Detection of creatinine using surface-driven ordering transitions of liquid crystals

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ABSTRACT
Determining creatinine levels in blood is of great importance in the detection of high risk for renal failure. Here, we report a simple methodology for real-time monitoring of creatinine employing surface-driven ordering transitions in liquid crystals (LCs) by changing pH in presence of creatinine deiminase enzyme. It is found that when 5CB (4-Cyano-4ʹ-pentylbiphenyl) LC doped with 4ʹ-hexyl-biphenyl-4-carboxylic acid, a bright optical appearance was observed (at aqueous–LC interface) which is not disturbed in presence of creatinine, consistent with a planar/tilted orientation of the LC molecules at those interface. Interestingly, in presence of creatinine deiminase, an ordering transition was observed resulting from enzymatic reactions (giving rise to NH₄⁺ ions) that can change the local pH values and lead to dark optical appearance of the LC. Presence of different amounts of creatinine would lead varied ordering transition that can be monitored in real time in presence of creatinine deiminase. Our approach could detect the creatinine levels as low as that of the healthy adult (~50 µM) and can be successfully applied to measure higher concentration of creatinine in real time using dynamic optical response of the LC.

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**Introduction**

Creatinine is an important indicator of renal health.[1–3] Being a waste product (with no further biological function), it is removed by the kidneys from the circulatory system via glomerular filtration. The creatinine level in body fluids ranges from 40 to 150 µM in healthy adults and 1000 µM or more in patients with renal dysfunction,[4] while level less than 40 µM may indicate decreased muscle mass. The conventional employed methods for the laboratory analysis of creatinine are based on colorimetry using the Jaffe’s reaction[5] or the enzymatic colorimetric method where creatinine is enzymatically catalysed in multiple steps to release H₂O₂ that is further quantified.[6] However, most of these methods have their own limitations. For example, colorimetric methods suffer interference from numerous metabolites and drugs found in biological samples.[7] On the other hand, enzymatic assays are complex, costly and time-consuming.[8] Therefore, point-of-care creatinine testing for the detection and monitoring of chronic kidney disease is necessary to have fast and reliable results.[9] These testing are generally based on potentiometric and amperometric detection systems. Potentiometric sensors are based on the hydrolysis of creatinine by creatinine iminohydrolase or creatinine deiminase, which generates ammonia that can be detected,[10–12] while amperometric biosensors involve conversion of creatinine to glycine (three-stage conversion) followed by the detection of hydrogen peroxide liberated via electrode.[13] Although these detection systems have excellent operational stability, short response time and high sensitivity, the operation system is complex and limits their widespread use. The majority of the currently available techniques are not applicable to point-of-care treatment for chronic kidney disease. This is because the kits available in the market are simply too large to carry and therefore, cannot be used as a portable device.[14] However, capillary electrophoresis as scale down version of laboratory technique has been developed but currently have the drawback of being unable to detect creatinine levels as low as that of a healthy adult (50 µM).[15] Therefore, it is important to develop a new label-free biosensor which is simple, easy to use and specific to creatinine with short response time and can detect creatinine levels as low as 50 µM.

Liquid crystals (LC) have been known to transduce and amplify the chemical and biological molecular events (at aqueous interfaces) into visual outputs detectable by naked eye.[16–31] For instance, past studies have demonstrated that a range of amphiphiles such as surfactants [18,21] and phospholipids [16,22,23] at aqueous–LC interface leads to the changes in the optical appearance of the LC, consistent with an ordering transition of the LC. The changes in the alignment of the LC molecules result from the coupling between the aliphatic tails of the adsorbed amphiphiles and the mesogens of the LCs. [16] Zhong et al. reported a new experimental system based on micrometre-sized LC droplets in which the bright or blue coloured droplets show dark or orange coloured optical response when come in contact with bulk surfactant solutions.[28] Micrometre-scale LC droplets supported on a solid surface have been used to determine lipase activity which can detect 0.1 µg/mL lipase in the aqueous solution within 6 min.[29] Anil et al. reported the textures of 4-Cyano-4’-pentylbiphenyl (5CB) and DSCG, a lyotropic LC, on modified surfaces depending upon the alignment of the LCs which can detect the low concentration of protein.[30] Dingdong et al. reported the simple and label-free technique for imaging catalase activity utilising the interactions between LCs and oil in water emulsions.[31]

