Talanta 206 (2020) 120246

Contents lists available at ScienceDirect

Talanta

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

# Design of a liquid crystal-based aptasensing platform for ultrasensitive detection of tetracycline

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ARTICLE INFO

*Keywords:*
Liquid crystals
Biosensor
Aptamer
Tetracycline
Polarized light microscopy

ABSTRACT

We develop a novel label-free liquid crystal (LC) aptasensor based on intrinsic properties of nematic LCs for ultra-sensitive detection of tetracycline. The aptasensor is assembled by immobilizing aptamers onto the glass slide modified with both homeotropic alignment and silane coupling agents. Designed aptasensor makes use of the target-induced aptamer conformational switching and disruption of the orientation of LCs which lead to an obvious change of the optical appearance from a dark to a bright response. We describe the optimized condition for maintaining the homeotropic orientation of LCs, which are suitable for the tetracycline detection. The average gray-scale intensities of polarizing optical microscopy images were calculated to quantitatively detect tetracycline concentrations. The aptasensor works especially at trace level of tetracycline as low as 0.5 pM. Moreover, the LC aptasensor was successfully used to detect tetracycline in the real milk sample. According to the results, the proposed LC aptasensor for tetracycline detection is simple, ultra-sensitive, label free and ease of preparation.

## 1. Introduction

Tetracycline is a broad-spectrum antibiotic used in the therapy of human and animal. The overuse of tetracycline, an antibiotics and growth stimulants, in veterinary medicine can lead to their accumulation in animal-based food [1,2]. The residue of tetracycline in food can lead to unpleasant side effects in human and animal health, including the emergence of antibiotic resistance, allergic reactions, liver and dental damage [3,4]. Furthermore, antibiotics in milk cause the elimination of beneficial bacteria used in the production of cheese, yogurt and other fermentation products [5,6]. Therefore, in addition to creating a health problem, the presence of antibiotics in milk also reduces the quality and quantity of products produced.

Different analytical and bioanalytical methods such as spectrophotometry, chromatography, electrophoresis, microbiology and immunochemical techniques are employed to determine the tetracycline residues in foodstuffs, which are unusable in the dairy industry due to their expensive and time-consuming [7–11].

Recently, commercial kits are used in the dairy industry to detect tetracycline. In such kits specific antibodies are usually used to detect the residue of antibiotics in raw milk [12,13]. Although antibodies are very sensitive and selective, their stability and expiration date are very short and their production and screening process are hard and expensive especially for small targets [14–16]. To resolve the limitations, aptamer-based biosensors have been developed as the promising alternatives, in which aptamers are used as the intelligent recognition elements [17]. Aptamer or synthetic antibodies are oligonucleotides which can recognize a wide range of contaminants, such as antibiotics, toxins, heavy metal ions, drug residues, microbial cells, and so on, owing to their special three-dimensional structure [18,19]. Compared to antibody-based biosensors, aptasensor have outstanding features, such as higher stability, reusability, low immunogenicity and more affordable. These features make the aptamers into unobtrusive recognition elements for the design of biosensors [20].

The label-free techniques has been greatly advanced by the combination of sensors with nanomaterial sciences [21,22]. These rapidly advancing sensors aim to provide data without the intervention of label molecules. The liquid crystal (LC) aptasensors have received substantial attention in the last few decades as new candidates of label-free optical biosensors due to low cost, rapid detection, wide response range, and facile operation [23,24]. LCs are soft matters with fluidity and long-range order in orientation of the molecules; and very low anchoring energy which make them extremely sensitive to external stimuli [25,26]. These responsive materials based on change of orientation LC molecules, amplify and transduce the binding of aptamer and target into optical signal visible by the polarized light microscope. This

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https://doi.org/10.1016/j.talanta.2019.120246
Received 2 June 2019; Received in revised form 4 August 2019; Accepted 8 August 2019
Available online 12 August 2019
0039-9140/ 

stimuli-responsive biosensors, allowing events that occur on the nanoscale to be observed easily even by the naked eye and can be portable, avoiding the complex and expensive laboratory-based equipment. In addition, LC-biosensors are label-free, making them an ideal candidate for the optical visualization and detection of many analytes [27–29]. It has been shown that this new generation of biosensors can be portable through a smartphone and are promise device for the practical application in industrial world and people's daily life, and will probably to be very popular in near future [30]. Only one commercialization of LC-based sensor for toxic gases was developed till now [31]. Recently, new trends were used to detection of some targets with LC-based aptasensor [32,33]. The combination of LC-based sensors technology and aptamer science has opened a new door in high sensitivity, label-free and real time, point of care (POC) biosensors.

