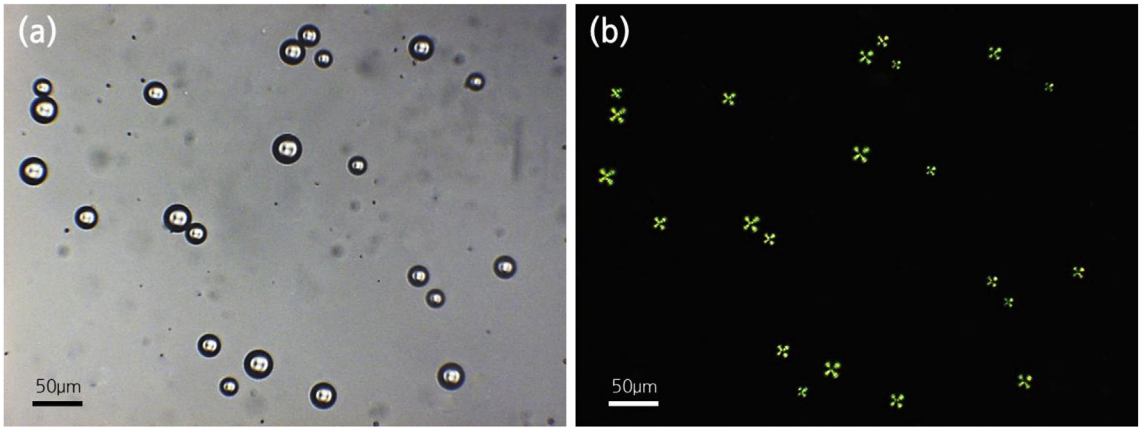


Fig. 1. Optical (a) and polarized light (b) micrographs of pristine LC microdroplets.

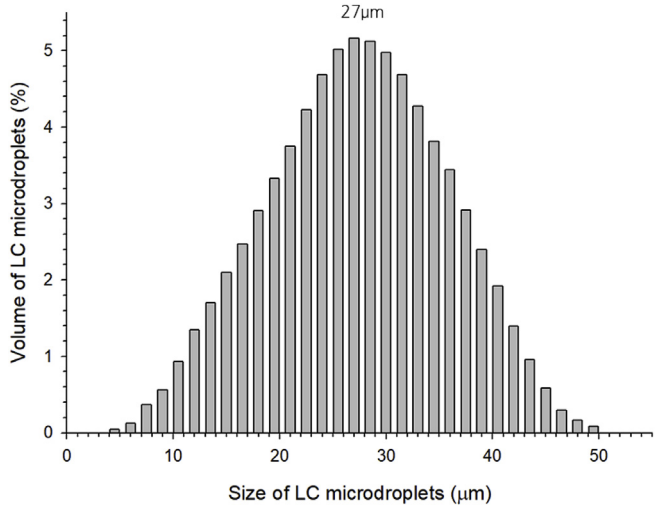


Fig. 2. Size distribution curve of LC microdroplets in PBS solution.

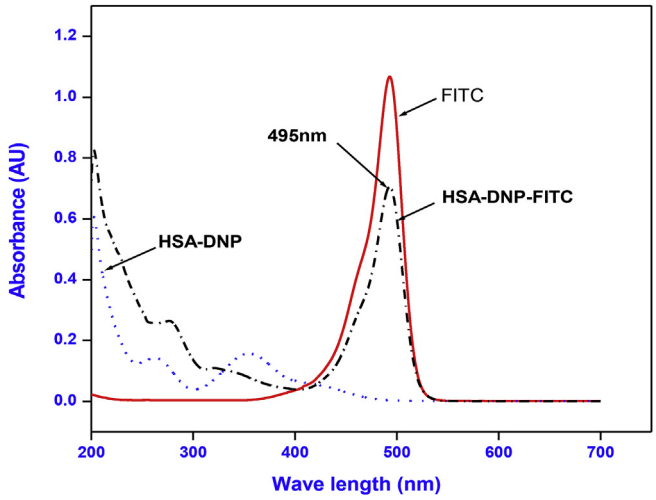


Fig. 4. UV–vis spectra of FITC, HSA-DNP, and HSA-DNP-FITC conjugate.

each of EDC and NHS in PBS solution. The formation of HSA-DNP-FITC conjugate was confirmed by analyzing its UV–vis spectrum (Fig. 4). The appearance of absorption peak at 495 nm in UV–vis spectra of HSA-DNP-FITC conjugate has confirmed the formation of FITC anchored HSA-DNP antigen. The formation of HSA-DNP-FITC conjugate has shown a blue shift in comparison to absorption band of pure FITC (Fig. 4). After confirming the formation of FITC conjugate with HSA-DNP antigen, 50 ng of HSA-DNP-FITC conjugate was used for anchoring on LC microdroplets (1×10^4 microdroplets/mL) in PBS solution.

To confirm the anchoring of HSA-DNP-FITC conjugate on LC microdroplets, the fluorescent microscopic images of HSA-DNP-FITC conjugated LC microdroplets were recorded using excitation and emission wavelengths as 492 nm (λ_{Ex}) and 515 nm (λ_{Em}) respectively. To confirm the orientation state of 5CB molecules in HSA-DNP-FITC conjugated LC microdroplets, the polarized light micrographs were recorded (Fig. 5).The polarized light micrographs of HSA-DNP-FITC conjugated LC microdroplets have shown radial state of 5CB molecules (Fig. 5a), as was observed with pristine LC microdroplets (Fig. 1b),

