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Highly Sensitive, Label-Free Detection of 2,4-Dichlorophenoxyacetic Acid using an Optofluidic Chip

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KEYWORDS: Optofluidic chip, microring resonator, silicon photonics, label-free detection, 2,4-dichlorophenoxyacetic acid.

ABSTRACT: A highly sensitive approach for rapid and label-free detection of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) using an optofluidic chip is demonstrated. The optofluidic chip is prepared by covalent immobilization of 2,4-D-bovine serum albumin (2,4-D-BSA) conjugate to an integrated microring resonator. Subsequent detection of 2,4-D carried out in a competitive immunoreaction format, enables selective detection of 2,4-D in different types of water samples, including bottled, tap and lake water, at a limit of detection (LOD) of 4.5 pg/mL and in a quantitative range of 15 – 10^5 pg/mL. The microring resonator-based optofluidic chip is reusable with ultra-high sensitivity that offers real-time and on-site detection of low molecular weight targets for potential applications in food safety and environmental monitoring.

Due to the growing public awareness of environmental and health safety issues, it has become increasingly important to develop analytical systems that enable rapid and sensitive detection of harmful compounds and pollutants. Herbicides, fungicides and pesticides constitute a family of low-molecular weight chemicals that are extensively used in agricultural and industrial practices, which may lead to the contamination of soil as well as ground and surface water. Most of them are known to affect the health of both human and wildlife, causing cholinergic dysfunction, carcinogenic activity or endocrine complications, etc.[1-3] 2,4-dichlorophenoxyacetic acid (2,4-D) is one of the most frequently used herbicide in agriculture and forestry. Its widespread use and high solubility has implication for natural water and soil resources.[4] The guidelines for 2,4-D in drinking water is 30 μg/L according to WHO[5] and European Union Regulations states that the level of 2,4-D in drinking water should be less than 0.1 μg/L.[6] The levels of herbicide is commonly determined by high-performance liquid chromatography (HPLC) and gas chromatography/mass spectrometry (GC/MS) methods. However, these techniques usually involve sophisticated laboratory-based instrumentations, skilled operators and are thus not suitable for on-site monitoring. Thus, the development of real-time, rapid, highly sensitive and low-cost methods for monitoring 2,4-D in water samples is of prime concern for the analytical and environmental communities.

Numerous efforts have been made to develop 2,4-D immunosensors based on antigen-antibody bioaffinity interaction with the merits of high specificity, which include enzyme-linked immunosorbent assay (ELISA),[7] quartz crystal microbalance (QCM),[8] evanescent wave all-fiber immunosensor (EWFI),[9] field effect transistor,[10] and surface plasmon resonance (SPR) immunosensor.[11-13] These methods offer a limit of detection (LOD) in the range of 0.008 – 10 μg/L depending on the level of instrumental sophistication and whether labels are employed to amplify the binding event. To further improve LOD for robust on-site detection of toxic chemical pollutants like 2,4-D, a new sensing platform based on a silicon photonic microring resonator is proposed. As a refractive index sensitive optical sensor, silicon photonic microring resonator has proven to be a promising platform for label-free detection. The sensing platform enables monitoring of chemical reactions and molecular binding events without chromophoric, fluorescent or enzymatic tags, thus eliminating the cost and complexity, preventing the labeling heterogeneity and perturbative binding interactions that are associated with the labeling process.[14] The microring resonators[15-22] mainly have been used for the detection of macromolecular targets such as proteins,[15,16] microRNAs,[17] cancer biomarkers,[14,23] etc. The detection of low molecular weight compounds remains challenging using microring resonators because of the minute shifts of the resonance wavelength caused by direct binding of such molecules at low concentrations.

In this paper, we report a real-time and highly sensitive indirect competitive immunoassay for 2,4-D detection using a silicon photonic microring resonator in an optofluidic chip. The performance of the microring resonator for 2,4-D detection, in terms of LOD, dynamic range and cross-reactivity is investigated and benchmarked against alternative detection methodologies. Regeneration of the chip

surface for multiple use is also demonstrated. The microring resonator also has been applied to the detection of 2,4-D spiked in different water samples, including bottled water, tap water, artificial and natural lake water. Our findings demonstrate that the proposed sensing platform offers great potential for detection and quantification of low molecular weight targets by employing an indirect competitive immunoassay.

