Blood typing chip using human whole blood sample

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Blood typing is a critical test for blood transfusion and many medical procedures. Described is a disposable microfluidic blood typing chip with a simple fabrication process. The chip allows one to use human whole blood directly so that the operation sequence of a manual polybrene technique for blood typing can be conducted automatically. In addition, the determination of the extent of red blood cell agglutination can be analysed by software. The feasibility of this chip was demonstrated by ABO blood typing tests using antibody screening cells and freshly drawn venous blood samples. From the pixel count of agglutination, the chip provided correct blood typing results. This proposed microfluidic chip has the merits of smaller consumption volumes of reagents. Moreover, the simple fabrication process of the chip results in low fabrication cost.

1. Introduction: Blood transfusion is a medical treatment to replace the lost blood components. Before the transfusion, blood typing is a critical test to ensure the serological compatibility of a donor and a recipient. Blood typing is based on the antigen–antibody interaction. If antigens on the surface of red blood cells (RBCs) correspond to antibodies in the serum, agglutination of RBCs occurs. Incompatible RBCs in transfusion, that is, mistyping of the ABO blood group, might result in intravascular haemolysis, renal failure and shock, and even cause the recipient's death [1].

There are many stages to examining blood products for the sake of successful transfusion. In blood banks, considerable quantities of routine blood typing tests are also executed to ensure that all individual recipients in scheduled transfusions are given blood that is compatible. Thus, fully automated blood typing systems are required [2–4]. These systems are expensive with a large footprint. In hospital transfusion services, a cross-matching test is performed before transfusion to check the degree of agglutination by mixing a sample of the recipient's serum with a sample of the donor's RBCs. In the case of agglutination, transfusion using the donor's blood to that particular recipient is not allowed. Currently, the microplate method, which is carried out in a 96-well microtitre plate, is usually used by manual operation followed by a visual determination of the extent of RBC agglutination. This assay is tedious and laborious. Moreover, it is not amenable to giving an automated optical readout of agglutination.

In this regard, we propose a disposable and low-cost microfluidic blood typing chip in this Letter, allowing us to use human whole blood directly. Microfluidic systems can handle and manipulate minute amounts of microfluids, such as reagents and samples, to provide transportation in the microchannels for the purposes of dilution, particle separation, mixing and incubation. Hence, microfluidic techniques have been successfully applied to numerous biochemical analysis procedures [5–7]. Inheriting the merits of microfluidic platforms, microfluidic blood typing systems can provide an elegant solution for assays to be conducted in an automated manner and miniscale volumes. Kim *et al*. [8] presented a disposable blood typing biochip by integrating flow splitting microchannels, chaotic micromixers, reaction microchambers and detection microfilters, allowing the observation of agglutination with the naked eye. Kline *et al*. [9] utilised the microfluidic droplet technique to encapsulate RBCs, antibodies and buffers in droplets to perform multiple agglutination assays. Makulska *et al*. [10] presented a method to determine agglutination by measuring the speed of flow of microdroplets, containing RBCs and antibodies, in the microchannel. Alternatively, paper-based microfluidics is another branch developed for blood typing [11–13].

This proposed blood typing chip was designed to have a simple structure. Furthermore, the assay result can be analysed by software. In this reported study, the functionality of this chip was verified by assays using ABO blood groups.

2. Experimental: The manual polybrene (MP) technique for blood typing is operated using a 96-well microplate and widely adopted in Asia. The MP technique is a rapid and sensitive method used to detect a wide range of RBC antibodies. The test takes 5–10 min and requires a blood sample of 200 µl. The test protocol involves a series of mixing, incubation and centrifugation steps [14, 15]. First, RBCs, test serum (or plasma) and low ionic medium (LIM) are added to the microplate. The LIM can promote the reaction between RBC antigens and antibodies if they are associated. After 1 min of incubation at room temperature, the plate is centrifuged. Then, the supernatant is flicked out of the plate and one drop of 0.05% polybrene solution is added. The function of polybrene is to cause non-specific RBC aggregation. After mixing and centrifugation, the supernatant is discarded and one drop of resuspension solution, which is for reversing the non-specific polybrene-induced aggregation, is added. Agitation is thus performed. Finally, agglutination is observed.

Based on the test protocol, this proposed microfluidic blood typing chip was designed. This chip allows us to use the whole blood for experiments with miniscale volumes.

