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# Direct and ultrasensitive optofluidic-based immunosensing assay of aflatoxin $M_1$ in dairy products using organic solvent extraction

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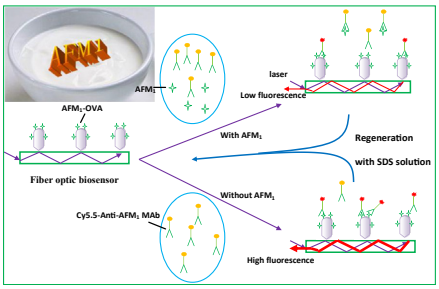
## HIGHLIGHTS

- A simple, low-cost, and reusable optofluidic platform was developed for direct and ultrasensitive immunoassay of $AFM_1$.
- Effect of organic solvents on interaction of antibody-antigen in heterogeneous and homogeneous solutions was evaluated.
- $AFM_1$ can be directly quantified by the proposed platform even if the samples contain a certain organic solvent.
- This method provides the LOD of 5 ng/L for $AFM_1$ in dairy products.
- This method provides a tool for the rapid, accurate, and reliable detection of $AFM_1$ with a simple sample pretreatment.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Aflatoxin $M_1$ ($AFM_1$), a highly toxic secondary metabolite, is present in a wide range of dairy products. In this study, we designed a simple, low-cost, reusable, and easy-to-operate immunosensing method for ultrasensitive detection of $AFM_1$ in dairy products by using a portable evanescent wave-based optofluidic biosensing platform (EOBP). The developed method provides the minimum detection limit of 5 ng/L, which is below the most restrictive standard imposed by the current regulations for $AFM_1$ in dairy products. The effect of several organic solvents, such as methanol, acetone, and acetonitrile, on the binding reaction of antibody-antigen in heterogeneous and homogeneous solutions was evaluated. Although the effect of organic solvents on the homogeneous binding reaction between antibody and antigen is more significant than that of heterogeneous binding reaction between antibody in solution and antigen immobilized onto the sensor surface, the fluorescence signal detected by EOBP is linearly dependent on $AFM_1$ concentration. Therefore, $AFM_1$ can be directly quantified even if the samples contain a certain organic solvent concentration. The robustness and stability of $AFM_1$-ovalbumin conjugate allow the regeneration of modified biosensor surface for more than 200 times, thereby achieving a cost-effective and reliable $AFM_1$ determination. The proposed method provides a rapid, ultrasensitive, and reliable $AFM_1$ determination in dairy products without complicated sample pretreatment process.



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## 1. Introduction

Aflatoxins, which occur naturally in a wide variety of agricultural products, are highly toxic, mutagenic, carcinogenic, and teratogenic mycotoxins [1]. Aflatoxin $B_1$ ($AFB_1$), one of the most widespread aflatoxins, is first class human carcinogen [2–4]. Aflatoxin $M_1$ ($AFM_1$), the hydroxylated metabolite of $AFB_1$, can be found in milk, urine, blood, and internal organs after ingestion of $AFB_1$-contaminated feed by animals [5]. Consumption of milk and milk products by human beings is relatively high, particularly among infants and children, thereby increasing the risk of exposure to $AFM_1$ [6,7]. The maximum residual levels of $AFM_1$ in milk are established at 0.5 μg/kg in China and USA, and 0.05 μg/kg in the European Union (EU) because of its potential hepatotoxicity and carcinogenicity [8–10]. The most restrictive levels are established for baby food in the EU at 0.025 μg/kg for $AFM_1$ [8–10]. The adoption of regulatory limits requires the development of validated analytical methods for rapid and cost-effective screening of $AFM_1$, specifically for dairy farms and milk industries.

To satisfy the regulatory levels and ensure the quality of dairy products and therefore, human health, many analytical methods and strategies for $AFM_1$ have been developed, such as HPLC, HPLC-MS, thin-layer chromatography (TLC), ELISA, and various biosensors [11,12]. However, the chromatographic methods not only require expensive equipment and trained personnel, but also they are time/labor-consuming, thus limiting their practical application. With the emerging trend of on-site $AFM_1$ detection, the sample pretreatment process of dairy products should be simplified. The current sample preparation of dairy products, which includes extraction into organic solvent, solvent evaporation, partitioning of the reconstituted residue with hexane, and subsequent $AFM_1$ quantification using HPLC or HPLC-MS, is rather complicated [11]. Therefore, the chromatographic methods could not be suitable for the on-site $AFM_1$ determination because dairy farms and milk industries require real-time and cost-effective screening of $AFM_1$ in dairy products. The TLC has poor sensitivity with obvious cross-reactivity from other pollutions with resembling structure [11]. Although ELISA is simple, highly sensitive, and suitable for batch tests, it needs relatively large amounts of reagents and long assay time (>2 h) [11]. Several immunosensors based on fluorescence, electrochemistry, and nanomaterials have been developed for $AFM_1$ detection [13–16]. The need to develop rapid, sensitive, cost-effective, and suitable on-site methods for $AFM_1$ detection has been highlighted to protect consumers from adverse effects [17].

