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Self assembled monolayer based liquid crystal biosensor for free cholesterol detection

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A unique cholesterol oxidase (ChOx) liquid crystal (LC) biosensor, based on the disruption of orientation in LCs, is developed for cholesterol detection. A self-assembled monolayer (SAM) of Dimethyloctadecyl[3-(trimethoxysilyl)propyl]ammonium chloride (DMOAP) and (3-Aminopropyl)trimethoxy-silane (APTMS) is prepared on a glass plate by adsorption. The enzyme (ChOx) is immobilized on SAM surface for 12 h before utilizing the film for biosensing purpose. LC based biosensing study is conducted on SAM/ChOx/LC (5CB) cells for cholesterol concentrations ranging from 10 mg/dl to 250 mg/dl. The sensing mechanism has been verified through polarizing optical microscopy, scanning electron microscopy, and spectrometric techniques. © *2014 AIP Publishing LLC*. [http://dx.doi.org/10.1063/1.4871704]

Cholesterol is a fat like substance (lipid) that is found in all cells of the body. It is required to provide fluidity to cell membranes and acts as an antecedent of hormones, vitamin D, and bile acids. However, it causes trouble for our body when its amount exceeds through a certain level. High serum cholesterol levels >200 mg/dl leads to hardened arteries, coronary heart disease, and even strokes. Thus, cholesterol detection in serum and food is very important for diagnosis and treatment. Several methods such as fluorescence methods, electrochemical methods, and calorimetric methods have been developed for cholesterol detection in serum.[1–3] Immobilization of enzymes to solid electrode surface is a key step for the design, fabrication, and performance of the biosensor. Cholesterol oxidase (ChOx) is a water-soluble enzyme and cholesterol is oxidized to cholest-4-en-3-one in presence of ChOx. Various studies have been performed using ChOx to precisely monitor the cholesterol level in its biological environment.[4] Moreover, self-assembled monolayers (SAMs) of various polymers and carbon nanotubes have been employed as a matrix for enzyme immobilization and detection of cholesterol.[5–7]

The Liquid crystals (LCs), fascinating materials emerging from the field of soft condensed matter, have been extensively used in a variety of display and non-display applications because they are susceptible to small external influences such as electric, magnetic, and surface forces.[8,9] Recently, liquid crystals have proved themselves as a fresh entrant for developing innovative sensing systems with advanced and dominant functions. LC based biosensors have attracted particular attention due to their unique sensing properties. The alignment of LC molecules is extraordinarily sensitive to biomolecular and chemical bounding events and the inherent long range orientational order in LC materials serves to transform chemical and biomolecular binding events into amplified optical signals.[10,11] The detections through LC based biosensor can be carried out in ambient light without applying electrical current or molecular labeling. Moreover, their optical signals can be easily observed with naked eyes.[10,12–15] These important characteristics of LC based biosensors make them well suited for simple detection, direct transduction, high sensitivity, and low-cost bioassays.[16–19]

For the detection of enzymatic events, few LC biosensors have been reported because most of the enzymatic reactions catalyze substrate to generate some small-molecule products, whose disruption behaviour to the orientation of LC molecules is far smaller than that of macro-biomolecules.[13,20–22] Recently, Liao and co-workers developed a LC biosensing system based on the enzymatic growth of gold nanoparticles (Au NPs) for the detection of acetylcholine (ACh) and acetylcholinesterase (AChE) inhibitor.[23] In their method the product of AChE enzymatic reaction, thiocholine molecules were attached to large sized Au NPs, which could remarkably change the orientation of LCs leading to an amplified optical signal and improves the detection sensitivity of the LC enzymatic biosensor. Tan *et al*. reported a signal enhanced LC biosensing system based on enzymatic silver deposition for highly sensitive DNA detection.[24] Later, the same group developed signal enhanced LC biosensor based on using Au NPs for highly sensitive DNA detection.[25]

In the present study, we report a simple LC biosensing strategy for highly sensitive detection of cholesterol by using cholesterol oxidase based enzymatic reaction for the signal amplification. The sensing mechanism has been verified through polarizing optical microscopy (POM), scanning electron microscopy (SEM), and spectrometric techniques. The samples with different concentrations of cholesterol have been tested to validate the sensing mechanism. This

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study provides a simple and sensitive ChOx-LC biosensing approach and offers effective signal enhanced strategies for the development of LC based biosensors.