In this study, we have designed and fabricated a new and label-free LC-based aptasensor for tetracycline. In the developed biosensor, aptamer was immobilized as the recognize element on a modified glass substrate via an amide chemical-bond. The binding of aptamer and tetracycline can dramatically disrupt the orientation of LCs bar-shape molecules, resulted in visible color images. The designed aptasensing test platform is a promising candidate for the tetracycline screening in the dairy industry.

## 2. Materials and methods

### 2.1. Materials and reagents

The truncated HPLC purified DNA aptamer which successfully recognized four different tetracyclines with high affinity were purchased from Bioneer, South Korea [34,35]. The following sequence of DNA aptamer: 5′-$NH_2$-$(CH_2)_6$-GAG AGA CGG TGG TG-3′ was dissolved in a Tris-HCl buffer (pH 7.5) containing 10 mM NaCl and 5 mM $MgCl_2$. The concentrations of aptamer is determined from the nearest-neighbor model [36]. Homeotropic alignment agent N,N-dimethyl-N-(3 (trimethoxysilyl) propyl)-1 octadecanaminiuchloride (DMOAP), (3-aminopropyl)triethoxysilane (APTES) as silane coupling agent, and 4′-Pentyl-4-biphenylcarbonitrile (5CB) nematic liquid crystal (TNI = 37 °C) were purchased from Sigma-Aldrich. HDA standard disposable glass slides were obtained from China. Copper grids (150 meshes, 20 μm in thickness) and tetracycline, oxytetracycline, doxytetracycline, cefixime, carbamazepine, fluoxetine hydrochloride, benzyl butyl phthalate (BBP) and dioctyl phthalate (DOP) were provided by Sigma. Glutaraldehyde solution (GA) and glycine were purchased from Merck. Other chemicals such as acetonitrile, ethanol, methanol, hydrogen peroxide, sulfuric acid and sodium dodecyl sulfate (SDS) were of analytical grade and obtained from commercially available suppliers, applied without the further purification.

### 2.2. Apparatus

Polarizing optical microscopy (POM) images were taken using an Olympus BH2-UMA polarizing optical microscope equipped with a crossed polarizer in a transmission mode. The images were obtained using a digital camera (KECAM). The morphology of substrates was characterized using the atomic force microscope (JPK-NanoWizard II).

### 2.3. Cleaning of glass substrates

First, the glass slides were cut into the size of 2 cm × 2 cm pieces, and then were incubated in fresh prepared piranha acid mixing (30% $H_2O_2$ and 70% $H_2SO_4$) about 2 h at 80 °C to remove all organic contaminants [*warning: piranha solution reacts heatedly with organic chemicals and should be worked with excessive caution; do not be kept it in closed containers!*]. The cleaned glass substrates were rinsed with HPLC-grade water and ethanol several times, then dried with nitrogen stream, and it placed in a 110 °C oven about 3 h before modification.

### 2.4. Modification of glass substrate

#### 2.4.1. Modification with DMOAP

Cleaned glass slides were submerged in an aqueous solution of 0.35% (v/v) DMOAP for 30 min at room temperature and then rinsed four times with ethanol and HPLC-grade water respectively. At last, The DMOAP-modified substrate were dried via nitrogen and baked in an 110 °C oven for an hour.

#### 2.4.2. Modification with DMOAP/APTES

Half of cleaned glass slides modified with both DMOAP and APTES. They were submerged in ethanol solution containing 1.5% (v/v) APTES and 0.3% (v/v) DMOAP at 80 °C for 2 h, then rinsed with ethanol and HPLC-grade water, respectively and baked at 110 °C for 1 h after drying under nitrogen stream.

### 2.5. Functionalization of the substrate

DMOAP/APTES modified slides were immersed in 0.5% (v/v) GA aqueous solution for 45 min at room temperature and rinsed with HPLC-grade water, then dried via nitrogen gas, glass/DMOAP/APTES/GA as lower substrate.