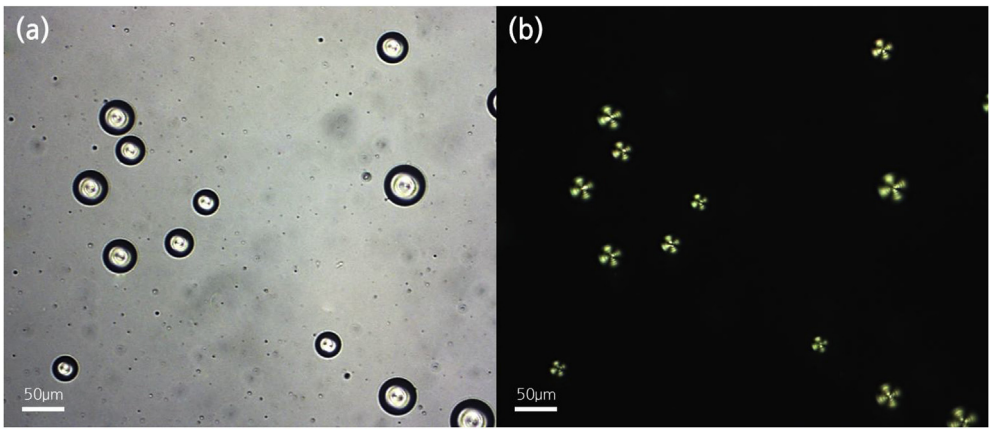


Fig. 3. Optical (a) and polarized light (b) micrographs showing orientational state of 5CB molecules in LC microdroplets after conjugated with HSA-DNP antigen.

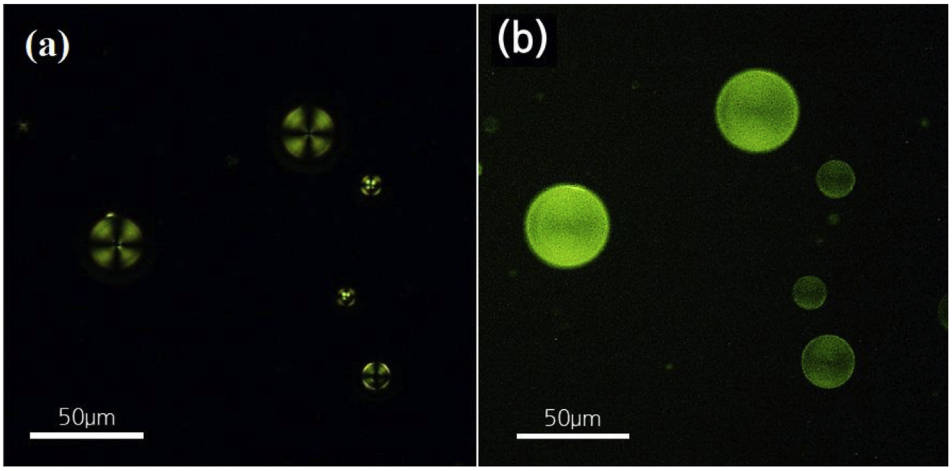


Fig. 5. Polarized light (a), and fluorescent microscope (b) images of HSA-DNP-FITC conjugated LC microdroplets.

which clearly indicated that the interactions forces of HSA-DNP-FITC conjugate was not sufficient to bring any orientational transition in 5CB molecules; hence, the 5CB molecules remained in radial state as they were in pristine LC microdroplets. The green fluorescent image of HSA-DNP-FITC conjugated LC microdroplets has further confirmed the anchoring of HSA-DNP-FITC conjugate on LC microdroplets (Fig. 5b).

These results have clearly indicated that the interaction forces of HSA-DNP antigen and its FITC conjugate were very weak at the interface of LC microdroplets and not sufficient to cause orientational transition in 5CB molecules in LC microdroplets.

In vitro detection of basophils using antigen anchored LC microdroplets

The presence of anti-IgE receptor antibody on sensitized basophils in allergic reactions is highly useful for their detection by recording optical transition in 5CB molecules confined in HSA-DNP antigen anchored LC microdroplets. To confirm the importance of anti-IgE receptor in *in vitro* detection of basophils by HSA-DNP antigen anchored LC microdroplets, first of all sensitized basophils expressed with different amounts of anti-IgE receptor were obtained by sensitizing basophils in PBS solution taking different amounts of HSA-DNP antigen ranging from 0 to 50 ng/mL. After sensitization, the anti-IgE receptor bearing basophils were allowed to interact at 30° C for 30 min with LC microdroplets (1×10^4 microdroplets), which were conjugated with 100 ng/mL of HSA-DNP antigen (8.5 pg HSA-DNP antigen/microdroplet). To determine the optical response of HSA-DNP antigen anchored LC microdroplets for basophils with different amounts of anti-IgE receptor, the bright fields and polarized light micrographs of LC microdroplets were recorded after seeding for 30 min at 30 °C in a dish along with basophils (Fig. 6a and b). The optical light micrographs recorded for basophils sensitized with 50 ng/mL of HSA-DNP antigen (i.e., basophil expressed with 4.5 pg of anti-IgE-receptor/basophil) have indicated that one basophil was contacting with LC microdroplet as marked with red circle (Fig. 6a) and contacting basophil was able to induce orientational transition in 5CB molecules in LC microdroplet as indicated by red circle in its polarized light micrograph (Fig. 6b). But on using basophils sensitized with 0 ng/mL of HSA-DNP antigen (i.e., basophils without anti-IgE receptor) and recording their optical and polarized light micrographs, it was clear that there was a contact of basophil with LC microdroplet as shown with red circle in its optical light micrograph (Fig. 6c) but this contacting LC microdroplet was found to be with radial orientational as shown with a red circle in its polarized light micrograph (Fig. 6d).

This has clearly indicated that LC microdroplet, which was contacting with basophil sensitized with 50 ng/mL of HSA-DNP antigen (i.e., basophil expressed with 4.5 pg of anti-IgE receptor/basophil) was able to show orientational transitions in 5CB molecules from radial to bipolar (Fig. 6b). These basophils were having sufficient density of anti-IgE receptors on their surfaces (4.5 pg of anti-IgE receptor/basophil), which was able to cause enhanced interactions with HSA-DNP antigen anchored LC microdroplets and were able to cause efficient interactions to show orientational transition in 5CB molecules in contacting LC microdroplet. In other case, the interactions of basophils (Fig. 6c) sensitized with 0 ng/mL of HSA-DNP antigen was poor and was not able to show orientational transition in 5CB molecules in LC microdroplets. These basophils were not having anti-IgE receptor on their surfaces, hence their interaction forces with HSA-DNP antigen anchored LC microdroplets were not sufficient to cause orientational transition in 5CB molecules in LC microdroplets (Fig. 6d). These results have suggested that LC microdroplets anchored with 100 ng/mL amount of HSA-DNP antigen (8.5 pg HSA-DNP antigen/LC microdroplet) could be used for the detection of basophils sensitized with $\geq$50 ng/mL of HSA-DNP antigen. The basophils sensitized with $\geq$50 ng/mL of HSA-DNP antigen were expressed with sufficient amount of anti-IgE receptor (i.e., expressed with 4.5 pg of anti-IgE receptor/basophil) on their surfaces; otherwise their interactions with LC microdroplets would have been poor to cause orientation transition in 5CB molecules in LC microdroplets (Fig. 6d).

