Preferred citation: L. Li, D. Ballerini, M. Li, J. Tian and W. Shen. Paper-based blood grouping; exploring the mechanisms of red blood cell agglutination in antibody-treated paper via confocal microscopy. In Advances in Pulp and Paper Research, Cambridge 2013, *Trans. of the XVth Fund. Res. Symp. Cambridge, 2013*, (S.J. l'Anson, ed.), pp 929–944, FRC, Manchester, 2018. DOI: 10.15376/frc.2013.2.929.

PAPER-BASED BLOOD GROUPING; EXPLORING THE MECHANISMS OF RED BLOOD CELL AGGLUTINATION IN ANTIBODY-TREATED PAPER VIA CONFOCAL MICROSCOPY

Lizi Li, David Ballerini, Miaosi Li, Junfei Tian, Wei Shen

Department of Chemical Engineering, Monash University, Clayton Campus, Victoria 3800, Australia

ABSTRACT

The recent invention of paper-based blood typing devices which are inexpensive, but also accurate and easily interpreted, has shown great promise for the future. Despite the efficacy of these devices, the underlying mechanisms responsible for how they function have remained largely unknown. This work illuminates these mechanisms by using the technique of confocal microscopy to delve into the behaviour of red blood cells at the micro-scale and view exactly what is happening as blood samples interact with antibody treated paper substrates. The underlying mechanisms responsible for the phase separation of red blood cells and plasma from whole blood on paper are elucidated for the first time, opening the door to future enhancements to such devices. It was revealed that the dominant mechanism responsible for the separation of whole blood into its respective phases was the physical entrapment of large red blood cell aggregates following their agglutination. Understanding these mechanisms and the effects of the paper structure makes optimization of paper-based blood diagnostics possible. Further investigations of optimal pore sizes, tortuosity or fibre size may lead to significant improvements in the sensitivity and accuracy of this important diagnostic platform.

INTRODUCTION

Recent research has shown the tremendous potential of using low-cost, bio-active paper and thread to construct diagnostics to determine human blood type [1–4]. Blood typing is a routine assay in modern medicine and advanced technologies are available, however, in situations of medical emergency where speed and accuracy are critical such as during disaster response or on the battlefield, mainstream technology and trained medical personnel are generally not available. Furthermore, the lack of advanced technology and trained medical professionals poses a significant challenge to large scale disease screening and blood banking projects in developing regions. Such situations make the development of low-cost and easy-to-use blood typing assays absolutely essential.

Blood grouping is the classification of blood based upon the presence of certain antigens on the surface of red blood cells (RBCs) [5]. In the plasma phase of blood, antibodies exist which attack foreign antigens to protect the body from perceived threats. As a result, a reciprocal relationship exists where individuals who lack a RBC antigen (A, B or D for example) will possess the corresponding serum antibody, whilst those who possess the antigen will not. When serum antibodies bind to corresponding antigens on the RBCs, haemagglutination occurs, leading to the clumping of RBCs to form larger aggregates that cannot be stably suspended in the plasma [2, 6, 7]. The majority of techniques for grouping blood are based upon the observation of haemagglutination reactions between RBCs and synthetic serum antibodies in vitro [8].

Paper has been used as a substrate for blood typing in the past [9], however it merely functioned as a non-absorbent surface to support the occurrence of agglutination reactions, without any fluid penetrating into the paper itself. In such applications the assay is carried out by observing RBC agglutination in antibody solutions, which causes the formation of aggregates which are visible to the human eye. This type of blood typing device is convenient, provided that the user can interpret the visual change due to agglutination. Therefore this type of device is suitable for laboratory and hospital use, but less so for untrained users in the home or in the developing countries, since it is not feasible to assume that users can interpret the subtle changes in visual appearance due to agglutination which differentiate blood types.

