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Short communication

Visible paper chip immunoassay for rapid determination of bacteria in water distribution system

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ABSTRACT

Paper chips for immunoassay were patterned by screen printing of polydimethylsiloxane (PDMS) or wax pencil drawing. The methods for paper chip patterning are cheap, convenient, rapid and suitable for most laboratories. The whole time for patterning a paper chip is no more than 10 min. Visible immunoassay for the detection of bacteria (*Escherichia coli*) has been realized using the paper chip, on which the antibody for capturing *E. Coli* was immobilized on the detection zones of the paper chip, while the detection antibody was labeled with gold nanoparticles (AuNPs) as a signal reporter. After an immunological reaction, the AuNPs bound on the paper chip can effectively catalyse the reduction of silver ions during the silver enhancing step, generating a visible result that can be read by naked eyes. The quantitative results can be acquired by scanning the silver stained paper chip with a commercial scanner/or digital camera. The density of *E. coli* in water samples can be measured after calibrating the gray value of silver stained spots with the logarithmic number of bacteria. The time and reagents consumed on the paper chip immunoassay is much smaller than those of conventional ELISA, while the sensitivity of the paper chip immunoassay is comparable to conventional ELISA. The technology proposed in this work displays a great potential in the in-situ analysis when daily monitoring of water quality are required.

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1. Introduction

The quality of drinking water is essential to human health. An adequate, safe and accessible water supply must be available to all residents. In developing countries, waterborne infections are common [1]. According to the WHO, the mortality of water associated diseases exceeds 5 million people per year. Among them, more than 50% are microbial intestinal infections. In general terms, the microbial risks are associated with ingestion of water that is contaminated with pathogens [2–4]. The infection caused by these pathogenic bacteria has been related to hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenia [5]. Therefore, a reliable and fast monitoring method is required to control the spread of pathogens in water distributing systems.

Conventional method to detect bacteria relies on selective culturing technique in a specific medium, followed by biochemical isolation and morphological identification [6]. These standard identification methods are sensitive and can be specifically used for the detection of bacteria in unknown complex samples such as foods, water and clinical samples [7]. However, conventional methods require well-trained experts and involve long assay times. To overcome these disadvantages, immunological- and gene-based analytical methods have been developed. The gene-based methods can display high sensitivity with the assistance of PCR technologies. However, the gene-based assay usually requires sequential complex processes including cell extraction, nucleic acid purification, amplification and detection. Immunological methods for pathogen detection use antibodies to selectively bind with bacterial antigens located on their cell membrane, and usually do not require complex sample extraction and purification. During the past decade, several types of immunosensors for pathogen detection have been developed using label-based or label-free techniques [8–12]. However, low price portable devices allowing field analysis are still urgently needed.

Microfluidic chips have has raised great interest since Manz came up with this idea [13]. One of the main purposes for developing microfluidic chips is to find a cheap tool for point-of-care test (POCT). However, in most situations, using microchip is still expensive, since most of microfluidic chips cannot be repeatedly used. In addition, microfluidic chip technology usually need a power source, such as micro-injector, pneumatic pump and piezoelectric element, to drive the solution in micrometer scale channels. Meanwhile, a large detector compared with the small chip is also needed to get the results of the experiments. These equipment may

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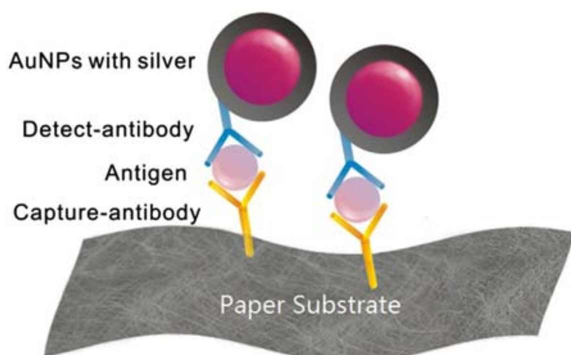
be accessible in a laboratory, but it is not convenient for field analysis. Paper microfluidic chip (PμFC) proposed by Whitesides and coworkers [14] might be a promising chip that can overcome the disadvantages of expense and large external control. The paper chip can be patterned by hydrophobic materials, such as wax [15], SU-8 photoresist [14], PDMS [16] and alkyl ketene dimer [17]. Solutions can be driven to specific areas by capillary force so that no external equipments are needed for PμFC. In addition, the chemical nature of paper and its porosity make it an ideal substrate to absorb hydrogel [18], indicators [17], nano-particles [19] and biomolecules [20].

