One-sampling and Rapid Analysis of Cancer Biomarker on A Power-free and Low-cost Microfluidic Chip

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Abstract

Alpha-fetoprotein (AFP) is an important disease biomarker, related to cancers such as hepatocarcinomas and gastric cancer. However, traditional methods are time-consuming, relied on bulky instruments and trained professionals, cannot satisfy the demand for low cost and point-of-care testing (POCT). In this study, a power-free POCT device is developed for rapid and low-cost detection of AFP via one-sampling. Based on the principle of sandwich immunofluorescence, the chip is capable of automatically accomplishing on-chip mixing, labeling and capturing procedures, which only requires the operator add 40 μl sample into the chip one time. The proposed device is capable of sensitively detecting human AFP in FBS with a dynamic ranging from 10-1,000 ng/mL and LOD (1.88 ng/ml) within a short time of 3 min. Predictably, our method holds the great potential to be applied in POC diagnostics of proteins, especially for some regions with resource-limited.

Key words: power-free, one-step, cancer biomarker, POCT, microfluidic, immunoassay microchip
Introduction

Cancers are the second cause of death worldwide, 14 million new cancer cases being estimated in 2035\textsuperscript{1,2}. Early diagnosis through cancer biomarker is crucial for cancer monitoring, improvement of efficacy and survival rate\textsuperscript{2}. Among the cancer biomarkers, Alpha-fetoprotein (AFP, a kind of glycoprotein) is widely applied for the diagnosis of many cancers\textsuperscript{3}. Due to the high selectivity, immunoassays-based methods are the predominant for AFP determination, such as enzyme-linked immunoassorbent assay (ELISA), radioimmunoassay and electrochemiluminescence immunoassay\textsuperscript{3-5}. However, traditional immunoassays are time-consuming and costly, and require professional operators and complicated operation steps\textsuperscript{6,7}. Therefore, developing an easy-to-operate, rapid and low-cost method is significant for sensitive AFP determination.

Due to the efficiently and effectively on-site diagnosis without professionals and bulky instruments, point-of-care testing (POCT) analytical device has attracted attention for biomarker
detection\textsuperscript{7-9}, thus being an ideal for AFP detection. Typical POCT analytical devices are various lateral flow immunoassays (LFIA)\textsuperscript{10-12}, such as paper-based commercially pregnancy test strips, presenting test results with visible color changes\textsuperscript{13}, and AFP testing\textsuperscript{2}, accomplishing AFP detection within 5 min. However, the porosity of paper and batch difference will affect the precise flow of fluid, potentially resulting in inaccurate result and poor repeatability, thus there are great detection hazards\textsuperscript{13, 14}. In contrast, microfluidic chips have better flow control and repeatability over LFIA due to the uniform materials (e.g., polymer\textsuperscript{15}), highly controllable fabrication (e.g., lithography\textsuperscript{16}, injection molding\textsuperscript{17}) and more flow control elements (e.g., delay valves\textsuperscript{18,19}).

In recent years, there were several microfluidic-based POCT devices for AFP detection\textsuperscript{19-21}. However, most of them still involved multi-step operations to introduce sample, which is unfriendly to untrained people. For example, the chip prepared by Ning Chang \textit{et al.} could
simultaneously detect multi-protein such as AFP with the limit of detection (LOD) 18.92 ng/ml\textsuperscript{22}. However, it was necessary to mix the target solution with fluorescence antibody in advance. To avoid the multi-step, almost all utilized extra power supply to propel reagents, such as syringe pumps\textsuperscript{23} and centrifugal instruments\textsuperscript{6}, which undoubtedly increased test costs and equipment dependence. Recently, Chao Liang, \textit{et al.} successfully developed a one-step power-free device for cardiac troponin I detection: they immobilized the fluorescence antibody on the surface of the microchannel in advance, which would combine with targets when the sample flowed through\textsuperscript{13}. Although it took 12 minutes to complete the test due to the low labeling efficiency on planar\textsuperscript{13}, it provided an important reference for the one-step power-free detection of protein.

With the benefits of good stability, easy-to-fabricate and high specific surface, micropillar can not only be used as an immuno-capture structure for sensitive detection\textsuperscript{24, 25}, but also as a
micromixer for efficient mixing\textsuperscript{26, 27} in recent years. For example, Roer Eka Pawinanto \textit{et al.} prepared miropillar-based active electromagnetic microfluidic mixer, accomplishing efficient mixing within 4 s under a low electrical current\textsuperscript{27}. Therefore, if replacing planar with micropillar for mixing and labeling, their efficiency should be significantly increased. However, as far as we know, such method has not yet appeared in the one-step power-free POCT device, especially for labeling antibodies.

