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Liquid-crystal-based immunosensor for detection of cardiac troponin I

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Abstract

Cardiac troponin I (cTnI) is one of the most sensitive and specific markers of myocardial cell injury, which can detect even minor myocardia damage. It is recognized as the main biochemical marker of rapid diagnosis of acute myocardial infarction (AMI) and acute coronary syndromes (ACS). In this study, a label-free biosensor that utilizes the birefringence property of nematic liquid crystal (LC) for detection of cTnI is demonstrated. A chemically sensitive film with specific molecular recognition ability is decorated on the surface of the substrate, the LC molecules are arranged in a vertically oriented order under the influence of the sensitive film and a dark background signal is obtained under polarizing optical microscope. When the antigen-antibody specifically binds to form a stronger acting force, the orientation of LC molecules changes, resulting in a bright optical appearance. This LC-based immunosensor has not only the advantages of facile structure, low cost and excellent specificity, but also high sensitivity (low detection limit of 1 pg/ml), which has a promising future in biomedical related fields.

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1. Introduction

Cardiac troponin I (cTnI) is a kind of regulatory protein peculiar to myocardial tissue, which inhibits the binding of myosin to actin and plays an important role in myocardial contraction [1-3]. A large number of studies have shown that cTnI level rises instantly with high amplitude variation in the early acute myocardial infarction (AMI) and have high sensitivity. In addition, cTnI possesses high cardiomyocyte specificity, it is not functioned in any type of skeletal muscle [4-6]. The specificity of cTnI for the diagnosis of myocardial infarction (MI) is 96% and the sensitivity is 97% [7-9]. Therefore, cTnI is one of the most sensitive and specific serum markers of myocardial cell injury [10-11]. Moreover, cTnI has the advantages of definite diagnostic threshold and rapid detection [12-13], which has gradually become the main biochemical indicator for judging myocardial cell injury in AMI patients [14-15].

At present, a lot of methods has been studied to monitor cTnI levels, such as enzyme linked immunesorbent assay (ELISA) [16-17], fluorescent immunoassay [18], colorimetry [19], electrochemistry [20], paramagnetic [21], surface plasma resonance (SPR) [22], etc. These methods have specificity for cTnI and play an essential role in detecting cardiac biomarkers. However, there are some obvious shortcomings in these traditional methods, such as the need for complicated instruments and cumbersome detection steps. Furthermore, complex and professional labeling processes require long time and extra cost. Recently, Tong Liu et al [23] reported a compact biosensor for detecting cTnI based on the phase-shifted microfiber Bragg grating probe. Deepika Sandil et al [24] demonstrated a biofunctionalized tungsten trioxide-reduced graphene oxide nanocomposites for sensitive electrochemical immunosensing of cTnI. Nevertheless, we found that whether it uses microfiber Bragg grating or nanocomposites for sensing, it is not easy to manufacture the sensing structure, and the production requirements are relatively high. As a result, it is of paramount importance to establish a fast, sensitive, low-cost and label-free method to detect cTnI.

As a material with many excellent properties, liquid crystal (LC) has become a research hot spot. As a special material form between disordered liquid and solid crystal, it possess the orderliness and optical anisotropy of crystal molecular arrangement [25-26]. The orientation of the LC molecules is highly sensitive to surface molecular binding events, and the binding of molecules to the surface would induce a change in the LC optical image, which provides a possibility for sensitive detection of biomolecule [27-28] or chemical reaction [29]. The LC biosensor utilizes the birefringence characteristics of the LC on the polarized light to monitor the change of the LC orientation caused by the biomolecule, thereby causing change of the polarization signal. This change can be observed with

the naked eye and does not require other expensive and high-end instrument. As a label-free sensor, the LC-based biosensor possesses simple structure, easy operation, outstanding portability, no need to special light source, and point-of-care (POC) diagnosis. It turns out LC-based biosensor has great potential for the detection of cardiac biomarkers of myocardial infarction. Over the past decades, LC sensors have been applied in the domain of chemical and biological sensing systems [30-31]. However, the methods they used almost utilize one-sided sensing, which limit its sensing effect to some extent, and we believe that the effective use of the other side to sense simultaneously can improve its detection efficiency and further increase its detection sensitivity.