As an emerging advanced assay method, immunosensors have attracted considerable attention for rapid and sensitive detection of trace targets in a complicated matrix. When immunosensors are applied for the detection of $AFM_1$ in dairy products, the pretreatment process using organic solvent extraction is necessary because the dairy products are generally highly viscous or solid. In the detection of $AFM_1$ in dairy products, the following questions need to be addressed. 1) How high organic solvent concentration affects the antibody-antigen binding performance? 2) How does the organic solvent change the detection ability of the immunosensor? Can the $AFM_1$ extracted from the samples using organic solvent be directly detected using immunosensor without complicated pretreatment process? To address these questions, a portable miniaturized evanescent wave-based optofluidic biosensing platform (EOBP) developed by our research group was used to detect $AFM_1$ in dairy products [18,19]. The EOBP demonstrates several advantages in detection of trace targets, such as simplification, rapidness, reliability, ease of use, and capability for on-site detection. Several organic solvents were used to extract $AFM_1$ in dairy products and their extraction effects were compared. The effect of several organic solvents on the immunoassay was also evaluated. Three commercially available dairy products, namely, liquid milk, cheese, and milk tea, were detected by the portable EOBP using organic solvent extraction.

## 2. Experimental sections

### 2.1. Materials and chemicals

Glutaraldehyde (GA), bovine serum albumin (BSA), $AFM_1$, (3-Aminopropyl)-trimethoxysilane (APTES), and N.N′-Disuccinimidylcarbonate (DSC) were purchased from Sigma-Aldrich (China). Monoclonal anti-$AFM_1$ antibody (Guo et al., 2016) and $AFM_1$-ovalbumin ($AFM_1$-OVA) were purchased from Shijiazhuang Solarpex Biotechnology Co., Ltd. The estimated number of hapten molecules attached to the carrier protein (hapten-to-protein molar ratio, MR) OVA determined by MALDI-TOF MS is 5. Methylbenzene, $H_2SO_4$, $H_2O_2$, methanol, acetone, acetonitrile, and all other reagents, unless specified, were supplied by Beijing Chemical Agents (China). 0.5% sodium dodecyl sulfate (SDS) solution (pH = 1.9) was used for biosensor regeneration. 0.01 M phosphate buffer solution (PBS, pH = 7.4) was used to prepare various solutions. 10 μg/mL of $AFM_1$ stock solution was serially diluted by PBS to produce standard solutions.

Anti-bisphenol A (BPA) antibody and anti-microcystin-LR (MC-LR) antibody were prepared by our group [18,21]. All antibodies were labeled by Cy5.5 as previously described [16,17].

### 2.2. Instrument: portable evanescent wave-based optofluidic biosensing platform

A portable EOBP was used for the rapid and sensitive immunoassay of $AFM_1$ in dairy products [19]. The pulse diode laser (635 nm, 10 mW) (HuayuanStar Ltd., China) with pigtail was used to excite the fluorescence molecule labeled on antibodies. A poly (methyl methacrylate)-based optofluidic cell with a channel length of 30 mm and a diameter of 500 μm (Suzhou Wenhao Chip Technology Co., Ltd., China) was used for $AFM_1$ immunoassay. The optical fiber biosensor modified by $AFM_1$-OVA was embedded into the optofluidic cell. A single-multi silica fiber optic coupler, combining a single mode fiber optic and a multi-mode fiber with a diameter of 600 μm (NA = 0.22), facilitated the transmission of the excited light as well as the collection and transmission of fluorescence. The pigtail of the diode laser was connected with the single mode fiber optic of the single-multi fiber optic coupler using a fiber optic connector. The laser beam was directly launched into the single multi-mode fiber optic coupler, and then entered the fiber optic biosensor, which linked to the multi-mode fiber optic coupler. When the fluorescence-labeled antibodies were excited and some of the fluorescence coupled into the fiber optic biosensor, the fluorescence was collected by the multi-mode fiber of the single-multi mode fiber optic coupler. The collected fluorescence was filtered through a bandpass filter (FF01-710/40, Semrock, US) and detected with a photodiode. The photodiode converted the fluorescence signal into an electric signal, which was amplified and converted to digital signals with a digital lock-in amplifier. A user-friendly interface on-time displays the fluorescence signal value, and the binding kinetic process between fluorescence-labeled antibody and antigen immobilized onto the biosensor surface can be obtained, which benefits for better studying the immunochemical interaction and the effect of organic solvents [21,22].

### 2.3. Immobilization of $AFM_1$-OVA onto the surface of fiber optic biosensor

To achieve the detection of $AFM_1$, $AFM_1$-OVA conjugate, served

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as bio-recognition molecules, were modified onto the surface of the fiber optic biosensor using the glutaraldehyde-covalent-coupling strategy as previous described (Details shown in Fig. S1) [19,22].

### 2.4. Immunoassay of $AFM_1$ using EOBP

Indirect competitive immunoassay was applied for $AFM_1$ determination (Fig. 1). $AFM_1$ standard solutions within the range of 0.01–100 μg/L were prepared from the stock solution using PBS buffer. For the inhibition assays, 200 μL of fluorescence-labeled anti-$AFM_1$ antibodies with a fixed concentration (0.5, 1, or 3 μg/mL) were mixed with 200 μL of different concentrations of $AFM_1$ solutions. The actual concentration of antibody used in the experiments was 0.25, 0.5, or 1.5 μg/mL. After pre-incubation for 5 min at room temperature, the mixture was introduced into optofluidic cell for the $AFM_1$ detection. During the pre-incubation period, anti-$AFM_1$ antibody binding sites were occupied depending on the concentration of the $AFM_1$ in the samples. When the mixture entered the optofluidic cell, antibodies left with free binding sites were able to bind to the $AFM_1$-OVA conjugate. The fluorescence molecule labeled on the antibodies bound with $AFM_1$-OVA conjugate could be excited by an evanescent wave, and real-time monitoring of the fluorescence signal was conducted as binding occurred between the antibodies with free binding sites and immobilized conjugate of the biosensor. The increasing $AFM_1$ concentrations reduced the EOBP signal because the $AFM_1$ in the solutions inhibited the fluorescence-labeled antibodies to bind with the $AFM_1$-OVA conjugate. For the regeneration of the active biosensor surface, a SDS (pH = 1.9) regeneration solution was used to break the antibody-analyte association on the biosensor surface.

