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Direct analysis of trichloropyridinol in human saliva using an Au nanoparticles-based immunochromatographic test strip for biomonitoring of exposure to chlorpyrifos

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ABSTRACT

A portable immunochromatographic strip-based biosensor for direct detection of trichloropyridinol (TCP), a specific biomarker of exposure to chlorpyrifos, in human saliva sample is presented. In this approach, a series of immunoreactions was performed on the test strip, where the targeted analytes (TCP) bound to the Au nanoparticles-labeled antibodies on the conjugate pad to form analyte–Au–antibody conjugates, and then free Au-labeled antibodies were captured by TCP–BSA in the test zone. Captured Au nanoparticles, increased with decreased levels of analytes, can be observed visibly without any equipment and later quantified by a colorimetric reader. Several experimental parameters were optimized including Au nanoparticle-to-TCP antibody coupling ratio, the amount of Au-labeled TCP antibody, immunoreaction time, the pretreatment of sample pad and the preparation of stock solution of Au–TCP antibody that realize sensitivity, selectivity and direct detection of TCP. Under optimal conditions, this biosensor displays a highly linear range of 0.625–20 ng/mL TCP, with a detection limit of 0.47 ng/mL. Moreover, the immunosensor was successfully used for direct analysis of human saliva sample without any pretreatment. These results demonstrate that this Au nanoparticles-based immunochromatographic test strip (ITS) provides a simple, accurate, and quantitative tool for TCP detection and holds a great promise for point-of-care and in-field analysis of other biomarkers.

1. Introduction

Organophosphorus (OP) pesticides have broad household and agricultural applications worldwide. These OP compounds inhibit acetylcholinesterase (AChE) and can be acutely toxic to humans [1–3]. Chlorpyrifos (CPF), one of the most extensively used OP insecticides, can affect the central nervous system, the cardiovascular system, as well as the respiratory system due to its high acute toxicity [4,5]. CPF is metabolized to form chlorpyrifos-oxon (CPF-oxon) or 3,5,6-trichloro-2-pyridinol (TCP) when ingested, inhaled, or absorbed into the bloodstream [6]. CFP-oxon can be further metabolized to TCP and diethylphosphate. Since TCP is the primary excreted metabolite of CPF exposure, it is one of the most commonly used biomarkers for biomonitoring CPF exposure [7]. Since TCP is present in the environment and is a metabolite of other pesticides (ex. trichlopyr), TCP is not a specific biomarker for CPF exposure [8,9]. In 1995, it was estimated that up to 82% of adults in the United State contained detectable levels of TCP in their urine [10], indicating exposure to CPF or TCP. Though the United States restricted CPF from home use in 2002, it is still widely used in agriculture. Therefore, it is necessary to develop a simple, portable and sensitive analytical instrumentation for rapid in-field biomonitoring and screening the metabolite TCP for the exposure to CPF.

Several different analytical approaches have been developed to quantify exposure to OP pesticides such as the Ellman colorimetric assay [11], gas chromatography–mass spectroscopy [12], or liquid chromatography–mass spectrometry [13] and electrochemical detection [14,15]. However, these conventional strategies have some disadvantages such as the high-cost and the well-trained technicians should be needed. Recently, a host of various immunoassays for protein detection have been established such as surface-enhanced Raman scattering [16], lateral flow test strip [17], enzyme-linked immunosorbent assay (ELISA) [18], electrochemical immunosensor [19,20] and microsphere-based arrays [21]. In comparison to the

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traditional methods above, these approaches exhibit promising results for sensitive detection of protein, however there are still some formidable challenges including time-consuming sample treatment before analysis, the utilization of radioactive substances, and specialized equipment. For the insecticide CPF, immunoassays have been developed for analysis of the parent compound or metabolite residues in food, agrochemicals and environmental samples [22]. The development of immunochromatographic biosensors to identify individual exposure to OPs is attractive in recent years due to its promising potential as an alternative approach for rapid, inexpensive, sensitive and accurate biomedical diagnostic tools [23,24]. Previously, a detection method for TCP was developed in our group that relied on a quantum dot (Qdot)-based immunochromatographic fluorescent test strip, followed by fluorescent signals detection [25]. In this method, pretreatment steps were needed for the plasma samples, which was not convenient for in-field rapid diagnosis.

