Talanta 141 (2015) 279–287

Contents lists available at ScienceDirect

Talanta

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

Detection of 17β-estradiol in water samples by a novel double-layer molecularly imprinted film-based biosensor

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ARTICLE INFO

Article history:
Received 30 January 2015
Received in revised form
2 April 2015
Accepted 5 April 2015
Available online 10 April 2015

Keywords:
Double-layer MIF
17β-Estradiol
Surface plasmon resonance (SPR)

ABSTRACT

This study reports a novel double-layer molecularly imprinted film (MIF)-based biosensor for rapid, sensitive and highly selective detection of small molecule 17β-estradiol (E2) that is frequently detected in environmental water samples. In this system, the modification of gold surface of SPR chip was performed by 1-dodecanethiol. Then double-layer MIF was generated on the 1-dodecanethiol modified gold surface. The non-modified and imprinted surfaces were characterized by atomic force microscopy (AFM), scanning electron microscope (SEM) and contact angle measurements. Analysis of surface plasmon resonance (SPR) spectroscopy showed that the imprinted sensing film displayed good selectivity for E2 compared to other analog molecules and NIF. A good linear relationship was obtained between the SPR angle and E2 concentrations over a range of 2.50×10^{-13}–2.50×10^{-9}mol/L ($R^2=0.993$) with the lowest measurable concentration of 2.50×10^{-13} mol/L. The sensor can be regenerated with the mixture of acetic acid and PBS buffer (v/v=1:9) as a desorption agent over tens of times without significant deterioration of the sensor performance. Potential interference of real environmental sample matrix was assessed by spiked samples in several waste seawater effluents. This portable sensor system can be successfully applied for on-site real-time inexpensive and easy-to-use monitoring of E2 or other small molecule pollutants in environmental samples such as effluents or water bodies.

1. Introduction

Endocrine disrupting compounds (EDCs) are contaminants of emerging concern due to their harmful effects on endocrine function of human and aquatic organisms [1]. The steroid 17β-Estradiol (E2) is a category of natural EDCs that has also been found frequently in various water sources [2–4]. High amounts of E2 may interfere with the normal physiological processes and induce sexual abnormalities, a declined male birth rate, and even cancer development [5,6]. The EPA Unregulated Contaminant Regulation has proposed to require monitoring of a number of EDCs, including E2 in wastewater samples. Therefore, it is of great need to develop rapid and sensitive methods to detect E2 for environmental monitoring and clinical diagnostics. Considerable research interests have risen for the detecting of E2 by both advanced instrumental analysis and immunoassay methods [7]. Instrumental analysis methods, such as HPLC and GC/MS are very sensitive. However, the analytical procedures are rather complicated, therefore labor-intensive and time-consuming [8,9]. Also various immunoassays have been explored for detection of E2, including fluorescence immunoassays [10,11], chemiluminescence immunoassays [12–14] and so on [15–18], these methods are accurate and highly sensitive. However, they require expensive and sophisticated instruments, multi-step complicated sample preparation procedures and long analysis times.

Recently, many biosensors have been developed for determination of biomolecules [19–21]. SPR sensors have attracted attention for last two decades [22,23]. It is an optical technique that measures changes in the refractive index occurring within approximately a few hundred nanometers from the sensor surface [24]. Now SPR sensors have become a widely used analytical technique to determinate affinity-binding constants [25,26], monitor environmental contaminants [27,28], diagnose [23] and genotype analyze [29].

Although various methods are used to generate the sensitive SPR sensor, the most effective method is molecular imprinting technique. The method involves the synthesis of molecular imprinted polymers (MIPs), which were templated with target analytes and yielded recognition sites capable of selectively rebinding the analytes [30]. MIPs have various applications, such as artificial antibodies [31], chromatographic separations [32], solid-phase extraction [33,34], enzyme catalysis [35] and sensor devices [36–38].

In this study, we firstly prepared E2-imprinted film on gold surface of SPR chip by the double-layer MIFs. The developed SPR

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http://dx.doi.org/10.1016/j.talanta.2015.04.019
0039-9140/

sensors show high sensitivity, high selectivity and good reproducibility for determination of E2 in various water samples. Finally, the developed portable biosensor was successfully developed and applied to the seawater detection of E2. This portable sensor system can be used for on-site real-time inexpensive and easy-to-use monitoring of E2. Other small molecular pollutants in environmental aqueous samples also are possibly detected by the same strategy.

2. Materials and methods

2.1. Material

17β-Estradiol, progesterone, estriol, estrone, cinchonine and ethylene glycol dimethacrylate (EGDMA) were purchased from Aladdin Regent Company, testosterone and benzophenone (BP) were purchased from Acros Organics. Methacrylic acid (MAA) and 1-dodecanethiol were all purchased from J and K Scientific Ltd. (China). Ethanol and acetic acid were analytical grades and purchased from Beijing Tongguang Fine Chemicals Company. MAA and EGDMA were distilled under reduced pressure before used. All the other reagents except MAA and EGDMA were used as received. Phosphate buffered saline (PBS) used in the experiments was prepared from 140 mM NaCl, 10 mM phosphate and 3 mM KCl, and pH 7.4 was adjusted by HCl and NaOH.

2.2. Surface modification of the SPR chip

2.2.1. 1-Dodecanethiol modification of the SPR chip

To modify gold surface of the SPR chip with $C_{12}H_{25}SH$, the chip was washed with 30 ml of acidic piranha solution (3:1 H_2SO_4:H_2O_2, v/v). After the SPR chip was dipped in cleaning solution for 5 min, it was washed with ethanol and dried in a vacuum oven for 2 h. To let the gold surface react with the benzophenone to form free radicals for anchoring polymers better, the chip was dipped in an ethanol solution (1 mmol/L) of 1-dodecanethiol for 24 h in order to form a self-assembled monolayer (SAM). Then it was cleaned with ethanol and dried with nitrogen gas. The alkanethiolate self-assembled monolayer on gold substrates will allow subsequent growth of surface-confined polymer.