Detection of sensitized basophils and macrophages

To evaluate the selectivity of HSA-DNP antigen anchored LC microdroplets in detection of sensitized basophils, the detection of sensitized basophils by HSA-DNP antigen anchored LC microdroplets has been carried out in presence of macrophages. To carry out these detections of sensitized basophils by LC microdroplets, the macrophages and basophils were seeded at 30 °C in separate culture dishes and after washing the seeded cells with PBS solution, 1.0 mL of HSA-DNP antigen anchored LC microdroplets emulsion (1×10^4 microdroplets/mL) was added. After a contact time of 30 min, the optical and polarized light micrographs were recorded at 3–4 places of the culture dishes to get an average view of contacting HSA-DNP antigen anchored LC microdroplets with seeded basophils or macrophages (Fig. 7). The optical micrographs of basophils (Fig. 7a) and macrophages (Fig. 7c) in culture dish have indicated that one basophil was contacting with LC microdroplet as shown by red circle (Fig. 7a) and three macrophages also became successful in making contacts with LC microdroplets as shown by red circles (Fig. 7c). But the orientation state of 5CB molecules in LC microdroplets after interactions with macrophages remained in radial state (Fig. 7d) in comparison to LC microdroplet, which was contacting with basophil (Fig. 7a) and shown orientation transition from radial to bipolar in 5CB molecules as shown by red circle (Fig. 7b).

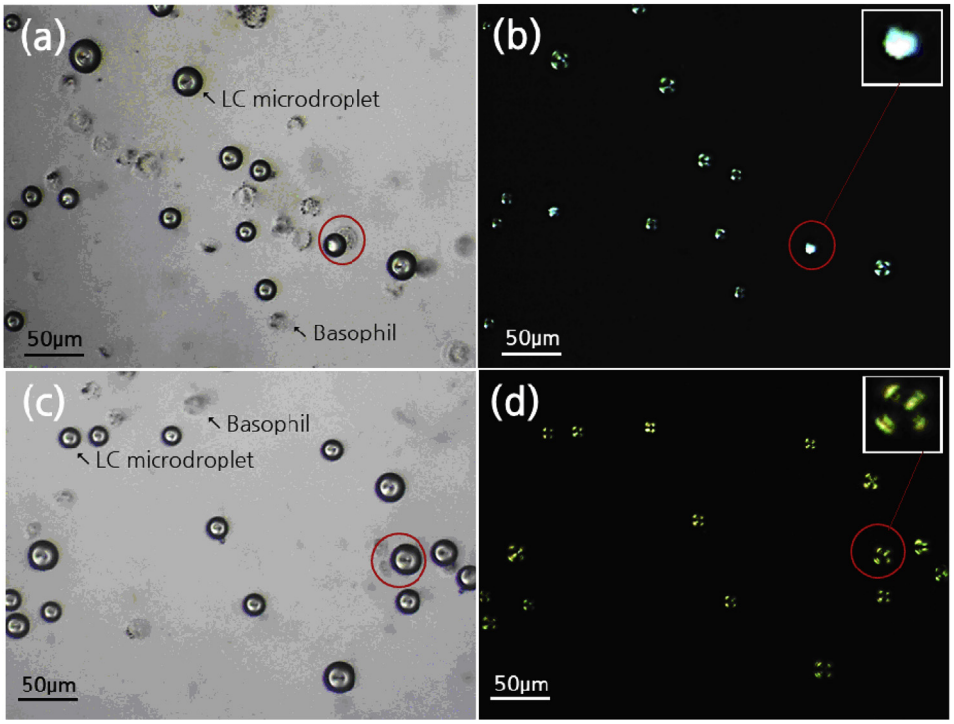


Fig. 6. Optical (a, c) and polarized light (b, d) micrographs showing orientational transitions in HSA-DNP anchored LC microdroplets on contacting with basophils sensitized with 50 ng/mL (a, b) and 0 ng/mL (c, d) of HSA-DNP antigen for a contact time of 30 min.

Though LC microdroplets anchored with HSA-DNP antigen (8.5 pg HSA-DNP antigen/microdroplet) were allowed to interact equally for a contact time of 30 min but contacting macrophages were not able to cause any orientational transition in 5CB molecules confined in LC microdroplets due to the absence of anti-IgE receptor on their surfaces in comparison to sensitized basophils, which were sufficiently expressed with anti-IgE receptor (4.5 pg anti-IgE receptor/basophil) to interact with HSA-DNP antigen anchored LC microdroplet and to produce sufficient interaction forces to cause orientational transition in 5 CB molecules. These results have suggested that HSA-DNP antigen anchored LC microdroplets were sensitive in detection of those basophils, which were expressed sufficiently with anti-IgE receptor but remained