Human saliva is an attractive detection biofluid [26,27] because its collection is safe, convenient, non-invasive and real-time, which is more preferable than conventional matrices such as blood and urine. Furthermore, saliva contains a large array of proteins, many of which have been shown to be informative for the detection of drugs of abuse [28], hormones [29], heavy metals [30], systemic diseases [31] and pesticides [32,33]. Pesticides, like CPF, may affect salivary glands directly or indirectly, and may influence the composition of saliva [9,34]. Timchalk evaluated the pharmacokinetics of the CPF in blood and saliva, and a good correlation between them was reported, suggesting that saliva could be used as a biomonitoring matrix [9].

In the present work, the sample pad of ITS was pretreated with the pretreatment buffer developed in our lab, and the stock solution of Au–TCP antibody was optimized, which realized direct detection of the analyte in the biological sample. Meanwhile, in order to meet the requirement for in-field analysis, instead of Qdot, Au nanoparticles were employed as reporters for visible qualitative or semiquantitative detection without using a test-strip reader. Moreover, with a colorimetric reader, the Au nanoparticles-based ITS has been developed for directly detecting saliva TCP. Experimental results demonstrate that the Au nanoparticles-based ITS is simple, highly sensitive and selective, and holds a great promise for point-of-care and in-field diagnosis of exposure to pesticides.

2. Experimental

2.1. Reagent and materials

Mouse monoclonal anti-TCP antibody (2 mg/mL) was obtained from Strategic Diagnostics Inc. (Newark, Delaware). The HTCP, TCP derivatized with a carboxyl functional group, was prepared according to previous literature [25]. Au nanoparticles (~0.01%, $HAuCl_4$, 30 nm), phosphate buffer saline (PBS, 0.01 M), bovin serum albumin (BSA), *N*-hydroxy-succinimide (NHS), polyethylene glycol (PEG) 6000, Triton X-100, Tween-20, sucrose, trehalose and *N*,*N*′-dimethylformamide (DMF, 99.8%) were purchased from Sigma-Aldrich (St. Louis, MO). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was purchased from Fluka (St. Louis, MO). Casein was purchased from Bio-Rad (Hercules, CA). PD-10 columns were purchased from GE Healthcare UK Limited. Nitrocellulose membrane, absorbent pads, sample pads, conjugation pads, and backing cards were purchased from Millipore (Temecula, CA). All chemicals used in this study were analytical reagent grade. All stock solutions were prepared using deionized water purified with the Nanopure System (Barnstead, Kirkland, WA).

Saliva was collected from employees of a chlorpyrifos manufacturing and formulating facilities and employees of another facility that does not involve chlorpyrifos processing [35]. Saliva was collected using a plain Salivette® collection device (Sarstedt, Numbrecht, Germany). Briefly, volunteers were instructed to rinse out their mouths with water and then insert the cotton swab into their mouth. For 1 min, the swab is to be chewed, and then replaced back into the plastic receptacle once it is saturated with saliva. The cap was replaced; the vial was sealed with tape and frozen. At the time of measurement, the vial was brought to room temperature and centrifuged for 2 min at 1000 × *g* to extract saliva from the cotton swab. Saliva samples were collected from each individual on consecutive days, the first after a work day, the second after a non-work weekend. Samples were not identifiable back to the donating patient, thus this study was determined to be exempt status by the Institutional Review Board (IRB) at Battelle (2011-14 EXP).

2.2. Instrumentation

The ITS fabrication system consists of a XYZ-3050 Dispenser, LM5000 Laminator, and the Guillotine Cutting System CM4000 which were purchased from BioDot LTD (Irvine, CA). The XYZ-3050 Dispenser includes an AirJet Quanti 3000 dispenser and a BioJet Quanti 3000 dispenser. A portable test strip reader ESE-Quant GOLD, which can be connected to a laptop, was purchased from DCN Inc. (Irvine, CA) to collect the signal of the assay on the ITS. A centrifuge Eppendorf 5418 was purchased for concentration of Au nanoparticles and separation of Au nanoparticles-labeled TCP antibody. A bench-top centrifuge Eppendorf 5804 (Eppendorf, Germany) and Amincon Ultra 5 K centrifugal tubes (Millipore, Billerica, MA) were purchased for concentration and separation of TCP–BSA conjugate. Zeba spin 7 K columns were used for removing salt ions from TCP antibodies. The ELISAs were carried out on the Coring® 96 well High-binding EIA/RIA plates (Product# 3590). Absorbance was measured with a TECAN's Monochromator Microplate Reader connected to a personal computer.