2.2.2. In-situ preparation of MIF

The MIF on $C_{12}H_{25}SH$ modified SPR chip was prepared as follows (Scheme 1). Firstly, E2 (0.025 mol/L) and MAA (0.1 mol/L) monomer were mixed with acetonitrile (4 mL) at room temperature for 3 h. The cross-linker ethylene glycol dimethacrylate (EGDMA, 0.375 mol/L) and initiator benzophenone (0.02 g/mL) were then added to the pre-polymerization solution, followed by ultra-sonication for 10 min. The SPR substrate was immediately placed on the glass slide with the Au-SAM surface facing downwards and fastened tightly with chips. Parafilm was sandwiched between the two glass slides as a spacer. The first layer of MIF was carried out by UV photo-polymerization for 86 min with UV light set at 10 mm.

To obtain the second layer of MIF, the same protocol was used except the concentration of the cross-linker ethylene glycol dimethacrylate (EGDMA) was 0.45 mol/L and the time of the UV photo-polymerization was 15 min. Then the double-layer MIF was obtained.

The NIF was synthesized as a reference under exactly the same conditions without the template. Also, the single-layer MIF was prepared without the second layer MIF as the comparison.

2.3. E2 removal from SPR chip surface

There are electrostatic interactions and hydrogen bonding between the carboxylic acid groups of MAA monomer and polar groups of E2 molecules. In order to break the interactions, we used the mixture of acetic acid and PBS buffer (v/v=1:9) as a desorption agent. First, the removal study of E2 was performed by pumping the rinsing solution to the flow-cell for 10 min at the flow rate about 1 ml/min. The SPR chip was rinsed at room temperature. After E2 removal, the SPR chip was washed with PBS buffer.

2.4. Characterization method

Contact angle of the surfaces was obtained with POWEREACH JC2000C (Shanghai, China) instrument. Contact angles were measured with the Sessile Drop method by dropping one water drop. The five photos were obtained from the different parts of SPR chips. The calculated values for the SPR surfaces were the mean of the five measurements.

Tapping mode atomic force microscope (AFM) was used (Veeco Innova Instruments, Germany) to characterize the surfaces. SPR chip was installed on sample holder. The scan rate was 2 μm s^{-1}. The studies were performed in air atmosphere.

In order to observe the longitudinal section of the MIF, the biosensor chip surface was characterized using a scanning electron microscope imaging (SEM) (Hitachi S-4800, Japan) microscopy.

2.5. Procedure of the analysis

The detection of E2 was performed using an SPR system. The working solutions were prepared by diluting the stock solution with 0.10 M phosphate buffer (pH 5.0). After the MIF surface was washed with phosphate buffer (5.0 mL), the affinity binding of template molecules was observed by injection of a series of samples with E2 dissolved in PBS buffer (pH 5.0) at concentrations ranging from 2.5×10^{-13} to 2.5×10^{-9} mol/L. Each sample of 3 mL was injected circulated through the flow cell for 600 s. The changes in reflectivities were monitored in the process.

The desorption studies were carried out using a rinsing solution (5.0 mL) with 2.0 mL/min of flow rate. After that, the E2 (2.5×10^{-8} mol/L) imprinted SPR chip was washed with phosphate buffer. The steps of adsorption–desorption were repeated for the 2.5×10^{-8} mol/L E2 PBS solutions (pH 5.0).

To investigate the selectivity of the MIF, five analog molecules, testosterone, progesterone, estriol, estrone and cinchonine, at a concentration of 2.5×10^{-8} mol/L in PBS buffer (pH 5.0) were, respectively, incubated with the MIF. For the control experiment, the 2.5×10^{-8} mol/L E2 in PBS buffer (pH 5.0) was incubated with the NIF and conventional MIF.

To show the necessity of the 2nd MIF, E2 at a concentration of 2.5×10^{-8} mol/L in PBS buffer (pH 5.0) was incubated with this double-layer MIF and the single-layer MIF.

2.6. Analysis of spiked waste seawater

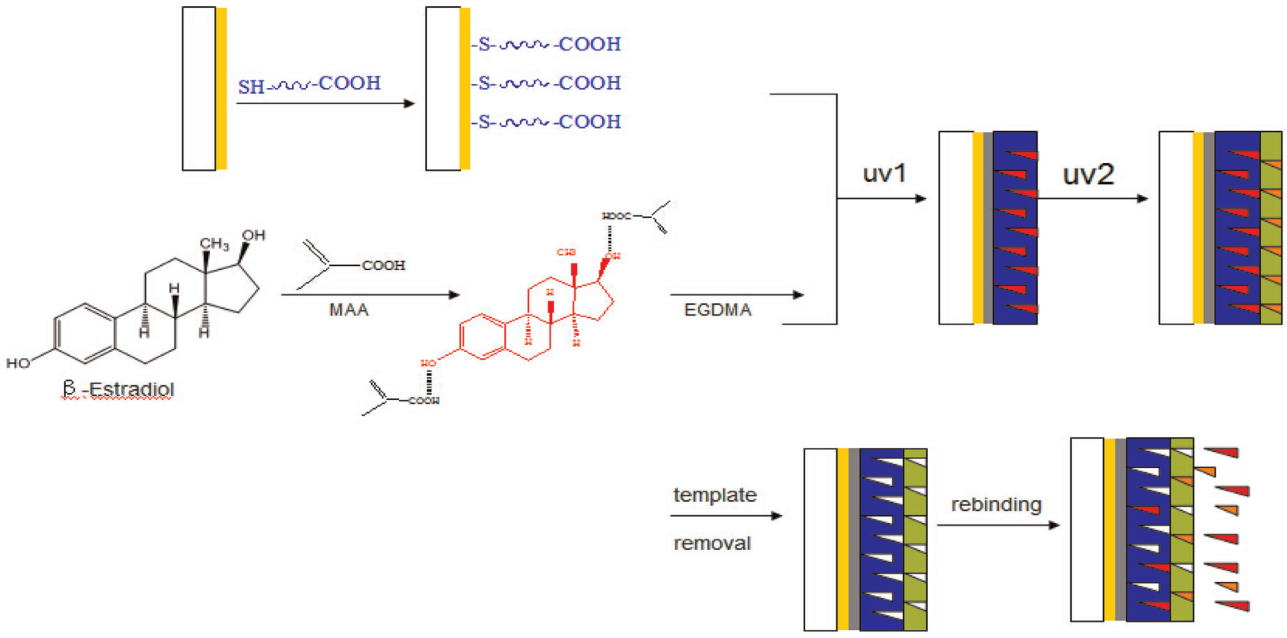
To detect E2 in real environmental water, we analyzed different waste seawater effluents to detect E2. The waste seawater effluent samples were filtered through 0.22 μm filters to remove all particulates before they were spiked with E2. Three duplicate experiments were performed for all samples. Similar analytical procedures were followed as described above.