During recent research we recognized that if low-cost devices are used in developing regions for large-scale disease screening, even if they are robust enough to function under unsupported field conditions, misinterpretation of the assay results may still be a significant factor that could compromise the efficacy of low-cost diagnostics. In response to this concern we developed a text-reporting blood typing device using bioactive paper. As shown in Fig. 1, This device reports the patient's blood type in straightforward and unambiguous letters and symbols,



Figure 1. The text-reporting blood typing diagnostic. (a) The device pre-use, showing some symbols and letters permanently stained in red ink; (b) The device after blood of type A+ has been added to all bioactive zones; (c) The device following rinsing with saline to reveal the patients' blood type in unambiguous text.

patterned into the paper using inkjet printed alkenyl ketene dimer (AKD), so that users do not have to identify and interpret agglutination of RBCs [10].

A major difference between our blood typing device and previous incarnations is that the biochemical process of blood agglutination occurs within the paper, not just on its surface. In our design paper is an integral part of the device, facilitating the haemagglutination reactions. The interfibre pores within paper are used to discriminate agglutinated RBCs from non-agglutinated RBCs, and this sizediscrimination between RBC clusters allows non-agglutinated RBCs to be washed out of the paper, while larger aggregates of agglutinated cells remain trapped in the fibre matrix of the paper, permanently staining the paper and allowing easy identification of the blood type.

It is therefore important for us to clearly understand the behaviour of RBCs inside the paper structure and interfibre pores, as well as the mechanisms of RBC agglutination in fibre structures inside paper and the mechanisms of how paper structures provide size separation to the agglutinated RBCs. The use of confocal microscopy has allowed the visualisation of the RBCs and their aggregates at the scale of the paper fibres, giving us a window into exactly what is occurring during the assay on the micro-scale. This understanding will be essential to the design of high performance paper-based devices for blood typing in the near future.

EXPERIMENTAL

Materials

Blood samples with anticoagulant were acquired from adult volunteers of known blood group on our behalf by Dorevitch Pathology, Australia. All blood samples were stored in Vacutainer® test tubes containing heparin, citrate, and

ethylenediaminetetraacetic acid (EDTA) at 4°C, and used within 5 days of withdrawal. EpicloneTM anti-A, anti-B and anti-D monoclonal grouping reagents were sourced commercially from Commonwealth Serum Laboratories (CSL), Australia. Anti-A and anti-B are a transparent cyan and a yellow solution respectively, while anti-D is a transparent colorless solution. Monoclonal grouping reagents were also kept at 4°C. Kleenex paper towel with basis weight of 34 g/m² and measured apparent thickness of 140 µm was purchased from Kimberly-Clark, Australia. Analytical grades of NaCl, KCl, Na₂HPO₄, and KH₂PO₄ were purchased from Sigma-Aldrich for preparation of physiological saline solution (PSS) and phosphate-buffered saline solution (PBS, pH 7.4). Fluorescein isothiocyanate (FITC, isomer I, product number: F7250) from Sigma-Aldrich was used for labeling red blood cells. Anhydrous dimethyl sulphoxide (DMSO, from MERCK Chemicals Ltd., Australia) was employed to dissolve FITC. Anhydrous D-glucose was provided by AJAX Chemicals Ltd., Australia. Immersion oil was purchased from Sigma-Aldrich, Germany.

Methods

Red blood cells were labeled according to Hauck et al. [11] and Hudetz et al. [12]. Firstly, whole blood was centrifuged at 800 rpm for 10 minutes and plasma was removed. RBCs were then washed in PSS and incubated in PBS with glucose (0.5 mg/ml) and FITC (0.4 mg/ml) for 3 hours at room temperature. The labeled cells were then washed with PSS twice and resuspended at a hematocrit of 45%. The testing papers were prepared by adding 10 µL of antibody solution onto 10 mm \times 10 mm paper squares and allowing the antibody to penetrate and dry for 1 minute. In order to form agglutinated blood lumps properly within the paper sheet, 8 µL blood was added onto the paper from the opposite side to which the antibody was introduced. Thirty seconds were given for the interaction between the RBCs and the antibody to occur within the paper. The sample was subsequently washed with saline solution or left unwashed depending on the experimental design. It was then transferred onto a microscope slide for confocal imaging. Images of the red blood cells in the antibody-treated paper were captured using the confocal microscope (Nikon Ai1Rsi) at the Melbourne Centre for Nanofabrication. The magnifications of the objective lens used with the microscope were $20 \times \text{air}$ and $60 \times \text{oil}$ as these were deemed to give the most suitable resolution images for our study. RBCs within the paper could be visualised through crosssectional images or a series of x-y images varying in the z-direction to create 3D constructions, known as z-stacks. The images were taken with a resolution of 1024×1024 pixels and the step width for the 3D images was 0.125~0.250 µm.