2.3. Preparation of Au nanoparticles-labeled TCP antibody conjugate

TCP antibody was labeled by Au nanoparticles through the surface adsorption. The mechanism of adsorption is that the positive charge of the antibody formed static adsorption with the negative charge took by the surface of Au nanoparticles. First, the Au nanoparticles was five times concentrated (13,200 × *g*), and adjusted to pH 8.2. Then, 1.25 mL of the above concentrated Au nanoparticles solution was added to 15 μL of 2 mg/mL desalted TCP antibody, and the resulting solution was incubated at room temperature under gentle shaking for 1 h. To reduce the non-specific binding, an appropriate volume (~120 μL) of 10% BSA, was added to the above solution to a final concentration of 1% BSA, and followed by incubation under the same conditions for 30 min. The resulting solution was centrifuged at 13,200 × *g* for 15 min. The pink supernate was removed, and the dark red soft sediment that was washed by 1 mL of 1% BSA–PBS. Finally, the suspension was re-centrifugation (13,200 × *g*) for 15 min, and the pellet containing the Au nanoparticles-TCP antibody conjugate was suspended to a final volume of 1 mL PB buffer (0.01 M, pH 7.4) containing 5% trehalose, 0.5% casein, 15% sucrose and 1% BSA and stored at 4 °C.

2.4. Preparation of TCP–BSA

TCP–BSA was prepared by EDC-facilitated conjugation. First, TCP was preactivated by adding 500 μL of 0.2 M NHS and 500 μL of 0.2 M EDC to 1 mL of 0.1 M HTCP in dry DMF, and the resulting solution was incubated at room temperature under gentle shaking for 1 h. Then, 100 μL of the above peractivated HTCP solution was added to 1 mL sodium bicarbonate buffer (0.1 M, pH 8.1)

containing 10 mg/mL BSA, and the resulting solution was incubated under the same conditions for 6 h. The solution was subjected to PD-10 column separation to remove excess unconjugated HTCP and other small molecules. Finally, the collected TCP–BSA solution was concentrated by a 5 K centrifugal tube (7000 rpm) for 15 min and reconstituted to a final volume of 1 mL with 0.01 M PBS and stored at 4 °C, as a final concentration of 10 mg/mL.

2.5. Preparation and pretreatment of ITS

Au nanoparticles-based ITS is composed of a sample application pad, conjugate pad, nitrocellulose membrane, absorbent pad, and backing card. Both the sample pad (20 mm × 30 cm) and conjugation pad (8 mm × 30 cm) were made of glass fiber. The sample pad was pretreated with pretreatment PBS (0.01 M, pH 7.4) containing 2% PEG 6000, 2% Tween-20, 1% BSA, 2% Triton X-100 and 0.5% casein. Then it was dried at room temperature overnight and stored under the same condition. Meanwhile, a desired volume of TCP antibody-Au nanoparticles conjugate was dispensed on the conjugation pad with the dispenser XYZ-3050 AirJet Quanti 3000, dried at room temperature for 2 h, and stored at 4 °C. In addition, the test line of the ITS was prepared by dispensing 40-fold dilution of TCP–BSA conjugate (250 μg/mL) onto the nitrocellulose membrane (40 mm × 30 cm) using the dispenser XYZ-3050 BioJet Quanti 3000, dried and stored in the same condition with a conjugation pad. The absorbent pad was stored at room temperature without any treatment. All of the above parts were assembled on a plastic adhesive backing card using the Batch Laminating System LM5000, and each part overlapped 2 mm to ensure the solution migrating through the strip during the assay. Finally, the ITS with a 4 mm width was cut using the Guillotine Cutting System CM 4000 and assembled into strip cassettes for the following assay.

2.6. Immunochromatographic assay of TCP

An amount of 100 μL of sample solution containing a desired concentration of TCP in PBS was applied to the sample pad. PBS or saliva without TCP was used as a control. After the desired time (e.g., 15 min), immunoreactions among the Au nanoparticles-labeled TCP antibody, TCP and TCP–BSA, were completed as a red line or a colorless line appeared at the test zone. The images were captured directly by a Sony DSLR-A300 digital camera. Meanwhile, the cassette was inserted into the reader ESE-Quant GOLD, and the signal from the Au on the test zone was recorded accordingly to quantify the analytes. For detecting human concentration of TCP, 70 μL of saliva filtrate extruded from the Salivette® collection device was added to the sample pad. After about 15 min, results were observed visually, and quantified by reading the optical response with reader ESE-Quant GOLD.