3. Results and discussion

3.1. Characterization of MIF

As seen in Fig. 1, contact angle values of non-modified (Fig. 1A) and $C_{12}H_{25}SH$ modified SPR surfaces (Fig. 1B) were obtained as 88.70 ± 1.21 and 104.60 ± 1.92, respectively. The increase in surface contact angles indicated that hydrophobicity of surface increased, as a result of surface containing the hydrophobicity structure of $C_{12}H_{25}SH$. The contact angle value of MIF on $C_{12}H_{25}SH$ modified SPR chip was obtained as 120.40 ± 1.40 (Fig. 1C). The fluid spreads more difficulty over the surface of the MIF. The increase of hydrophobicity character of surface is expected due to the recognition cavities in the MIF. These results show that the polymerization could be accomplished on the SPR chip.

The surface morphology of MIF was studied by AFM. Compared to non-modified SPR chip (Fig. 2A), it was obvious that the MIF surface was covered by many distributed cavities (Fig. 2C), which were formed by removing the template molecules from the MIF and were very important channels for template taking the binding sites that locate in the inside of MIF. As seen in Fig. 2C and D, the cavities had diameters from 600 nm to 2 μm and depth about 100 nm. Moreover, the values of surface deepness for non-modified SPR chip (seen in Fig. 2A and B) varied from 5 to 13 nm, and the height of MIF on $C_{12}H_{25}SH$ modified SPR chip surface (seen in Fig. 2C and D), which indicated that the surface depth of the MIF



Scheme 1. Illustration of the formation of double-layer MIF on the SPR substrate.

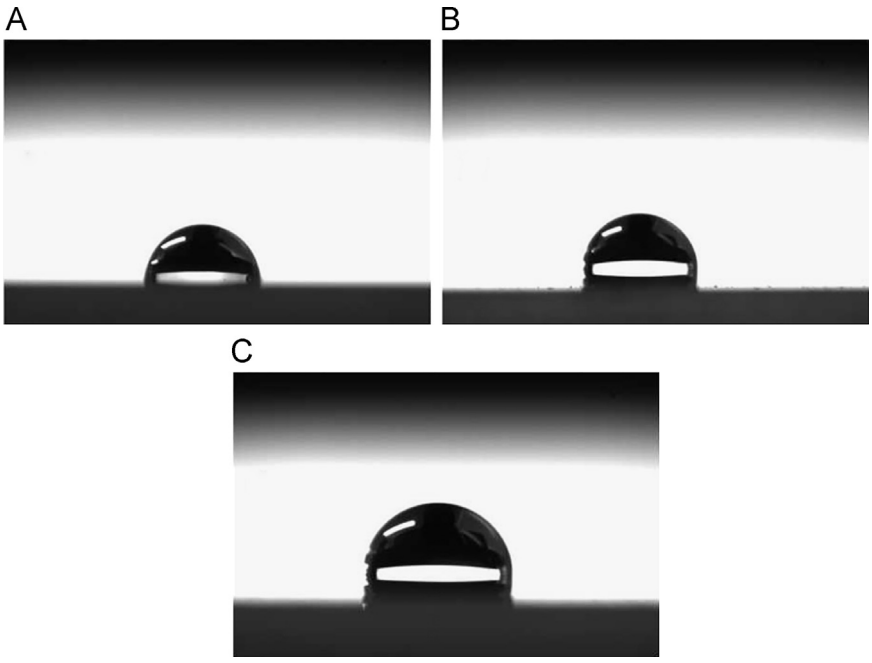


Fig. 1. Contact angle measurements of (A) non-modified; (B) $C_{12}H_{25}SH$ modified; and (C) MIF on SPR chip.