### 2.6. Immobilization of aptamer

The stock aptamer solution was first diluted with a Tris-HCl buffer (pH 7.5) containing 10 mM NaCl and 5 mM $MgCl_2$, then 200 μL from aptamer 100 nM was dropped onto the surface of the glass/DMOAP/APTES/GA substrate and incubated 2 h at 37 °C. After incubation, the substrate rinsed with a solution containing of 0.3 M sodium chloride/0.03 M trisodium citrate containing SDS 0.01% (v/v) and HPLC-grade water to remove the unbonded aptamers. Finally, in order to block any residual aldehyde groups, the glass/DMOAP/APTES/GA/aptamer substrates were treated with glycine solution (80 mM) for 1 h and after rinsed with HPLC-grade water, dried with nitrogen stream.

### 2.7. The fabrication process of the LC-based aptasensing platform

The copper grids (150 meshes) were first placed onto the center of glass/DMOAP/APTES/GA/aptamer substrate. After that, 2 μL of 5CB (were heated > 40 °C in advance) was spotted onto the meshes of the copper grid and the excess of LCs collected with a clean capillary tube. Then, the upper slide was put onto assembled biosensor and fixed by two binder clips. The LC biosensor was assembled by sandwiching of aptamer and LCs between two glass slides.

### 2.8. Assay procedure for the detection of tetracycline

200 μL solutions contain the series of gradient concentration of tetracycline were dipped on glass/DMOAP/APTES/GA/aptamer substrate and were incubated for 2 h at room temperature, then rinsed with HPLC-grade water to remove nonspecific adsorbed molecules. Finally, it was dried under a stream of nitrogen. The copper grids were placed on to the modified substrate and filled by 5CB. It was sandwiched with the upper substrate, glass/DMOAP, and secured with three binder clips.

### 2.9. Selectivity of the LC-based aptasensing test kit

The selectivity was analyzed in the presence of oxytetracycline, doxytetracycline, cefixime, carbamazepine, fluoxetine hydrochloride, benzyl butyl phthalate and dioctyl phthalate using the POM image analysis. Data are means ± SD, n = 4.

### 2.10. The real sample analysis

To investigate the application of the fabricated aptasensors in milk

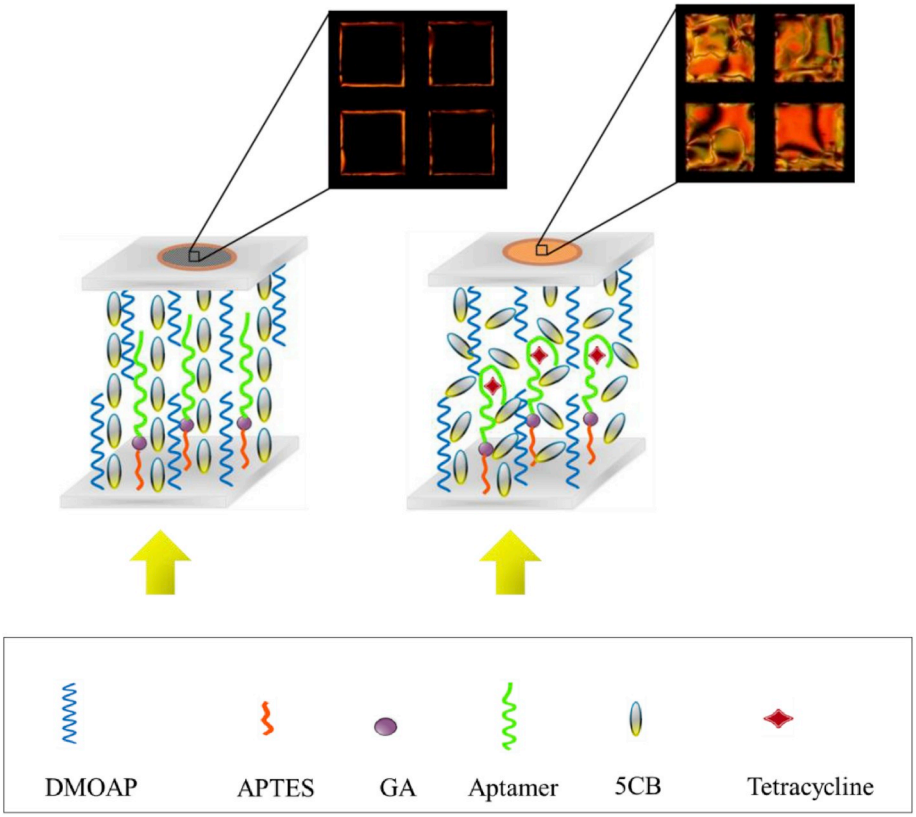


**Fig. 1.** Schematic representation of aptamer-based LC biosensor for tetracycline. In the presence of tetracycline, target binds to the aptamer immobilized on the substrate and considerably disrupts the homeotropic orientation of LC molecules.