In this paper, a new immunosensor based on LC cell for sensitive detection of cTnI is reported. Based on one-sided sensing LC cell method, a double-sided sensing LC cell biosensor is proposed. When LC molecules is vertically oriented with the function of surfactant, the all black appearance is observed under a polarizing optical microscope (POM), and the interaction between cTnI and its corresponding antibody may disturb the perpendicular alignment of LC molecules. The sensor shows a fast optical response to orientation change of LC occurring on the surface, a random optical texture of the surface can be observed using a POM. This new simple LC-based biosensor exhibits high specificity for cTnI compared to other proteins, and detection sensitivity is also very high, which has a promising prospect in biomolecular detection.

2. Experimental section

2.1. Materials

Nematic liquid crystal 4-cyano-4'-pentylbiphenyl (5CB) was purchased from Beijing Bayi Space LCD Technology Co.,Ltd.,China. N, N-dimethyl-N-octadecyl (3-aminopropyl) trimethoxysilyl chloride (DMOAP) was purchased from Sigma-Aldrich, Cardiac troponin I antigen was bought from Wondfo Bio-Technology Co., Ltd. Phosphate buffered saline (PBS), (3-aminopropyl) triethoxysilane (APTES), glutaraldehyde (GA) solution and glycine were obtained from Beijing Innochem Technology Co.,Ltd. Cardiac troponin I antibody was purchased from Hytest Bio-Technology Co., Ltd. Human Immunoglobulin G (IgG) and human serum albumin (ALB) were bought from Beijing Bioss Bio-Technology Co., Ltd. Deionized (DI) water was from a Milli-Q system form Millipore, USA, and used in subsequent experiment.

2.2. Pretreatment of glass slides

The slides were cut into 2cm × 1cm firstly, and soaked them in the newly prepared Piranha solution

(70% H_2SO_4 and 30% H_2O_2, Notice: Piranha solution has strong oxidizing property and can react drastically with organic matters. Don't store it in a closed container) at 80 °C for 1 h. Then, rinsed with absolute ethanol and a large amount of DI water, blow dry with nitrogen and put them in an oven at 110 °C for at least 3 h.

2.3. Silylanization of glass slides

The slides were then decorated by a mixture solution of 1% (v/v) APTES and 1% (v/v) DMOAP, soaked them in the mixed solution at 80 °C for 1h (turn them over 30 minutes later), then, rinsed them with absolute ethanol and a plenty of DI water after the reaction. Finally, the slides were heated in an oven at 110 °C for 1h after dried under a stream of nitrogen gas.

2.4. Coupling of GA

The slides decorated with APTES/DMOAP self-assembled film were immersed in 2% GA solution (v/v), and reacted for 1 hour at room temperature. After the reaction was completed, the slides were washed with a large amount of DI water, and dried under a stream of nitrogen gas.

2.5. Immobilization of cTnI antibody

The slides were then incubated with a cTnI antibody solution (1μg/ml) in PBS for 2 hours at room temperature, washed the slides with a plenty of water after the incubation. Subsequently, the slides assembled with anti-cTnI were soaked in a glycine solution (80 mM) at room temperature for 1 h with the purpose of blocking any unreacted aldehyde groups. The slides were finally rinsed with DI water, and then dried with a small stream of nitrogen gas.

2.6. Detection of cTnI and fabrication of LC cells

A cTnI solution in PBS was incubated on the anti-cTnI immobilized surfaces at 37 °C. After 1h of incubation, the slides were sequentially rinsed with DI water and dried under a stream of gaseous nitrogen. LC optical cells were fabricated by pairing the deposited bottom slide with deposited top glass slide with two thin polyester films as spacer (thickness is approximately 12 μm) on left and right sides. 5CB was heated to its isotropic phase (above 35.3 °C) and loaded into an empty space between the two slides using the capillary force. When cooled to room temperature, the liquid crystals are nematic (5cb is nematic at 12 °C~35.3 °C), in the meanwhile, the enthalpy of the LC phase transition is 17.15kJ/mol.

2.7. Examination of optical images

A POM (CX31 Olympus, Japan) was utilized to examine all the optical images of LC cells. All POM images under cross polarizers were obtained by a CCD camera (DP21, Olympus, Japan) at room temperature.