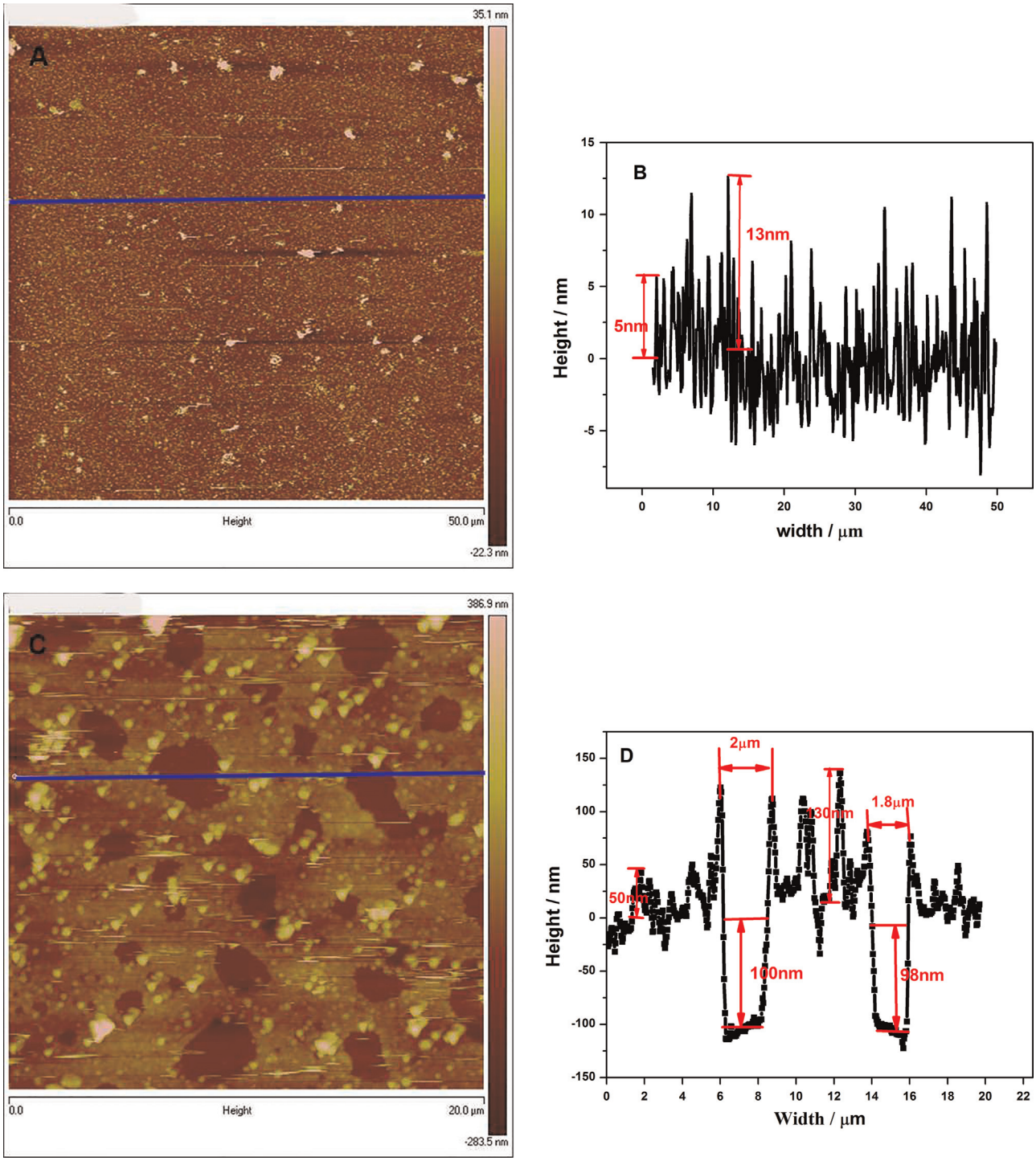


Fig. 2. AFM of (A) non-modified SPR chip; (B) the height and width of the underlined part in (A); (C) MIF on $C_{12}H_{25}SH$ modified SPR chip; and (D) the height and width of the underlined part in (C).

increased. These results confirmed that polymerization could be accomplished on the SPR chip.

In order to observe the longitudinal section of the MIF, the film was characterized by SEM. As seen in Fig. 3A, the thickness of the MIF is about 100 nm. SEM image of the coated polymer film (Fig. 3B) showed the surface of the MIF was very rough. The MIF has double layer structures and the first layer (about 80 nm) was thicker than the second layer (20 nm) (Fig. 3C). These images confirmed that polymerization was accomplished on the gold substrate.

We used the polymerization dynamics to monitor whether the two layers MIF were formed (Fig. 4A shows the first MIF polymerization dynamics, Fig. 4B the second MIF polymerization dynamics). Also, the SPR angular reflectivity spectra were measured. As seen in Fig. 4C, 57.9° is the angle of Au-SAM film. The SPR resonance angle shifted from 57.9° to 71.2° after the formation of

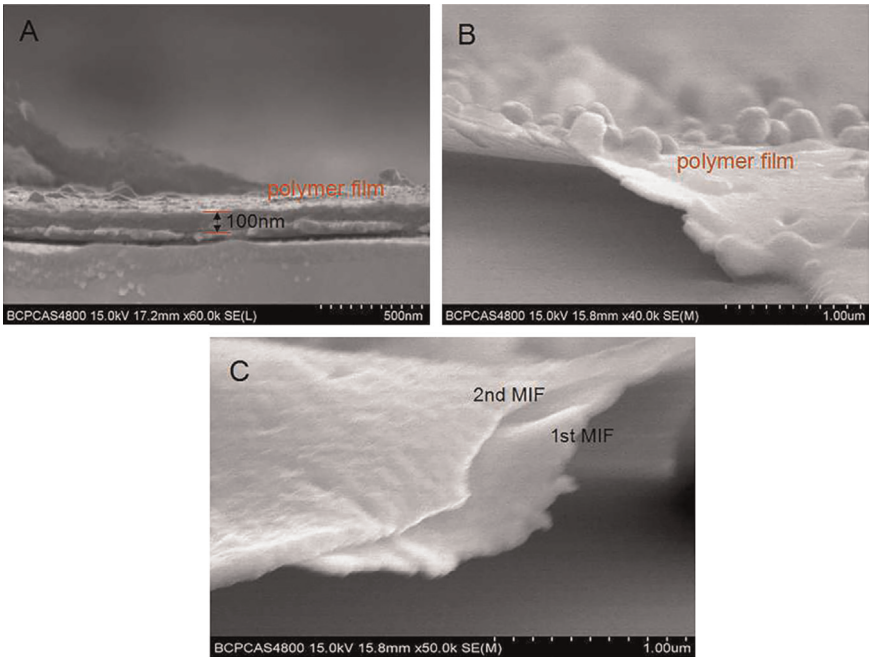


Fig. 3. (A, B and C) SEM images f the E2-imprinted MIF.