Reagents and materials: Glass microslides were purchased from JSGW-India, Dimethyloctadecyl[3-(trimethoxysilyl)-propyl]ammonium chloride (DMOAP), (3-Aminopropyl)-trimethoxysilane (APTMS), 4′-Pentyl-4-biphenylcarbonitrile (5CB) was purchased from Sigma-Aldrich, USA and used as received. Cholesterol Oxidase (ChOx) with a specific activity of 22.7 Umg^{-1} was procured from Sisco Research Laboratory, Mumbai. All other chemicals used for preparation of phosphate buffer are of analytical grade.

The micrographs of the sample cells were taken with the help of polarizing optical microscope (Ax-40 Carl Zeiss, Germany) fitted with charge coupled device (CCD) camera. High resolution SEM experiments were performed by using a SEM—model Zeiss EVO® MA10 (Carl Zeiss, Oberkochen, Germany) operated at the electron accelerating voltage of 10 kV using electron source as field emission gun. The intensity of dark and bright state of reference and biosensing LC cells was compared using high resolution spectrometer, Ocean Optics, Inc., HR 4000, Germany.

Preparation of self assembled monolayer: The glass slides were cleaned using piranha solution. A SAM of DMOAP/APTMS was prepared by dipping previously cleaned glass slides in 0.5% ((v/v) DMOAP and 1% (v/v) APTMS solution in sodium acetate buffer (0.2M, pH 5) at 80 °C for 1 h, followed by drying in a vacuum chamber.[23]

Immobilization of Cholesterol Oxidase enzyme: The enzyme Cholesterol Oxidase has been covalently immobilized to DMOAP/APTMS film using glutaraldehyde as a cross linker.[26] SAM of glutaraldehyde over DMOAP-APTMS monolayer was prepared by dipping the glass slides in 1% (v/v) glutaraldehyde prepared in de-ionized water (18.2 MΩ cm) for 4 h, followed by washing with de-ionized water. ChOx solution (50 U/ml) prepared in Phosphate buffer saline (PBS; 50 mM, pH 7.0, 0.9% NaCl) was spread over glutaraldehyde SAM, and kept at 25 °C for ∼12 h in a humid chamber to facilitate immobilization, loosely bound enzyme was washed with PBS solution.

The LC cells were prepared by combining two glass slides and a finite gap is maintained by using 3 μm Mylar spacers. The cells are sealed by using a UV curable sealant. The dimensions of our cell were 1.5 cm × 1 cm, thus enabling a portable set up. The 5CB nematic liquid crystal was filled at room temperature by means of capillary action. Three types of LC cells were prepared to facilitate our scheme. First, a reference cell was prepared by coating the top and bottom glass slides with DMOAP-APTMS SAM. Subsequently, a counter cell or enzyme cell was prepared by loading Cholesterol oxidase solution (20 μl) on top of DMOAP-APTMS-Glu SAM as mentioned above. The enzyme cells were stored under refrigeration at 4 °C for future applications. Finally, a biosensing cell, free Cholesterol solution was prepared in Triton X-100 (10%, v/v, Sigma). Cholesterol (20 μl) was loaded on enzyme cell surface and kept at room temperature for 1 h to complete the reaction. In the present experiments, we have prepared four different cells with concentration of cholesterol varying from 10 mg/dl to 250 mg/dl.

The scanning electron micrographs shown in Fig. 1 demonstrate changes in the surface morphology on introduction of DMOAP, APTMS, ChOx, and Cholesterol. Figure 1(a) depicts homogenous morphology of DMOAP monolayer. Figure 1(b), a self-assembly of DMOAP/APTMS, illustrates dense web like morphology owing to presence of APTMS. A smooth morphology with some globular structures is observed after the immobilization of enzyme in Fig. 1(c). Cup-like structures are observed after cholesterol is introduced to DMOAP/APTMS/Glu/ChOx layer in Fig. 1(d). The origin and role of these structures are discussed in subsequent sections.

The schematic of whole procedure is shown in Fig. 2, which highlights the design of the current LC based biosensor. It is well known that alignment of LC is highly