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3. Results and discussion

3.1. Sensing strategy for detection of cTnI

The sensing strategy of the proposed LC-based immunosensor is illustrated in Fig. 1. Firstly, a chemically sensitive membrane consisting of a mixture of DMOAP/APTES was assembled on the surface of the slide (Fig. 1a), which induced homeotropic alignment of LC molecules. Then the cTnI antibody was immobilized on the surface of the glass substrate by using the bifunctional cross-linking agent GA (Fig. 1b and c). The LC molecules adopt a homeotropic orientation on the slide when rationally controlling the DMOAP/APTES ratio and the amounts of cTnI antibody immobilized on the slide (Fig. 1e) and thus the image under POM appears uniformly dark (Fig. 1g). When the cTnI antibody immobilized on substrate specifically reacted with the cTnI antigen (Fig. 1d), it can disturb the homeotropic alignment of the LC molecules (Fig. 1f), thereby generating different optical response signals (Fig. 1h), and the higher the concentration of the specific molecule cTnI, the greater the disturbance and the brighter the optical image, so as to realize the detection of specific biomolecules cTnI. This change of orientation can be detected by a POM on account of the birefringence of LC molecules to polarized light, and this change is directly observed by the naked eye.

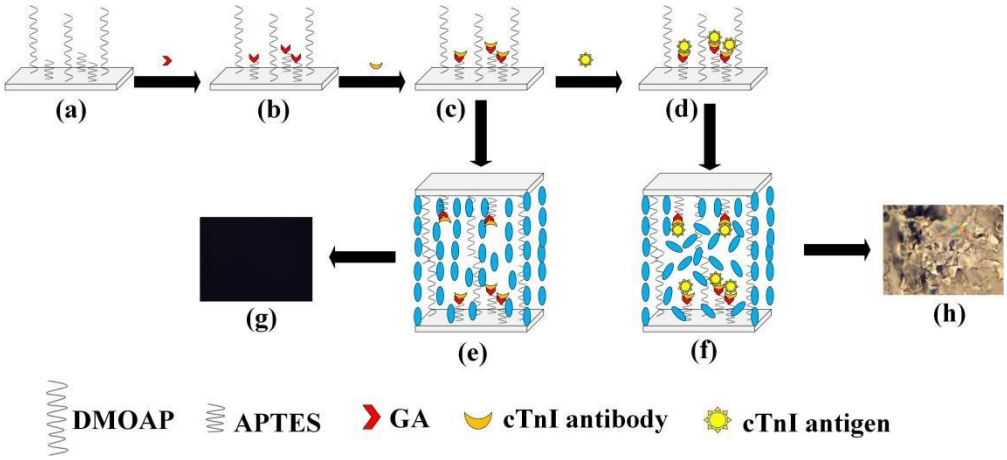

Fig. 1. Schematic illustration of the proposed sensor and sensing strategy.

3.2. Optimization of APTES/DMOAP/GA ratio

The main component of the LC-based biosensor is a LC cell. We initially selected the APTES containing the amino functional group at the end to assemble, because the biomolecule can be bonded to APTES by the bifunctional cross-linking agent GA. However, APTES is a silane reagent with a short carbon chain. If the slides are assembled only with it, it can not effectively induce the homeotropic alignment of LC molecules. In order to achieve the purpose of obtaining a dark

background and fixing biomolecules on the slide, we used a mixed solution of APTES/DMOAP to assemble the slides.

As shown in Fig. 2, the ratio of APTES/DMOAP were related to the background brightness value. The images under POM show a bright-to-dark change with the decrease of the APTES/DMOAP ratio. When the ratio of APTES/DMOAP was 1:1 (v/v) or lower, a completely dark stable background was observed, indicating that the LC molecules exhibited a uniform vertical orientation (Fig. 2c). The results show that the presence of a small amount of APTES does not disturb the vertical alignment of the LC in the cell. In Fig. 2b, a dark texture with some bright spots was observed in a ratio of 5:1 (v/v) for the APTES/DMOAP solution. In addition, more bright spots were observed when the APTES/DMOAP volume ratio was 10:1 (v/v) (Fig. 2a). In order to obtain sufficient dark background signal and a good self-assembly efficiency of anti-cTnI, a 1:1 (v/v) APTES/DMOAP ratio was chosen to immobilize the GA.

The background signal is also affected by the amount of GA. Optimization experiments were carried out at different concentrations of GA and the results are shown in Fig. 2. We found that the optical appearance of the LC cell showed a uniform black image at a concentration of GA of 1% and 2% (Fig.2h and g). In contrast, when the concentration of GA reached 5% (Fig. 2f) or 10% (Fig. 2e), the LC molecules alignment was disturbed and some bright spots appeared in the POM image. To ensure maximally immobilized anti-cTnI and a dark background signal, 2% GA was used in subsequent experiments.