### 2.5. Direct detection of $AFM_1$ in dairy products using organic solvent extraction

To evaluate the potential matrix effect of real samples on the performance of the proposed biosensor and recovery of the developed method using organic solvent extraction, three commercially available dairy products, namely, liquid milk, cheese, and milk tea, were purchased from the local supermarket (Beijing) and analyzed using EOBP. Three organic solvents, namely, methanol, acetone and acetonitrile, were used for simultaneous precipitation of protein and extraction of $AFM_1$ in dairy products. All samples were initially measured to determine if they were contaminated by $AFM_1$. Afterward, different concentrations of $AFM_1$ standards were spiked into the samples. These samples were extracted using organic solvents, and directly measured by the proposed immunosensing method after simple dilution with PBS buffer.

To achieve an on-site, fast, and accurate determination of dairy products, we simplified the pretreatment steps of dairy products (Details shown in Supporting information). The centrifuge and concentration process that is necessary in common detection methods was eliminated. The liquid samples (liquid milk and milk tea) were initially mixed with 10 mL of organic solvent. After 20 min shaking at room temperature, the mixture was filtered using 0.22 μm filter membrane. The filtrate was left standing for 10 min, and the supernatant was taken out and diluted with PBS buffer (1:4, v/v). The diluted sample was mixed with fluorescence-labeled anti-$AFM_1$ antibody (1:1, v/v) for 5 min pre-incubation. Subsequently, the mixture was introduced into the optofluidic cell to detect $AFM_1$ content. The cheese samples were powdered before detection, and similar analytical procedures were followed as described above. Two duplicate experiments were performed for all samples.

## 3. Results and discussions

### 3.1. Characterization of $AFM_1$-OVA conjugate modified biosensor

$AFM_1$-OVA conjugate, regarded as bio-recognition molecules, was immobilized onto the optic fiber biosensor as described above. To evaluate the modification effect of biosensor, we performed several control experiments. First, 1 μg/mL Cy5.5 labeled anti-BPA antibody (anti-mouse IgG), 1 μg/mL Cy5.5 labeled anti-MC-LR antibody (anti-mouse IgG), and 1 μg/mL Cy5.5 labeled anti-$AFM_1$ antibody (anti-mouse IgG) were introduced into the optofluidic cell, respectively. As shown in Fig. 2a, when anti-BPA antibody and anti-MC-LR antibody were added, the fluorescence signal slightly