2.7. Enzyme-linked immunosorbent assay (ELISA)

The one-side competitive ELISA experiments were carried out with the Coring® high-binding microplates. Concentrations of analyte (TCP) and TCP–BSA were the same as the one used in ITS detection. Responses were recorded using a Tecan microplates reader.

3. Results and discussion

3.1. Principle of the method

Fig. 1 schematically illustrates the configuration and measuring principle of the Au nanoparticles-based ITS. It is based on the immunoreaction that the antibody (Au nanoparticles-labeled TCP antibody) was captured separately by the analyte (TCP or saliva) dropped into the sample pad and TCP–BSA immobilized on the test zone. During the detection, 100 μL of aqueous sample containing TCP was applied onto the sample pad as shown in Fig. 1A. Then capillary action causes the liquid sample to migrate toward the other end of the strip. When the sample reached the conjugated zone, the Au–TCP antibody pre-loaded in the conjugation pad bound with it according to the specific antibody-antigen interaction. Then TCP, Au–TCP antibody and TCP–Au–TCP antibody complexes continue to migrate along the strip (Fig. 1B). As these mixtures reach the test zone, unbound Au–TCP antibody was recognized by TCP–BSA, which resulted in the accumulation of Au nanoparticles on the test line. The excess TCP and TCP–Au–TCP antibody complexes continue to flow into the absorbent pad to the end of the strip. For the control experiment (no analyte), the Au–TCP antibody was fully bound to the TCP–BSA in the test zone (Fig. 1C). After the completion of immunoreaction, the Au nanoparticles-based ITS® can be observed by the naked eyes, and subjected to quantitative analysis by reading the signal of the test line with a portable strip reader (Fig. 1D). The more analyte in the sample, the less the Au–TCP antibody would bind to the TCP–BSA in the test zone, which leads to the decrease of signal. According to the principle described above, the signal will be inversely proportional to the concentration of the TCP in the samples, which can be widely used for quantitation of other analytes.

3.2. Optimization

Certain parameters of the assay affected the response of the immunosensor. To increase the sensitivity and selectivity of immunosensor, several parameters such as Au-to-TCP antibody coupling ratio, immunoreaction time and the amount of Au-labeled TCP antibody pre-loaded on the conjugation pad were optimized. The Au-to-TCP antibody coupling ratio directly affects the sensitivity of immunosensor since the signal mainly depends on the amount of Au-labeled TCP antibody conjugate. We studied the influence of the coupling ratio using 0.01 M PBS. As shown in Fig. 2A, the highest absorption signal can be obtained with the coupling ratio of 15 μL TCP antibody/1.25 mL Au. In order to obtain the best competitive purpose, the influence of the amount of the antibody on the conjugation pad was also examined using 0.01 M PBS. As shown in Fig. 2B, the absorption signal increases with the amount of antibody up to 5 μL/cm dispensed on the conjugation pad, then begins to level off, indicating that the competitive immunoreaction between the Au–TCP antibody and the competitor on the test zone is saturated. Therefore, 5 μL/cm of stock Au–TCP antibody conjugate was dispensed in the conjugation pad throughout this study.

The influence of the immunoreaction time was studied with 5 ng/mL TCP, while PBS was determined in parallel as a control (Fig. 2C). The absorption signal increases with reaction time up to 15 min, after which the absorption signal forms an asymptote. Therefore, 15 min was the optimal time routinely used for this immunosensor.

Furthermore, in order to make the immunosensor a great convenience for point-of-care and in field analysis of TCP without any pretreatment of biological sample, we studied the pretreatment of sample pad of the Au nanoparticles-based ITS and the preparation of the stock solution of Au–TCP antibody. Due to its