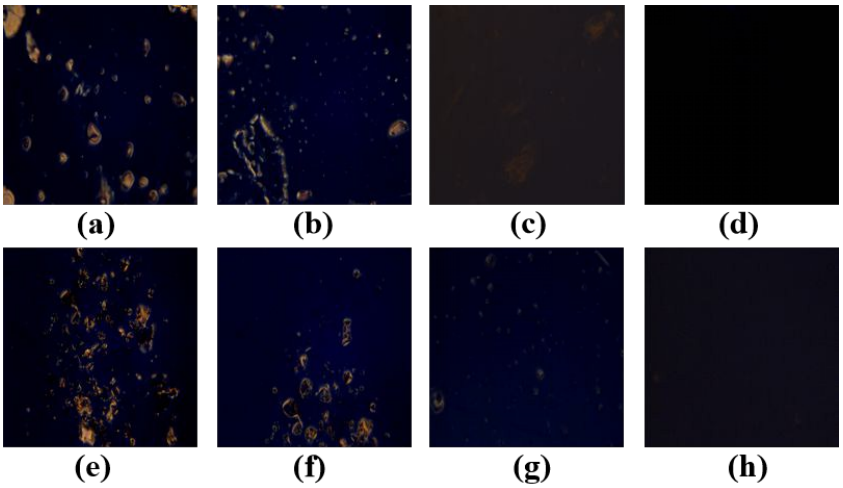

Fig. 2. POM images of LC cells with 5CB in different ratios of DMOAP/APTES. The ratio of APTES/DMOAP was: (a) 10:1; (b) 5:1; (c) 1:1; (d) 0.5:1, respectively. And, after incubation with different ratios of GA (DMOAP/APTES=1:1): (e) 10%; (f) 5%; (g) 2%; (h) 1%.

3.3. Effect of anti-cTnI concentration

The orientation of LC molecules is also closely connected with the concentration of assembled anti-cTnI. In order to obtain the optimal concentration of anti-cTnI, we explored several different concentrations of the anti-cTnI. As shown in Fig. 3, when the concentration of the anti-cTnI is 10μg/ml or higher, a bright large area was observed, indicating the alignment of LC molecules was disturbed to a large extent by the immobilized anti-cTnI. An almost black optical imagine was observed when the concentration was equal to or less than 1μg/ml, indicating that the LC molecules were arranged neatly. In order to provide as many active groups as possible on the slide substrate and increase the sensitivity of subsequent detection, the anti-cTnI concentration was selected as 1μg/ml.

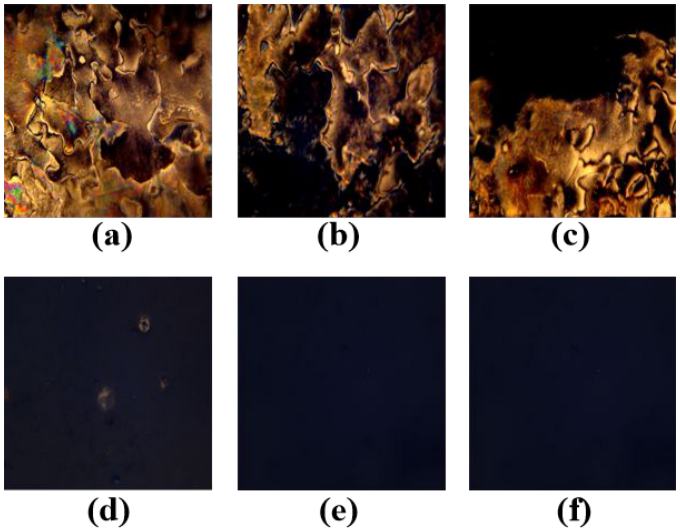

Fig.3. POM images of LC cells with 5CB after the immobilization with different concentrations of anti-cTnI: (a) 1000μg/ml; (b) 100μg/ml; (c) 10μg/ml; (d) 1μg/ml; (e) 0.1μg/ml; (f) 0.01μg/ml.

3.4. The detection of cTnI

Under the optimal experimental conditions, we examined the detection characteristics of cTnI in the LC biosensor. We initially used the one-sided sensing LC cell to detect cTnI. Specifically, the top slide was treated with only 0.5% DMOAP, and the bottom slide was treated to specifically bind the biomolecule to be detected. The results are shown in the Fig. 4. As the antigen cTnI concentration increases, the intensity and extent of the damage increase gradually, presenting a bright texture under POM. This result indicates that the antigen-antibody binding is stronger than the effect of the vertical surfactant DMOAP on the LC molecules. As the concentration gradually decreases to 0.1ng/ml, the bright area is already very small, and when the antigen concentration is further lowered, only some bright spots or even all-black images are observed. It indicates that too little antigen is bound, and it can not disturb the vertical orientation of the LC molecules.