In order to get a better understanding of the lateral transportation behaviour of agglutinated and non-agglutinated RBCs in the paper, an experiment of

chromatographic elution by PSS was performed. Briefly, 10 μ L of antibody solution and 3 µL of blood sample were dropped on a glass slide and allowed to react for 30 seconds. The mixture of blood and antibody was then transferred from the glass slide onto a piece of Kleenex paper 2 cm from the lower edge of paper and allowed to absorb completely for 1 minute. The Kleenex paper was then suspended in PSS in a chromatography tank about 1 cm from the lower edge, ensuring the blood spot remained above the buffer surface. The saline solution was allowed to wick into the paper by capillary action for 10 minutes. The paper was then allowed to dry at room temperature on a piece of blotting paper for another 10 minutes and the elution patterns of RBCs were characterized by observing the presence of RBCs at different distances from the original dosing point of the blood sample, with the confocal microscope. To study the vertical transportation behaviour of agglutinated and non-agglutinated RBCs through the paper, paper squares with RBCs were washed with a small amount of saline solution; the saline solution then penetrating through the paper and transporting the RBCs. We refer this mode of saline washing as the "vertical washing". Confocal microscopic images were then taken to analyze the RBC transport behaviour inside paper sheet.

RESULTS AND DISCUSSION

Possibility of observing red blood cells in paper by confocal microscopy

The overall view of cellulosic fibres and red blood cells in paper can be seen in Fig. 2. This figure demonstrates that the confocal microscope is a useful imaging technique for giving a detailed view of RBCs in cellulosic paper. The Nikon Ai1Rsi Confocal Microscope is equiped with a laserline which is capable of



Figure 2. Confocal images of lignocellulosic fibres (a) fibre network of the Kleenex paper towel; (b) FITC-labeled RBCs within paper captured by 20× air lens.



Figure 3. Chemical structure of FITC isomer I.

generating fluorescent emission spectra with an excitation wavelength of 405 nm, which is similar to the absorption peak of the cellulosic fibres used. This enabled the generation of clear and detailed images, where the cellulose fibres of the Kleenex paper towel were visible in a blue colour. The image in Fig. 2(a) was captured by the $20\times$ air lens, from which it can be clearly observed that cellulosic fibres are dispersed evenly and are oriented in all directions. The spaces between fibres form the porosity within the structure of paper. The widths of the cellulose fibres are around 20 to 30 μ m.

Fluorescein isothiocyanate (FITC) is widely used as a fluorescent label to attach on proteins via its amine group. The isothiocyanate group reacts with the amino terminal and primary amines in proteins. Isomer I of FITC was used in the present study, which has the thiocyanate group on the fourth carbon of the benzene ring, as shown in Fig. 3. It has been reported that FITC is a suitable fluorescent agent to label RBCs [11, 12], the results of our research strongly support this point. A fluorescent emission spectrum was generated with an excitation wavelength of 488 nm by the multiline argon-ion laser of the Nikon Ai1Rsi Confocal Microscope used, which is close to the absorption peak of FITC. As a result, after labeling RBCs with FITC, the red blood cells located within the voids between the cellulose fibres could be visualised. Fig. 2(b) illustrates the uniform distribution of the RBCs labeled with FITC within paper, which was acquired by $20 \times$ air lens.

In addition, it was confirmed that the FITC labeling of the RBCs does not have a noticeable effect on the activity of RBC antigens on the surface of the red cells. This is an important condition for the use of FITC as a fluorescent label of RBCs in the confocal method. By comparing the reactions of blood and FITC-labeled RBCs drawn from the same source with the corresponding antibodies on glass slides, it was shown that FITC labeling does not cause discernable differences in the sizes of the agglutinated RBC aggregates nor in the time required for the RBCs to agglutinate after contacting their corresponding antibodies under the same assaying conditions.