It is also reported that when polymers and surfactants containing pH sensitive functional groups are adsorbed at the aqueous–LC interface, the ordering of LCs becomes responsive as a function of pH.[32–38] For example, Kinsinger et al. designed an amphiphilic polymer (by conjugation of poly(ethylene imine) with N-[3-(dimethylamino)-propyl]acrylamide) and demonstrated that assembly of this functionalised polymer at aqueous/LC interface responds reversibly to pH changes in the aqueous phase (although the response time is very long ~10 h).[32] They concluded that ordering of the polymer at the interface is responsible for the pH-dependent changes in the orientation of the LCs. Yet, it is still unclear whether the system can be employed to detect small pH changes as they only observed different orientational response of the LCs at pH 9.0 and 5.0. Dong-Yul et al. reported the pH responsive aqueous–LC interface functionalised with pH-responsive poly(acrylicacid-b-4-cyanobiphenyl-4-oxyundecylacrylate) (PAA-b-LCP). At low pH, the PAA block collapses showing the planar alignment tendency of 5CB at an aqueous interface. As pH increases, the PAA chains become increasingly charged and swell, producing a change to homeotropic anchoring of the LCs.[33] Polyyacrylic acid block liquid crystalline polymers were further exploited for detecting proteins through changes in pH at water/LC interface.[34] Using the similar strategy, glucose
sensor has been developed by coating microsized droplets with PAA-b-LCP and covalently immobilising glucose oxidase to the PAA chains. The functionalised LC droplets change from radial to bipolar configuration when come in contact with glucose.

Bi et al. reported a LC-based sensor in which when LC is doped with 4ʹ-pentyl-biphenyl-4-carboxylic acid (PBA), it shows change in its optical appearance from bright to dark when pH of the aqueous phase changes from 6.9 to 7.0. They suggested that orientational transitions of 5CB can be the result of the protonation and deprotonation of PBA at the aqueous/LC interface.

In this paper, we utilised similar strategy that employed simple 5CB LC doped with nearly same molecular structure having pH-sensitive functional group for the detection of creatinine.

In the present study, the creatinine biosensor is based on the change in the optical properties of the nematic 5CB that exploit long-range orientational ordering of LC molecules and the enzymatic hydrolysis of creatinine to ammonia and N-methylhydantoin (Figure 1). The designed biosensor could detect the creatinine level as low as that of a healthy adult (50 µM). We have used a well-known nematic 5CB LC which is doped with 0.3 wt% of 4ʹ-n-Hexylbiphenyl-4-carboxylic acid (HBA) for our study. The change in the protonated state of doped 5CB could be tuned during enzymatic hydrolysis of creatinine (by creatinine deiminase) that resulted different optical signal at aqueous–LC interface.

**Experimental section**

**Materials**

Creatinine deiminase microbial, creatinine, Human haemoglobin, α-D-Glucose, 5CB, N,N-dimethyl-N-octadecyl-3-aminopropyltrimethoxysilyl chloride (DMOAP), hydrochloric acid and sodium hydroxide were purchased from Sigma-Aldrich (St. Louis, MO). l-Ascorbic acid was obtained from HiMedia (Mumbai, India). 4ʹ-n-Hexylbiphenyl-4-carboxylic acid, 99% was purchased from Alfa Aesar (Heysham, England). Sulphuric acid and hydrogen peroxide (30% w/v) were purchased from Merck (Mumbai, India). Ethanol was obtained from Jebsen & Jenssen GmbH and Co., Germany (S D Fine-Chem, Ltd.). Deionisation of a distilled water (DI water) source was performed using a Milli-Q-system (Millipore, Bedford, MA). Fisher’s Finest Premium grade glass microscopic slides and cover glass were obtained from Fischer Scientific (Pittsburgh, PA). Gold specimen grids (20 µm thickness, 50 µm wide bars, 283 µm grid spacing) were obtained from Electron Microscopy Sciences (Fort Washington, PA).