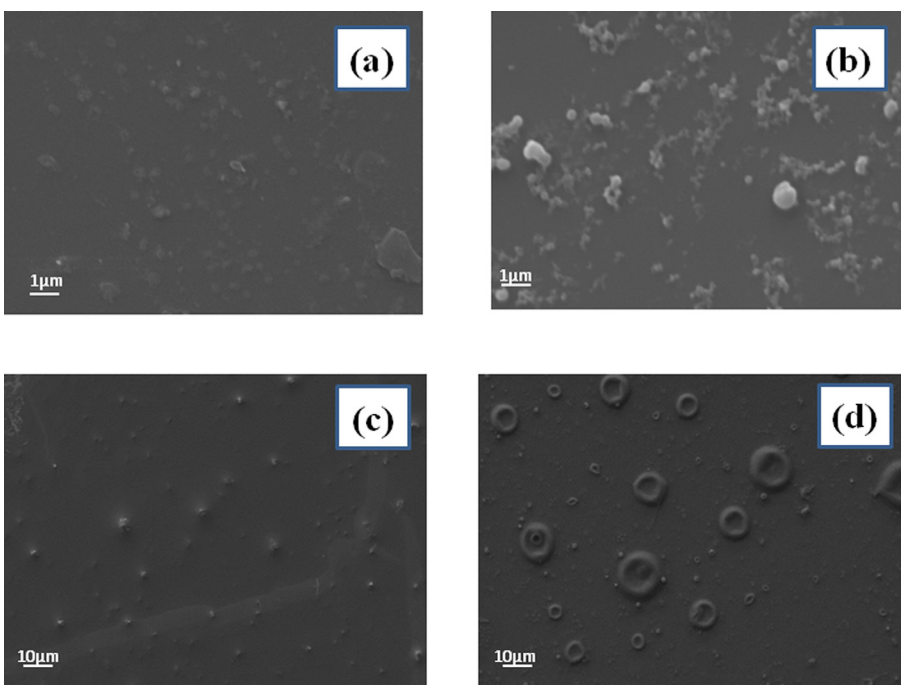

FIG. 1. SEM images of various layers deposited on glass substrates (a) DMOAP, (b) APTMS, (c) ChOx, and (d) cholesterol.

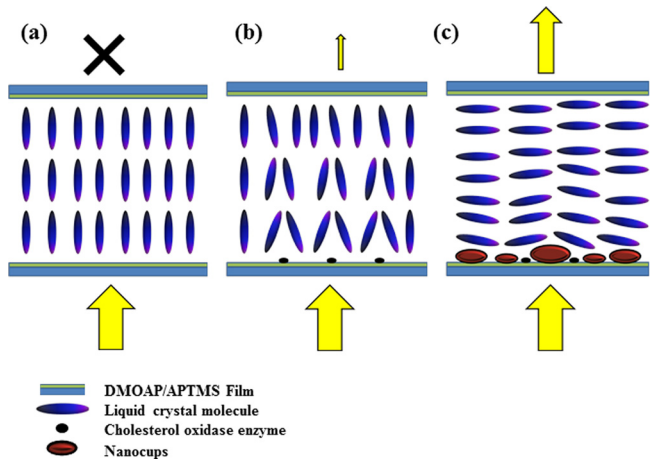

FIG. 2. Schematic illustration of various stages of LC biosensor (a) no light transmission in case of homeotropic alignment, (b) slight transmission of light due to the presence of ChOx, (c) maximum light transmission in case of homogeneous alignment caused due to the presence of cholesterol.

dependent on the surface effects.[27] A slight change on the surface leads to change in the alignment of LC molecules, which can be detected through POM. The present study is based on the above concept. For this purpose, a reference cell is used to achieve the homeotropic alignment of 5CB using above mentioned SAM. In this configuration, no light is passed through the LC cell. The presence of cholesterol can be detected in terms of non-zero transmitted light intensity through the LC cell containing cholesterol solution.

The homeotropic alignment is confirmed through POM and the corresponding texture is shown in Fig. 3(a). The upright orientation of 5CB molecules results into extinction of light between the crossed polarizers owing to lack of birefringence. Now ChOx enzyme is loaded over the prepared SAM and bonded through glutaraldehyde. After immobilization of enzyme, the cell is assembled and 5CB is filled at room temperature. The corresponding texture is shown in Fig. 3(b). There is a slight deviation from homeotropic alignment as compared to Fig. 3(a), but overall the colour does not change on rotating the sample cell, so the alignment can still be termed as homeotropic. Finally, various concentrations of cholesterol are loaded over ChOx and LC cells are prepared and 5CB is filled in these cells to analyse the change in the texture due to the presence of cholesterol. Figure 3(c) shows the optical micrograph of biosensing cell having 10 mg/dl concentration of cholesterol. It is clear that the presence of cholesterol causes a drastic change in the alignment of 5CB. It induces a definite birefringence by disrupting the orientation of LC molecules at the interface. The alignment changes from homeotropic to homogeneous (in which LC molecules align parallel to the substrate). This change in the alignment is related to change in the surface topology, which is governed by surface energy.[28,29] As it is established that a small volume of cholesterol is able to change the LC alignment, the concentration of cholesterol is increased. Figure 3(d) shows the optical micrograph of cell with 50 mg/dl concentration of cholesterol. Again the alignment is homogeneous, but with improved contrast, which means higher concentration of cholesterol results into more disruption and more tilting of LC molecules. This change in the contrast is a key factor to distinguish between two different concentrations of cholesterol. Nevertheless, the change in contrast is visible through naked eyes (or under the microscope); it requires a systematic determination and quantization of intensity of transmitted light through sample cell. We further take two more concentrations 150 mg/dl and 250 mg/dl to see the change in the LC alignment. An optical micrograph of concentration 150 mg/dl is recorded with vivid colour features as compared with previous two concentrations as shown in Fig. 3(e). In the last part, the concentration of cholesterol is increased to 250 mg/dl and the corresponding optical micrograph is shown in Fig. 3(f). It is observed that the alignment and its contrast follow a regular trend. The highest contrast is observed in the LC cell having highest concentration of cholesterol, suggesting that maximum birefringence is achieved in such cell.