Mechanisms of red blood cell agglutination in antibody-treated paper

In this study, we prepared paper squares pre-treated with different antibody reagents, and then dosed corresponding and non-corresponding blood samples labeled by FITC onto the squares, allowing us to visualise the distribution and agglutination behaviour of RBCs with and without haemagglutination. Figs. 4(a) and (b) display two-dimensional confocal micrographs of RBCs of type O+ introduced onto paper squares pre-treated by anti-A reagent, obtained by 20× air lens and $60 \times$ oil lens respectively, which show the distribution and behaviour of RBCs within paper in the absence of a haemagglutination reaction. When the type of blood added does not correspond to the antibody present in the fibre network, the RBCs disperse uniformly in the fibre network. The RBCs move independently and do not appear to affect each other. In addition, there is no change in the morphology of the RBCs as a result of the presence of the non-corresponding antibody reagent and RBCs maintain their disk-like appearance. A threedimensional confocal micrograph of RBCs of type O+ in paper containing anti-A solution is given in Fig. 4(c). It can be seen from this figure that the RBCs penetrate to a distance vertically into the paper and finally come to rest in the same plane, almost at the exact centre of the papers thickness.

Figs. 4(d) and (e) provide images of two-dimensional confocal micrographs of RBCs of type O+ introduced onto paper squares pre-treated by anti-D reagent, obtained by $20 \times$ air and $60 \times$ oil lens respectively, which show the distribution and behaviour of RBCs within paper when the haemagglutination reaction has occurred. When antibody molecules bond to the specific binding sites on the antigens of adjacent red blood cells, the aggregation of RBCs leads to the formation of much larger aggregates that cannot be stably suspended in the plasma phase. Since the paper square has been treated with antibody solution, there is a large number of antibody molecules adsorbing on the surface of the cellulose fibres. In addition, some of the antibody molecules also remain in the porous voids between fibres. When the blood sample is introduced onto the paper and wicks through the voids, there is a simultaneous desorption of antibody molecules from the fibre surface back into the blood sample due to the effect of flushing. The antibody molecules remaining between fibres as well as the ones desorbed from the surface of fibres act to agglutinate the RBCs, and there is great variation in the size of the agglutinated RBC aggregates formed. Some aggregates contain hundreds of RBCs, whilst others consist of only two or three RBCs. Due to the great number of antibody molecules adsorbed on the surface of the fibres, RBC aggregates can attach to the fibres and become immobilized; this is the result of the specific binding of antibody molecules on the fibre surface to the antigens of the RBCs located on the outer surface of the aggregate. While some blood aggregates just adhere to a single fibre, others form bridge like structures between many adjacent

Lizi Li, David Ballerini, Miaosi Li, Junfei Tian, Wei Shen

fibres, which further restricts their mobility. In addition, small quantities of individual RBCs react directly with the antibody molecules on the fibre surfaces and become fixed. From Fig. 4(e), it can be observed that many of the RBCs become deformed during the process of agglutination; this is likely caused by the strong binding force existing between the multiple antigens on the RBC surface and the antibody molecules. Fig. 4(f) shows a 3-dimensional confocal micrograph of RBCs in paper which have been subject to a haemagglutination reaction, from which we can observe the adhesion of agglutinated RBC aggregates to the fibre surface randomly at different positions, such that the distribution of aggregates is not uniform vertically.

Comparing the confocal results in Fig. 4 shows that in the cases when no reaction occurs, the red blood cells disperse evenly and have high mobility within the paper substrate; however, in the cases where a reaction has taken place, the agglutinated blood aggregates attach on the surface of cellulose fibres and are immobilized. This provides the basis for the design of paper-based blood typing devices.



Figure 4. Confocal images of non-agglutinated and agglutinated RBCs within paper.
(a) 2D image of non-agglutinated RBCs captured by 20× air lens.
(b) 2D image of non-agglutinated RBCs captured by 60× oil lens.
(c) 3D image of non-agglutinated RBCs captured by 60× oil lens;
(d) 2D image of agglutinated RBCs captured by 20× air lens.
(e) 2D image of agglutinated RBCs captured by 60× oil lens.
(f) 3D image of agglutinated RBCs captured by 60× oil lens.