In this study, a new low-cost and power-free microfluidic device \textit{for rapid detection} of cancer biomarker AFP has been presented. Based on the sandwich immunofluorescence principle, the fluorescence-labeled antibody and the capture antibody were pre-immobilized on the different functional regions via physical adsorption and oriented immobilization, respectively. With once adding of 40 μl sample into the chip, the labeling and capturing steps for the immunoassay will be automatically completed. For lower cost, the designed chip is
fabricated from PMMA via CNC machine. The develop method is successfully applied to detect **AFP in fetal bovine serum (FBS)** with a dynamic ranging from 10-1,000 ng/mL and LOD (1.88 ng/ml) within 3 min, which is more rapid than the previous studies\textsuperscript{13, 28}. It can be foreseeable that this method has great potential in protein one-step detection.

**Experimental**

*Reagents and chemicals*

Polymethyl methacrylate (PMMA) sheets with different thickness were purchased from XinTao Acrylic (Shenzhen, China). Double sided adhesive tape with a thickness of 60 μm were purchased from Wenhao (Suzhou, China). Glutaraldehyde (GA, 50% in water) were purchased from Aladdin Reagent (Shanghai, China). Poly (ethylenimine) (PEI, Mw = 70,000 g mol\(^{-1}\), 50 wt% aqueous solution), Human immunoglobulin (IgG), Protein A from *Staphylococcus aureus* (SPA) were purchased from Sigma-Aldrich (Montana, USA). Human AFP recombinant protein were purchased from ARP Inc. (USA). Goat anti-Chicken IgY (IgG) H&L (Alexa Fluor\textsuperscript{®}680)
and Rabbit anti-chicken IgG were purchased from Abcam (England). Chicken anti-human AFP antibody were purchased from R&D (USA). Albumin bovine V (BSA), Human Serum Albumin (HSA) were purchased from Solarbio (Beijing, China). Mouse anti-human AFP antibody (C3) (Alexa Fluor®700) were purchased from Novusbio (USA). Fetal Bovine Serum (FBS) were purchased from Lonsa (South America). Human Fibrinogen (HF), Mouse IgG (H+L), Chicken IgY (IgG) (H+L), Ready-to-use PBS powder, Tris (hydroxymethyl) aminomethane (Tris) were purchased from Sangon Biotech (Shanghai, China).

**Apparatus**

The fluorescence signal was detected by Odyssey Clx, purchased from Li-Cor (Nebraska, USA). The fluorescence image was analyzed with the ImageJ software to calculate the average value of the fluorescence signals from all pixels in each boss.
Microchip design and fabrication

A schematic of the design is shown in Fig. 1a, and a photograph of a completed chip is shown in Fig. 1b, which comprises three parts: a cover layer with inlets to introduce the sample and air holes (diameter (D), 1.40 mm) to assist the air vent; a middle layer (double sided adhesive tape) used for assembling and fixing the whole chip including the cover and substrate layers together; a substrate layer with four functional regions along the longitudinal axis of the main microchannel, including labeling region (micropillars array) and capture region (boss array), time valve (gradient stairs) and waste reservoir. The cover and substrate layer are fabricated from PMMA sheets using the CNC milling machine. The length (L) and width (W) of the microchip are 59.00 and 35.00 mm respectively. The thickness of the cover layer, middle layer and substrate are 1.50, 0.06 and 3.00 mm respectively. For the substrate layer, the depth of inlet and main microchannel are both 0.18 mm; the height (H) of micropillar and boss are 0.18 and 0.15 mm, respectively; the interval between micropillars (D, 0.58 mm) and bosses (L × W,
1.40 \times 1.40 \text{ mm} \) are 0.58 and 0.70 \text{ mm}, respectively; the size of waste reservoir is 5.40 \text{ (L)} \times 4.00 \text{ (W)} \times 0.30 \text{ mm (H)}. The whole fabrications are easy, low cost and with high production efficiency.