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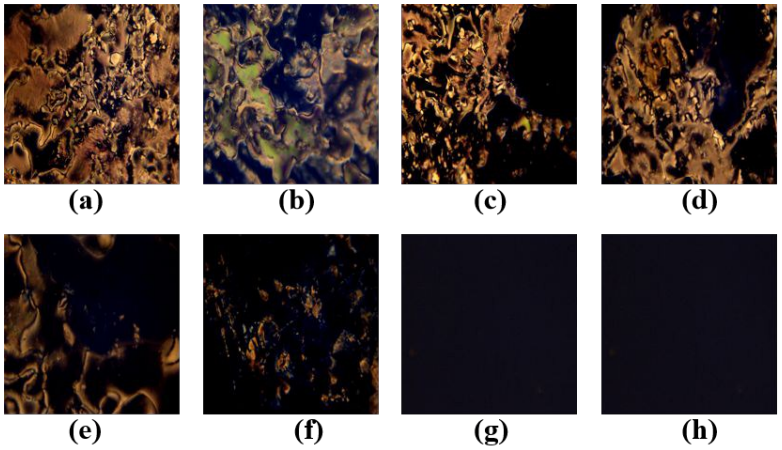

Fig. 4. POM images of LC cells with 5CB with different concentrations of cTnI through one-sided sensing LC cell: (a) 1000ng/ml; (b) 100ng/ml; (c) 10ng/ml; (d) 1ng/ml; (e) 0.1ng/ml; (f) 0.01ng/ml; (g) 0.001ng/ml; (h) 0.0001ng/ml.

For one-sided sensing LC cell, only one slide is utilized to bond specific molecule. We conjecture that double-sided sensing will be more efficient. Thus, we further explored the sensing of the double-sided liquid crystal cell. AS shown in Fig. 5, the addition of cTnI can largely change the optical response and orientation of LC. When the concentration is 0.001ng/ml, some bright spots are observed. When the concentration is higher, the brighter appearance of the LC cell can be observed. We found that the optical appearance of double-sided sensing is different from that of previous one-sided sensing LC cell sensor. On the one hand, the brightness of double-sided sensing is greater at the same concentration. On the other hand, the detection limit of double-sided is lower and the sensitivity is improved by at least one order of magnitude. Thus, we measured the gray value of the POM images through two sensing methods respectively and explored their respective relationship between the cTnI concentration and the gray level of its corresponding POM images. As clearly shown in the left graph of Fig. 5, as the concentration of cTnI increases, the average gray value of the images increases accordingly for these both methods. When it increases to a certain value, the gray value changes tend to gentle, this is because the bound antigen is already close to saturation value. Besides, as we expected, at the same concentration, its gray value is indeed much higher than the one-sided sensing LC cell detection method. Then, we take the logarithm of the cTnI concentration and find that it has a good linear relationship with the average gray level of the POM image and the linearity reaches 0.992 for double-sided sensing LC cell detection, as shown in the right graph of Fig. 5. However, the linearity reaches 0.962 for one-sided sensing LC cell detection. Through analysis, our proposed double-sided sensing LC cell is superior to the previous one-sided sensing LC cell. Moreover, Based on Fig. 5, the

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limit of detection (LOD) for cTnI was determined to be 1 pg/ml, which is competitive to the existing assays (Table 1).