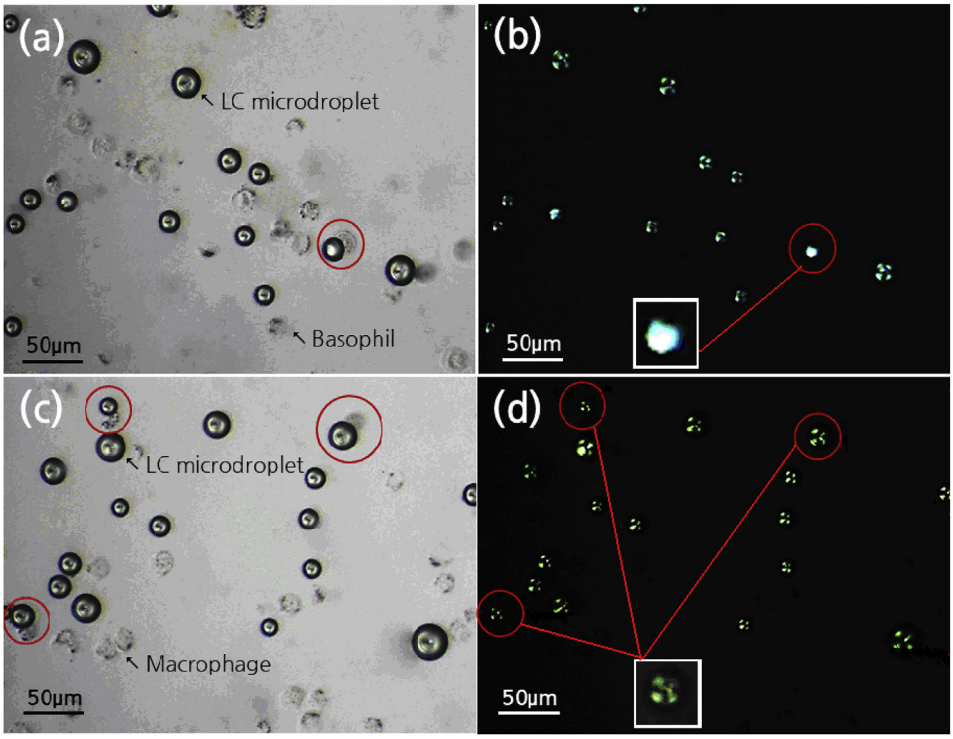


Fig. 7. Optical (a, c) and polarized light (b, d) micrographs of LC microdroplets seeded with basophils (a, b) and macrophages (c, d) in PBS with 1×10^4/mL of LC microdroplets for a contact time of 30 min.

insensitive to cells without anti-IgE receptor such as macrophages or un-sensitized basophils.

Selective detection of basophils co-cultured with control cells

To evaluate the selectivity of LC microdroplets for sensitized basophils in complex environment, the detection of sensitized basophils has been carried out in presence of control cells such as; non-sensitized basophils or macrophages. The sensitized basophils and methylene blue stained control cells (each 5×10^3 cells/mL) were cultured together in a dish in presence of 5% CO_2 at 37 °C in α-DMEM medium. After co-culturing these cells for 24 h, the culture dish was washed with PBS solution. After removing free cells from the culture dish, 1.0 mL PBS solution containing HSA-DNP antigen anchored LC microdroplets (1×10^4 microdroplets/mL) was added gently. After contacting for 30 min, the optical and polarized light micrographs were recorded to visualize the contacts of seeded cells with HSA-DNP antigen anchored LC microdroplets and to analyze the effect of interactions of seeded cells on orientational state of 5CB molecules in HSA-DNP antigen anchored LC microdroplets. The optical micrograph has indicated that a non-sensitized cell marked as 1 by red circle was able to contact with LC microdroplet (Fig. 8a1) but it could not cause orientational transition in 5CB molecules in LC microdroplet as indicated by its polarized light micrograph (Fig. 8 b1). The orientation state of 5CB molecules in LC microdroplet remained in radial state as similar to pristine LC microdroplets (Fig. 1b) as shown by magnified subset in Fig. 8b.

On the other hand, the contacts of HSA-DNP anchored LC microdroplets with sensitized basophils marked as 2, 3, 4 by red circles in their optical micrograph (Fig. 8a 2, 3, 4) found to be effective in causing orientational transition from radial to bipolar in 5CB molecules in LC microdroplets as indicated by red circles in their polarized light micrograph (Fig. 8 b 2, 3, 4), which was recorded to visualize the optical state of LC microdroplets on interactions with seeded sensitized and macrophages. This has clearly indicated that the interactions of HSA-DNP antigen anchored LC microdroplets were quite selective and sensitive in showing orientational transition in 5CB molecules on contacting with sensitized basophils, which were expressed sufficiently with anti-IgE receptors at their surfaces; otherwise the interactions of HSA-DNP antigen anchored LC microdroplets remained insensitive as found on interaction with non-sensitized basophils (Fig. 8 b1). Similar to non-sensitized basophils, the selectivity of HSA-DNP antigen anchored LC microdroplets for sensitized basophils was also determined in presence of macrophages by co-culturing sensitized basophils along with macrophages at same cell density (5×10^3 cells/mL). The detection of sensitized basophils in presence of macrophages was carried out by adding 1 mL of PBS solution containing HSA-DNP antigen anchored LC microdroplets (1×10^4microdroplets/mL) in a culture dish. After seeding for 30 min, the optical and polarized light micrographs were recorded (Fig. 9) to confirm the contacts of macrophages with HSA-DNP antigen anchored LC microdroplets and to analyze the effect of their interactions on orientational state of 5 CB molecules in contacting LC microdroplets. The red circle 1 as shown in optical micrograph (Fig. 9 a1) has indicated that a HSA-DNP antigen anchored LC microdroplet was contacting with a macrophage cell but this contact of macrophage cell with HSA-DNP antigen anchored LC microdroplet did not cause any orientational change in 5CB molecules, hence 5CB molecules in LC microdroplet remained in pristine radial configuration as shown by red circle 1 in polarized light micrograph (Fig. 9 b 1). On the other hand, the contacts of HSA-DNP antigen anchored LC microdroplets with sensitized basophils as shown by red circles 2 and 3 in optical micrograph (Fig. 9a 2, 3) have caused orientational transition from radial to bipolar in 5CB molecules in LC microdroplets as shown by red circles 2 and 3 in polarized light micrograph (Fig. 9 b 2, 3) and by attached magnified subsets.