CHIP DESIGN AND DETECTION MECHANISM

Figure 1 shows the schematic design of the optofluidic chip, which consists of a microring resonator system for optical detection and a microfluidic system for buffer manipulation. The sensing unit consists a bus waveguide and a micrometer-sized ring-shaped optical waveguide fabricated on a silicon-on-insulator (SOI) chip.[24,25] The cross-section of the waveguide and the ring is 450 × 220 nm (width × thickness) while the ring radius is 20 µm. The coupling gap between the linear waveguide and the ring is 200 nm. Light is coupled into the bus waveguide through the tapered lensed fiber. Specific wavelengths of light are resonantly confined in the microcavity, i.e. $m\lambda = 2\pi r n_{eff}$, where m is an integer, λ is the wavelength of light, r is the radius of the ring and n_{eff} is the effective refractive index (RI) of the ring resonator and the bounding dielectric medium.[26,27] The wavelength of light that resonates within the microring is extremely sensitive to the local changes in the refractive index, which is employed as a sensitive label-free biosensor to monitor biomolecular binding events occurring at the surface of the microring resonator.[17] The device offers an estimated Q-factor > 5 ×10^4. The narrow resonance wavelengths along with the high-Q factor of the resonator offer the possibility to resolve minute spectral shifts induced by effective RI changes at the surface of the microring resonator.

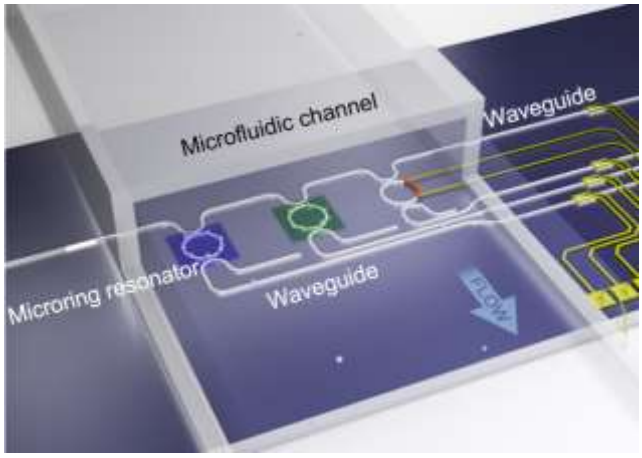


Figure 1. Schematic illustration of the optofluidic chip, that consists of a microfluidic system to facilitate buffer exchange and sample injection, and a microring resonator photonic system for 2,4-D detection.

The detection mechanism for small molecules such as 2,4-D is based on a heterogeneous competitive immunoassay, whereby the detection of 2,4-D in water sample is indirectly based on the binding of free monoclonal anti-2,4-D on the microring resonator. The surface of the microring resonator is first decorated with 2,4-D-BSA conjugates by covalent immobilization (Figure 2a). Then, a fixed volume of water sample is added to a fixed concentration of monoclonal anti-2,4-D. In the presence of 2,4-D in the water sample, some of the monoclonal anti-2,4-D binding sites are occupied with 2,4-D. Subsequently, when the mixture is injected into the microring resonator using the microfluidic platform, free monoclonal anti-2,4-D binds to the 2,4-D-BSA conjugates on the surface, leading to a certain wavelength shift. The resonance wavelength shift of the microring resonator due to binding of the monoclonal anti-2,4-D is shown in Figure 2b. The shift is correlated with the amount of monoclonal anti-2,4-D bound on the surface, which serves as the basis for sensor calibration and analyte concentration determination. Thus, higher analyte concentration results in less free monoclonal anti-2,4-D available for binding to the chip surface, and consequently, the wavelength shift decreases. To realize multiple usage of the optofluidic chip, non-covalent interaction between the antibody and the antigen is disrupted during rinsing in the regeneration buffer and moves the wavelength back to the original level obtained upon immobilization of the 2,4-D-BSA conjugate. The employed covalent immobilization strategy of the 2,4-D conjugate to the chip surface offers improved stability compared to immobilized antibodies, particularly during the surface-regeneration process.