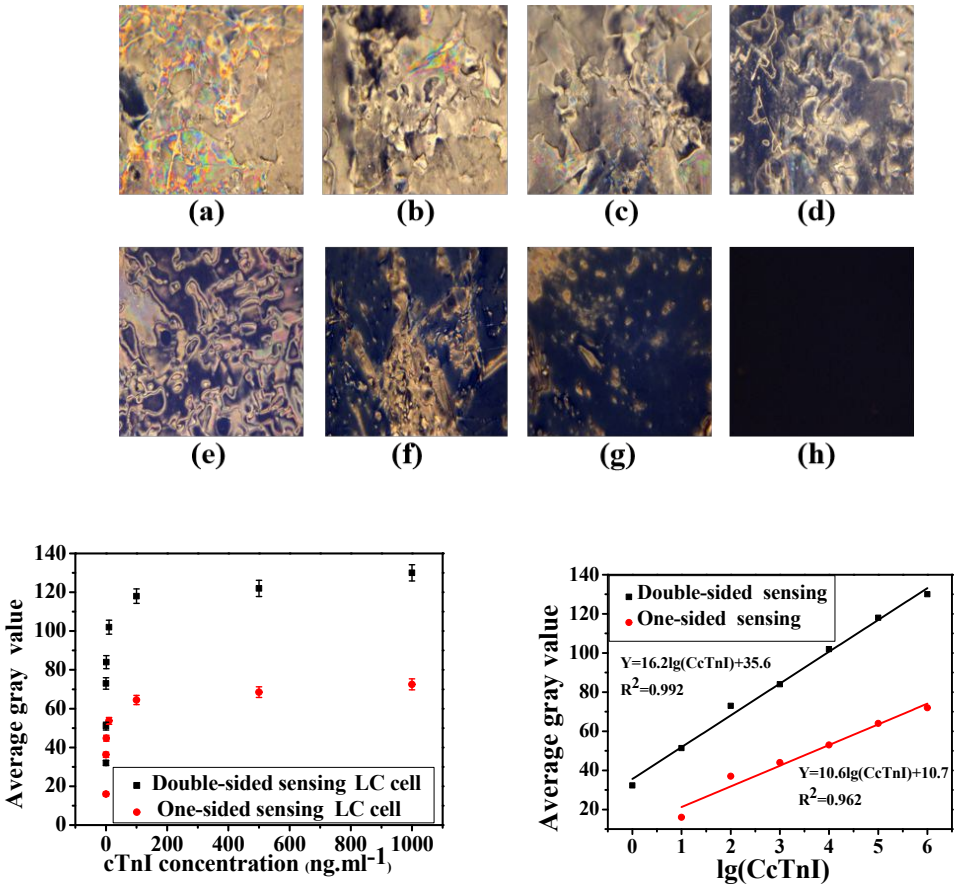

Fig. 5. POM images of LC cells with 5CB with different concentrations of cTnI through double-sided sensing: (a) 1000ng/ml; (b) 100ng/ml; (c) 10ng/ml; (d) 1ng/ml; (e) 0.1ng/ml; (f) 0.01ng/ml; (g) 0.001ng/ml; (h) 0.0001ng/ml. The graph on the left is the relational graph between the average gray value of the image and the cTnI concentration (10^1-10^6pg/ml for one-sided sensing LC cell method and 10^0-10^6pg/ml for double-sided sensing LC cell method), and the right one is a liner fitting between the logarithm of the cTnI concentration (10^1-10^6pg/ml for one-sided sensing LC cell method and 10^0-10^6pg/ml for double-sided sensing LC cell method) and the average gray level of the POM images.

Table 1. Comparison of the cTnI biosensors.

Detection method	Strategy	LOD	Ref.
ELISA	Cross-flow chromatography	0.027ng/ml	[17]
Colorimetry	Au nanoparticles composite	0.01ng/ml	[19]
Electrochemistry	Electrochemical impedance spectroscopy	0.11μg/ ml	[20]
Paramagnetic	Paramagnetic particle	0.5ng/ml	[21]
SPR	Resonant circuit	1.4ng/ml	[22]

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Phase-shifted microfiber Bragg grating	Notch signal	0.03ng/ml	[23]
Tungsten trioxide-reduced graphene oxide nanocomposite	Differential pulse voltammetry	0.01ng/ml	[24]
LC-based sensor	Polarizing microscopy	0.001ng/ml	This work

In order to confirm the LC molecular perturbation was caused by antibody antigen-specific binding, we performed a series of control experiments in the absence of antibodies, only the antigen was added in various concentration, as shown in Fig. 6, we observed almost all black image when cTnI concentration is 0.01mg/ml, and complete black background images are obtained when it's below that value. However, it turns out this concentration value is many times higher than the concentration we detected before, so the influence of antigen can be completely excluded and cTnI antigen cannot function alone at low concentrations when anti-cTnI wasn't added. Therefore, the change in the alignment of the LC molecules of LC cell is due to the specific binding of the anti-cTnI and cTnI.