samples, milk with a fat content of 3.2% (w/v) was provided from a local market. Acetonitrile was used to eliminate the matrix effect of interference proteins. The cold acetonitrile and diluted milk sample (3:1) were gently mixed and incubated for 1 h at refrigerator. The samples were centrifuged at 9500 g for 15 min, and the upper supernatant stacked up. Then, the increasing concentrations of tetracycline (5-200 pM) were spiked to diluted milk and its recovered concentrations were recorded.

## 3. Results and discussions

### 3.1. Sensing strategy of the designed LC-based aptasensor

Fig. 1 schematically illustrates the sensing strategy of the offered LCs-based aptasensor. The lower substrate, glass/DMOAP/APTES/GA, possesses active aldehyde groups for amine-modified aptamer binding. The DMOAP molecules as the orientation agents induced homeotropic orientation of LCs molecules on the slide surface. An appropriate ratio of the DMOAP/APTES/GA and the amounts of immobilized aptamers on the substrate can induce homeotropic orientation to the LC molecules. Also, with the synergistic effect of the DMOAP orientation agents on upper substrate, a dark polarized light image appears. However, in the presence of tetracycline, target binds to the aptamer immobilized on the substrate and considerably disrupts the homeotropic orientation of LC molecules. Therefore, an optical appearance transition of polarized image from dark to the bright image observed.

### 3.2. Optimization of the detection conditions

#### 3.2.1. Optimization of the APTES/DMOAP (v/v) ratio

In previous studies, the orientation of LCs was changed by varying the chemical compositions of the substrates [37,38]. To avoid the background interference and attain to the best sensing efficiency, the ratio of the APTES to DMOAP immobilized on sensor substrates was optimized. APTES offer amino groups for immobilization of the aptamers with GA crosslinks, but an inappropriate ratio can disrupt the LC molecules orientation. Therefore, we studied the effect of different DMOAP/APTES volume ratio on output signals. The image analysis show with the decline of APTES/DMOAP ratio, the number of colored pixels decrease and it becomes a uniform dark image when the APTES/DMOAP ratio reaches to 5:1 (Fig. 2). The mean brightness values corresponding to the POM images shows in Fig. S1. As a result, the APTES/DMOAP ratio of 5:1 is used in the all experiment.

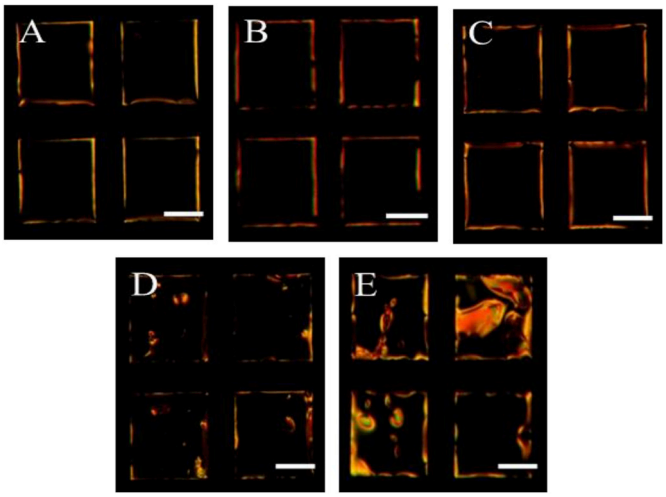


**Fig. 2.** POM images of LC aptasensor cells with different APTES/DMOAP volume ratio: (A) 1:1; (B) 2:1; (C) 5:1; (D) 10:1; (E) 20:1. Scale bar: 100 μm.

