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Real time observation and automated measurement of red blood cells agglutination inside a passive microfluidic biochip containing embedded reagents

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ABSTRACT

The process of agglutination is commonly used for the detection of biomarkers like proteins or viruses. The multiple bindings between micrometer sized particles, either latex beads or red blood cells (RBCs), create aggregates that are easily detectable and give qualitative information about the presence of the biomarkers. In most cases, the detection is made by simple naked-eye observation of agglutinates without any access to the kinetics of agglutination. In this study, we address the development of a real-time time observation of RBCs agglutination. Using ABO blood typing as a proof-of-concept, we developed i) an integrated biological protocol suitable for further use as point-of-care (POC) analysis and ii) two dedicated image processing algorithms for the real-time and quantitative measurement of agglutination.

Anti-A or anti-B typing reagents were dried inside the microchannel of a passive microfluidic chip designed to enhance capillary flow. A blood drop deposit at the tip of the biochip established a simple biological protocol. *In situ* agglutination of autologous RBCs was achieved by means of embedded reagents and real time agglutination process was monitored by video recording. Using a training set of 24 experiments, two real-time indicators based on correlation and variance of gray levels were optimized and then further confirmed on a validation set. 100% correct discrimination between positive and negative agglutinations was performed within less than 2 min by measuring real-time evolution of both correlation and variance indicators.

1. Introduction

The process of agglutination is commonly used for the detection of biomarkers like proteins or viruses present in solution or in biological samples. The multiple bindings to micrometer sized particles, either latex beads or red blood cells (RBCs), create aggregates that are easily detectable and lead to qualitative information about the presence or not of the biomarkers (Golchin et al., 2012; Jemima et al., 2014). Hemagglutination assays are widely employed to characterize viruses and bacteria that naturally agglutinate RBCs, especially for influenza and veterinary diagnosis (Fan et al., 2012; Gopinath and Kumar, 2013; Horie et al., 2009; Idelevich et al., 2014). In addition, hemagglutination assays can also be used to detect the presence of antigens on RBCs by specific probes. In this case agglutinins such as IgM antibodies may be used as reagents. An example of such assays is forward blood typing (Li et al., 2015; Noiphung et al., 2015; Voak, 1990). Moreover, some studies focused on the detection of biomarkers *via* hemagglutination, using a bispecific reagent. The latter is able on one hand to interact with red blood cells and on the other hand to recognize the targeted biomarker (Chen et al., 2007; Gupta and Chaudhary, 2006; John et al., 1990). Hemagglutination Inhibition Assays (HIA) are also considered to determine the affinity of inhibitors of agglutinins (Cecioni et al., 2015).

Agglutinates can be generated using various protocols, based on slide (Pandya and Kirby, 1981), tube (Li et al., 2005), microplate (Spindler et al., 2001), gel column techniques (Seth et al., 2012) or on paper based devices (Noiphung et al., 2015). Usually, these protocols are performed manually. In most cases, the detection of the agglutination is made by simple naked-eye observation (Chattopadhyay et al., 2014; Gupta and Chaudhary, 2003). Sometimes, automation of the analysis is included to overcome bias and variability induced by manual protocols (Charrière et al., 2015; Nguyen et al., 2016). Nevertheless, in

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all cases end-point measurements without any access to the kinetics of agglutination are performed. In order to obtain absolute quantitation of a biomarker, qualitative or semi-quantitative end point measurements may not be sufficient. Consequently, additional information would be an asset. In this context, we address in this study the development of a real-time observation of RBCs agglutination.

Using ABO blood typing as a proof-of-concept, we developed an integrated biological protocol suitable for further use as point-of-care (POC) analysis. An injection molded microfluidic chip, designed to enhance capillary flow, contains anti-A or anti-B dried typing reagents inside its microchannel. The biological protocol simply consists of the deposition of a blood drop at the tip of the biochip. Then, imaging of the agglutination inside the biochip is achieved by video recording. The embedded reagents are able to trigger autologous RBCs agglutination *in situ*, allowing us to monitor in real time the whole agglutination process. Two dedicated image processing algorithms are specifically designed for the real-time and quantitative measurement of agglutination. Those two agglutination indicators, based on correlation and variance of gray level, are optimized using a first training set of 24 experiments and are further confirmed on a validation experimental set. Both correlation and variance indicators monitor the kinetics of agglutination in real-time and give 100% correct discrimination between positive and negative agglutinations within less than 2 min.

2. Material and methods

2.1. Microfluidic chip

The microfluidic chip (Cema, Le Mans, FRANCE), represented in Fig. 1, is an injection molded chip made of cyclic olefin polymer (COP). It was specifically designed for passive microfluidic by simple capillary flow (Berthier et al., 2015). To increase the wettability of the COP, the chip was exposed to O_2 plasma using a MVD100 (Applied Microstructures, San Jose, CA, USA) for 10 min, at a flow rate of 450 cm^3/min, with 200 W power. The hydrophilic treatment efficiency was then checked by measuring the contact angle of water on the COP. The measure was performed using a DSA100 (Krüss, GERMANY) by deposing a 2.5 µL drop of water on the elbow part of the microfluidic chip. The hydrophilic treatment is performed before the deposit of the reagent solution to be dried. The enhanced hydrophilicity lasts at least 6 months without any noticeable increase in water contact angle..