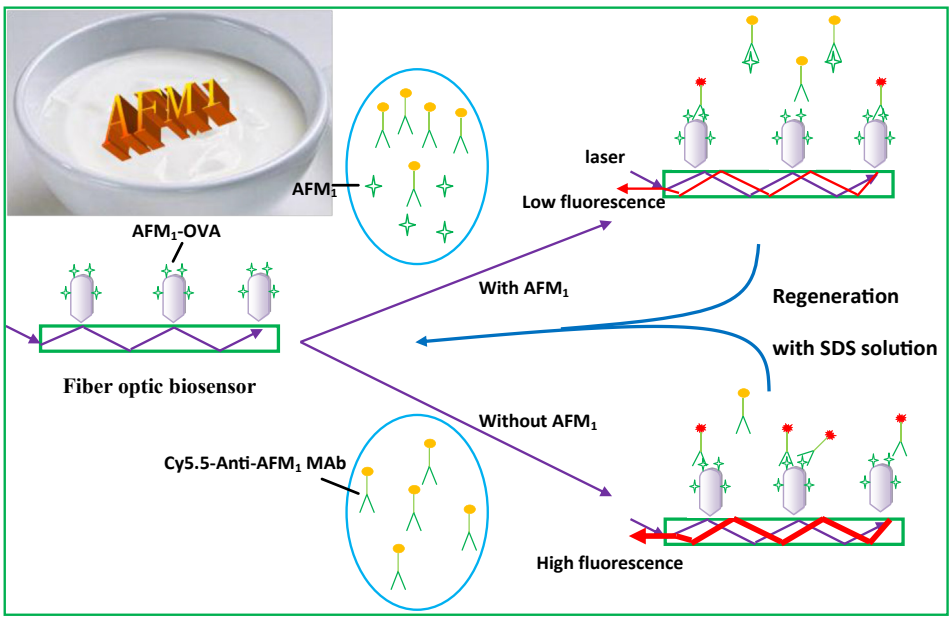


**Fig. 1.** Immunoassay mechanism of $AFM_1$ based on EOBP.

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increased and rapidly reached a balance. Obviously, these two antibodies could not bind with $AFM_1$-OVA conjugate immobilized onto the sensor surface. When anti-$AFM_1$ antibody was added, the fluorescence signal rapidly increased with time, which attributed to the specific binding between anti-$AFM_1$ antibody and the $AFM_1$-OVA conjugate modified onto the sensor surface. The effective signal for each assay was defined as the difference ($S_e$) between Fluorescent intensity at the peak value and signal intensity at the baseline (Fig. 2). The effective signal of specific binding between anti-$AFM_1$ antibody and the $AFM_1$-OVA conjugate was by far higher than that of adsorption of non-specific antibodies. Furthermore, after anti-$AFM_1$ antibody bound with $AFM_1$-OVA conjugate, only a little decrease of fluorescence signal was observed using PBS washing (Fig. S2). When the SDS solution was used to regenerate the biosensor, the fluorescence signal could rapidly return to baseline. These results demonstrated that the $AFM_1$-OVA conjugate was successfully modified onto the sensor surface and had highly reactive activity with anti-$AFM_1$ antibody. Only few anti-BPA antibody and anti-MC-LR antibody nonspecifically adsorbed onto the sensor surface, and the free fluorescence in solution had few contributions for the detection fluorescence signal. Because the evanescent wave only has hundreds nanometer of effective penetrate depth, few free fluorescence molecule is excited [23].

Furthermore, the stability and reusability of biosensor is essential for obtaining reliable results and reducing the detection cost. After the 10 min reaction between anti-$AFM_1$ antibody and $AFM_1$-OVA on the sensor surface, the biosensor was regenerated by 0.5% SDS solution (pH 1.9). From Fig. 2b, the Cy5.5 labeled anti-$AFM_1$ antibody can be completely removed from the sensor surface and the signal curve comes back to the baseline. After 200 times replication, the fluorescence signal detected decrease 85% of initial value when the same concentration of anti-$AFM_1$ antibody was used. More importantly, even though many samples containing different organic solvents (e.g. methanol, acetone, and acetonitrile), even up to 10% (v/v), were introduced onto the biosensing surface, the bioreaction activity of $AFM_1$-OVA on the sensor surface after regeneration was not still significantly changed. Most of previous studies usually immobilized antibody onto the sensor surface to consider the effect of organic solvent on immunoassay. Although the effect of low concentration of organic solvent on antibody is low, the sensor surface modified by antibody can rarely be reused more than fifteen times when many actions of organic solvent and harsh regeneration conditions (strong acid and strong base) were performed [12,24]. This results in the decrease of antibody activity with each cycle, thus leading to inaccurate detection results. In the proposed immunosensing technology, hapten-carrier protein ($AFM_1$-OVA conjugate) modified the biosensor surface could address the above problems thanks to the robust and stability of $AFM_1$-OVA conjugate. The good regeneration performance indicated the cost-effective and reliable determination of $AFM_1$ using the proposed biosensor.

### 3.2. Dose-response curves of $AFM_1$ using different concentrations of anti-$AFM_1$ antibody

The indirect competitive immunoassay mechanism was applied for the $AFM_1$ detection. To achieve the sensitive detection of $AFM_1$, different concentrations of $AFM_1$ standard solution were initially mixed with a certain concentration of fluorescence-labeled anti-$AFM_1$ antibody, and this mixture was introduced into the optofluidic cell. Parts of the binding sites of fluorescence-labeled anti-$AFM_1$ antibody were occupied by the free $AFM_1$ in the solution, and the other fluorescence-labeled antibodies containing free binding site could bind with $AFM_1$-OVA conjugate immobilized onto the sensor surface. Fluorescence molecules labeled on the antibody, which showed binding with $AFM_1$-OVA, could be excited by evanescent wave. Part of fluorescence coupled into the fiber optic biosensor, which was probed by a photodiode through transmission of single-multi mode fiber optic coupler. The fluorescence signal trace was shown on-time in a user-friendly interface (Fig. 3a).

In the present study, three concentrations of Cy5.5-labeled anti-$AFM_1$ antibody (0.5, 1, and 3 μg/mL) were used to obtain optimal detection performances, such as sufficiently high fluorescence signal, optimal limit of detection (LOD), and linear detection range. Fig. 3a shows that the fluorescence signal of biosensor inversely decreases with increasing of $AFM_1$ concentration (0–100 μg/L) using 1 μg/mL Cy5.5-labeled anti-$AFM_1$ antibody. For each $AFM_1$ concentration, the fluorescence signal of biosensor rapidly increased when the mixture of $AFM_1$ and anti-$AFM_1$ antibody was introduced into the optofluidic cell. After a 5 min reaction, the fluorescence signal lightly increased and reached an equilibration after reaction for 10 min. For the two other concentrations of fluorescence-labeled anti-$AFM_1$ antibody, similar appearances were observed (data not shown). To shorten the detection period, the fluorescence signal at 8 min reaction was considered an effective signal value.