**Antibody immobilization and chip assembling**

A schematic of the process is shown in Fig. 1c. First, the cover and substrate layer were cleaned with ultrapure water via ultrasonic for 30 min to wash away surface impurities. Second, soak the chip in 1M NaOH and place it in a constant temperature shaker (50 rpm/min, the same below) for 30 min at 60 \degree \text{C}. Third, soak it in 0.2\% PEI for 1 h at 25 \degree \text{C}. Fourth, soak it in 2\% GA for 2 h at 25 \degree \text{C}. Fifth, add 1 \mu l of 52 \mu g/ml SPA onto the modified region via pipette, incubating for 12 h at 4 \degree \text{C}. Sixth, remove residual aldehyde groups with 0.2M Tris-HCl (pH, 8.0) at 25 \degree \text{C} for 10 min. Seventh, 1 \mu l of 20 \mu g/ml of Chicken anti-human AFP antibody or 1 \mu g/ml of Chicken IgG (for feasibility assay) or 10 \mu g/ml Rabbit anti-chicken IgG (for one-multi
step assay) as the capture antibody was added onto the modified region, incubating at 4 °C for 12 h. Finally, block the chip with 2% BSA at 25 °C to minimize the non-specific absorption and discard the solution after 2 h. During the whole process, the chip were thoroughly washed with 1×PBS (pH 7.4) after each step and sucked with a vacuum pump, except the first three steps (washed with ultrapure water).

When the chip is dry after blocking, 4 μl of 20 μg/ml of Mouse anti-human AFP antibody or 10 μg/ml of Goat anti-Chicken IgG (for feasibility and one-multi-step assay) was dropped on the labeling region and incubated at 4 °C for 12 h, then assembling via double sided adhesive tape when the substrate is dry and storing in a dry environment for later testing.

(Figure 1)

One-multi step fluoroimmunoassay

To compare the developed method with common multi-step detection, the fluoroimmunoassay for chicken IgG are carried out as follows. First, add 4 μl of 20 μg/ml of
labeling antibody onto the second, fourth and sixth labeling region before assembling. Then, prepare two identical chicken IgG solutions, one is mixed with the labeling antibody, incubating at 25 or 37 °C and the other with the PBS buffer, and introduce 40 μl sample into the ordered inlet via pipette. After 3 min, throw the waste liquid out by hand, from the opening at the end of the chip. Or open the chip from the opening, wash the substrate layer 30 s in PBS for a lower background, especially for low concentration assays. Finally, obtain fluorescence image via the Odyssey.

**Fluoroimmunoassay for AFP via one-sampling**

The fluoroimmunoassay detection of AFP in the developed chip is carried out as follows (Fig. 1d). 40 μl sample with different concentration of AFP in FBS (for sensitivity assay) or 20 ng/ml AFP in FBS and 400 ng/ml other proteins in FBS (for specificity assay) or 50 ng/ml and 200 ng/ml AFP in FBS (for repeatability assay) were introduced into the inlet, and after 3 min,
open and wash the chip 30 s in PBS. Finally, obtain fluorescence image via the Odyssey.

**Results and Discussion**

*Feasibility assay of on-chip labeling based on micropillars*

Recently, micropillars have been successfully used for active liquid mixing in microfluidic chip, however, there is no research on power-free chip, especially for one-step detection, as far as we know. Therefore, after referring to the structure parameters of micropillars used for mixer and capture \(^{24, 26, 27}\), we first verified the feasibility on our designed chip by using the pair of chicken IgG/goat anti-chicken IgG antibody, as the capture and labeling antibody respectively.

Taking into account the different functional requirements of labeling and capture antibody, the former was immobilized on the micropillars region via physical method to ensure that they were able to flow downstream with the sample and the latter were immobilized on the detection region via chemical method to improve stability and sensitivity: for the latter, when the surface of the material is treated with chemical reagents, specific functional groups are exposed, such as
aldehyde group, which can bind the antibody’s Fc fragment via covalent coupling, and exposing

the Fab fragment for better binding with the antigen, thus not only improving the binding
efficiency, but also reducing the amount of antibody lost during washing\textsuperscript{9}. To avoid introducing

other interference factors, PBS was used as the sample. At the same time, we hoped that this

method had a shorter time than previous studies. Therefore, after 5 min, we stopped the reaction

and read the fluorescence signal. As shown in Fig. 2a, significant fluorescence appeared in the

test group, indicating that the proposed method could be used for rapid detection via

one-sampling.