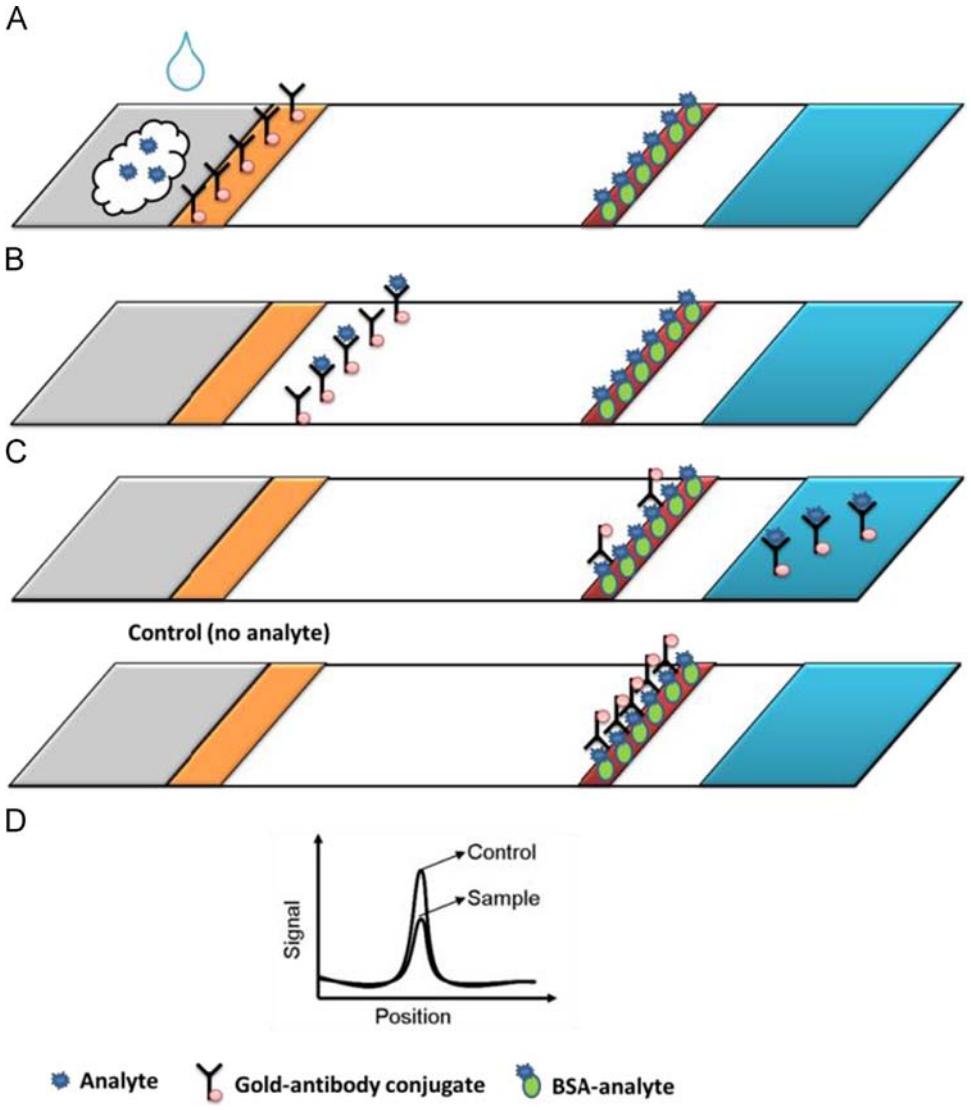

Fig. 1. Schematic illustration of the principle of Au nanoparticles immunochromatographic test strip assay. (A) Aqueous sample containing analytes was applied to the sample zone. (B) Analytes bound to part of the Au-labeled antibodies to form analyte–antibody conjugates, and migrated together with free analytes and free Au-abeled antibodies towards the other end of the test strip by capillary force. (C) Free Au-labeled antibodies were recognized by BSA-analyte in the test zone. The analyte–antibody conjugates and excess analytes continue to migrate towards the absorption pad. In a control assay (no analyte), the Au-labeled antibodies are fully bound to the BSA-analyte in the test zone. (D) Absorption detection on test strip reader.

favorable bio-compatibility and non-toxicity, PEG 6000 is used as an ideal vector for the sample releasing. Triton X-100 is a nonionic surfactant, which could dissolve the lipid to yield an increase of eukaryotic cell membrane permeability. The addition of casein (0.5%) could eliminate the influence of nonspecific binding. Therefore, the sample pad pretreatment buffer was prepared as described above (2.5). Meanwhile, trehalose, which could protect the protein from degeneration or inactivation, was used in the stock solution of Au–TCP antibody as described in Section 2.3.

