A micro-rheological method for determination of blood type†

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The measurement of time and distance can be used for determining agglutination in small (nL) samples of liquid. We demonstrate the use of this new scheme of detection in typing and subtyping blood in a simple microfluidic system that monitors the speed of flow of microdroplets. The system (i) accepts small samples of liquids deposited directly onto the chip, (ii) forms droplets on demand from these samples, (iii) merges the droplets, and (iv) measures their speed in a microchannel. A sequence of measurements on different combinations of blood and antibodies can thus be used to determine blood type with the estimated probability of mistyping being less than 1 in a million tests. In addition, in the agglutinated samples, red blood cells concentrate at the rear of the droplets yielding an additional vista for detection and suggesting a possible mechanism for separations.

Classification of human blood is based on the presence or absence of specific antigens on the surface of red blood cells. The body only produces antibodies against antigens that are not its own: if a particular antigen is present in an organism, its specific antibody is absent. Each antigen is assigned to one of ca. 30 human blood group systems, of which only few are clinically important. Full characterization of a blood type (group) requires determination of antigens in each of the systems. Of these, the most important reduce to ABO, distinguishing A, B, AB and O blood groups, determined by the presence of either A or B antigens, both or none of them respectively. The additional key determinant is the Rh factor, which can be either positive (presence of antigen D) or negative (absent).

Technically the determination of the ABO/Rh blood type requires the following procedures: the patient’s red blood cells are contacted with standard sera containing specific antibodies while in parallel, and the patient’s serum is mixed with reference blood cells containing known antigens. Standard tests are done on glass substrates or in test tubes, require ca. 15 min, and use visual detection of agglutination to assign the blood types: (i) A, if red blood cells agglutinate with serum containing only anti-A antibodies; (ii) B, if red blood cells agglutinate with serum containing only anti-B antibodies; (iii) AB, if red blood cells agglutinate with both sera containing anti-A and anti-B antibodies, and (iv) O, if red blood cells agglutinate with none of the sera. There are also subtypes that differ in the degree of expression and antigenicity. Less expressive antigens exhibit weaker agglutination. The strongest antigen-A subtype is A1, A2 and others (A3 etc.) result in weak agglutination and can be mistyped as O. This misassignment may result in a mismatched transfusion. Subtypes of antigen B have no diagnostic significance. Similarly, Rh

Introduction

Here we demonstrate a microfluidic method for reproducible and objective determination of agglutination in small, nanoliter quantities of liquid. We show for the first time, that agglutination of particles (here red blood cells) within a microdroplet can produce a reliable measurable change in their mobility in the microchannels. Here we focus the demonstration of the reliability of the method on the model example of determination of blood type, yet the method should also be possible to use with the wide range of agglutination assays used in in vitro diagnostics.

Blood is the most important physiological fluid both diagnostically and therapeutically, and is used in transfusions in life threatening situations that often necessitate swift treatment. Transfusion requires serological compatibility of a donor and an acceptor. Optimum blood typing assays should be swift, objective and use small samples, a feature that apart from being attractive in standard assaying is critical in the treatment of infants or seriously ill patients. Standard blood typing and cross-testing assays are not optimized for miniaturization of sample volume and are based on subjective optical indicators of agglutination. The method that we describe (i) is based on an objective measurement of the speed of flow of microdroplets containing blood, (ii) uses single micro-liters of blood per typing assay, (iii) distinguishes subtypes of blood, and (iv) is amenable in the development towards either point-of-care devices or towards multiplexing.

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factor is determined by mixing red blood cells with serum containing antibodies against antigen D. Agglutination signifies Rh+ and vice versa.

Transfusions require more extensive testing for serological compatibility of donor and recipient. Tests for the presence of many antibodies require up to an hour of analysis time and extensive human manipulation. Except for emergency situations, the compatibility is typically tested beyond the ABO/Rh system. The most routinely tested are antigens from Kell, Lewis, Kidd and Duffy systems. Red blood cells containing these antigens are added to the recipient’s serum. Agglutination assays determine which antigens should not be present in the donor’s blood. The last step comprises of verification of whether the recipient serum is immune against any donor antigens. Importantly, these often subjective procedures are critical as any mistakes in transfusion can have fatal consequences.

Development of microfluidic blood-typing assays can reduce the time of analysis, enable simultaneous determination of the most clinically important antigens and decrease the requirement for human intervention. The usage of a small volume of blood can eliminate the necessity of storage and minimize handling and recycling of biological material. Kim et al. described a compact biochip requiring ca. 1 μL of blood passed through a series of microfilters, allowing for simple subjective assessment of agglutination with the naked eye. Ramasubramanian et al. automated the detection of agglutination in a system equipped with optical fibers, while Kline et al. used droplets in microchannels for blood typing: droplets containing mixtures of RBCs and antibodies were tested for agglutination via measurement of the change in uniformity of brightness across any given droplet over time. This technique allowed for resolving not only ABO/Rh groups, but also for detection of rare A subtypes and for detecting bacteria. However, image analysis is laborious and time-consuming. Also paper and thread-based microfluidics have been used for blood typing on the basis of chromatographic separation of blood components and changes in kinetics of wicking via detection with the naked eye.