Enzyme-linked immunosorbent assays (ELISA) has been widely used in biochemical analyses. These assays are typically carried out in microtiter plates or small vials. ELISA combines the specificity of antibodies with the high-turnover catalytic property of enzymes, resulting in high specificity and sensitivity towards target analytes. Paper-based ELISA has been demonstrated by Whitesides and coworkers [21]. However, in most of paper-based ELISA, the signal amplification strategies usually rely on the enzyme labeled antibody for catalyzing the turnover of substrate to color product. In this article, the traditional silver staining technology was employed for amplifying the signal of paper-chip based immunoassay. As illustrated in Scheme 1, a classical sandwich mode involving the formation of antibody–antigen–antibody complex was employed in our paper-based microfluidic chip immunoassay (PμFC-immunoassay). The capture antibodies (*Escherichia coli* antibody) were immobilized on the patterned detection zones on a paper chip though physical adsorption. The non-binding sites were then blocked by bovine serum albumin (BSA) with relatively high concentration. Water sample containing *E. coli* bacteria and detection antibody labeled with gold nanoparticles (AuNPs) were sequentially introduced to the detection zones with a defined time interval. AuNPs is a common material to label biomolecules due to its good biocompatibility. Meanwhile, antibody can be conveniently linked to the AuNPs by electrostatic interactions or thiol chemistry. However, the results of immunoassay cannot be detected by naked eyes when the concentration of AuNPs is too low. For improving the sensitivity, silver enhancing step was employed in this work. The AuNPs bound on the PμFC can effectively catalyse the reduction of silver ions. The reduced Ag atoms deposited around the AuNPs core can form a gray, even black spot in the sample area, causing a strong light scattering effect that can be readable with naked eyes. Methods for quantitation of PμFC-immunoasay result are versatile. For example, the image of detection zones can be conveniently acquired by a commercial scanner, a digital camera, or portable device such as a cell phone camera. Subsequently, the mean value of gray level at each detection zone can be obtained by imaging processing software.



Scheme 1. Principle of PμF C-immunoassay based on gold nano-particles labeling and silver enhancing. Capture-antibody was absorbed on the porous structure of paper substrate by physical absorption. Bacteria was captured by attached antibody and followed by binding with AuNPs labeled antibody based on sandwich immunoassay mode. AuNPs is a catalyst so that silver can be reduced around the core of AuNPs and make the visualized black spot.

This work is helpful to develop a new method for field analysis, especially for daily monitoring of water contamination. The PμFC-immunoassay has significant advantages over conventional immunoassay. For example, PμFC-immunoassay costs less time and consumes fewer reagents compared with those of conventional ELISA. Furthermore, external equipments are simplified and the power source can be even avoided. The silver enhancing method for visualizing immunoassay result on a PμFC is more robust than previously reported work, because the deposited silver spot are relatively stable and will not be washed away easily. Most importantly, the small volume and weight of paper make it unique compared to commercial immunoassay products. The features make it especially suitable for in-situ environmental anaysis and drinking water quality monitoring.

2. Experimental section

2.1. Reagents

Whatman filter paper (no. 1) and wax pencil was used as received, *E.coli* antibody (Goat polyclonal to *E.coli*) were purcased form Abcam Co. Silver enhancer solution A and B were purchased from Sigma-Aldrich Co. PDMS was from Dow Corning Corporation. All reagents were used without further purification.

2.2. Patterning paper with wax pencil or PDMS

Paper chips used in this work were patterned with wax pencil drawing and screen printing method. Detail procedure is illustrated in Fig. 1a. Wax pencil drawing is a prototype method to pattern a paper chip that could be used in optimization of experimental condition. As illustrated in Fig. 1b, the whole filter paper except eight identical round sample areas were coated by a wax pencil. The paper was heated at 180 °C on a hot plate for less than 1 min. After wax melting and penetrating through the filter paper, a sharp edge defining the detection zone appears. Screen printing is another effective method for mass production. Device for performing screen printing is purchased from Ridongsheng Co., China. The printing mold was designed by AutoCAD 2007 (Fig. 1c). PDMS pre-polymer, a mixture containing the silicone elastomer and the curing agent (10:1 weight ratio) was diluted with N-hexane to adjust the viscosity of pre-polymer. The mixture was left to stand for a while under vacuum to remove bubbles and then was poured onto the mold, while the filter paper was pressed under the mold. A rubber brush was used to coat the PDMS pre-polymer uniformly on the paper to pattern the exposed channel and detection zone. Finally, the screen printed paper was baked at 110 °C from 30 min to 1 h to allow the pre-polymer cured. A photo of paper chip patterned by screen printing method is shown in Fig. 1d.