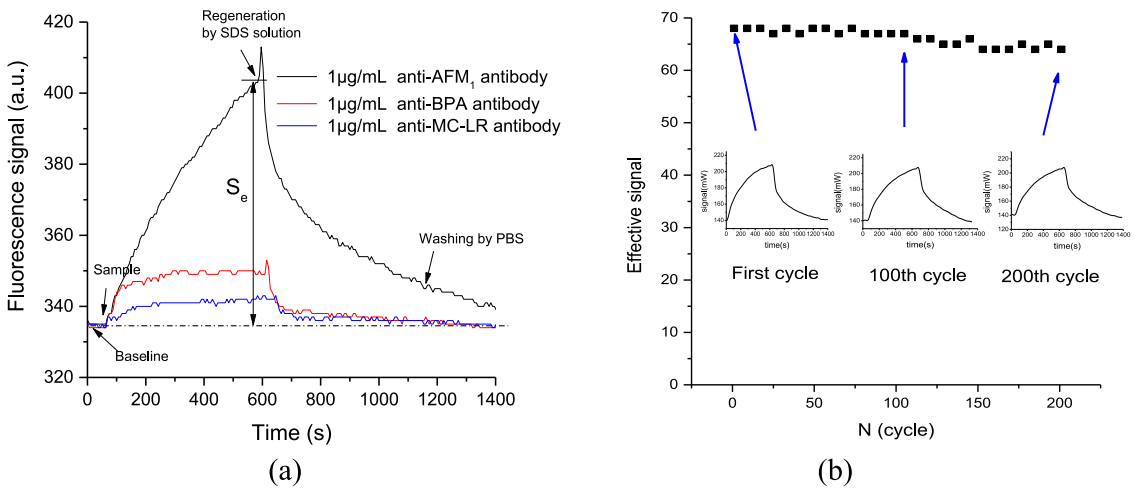


**Fig. 2.** Characterization of $AFM_1$-OVA modified biosensor. (a) Assessment of the immobilization effectiveness. The Cy5.5-labeled anti-$AFM_1$ antibody, Cy5.5-labeled anti-BPA antibody, or Cy5.5-labeled anti-MC-LR antibody was pumped over the fiber optic sensor surface modified by $AFM_1$-OVA, respectively. (b) Stability and reusability of biosensor (1 μg/mL Cy5.5-labeled anti-$AFM_1$ antibody in PBS solution was used and part of results were shown).

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Fig. 3b demonstrates the three dose-response curves of $AFM_1$ using three concentrations of anti-$AFM_1$ antibody, which were normalized by expressing the signal of each standard point (S) as the ratio of that of the blank sample without $AFM_1$ ($S_0$). The signal intensities were fitted to the four parameter logistic equation [25]. The error bars in the figure correspond to the standard deviation (<6.8%) of the data points in triplicate experiments. With increasing the antibody concentration, the fluorescence signal detected by EOBP increases, however, the LOD of biosensor increased. The linear detection range of $AFM_1$ was 0.045–5.1, 0.10–10.5, and 0.15–10.0 μg/L for 0.5, 1.0, and 3.0 μg/mL of fluorescence-labeled anti-$AFM_1$ antibody, respectively (Fig. 3b). The LODs were determined as 0.005, 0.009, and 0.02 μg/L for 0.5, 1.0, and 3.0 μg/mL of anti-$AFM_1$ antibody, respectively, based on the average standard deviation of the measurements (σ) and slope of the dose-response fitting curve (S) as 3σ/S [26,27]. The LODs improved with the decreasing of anti-$AFM_1$ antibody concentration, and could satisfy even the most stringent requirements demanded by the EC. This LODs are initially comparable with that of other immunoassay methods, such as the time-resolved fluorescence competitive immunoassay (0.030 μg/L) [14], nanogold strip immunoassay (0.30 μg/L) [15], impedimetric immunosensor based on silver wire amplification (0.0063 μg/L) [16], and electrochemical immunochip (0.008 μg/L) [28]. A long-range surface plasmon-enhanced fluorescence spectroscopy exhibited a very low LOD of 0.6 ng/L, but the assay time was too long (about 1 h) [29]. Compared with these methods, the proposed immunosensor is simpler and faster (less than 20 min, including measurement and regeneration). In addition, the portable optofluidic biosensing platform allows for potential on-site or real time measurements. When 0.5 μg/mL anti-$AFM_1$ antibody was used, the fluorescent signals were low, which may reduce the accuracy of the detection results. Therefore, we used 1 μg/mL Cy5.5-$AFM_1$-antibody for the following experiments. In the following experiments, all measurements were normalized with respect to the blank signal at the beginning of the daily analysis to reduce the effect of the signal shift on the sample measurement.

### 3.3. *Effect of organic solvents on the binding interaction of antibody-antigen*

The organic solvent extraction of $AFM_1$ in dairy products is essential for $AFM_1$ detection using biosensor. Aiming for practical applications, the immunoassays for the targets in dairy products should tolerate at least some organic solvents in the immunoreaction mixture. Accordingly, the organic solvent extracts of the dairy products could be analyzed either directly or after dilution with aqueous phase. Several organic solvents commonly used for extractions, including methanol, acetone, and acetonitrile, were chosen as typical examples to evaluate the effects of organic solvents on the binding interaction between anti-$AFM_1$ antibody and $AFM_1$. First, the effect of organic solvents on the heterogeneous binding interaction between anti-$AFM_1$ antibody in solution and $AFM_1$ immobilized onto the sensor surface was tested. All fluorescence signal values were normalized for comparison by expressing the signal of each point as the ratio of the signal of the blank sample (without organic solvents). The effect of methanol on the heterogeneous binding interaction of antibody-antigen was the lowest among three organic solvents (Fig. 4a). The concentration of methanol increased from 0 to 10%, the fluorescence signal slightly decreased. However, the effect of acetone and acetonitrile on the heterogeneous binding interaction of antibody-antigen was obvious. With increasing acetone and acetonitrile concentration (except for 5%), the fluorescence signal detected by EOBP significantly decreased, thus indicating that less fluorescence-labeled anti-$AFM_1$ antibody bound with the $AFM_1$-OVA immobilized onto the sensor surface.