3.3. Analytical characterization

To investigate the performance of immunosensor for detecting TCP, the assay was examined with different concentrations (0, 0.625, 1.25, 2.5, 5, 10, 20, 40 ng/mL) of standard TCP in PBS. The signal on the test line was recorded by the test strip reader and digital camera after 15 min. As shown in Fig. 3A, well-defined peak intensity decreased along with the increase of TCP concentrations (from a to h). Normalized signals expressed as 100 ($A/A0$) (where A and $A0$ are the peak intensity obtained with the TCP analyte and the blank sample, respectively) were plotted versus the logarithm of TCP concentration. An obvious sigmoidal shape of the calibration curve of TCP was obtained at the concentration range from 0.625 to 40 ng/mL, with a linear range from 0.625 to 20 ng/mL (Fig. 3B). The detection limit of the immunosensor was estimated to be 0.47 ng/mL according to the linear equation based on 90% of $A/A0$. Error bars are based on four duplicated measurements of TCP at different concentrations.

Due to the superior signal brightness and high photostability of Au nanoparticles, images of this biosensor after assay could be employed as a conventional approach to qualify or semi-quantify TCP visually and rapidly. By observing the Au imaging directly, we can easily judge the existence or not of target analyte. As shown in Fig. 3C, the brightness of the red line decreases clearly with the increase of TCP concentrations. Such an observation demonstrates that the competitors on the test line capture less Au-labeled antibody conjugates when the analyte concentration is higher. In the presence of 40 ng/mL TCP, no obvious band appeared, indicating very low nonspecific absorption. Combined with the well-defined peaks shown in Fig. 3A, the strip reader and images can be