It is interesting that as the concentration of cholesterol is increased, surface topography is influenced to a great extent, which causes more and more disruption of LC molecules. The various concentrations of cholesterol layer are again examined through SEM. Figure 4 shows SEM images of various concentrations of cholesterol. It is observed that some cup-like structures are formed over the cholesterol layer. These structures grow with increasing concentration of cholesterol in each layer and appear like bulging of the flat surface as shown in Fig. 4(d). These nano-cups are created due

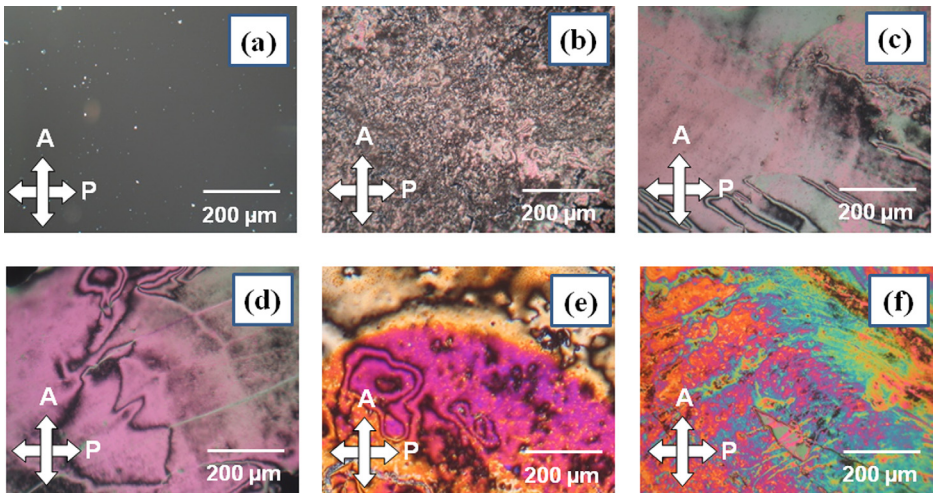

FIG. 3. Polarizing optical micrographs showing optical textures of various LC cells geometry (a) reference cell showing homeotropic alignment, (b) enzyme cell, (c) biosensing cell with cholesterol concentration as 10 mg/dl, (d) 50 mg/dl, (e) 150 mg/dl, and (f) 250 mg/dl.