This has clearly suggested that HSA-DNP antigen anchored LC microdroplets were only interacting effectively with sensitized basophils and interaction force between HSA-DNP antigen anchored LC microdroplet and anti-IgE receptor expressed on sensitized basophils were sufficiently enough to cause orientational transition in 5CB molecules in LC microdroplets (Fig. 9 b 2, 3). The interaction forces produced by the contacts of macrophage cell (Fig. 9 a1) with HSA-DNP antigen anchored LC microdroplets were weak and could not cause orientational transition in 5CB molecules in LC microdroplets. These results have suggested that HSA-DNP antigen anchored LC microdroplets were able to interact selectively with sensitized basophils and were able to show optical transition in 5CB molecules in presence of macrophages as they were able to detect sensitized basophils in presence of non-sensitized basophils (Fig. 8). The macrophages were similar to non-sensitized basophils as both were lacking detectable anti-IgE receptor at their surface, hence their interactions with HSA-DNP antigen anchored LC microdroplets were ineffective to cause orientation transition in 5CB molecules.

Detection of sensitized basophils by HSA-DNP anchored LC microdroplets in PBS solution containing human blood plasma

To simulate the selectivity and response time of HSA-DNP antigen anchored LC microdroplets in detection of sensitized basophils in real system, the selective detection of sensitized basophils (4.5 pg anti-IgE receptor/basophil) by LC microdroplets (8.5 pg HSA-DNP antigen/LC microdroplet) has been carried in PBS solution containing 10% blood plasma and macrophages. To carry out the detection of basophils, 1.0 mL solution of PBS solution containing HSA-DNP antigen anchored LC microdroplets (1×10^4 microdroplets/mL) was added in a culture dish containing basophils and methylene blue stained macrophages in PBS solution containing 10% human blood plasma. The HSA-DNP antigen anchored LC microdroplets were allowed to interact with seeded cells for different time intervals at 30 °C and then optical and polarized light micrographs were recorded (Fig. 10).

The optical light micrographs of HSA-DNP antigen anchored LC microdroplets for contacting basophils as marked by red circle 2 (Fig. 10 a 2) or contacting macrophages as marked by red circles 1 and

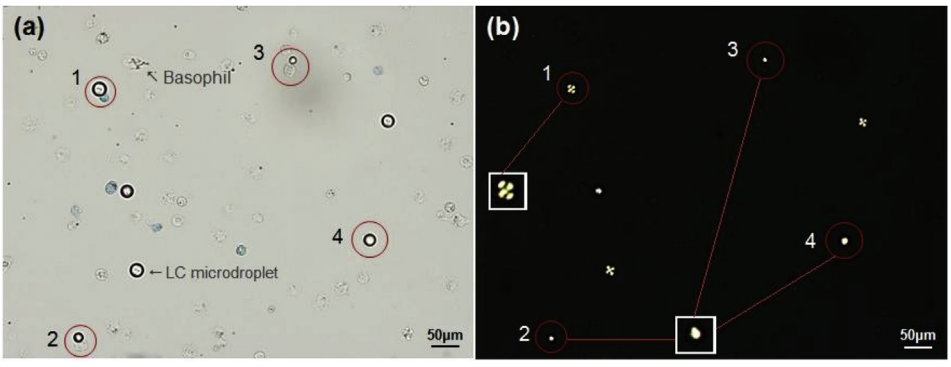


Fig. 8. Optical (a) and polarized light (b) micrographs of HSA-DNP anchored LC microdroplets on contacting for 30 min with co-cultured basophils sensitized with 50 ng/mL (white) and 0 ng/mL (blue) of anti-IgE receptor. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

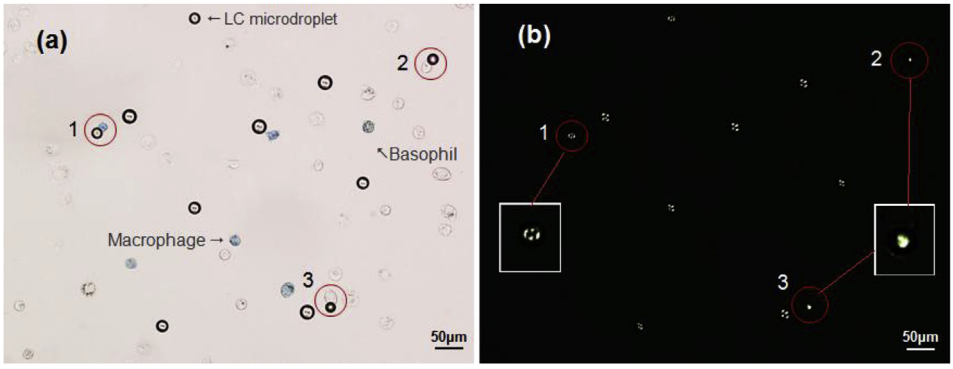


Fig. 9. Optical (a) and polarized light (b) micrographs of HSA-DNP antigen anchored LC microdroplets after contacting for 30 min either with basophils (white) or macrophage cell (blue) in PBS solution. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3 (Fig. 10a 1, 3) have indicated that sensitized basophils co-cultured with macrophages in PBS solution containing 10% human blood plasma were able to show orientation transition in 5CB molecules but at a delayed contact time of 40 min (Fig. 10 b 2). On the other hand, the contacting macrophages cells (Fig. 10a 1, 3) remained ineffective in causing any orientation transition in 5CB molecules in HSA-DNP antigen anchored LC microdroplets at 40 min (Fig. 10 b 1, 3) and even could not cause orientation transition in HSA-DNP antigen anchored LC microdroplets on keeping the macrophages in contact for 24 h. These results have suggested that HSA-DNP antigen anchored LC microdroplets also remained selective in detection of sensitized basophils in presence of macrophages in PBS solution containing 10% human blood plasma as they were selective in PBS solution except variation in response time from 30 min to 40 min in detection of sensitized basophils in 10% human blood plasma. This variation in contact time from 30 min to 40 min might be due to the interference from human blood plasma proteins that reduced the migration of sensitized basophils. These results have suggested that HAS-DNP anchored LC microdroplets can be used in detection of allergen sensitized basophils in biological fluids without any interference from macrophages or plasma proteins. The characteristics of prepared HAS-DNP antigen anchored LC microdroplets biosensor are summarized in Table 1.