**Treatment of glass microscope slides with DMOAP**

Glass microscope slides were cleaned with piranha solution (70:30 (% v/v) H₂SO₄:H₂O₂) for 1 h at 100°C according to the published procedures.[16] They were then rinsed with water, ethanol and methanol and dried under a stream of gaseous N₂, followed by heating to 120°C overnight. The ‘piranha-cleaned’ glass slides were immersed into 0.1% (v/v) DMOAP solution in DI water for 30 min at room temperature. The slides were then rinsed with DI water to remove the unreacted DMOAP from the surface. The DMOAP-coated slides were dried under a stream of N₂ and kept at 100°C.

**Preparation of optical cells**

Cleaned gold TEM specimen grids were placed on DMOAP-coated glass slides. The grids were filled with approximately 0.2 µL of 0.3% HBA-doped 5CB, and the excess LC was removed with the help of a syringe to produce a planar interface. Subsequently, the grid was immersed in 2.5 mL optical cell containing creatinine solution of a known concentration in DI water at room temperature. After few minutes, required aqueous solution of creatinine deiminase from the stock solution of 1 mg mL⁻¹ was introduced into the optical well to make the final concentration of 0.04 mg mL⁻¹ enzyme as and when needed. Therefore, the detection is strongly dependent on the volume of enzyme solution passing through the cell.

**Optical characterisation of LC films**

The orientational ordering of the LC was determined using a Zeiss polarising microscope Scope.A1 with cross polars (x50). For investigation of greyscale intensity of the images, images were processed using Image J free access software (developed by U. S. National Institutes of Health, Bethesda, MD).
Results and discussion

Our first experiment employed polarising optical microscopy (POM) to determine the ordering of nematic 5CB in presence of 0.3% HBA at aqueous–LC interface. First, we confined doped 5CB into a TEM grid supported on DMOAP-coated glass slides followed by immersion onto water. We observed a bright optical appearance of the LC under crossed polarisers as expected. Second, we immersed the grid containing HBA-doped 5CB in Millipore water (pH 7.2) in presence of 1000 µM creatinine (Figure 2(a)). Inspection of Figure 2(a) demonstrates that the optical appearance of the LC remains invariant (i.e. tilted/planar orientation) in presence of creatinine. Interestingly, when we introduced creatinine deiminase of concentration 0.04 mg mL$^{-1}$, the LC image became dark immediately (Figure 2(b)). In contrast, when undoped 5CB was used in the experiment, 5CB appeared bright (see below).

From these results, it can be concluded that the released ammonium ions from enzymatic hydrolysis of creatinine have increased the pH of the system which deprotonates the pH-sensitive functional group of HBA resulting an ordering transition of the LC. It is also evident that HBA played an important role in the observed ordering transition. Hydrolysis of 1000 µM creatinine produces enough amount of ammonia which deprotonates HBA acid molecules in 5CB causing them to self-assemble at the LC–water interface and thus we observed change in the interfacial phenomena. It should be noted that we have selected enzymatic pathway to detect creatinine due to the high specificity of the enzyme-based reaction which also eliminates the possibility of interference from other biomolecules present in the specimen.

In order to verify that the change of ordering of LCs is not caused by the creatinine deiminase alone, we performed a control experiment. The HBA-doped 5CB grid was immersed in the aqueous solution of 0.04 mg mL$^{-1}$ enzyme (without creatinine). Figure 3a shows that the optical micrographs of the LC at aqueous–LC interface which shows bright optical appearance consistent with the planar orientation of the LCs at aqueous interface. We observed that the optical image remains bright in presence of the enzyme suggesting that enzyme is unable to trigger an ordering transition (Figure 3(b)). Second, to further confirm that HBA molecules when doped in 5CB are responsible for the ordering transition (homeotropic), TEM grid was filled with pure 5CB followed by immersion in an aqueous solution of creatinine. Figure 4(b) shows that there was no change in the optical appearance of the LCs. When the enzyme was added subsequently in the system, the hydrolysis of the creatinine was also not observed to be able to change the orientation of LCs from planar to homeotropic at the LC–aqueous interface (Figure 4(c)).