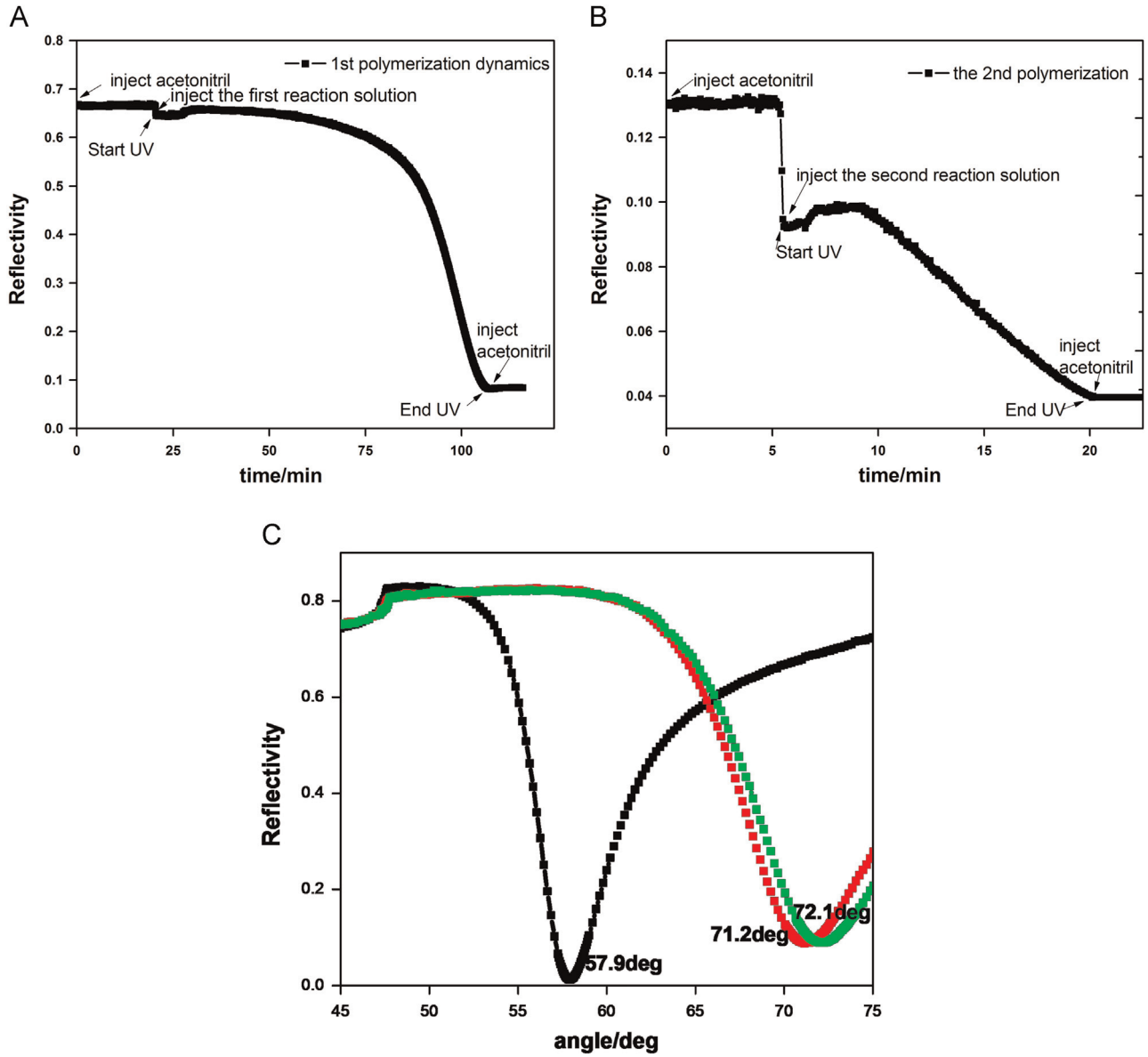


Fig. 4. (A) The 1st MIF polymerization; (B) the 2nd MIF polymerization; and (C) the angle of double-layer MIF.

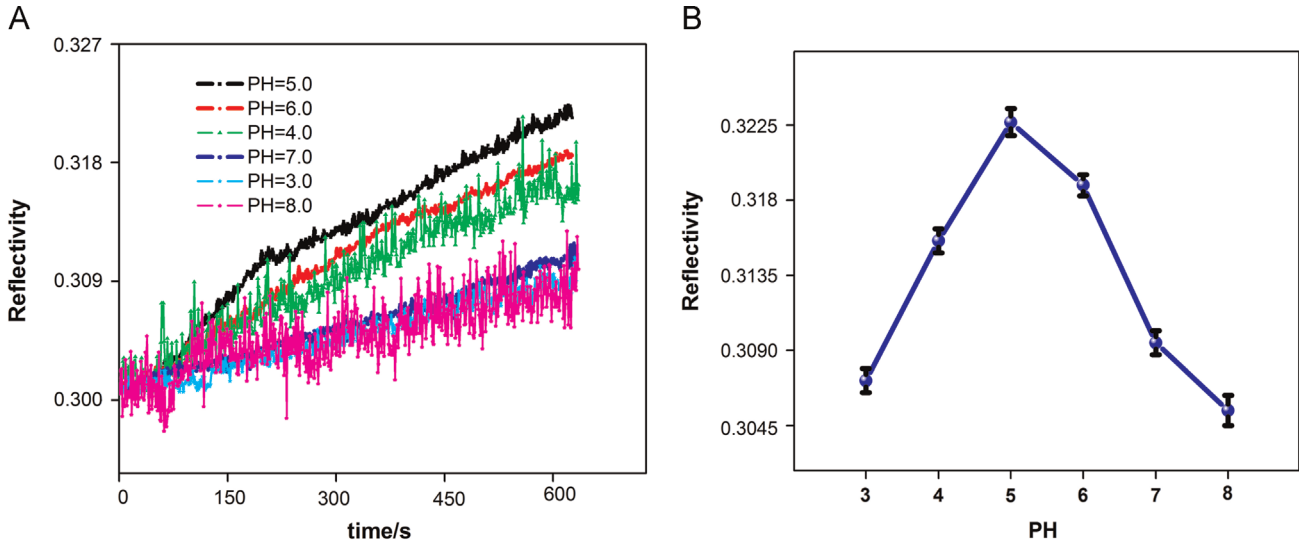


Fig. 5. (A) The sensorgrams for the interaction between 2.5×10^{-8} mol/L E2 in different pHs and E2-imprinted SPR chip and (B) effect of pH on E2-imprinted SPR biosensor.