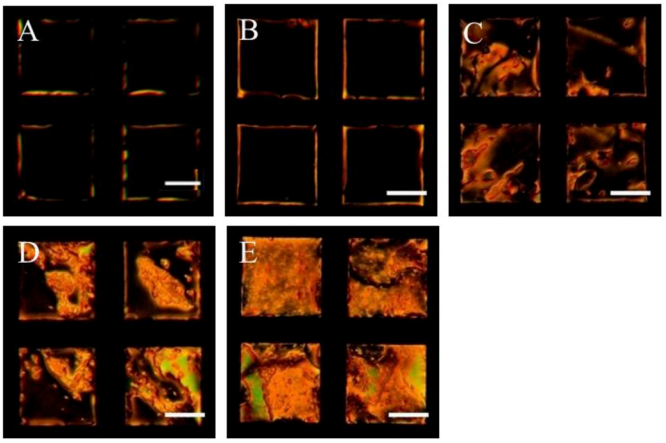


**Fig. 3.** POM images of LC aptasensor cells with different percent of GA: (A) 0.25% (B) 0.5%; (C) 1%; (D) 2%; (E) 5%. Scale bar: 100 μm.

### 3.2.2. Optimization of the GA

The GA concentration also should be optimized. Where, the GA concentration was more than optimal, a cross-linking reaction happened between the APTES molecules, which block the binding active sites [39]. Therefore, to ensure the best sensor performance, the concentration of GA as cross-linking agent was further optimized. As shown in Fig. 3 a uniform dark background was optimized by 0.5% GA. The mean brightness values corresponding to the POM images shows in Fig. S2.

### 3.2.3. Optimization of the aptamer

Similarly, since the concentration of the tetracycline aptamer was influence the surface density and homeotropic orientation of LCs, thereby the appropriate amount of aptamer was optimized.

As shown in Fig. 4, with the decline of the aptamer concentration from 300 nM to 50 nM, the numbers of bright spots decreased and the uniform dark background of the LC cells achieved when the concentrations of the tetracycline aptamer were less than 100 nM. Based on these results, we select the concentration of 100 nM as the optimal aptamer concentration for the all experiments to ensure adequate quantities of aptamers to immobilize on the substrate.

### 3.3. The target detection with applying the designed aptasensing platform

After determining the optimal conditions, we confirmed the feasibility of the presented aptasensor for detection of target with incubation of the series concentrations of tetracycline with LC prepared cells. The resultant polarizing optical microscopy images are shown in Fig. 5. The mean brightness values corresponding to the POM images shows in Fig. S3.

The bright areas in the images become larger with the concentrations of tetracycline increasing from 0.1 to 500 pM, confirms that the binding of tetracycline to aptamer on the substrate is capable of disturbing the homeotropic orientation of LC molecules. The atomic force microscope (AFM) images confirmed this conclusion. The comparison of the surface topography after and before adding tetracycline shows the great changes of the topography happened on the surface a negligible after binding with tetracycline (Fig. 6). When the concentration of target reaches 0.1 pM, the optical image become almost dark which shows that such concentration of tetracycline is insufficient to disrupt the orientation of LCs molecules. So, the sensitivity of this aptasensor negligible detection is as low as 0.5 pM.

To further quantitatively analyze of the aptasensor performance, we evaluated the correlations between the tetracycline concentrations and the average gray-scale intensities (GIs) of POM images using ImageJ software (NIH Freeware). As shown in Fig. 7, the calibration curve showed a linear relationship between GIs value of the POM image and the logarithm of tetracycline concentration in the range from 0.5 to 500 pM and the detection limit of tetracycline in our study is about 0.5 pM.

It is worthwhile noted that the proposed LC aptasensor could quantitatively detect tetracycline with an ultra-low detection limit. Compared with other reported aptasensors for the detection of the trace level of tetracycline, our assay exhibits relative high sensitivity with an excellent performance as shown in Table 1.

### 3.4. Selectivity

The specificity of the LC-based aptasensor was investigated by comparing the POM images induced by tetracycline and several counter targets, including oxytetracycline, doxytetracycline, cefixime, carbamazepine, fluoxetine hydrochloride, benzyl butyl phthalate and dioctyl phthalate under the same experimental status, as described above. Fig. 8 indicates that the significant bright areas in the polarized image of LCs could only be observed by tetracyclines (tetracycline, oxytetracycline, and doxycycline). But the POM images of LCs for other control groups were dark. The weak bright areas in Fig. 8 may cause by