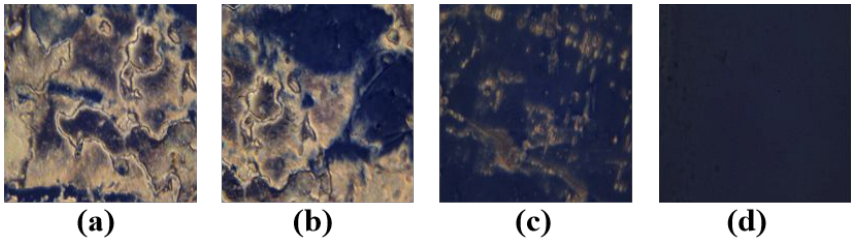

Fig. 6. POM images of LC cells with 5CB with different concentrations of cTnI without anti-cTnI: (a) 1mg/ml; (b) 0.1mg/ml; (c) 0.01mg/ml; (d) 0.001mg/ml.

3.5 Specificity

In addition to sensitivity, selectivity and specificity are also of great importance for detecting cTnI among various biomolecules in human plasma. The specificity of this LC-based immunosensor was assessed by measuring two-kinds of non-specific proteins IgG and ALB. These proteins and cTnI were diluted in the PBS with the same concentration of 1μg/ml. Then, they were incubated on the DMOAP/APTES/GA/anti-cTnI-coated slides, and a series of LC cells were prepared. From the experimental results illustrated in Fig. 7, the biosensor responds differently to other proteins in contrast with cTnI. The optical image appeared bright when cTnI was incubated on the slides (Fig. 7d). On the contrary, the optical image appears almost all-dark when other proteins were decorated on the slides (Fig. 7a-b). There are few bright spots in Fig. 7b due to the non-specific binding of the protein [32] or the tiny dust effect during the experiment. Meanwhile, we took PBS buffer solution as the control

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group (Fig. 7c) and found that PBS had almost no effect on the experimental result. These results indicate that no biological binding event occurred on the slides except for incubation with cTnI, demonstrating its high specificity.

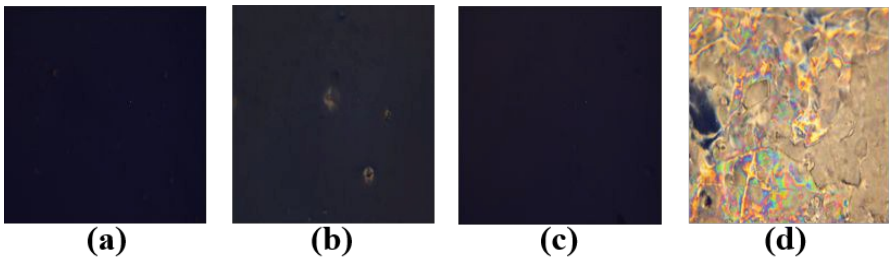

Fig. 7. POM images of LC cells with 5CB with different proteins: (a) 1μg/ml IgG; (b) 1μg/ml ALB; (c) PBS buffer; (d) 1μg/ml cTnI.

4. Conclusion

In conclusion, a novel LC immunosensor for detecting cTnI antigen based on the special birefringence effect of LC molecules was established in this paper. The sensor utilizes DMOAP/APTES mixed silylation reagent to self-assemble sensitive membranes to modify the glass substrate, which induces the LC molecules to be arranged in an ordered space. The specifically binding between cTnI antigen and anti-cTnI can destroy the ordered LC molecules alignment on the surface of the substrate, thereby causing the change of the POM images to realize the detection of cTnI. The minimum detection limit of the LC biosensor is 1pg/ml. Different from the one-sided sensing LC cell, we used the double sides of the LC cell for sensing, which improved the sensitivity and detection range of the sensor greatly. Besides, we found that the sensor showed a good selectivity by IgG and ALB. The method is simple in operation and high in sensitivity, and is expected to be popularized for rapid detection of other biomolecule.

Conflicts of interest

The author(s) declare that they have no competing interest.

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1. M. V. Westfall, E. M. Rust and J. M. Metzger, P. Natl. Acad., 1997, **94**, 5444-5449.
2. S. I. Yasuda, P. Coutu, S. Sadayappan, J. Robbins and J. M. Metzger, Circ. Res., 2007, **101**, 377-386.
3. K. M. Eggers, L. Lind, H. Ahlstrom, T. Bjerner, C. Ebeling Barbier, A. Larsson, P. Venge and B. Lindahl, Eur. Heart. J., 2008, **29**, 2252-2258.