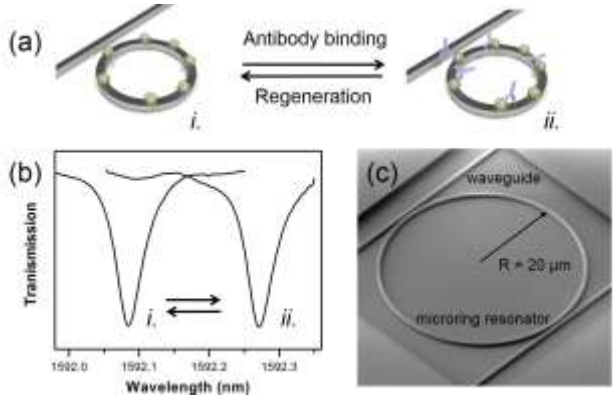


Figure 2. (a) Schematic illustration of the biosensing principle based on the microring resonator and (b) the corresponding transmission spectra obtained before (i) and after (ii) antibody (5 µg/mL) binding to the 2,4-D-BSA conjugate. (c) SEM image of the fabricated microring resonator structure.

EXPERIMENTAL SECTION

The silicon photonic chip was fabricated on a silicon-on-insulator (SOI) wafer. Both the ring resonator and the bus waveguide were silicon patterned by Deep-UV lithography followed by reactive ion etching (RIE) (Figure S1). Figure 2c shows the scanning electron microscope (SEM) image of the fabricated ring resonator structure. For surface modification, the chip surface was cleaned with O_2 plasma for 5

min prior to immersion in 1 % 3-mercaptopropyl-trimethoxysilane (MPTMS, Sigma-Aldrich) in toluene for 4 hours to introduce free thiol-groups. Subsequently, the chip was thoroughly washed with copious amounts of toluene and ethanol to eliminate excess MPTMS. The chip was then dipped into 2mM *N*-(4-maleimidobutyryloxy)succinimide (GMBS, Sigma-Aldrich) ethanol solution for 1 hour to introduce the NHS groups. After rinsing the chip with ethanol, the conjugate 2,4-D-BSA (J&Q Environmental) with approximately 20 haptens/BSA was dissolved in PBS solutions (100 μg/mL), drop-casted on the chip and incubated overnight at the temperature of 4°C. To block non-specific binding sites and remove weakly bound conjugates, the chip was immersed in 1 mg/mL bovine serum albumin (BSA, Sigma-Aldrich) in PBS (pH 7.4) for 20 min. In the experiment, the silicon photonic chip is attached to a home-made cell with a microfluidic flow channel defined by a laser-cut gasket (perfluoroelastomer, Kalrez), which is mounted on top of the microring arrays and sandwiched between the aluminum chip holder and lid, forming the integrated optofluidic chip. An Amplified Spontaneous Emission (ASE) broadband Light Source (Amonics, ALS-CL-13-B-FA) operating with a center wavelength of 1550 nm is coupled into the bus waveguide through a tapered lensed fiber. A polarization controller is used to tune and select Transverse Electromagnetic (TE) component of the light for injection into the waveguide. Subsequently, a tapered lensed fiber is used to couple the transmitted light out from the microring resonator and an Optical Spectrum Analyzer (Yokogawa, AQ6370D) is employed to capture the transmitted light spectrum with resonance wavelengths. The alignment of the fibers and the optofluidic chip is precisely controlled by a nano-positioning system (Figure S2).