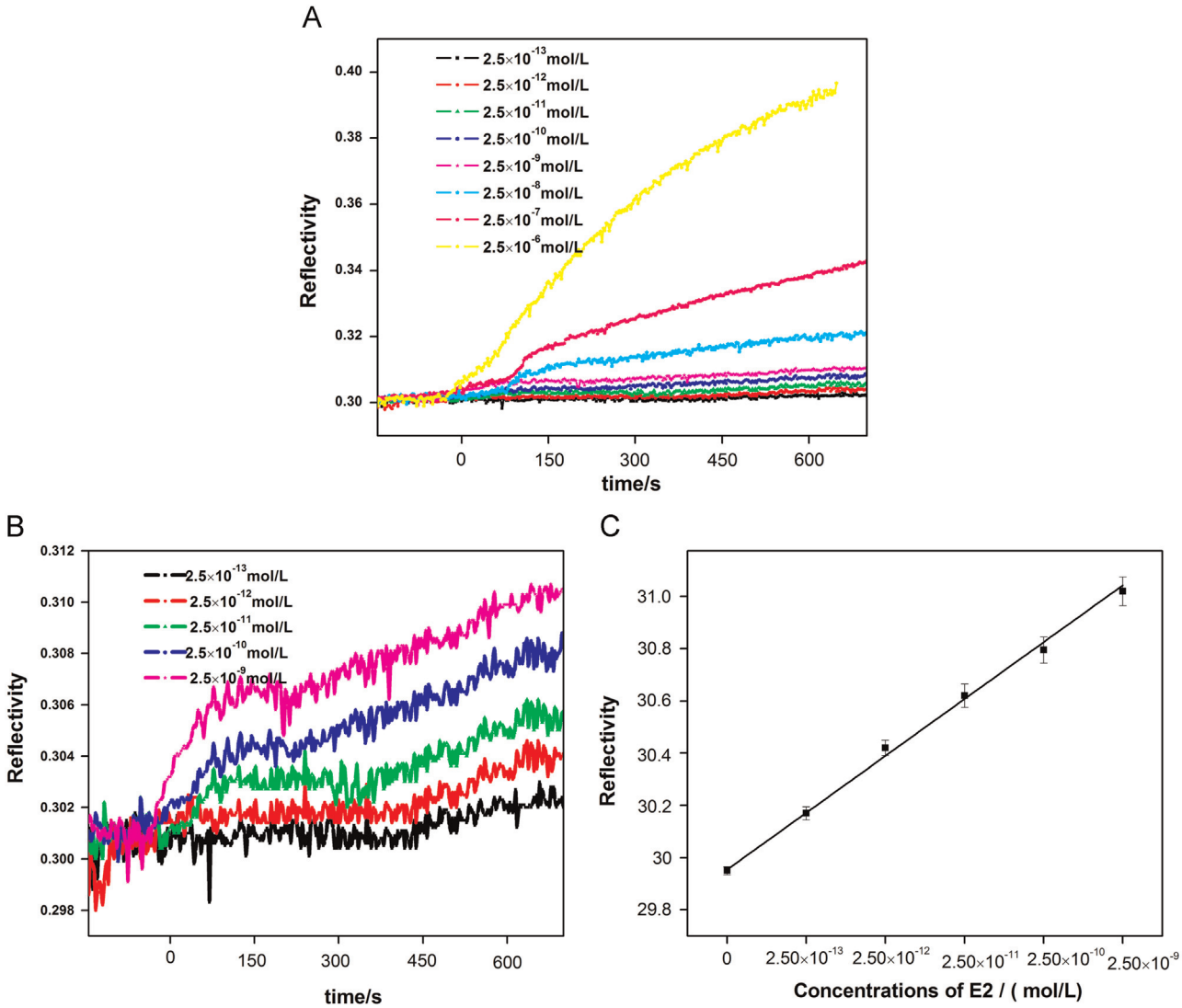


Fig. 6. (A) and (B) Adsorption dynamics to E2. (C) The linearity between the reflectivity changes and concentrations of the E2 concentrations from 2.50×10^{-13} to 2.50×10^{-9} mol/L.

Table 1
Comparison with other reported methods for the detection of E2.

Analytical methods	Environmental sample	Detection limit	Ref.
GC–MC	Human serum	1 pg/ml	[42]
SPE–LC–UV–ESI–MS	River water	1.8 pg/ml	[43]
SPE–HPLC–MS/MS	Wastewater treatment plan	0.4–2 pg/ml	[44]
Estrogen receptor biosensor	Buffer solution	0.6 ng/ml	[19]
SPR based immunoassay	Buffer solution	1.5 ng/ml	[45]
In-tube SPME–SPR immunoassay	Seawater/serum	0.17 ng/ml	[46]
HOWS biosensor	Buffer solution	50 pg/ml	[47]
Aptamer-based optical biosensor	Wastewater	0.6 ng/ml	[20]
Electrochemical biosensor	Human urine	2.0×10^{-12} mol/L (0.6 pg/ml)	[21]
Double-layer MIF-based biosensor	Seawater	0.07 pg/ml (2.5×10^{-13} mol/L)	This study

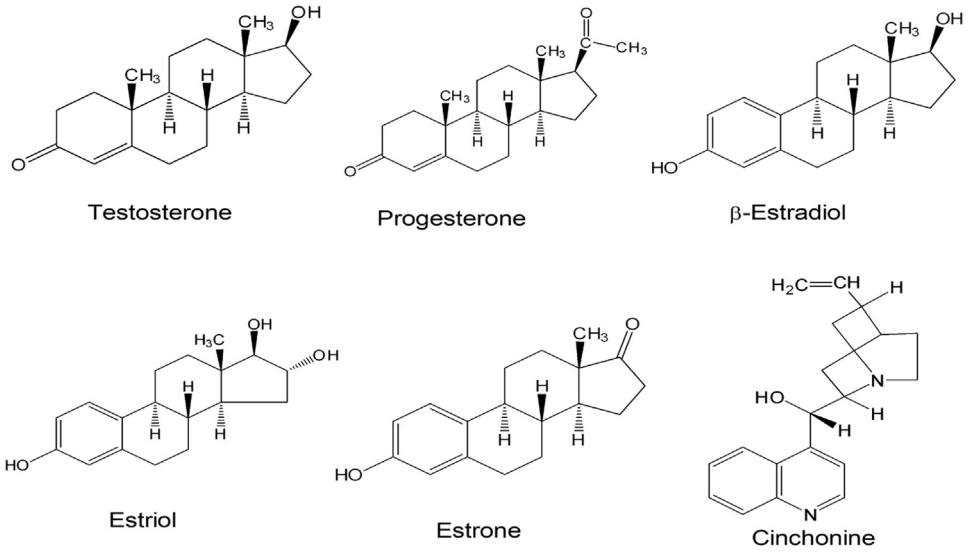


Fig. 7. Chemical structures of the six compounds investigated.