Table 1
Specifications of HSA-DNP antigen anchored LC microdroplets biosensor*.

Specification of HSA-DNP antigen anchored LC microdroplets	PBS solution without blood plasma	PBS solution with blood plasma
1 Lower detection limit for anti-IgE receptor on basophils	≥2.5 pg IgE receptor/basophil	≥5.5 pg IgE receptor/basophil
2 Detection range for IgE receptor on basophils\	4.5–10 pg IgE receptor/basophil	4.5–10 pg IgE receptor/basophil
3 Response time	30 min	40 min
4 Selectivity in presence of control or macrophage cells\	High	High

Average size of LC microdroplets = 27 μm, HSA-DNP antigen = 8.5 pg per LC microdroplet. Initial orientations of 5CB molecules in LC microdroplets were radial.

Conclusions

The HSA-DNP antigen anchored LC microdroplets with radial orientation of 5CB molecules have been prepared successfully using PS-b-PA block copolymer and SDS surfactant as surface modifiers. The prepared LC microdroplets were characterized successfully for their size distributions by particle size analyzer, which shown maxima at ~27 μm. The LC microdroplets anchored with sufficient amount of HSA-DNP antigen (8.5 pg HSA-DNP antigen/LC microdroplet) were able to detect allergen sensitized basophils expressed with detectable amount of anti-IgE receptor (4.5 pg anti-IgE receptor/basophil) both in PBS solution and in presence of 10% human blood plasma proteins. Though detection of anti-IgE receptor by HSA-DNP antigen anchored LC microdroplets is quite comparable to other methods [20] but they were found capable in detection of sensitized basophils within a contact time of 30 min. The selective interactions of HSA-DNP antigen anchored LC microdroplets with sensitized basophils have produced naked eye detectable optical signal by showing radial to bipolar transition in 5CB molecules in LC microdroplets. The basophils expressed with minimum amount of anti-IgE receptor (≥2.5 pg) at their surfaces were able to cause orientation transition in 5CB molecules in prepared HSA-DNP antigen anchored LC microdroplets through antigen-antibody interactions. The HSA-DNP antigen anchored LC microdroplets remained insensitive to basophils, which were expressed poorly with anti-IgE receptor (< 2.5 pg of anti-IgE receptor/basophil) or to non-sensitized basophils in PBS solution. The HSA-DNP antigen anchored LC microdroplets retained selectivity in detection of sensitized basophils in presence of macrophages or in presence of non-sensitized basophils in BPS solution as well as in presence of 10% human blood plasma. These

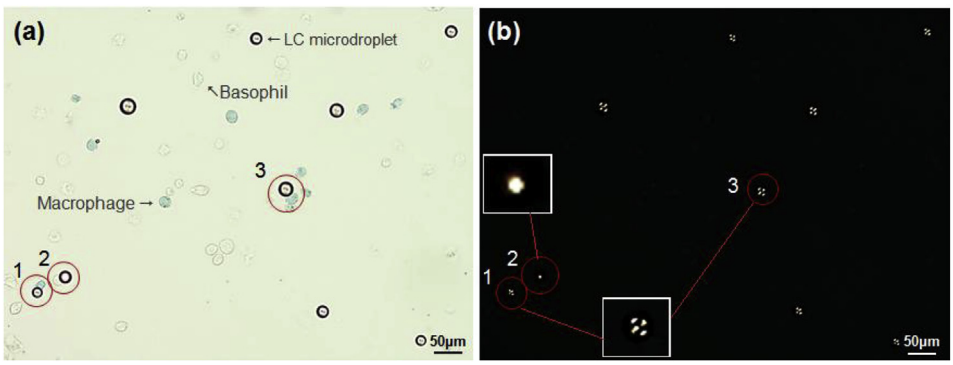


Fig. 10. Optical (a) and polarized light (b) micrographs showing orientation transitions in HAS-DNP antigen anchored LC microdroplets (1×10^4 microdroplets/mL) on contacting for 40 min with co-cultured basophils (white) and macrophages (blue) in PBS solution with 10% human blood plasma. The sensitized basophils were expressed with 4.5 pg anti-IgE receptor/basophil. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

studies have clearly indicated that HSA-DNP antigen anchored LC microdroplets could be used as economical and time saving biosensor for the detection of sensitized basophils in biological fluids in place of time consuming and costlier methods based on enzymatic immunoassaying of sensitized basophils. The prepared HSA-DNP antigen anchored LC microdroplets is found to be quite sensitive in detection of one basophil per milliliter of liquid. However, there are still sufficient scopes to improve the sensitivities of LC microdroplets for the detection of basophils which are expressed poorly with anti-IgE receptor (< 2.5 pg of anti-IgE receptor/basophil) or for the detection of sensitized basophils in presence of other control cells. These improvements are possible by anchoring basophils specific proteins on LC microdroplets and by controlling their surface properties.

Authors' contributions

Ms Hanbyeol Shin is a student of Master of Engineering at the Department of Polymer Science and Engineering, Kyungpook National University, Daegu, South Korea, made substantial contributions in designing the experiment and acquisition of data. Ms Sojung Park has also partly helped in acquisition of these data. Prof. Kailash Chandra Gupta participated in critical discussions on the contents and drafting of the manuscript for publication. Prof. Dong Yun Lee, Soo-Young Park and Inn-Kyu Kang gave the final approval after evaluating and discussing the contents.

Conflicts of interest

Authors declare no conflict of interest.

Acknowledgements

This work was supported by the National Research Foundation funded by the Ministry of Education, South Korea (No. 2016R1D1A1A09918166 and No. 2011-0020264). One of the authors, Prof. K.C. Gupta, would like to thank Prof. Inn-Kyu Kang for sponsoring his visit to Department of Polymer Science and Engineering at Kyungpook National University as visiting professor for collaborative research work.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ab.2018.07.025.