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DOI: 10.1039/D0AN00425A

4. J. L. Kim, Circulation, 2002, **106**, 2366-2371.
5. P. A. Kavsak, A. R. Macrae, M. J. Yerna and A. S. Jaffe, Clin. Chem., 2009, **55**, 573-577.
6. S. Melanson, D. Morrow and P. Jarolim, Am. J, Clin. Pathol., 2007, **128**, 282-286.
7. R. Labugger, L. Organ, C. Collier, D. Atar and J. E. Van Eyk, Circulation, 2000, **102**, 1221-1226.
8. T. Keller, T. Zeller, F. Ojeda, S. Tzikas, L. Lillpopp, C. Sinning, P. Wild, S. Genth-Zotz, A. Warnholtz, E. Giannitsis, M. Möckel, C. Bickel, D. Peetz, K. Lackner, S. Baldus, T. Münzel and S. Blankenberg, Jama-j. Am. Med. Assoac., 2011, **306**, 2684-2693.
9. B. Zethelius, N. Johnston and P. Venge, Circulation, 2006, **113**, 1071-1078.
10. T. B. Horwich, J. Patel, W. R. Maclellan and G. C. Fonarow, Circulation, 2003, **108**, 833.
11. N. J. Mehta, I. A. Khan, V. Gupta, K. Jani, R. M. Gowda and P. R. Smith, Int. J. Cardiol, 2004, **95**, 0-17.
12. H. J. He, M. S. Lowenthal, K. D. Cole, D. Bunk and L. Wang, Clin. Chim. Acta., 2011, **412**, 0-111.
13. F. S. Apple and P. O. Collinson, Clin. Chem., 2012, **58**, 54-61.
14. F. S. Apple, L. A. Pearce, S. W. Smith, J. M. Kaczmarek and M. M. Murakami, Clin. Chem., 2009, **55**, 930-937.
15. Y. Li, X. Wang, L. Xu and X. Wen, Lab. Med., 2014, **45**, 82-85.
16. X. Ji, R. Takahashi, Y. Hiura, G. Hirokawa, Y. Fukushima and N. Iwai, Clin. Chem., 2009, **55**, 1944-1949.
17. I. H. Cho, E. H. Paek, Y. K. Kim, J. H. Kim and S. H. Paek, Anal. Chim. Acta., 2009, **632**, 247-255.
18. M. Imazio, B. Demichelis, E. Cecchi, R. Belli, A. Ghisio, M. Bobbio and R. Trinchero, J. Am. Coll. Cardio, 2003, **42**, 2144-2148.
19. W. Y. Wu, Z. P. Bian, W. Wang, W. Wang and J. J. Zhu, Sensor. Actuator. B Chem., 2010, **147**, 298–303.
20. J. Wu, D. M. Cropek, A. C. West and S. Banta, Anal. Chem., 2010, **82**, 8235-8243.
21. J. Kiely, P. Hawkins, P. Wraith and R. Luxton, Iet. Sci. Meas. Technol., 2007, **1**, 270-275.
22. J. F. Masson, L. Obando, S. Beaudoin and K. Booksh, Talanta, 2004, **62**, 865-870.
23. T. Liu, L. L. Liang, P. Xiao, L. P. Sun, Y. Y. Huang, Y. Ran, L. Jin and B. O. Guan, Biosens. Bioelectron., 2018, **100**, 155-160.
24. D. Sandil, S. Srivastava, B. D. Malhotra, S. C. Sharma and N. K. Puri, J. Alloy. Compd., 2018, **763**, 102-110.
25. A. Hussain, A. S. Pina and A. C. A. Roque, Biosens. Bioelectron., 2009, **25**, 1-8.
26. R. A. M. Hikmet and J. Lub, Prog. Polym. Sci., 1996, **21**, 1165-1209.
27. S. Liao, Y. Qiao, W. Han, Z. Xie, Z. Wu, G. Shen and R. Yu, Anal. Chem., 2012, **84**, 45-49.
28. J. Y. Du, Q. F. Jiang, X. C. Lu, L. C. Chen, Y. Zhang and X. L. Xiong, Analyst, 2019, **144**, 1761-1767.
29. Y. Wang, L. Zhao, A. Xu, L. Wang, L. Zhang, S. Liu, Y Liu and H. Li, Sensor. Actuator. B Chem., 2018, **258**, 1090-1098.
30. S. Yang, C. Wu, H. Tan, Y. Wu, S. Liao, Z. Wu, G. Shen and R. Yu, Anal. Chem., 2013, **85**, 14-18.
31. J. M. Brake and N. L. Abbott, Langmuir, 2007, **23**, 8497-8507.
32. Y. Wang, Q. Hu, T. Tian, Y. Gao, and L. Yu, Anal. Chim. Acta., 2016, **937**, 119-126.