2.3. Label detection antibody with AuNPs

Colloidal gold was prepared with the following procedure: An aliquot of 0.2 mL trisodium citrate (0.1%, W/V) was added drop by drop to 10 mL 0.01% $HAuCl_4$ solution, which is heated to boil until the colloidal of AuNPs turned to red color. An aliquot of 0.5 mL 0.1 mol/L K_2CO_3 was then added into the mixture after it cools down.

To label the *E. coli* antibody with AuNPs, 10 μL *E. coli* antibody with concentration of 100 μg/mL was incubated with 60 μL of AuNPs overnight at 4 °C. Around 30 μL of BSA solution (50 mg/ml)

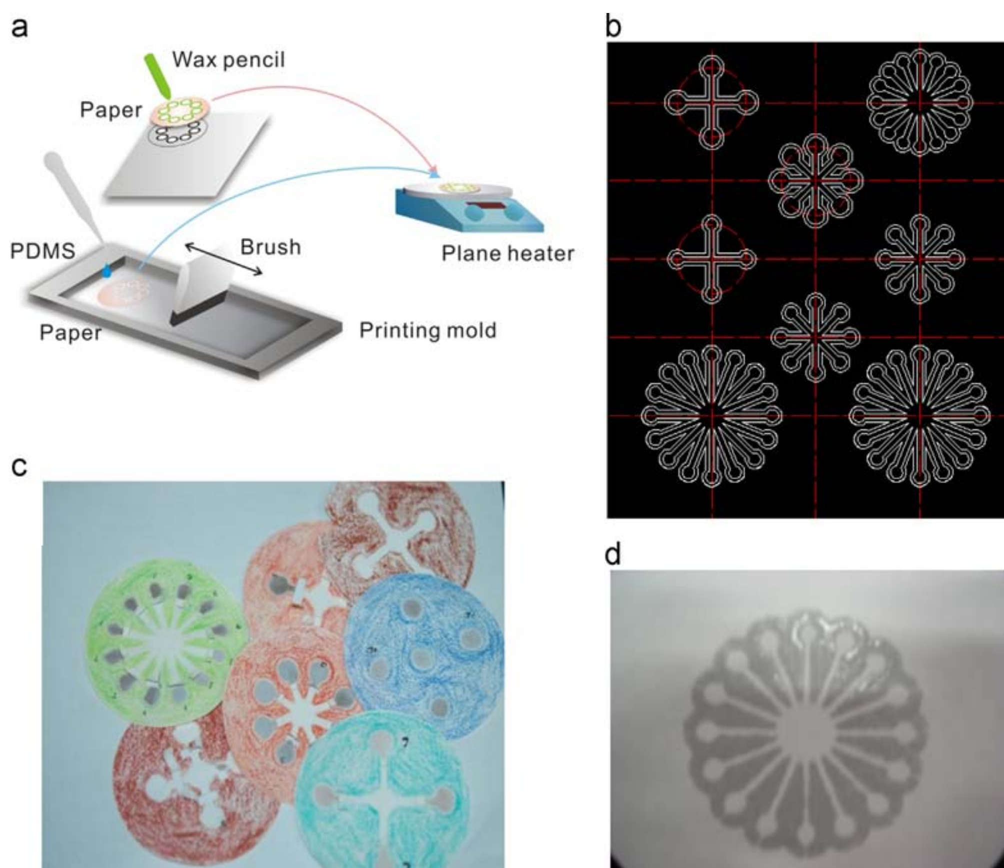


Fig. 1. (a) The schematic process of making a paper microfluidic chip by PDMS pattern and wax pencil pattern. (b) The photo of a PDMS patterned chip. (c) The photo of wax pencil patterned chips. (d) Various molds for screen printing of PDMS pattern designed by AutoCAD 2007.

was added into the AuNPs labled antibody solution, and the mixed solution was allowed to stand for another 12 h to prevent the non-specific absorption of AuNPs on filter papers.