Lateral transportation behaviour of RBCs on paper

In order to investigate the transportation mechanism of agglutinated and nonagglutinated RBCs on the surface of the paper substrate, an experiment of chromatographic elution by PSS was conducted. In this study, two drops of anti-B solution of 10 μ L volume were introduced to glass slides followed by 3 μ L volumes of either A+ or B+ blood, and then allowed to react for 30 seconds. The antibody sample mixed with B+ blood appeared to undergo a haemagglutination reaction, while the other sample mixed with A+ blood did not. The mixtures of blood and antibodies were then transferred from the glass slides to the paper surface and allowed to be absorbed completely. Afterward, the paper strips were suspended vertically with their lower edge submerged in PSS for 10 minutes so that chromatographic elution was achieved. The saline solution penetrates through the interfibre pores of the paper towel, wicking across the dosing spots of grouping antibody and blood mixture. If haemagglutination has occurred in a sample spot, the agglutinated blood sample will not be eluted up by the rising saline solution. If haemagglutination has not occurred, the blood sample will be eluted out of the sample spot, forming a visible, elongated chromatographic track of blood.

However, in practice, two elution bands may be observed. The first is seen close to the wicking front of the eluent and the second is observed behind the first band. In assays which were negative, these two bands could be simultaneously observed, as shown in Fig. 5(a); while in the positive assays, only one was observable, as shown in Fig. 5(b). Fig. 5(d) shows a schematic description of this phenomenon. The confocal microscopy method was employed to investigate the nature of the two bands in order to explain this phenomenon.

Fig. 6 shows the confocal micrographs captured at different wicking distances for both agglutinated and non-agglutinated samples. Fig. 6(d) displays the confocal micrograph captured from the antibody and blood sample dosing zone of the positive assay. Large aggregates of agglutinated RBCs are observed within the pores formed by the paper fibres; this observation is consistent with the findings discerned from Fig. 4. Another confocal image was captured of the second band of the negative assay, which shows the substrate was sparsely populated by individual red cells and small clusters formed by a few RBCs on the fibre surfaces (Fig. 6(b)). It shows that this band consists of non-agglutinated RBCs which have been eluted through the paper. Further confocal micrographs of the second band of a positive assay did not show any RBCs (Fig. 6(e)). This is because most of RBCs have reacted and agglutinated into aggregates at the original dosing point, leaving only a very small number of free RBCs available in this zone for chromatographic elution. It is therefore very difficult to locate any free RBCs in the vicinity of the second band. The confocal result is in good agreement with the elution pattern of the positive assay in Fig. 5(b).



Figure 5. Chromatographic Elution bands of RBCs on paper. (a) Elution bands of A+ blood sample mixed with anti-B reagent. (b) Elution bands of B+ blood sample mixed with anti-B reagent. (c) Elution bands of haemolysed blood sample of A+. (d) The schematic diagram of the observed elution bands of RBCs on paper.

Confocal micrographs taken of the area of the first band for both positive and negative assays failed to show any RBCs, despite Fig. 5 clearly showing that the first bands of all assays possesses a weak blood colouring. An explanation is that this first band is actually formed by the elution of haemoglobin from the internal fluid of ruptured RBCs; hence the band possesses the colour of blood but this is not due to the presence of non-ruptured RBCs. To verify this explanation, chromatographic elution of haemolysed blood samples of type A+ was conducted under the same conditions. Haemolysed blood samples were obtained by mixing 3 μ L blood and 10 μ L distilled water for 5 minutes. The activity of the haemolysed blood was assessed by adding the corresponding antibody reagent. The lack of any haemagglutination reaction proved that haemolysis of RBCs in the sample had occurred. The chromatographic elution pattern of the haemolysed A+ blood sample can be seen in Fig. 5(c). The presence of only the first band confirms the hypothesis that it has been caused by the haemoglobin released after haemolysis of the RBCs.

This study made use of the optical technique of confocal microscopy to investigate the two chromatographic elution bands and the spotting zone of blood on paper for their composition details. Confocal micrographs revealed that the rupture of RBCs occurred in the spotting zone. Haemoglobin released from the



Figure 6. Confocal images of chromatographic elution patterns of non-agglutinated RBCs at elution distance of (a) 0 cm, (b) 5 cm, (c) 8 cm and agglutinated RBCs at elution distance of (d) 0 cm, (e) 5 cm, (f) 8 cm.

ruptured cells is eluted by the saline solution faster than the free RBCs and small cell clusters forming the first visible band close to the elution front, while the free RBCs form the second elution band.