\textit{Parameter optimization for immunoassay}

On the one hand, optimize the volume of sample. Based on the cubage of the microchip

and previous reaction time, different volumes (25, 30, 35, 40, 45, 50 μl) of sample (1 μg/ml of

chicken IgG) were added into the chip to find suitable volume. In the assay, rabbit anti-chicken

IgG (10 μg/ml) and goat anti-chicken IgG (10 μg/ml) were immobilized on the surface of
detection and labeling region, respectively. As shown in Fig. 2b, when the sample volume ≥ 40 μl, the fluorescence signal began to stabilize. Therefore, it could be used as the most suitable sample volume for this chip.

On the other hand, optimize the amount of capture antibody. Considering the final purpose, this assay directly used AFP-related antibodies. First, different concentrations of chicken anti-AFP were immobilized in the detection region, and 50 μg/ml of mouse anti-human AFP and 20 ng/ml AFP in PBS were used as labeling antibody and sample, respectively. As shown in Fig. 2c, i, when the concentration ≥ 10 μg/ml, fluorescence intensity (blue pillar) began to increase significantly. However, the blank control group also had a strong signal from non-specific binding, thus, the T/C values (the ratio of the fluorescence intensity of test to control, T/C°) were introduced to reduce the impact and further ensure the reliability and accuracy (red pillar in Fig. 2c. ii). It could be seen that as the concentration increased, the T/C value gradually increased.
and starting to become stable at 20 μg/ml, indicating the optimal capture antibody parameter. At the same time, high background fluorescence may mean excess fluorescence antibody, thus, different lower concentrations of fluorescence antibody were immobilized on the labeling region to find the appropriate amount of labeling antibody (Fig. 2d). As the concentration of fluorescence antibodies decreased, the background fluorescence did decrease, and the maximum T/C value appeared at 20 μg/ml, indicating the optimal labeling antibody parameter.

(Figure 2)

The shortest time required for detecting AFP

To investigate the shortest time required, 20 ng/ml AFP in FBS was detected at different incubation time. Taking the previous 5 min as a reference, a time gradient (1, 3, 5 and 7 min) assays were used to analyze the shortest time. Start from adding the first AFP solution into the chip, adding samples every two minutes for a total of four times. As shown in Fig. 2e, there was a significant difference in fluorescence between the 1 and 3 min groups, but after 3 min, the
fluorescence intensity change was not very obvious, indicating that the reaction may have been almost completed in 3 min.

To confirm the shortest time, we further explored the time within 3 min (1, 1.5, 2, 2.5 min). As shown in Fig. 2e, the longer the incubation time, the stronger the fluorescence intensity. To accurately analyze the trend of fluorescence intensity over time, “1 min” was used as a reference to analyze the two sets of time gradients together (Fig. 2e), when the time≥2 min, the T/C value increased rapidly and tended to be stable after 3 min, indicating that the detection could be accomplished within 3 min. Therefore, we demonstrated that this method could be used for rapid detection of proteins such as AFP.

**Comparison with multi-step detection**

To verify that this rapid detection benefited from the labeling method, we compared it with traditional multi-step at different temperature, which accomplishes the labeling process by
mixing the solution of label and target in a tube. To further enhance the reliability and accuracy, 1 ng/ml, 100 ng/ml and 10 μg/ml of chicken IgG were respectively analyzed. First, the temperature was set at 25 ℃ and the incubation time of multi-step was 60 min. As shown in Fig. 3a, regardless of high, medium, or low concentrations, on-chip labelling-based detection gave a stronger fluorescence signal than 60 min multi-step. However, the chip had obvious background fluorescence by throwing the liquid out through the tail opening with hand. Therefore, for more significant analysis, the chip would be washed by PBS buffer.

Considering the activity of the antibody and the free diffusion rate of the molecule, we changed the temperature to 37 ℃ and compared it again. As shown in Fig. 3b, although the fluorescence intensity has increased a lot, the on-chip method still has absolute advantages, indicating that the static labeling method based on the free diffusion of molecules has much lower labeling efficiency than dynamic labeling in a microfluidic environment. Furthermore,
different from the performance of multi-step at low concentration, the T/C values between

different concentrations could be well distinguished via on-chip, indicating that both time and

sensitivity, the proposed method had significant advantages over multi-step.