To probe the detection limit of the creatinine in the system, we investigated the effect of 0.04 mg mL$^{-1}$ enzyme and different concentrations of creatinine to the response of the LC. Figure 5 shows that the optical appearance of the LC changes from bright to dark and its orientation from planar to homeotropic in 3 min when 1000 µM creatinine gets hydrolysed in the presence of 0.04 mg mL$^{-1}$ enzyme. The changes in the optical appearance of the LC from bright to dark occur over a period of 5–25 min depending upon the concentration of the creatinine present in the system. We make two other additional observations regarding this result. First, we note that LC is able to respond up to a
concentration of 50 µM. Second, below 50 µM no ordering transition was observed even after a period of 1 day. It should be noted that even after increasing the concentration of the creatinine deiminase in the system from 0.04 to 0.08 mg mL\(^{-1}\), hydrolysis of 40 µM creatinine did not affect the orientation of the HBA-doped LCs and the system remains bright (Figure S1). One of the possible reasons is that the hydrolysis of 40 µM creatinine is not able to produce the enough amount of ammonia which could deprotonate the HBA molecules and show the ordering. The ammonia being released from the enzymatic hydrolysis of 40 µM creatinine is neutralised by the bulk solution before it could deprotonate the HBA molecules. These results suggest that the system is capable of detecting the creatinine up to 50 µM which is close to the creatinine level in a healthy human. It also suggests that concentration of creatinine can be estimated from the kinetic response of the LCs.

Concentration of creatinine present in specimen can be estimated by measuring average greyscale intensity of the optical images with respect to the time taken by LCs for the optical transition from bright to dark. Figure 6 shows the graphs of greyscale intensity of the POM images taken at different interval times after addition of enzyme in the system containing different concentration of creatinine. Figure 6(a) shows that hydrolysis of 1000 µM creatinine produces enough amount of ammonia causing very fast change (in 3 min) in the optical appearance of the LC from bright to dark. Decreasing concentration to 500 µM does not have much effect on the response time of LCs anchoring transition as greyscale intensity dropped to its minimum in 5 min (Figure 6(b)). Further decreasing concentration of creatinine present in specimen can be estimated by measuring average greyscale intensity of the optical images with respect to the time taken by LCs for the optical transition from bright to dark. Figure 6 shows the graphs of greyscale intensity of the POM images taken at different interval times after addition of enzyme in the system containing different concentration of creatinine. Figure 6(a) shows that hydrolysis of 1000 µM creatinine produces enough amount of ammonia causing very fast change (in 3 min) in the optical appearance of the LC from bright to dark. Decreasing concentration to 500 µM does not have much effect on the response time of LCs anchoring transition as greyscale intensity dropped to its minimum in 5 min (Figure 6(b)). Further decreasing...
the concentration to 100 and 50 μM increases the response time to 13 and 25 min, respectively. Concentration less than 50 μM does not show any significant changes in the values of greyscale intensity of the micrographs obtained at certain time intervals. Since the response time of LCs is proportional to the amount of ammonia released from the enzymatic reaction, the results can be correlated with the creatinine concentration present in the system. Therefore, concentration of creatinine can be calculated as a function of time taken by the LC to change their orientation from planar to homeotropic (see below).

Figure 7 describes the correlation between the concentration of creatinine and the time taken by the LC to show an optical response from bright to dark. We mark two observations from this figure. First, when the concentration is high as 1 mM (a sign of renal failure), the LC optical change is as fast as 2.5 min with a standard deviation of 0.8 min. Second, when the concentration lies between 50 to 100 μM (which is considered as healthy), the transition becomes comparatively slower and ranges from 13.9 to 24 minutes. So our system is quite capable of measuring the creatinine concentration in real time quantitatively and able to differentiate between healthy human and human seeking medical attention for renal dysfunction.