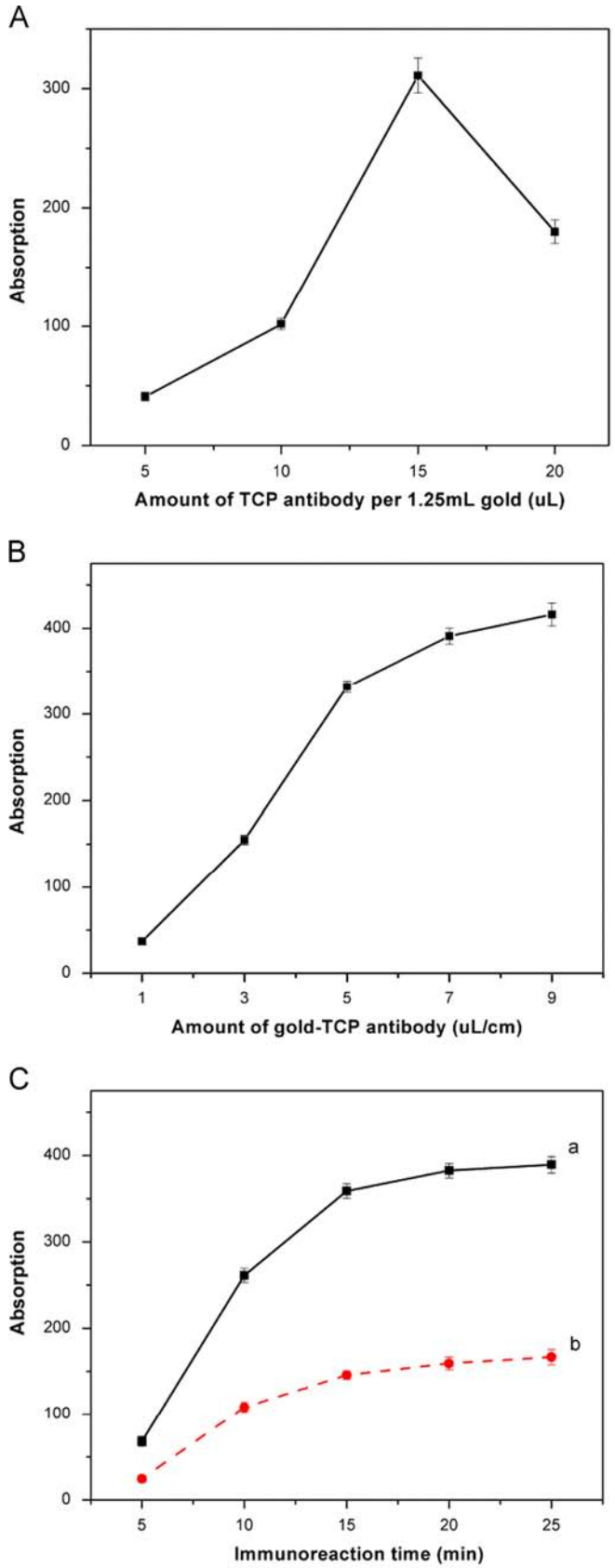

Fig. 2. Optimization of parameters of the immunosensor. (A) Effect of the coupling ratio between Au nanoparticles and TCP antibody of the sensor. (B) Effect of the amount of the Au–TCP antibody on the signal responses of the sensor. Absorption detection in both (A) and (B) were conducted after 15 min of immunoreaction. (C) Effect of immunoreaction time on the signal responses of sensor. It was performed by applying 100 μL of (a) PBS (control) or (b) 5 ng/mL TCP to sample application pad.

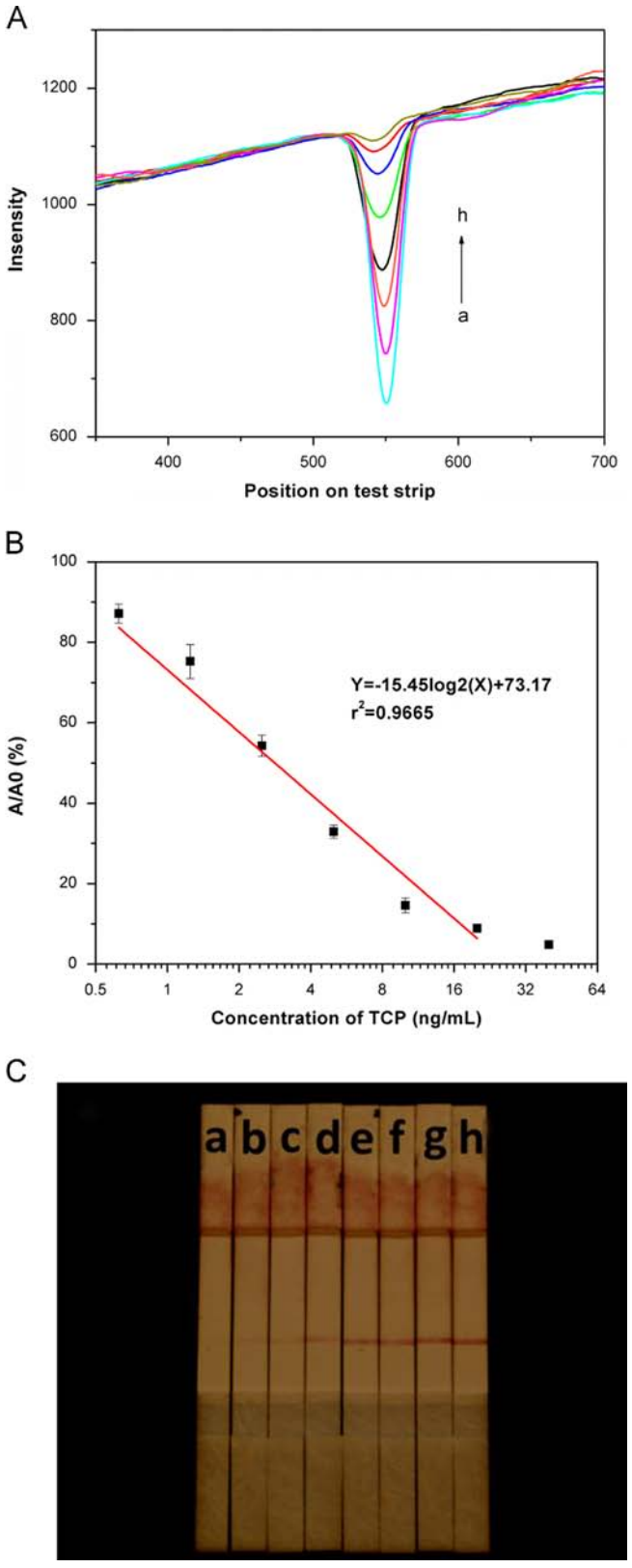

Fig. 3. (A) Typical signal responses of the immunosensor with increasing TCP concentrations, from a to h, 0, 0.625, 1.25, 2.5, 5, 10, 20, 40 ng/mL, respectively. Immunoreaction time was 15 min. (B) The resulting calibration curve of TCP. Normalized signals expressed as 100 (*A*/*A*0) (where *A* and *A*0 are the peak absorption intensity obtained with the TCP analyte and the blank sample, respectively) were plotted versus the logarithm of TCP concentration. (C) Images of Au-based immunochromatographic test strips for 40, 20, 10, 5, 2.5, 1.25, 0.625, 0 ng/mL TCP (from a to h), respectively.

used together to show assay results of this Au nanoparticle-based immunosensor with high sensitivity and specificity. Either of them could also be used alone depending on the conditions and requirements. By employing the dual reading approaches, a rapid and simple strategy for the analysis TCP analysis has been developed.