2.2. Embedded reagents

GROUPAKIT (Diagast, Loos, FRANCE) anti-A and anti-B blood typing reagents were used. These two liquid reagents were reformulated by Avalun (Grenoble, FRANCE). The reformulated solutions were separately embedded in microfluidic chips to make anti-A biochips or anti-B biochips by drying. Drying was achieved in a vacuum desiccator (DURAN, Mainz, GERMANY), all the biochips were held firmly in the same position (V-groove pointing downward, see Fig. 1) and filled completely by 6.5 µL of reformulated solution. The drying process occurred overnight at room temperature. The storage of the biochips does not exceed one week after reagent drying. The biochips are stored in 15 mL Falcon tubes (BD Biosciences, Le Pont de Claix, FRANCE) away from light at room temperature.

Since the reagent solution contains a dye, its resuspension after drying inside the biochip was verified by microscope observation after deposition of PBS solution. The reagent activity was confirmed after the experiments by recovering the blood from the biochip by centrifugation and by observing the agglutination status under a microscope. Furthermore, comparison between embedded reagents and solution based reagent deposit (2 µL of blood followed by 2 µL of reagent solution) have been performed to confirm *in situ* agglutination inside the microfluidic channel.

2.3. Blood samples

Blood samples from healthy donors (Etablissement Français du Sang (EFS), Grenoble, FRANCE) collected in EDTA vacutainer tubes (Becton Dickinson, Le Pont de Claix, FRANCE) were used after 1:5 dilution with PBS (Sigma-Aldrich, Steinheim, GERMANY). According to the ethical and legal standards of our blood supplier (EFS), informed consent was given by blood donors. Blood usage was allowed by Health Department of Research Ministry as described in the delivered French directive #DC-2008-334. The blood tubes were delivered 3 days after withdrawal and were kept at 4 °C in a fridge for storage. The experiments were performed within 3 days of delivery. Before experiments, 500 µL of each blood samples were pre-warmed at room temperature. With room temperature set at 25 °C, the temperature of the blood inside the biochip measured with a thermocouple (ThermoCoax, Suresnes, FRANCE) never exceeded 27 °C when exposed to the microscope lamp for the whole duration of an agglutination test.

For research purpose, venous blood collected in a vacutainer is a convenient solution to ensure consistency of the sample for several experiments. However the Point-Of-Care application described in the present study requires only a small amount of blood that can be obtained by a finger prick. The quick filling of the biochip and the rapid agglutination process would not require the suppression of the coagulation capability of the blood using an anticoagulant.

The blood supplier, EFS, determines the blood group of the donors as a routine test. Those results serve as controls for our blood typing test. A first training set of 24 experiments was performed, and used as an optimization set, to determine the parameters for the calculation of the agglutination indicators. Then a second set of eight experiments was performed, as a validation set, to check that the selected parameters are still relevant to discriminate positive and negative agglutination using the indicators for new blood samples. For the optimization set, blood samples from four different donors were selected with respectively A, A, B and O blood groups. For the validation set, blood samples from two donors with blood groups A and O were selected. Optimization and validation sets were performed at three months distance using different blood samples and biochips from different batches of embedded reagent.

2.4. Biological protocol and video recording

The non-functional end of the biochip (elbow part marked “B” in Fig. 1a) was fixed with double sided tape on a microscope glass slide. A volume of 6.5 µL of 1:5 diluted blood is deposited using a P10 micropipette (Eppendorf, Hamburg, GERMANY) at the curved tip of the biochip containing an anti-A or anti-B reagent. Capillary forces drained the blood inside the biochip (Berthier et al., 2015). The microfluidic channel is filled in less than 5 s.

Video recording of the agglutination process was made with an optical upright microscope, Olympus BX60 (Olympus, Shinjuku, Tokyo, JAPAN) with a ×5 objective lens. The transmitted light was recorded by a monochrome camera Mightex BTE-B050-U (Mightex, CANADA) in 8 bit mode. The images were recorded using 1:2 decimation; the resulting resolution being 1296×926 pixels. For each experiment an image sequence was collected during two minutes at one frame per second. The recording started roughly ten seconds before the blood was deposited at the tip of the biochip with t=0 s corresponding to the beginning of the acquisition. Solution injection is complete inside the microfluidic channel after t=20 s. Thus, images before t=20 s were discarded to avoid incorrect image processing due to the blood meniscus flowing through the channel.

The video recording of the blood filling inside the channel by spontaneous capillary flow was made with the same Mightex camera at 40 frames per second, with a 5–40 mm C-mount objective (COMPUTAR, Japan). Blood filling of a biochip containing embedded

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