Furthermore, the influence of organic solvent on the interaction of antibody and free $AFM_1$ in solutions should be considered to achieve $AFM_1$ detection in dairy products. According to the dose-response curve obtained above, the quantitative detection of $AFM_1$ ranged from 0.1 μg/L to 10.5 μg/L. Therefore, the effect of organic solvent on the detection of $AFM_1$ at different concentrations (0.1, 1, and 10 μg/L) was studied. The appropriate concentration of $AFM_1$ was mixed with 1 μg/mL of fluorescence-labeled anti-$AFM_1$ antibody in the PBS/organic solvent solution. After pre-incubation for 5 min, the mixture was introduced into the optofluidic cell, and the binding reaction curve was recorded. All fluorescence signal values were normalized by expressing the signal of each point as the ratio of the signal of the blank sample (without organic solvents and $AFM_1$) (Fig. 4b–d). At a concentration of 1%, the organic solvents, methanol and acetone, almost did not affect the binding reaction because the normalized value of fluorescence signal of each $AFM_1$ concentration in organic solvent/PBS solution is consistent with that of each $AFM_1$ concentration in PBS (Fig. 4b–c). With increasing methanol and acetone concentrations, the fluorescence signal of each $AFM_1$ concentration in organic

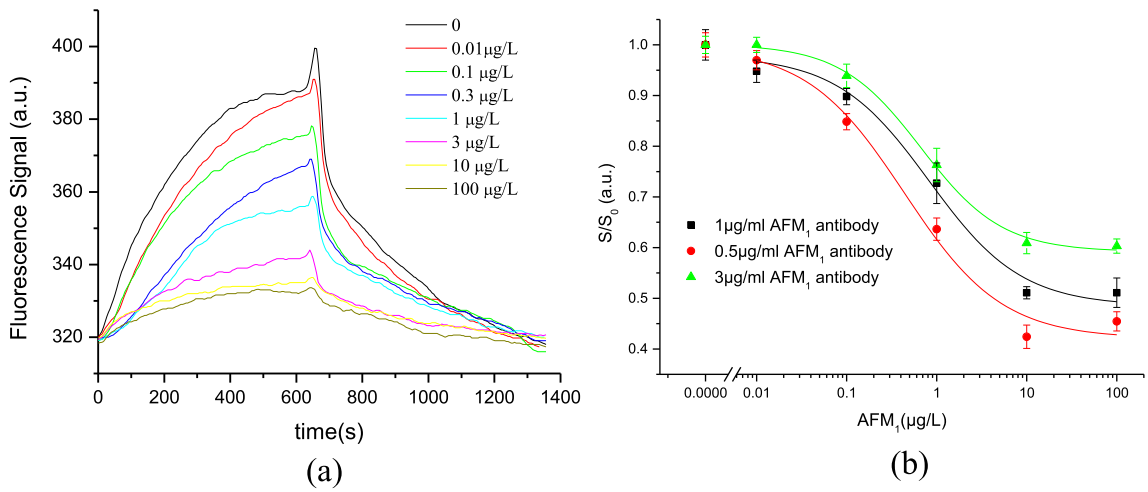


**Fig. 3.** Dose-response curves of $AFM_1$ using different concentrations of anti-$AFM_1$ antibody. (a) Typical fluorescence signal trace observed in the flow of 1 μg/mL Cy5.5-$AFM_1$-antibody mixture with different $AFM_1$ concentrations in the range 0–100 μg/L; (b) Standard curves of EOBP on the competitive immunoassay of $AFM_1$ mixed with the antibody at three concentrations (0.5, 1, and 3 μg/mL).