2.4. Preparation of bacterial cell culture and suspension

E.coli strain (ATCC no. 25922) was used as the model bacteria in this study. It was obtained by scrapping the colony from an overnight cultured sample in LB agar plate. The *E.coli* suspension was prepared by diluting the bacterial cell with fresh Mueller–Hinton broth(MHB) with 1% glucose (w/v). The cell density was adjusted to match the turbidity of a 0.5 McFarland Standard (10^8 CFU/ml) using MHB (with 1% glucose). Then the suspension was further quantitatively diluted to reach an appropriate cell density with MHB (with 1% glucose).The bacterial suspension with different cell density was used in subsequent experiment.

2.5. Immunoassay procedure

The antibody for capturing *E.coli* was immobilized on the PμFC by physical absorption. Briefly, 5 μL antibody solution (10 μg/mL) was dropped onto detection zone of the PμFC. After 10 min, 5 μL BSA solution (50 mg/mL) was added to each detection zone to block the non-specific binding sites. Sample solution containing *E. coli.* with different cell density was added onto each detection zone. After incubation for 15 min, the PμFC was dried under stream of ambient air. The dried chip was then immersed in PBST buffer (PBS solution containing 0.5% Tween-20) for 45 s to remove the non-specific adsorbed bacteria. Afterwards, 5 μL AuNPs labeled antibody was added to each detection zone and incubated for another 10 min, followed by washing and drying step as described above. The silver enhancing step was performed in the darkroom. The silver enhancer solution (solution A and solution B freshly mixed in a ratio of 1:1) was added onto each detection zone or the sample loading zone located in the center of PμFC. After 5 min, the chip was immersed in PBST buffer to stop the reaction. After drying, the paper chip was scanned by a commercial scanner (HP LaserJet Professional M1136). The gray value of each detection zone was acquired by Image J 1.42q software.

3. Results and discusion

3.1. Methods for paper patterning

Using wax pencil is one of the most convenient ways to pattern a paper, since the only necessary equipment used in this method is a hot plate, and the pattern can be changed easily. The wax should penetrate through the paper to build up hydrophobic barrier. When the paper was heated at 120 °C, around 5 min was needed for wax to penetrate through the paper and build up hydrophobic barrier. However, the space resolution of this pattern method is somewhat low because the wax could also diffuse on the surface of paper. The most effective way to balance the situation is to control the temperature of hot plate or using a thinner filter paper to minimize the time for wax penetration. Wax diffusion on the paper surface will not only destroy the designed channels, but also break the hydrophobic barrier. The extent of wax diffusion depends on the heating temperature and time. In this work, the optimal condition is to heat the wax at 180 °C in less than 1 min.

Using wax pencil is a convenient way to pattern a paper, but there are some limitations such as bad repeatability and difficult to

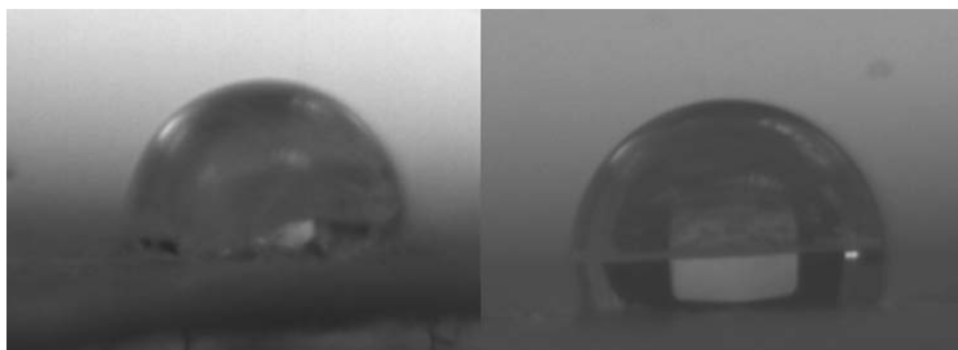


Fig. 2. The measurements of contact angel on (a) wax patterned area of paper chip, and (b) PDMS patterned area of paper. The contact angel for wax patterned paper is 92.5–96.5°, while it is 105.5–108.5° for PDMS patterned chip. Obviously, PDMS patterned area is more hydrophobic than the other one and can hold more solution within the sample spot.