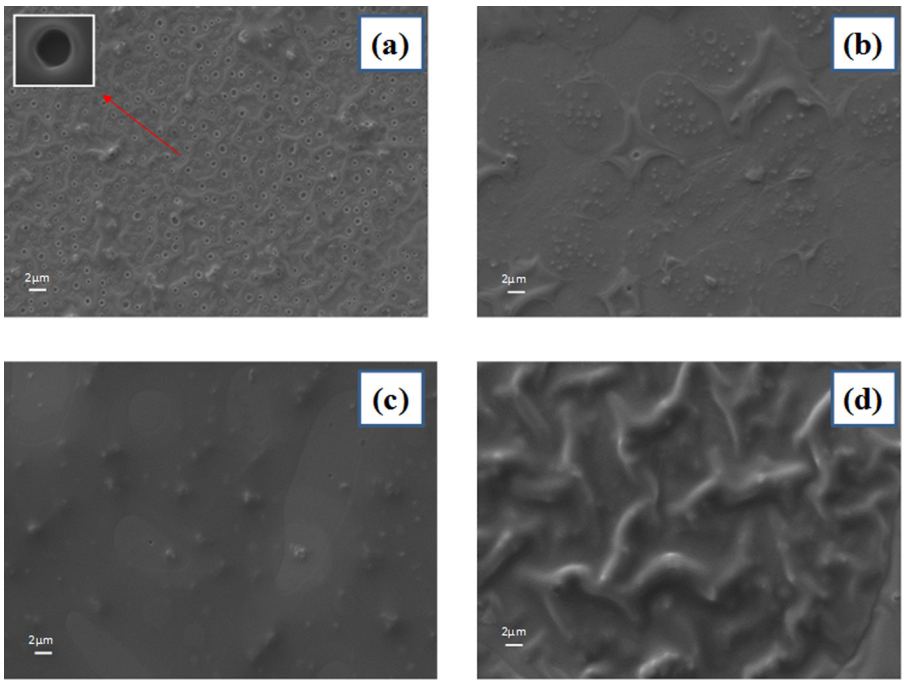

FIG. 4. Surface morphology examination through scanning electron microscopy of various concentrations of cholesterol (a) 10 mg/dl (magnified image of nano-cup (inset)), (b) 50 mg/dl, (c) 150 mg/dl, and (d) 250 mg/dl.

to the enzymatic reaction occurring between cholesterol oxidase and cholesterol releasing cholest-4-en-3-one and H_2O_2 [Eq. (1)], which results in enhanced non-uniform surface of self assembled monolayer.

$$\text{Cholesterol} + O_2 \overset{\text{ChOx}}{\rightleftharpoons} \text{Cholest}-4-\text{en}-3-\text{one} + H_2O_2. \quad (1)$$

The uniform vertical alignment of LC molecules becomes disordered around these surface defects. Therefore, the extent of light getting transmitted through biosensing cells enhances proportionally as the concentration of cholesterol is increased. Figure 4 present a direct evidence of different surface topology of cholesterol layer, which is responsible for observed changes in optical textures of 5CB under crossed polarizers. The study was repeated many times before making a conclusion. It is ensured that the results are highly reproducible, which is essential for the robust device.

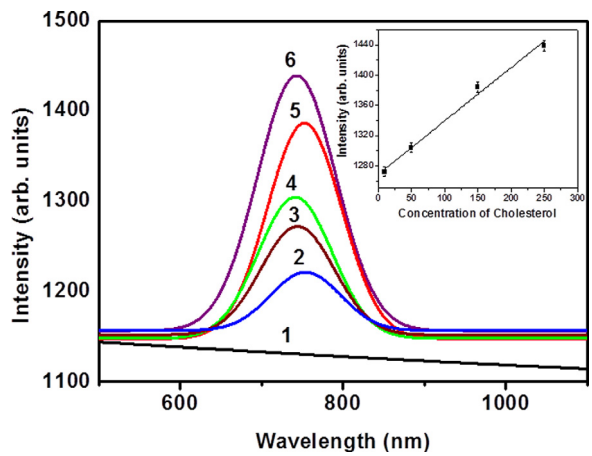

FIG. 5. Intensity of transmitted light through liquid crystal cells as a function of wavelength recorded using a spectrometer for (1) reference cell, (2) enzyme cell, (3) biosensing cell with cholesterol concentration as 10 mg/dl, (4) 50 mg/dl, (5) 150 mg/dl, and (6) 250 mg/dl.

The intensity of bright and dark states of an LC cell can be quantified by using a spectrometer.[30] Here, the intensity of transmitted light through different surface treated LC cells is measured with a spectrometer and plotted as a function of wavelength. The various plots are shown in Fig. 5. It has been inferred that intensity increases with an increase in cholesterol concentration. The homeotropic alignment resulting from the reference cell shows the lowest intensity as shown in Fig. 5-1. However, immobilization of ChOx enzyme itself brings change in the alignment and thus shows higher intensity relative to reference, which is shown as Fig. 5-2. The various cholesterol concentrations show regular increment in intensity Fig. 5-3–5-6, which is the basis of cholesterol sensing. The intensity versus concentration of cholesterol is plotted and shown as inset of Fig. 5. The spectrometer graphs summarise the working proposal of LC based cholesterol biosensor.

In summary, a unique enzyme based liquid crystal biosensor is developed for cholesterol detection. The cholesterol induced LC alignment conversion from homeotropic to homogeneous builds the foundation for this development. The change in surface topology and surface energy results into change in orientation of liquid crystal molecules and their alignment. The changes in alignment are realized through polarizing optical micrographs and a spectrometer. The results are reproducible, motivating, and offer an easy way to design portable biosensors based on liquid crystal, which are highly sensitive to the amount of cholesterol. Besides this, the present work opens up further scope in the area of enzyme based liquid crystal biosensing. The role of nano-cups is very crucial and it needs further research to find the origin and control over these nano-cups. The shelf life of the device is 10–15 days; efforts are being made to improve it further.

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