Vertical transportation behaviour of RBCs through paper

In certain designs of paper-based blood typing devices, vertical washing is adopted [3, 4, 10]. Vertical washing has the advantage over lateral washing in that the saline solution has a shorter penetration distance to travel to wash the free RBCs out of the fibre network of the paper. In performing the confocal imaging, agglutinated RBC aggregates were first formed within the assaying paper substrate as was described above in the previous section. A piece of dry filter paper was placed underneath this assaying paper substrate and in contact with it. Fifty micro litres of PSS was then used to wash the blood spot in the paper; the saline solution penetrated through the assaying paper and was absorbed by the filter paper below. From confocal micrographs of the washed assaying paper (Fig. 7), we can observe blood aggregates of different sizes, as well as even a few single RBCs. The presence of large blood aggregates confirms that their mechanical entrenchment by the paper fibres is strong enough to prevent them from being



Figure 7. Confocal images of agglutinated RBCs within paper vertically washed by 50 μL saline solution. (a) Large blood aggregates within paper. (b) Small blood aggregates and single RBCs within paper.

washed away. The confocal results show that RBC aggregates smaller than the pore size of fibre network can also be retained after washing within the corners and crevices formed by two or more fibres. These large and small RBC aggregates contribute to the visual identification of the final assaying results. The presence of a small number of single RBCs on the fibre surface, however, suggests that antibody molecules adsorbed to fibre surface may contribute to the immobilization of RBCs on the fibre surface, but they contribute negligibly to the visual assay result.

Since vertical washing can be achieved more rapidly than lateral chromatographic elution, vertical washing is therefore a desirable mechanism to be considered for device design where high assaying speed is the principal design target [10].

Washing the agglutinated RBCs in paper by submerging the paper in saline solution

When the paper blood typing assay is submerged into the saline solution, capillary action is eliminated. A paper sample is saturated with saline solution and the washing effect is provided by the saline solution moving in and out of the paper in a more free manner. This washing condition is expected to allow saline to have even stronger interactions with agglutinated and non-agglutinated RBCs inside the fibre network. The purpose of this study is to understand the effect of this

washing condition on the agglutinated and non-agglutinated RBCs in fibre network.

A paper square of a positive blood type assay was prepared; it was then held by a pair of tweezers and submerged into saline solution. Gentle agitation was provided by slowly moving the paper square in the saline solution for 1 minute.

The confocal micrographs of this paper square show that there were both large and small aggregates within the fibre network of the paper square (Fig. 8). The large RBC aggregates locked inside inter-fibre pores were not significantly affected by this washing condition, since their sizes are much larger than the interfibre pores. However, the number of large RBC aggregates reduced after washing by submerging. This observation suggests that some large RBC aggregates could have been dissociated under this washing condition. This is likely, since the formation of RBC aggregates caused by specific RBC antigen and antibody interactions is governed by an agglutination and dissociation equilibrium [5]. The submerged washing condition significantly dilutes the antibody concentration, therefore weakening the antibody activity. This effect would encourage the dissociation of the agglutinated RBC aggregates [5]. The gentle agitation employed in the submerged washing may also contribute to the removal of the aggregates from the fibre surface, since the shear force may assist large aggregates to detach from the fibre surface. Fig. 8 (b) shows that some smaller aggregates and single RBCs remain in inter-fibre pores and on the surface of fibres, and their numbers appear to be greater than on the paper samples that were washed using the lateral



Figure 8. Confocal images of agglutinated RBCs within paper washed by unlimited amount of saline solution. (a) Large blood aggregates within paper. (b) Small blood aggregates and single RBCs within paper.

and vertical methods. This could be caused by the bonding between these cells and antibody molecules adsorbed on the fibre surface.