reproduce the paper chip with an identical pattern. In addition, the wax pencil is not suitable to pattern small channels, since the channels may be blocked by the wax due to the diffusion mentioned above. PDMS screen printing is a rapid and effective printing method for patterning a paper. Since the commercial PDMS pre-polymer (the silicone elastomer: the curing agent = 10:1) is too viscous to penetrate the paper, solvent (n-hexane) should be used to help lowing the viscosity of the pre-polymer, but the amount of n-hexane added in the pre-polymer should be carefully controlled. Too much n-hexane will produce a lot of small holes in the PDMS hydrophobic barrier after cured. On the contrary, low content of n-hexane will not significantly improve the permeation of pre-polymer. The curing temperature should also be considered. The high temperature (more than 120 °C) may cause the quick evaporation of solvent and produce vapor bubbles that may demolish the smoothness of the surface. However, If the temperature is lower than 80 °C, the polymer will not be cured in time, resulting in the spread of the pre-polymer and destroying the channel. PμFC prepared by PDMS screen printing method is shown in Fig. 1d.

The hydrophobic nature of wax patterned paper and PDMS pattern paper are measured by POWEREACH JC2000D Contact Angle Meter. For wax patterned paper, the contact angle is around 92.5–96.5°, while for the PDMS patterned chip, the contact angle is around 105.5–108.5° (Fig. 2). The results indicate that PDMS is more hydrophobic than wax, so it can contain more solution in the limited area without leakage.

3.2. Immunoassay on paper chip

E. coli was chosen as the target analyte to test the performance of paper based immunoassay. Procedure for the PμFC-immunoassay is almost the same as the common immunoassay, except that the patterned paper chip consists of detection zones and channels for reagents loading and distribution. *E.coli* capture antibody should be first immobilized on the detection zones before detection. Due to the large surface area of cellulose paper, the antibodies can be simply immobilized on the cellulose fiber through physical adsorption. Multiplex analysis can be achieved if each detection zone was attached with different kinds of antibodies. Sample dropped on the loading area can be automatically distributed to each detective area through the patterned channel. However, if samples with different concentrations need to be analyzed, they should be sequentially added onto each detection zone, so that the cross-contamination can be avoided. After the antibodies immobilized on the detection zones specifically capture the analytes, the detection antibody (AuNPs labeled *E. coli* antibodies) was applied onto the detection zone, forming a sandwich complex as shown in Scheme 1. Finally, the signal of immunoassay was amplified by the silver enhancing step,

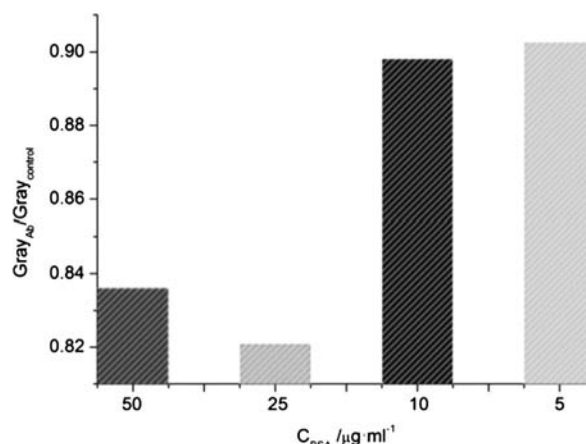


Fig. 3. The effect of the amount of BSA on the blocking efficiency. The results demonstrated that the lowest ratio of gray value between control and sample was found when the BSA solution with concentration of 25 μg/mL was used as blocking agent.

in which the bound AuNPs catalyzed the reduction of silver ions. The reduced silver atom deposited around the AuNPs could significantly enhance the light scattering effect, leading to the visualization of immunoassay results. The detection antibody and silver enhancing reagent can be either driven by capillary force through the patterned channel or directly added onto each detection zone. The choice of liquid driving method depends on the size of channel and detection zone on a PμFC. For a PμFC with wide channels and large detection zones, sequential addition of each reagent is more practical, because solution transportation in a wide channel through capillary force not only take longer time but also causes inhomogeneous distribution. In contrast, for a PμFC with narrow size channel and detection zones, solution distribution through capillary force is suggested.