Prior to 2,4-D detection, 0.1 mg/mL BSA in PBS (pH 7.4) buffer was used as a calibration buffer (running buffer) to decrease the nonspecific adsorption of antibodies in the flow cell and tubings. Then, different concentrations of 2,4-D (Sigma-Aldrich) solutions were prepared by spiking it in Milli-Q water. Subsequently, monoclonal anti-2,4-D (J&Q Environmental, 10 μg/mL in 10× concentrated PBS buffer, containing 1 mg/mL BSA) were mixed with the 2,4-D samples in 1: 9 (v/v) ratio and incubated at room temperature for 10 min before injecting the mixture into the optofluidic chip. The optical spectrum was recorded online for 15 min. Afterwards, the chip surface was washed with the running buffer and the resonance peak shift was calculated. After each detection cycle, the surface was regenerated by incubating the microring resonator with a regeneration buffer of 10 μg/mL pepsin (Type A from porcine gastric mucosa; ≥ 250 U/mg, Sigma-Aldrich) in 0.2 M glycin-HCl (pH = 2, Sigma-Aldrich) for 30 s, breaking the antibody-antigen association. The running buffer was then introduced into the channel again to stabilize the signal, and the chip is ready for the next detection cycle. In addition, 2,4-dichlorophenol (2,4-DCP, Sigma-Aldrich) and 4-chlorophenoxyacetic acid (4-CPA, Sigma-Aldrich), whose chemical structures are similar to 2,4-D, were measured using the optofluidic chip to evaluate the immunosensor performance in term of cross-reactivity. The standard curves for the compounds were executed under the same conditions and compared with the value obtained for the 2,4-D. The cross-reactivity (CR) is calculated as in Eq. 1,

$$CR = C_{I\text{-}50\ (2,4\text{-}D)} / C_{I\text{-}50\ (cross\text{-}reactant)} \times 100\%, \quad (1)$$

where $C_{I\text{-}50}$ is the concentration of analyte exerting 50% inhibition.

To further evaluate the performance of the microring resonator in real water samples, 2,4-D solutions prepared using tap water, bottled water and lake water were analyzed. For example, the tap water directly collected in laboratory was spiked with 2,4-D at 0.05, 1 and 10 ng/mL from a 0.8 mg/mL 2,4-D stock solution. Monoclonal anti-2,4-D (10 μg/mL in 10X concentrated PBS buffer, contain 1 mg/mL BSA) were then mixed with the spiked water samples in 1: 9 (v/v) ratio and incubated at room temperature for 10 min before injecting it into the optofluidic chip. The lake water samples obtained from an artificial and a natural lake were filtered through 220 nm microporous syringe filters before being spiked with 2,4-D.

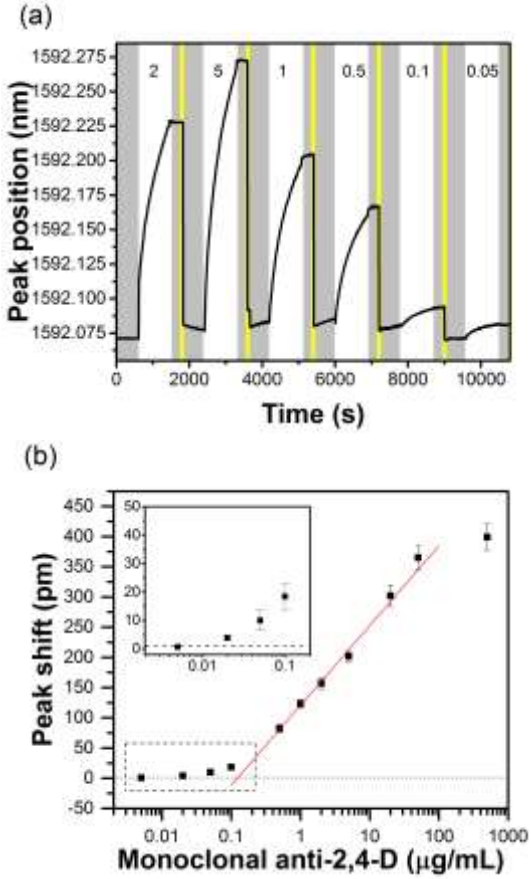


Figure 3. (a) Resonance wavelength for the binding of monoclonal anti-2,4-D at various concentrations (2, 5, 1, 0.5, 0.1 and 0.05 μg/mL)(white areas) followed by rinsing in the calibration buffer (narrow gray areas) and regeneration of the active chip surface using a pepsin containing regeneration buffer (yellow areas); Wide grey areas represent exposure the calibration buffer. (b) Wavelength shifts for the binding of monoclonal anti-2,4-D to the 2,4-D BSA modified sensor chip. The dashed line at y=0.97 pm (see inset) indicates resolution of the sensor with the signal-to-noise ratio of 3.