2.1. Chip design: The microfluidic blood typing chip is comprised of a microfluidic layer bonded on the glass microscope slide. The main microfluidic components include the blood separation microchannels, microfilters, the mixing microchannel, the detection chamber and the waste reservoir, as shown in Fig. 1. Their depth, except that of the microfilters, are all 100 µm. Using a syringe pump, the 3% diluted whole blood is injected into the inlet (A) and flows through the separation microchannels. Since plasma has less mass than blood cells, the centrifugal force produced in the curved separation microchannel results in blood cell flow and plasma flow as a double-layer laminar flow [16, 17]. Then, the blood plasma is drawn out and collected in the plasma chamber (B), whereas erythrocytes and leucocytes are driven forward to the microfilters. The microfilters aim to block leucocytes and to allow erythrocytes to pass through. Thus, the

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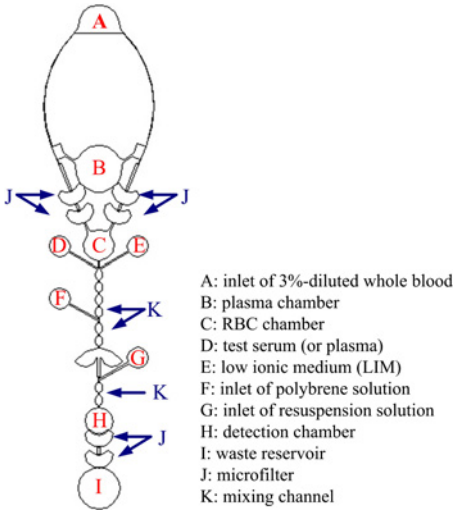

Figure 1 *Design of microfluidic blood typing chip*

microfilters are designed to have a gap of 5 μm since the average size of leucocytes is 8–15 μm. The second microfilter is for capturing any escaped leucocytes. The width of the microfilters is larger than the linking microchannel of 100 μm for the sake of mask alignment. Hence, a precise photolithography aligner is not required. In addition, because of the half-moon shape of the microfilters, erythrocytes flowing through the microfilters can be gathered together and completely drawn out to the connected microchannel without remaining at any dead space. Then, erythrocytes are collected in the RBC chamber (C) and impelled into the mixing channel. In the meantime, test serum (or plasma) and LIM originally stored in the chambers (D) and (E), respectively, are introduced into the mixing channel to mix with erythrocytes.

In a straight microchannel, two fluids flowing alongside keep in parallel layers because of the low Reynolds number and can mix only by diffusion. Hence, in this study the mixing microchannel is designed as a string of beads to force the fluids to mix because of the change in channel width. Then, polybrene solution is added from chamber (F). After mixing, inertia carries RBCs forward but the supernatant flows mostly into the wing-like chambers. Next, a resuspension solution is added from chamber (G). The final section of the mixing microchannel provides the necessary mixing and agitation. The fluid is eventually driven into the detection chamber (H). If no agglutination occurs, the fluid is continuously impelled to pass the microfilters behind the detection chamber to the waste reservoir (I). Otherwise, agglutination is blocked by the microfilters and the fluid stays inside the detection chamber, where agglutination is recorded using a digital charged-coupled device (CCD) for further image analysis.

2.2. Fabrication process: The microtransfer moulding technique was adopted in this study. A mould with the opposite pattern of all microfluidic components must be prepared first and then the microfluidic layer can be formed by casting polydimethylsiloxane (PDMS) in the mould. The fabrication of the mould started from the structure of the microfilters. A commercially available printed circuit board (PCB) was trimmed to a size of $3'' \times 1''$ as the substrate of the mould. Besides the consideration of cost, the main reason to adopt the PCB is that its conducting layer, typically made of thin copper foil, can serve as the plating seed layer directly. Thus, the fabrication process becomes simple. On the PCB substrate, the pattern of the microfilters was defined by photolithography process. After stripping the photoresist of these regions, the PCB substrate was washed thoroughly with deionised water. Therefore a Ni–Co film with a thickness of 5 μm was fabricated by electrodeposition. The thickness of the Ni–Co film becomes the gap of the microfilter after PDMS casting.

The electroplating process was carried out in a W 18 cm × L 25 cm × H 18 cm electroplating tank. A 100 V/35 W pump which was equipped at the bottom of the tank provided a flow upwards to the metal substrate to enhance the turbulence for promoting the uniformity of the plating bath. To eliminate impurities and crystalline solids, a filter with 1 μm pores was installed above the pump. Furthermore, a digital power supply controlled by a PID controller supplied a 100 V/100 W output to a quartz heater for temperature control of the plating bath.