Our next goal is sought to determine the detection limit of the enzymatic activity of the creatinine deiminase which is responsible for the observed ordering transition. First, a 2.5 mL optical cell (homemade) was filled with aqueous solution of 1 mM creatinine followed by the addition of creatinine deiminase (from the stock of 1 mg mL\(^{-1}\)) to have required final concentration for the experiment. Figure 8 shows the effect of varying concentration of the creatinine deiminase on the optical appearances of HBA-doped LCs when introduced in the optical cell containing aqueous solution of 1 mM creatinine. After addition of 80 μg mL\(^{-1}\), the transition of LCs from planar to homeotropic was as fast as 1.5 min (Figure 8(a)). As the concentration of the creatinine deiminase gets decreased in the system, response of LCs for the optical transition becomes slow and changes to dark in 3, 15 and 25 min when 40, 10, 7 μg mL\(^{-1}\) creatinine deiminase were used, respectively (Figure 8(b)–(d)). On further decreasing the concentration of creatinine deiminase to 5 μg mL\(^{-1}\) did not induce changes in the optical appearance of the LCs and the LCs remains bright, owing to their planar orientation. For all experiments the concentration of 40 μg mL\(^{-1}\) of the enzyme was used which shows ordering transition within 3 min.

Next, we explored whether it would be possible to determine creatinine in presence of human haemoglobin, L-ascorbic acid and glucose. For this, in the optical cell, the mixture of 0.1 μM haemoglobin, 5 mM glucose, 0.08 mM ascorbic acid and 1 mM creatinine was filled in the optical cell containing HBA-doped 5CB grid (Figure 9(a)). The initial bright appearance of the LC changed to dark in 11 min upon the addition of the enzyme (Figure 9(b)), consistent with the ordering transition. However, in the absence of creatinine no change in the ordering transition was observed for 3 h (Figure S2).

To establish that deprotonation of HBA molecules is responsible for the orientational transitions of LC
molecules, we sought to observe the effect of the addition of exogenous acid on LC anchoring after enzymatic hydrolysis of creatinine at aqueous–LC interface. We introduced different concentrations of HCl in the LC-filled grid (doped with HBA) after enzymatic hydrolysis with 1 mM creatinine. Figure 10 shows the effect of addition of different concentrations of exogenous HCl on the orientational ordering transition of the LC. We observed that addition of 1 mM HCl leads to the rapid change in optical appearance of the LC from dark to bright which remained stable over several hours (Figure 10(a) and (b)). This concludes that 1 mM HCl is able to neutralise the entire ammonium ions produced from the hydrolysis of 1 mM creatinine.

When we introduced 0.5 mM HCl, it was found that an immediate ordering transition of the LC to the bright optical appearance within 30 s followed by dark image as it was in original (Figure 10(c) and (d)). This suggests that 0.5 mM HCl is able to neutralise the effect of only 0.5 mM of the ammonium ions which could protonate the dissociated HBA molecules leading to the transient planar orientation of the LC molecules followed by original dark appearance within 25 min. When 0.1 mM HCl is added to the system, the sudden bright appearance turned to dark in 4 min suggesting that the effect of HCl was compensated by the bulk water (Figure 10(e) and (f)). Further decrease in concentration of HCl leads to similar interfacial events as expected (Figure 10). Considering the amount of HBA molecules at the LC–aqueous interface, concentration of HCl was reduced to 0.01 nM which does not have any effect to the dissociated HBA molecules at the interface (Figure 10(k) and (l)). A schematic of change in orientation of the LC in presence of enzymatic hydrolysis of creatinine is shown in Figure 11.
Conclusions

We have been successful in developing a LC-based label-free biosensor for creatinine which exploits the optical properties of the LC and shows the change in visual appearances from bright to dark during enzymatic hydrolysis. As the ammonia is released during the hydrolysis, the LC molecules change their orientation from planar to homeotropic. This transition can be attributed to the deprotonation of HBA molecules at the LC–aqueous interface. The LC-based creatinine biosensor being able to detect the creatinine levels as low as that of a healthy adult can be promising system for differentiating between the creatinine level of a healthy adult and a person with renal dysfunction. Along with this, the device is specific, fast, handy and can be made easily available to the patient.

Disclosure statement

No potential conflict of interest was reported by the authors.

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