RESULTS AND DISCUSSION

Antibody binding and surface regeneration

Specific immunobinding of monoclonal anti-2,4-D to the 2,4-D-BSA decorated optofluidic chip surface is shown in Figure 3a (white areas). Different concentrations of monoclonal anti-2,4-D solutions, ranging from 5 ng/mL to 500 μg/mL, were injected in a random sequence for 15 min and the resonance peak positions were monitored in real time as shown in Figure 3a and Figures S3 and S4. Then, the calibration buffer was injected for 5 min (narrow grey areas) to remove any physically absorbed or non-specifically bound monoclonal anti-2,4-D from the chip surface to yield the final wavelength shifts. To allow for multiple detection cycles with the same optofluidic chip, the monoclonal anti-2,4-D specifically bound on the 2,4-D-BSA-modified surface had to be dissociated without disrupting the binding of the antigen conjugate to the surface. Pepsin in glycin-HCl buffer (pH = 2.0) was used as the regeneration buffer because pepsin is found to be suitable for the elution of the antibody without affecting the underlying antigen conjugate.[28] The regeneration buffer was injected for 30 s (yellow areas in Figure 3a) to restore the signal to the original level. The 2,4-D-BSA decorated optofluidic chip could be regenerated more than 30 times without substantial loss of the signal, thus enabling consecutive analyses using the same chip. These binding/regeneration experiments were repeated at least 3 times for each antibody concentration. In between each detection cycle, BSA in PBS (calibration buffer) was injected for 10 min to stabilize the signal (wide grey areas in Figure 3a). For consecutive injections of the same concentration of monoclonal anti-2,4-D, an almost identical resonance peak shift was obtained (see error bars in Figure 3b), which confirms a reproducible recovery of the binding capacity of the 2,4-D-BSA coating upon surface regeneration. It is worthwhile mentioning that higher pepsin concentrations and/or extended exposure to the regeneration solution induced irreversible functional damage to the sensing layer. The wavelength shifts versus monoclonal anti-2,4-D concentration are plotted in Figure 3b. The wavelength shift increases exponentially with the concentration of monoclonal anti-2,4-D in the region 0.2-50 μg/mL, above which the wavelength shift levels off. For lower concentrations (<0.5 μg/mL), the wavelength shift increases linearly with the concentration of monoclonal anti-2,4-D (Figure S6 inset). The baseline of the microring resonator was recorded and the noise level was calculated (Figure S5). Finally, the LOD of monoclonal anti-2,4-D on the optofluidic chip was estimated to be 7 ng/mL at a signal-to-noise ratio of 3.

2,4-D competitive immunoassay

Competitive immunoassay is employed for the quantification of the 2,4-D analyte at low concentrations. During the immunoassay measurement, standard solutions of 2,4-D in Milli-Q water at various concentrations were premixed with 10 μg/mL of monoclonal anti-2,4-D in 1 mg/mL BSA 10× PBS at a volume ratio of 9: 1 for 10 min. This recipe was chosen to decrease the matrix effect and keep the pH and buffer conditions of the mixture. Then, the pre-reacted mixture was delivered into the optofluidic chip. The antibodies with the unoccupied binding sites bound to the chip surface with immobilized 2,4-D-BSA antigen. The resonance wavelength was recorded for 15 min, followed by a 5 min injection of the calibration buffer. Regeneration of the chip surface was carried out with the regeneration buffer for 30 s. Subsequently, the chip surface was stabilized for 10 min in the calibration buffer prior to next sample injection.