After the electrodeposition process, the photoresist of the PCB substrate was completely removed. Instead, a layer of SU-8 2050 with a thickness of 100 μm was coated. Then, the pattern of the microfluidic components was defined by the photolithography process to finish the SU-8 mould.

The microfluidic layer was formed by casting PDMS in the SU-8 mould. PDMS is one of the extensively used materials in microfluidic chips and miniaturised devices because of its distinguishing features of transparency, shaping and adhesion ability. It is a silicone elastomer (Sylgard®184, Dow Corning), with the supply of a base and a curing agent as a two-pair kit, comprising liquid components. The base, curing agent and silicon oil with a volume ratio of 10:0.75:1 were thoroughly mixed, poured into the SU-8 mould and placed under vacuum for 45 min to remove entrapped air. Then, the PDMS microchannel layer was left to cure at 95°C in an oven for 1.5 h. Finally, it was carefully peeled from the SU-8 mould.

For any microfluidic applications, leakage is not allowed. Although PDMS possesses as excellent physical absorption property so as to adhere to the surface of many substrates, oxygen plasma treatment was adopted in this study to modify the surfaces of both the PDMS layer and the microscope slide. The process was conducted at a pressure of 1.2×10^{-1} torr for 3 min by decomposing oxygen into OH– to deposit on both surfaces to be bonded. The PDMS layer was thus placed on the PCB substrate and the chip was heated in a 95°C hot circulator oven for 5 min to complete the bonding process.

2.3. Bioexperiments: Two stages of bioexperiments were carried out. In the first stage, antibody screening cells (purchased from the Formosa Biomedical Technology Corp., Taiwan) were adopted to test the functioning of this proposed microfluidic blood typing chip. In the second stage, freshly drawn venous blood samples from five healthy donors and two informed and consenting patients were used for the experiments. In one experiment, 5 μl of 3% diluted whole blood, 10 μl of antibody, 10 μl of LIM, 5 μl of polybrene and 5 μl of resuspension solution were used. A KDS Legato 270 programmable syringe pump (manufactured by KD Scientific Inc.) was utilised to manipulate the blood sample and the reagents.

Agglutination was recorded by the CCD. The recorded colour images were first transformed into greyscale images and analysed using MATLAB. For each greyscale image, a thresholding technique was used to create its corresponding binary image with a monochrome black and red colour. The red portion indicates the RBC agglutination. Furthermore, a bypass filtering technique was used to eliminate any small spots on the image, which is usually regarded as noise.

3. Results and discussion: Before proceeding to the blood typing experiments, different settings of the syringe pump were tested to determine the proper flow rate under which the blood cells and agglutinated RBCs would not be broken up. The optimal flow rate was finally chosen to be 30 μl/min after a series of tests. The experimental results are elaborated as follows.

3.1. Blood separation: The role of the separation channel is to separate blood components. In addition, the microfilters are designed to block leucocytes for extracting erythrocytes. To test the separation effect, 1 ml of 3% diluted whole blood was employed to flow through the microfluidic chip. Three kinds of flow rates, involving 10, 15 and 30 µl/min, were set, respectively. For each flow rate, the experiment was carried out five times. To check whether any leucocytes pass through the microfilter, 20 µl of the substance was drawn from the RBC chamber (C) using a pipette and mixed with 180 µl of Türk's solution (Merck KGaA, Germany). Then, 20 µl of the mixture was drawn to the haemocytometer for leucocyte counting. The mixture drawing and leucocyte counting were performed five times.

Similarly, 20 µl of the substance was drawn from the microchannel in front of the first microfilter to count the blocked leucocytes. In addition, we drew 20 µl of blood plasma each time from the plasma chamber (B) until the chamber was empty to count the total volume collected.

