An integrated microfluidic device for rapid serodiagnosis of amebiasis

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A microfluidic device was successfully fabricated for the rapid serodiagnosis of amebiasis. A micro bead-based immunoassay was fabricated within integrated microfluidic chip to detect the antibody to *Entamoeba histolytica* in serum samples. In this assay, a recombinant fragment of C terminus of intermediate subunit of galactose and *N*-acetyl-D-galactosamine-inhibitable lectin of *Entamoeba histolytica* (C-Igl, aa 603-1088) has been utilized instead of the crude antigen. This device was validated with serum samples from patients with amebiasis and showed great sensitivity. The serodiagnosis can be completed within 20 min with 2 μ l sample consumption. The device can be applied for the rapid and cheap diagnosis of other infectious disease, especially for the developing countries with very limited medical facilities. © 2013 American Institute of Physics. [<http://dx.doi.org/10.1063/1.4793222>]

Entamoeba histolytica is the causative agent of amebiasis and is globally considered a leading parasitic cause of human mortality.¹ It has been estimated that 50×10^6 people develop invasive disease such as amebic dysentery and amebic liver abscess, resulting in 100 000 deaths per annum.^{2,3} High sensitive diagnosis method for early stage amebiasis is quite critical to prevent and cure this disease. To date, various serological tests have been used for the immune diagnosis of amebiasis, such as the indirect fluorescent antibody test (IFA) and enzyme-linked immunosorbent assay (ELISA).

We have recently identified a 150-kDa surface antigen of *E. histolytica* as an intermediate subunit (Igl) of galactose and *N*-acetyl-D-galactosamine-inhibitable lectin.^{4,5} In particular, it has been shown that the C-terminus of Igl (C-Igl, aa 603-1088) was an especially useful antigen for the serodiagnosis of amebiasis. ELISA using C-Igl is more specific than the traditional ELISA using crude antigen.⁶ However, the ELISA process usually takes several hours, which is still labor-intensive and requires experienced operators to perform. More economic and convenient filed diagnosis methods are still in need, especially for the developing countries with limited medical facilities.

Among all the bioanalytical techniques, microfluidics has been attracting more and more attention because of its low reagent/power consumption, the rapid analysis speed as well as easy automation.^{7–11} Especially with the development of the fabrication technique, microfluidics chip can include valves, mixers, pumps, heating devices, and even micro sensors, so many traditional bioanalytical methods can be performed in the microfluidics. Qualitative and quantitative immune analysis on the microfluidic chip was successfully proved by plenty of research with improved sensitivity, shorten reaction time, and less sample consumption.^{8,10–17} Moreover,

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FIG. 1. Structure illustration of microfluidic chip.

with the intervention of other physical, chemical, biology, and electronic technology, microfluidic technique has been successfully utilized in protein crystallization, protein and gene analysis, cell capture and culturing and analysis as well as in the rapid and quantitative detection of microbes.^{13–20}

Herein, we report a new integrated microfluidic device, which is capable of rapid serodiagnosis of amebiasis with little sample consumption. The microfluidic device was fabricated from polydimethylsiloxane (PDMS) following standard soft lithography.^{21,22} The device was composed of two layers (shown in Figure 1) including upper fluidic layer (in green and blue) and bottom control layer (in red).

To create the fluidic layer and the control layer, two different molds with different patterns have fabricated by photolithographic processes. The mold to create the fluidic channels was made by positive photoresist (AZ-50 XT), while the control pneumatic mold was made by negative photoresist (SU8 2025). For the chip fabrication, the fluidic layer is made from PDMS (RTV 615 A:B in ratio 5:1), and the pattern was transferred from the respective mold. The control layer is made from PDMS (RTV 615 A:B in ratio 20:1). The two layers were assembled and bonded together accurately, and there is elastic PDMS membrane about 30 μm thick between the fluidic layer channels and control layer.^{21,22} The elastic membrane at the intersection can deform to block the fluid inside the fluidic channels, functioning as valves under the pressures introduced through control channels. There are two types of channels in fluidic layer, the rectangular profiled (in green, 200 μm wide, 35 μm thick) channel and round profiled channels (in blue, 200 μm wide, 25 μm center height). Because of the position of the valves on the fluidic channels, two types of valves (Figure 2(a)) were built, working as a standard valve and a sieve valve. The standard valves (on blue fluidic channels) can totally block the fluid because of the round profile of fluidic channel; the sieve valve can only half close because of the rectangular profile. The sieve valve can be used to trap the microspheres (beads) filled inside the green fluidic channels, while letting the fluid pass through. By this sieve valve, a micro column (in green) is constructed, where the entire ELISA reaction happens. The micrograph of the fabricated micro device is shown in Figure 2(b). The channels were filled with food dyes in different colors to show the relative positions of the channels. The pressures through different control channels are individually controlled by solenoid valves, connected to a computer through relay board. By programming the status (on/off) of various valves at different time periods, all the microfluidic chip operation can be digitally controlled by the computer in manual, semi-automatic, or automatic manner.