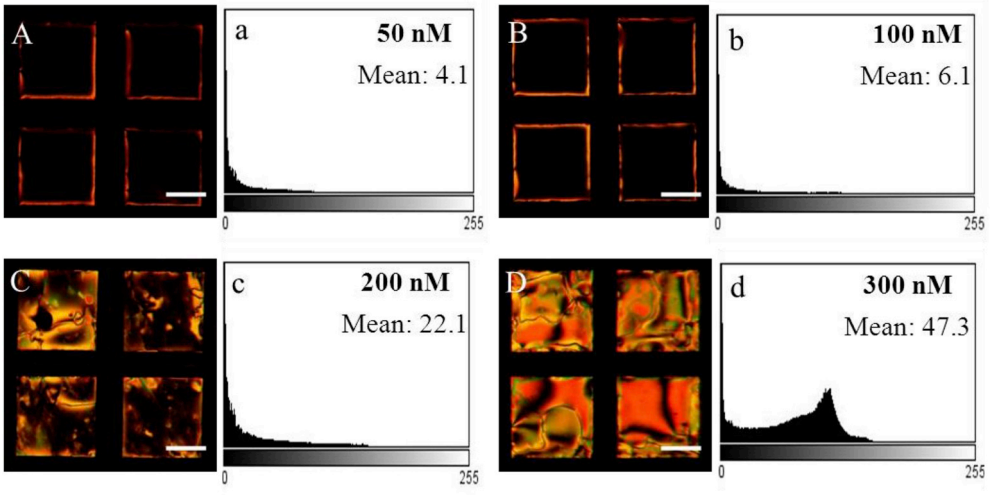


**Fig. 4.** POM images of LC aptasensor cells with different concentration of aptamer:(A) 50 nM; (B) 100 nM; (C) 200 nM; (D) 300 nM; and the different mean brightness values corresponding to the different polarized images. Scale bar: 100 μm.

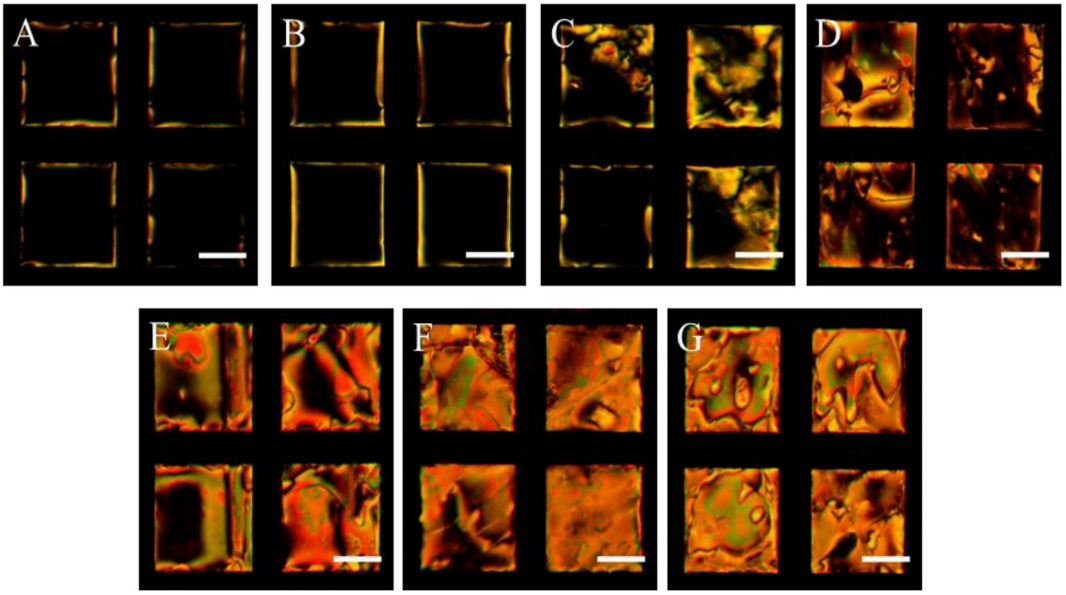


**Fig. 5.** POM images of LC aptasensor cells with tetracycline at the concentration of: (A) 0.1 pM; (B) 0.5 pM; (C) 5 pM; (D) 10 pM; (E) 100 pM; (F) 250 pM; (G) 500 pM. Scale bar: 100 µm.