References

[1] P. Valent, A.W. Hauswirth, S. Natter, W.R. Sperr, H.J. Buhring, R. Valenta, Assays for measuring in vitro basophil activation induced by recombinant, Allergens, Methods 32 (2004) 265–270.
[2] J. Sainte-Laudy, A. Sabbah, C. Vallon, J.C. Guerin, Analysis of anti-IgE and allergen induced human basophil activation by flow cytometry. Comparison with histamine release, Inflamm. Res. 47 (1998) 401–408.
[3] M. Mack, M.A. Schneider, C. Moll, J. Cihak, H. Bruhl, J.W. Ellwart, P.M. Hogarth, M. Stangassinger, D. Schlondorff, Identification of antigen- capturing cells as basophils, J. Immuonl. 174 (2005) 735–774.
[4] J.T. Schroeder, D.W. MacGlashan Jr., L.M. Lichtenstein, Human basophils: mediator release and cytokine production, Adv. Immunol. 77 (2001) 93–122.
[5] K. Chen, W. Xu, M. Wilson, B. He, N.W. Miller, E. Bengten, E.S. Edholm, P.A. Santini, P. Rath, A. Chiu, Immunoglobulin D enhances immune surveillance by activating antimicrobial, proinflammatory and B cell stimulating programs in basophils, Nat. Immunol. 10 (2009) 889–898.
[6] D.B. Golden, What is anaphylaxis? Clin. Immunol. 7 (2007) 331–336.
[7] D.E. Sloane, D. MacGlashan, Basophils in Anaphylaxis, Anaphylaxis and Hypersensitivity Reactions vol. 87, (2011), pp. 69–470.
[8] J. Schmitz, A. Owyang, E. Oldham, Y. Song, E. Murphy, T.K. McClanahan, IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor- related protein ST2 and induces T helper type 2-associated cytokines, Immunity 23 (2005) 479–490.
[9] J.G. Perrigoue, S.A. Saenz, M.C. Siracusa, E.J. Allenspach, B.C. Taylor, P.R. Giacomin, M.G. Nair, Y. Du, C. Zaph, N. van Rooijen, M.R. Comeau, E.J. Pearce, T.M. Laufer, D. Artis, MHC class II-dependent basophil-CD4 + T cell interactions promote T(H)2 cytokinedependent immunity, Nat. Immunol. 10 (2009) 697–705.
[10] X. Bosch, F. Lozano, R. Cervera, M. Ramos-Casals, B. Min, Basophils, IgE, and autoantibody-mediated kidney disease, J. Immunol. 186 (2011) 6083–6090.
[11] P.B. Williams, W.K. Dolen, J.W. Koepke, J.C. Selner, Comparison of skin testing and three in vitro assays for specific IgE in the clinical evaluation of immediate hypersensitivity, Ann. Allergy 68 (1992) 35–45.
[12] S. Hayashi, H. Suto, N. Wada, H. Ogawa, K. Okumura, C. Ra, Detection of anti-IgE and anti-FcεRIα chain auto-antibodies in patients with atopic dermatitis, Allergol. Int. 49 (2000) 47–54.
[13] M. Hide, D.M. Francis, C.E.H. Grattan, J. Hakimi, J.P. Kochan, M.W. Graves, Autoantibodies against the high-affinity IgE receptor as a cause of histamine release in chronic urticarial, N. Engl. J. Med. 328 (1993) 599–604.
[14] M.L.B. Souza-Atta, M.I. Araújo, A. D'Oliveira Júnior, A. Ribeiro-de- Jesus, R.P. Almeida, A.M. Atta, E.M. Carvalho, Detection of specific IgE antibodies in parasite diseases, Braz. J. Med. Biol. Res. 32 (1999) 1101–1115.
[15] D. Furuya, A. Yagihashi, T. Yajima, D. Kobayashi, K. Orita, M. Kurimoto, N. Watanabe, An immuno-polymerase chain reaction assay for human interleukin-18, J. Immunol. Meth. 238 (2000) 173–180.
[16] E.F. Knol, F.P. Mul, H. Jansen, J. Calafat, D. Roos, Monitoring human basophil activation via CD63 monoclonal antibody 435, J. Allergy Clin. Immunol. 88 (1991) 328–338.
[17] F.H. Falcone, G. Telford, D. Hooi, A.P. Brown, R. Seabra, J. Feary, A. Venn, J. Britton, D.I. Pritchard, Antigen-driven basophil activation is indicative of early necator americanus infection in IgE-seronegative patients, J. Clin. Immunol. 124 (2009) 1343–1350.
[18] M.L. Sanz, G. Sanchez, P.M. Gamboa, L. Vila, C. Uasuf, M. Chazot, I. Die´- guez, A.L. De Weck, Allergen-induced basophil activation: CD63 cell expression detected by flow cytometry in patients allergic to dermatophagoides pteronyssinus and lolium perenne, Clin. Exp. Allergy 31 (2001) 1007–1013.
[19] N.E. Aerts, E.J. Dombrecht, C.H. Bridts, M.M. Hagendorens, L.S. de Clerck, W.J. Stevens, D.G. Ebo, Simultaneous flow cytometric detection of basophil activation marker CD63 and intracellular phosphorylated p38 mitogen-activated protein kinase in birch pollen allergy, Cytometry B Clin. Cytometry 76B (2009) 8–17.
[20] K. Mukai, N. Gaudenzio, S. Gupta, N. Vivanco, S.C. Bendall, H.T. Maecker, R.S. Chinthrajah, M. Tsai, K.C. Nadeau, S.J. Galli, Assessing basophil activation by using flow cytometry and mass cytometry in blood stored 24 hours before analysis, J. Allergy Clin. Immunol. 139 (2017) 889–899.
[21] Y. Yanase, H. Suzuki, T. Tsutsui, T. Hiragun, Y. Kameyoshi, M. Hide, The SPR signal in living cells reflects changes other than the area of adhesion and the formation of cell constructions, Biosens. Bioelectron. 22 (2007) 1081–1091.
[22] Y. Yanase, H. Suzuki, T. Tsutsui, I. Uechi, T. Hiragun, S. Mihara, M. Hide, Living cell positioning on the surface of gold film for SPR analysis, Biosens. Bioelectron. 23 (2007) 562–567.
[23] Y. Yanase, A. Araki, H. Suzuki, T. Tsutsui, T. Kimura, K. Okamoto, T. Nakatani, T. Hiragun, M. Hide, Development of an optical fiber SPR sensor for living cell activation, Biosens. Bioelectron. 25 (2010) 1244–1247.
[24] Y. Yanase, T. Hiragun, T. Yanase, T. Kawaguchi, K. Ishii, M. Hide, Evaluation of peripheral blood basophil activation by means of surface plasmon resonance imaging, Biosens. Bioelectron. 32 (2012) 62–68.
[25] Y. Yanase, T. Hiragun, T. Yanase, T. Kawaguchi, K. Ishii, M. Hide, Application of SPR imaging sensor for detection of individual living cell reactions and clinical diagnosis of Type I allergy, Allergol. Int. 62 (2013) 163–169.
[26] K.H. Ruan, R.J. Kulmacz, A. Wilson, K.K. Wu, Highly sensitive fluorimetric enzyme immunoassay for prostaglandin H synthase solubilized from cultured cells, J. Immunol. Meth. 162 (1993) 23–30.
[27] R.G. Hamilton, 3rd, R. Rich, T.A. Fleisher, W.T. Shearer, H.W. Schroeder, A.J. Frew, C.M. Weyand (Eds.), Clinical Immunology, vol. 102, Mosby Elsevier, Missouri, 2008, p. 1471.
[28] C.J. Stanley, A. Johannsson, C.H. Self, Enzyme amplification can enhance both the speed and the sensitivity of immunoassays methods, J. Immunol. 83 (1985) 89–95.
[29] A. Johannsson, D.H. Ellis, D.L. Bates, A.M. Plumb, C.J. Stanley, Enzyme amplification for immunoassays: detection limit of one hundredth of an attomole, J. Immunol. Meth. 87 (1986) 7–11.
[30] R.G. Hamilton, Accuracy of US food and drug administration-cleared IgE antibody assays in the presence of anti-IgE (omalizumab), J. Allergy Clin. Immunol. 117 (2006) 759–766.
[31] C.-Y. Xue, K.-L. Yang, Dark-to-bright optical responses of liquid crystals supported on solid surfaces decorated with proteins, Langmuir 24 (2008) 563–567.
[32] X. Bi, K.-L. Yang, Liquid crystals decorated with linear oligopeptide FLAG for applications in immunobiosensors, Biosens. Bioelectron. 26 (2010) 107–111.
[33] M.L. Bungabong, P.B. Ong, K.-L. Yang, Using copper perchlorate doped liquid crystals for the detection of organophosphonate vapor, Sensor. Actuator. B Chem. 148 (2010) 420–426.
[34] D. Hartono, S.L. Lai, K.-L. Yang, L.Y.L. Yung, A liquid crystal- basedsensor for real-time and label-free identification of phospholipase-like toxins and their inhibitors, Biosens. Bioelectron. 24 (2009) 2289–2293.
[35] S. Sivakumar, K.-L. Yang, J.K. Gupta, N.L. Abbott, F. Caruso, Liquid crystal emulsions as the basis of biological sensors for the optical detection of bacteria and viruses, Adv. Funct. Mater. 19 (2009) 2260–3265.
[36] S.H. Yoon, K.C. Gupta, J.S. Borah, S.Y. Park, Y.K. Kim, J.H. Lee, I.K. Kang, Folate ligand anchored liquid crystal microdroplet emulsion for *in vitro* detection of KB cancer cells, Langmuir 30 (2014) 10668–10677.
[37] K. Lee, K.C. Gupta, S.Y. Park, I.K. Kang, J. Mater. Chem. B 4 (2016) 704–711.
[38] F. Zuo, Z. Liao, C. Zhao, Z. Qin, X. Li, C. Zhang, D. Liu, An air- supported liquid crystal system for real-time reporting of host–guest inclusion events, Chem.