3.4. Detection of the concentration of TCP in human saliva

To explore the biomonitoring application feasibility of the sensor, the Au nanoparticles-based ITS was used to measure TCP levels in saliva collected from four workers from a chlorpyrifos manufacturing facility as well as one additional worker from a non-chlorpyrifos manufacturing facility. Without any pretreatment, 70 μL of saliva extruded from the Salivette® collection device was applied to the immunosensor. Absorption signals were recorded by the test strip reader in 15 min. Concentrations of TCP in human saliva were calculated from an external calibration curve of TCP. TCP was detected in all saliva samples tested with the sensor and the commercially available ELISA kit (Fig. 4). The quantified levels of TCP by the sensor were very consistent to those from a commercially available ELISA kit. Compared with traditional ELISA, the developed immunochromatographic strip is more suitable for fast quantitative analysis of TCP (the analytical time for test strip is 15 min vs. more than 6 h of the ELISA) and in-field screening of organophosphorus insecticides exposure. In addition, the prepared strip was very stable stored with desiccant at room temperature. In this way, over 90% of the initial response remained after three-month storage. Successfully detecting the TCP from human saliva displays promise of our device for various biomonitoring applications in the near future. The immunochromatographic strip detected TCP human saliva with relative standard deviations (RSD) in the range of 1.0–12.5%, indicating a good reproducibility of this strip.

TCP levels were highest in those that worked at the CPF manufacturing plant, and interestingly, TCP levels in saliva decreased from day one to day two of sampling, which indicates that occupational exposure to CPF or TCP was low on the day prior to the second sampling.

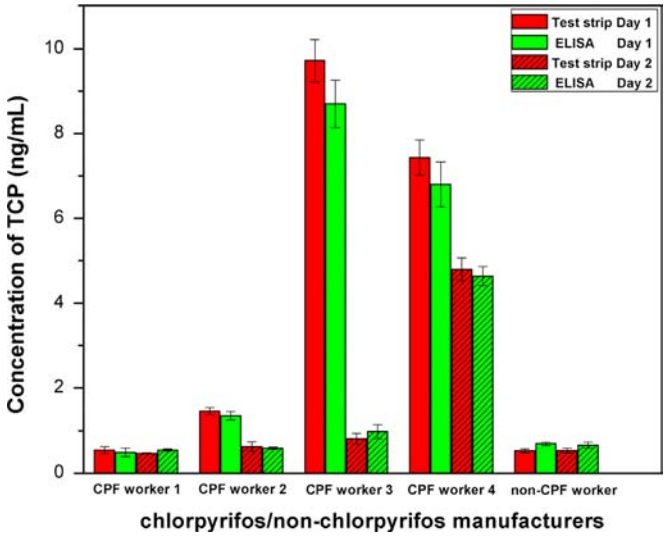

Fig. 4. Detection of TCP in the saliva samples collected from chlorpyrifos/non-chlorpyrifos workers. Red color: monitoring the TCP using the test strip; Green color: monitoring the TCP using the ELISA. The concentration of TCP was calculated from the calibration curve of the standard TCP sample. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Conclusion

In summary, we have successfully developed a test strip based visible immunosensor for quantitative detection of TCP in biological samples, a specific biomarker exposing to pesticide chlorpyrifos. This biosensor takes advantage of the speed and low cost of conventional ITS as well as sensitivity, high stability and easy preparation of the Au nanoparticles-based immunosensor. Furthermore, this sensor is convenient for directly detecting the concentration of TCP in complicated real samples such as human saliva without any pretreatment. Overall, the device developed in this study shows a great potential for rapid, sensitive and on-site analysis of TCP and other protein biomarkers in clinical diagnostics, basic discovery, and a variety of other biomedical applications.

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