(Figure 3)

Analytical performance of AFP detection

The detection sensitivity was investigated by a series of different concentrations of AFP in

FBS, 1 ng/ml, 10 ng/ml, 100 ng/ml, 1 μg/ml and 10 μg/ml, which were added to the designated

inlet on the same chip almost at the same time. After grayscale analysis and linear fitting, the

calibration plot of T/C value and AFP concentration was shown in Fig. 4a. When the

concentration belonged to 10-1000 ng/ml, a better trend could be seen from the curve and a

good linear relationship could be obtained ($R^2=0.982$) after linear fitting. When the

concentration≤10 ng/ml or≥1 μg/ml, the cure tended to be smooth, indicating that it was hard to
distinguish different concentration. After calculating, the LOD was 1.88 ng/ml, which was much
lower than the clinical standard (20 ng/ml). 

The specificity was investigated by introducing several proteins including mouse IgG, chicken IgG and main proteins in blood, HSA, HF and human IgG, whose final concentration were conducted at 20 times of AFP in each detection system. As shown in Fig. 4b, negligible interference was obtained after introducing these interfering substances, indicating that the perfect specificity and selectivity for this chip.

The stability and repeatability of this chip were tested from two aspects by detecting 50 ng/ml and 200 ng/ml of AFP. For coefficient of variation (CV) (Fig. 4c. i and ii), although inter-assay group showed higher CV value than intra-assay group due to processing errors in different batches, the CV value of intra-assay and inter-assay at different concentration were less than 5%, indicating that the proposed method had good repeatability and reliability. We have reason to believe that if chips are prepared by other methods such as hot embossing, the
instability caused by batch difference will be improved. For stability and reproducibility in long-term storage, two different storage types have been analyzed separately: One (split type) was to store only the substrate of the chip at 4 °C and complete assembling before use; the other (assembled type) was to store the assembled chip at 4 °C. As shown in Fig. 4d, both concentrations (50 ng/ml, i and 200 ng/ml, ii) had excellent stability and repeatability through split type. However, the T/C value was significantly reduced from the third day via the other, and the stability was extremely poor for both concentrations. This was due to the insufficient bonding strength of the double-sided tape in the low temperature environment, which caused the sample to leak from the chip's inlet, and the amount flowing through the detection region became unstable. Thus, based on the bonding method, split type will be more conducive to long-term storage.

Therefore, whether the sensitivity, specificity or repeatability, this chip can meet the needs
of rapid clinical detection, and furthermore, the microchip is cheap enough and can be well
applied to POC device.

(Figure 4)

Conclusions

In this study, a power-free microfluidic-based POCT device has been developed and
demonstrated for rapid detection of AFP via one-sampling. On the one hand, the entire
immunoassay for AFP could be completed within 3 min due to the steps of mixing, labeling and
capturing for the fluorescence immunoassay can be completed automatically on the developed
chip: a dynamic ranging from 10 ng/mL to 1 μg/mL and the LOD of 1.88 ng/ml of AFP in FBS
has been obtained, which is enough to meet the needs of AFP clinical diagnosis. Furthermore,
the high designability gives it greater application potential to a wide range of biomarkers. Thus,
a simple and ultrafast immunoassay system has been successfully developed.

On the other hand, using PMMA as the chip material reduces the production cost of the
chip, and fast detection saves time cost, however, the size of the chip, especially the detection
and the label region, can also be optimized to reduce the volume of reagents, thus to decrease
reagents cost in the future. At the same time, to achieve true “one-step”, we expect that
introducing smartphone for portable fluorescence analysis to reduce equipment costs, and
absorbent paper in waste reservoir for wash-free to further reduce operational requirements.

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**Figure Captions**

Figure 1. Design and fabrication of power-free immunoassay microchip. (a) Schematic diagram and (b) photograph of microchip. (c) Schematic flow diagram of the surface modification and capturing protein immobilization. (d) Schematic diagram of fluoroimmunoassay for biomarker detection.

Figure 2. (a) The feasibility of detection via on-chip labelling. (b) The optimization of sample volume. (c) The optimization of the amount of capture antibody. (d) The optimization of the amount of labeling antibody. (e) The minimum time required to achieve an effective detection of 20 ng/ml AFP in FBS. All data points are means ± standard deviations (n = 4).

Figure 3. The superiority of on-chip (one-sampling) over off-chip (multi-step) with different concentration at different temperature (a) 25 °C and (b) 37 °C. All data points are means ±
Figure 4. Analytical performance of detecting AFP. (a) The sensitivity to AFP in the range of 1-10 000 ng/ml. (b) Anti-interference performance of the chip. (c) CV value of 50 ng/ml (i) and 200 ng/ml (ii). (d) The stability and reproducibility in long-term storage (i, 50 ng/ml and ii, 200 ng/ml). All data points are means ± standard deviations (n = 4).
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