Commun. 50 (2014) 1857–1860.
[39] H.J. VanTreeck, D.R. Most, B.A. Grinwald, K.A. Kupcho, A. Sen, M.D. Bond, B.R. Acharaya, Quantitative detection of a simulant of oganophophonate chemical warfare agents using liquid crystals, Sensor. Actuator. B Chem. 158 (2011) 104–110.
[40] H. Xu, X. Bi, X. Ngo, K.-L. Yang, Principles of detecting vaporous thiols using liquid crystals and metal ion microarrays, Analyst 134 (2009) 911–915.
[41] X. Bi, K.-L. Yang, Real-time liquid crystal-based glutaraldehyde Sensor, Sensor. Actuator. B Chem. 134 (2008) 432–437.
[42] Y. Choi, K.C. Gupta, S.Y. Park, I.K. Kang, The role of ligand – receptor interactions in visual detection of HepG2 cells using liquid crystal microdroplets based sensor, J. Mater. Chem. B 4 (2015) 8659–8669.
[43] W. Ding, K.C. Gupta, S.Y. Park, I.K. Kang, In Vitro detection of Breast cancer cells (SK-Br3) using herceptin-conjugated liquid crystal microdroplets as sensing platform, Biomater. Sci. 4 (2016) 1473–1484.
[44] C.-S. Xue, S.A. Khan, K.-L. Yang, Exploring optical properties of liquid crystals for developing label-free and high-throughput microfluidic immunoassays, Adv. Funct. Mater. 21 (2008) 198–202.
[45] L. Birchall, R. Ulijn, S. Webb, A combined SPS-LCD sensor for screening protease specificity, Chem. Commun. (2008) 2861–2863.
[46] D. Hartono, W.J. Qin, K.-L. Yang, L.Y.L. Yung, A combined SPS- LCD sensor for screening protease specificity, Biomaterials 30 (2009) 843–849.
[47] X. Bi, D. Hartono, K.-L. Yang, Real-time liquid crystal pH sensor for monitoring enzymatic activities of penicillinase, Adv. Funct. Mater. 19 (2009) 3760–3765.
[48] V.J. Alino, J. Pang, K.-L. Yang, Liquid crystal droplets as a hosting and sensing platform for developing immunoassays, Langmuir 27 (2011) 11784–11789.