Blood typing and cross testing are not the only assays that use agglutination as the key factor for detection. Agglutination tests are typically quite simple to carry out and relatively sensitive, and are an important and common technique in *in vitro* diagnostics. Agglutination is widely used to diagnose various infectious diseases, especially those caused by bacteria, (e.g. brucellosis, by protozoa or parasites). Latex agglutination tests (LAT) are used in the detection of viruses. The event of agglutination is typically detected by visual inspection. Here we show that a more fundamental measurement can also be used to detect agglutination in microdroplets. In particular we demonstrate for the first time the use of the most fundamental measurements of time and distance to detect agglutination on the basis of it modulates the mobility of droplets traversing a microfluidic channel, thus providing a reliable and reproducible detection scheme that uses the simplest optical detection. Importantly, the system that we demonstrate accepts small samples of suspensions of RBCs and solutions of antibodies onto the chip, and subsequently uses these samples to generate microdroplets and measure their speeds in a completely automated protocol. In a model demonstration we used this method to characterize a set of samples against the ABO and Rh systems.

### Results

Fig. 1 illustrates the microfluidic chip that we used in our experiments. The chip is equipped with six T-junctions. Each T-junction comprises of two inlet ports for the continuous liquid (n-hexadecane, viscosity \( \mu_c = 3.0 \pm 0.1 \text{ mPa s} \), density \( \rho_c = 770 \text{ kg m}^{-3} \) at 25 °C; Alfa Aesar, as supplied) and one additional port for the deposition of the sample of red blood cells (RBCs) or of monoclonal reagents containing antibodies. These inlet ports allow for deposition of samples as small as a few μL from a pipette. In this work we used syringes to pump 200 μL of each sample onto the on-chip reservoir and then we clamped the tubing (O.D. 1.22 mm, I.D. 0.76 mm, Intramedic, USA) effectively closing the port.

Once all the T-junctions were loaded with the samples and the ports were closed we turned on the Lab View protocol that controlled the flow of oil through the junctions. We delivered oil from pressurized containers \( (p \in \{40, 500 \text{ mbar}\}) \) controlled with manual pressure regulators (Rexroth PR1-RGP that allowed for fluctuations of order of 1 mbar) through electromagnetic valves (V165, Sirai, Italy). The valves were connected to the chip with resistive steel capillaries (O.D 400 μm, I.D. 205 μm, length = 1 m, Mifam, Poland) as described by Churski et al. to regulate the range of flow rates \( Q \in \{50, 2000 \text{ μL h}^{-1}\} \) available via tuning of \( p \). We calibrated the relation between \( Q \) and \( p \) by weighing liquid flowing out from the chip onto a balance (WLC C/2, Radwag, Poland).

We used the previously described method for on-demand generation of droplets of predefined volumes and at predefined instants to simultaneously generate pairs of 256 N. droplets of human blood or standardized RBCs, and droplets of 64 N. of monoclonal reagents, yielding the total droplet volume of 320 N. These proportions were optimized for the
large differences in the time of flow of droplets containing different monoclonal reagents (see Fig. 1 in ESI†). That certain volume was chosen according to mobility observations in dependence of viscosity.16, Fig. 2 therein

Once formed, droplets flowed to the merging chamber and a pulse of an oscillating electric field merged them via electrocoalescence.17,18

In the experiments we loaded the T-junctions with A1−, A2+, B− and O− standardized RBCs and with anti-A and anti-B monoclonal reagents. In another experiment we loaded two T-junctions with RBCs with and without antigen D, and a third T-junction with anti-D BLEND monoclonal reagent. The same procedures were repeated while testing real human blood samples. For each sample we generated 50 droplets to obtain statistically significant results.

Once formed, merged and mixed, the droplet containing a given combination of RBCs and antibodies flowed into the detection area that comprised a long (10 cm) microfluidic channel equipped with two photo-diodes. Fig. 2, shows the measurements of time required for a droplet containing a particular combination of red blood cells and antibodies to flow between the detection points for the rate of flow of the continuous liquid. All values are calculated on the basis of 50 measurements for each data point.

Fig. 2 Time of flow of droplets containing different combinations of red blood cells and antibodies, with standard deviations and their triple values. A1−, A2+, B−, O− and O+ standardized RBCs (greyscale bars) and A1+, A2+, O− human blood (greyscale patterned bars) were combined with the antibodies anti-A, anti-B and anti-D. Agglutinated droplets flow slower than non-agglutinated, independently of blood group tested. The rate of flow of the continuous liquid was 400 μl h⁻¹.