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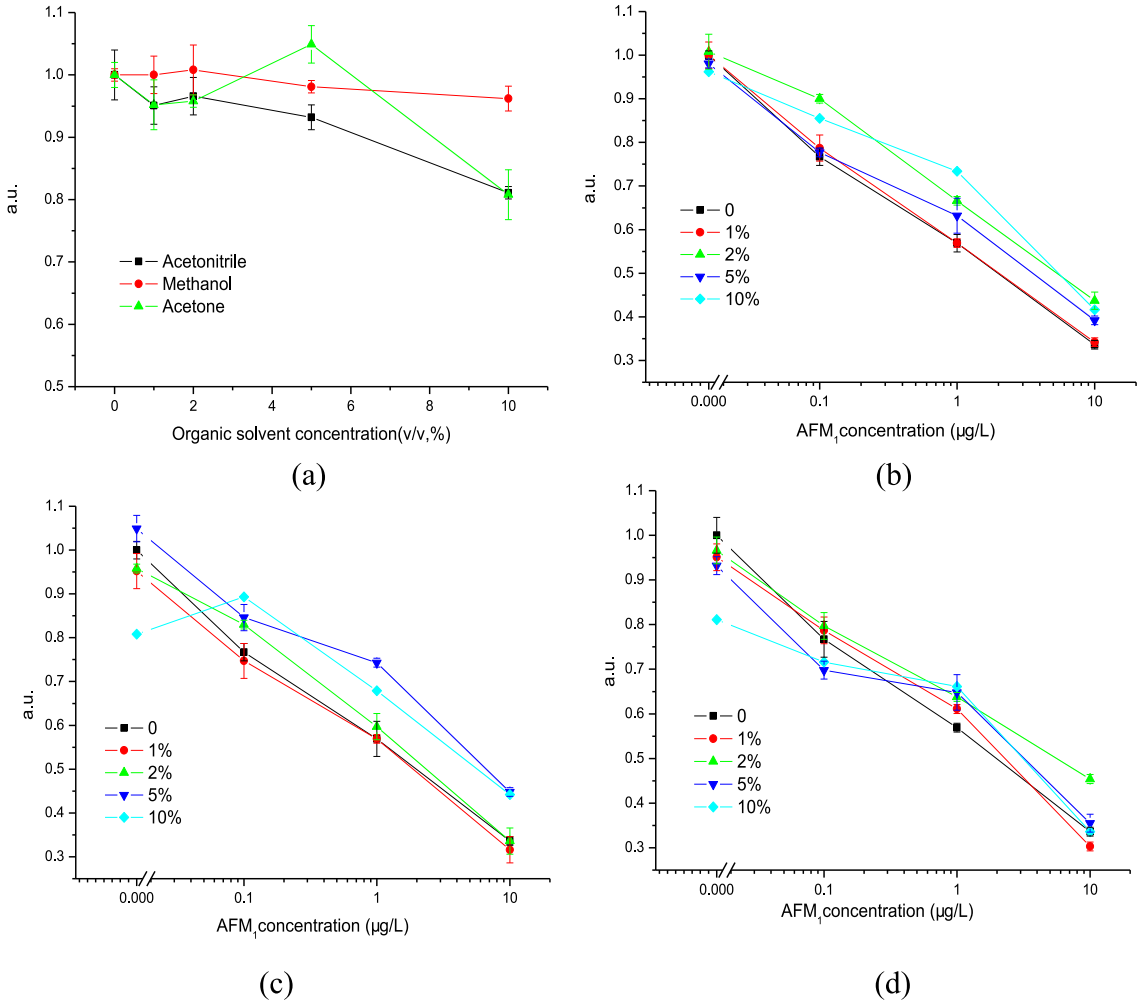


**Fig. 4.** Effect of organic solvents on the binding reaction between anti-$AFM_1$ antibody and $AFM_1$. (a) Effect of methanol, acetone, and acetonitrile on the heterogeneous binding interaction between anti-$AFM_1$ antibody in solution and $AFM_1$ immobilized onto the sensor surface. (b) Effect of different methanol concentrations (0%, 1%, 2%, 5%, and 10%, v/v) on the indirect competitive immunoassay of $AFM_1$. (c) Effect of different acetone concentrations (0%, 1%, 2%, 5%, and 10%, v/v) on the indirect competitive immunoassay of $AFM_1$. (d) Effect of different acetonitrile concentrations (0%, 1%, 2%, 5%, and 10%, v/v) on the indirect competitive immunoassay of $AFM_1$. The error bars represent standard deviation from two measurements.

solvent/PBS solution containing a certain $AFM_1$ concentration is higher than that in PBS solution because the normalized value of the former is higher than that of the latter. Because the methanol and acetone (<10%) had a little influence on the binding reaction between antibody in solution and $AFM_1$-OVA immobilized onto the sensor surface, we can assume that the effect of methanol and acetone on the homogeneous binding reaction between antibody and antigen in the solution is more significant than that of heterogeneous binding reaction between antibody in solution and immobilized antigen. The concentration of methanol increased from 0 to 10%, the fluorescence signal slightly decreased. However, the fluorescence signal detected by EOBP is linearly dependent on the $AFM_1$ concentration under different organic solvent concentrations. Table S1 summarizes the linear dependence coefficient. Therefore, the biosensor can be directly used to quantify the $AFM_1$ when the samples contain a certain methanol or acetone concentration. Nevertheless, the effect of acetonitrile on the homogeneous and heterogeneous binding reaction between antibody and antigen is similar under the experimental concentration range. Thus, the linear relation between $AFM_1$ concentration and the normalized value of fluorescence signal of each data point is almost consistent under different acetonitrile concentrations (Table S1).

All of the results presented herein demonstrate that immunochemical interactions can be carried out in the presence of organic solvents. The effects of organic solvents on antibody-antigen interactions can be mainly attributed to the conformation of antibody protein, which depends on the ratio of hydrophobic and hydrophilic regions on its surface [30,31]. The change of this ratio resulted in the rearrangement of hydrogen bonds, thereby causing conformational changes in the whole protein structure. Some studies showed that the less soluble the organic solvent in water, the lower its influence to the immunointeraction [31,32]. However, most of these studies applied ELISA to detect the targets, and the binding kinetics between antibody and antigen could not be obtained [31–33]. Horáček et al. demonstrated that immunochemical interaction could proceed in nonaqueous solvents using piezoelectric biosensor [34]. However, the different effects of organic solvents on homogeneous and heterogeneous binding interaction between antibody and antigen were not shown. Studies on the conformation of proteins in organic solvents are rare. Data on functional activation of antibodies after their structural perturbation are scarcer. Some findings in this study are difficult to be

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**Table 1**
Immunoassay of $AFM_1$ in dairy products using organic solvent extraction.