In the pre-reacted mixture, the 2,4-D molecules in the solution bind to monoclonal anti-2,4-D, inhibiting the antibody binding to the chip surface. A calibration curve is shown in Figure 4, which was normalized by expressing the peak shift of each concentration as the ratio of the maximum response. For each concentration, the sensor response was measured in triplicates and the standard deviation was determined. As anticipated in the competitive immunoassay, the sigmoidal analytical curve was obtained and the shift of resonance wavelength is inversely proportional to the concentration of 2,4-D. The curve was fitted to a four-parameter logistic equation, Eq. 2.

$$y = \frac{A_0 - A_1}{1+(\frac{[x]}{[x_0]})^p} + A_1, \qquad (2)$$

where A_0 and A_1 are the upper and lower asymptote to the titration curve, respectively; $[x]$ and $[x_0]$ represent the analyte concentration in the sample and at the inflection point, respectively, while p is the slope at the inflection point. The red curve in Figure 4 represents the fitting data with R = 0.9983. The $C_{I\text{-}50}$ is determined by the calibration curve as 1.42 ng/mL (6.42 nM). The linear region of the dose-response curve for 2,4-D spans the concentration range from 15 pg/mL (0.067 nM) to 100 ng/mL (0.45 μM) with R = 0.9988.

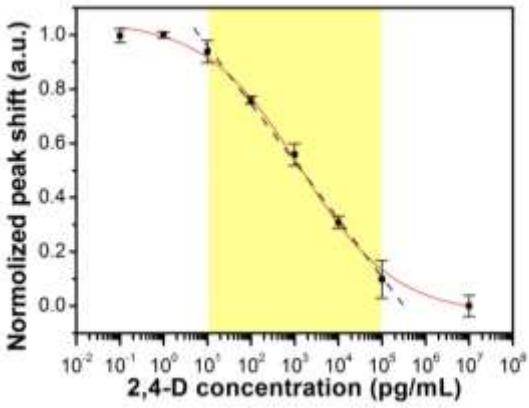


Figure 4. Calibration curve of normalized peak shift vs. 2,4-D concentration determined using silicon photonic microring resonator, red line represents the logistic fitting curve; the yellow regime indicates the quantitative detection range, showing the linear relationship (dash line) between the 2,4-D concentration and the shifts.

Table 1. C_{I-50} values and cross-reactivity detected by 2,4-D-BSA modified microring resonator sensor chip against organic compounds structurally analogous to 2,4-D.

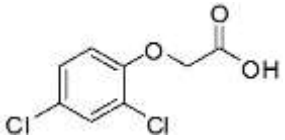
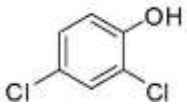
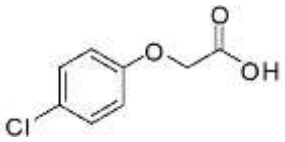
Compound	Structure	C_{I-50} (ng/mL)	Cross-reactivity (%)
2,4-D		1.42	100
2,4-DCP		300	0.47
4-CPA		400	0.36

Table 2. Detection results of four different water samples each spiked with three 2,4-D concentrations.

Source	**2.4-D spiked to the samples (pg/mL)**	**2.4-D determined by microring resonator (pg/mL)**	**Coefficient of Variation (CV, %)**	**Recovery (%)**
Tap water	50	56.4	6.7	112
	1,000	1,026.7	9.6	102.6
	10,000	10,834.4	6.8	108.3
Bottle water	50	49.8	8.9	99.6
	1,000	1,015.8	5.3	101.5
	10,000	10,659.4	9.8	106.6
Nanyang Lake*	50	57.5	10.2	114
	1,000	989.2	10.8	98.9
	10,000	10,203.7	11.2	102.3
Jurong Lake#	50	44.9	12.4	89.9
	1,000	1,053.9	14.9	105.4
	10,000	10,711.7	19.0	107.1

*Artificial lake on NTU campus; # Jurong lake is a natural lake in Singapore (freshwater lake and reservoir located in the western region of Singapore that connects with the damming of Sungei Jurong further downstream).