This finding suggests that saline washing conditions affect the number and morphology of large RBC aggregates within the fibre network. Washing using a large quantity of saline solution may cause the breakdown of RBC agglutination, which may reduce the colour intensity of the assay and therefore make the visual identification of the final assay results more difficult. This result provides useful information for the future design of paper-based blood typing devices and saline washing protocols; washing protocols involving the use of large quantities of saline solution for long washing times should be avoided.

CONCLUSIONS

A confocal microscopy method was developed to study the mechanism of agglutination of RBCs in paper-based blood typing devices. This work shows that confocal microscopy is a suitable technique that can provide details of RBC agglutination on the cellular level inside the fibre network of paper. Human RBCs can be labeled by FITC without the antigens on the surface of the red cells losing their activity. Two laser beams with different wavelengths were used to excite fluorescent signals with two different emission wavelengths of lignocellulosic fibres and FITC-labeled red cells. Two-dimensional and three-dimensional confocal images were formed for investigation of the fibre network, RBCs, and different distributions of free RBCs and agglutinated blood aggregates inside the fibre network. The non-agglutinated RBCs do not undergo morphological changes, and are distributed rather uniformly within the spaces of the fibre network. On the other hand, the agglutinated RBCs form aggregates containing many RBCs.

The results show that the dominant mechanism responsible for RBC aggregates immobilization in paper is the physical entrapment of large RBC aggregates formed within fibre network. These RBC aggregates containing hundreds RBCs are too large to move within the paper structure. At the same time, non-agglutinated RBCs can move freely in the fibre network and can be washed out of the fibre network by saline solution.

Separation of RBC aggregates from free RBCs in the paper structure can be achieved by lateral chromatographic elution or vertical washing with saline solution; both lateral elution and vertical washing lead to correct identification of the blood type. Vertical washing, however, has the advantage of having a shorter liquid wicking distance and is therefore a faster assay than the lateral flow method. This work shows that washing with large volumes or long time periods is not recommended, since the agglutinated RBC aggregates may dissociate, and this could affect the assay results.

Blood Cell Agglutination in Antibody-Treated Paper Via Confocal Microscopy

This work has for the first time obtained insights at the cellular level into the working principle of paper-based blood typing devices. It provides an effective and easy experimental method to guide the future development of new device designs.

ACKNOWLEDGEMENT

This work is supported by Australian Research Council Grants (ARC DP1094179 and LP110200973). Authors thank Haemokinesis for its support through an ARC Linkage Project. Authors also thank staff of MCN for Confocal training and usage, Dr M. Al-Tamini and Dr Emily Perkins of Department of Chemical Engineering for information on red cell tagging and for proof-reading the manuscript. Authors would like to thank Dorevich Pathology for supplying blood samples. Ms Lizi Li, Ms Miaosi Li and Mr David Ballerini thank Monash University Research and Graduate School and the Faculty of Engineering for postgraduate research scholarships.

REFERENCES

- M.S. Khan, G. Thouas, W. Shen, G. Whyte and G. Garnier. Paper Diagnostic for Instantaneous Blood Typing. *Anal. chem.* 82 (10): 4158–4164, 2010.
- 2. D.R. Ballerini, X. Li and W. Shen. An inexpensive thread-based system for simple and rapid blood grouping. *Anal. Bioanal. Chem.* **399** (5): 1869–1875, 2011.
- P. Jarujamrus, J. Tian, X. Li, A. Siripinyanond, J. Shiowatana and W. Shen. Mechanisms of red blood cells agglutination in antibody-treated paper. *Analyst.* 137(9): 2205–2210, 2012.
- M. Al-Tamimi, W. Shen, R. Zeineddine, H. Tran and G. Garnier. Validation of paperbased assay for rapid blood typing. *Anal. chem.* 84(3):1661–1668, 2012.
- 5. G. Daniels and I. Bromilow. Essential Guide to Blood Groups, 2nd ed., Wiley-Blackwell: Chichester, West Sussex, UK, 2010.
- 6. M.S. Khan, Bioactive Papers: Printing, Activity and Stability, Department of Chemical Engineering, Monash University, Melbourne, 2009.
- W. Malomgre and B. Neumeister. Recent and future trends in blood group typing. *Anal. Bioanal. Chem.* 393(5): 1443–1451, 2009.
- G. Daniels and M.E. Reid. Blood groups: the past 50 years. *Transfusion* 50 (2) 281–289, 2010.
- 9. Sifin, Serafol ® ABO and ABO+D monoclonal bedside cards. http://www. imeskosgeb.com/sanalfuar/images/userImages/sifin/Product%20Information%20 Serafol%20bedside%20cards.pdf (accessed 3/10/2012).
- 10. M. Li, J. Tian, M. Al-Tamimi and W. Shen. Paper-based blood typing device that reports patient's blood type "in writing". *Angew. Chem.* **51**:1–6, 2012.