To validate this device, 12 patient serum samples were collected. Sera from 9 patients (Nos. 1–9) with an amebic liver abscess or amebic colitis were used as symptomatic cases. The diagnosis of these patients was based on their clinical symptoms, ultrasound examination (liver abscess) and endoscopic or microscopic examination (colitis). We also identified the clinical samples using PCR amplification of rRNA genes.²⁴ As negative control, sera obtained from 3 healthy individuals with no known history of amebiasis were mixed into pool sera. The serum

FIG. 2. (a) Structure illustration of micro column, standard valve and sieve valve; (b) photograph of the microfluidic chip.

was positive for *E. histolytica* with a titer of 1:64 (borderline positive), as determined by an indirect fluorescent-antibody (IFA) test.^{23,24} In our previously study, the sensitivity and specificity of the recombinant C-IgI in the ELISA were 97% and 99%.^{6,25} In the current study, the serodiagnosis of amebiasis was also examined by ELISA using C-IgI.²⁶ The cut-off for a positive result was defined as an ELISA value > 3 SD above the mean for healthy negative controls²⁷ (shown in Figure 3). The seropositivity to C-IgI was 100% in patients with amebiasis.

In the diagnosis process with microfluidic chip, the 4 micro immuno-columns filled with C-IgI-coated microspheres were the key components of the device. The C-IgI was prepared in *E. coli* as inclusion bodies. After expression, the recombinant protein was purified and analyzed by SDS-PAGE. The apparent molecular mass was 85 kDa.²⁶

The immune-reaction mechanism is illustrated in Figure 4. The anti-His monoclonal antibody was immobilized onto the microspheres (beads, 9 μ m diameter) coated with protein A.

FIG. 3. ELISA reactivity of sera from patients against C-IgI. ELISA plate was coated with 100 ng per well of C-IgI. Serum samples from patients and healthy controls were used at 1:400 dilutions. The dashed line indicates the cut-off value. Data are representative of results from three independent experiments.

FIG. 4. Schematic representation of the ELISA in the chip.

The C-IgI was then immobilized onto the beads through the binding between the His tag and C-IgI. For the diagnosis, the microspheres immobilized with C-IgI and blocked by 5% BSA were preloaded into the columns for the rapid analysis of the patient serum samples. Generally, serum samples which were diluted 100 times were first loaded into the reaction column and incubated at room temperature for 5 min. After being washed by PBS buffer, FITC-conjugated goat anti-human polyclonal antibody was added into the column for 4 min incubation. The fluorescence image can be collected by the fluorescence microscope after the micro column was washed with PBS buffer. From loading diluted serum samples into column to collecting fluorescence images, the total time to complete the immunoassay is less than 10 min. The final fluorescence results were analyzed by Image Pro Plus 6.0.

FIG. 5. ELISA on the chip. The signals were collected by CCD of microscope. A: negative sample; B and C: positive samples.

TABLE I. ELISA on the chip (analyzed by IPP6.0).

Sample	Average scores	Standard deviation
1	33 790	368
2	23 269	271
3	39 598	307
4	7784	52
5	21 222	197
6	38 878	290
7	22 437	227
8	36 295	334
9	41 024	396
Negative	200	32

Different reaction conditions have been investigated to find the optimized ones. For each patient, 2 μ l sample is enough for the analysis. The designed microfluidic chip with 4 micro columns is capable for 4 parallel analyses at the same time. More micro columns can be integrated into the device if more parallel tests are needed.

Different incubating time for the diagnosis has also been investigated and no significant difference has been found for various time periods. It is enough to incubate the chip for only 5 min. The total diagnosis time for one sample is less than 10 min. The detection result appeared as the fluorescence intensity of the reaction column. As shown in Figure 5, the negative sample showed relatively low fluorescence intensity, because little FITC-conjugated goat anti-human polyclonal antibody could attach to the surface of microspheres; on the contrast, the positive sample showed much brighter fluorescence. The fluorescence intensity can be transferred to digital data (Table I) by IPP software. The results showed good correspondence with the traditional ELISA results. The positive samples showed a significantly higher number than the negative one, and the standard deviation was nearly 1% to the average scores.

For the heterogeneous immunoreactions, the immobilization of the immune molecules is essential for the reaction efficiency. Herein, we utilized micro columns filled with pre-modified microspheres (beads) instead of the direct surface modification for the ELISA analysis. Compared with the traditional method, diagnosis using the microfluidic device took less than 10 min with only 2 μ l sample consumption and little reagent consumption. The high efficiency might be attributed to the high surface modification efficiency by using beads as well as the advantages from microfluidic device itself. The C-IgI modified microspheres can be easily prepared in 1 h and preloaded inside the micro device for convenient application. The device is made from standard soft lithography by PDMS and its throughput can be easily improved by adding more micro columns into the microfluidic device in an economic manner, which is perfect for the onsite rapid and cheap diagnosis of amebiasis. Similar methodologies can be developed for diagnosis of other infectious disease, especially for the developing countries with very limited medical facilities.

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