The precision of the measurement decreases with increasing flow rate of the continuous liquid (Fig. 3), showing that the slowest flow provides the most reliable determination of the blood type of the sample. Fig. 3 shows how the difference Δt of intervals of agglutinated and non-agglutinated samples depends on the rate of flow Q of the continuous phase. Δt is largest for slow flows. Interestingly the ratio of Δt and the average time $t_{avr}$ – given by the average speed of flow of the continuous phase $t_{avr} = Ad/Q$, where A is the surface area of the cross-section of the channel and $d$ is the distance between the checkpoints – is largest for intermediate speeds of flow (ca. 1 mL h⁻¹). Still the ratio of Δt and the standard deviation $δt$ of intervals within the groups of agglutinated/non-agglutinated samples is again largest for slowest flows.

The measurement of the speed of droplets requires only a binary measurement of the presence of a droplet at the checkpoints and thus can be conducted with the simplest optical detectors (e.g. photodiodes). The light-to-voltage converter that we used allowed us to also measure the distribution of the intensity of light passing through the droplet.

Fig. 3 Differences Δt between the time of flow of droplets with agglutination and those without (a), the ratio Δt/avr (b) and the ratio Δt/δt (c) as a function of the rate of flow of the continuous liquid. All values are calculated on the basis of 50 measurements for each data point.
(attenuated via absorbance and scattering) as a function of position along the droplet passing over the detector.

Fig. 4 shows exemplary traces of the amplitude of the potential recorded from the converter as a function of time for two droplets, one containing a homogenous mixture of RBCs monoclonal reagents, and one containing agglutinates. A low level of potential corresponds to lower intensity of light reaching the detector, correlating with an opaque content of the portion of the droplet over the detector, while high values of the potential signify a transparent liquid. The non-agglutinated droplet shows a uniform level of intensity of transmitted light along the length of the droplet. At the same time, the trace recorded for the agglutinated droplet suggests separation of the agglomerates of RBCs at the rear of the droplet. Indeed micrographs of agglutinated droplets do show an effective separation of RBCs (Fig. 4 and supporting videos, ESIF!). This separation provides an additional approach for detection of agglutination, possibly allowing for faster flows through the device.

Discussion

The system that we demonstrated uses small samples of solutions and suspensions deposited directly on the chip to conduct a fully automated determination of blood type. The results that we report suggest that it is possible to detect agglutination of RBCs via a measurement of time of flow of the droplets containing RBCs and antibodies.

Agglutination occurs when specific antibodies recognize antigens presented at the surface of the RBCs. Apparently this changes the mobility of the droplet. It is known that the viscosity of the liquid inside the droplet affects its mobility.19–21 The mobilities of droplets containing suspensions are much less studied and the results that we present here form an interesting fundamental question in rheology.

An interesting observation is that the agglutinated agglomerates of RBCs concentrate at the rear of the droplets.22 The analysis of the profile of transmittance of light along the droplet may provide an additional handle for detection of agglutination, especially of weak antigens. The observation that the agglomerates concentrate in one part of a droplet could potentially be also used for separations.

The system that we describe here shows a proof-of-concept for detection of agglutination via a rheological measurement. Although it requires some off-chip elements, the method itself is faster, more reliable and needs fewer operations than other microfluidic solutions (e.g. centrifugation of blood is not necessary). For applications in point-of-care testing, the system can be greatly simplified: (i) instead of the high voltage generator a battery source of power23,24 can be used for electrocoalescence, (ii) detectors and NI card can be replaced by an electronic system based on a PIC or ATMEL processor connected to computer via a USB or directly displaying results on a small LCD screen, (iii) the samples can be deposited on the chip using standard pipettes.

The presented method is of very high sensitivity and reliability. The reliability is approaching 100% with an estimated chance of mistyping on the order of one per million samples. For comparison, the current bedside ABO agglutination tests performed with special cards vary in reliability from 93% to 99% (when combined with another test). The typical spot-assays are even more prone to detection of weak A antigens where the reliability is approximated to be less than 40%.25 Technical and clerical errors in standard methods represent 13% of all transfusion errors,26 and 1 to 250 transfusion units per 100 000 are burdened with ABO-incompatibility.27,28 In our technique the risk of a human error is practically eliminated, and potential measurement error can be even more minimized by elongation of the detection channel.