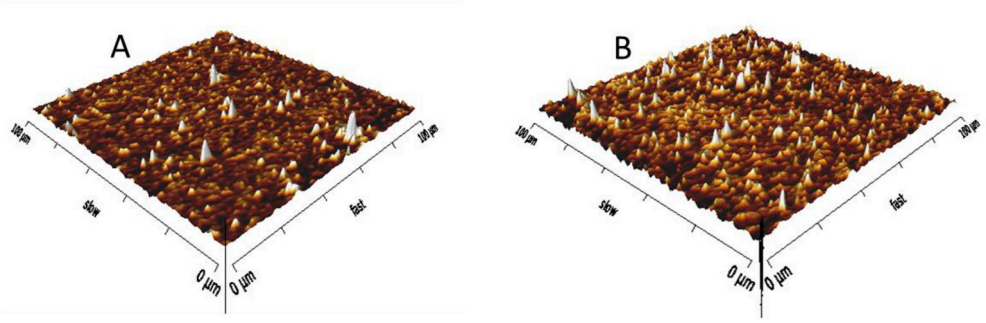


**Fig. 6.** (A) AFM images of surface modified with 100 nM aptamer; (B) AFM images of substrate modified with compounds induced by interaction between tetracycline (100 pM) and aptamer (100 nM).

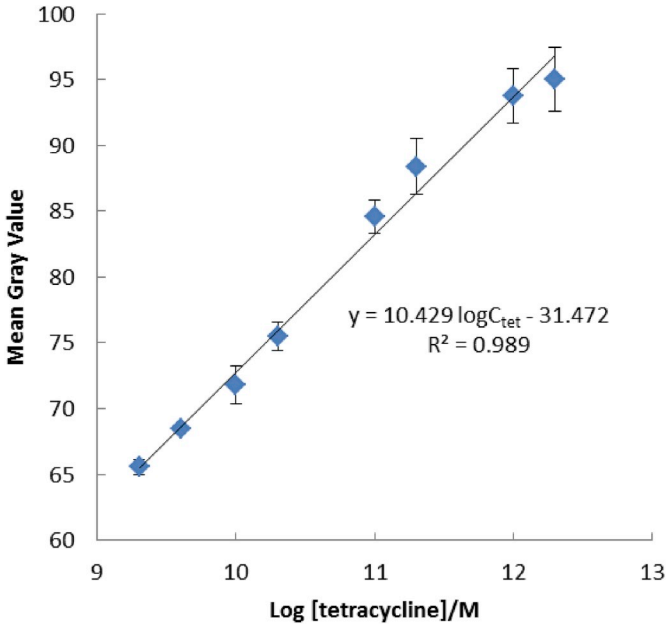


**Fig. 7.** The linear calibration curve of the average gray value of the POM image and the logarithm of the tetracycline concentration (n = 3).

the unbound molecules which could not washed away completely. The mean brightness values corresponding to the POM images shows in Fig. S4. Therefore, the developed LC aptasensor provided sensible selectivity for tetracyclines.

### 3.5. Real sample analysis

To assess the reliability and applicability of the LC-based aptasensor in practical analysis, the sensing platform was applied to analyze tetracycline in milk samples. The results are presented in Table 2 that indicates the recoveries of the spiked samples were between 95.6% and 108.7%. Hence, the proposed LC aptasensing platform can have an efficient applicability in milk analysis with high precision and accuracy.

## 4. Conclusions

Antibiotics are widely overused and cause the progress of antibiotic-resistant bacteria, allergic reactions, and different side effects on human health. Therefore, the ultra-sensitive assays for detection of tetracyclines are great interest. In this work, we have developed an ultra-sensitive aptasensor down to 0.5 pM, rely on the change in alignment and optical properties of the LCs for tetracyclines detection. The developed LC aptasensor was applied for the tetracycline determination in milk samples with satisfying results. Such advanced novel aptasensors hold great promise of overcoming many challenges because LCs