Non-specific absorption is one of the most difficult problems to be solved in immunoassay. Porous cellulose may non-specifically adsorb protein, antibodies and gold nanoparticles by hydrophobic interaction or electrostatic interaction. For example, antigens or antibodies absorbed on the cellulose fiber of filter paper will loss specificity of the immunoassay. In the meantime, filter paper may also adsorb AuNPs strongly. As observed in our experiment, the nonspecific adsorption was remarkable when the filter paper or AuNPs were not blocked by BSA. Under this situation, even the blank sample may become black after silver enhancing step owing to the non-specific adsorption of AuNPs labeled antibodies on the paper. To get better experimental results, both of paper and AuNPs used in this work should be blocked. As shown in Fig. 3, the amount of BSA used in the blocking step has significant impact on the blocking efficiency. The ratio of gray value between control sample

and real sample spot tends to decrease when the concentration of BSA increase. The lowest value was found when the concentrations of BSA reach ~25 μg/mL (Fig. 3). Accordingly, BSA solutions with concentration of 25.0 μg/mL were chosen as the blocking reagents. In addition, the non-specific adsorption can be further suppressed using a washing step, as is known for the conventional immunoassay. In this work, solution consist of PBS and 0.5% Tween-20 was proven to be the effective wash buffer to remove non-specific adsorbed bacteria and AuNPs labeled antibody.

Gray value represents the readout of the paper chip immunoassay. It was obtained from a scanned grayscale image of the paper chip, in which the value of each pixel is a single sample, that is, it carries only intensity information. Images of this sort, also known as black-and-white, are composed exclusively of shades of gray, varying from black at the weakest intensity to white at the strongest. No matter what pixel depth is used, the binary representations assume that 0 is black and the maximum value (255 at 8 bpp, 65,535 at 16 bpp, etc.) is white, if not otherwise noted.

With the assistance of silver enhancing step, the immunoassay readout can be well represented as the gray value, so that the results of the PμFC-immunoassay can be interpreted without the need for a specialized detector. By using a commercial scanner of a cell phone camera, the image of PμFC can be quickly acquired (Fig. 4a). Meanwhile, gray value assays are also well-suited for semi-quantitative assay by naked eye. However, the gray value of detection zones is dependent on many factors, such as the temperature, the intensity of light exposure, as well as the time for silver enhancement. Therefore, the procedure and conditions for performing PμFC-immunoassay should be precisely controlled. Under the conditions described in the experimental section, the gray value of the detective area has a linear relationship with the logarithmic number of *E. coli* (log *N*), and the limit of detection (3*S*/*N*) for *E.coli* was found to be 57 cfu/mL (Fig. 4b). This sensitivity is sufficient enough to monitor the outbreak of bacteria contaminations in water distributing system.

For evaluating the accuracy of the PμFC-immunoassay, water samples from eight sampling site across the rural area of Jiaxin city, China were measured, each sample was duplicated analyzed for three times and the results were compared with conventional plate counting method. As shown in the Table 1, the results obtained with PμFC-immunoassay have no significant difference from the results obtained with conventional plate counting method, indicating the PμFC-immunoassay method established in this work is reliable for the bacterial assay.

3.3. Comparison of cost and time consuming between PμFC-immunoassay and conventional ELISA

Paper-based immunology is quicker and consumes fewer reagents than conventional immunoassay methods. As shown in Table 2, the PμFC-immunoassay consumes only about 1/10