Lizi Li, David Ballerini, Miaosi Li, Junfei Tian, Wei Shen

- 11. E.F. Hauck, S. Apostel, J.F. Hoffmann, A. Heimann and O. Kempski. Capillary flow and diameter changes during reperfusion after global cerebral ischemia studied by intravital video microscopy. *J. Cerebral Blood F. Metab.* **24**(4):383–391, 2004.
- 12. A.G. Hudetz, G. Feher, C.G.M. Weigle, D.E. Knuese and J.P. Kampine. Video microscopy of cerebrocortical capillary flow: response to hypotension and intracranial hypertension. *Am. J. Physiol. Heart Circ. Physiol.* **268**(6): H2202–H2210, 1995.

Transcription of Discussion

PAPER-BASED BLOOD GROUPING; EXPLORING THE MECHANISMS OF RED BLOOD CELL AGGLUTINATION IN ANTIBODY-TREATED PAPER VIA CONFOCAL MICROSCOPY

Lizi Li, David Ballerini, Miaosi Li, Junfei Tian, and <u>Wei Shen</u>

Department of Chemical Engineering, Monash University, Clayton Campus, Victoria 3800, Australia

Pooya Saketi Tampere University of Technology

I can see that there is a very big potential in using paper for micro-fluidic applications. In our group we have been developing micro-fluidic chips made from different polymers for detecting some specific proteins after a heart attack. It took about three years to find and screen out from different types of polymers which polymer is the best to be used for this purpose, so that it always gives a consistent contact angle of blood serum. I see a major problem with using paper for this kind of application, and that is humidity. If you develop some kind of point-of-care diagnostic out of paper that it is possible to use, for example, somewhere very warm like the Sahara but it is not possible to use it in the UK (where it is raining all the time) because your blood might not diffuse far enough into the paper, this is a very major problem. I think you need to develop a type of paper which is stable for the full range of humidities and which works in different countries; or at least you need to develop a product for a specific country. Have you done anything on developing that type of paper?

Discussion

Wei Shen

We are doing that. The question that you just asked is the question that everyone working on this kind of project has to worry about. We have investigated the longevity of the paper devices; how long they are going to last in the field. We have a few options. I think, fortunately, in this application, we do not have to rely on the contact angle as one of the design criteria, so we have one less thing to worry about. We just need to soak the blood in and then wash it off. But the antibody longevity is the thing that we are working on.

Pooya Saketi

I have one more question. Do you add heparin to the blood when running this test? If you add heparin to the blood, the blood is not going to clot anymore; it will stay fluid, and the serum and the red blood cells will not separate. For example, if you go to donate blood for transfusion, they initially add heparin, or the container is already covered with heparin. Otherwise you immediately have a bulk solid piece of blood and it is useless for transfusion.

Wei Shen

Yes, I know what you mean. The samples that we got from the Red Cross had got EDTAC in them, so they have some protection. So, the blood does not really fall apart, not for a few days.

Anders Åström Aylesford Newsprint (from the chair)

We have been talking about the longevity; what life spans do you expect to be required for this paper to be of any use?

Wei Shen

The company that is supporting this work, is asking for 12 months. I think there is also, however, an industrial solution; freeze drying is going to solve quite a bit of the problem. The product is going to last for quite a while. But our research currently is trying to get away from having to use freeze drying; we do not know whether we can do it or not, but we are trying.

Anders Åström

You are working on developing the structure of the paper; what parameters are you working with to get the functionality you need?

Wei Shen

The structure of the paper, in my view, is important. We have investigated different papers of different structures which gave different results, and also when we used too much cationic polymer, it slowed down the migration of free red cells, and that is not good.