1st layer MIF. The SPR resonance angle shifted from 71.2° to 72.1°, indicating that the second layer of MIF was obtained.

3.2. Validation for the SPR method

3.2.1. Effect of pH on the response properties of MIF to E2

The pH effect on the sensor response was investigated in the 2.5×10^{-8} mol/L E2 various buffer solution from pH 3.0 to pH 8.0 at room temperature. The dependence of reflectivity with pH is shown in Fig. 5. It was found that the reflectivity of the MIF responsive to E2 increased with pH values increasing from pH 3 to pH 5. When the pH values ranged from 5 to 8, the adsorption of E2 on the imprinted film was decreased. As seen in Fig. 5B, the maximum reflectivity changes responding to E2 was up to 0.32 at pH 5.0–6.0. The high binding affinities at pH 5–6 can be explained because the pKa of MAA is 5.5 [39]. In the range of pH 5–6, both hydrogen bonding and electrostatic interaction are so strong that the uptake of E2 loading reaches the maximum for the imprinted polymer. Outside that range, the interaction between MAA and E2 will recede, even if not existing [40]. Therefore, optimum pH 5.0 was selected in the following experiments.

3.2.2. Real-time E2 monitoring

The effect of various concentrations of E2 on the SPR reflectivity on the MIF is shown in Fig. 6A and B. Obviously, the SPR responses gradually increase with the E2 concentrations increasing from 2.50×10^{-13} to 2.50×10^{-6} mol/L for which the cavities formed by departure of the template in the MIF were gradually taken by E2 analyte molecules. As shown in Fig. 6C, the reflectivity changes showed a good linearity ($R^2 = 0.993$) versus concentrations of the E2 concentrations from 2.50×10^{-13} to 2.50×10^{-9} mol/L, and the concentrations as low as 2.50×10^{-13} mol/L could be readily measured. The regression equation of E2 was $y = 26.3083 + 0.4353x$. Each point of the calibration graph corresponded to the mean value obtained from 5 independent measurements.

3.2.3. Precision and accuracy

Three different concentrations of E2 (2.50×10^{-9} and 2.50×10^{-10} mol/L) were evaluated in five independent series on the same day and five consecutive days. The relative standard deviation (RSD) values changed from 0.41 to 0.82 for intra-day and from 0.76 to 1.09 for inter-day precision. The low RSD values show that the proposed method has good precision [41]. In addition, both results obtained for intra-day and inter-day accuracy were $\leq 2.00\%$ [41].

3.2.4. Recovery

Table 1 gives the recent reported analytical methods for the determination of E2. Compared to the performance of electrochemical biosensor for the detection of E2 in aqueous solution [21], the presented double-layer MIF biosensor detection scheme offers about one order of magnitude higher sensitivity for the detection of E2.

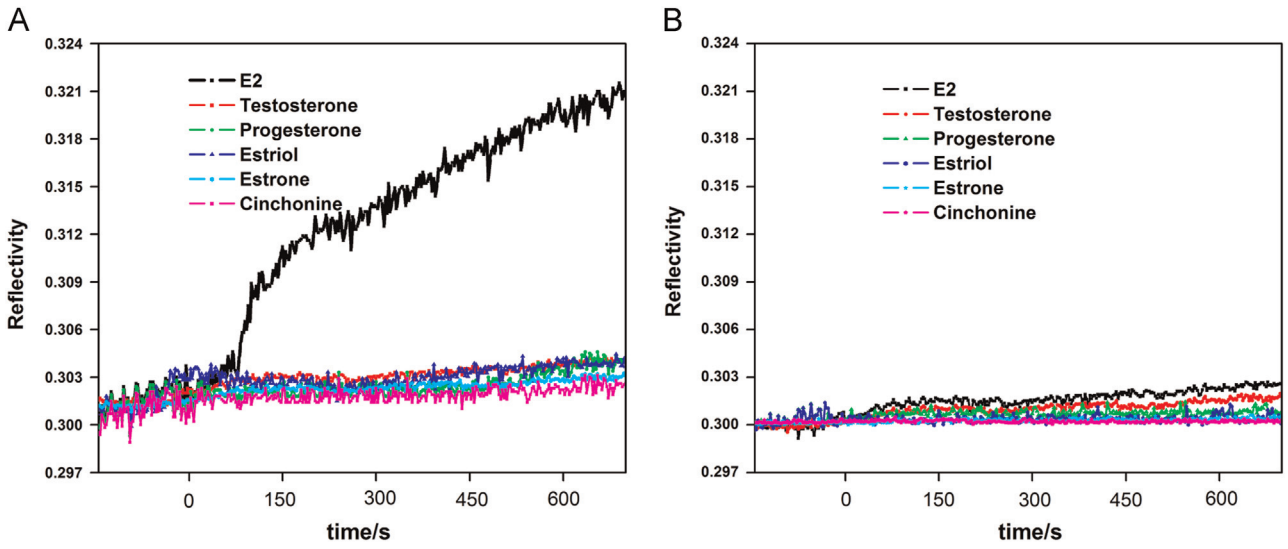


Fig. 8. The selectivity of MIF. The SPR response of (A) adsorption E2, testosterone, progesterone, estriol, estrone and cinchonine (each of 2.5×10^{-8} mol/L) on MIF; and (B) adsorption E2, testosterone, progesterone, estriol, estrone and cinchonine (each of 2.5×10^{-8} mol/L) on NIF.

3.2.5. *Selectivity of the MIF*

To confirm the selectivity of MIF, we used testosterone, progesterone, estriol, estrone and cinchonine as competing templates (The structures of them were shown in Fig. 7.). The results in Fig. 8A showed that because of selective cavities in the polymer structure, E2-imprinted SPR biosensor had higher adsorption capacity (ΔR=2.21) for E2 in comparison to testosterone, progesterone, estriol, estrone and cinchonine. E2-imprinted SPR biosensor shows low on-specific responses for testosterone (ΔR=0.418), progesterone (ΔR=0.400), estriol (ΔR=0.398), estrone (ΔR=0.301) and cinchonine (ΔR=0.24). These responses resulted from the structural and physico-chemical similarities between E2 and the analog molecules. To display the specificity of MIF, NIF was also prepared and the signals against E2, testosterone, progesterone, estriol, estrone and cinchonine were obtained as 0.252, 0.178, 0.096, 0.081, 0.035 and 0.031, respectively (Fig. 8B). These results demonstrated that the E2-imprinted films possessed good selectivity for E2 molecules.