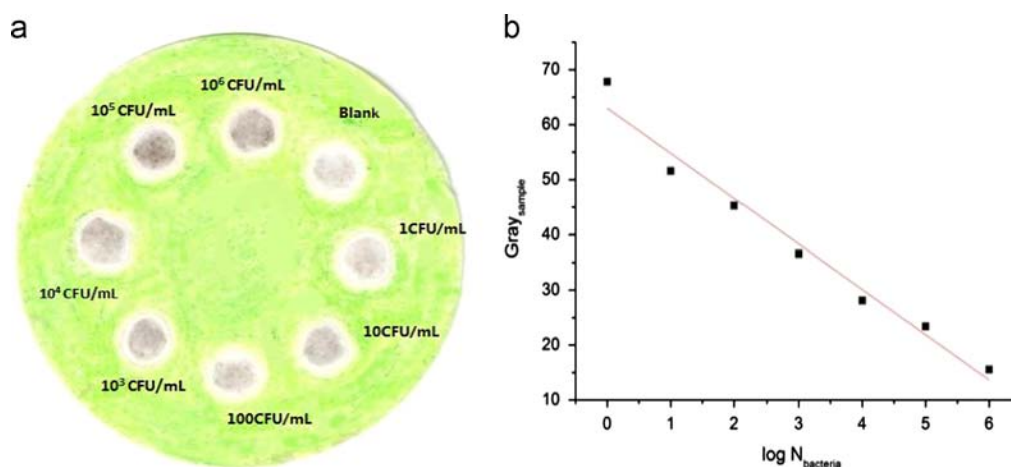


Fig. 4. (a) The scanned immunoassay result for *E. Coli.* detection on the patterned paper chip. The detective spot becomes darker with the concentration of *E. Coli* increase. (b) The calibration curve of immunoassay for *E. Coli* detection on PμFC. The correlation coefficient of calibration curve for bacteria detection is around 0.97.

Table 1
E. coli determination results obtained from PμFC-immunoassay and plate counting method.

Sampling site (No.)	1	2	3	4	5	6	7	8
PμFC-immunoassay (CFU/mL)	26 ± 3	–	–	–	123 ± 8	23	–	108 ± 6
Plate counting (CFU/mL)	28 ± 4	3 ± 1	2 ± 1	3 ± 1	120 ± 10	25	1 ± 1	110 ± 7

"–" indicate *E. coli* in the sample is undetectable by the method.

Table 2
Comparison of paper-based Immunoassay and conventional ELISA.

Time and reagents	Paper-based Immunoassay		Conventional ELISA	
	Volume (μl)	Time (min)	Volume (μl)	Time (min)
Antigen immobilization	5	15	100	120
Blocking	5	15	100	30
Immunology reaction	5	15	100	60
Signal amplification	10	10	100	15
Total per zone	25	55	400	225

Table 3
Cost for paper-based immunoassay and conventional ELISA. [a]

Reagent	Paper-based Immunoassay		Conventional ELISA	
	Amount	Price (dollar)	Amount	Price (dollar)
Container	Filter paper	0.2	ELISA plate	2.0
Capture-antibody	0.6 μg	2.1	2.4 μg	8.4
Detect-antibody	1.0 μg	2.6	2.4 μg	8.4
Total		4.9		18.8

[a] The cost was calculated assuming each paper chip or ELISA plat contain 8 sample zones.

reagents than conventional immunoassay (ELISA) and can be accomplished in one hour. These advantages may be related with the large surface-volume ratio of filter paper with porous structure, on which rapid immune-reaction between the surface attached antibody and analytes contained in sample droplet can take place. Due to the small amount of reagent consumed in the experiments, the price of a paper chip will be significantly cut down. The usual costs of a paper chip and conventional ELISA plate are compared in Table 3. The amounts of consuming reagents are calculated assuming that each paper chip or ELISA plate contain eight detection zones or micro-wells. Therefore, the estimated price for each paper chip is less than 5 dollars, which is significantly lower than a conventional ELISA plate.

4. Conclusions

This study demonstrates that wax drawing and screen printing with PDMS are cost effective and convenient methods for patterning a paper chip. The PμFC-immunoassay can be performed with a conventional sandwich mode, in which the capture-antibodies were physically adsorbed on the paper chip, while the detection antibodies were labeled with AuNPs, which can catalyze the reduction of silver ions. Therefore, the immunoassay results can be amplified and visualized by silver enhancing step, resulting in a good sensitivity of the method. The image of immunoassay results can be conveniently obtained by a scanner or cell phone camera. Quantitative signal can be represented as the gray value of each detection zone, which can be acquired with image processing software. As demonstrated in this work, the bacteria quantitation in municipal water sample has been achieved on the PμFC. Compared with conventional ELISA, the PμFC-immunoassay is cost-effective and time saving. Multiplex analysis will be conveniently realized if different kinds of capture antibody are immobilized on each detection zone. The PμFC-immunoassay coupled with silver enhancing step may find wide applications in the rapid detection of pathogen contained in water or food samples, as well as in the point of care test for medical usage.

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