Although there are other, sophisticated and efficient methods for point-of-care blood typing (see e.g. the recent work by Li et al.1) the method that we demonstrated here could also be used in more complex diagnostic setups, and be adjusted for detection of up to dozens of antigens within few minutes. It may be clinically useful in cases of cross testing, especially in emergency, when rapid analysis is crucial for saving a patient’s health and life. For such applications it would be necessary to pre-load the chips with a sequence of droplets of a metered volume containing a range of suspensions of RBCs and a range of solutions of antibodies. Then the procedure could be simplified to loading of the samples of patients RBCs and serum and a completely automated run comprising of multiple tests.

Conclusions

We presented a novel scheme for determination of blood types in the format of an automated microfluidic system. The procedure is based on discrimination of agglutinated from non-agglutinated solutions on the basis of the measurement of the speed at which the drops comprising the solutions flow in a microchannel. The system uses small samples of liquids and its operation relies on the simplest scheme of detection possible, requiring only a digital signal reflecting the presence
of droplets at predefined checkpoints and a measurement of time. This time of flow measurement has never been considered with reference to blood typing before.

Agglutination of RBCs causes a detectable and reproducible change in the mobility of droplets allowing for detection of blood types and subtypes, which may be too subtle for other blood typing assays. Our results show that two subtypes of antigen A – A1 and the weaker A2 – can be detected with the same reliability. In addition, we observe that agglutination causes separation of the agglomerates into the rear of the droplet. An effect that could add to the detection of agglutination, especially of weak antigens, and be potentially useful in separations.

The method of detection of agglutination that we presented could potentially be used for automating various agglutination assays, following the FDA regulations29 for automated systems. Besides the reliability of the new type of detection scheme, the method offers the potential benefits of (i) reduction of the volume of blood sample to single micro-liters for the whole test, (ii) lack of requirement for preprocessing (e.g. centrifugation), and (iii) eliminating any subjectiveness from the procedure. Importantly, the method that we demonstrated could be compliant both with a point-of-care format for the simplest blood-typing protocols and with multiplexed assays for complex analyses, such as cross testing or other multiple antigen determinations in a bench-top format.

Methods

Fabrication and experimental system

We fabricated the microfluidic chip (Fig. 1) via micro-milling in polycarbonate (PC) and bonding with a flat slab of PC.30 All of the channels had a square cross section of area $A = \mu^2 = 360 \pm 10 \times 360 \pm 10 \mu m^2$. We did not modify the surface of the channels before the experiments, in which standardized RBCs were tested, but we did modify channel surface to increase its hydrophobicity31 (contact angle for water: $125 \pm 1.8^\circ$ in $25^\circ C$) prior to human blood tests. Electromagnetic valves, digital manometers, LED white diode (OF-SMD1608W), ultrafast single-supply comparators (AD8611ARZ) and high-sensitivity light-to-voltage converter (TSL257), all enclosed in a steel box, were connected to appropriate I/O National Instruments cards (NI PCIe-6320, NI PCI-6703). The temperature of the chip was stabilized to $24 \pm 1^\circ C$ in all of the experiments. To verify the results obtained with photodiodes we recorded a number of videos (available in ESI) using a stereoscope (Nikon SMZ 1500) and a fast camera (Photron Fast-Cam 1000 k).

LIquids and samples

In all of the experiments we used n-hexadecane (viscosity $\mu_c = 3.0 \pm 0.1$ mPa s, density $\rho_c = 770$ kg m$^{-3}$ at $25^\circ C$; Alfa Aesar, as supplied) as the continuous liquid. Human blood (O Rh$^-$, A$_1$ Rh+, A$_2$ Rh$^+$), standardized red blood cells for detection of antibodies (O Rh$^-$, 0 Rh$^-$, A$_1$ Rh$^-$, A$_2$ Rh$^+$, B Rh$^-$; Regional Blood Center, Katowice, Poland; used as supplied) and corresponding monoclonal reagents for ABO and Rh system antigens determination (anti-A, anti-B and anti-D; Regional Blood Center, KATOWICE; used as supplied) served as the droplet phase.

Experiments

We performed the experiments in two series. The first series was done for testing agglutination in droplets with A, B and O blood groups, whereas the second series concerned the agglutination test in droplets with different Rh factors. Each series was performed both for standardized red blood cells and human blood samples (red blood cells were not removed from the whole blood before these tests). In each series a droplet with antibodies was linked in the merging chamber with a droplet containing red blood cells. The merging chamber was equipped with two electrodes connected to a source of AC electrostatic potential (1,5 kV/cm, 200 Hz, Trek). An oscillating electric field speeds up32 the coalescence of droplets arriving from the individual T-junctions. A created droplet was lead to the section of measurement for the time of flow. Formerly written software performed at least 50 repetitions of measurement for each blood group for a fixed flow of oil. The difference in arrival time of each droplet between the second and the first detector was automatically calculated by a computer. We checked all of the possible combinations of red blood cells and antibodies.

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Notes and references