3.2.6. *Compared to single-layer MIF*

The results in Fig. 9 showed that this double-layer MIF biosensor had much higher adsorption capacity ($\Delta R\%$=2.21) for E2 in comparison to the single-layer MIF ($\Delta R\%$=0.4). These responses may be because there were no recognition cavities in the single-layer MIF and a high fraction of a cross-linker in the second layer MIF can stabilize the binding sites better when the film detected E2 in the aqueous environment.

3.2.7. *Reproducibility of the MIF-SPR sensor*

In order to show the reproducibility of the MIF, about 10 adsorption–desorption cycles were repeated by 2.5×10^{-8} mol/L E2. As seen in Fig. 10, E2-imprinted SPR biosensor has demonstrated repeated reflectivity response during the cycles.

3.2.8. *Fabrication reproducibility and stability of the MIF*

The fabrication reproducibility was evaluated with five different SPR chips that were prepared independently by the same procedure as in Section 2.2.2. The RSD is 0.48% for the changes in reflectivity measuring for 2.5×10^{-8} mol/L E2 which demonstrate the reliability of the fabrication. In addition, the stability of E2-imprinted SPR biosensor was also investigated. After 90 days, the value of the reflectivity changes is approximately 98.91% of the original value.

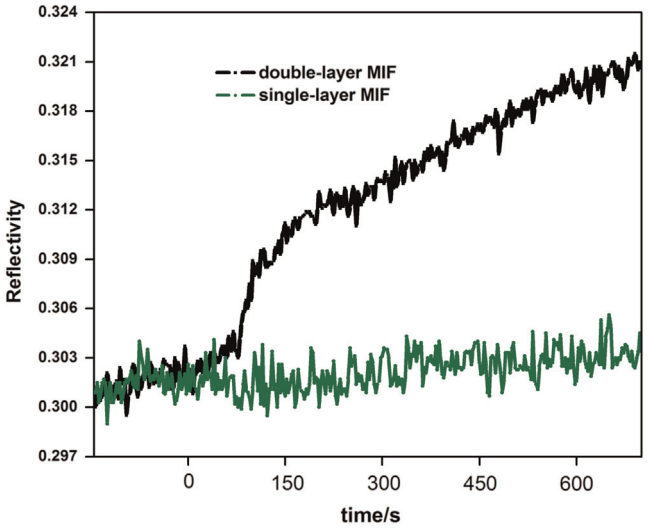


Fig. 9. The comparison of single-layer MIF.

3.3. *Wastewater samples analysis*

To study the influence of matrix effect on the biosensor response, several samples spiked with E2 were tested by spiking the filtered seawater samples. The concentration of E2 in the seawater samples (average of three determinations) and the recovery of E2 by the standard addition have been measured using the proposed biosensor. The results were summarized in Table 2. The recovery of all measured samples was between 96.1% and 101.4%. These results indicated that the proposed biosensor system could be applied to E2 analysis in real environmental water samples.

4. Conclusions

In conclusion, we have developed a novel double-layer MIF based biosensor for rapid and selective E2 using a portable and easy-to-use biosensing platform. The sensing process can be completed in less than 10 min, with a detection limit of 2.5×10^{-13} mol/L. The in-situ photo-grafting polymerization method used to prepare the MIF and

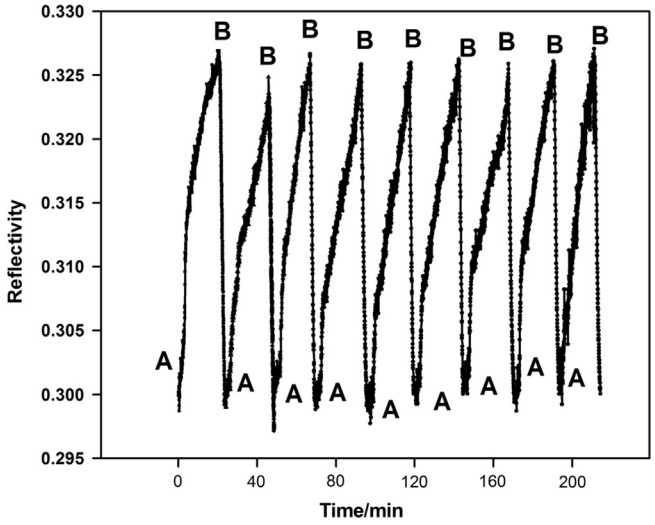


Fig. 10. Reproducibility of the MIF: (A) adsorption; and (B) desorption (for 2.5×10^{-8} mol/L E2).

Table 2
Detection results of E2 in waste seawater samples[a].

Sample	Detected (mol/L)	Added (mol/L)	Found after adding (mol/L)	Recovery (%)
Sample 1#	2.20×10^{-10}	2.55×10^{-10}	4.65×10^{-10}	96.1
Sample 2#	1.87×10^{-11}	2.10×10^{-11}	4.00×10^{-11}	101.4
Sample 3#	2.46×10^{-12}	1.60×10^{-12}	4.05×10^{-12}	99.4

[a] The sample from each sample had been detected separately.

the polymerization dynamics of MIF to monitor the course that the two layers MIF formed could monitor the whole course accurately. The effective surface regeneration procedures allow tens of times cycles without any significant loss of its performance. The performance of the biosensor evaluated in spiked seawater samples showed good recovery, precision and accuracy, indicating that it was not susceptible to water matrix interferences even without the need of complicate sample pretreatments. All of these results illustrated that the biosensor developed here could be readily extended toward the on-site monitoring of the other trace small molecular pollutants in environmental matrices with the employment of different template modified by the double-layer MIF.

Acknowledgment

This research was supported by the National Natural Science Foundation of China (No. 20771015) and the National "111" Project of China's Higher Education (No. B07012).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2015.04.019

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