**Table 1**
Comparision of the presented LC aptasensor with other reported tetracycline aptasensors.

| Ref. | Detection sample | Linear range | LOD | Strategy | Method |
|---|---|---|---|---|---|
| [40] | Milk and pork | 0.02–225 nM | 14 pM | Using a novel upconversion | Fluorescence |
| [35] | Serum and milk | 0.3–10 nM | 266 pM | Triple-helix molecular switch | Colorimetric |
| [41] | Milk | 0.45–4.5 μM | 87.7 nM | Cysteamine-stabilized gold nanoparticles | Colorimetric |
| [42] | Honey | 0.225 nM-2.25 μM | 0.22 nM | Direct competitive assay-based | DC-ELAA |
| [43] | Milk and serum | 1.5 nM-3.5 mM | 450 pM | Disassembly of M-shape structure | Electrochemical |
| [44] | Milk | 10–50000 nM | 5 nM | Multi-walled carbon nanotubes amplification | Electrochemical |
| [45] | Not reported | 1 nM-5 μM | 0.6 nM | Electrode modification with an alginate film containing reduced graphene oxide and magnetitenanoparticles | Electrochemical |
| [46] | Drug | 0.45 nM- 2.25 μM | 0.022 nM | Cerium doped CdS sensitized $BiYWO_6$ | Photoelectrochemical |
| [47] | Environmental Water | 10–250 nM | 5.3 nM | Graphitic Carbon Nitride Sensitized with CdS Quantum Dots | Photoelectrochemical |
| [48] | Milk | 2.25 pM-225 nM | 2.25 pM | Magnetite/PMAA nanospheres | Surface-enhanced Raman scattering (SERS) |
| Reported here | Milk | 0.5–500 pM | 0.5 pM | Target induced aptamer switching and disruption of the orientation of LC | LC-based aptasensor |

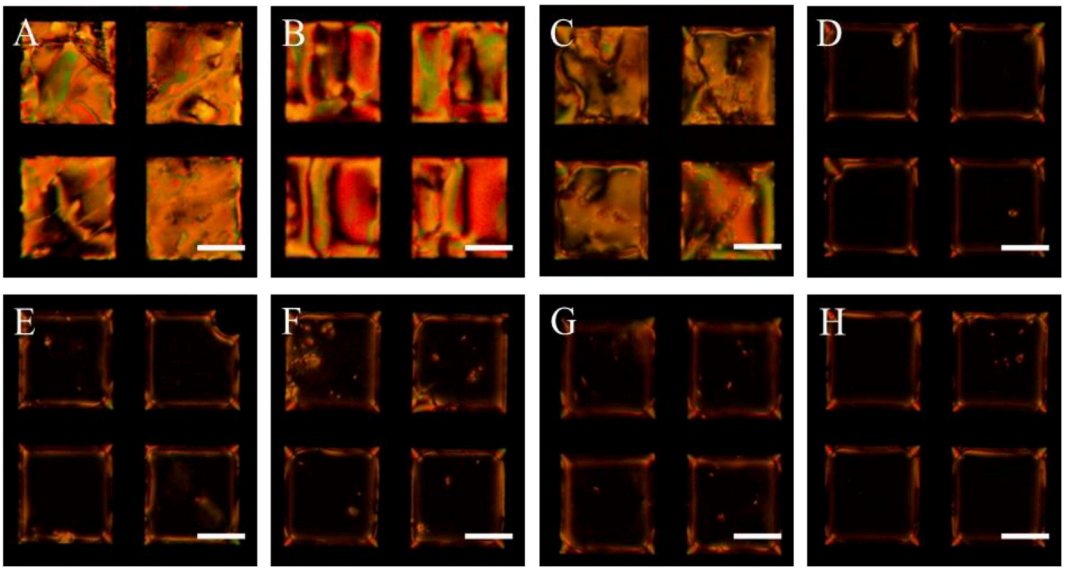


**Fig. 8.** POM images of LC aptasensor cells in the presence of different molecules: (A) tetracycline; (B) oxytetracycline; (C) doxytetracycline; (D) carbazpine; (E) fluxetine; (F) BBP; (G) cefixine; (H) DOP. Scale bar: 100 μm.

**Table 2**
Recovery of tetracycline from milk samples (n = 4). Data are mean ± standard deviation (SD).

| RSD (%, n = 4) | Recovery (%) | Found (pM) | Added Tetracycline (pM) | Milk samples |
|---|---|---|---|---|
| 2.9 | 95.7 | 4.8 | 5 | 1 |
| 5.3 | 95.6 | 24 | 25 | 2 |
| 7.3 | 108.5 | 54 | 50 | 3 |
| 4.7 | 108.7 | 109 | 100 | 4 |
| 5.3 | 98 | 190 | 200 | 5 |

biosensors are ultra-sensitive, easy-to-operate, and portability. These findings could contribute to improving the design of commercial tetracycline-kits in the future.

## Conflicts of interest

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

## Acknowledgement

This work was financed by the Research Institute of Food Science and Technology (RIFST), Mashhad, Iran, as a project (No. 26097003).

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2019.120246.

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