Table 1 Separation effect

Flow rate	10 µl/min	15 µl/min	30 µl/min
total collected volume of blood plasma, µl	508	460	428
number of leucocytes blocked by microfilter	9.2	8	8
number of leucocytes detected in RBCs	0	0	0

Table 1 shows the experimental results. For all designed flow rates, no leucocyte was observed to pass through the microfilter so that the number of leucocytes detected in the RBC chamber is zero. Since most of the blocked leucocytes are attached to the surface of the microchannel, it is difficult to draw them out completely. Nevertheless, some leucocytes were detected in front of the microfilter. Obviously, the microfilter achieved its function of completely separating erythrocytes and leucocytes for the blood typing test. However, the separation of plasma was affected by the flow rate. Blood plasma makes up about 55% of total blood volume. With the curved separation microchannel in this proposed chip, the amounts of collected blood plasma under the different flow rates reach 50.8, 46.0 and 42.8%, respectively, from 1 ml of whole blood. That is, the separation efficiencies, defined as the collected blood plasma over the value of 55%, are 92.36, 83.64 and 77.82%, respectively. At the lower flow rate, more blood plasma was collected but a few leucocytes entered the plasma chamber along the plasma flow. The average number of leucocytes detected in the plasma chamber at a flow rate of 10 µl/min is 0.5 cells. No leucocyte was observed at flow rates of 15 and 30 µl/min. As the flow rate increased, a greater amount of plasma was impelled to pass through the microfilter.

3.2. Mixing effect: To examine the mixing effect of the bead-type microchannel, white dye and red dye solutions were used as the materials for the mixing test. The straight microchannel was also employed for comparison. Both microchannels were 20 mm in length. The width of the straight microchannel was 200 µm, whereas the bead-type microchannel had a maximal width of 500 µm and a minimal width of 200 µm. For each type of microchannel, both dye solutions were injected into the channel and were driven by a syringe pump. The image of the flow was captured and analysed using software to investigate the mixing phenomenon.

The greyscale of white dye solution is in the range of 115–135, whereas the scale value of red dye lies in the range of 180–200. The change of greyscales within the mixing channel explains the mixing

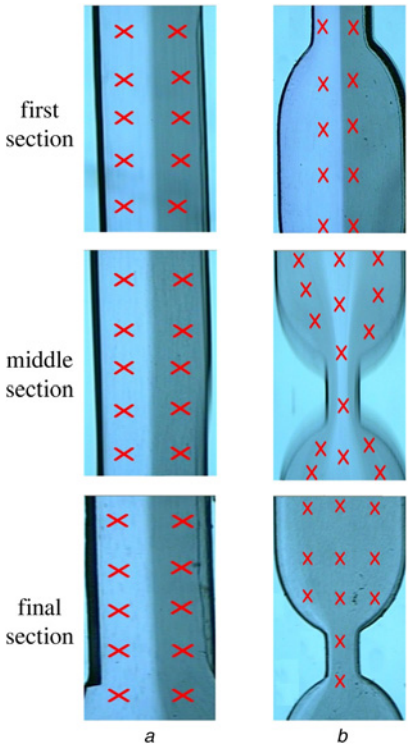

Figure 2 *Mixing phenomenon by greyscales observed at different locations of mixing channel*

effect. As shown in Fig. 2*a*, two dye solutions flowing alongside keep in parallel layers in the straight microchannel. We divided the straight microchannel into three sections and picked five locations, each section being for statistical study. In the first section, the average greyscales for both dye solutions are 190.0 and 125.8, respectively. In the middle section, the average greyscales become 189.2 and 124.7, respectively. In the final section, the average greyscales are 182.7 and 116.8, respectively. This result explains that in the straight microchannel mixing occurs at the interface of both dye solutions by diffusion. Hence, the greyscale of each dye solution changes slightly. However, the bead-type microchannel forces the two dye solutions to mix by the change of channel width. As shown in Fig. 2*b*, the marks indicate the locations at which the greyscales of mixture are calculated. In the first section of the bead-type microchannel, the two dye solutions are separated and obviously not mixed yet. Their average greyscales are 187.0 and 130.2, respectively. A certain degree of mixing is reached in the middle section of the bead-type microchannel, the average greyscales being 154.0, 188.0 and 143.8. Thorough mixing can be observed in the final section of the bead-type microchannel. This result is verified by the average greyscales being 146.6, 145.6 and 147.3, respectively.

3.3. Observation of aggregation: Agglutination and non-agglutination tests were conducted to verify the functionality of this microfluidic blood typing chip. To obtain the statistical results, three chips were prepared for repetitive experiments in each test.