| | Organic solvent | Original concentration (μg/kg) | Spiked concentration (μg/kg) | Measurement by biosensor (μg/kg) | Recovery (%) | CV (%) |
|---|---|---|---|---|---|---|
| Liquid milk | Acetone | <0.05 | 0.24 | 0.27 | 112.5 | 2.6 |
| | | | 1.39 | 1.17 | 84.2 | 3.8 |
| | Acetonitrile | | 0.24 | 0.23 | 95.8 | 4.1 |
| | | | 1.39 | 1.22 | 87.8 | 0.6 |
| | Methanol | | 0.24 | 0.05 | 19.2 | 5.3 |
| | | | 1.39 | 0.62 | 44.3 | 3.6 |
| Cheese | Acetone | <0.05 | 0.1 | 0.11 | 110.0 | 5.4 |
| | | | 1.0 | 0.87 | 87.0 | 3.2 |
| | Acetonitrile | | 0.1 | 0.107 | 106.7 | 3.7 |
| | | | 1.0 | 0.83 | 83.0 | 1.2 |
| | Methanol | | 0.1 | 0.056 | 56.4 | 0.9 |
| | | | 1.0 | 0.535 | 53.5 | 4.9 |
| Milk tea | Acetone | 0.58 | / | / | / | 5.6 |
| | Acetonitrile | 0.50 | / | / | / | 3.5 |
| | Methanol | 0.688 | / | / | / | 4.5 |

clearly explained if only the limited knowledge of conformational change mechanism of proteins in organic solvents is considered as a basis. Thus, more studies are necessary.

### 3.4. Direct determination of $AFM_1$ in commercial dairy products using organic solvent extraction

The evaluation of the matrix effect is essential for the assessment of the newly developed methods [20]. In an immunoassay, the interactions of antibody and antigen are affected by the pH, the ionic strength and the organic matters of real samples. The EOBP was applied to analyze three commercial dairy products, namely, liquid milk, cheese, and milk tea purchased from the local supermarket in Beijing, China. As shown in Table 1, the content of $AFM_1$ in liquid milk and cheese should be lower than the LOD of the proposed biosensor, whereas milk tea was contaminated by $AFM_1$. The concentration of $AFM_1$ in milk tea was >0.5 μg/kg as shown by different organic solvents extraction, and verified as 0.51 μg/kg by HPLC [35]. This value was higher than the limited value of China. Therefore, the spiked samples of milk tea were not used in this study. For the liquid milk and cheese samples, the recovery of all measured samples using acetone and acetonitrile extraction was within the range of 80%–110% when the samples were spiked by a certain $AFM_1$ concentration. Parallel test results showed that the coefficient of variation (CV) was no more than 6%. These experimental results definitely demonstrated that any possible interference from the different background compositions of liquid milk and cheese samples on the optofluidic biosensor analysis is negligible. The accuracy and reliability of the present method indicated that the developed biosensor system can be successfully applied to $AFM_1$ analysis in dairy products using acetone and acetonitrile extraction methods. However, the recovery of $AFM_1$ was low (<50%) in liquid milk and cheese samples when methanol extraction was performed. We assume that the complete miscibility between methanol and water is a reason to result in low $AFM_1$ extraction efficiency from high viscosity liquid or solid samples when methanol extraction was performed.

The performance of the EOBP assay was also evaluated through comparing the results obtained by EOBP using liquid milk samples (spiked concentration: 0.1, 0.3, and 1 μg/L) with those obtained from the analysis of similar samples by HPLC [35]. Comparison between methods yielded good correlation results ($y = 0.8335x+0.0224$) (Fig. S3, Table S3), and the correlation coefficient ($R^2 = 0.9999$) demonstrated satisfactory agreement between methods, which indicates adequacy of the optofluidic-based biosensing platform for accurate $AFM_1$ detection.

## 4. Conclusions

A simple, low-cost, reusable, and easy-to-operate immunosensing method has been developed for ultrasensitive detection of $AFM_1$ in dairy products using a portable EOBP. The LOD of the developed method is below the maximum levels imposed by the current regulations for $AFM_1$ in dairy products. The effect of organic solvents on the homogeneous binding reaction between antibody and antigen in the solution was significant than that of heterogeneous binding reaction between antibody in solution and antigen immobilized onto the sensor surface. EOBP can directly be used to quantify the $AFM_1$ even if the samples contain a certain concentration of organic solvent because the fluorescence signal detected by EOBP was linearly dependent on the $AFM_1$ concentration.

The accuracy and reliability of the present method indicated that the developed biosensor system can be successfully applied to $AFM_1$ analysis in dairy products using acetone and acetonitrile extraction methods. The experimental results definitely demonstrated that any possible interference from the different background compositions of liquid milk and cheese samples on the optofluidic biosensor analysis was negligible. Compared with traditional $AFM_1$ detection technologies, the proposed method provides a convenient tool for the rapid, accurate, and reliable determination of $AFM_1$ in dairy products using a simple and inexpensive sample pretreatment. In addition, the portable optofluidic biosensing platform allows for potential on-site or real time measurements of $AFM_1$ in milk products, which satisfies the requirement for rapid and sensitive detection of milk products in dairy farms and milk industries.

## Associated content

Additional information as noted in text. This material is available free of charge via the Internet.

## Notes

The authors declare no competing financial interest.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.aca.2016.08.020.

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