The limit of quantification for 2,4-D (10 % of inhibition calculated from the calibration curve) is approximately 15 pg/mL. The limit of detection (LOD) of this competitive immunoassay based on silicon photonic microring resonator for 2,4-D is determined to be 4.5 pg/mL, based on the 5 % of inhibition value obtained from the calibration curve.[29] This value is 20 times lower than the regulated concentration level for individual herbicide in drinking water demanded by the European Union. Moreover, the LOD of the microring resonator optofluidic chip is several orders of magnitude lower than those reported in the previous studies: commercial ELISA kit for 2,4-D exhibited a LOD of 1 ng/mL; electrochemical impedance showed a LOD of 10 ng/mL;[30] immunosensors employing fluorescent markers yielded a LOD as low as 70 pg/mL,[9,31] and conventional SPR provided a LOD of 0.1 ng/mL.[12] Improved detection sensitivity has been achieved by using a SPR method and an additional sandwich immunoassay signal enhancement strategy to yield a LOD of 8 pg/mL.[13] Thus, the LOD obtained in our microring resonator-based assay is competitive with the best immunoassays reported in the literature. To the best of our knowledge, this is the first demonstration of 2,4-D detection by employing silicon photonic microring resonator in a competitive immunoassay format. Importantly, the combination of microfluidic system in the optofluidic chip guarantees that less volume of solution is

required (~20 µL), as compared to conventional ELISA that requires at least 100 µL for each microwell.

Cross-reactivity and spiked environmental water samples

To explore the potential interference from other environmental pollutants, the response of the microring resonator against 2,4-DCP and 4-CPA were also examined. The chemicals chosen are potential endocrine disrupting pollutants that are structurally very similar to 2,4-D. Their wavelength shifts were monitored using the 2,4-D-BSA-decorated optofluidic chip using the same indirect competitive immunoassay method. The concentrations of the compounds that exerted 50 % inhibition were determined and summarized in Table 1 together with the corresponding cross-reactivities based on Eq. 1. The optofluidic chip exhibits excellent selectivity towards 2,4-D with respect to the other closely resembling pollutant molecules, which, to a large extent, is attributed to the excellent selectivity of the antibody used herein.

The influence of matrix effects on the detection of 2,4-D, water samples (tap water, bottle water, filtered lake water) spiked with 2,4-D with concentration ranging from 50 pg/mL, 1 ng/mL and 10 ng/mL were also investigated using the developed competitive immunoassay. The results from a series of experiments are summarized in Table 2. All samples were tested unspiked first to ensure that they did not contain any 2,4-D. The recovery of all measured samples was ranging in 90% - 115% and the samples of different origin displayed comparable levels of recovery. The CV values were essentially identical for bottled and tap water (<10 %). However, the CV values for water collected from the artificial and natural lakes gradually increased to double-digit values. Although the CV values increase with increasing complexity of the sample matrix, our findings clearly infer that the optofluidic chip with the integrated microring resonator can be applied for sensitive detection of 2,4-D in water of different origin.

CONCLUSIONS

A highly sensitive optofluidic chip based on silicon photonic microring resonator has been developed for rapid and label-free detection of 2,4-D. A competitive immunoassay was used for sensitive 2,4-D detection with a limit of detection of 4.5 pg/mL and quantitative detection range of 15 – 10^5 pg/mL. The optofluidic chip displayed numerous advantages including robustness, reusability, high sensitivity and applicability for analysis in complex water sample matrices. It also demonstrated excellent cross-selectivity values against structurally similar compounds. Taken together, the proposed assay format and chip design offer great potential for on-site analysis of environmental samples, especially for soil, food and water quality monitoring.

ASSOCIATED CONTENT

Supporting Information.
The Supporting Information is available free of charge on the ACS Publications website.

Description of chip fabrication process; schematic illustration of the sensing setup; real time monitoring of the 2,4-D-BSA functionalized microring resonator's responses on sequence sensing of a variety concentration of monoclonal anti-2,4-D; noise analysis; calibration curve of resonance wavelength shift vs. concentration of monoclonal anti-2,4-D; and the fitting result of the dose response curve of the competitive immunoassay.

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Notes

The authors declare no competing financial interest.

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