Since the ABO system is the most important blood group system in transfusion, assays using A, B and O antigens were performed in this experiment. If type A RBCs are exposed to anti-A, agglutination must occur. After the aforementioned blood typing procedure, the agglutinated blood cells will be blocked by the microfilter behind the detection chamber (H). A similar result was observed for the experiment of type B RBCs and anti-B. However, for non-agglutination situations, such as type A or type O RBCs being exposed to anti-B, most of the reactant solution in the mixer will

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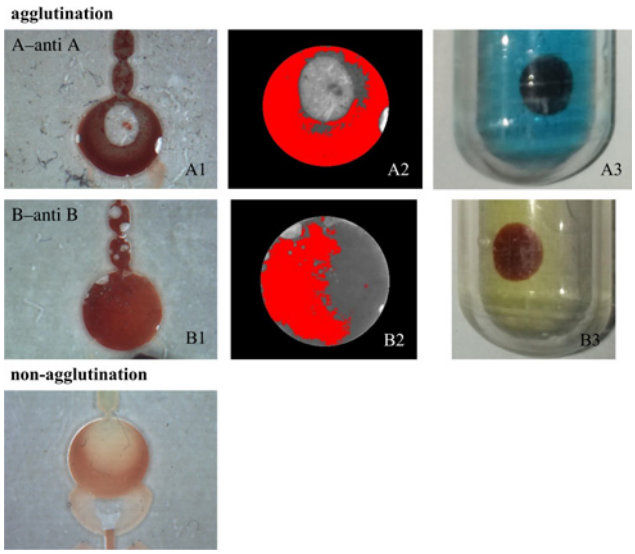

Figure 3 *Images of agglutination/non-agglutination phenomena*

be driven to pass through the microfilter. No evident agglutinated blood cells can be observed. Figs. 3 A1 and B1 show the images of agglutination phenomena in the blood typing chip. For comparison, both assays were conducted using tubes. Agglutinations can be clearly observed, as shown in Figs. 3 A3 and B3.

To obtain quantitative results, an image processing and analysis system was developed. The images of the agglutination tests were captured using a digital CCD. Then, the thresholding method was used to create binary images with only black and red colours, as shown in Figs. 3 A2 and B2. The area of red colour indicates the agglutinated blood cells. Next, the pixels of the red colour areas were counted. All small spots with <5 pixels were regarded as noise and eliminated. Thus, the pixel count of every clump represents its size. For type A RBCs agglutinating with anti-A antibodies, the average pixel count of agglutination is 17 647 with the standard deviation of 3855.8, as shown in Fig. 4. For type B RBCs with anti-B antibodies, the average pixel count of agglutination reaches 15 924 with the standard deviation of 2485.5. This is completely distinct from the results of the non-agglutination situations, where the pixel counts are almost 0. The determination of blood groups can be accurately and rapidly accomplished via the image processing and analysis system.

The influence of bubbles in the chamber, as shown in Figs. 3A1 and B1, deserves to be discussed. The bubbles came from the air entrapment during pumping. Air does not participate in the reaction. However, the bubbles decrease the space for the existence of blood agglutination in the chamber and affect the pixel count of agglutination. However, there is no influence on the result of the non-agglutination situation. In this study, a bigger chamber was designed to prevent a false result.

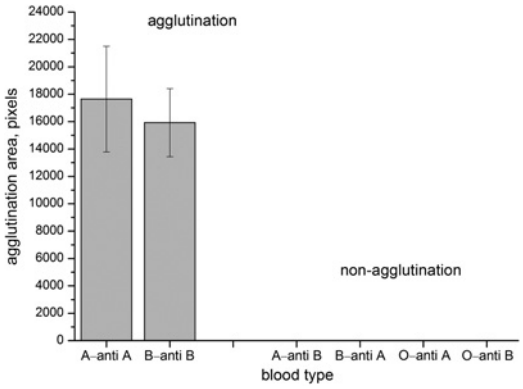

Figure 4 *Pixel count of agglutinated RBCs*

4. Conclusion: Blood centrifugation and microplate operation are required steps for blood typing tests before transfusion. Both steps are conducted using a centrifuge and a 96-well microplate, respectively. It is important to integrate the complicated and laborious steps into a single operation sequence, especially for emergency cases. In this study, a disposable microfluidic blood typing chip with the simple fabrication process has been developed. In addition, the determination of the extent of RBC agglutination can be analysed by software. ABO blood typing tests using antibody screening cells and freshly drawn venous blood samples were performed to verify the functionality of this microfluidic blood-typing chip. From the pixel count of agglutination, the chip provided correct blood typing results. Owing to the small volume of reagent consumption, the reagent cost can be significantly reduced, compared with the cost of traditional blood typing methods. The test takes <5 min. Moreover, the simple fabrication process of the chip results in a low fabrication cost.

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