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Understanding Thread Properties for Red Blood Cell Antigen Assays: Weak ABO Blood Typing

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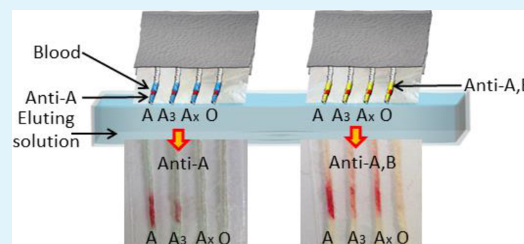
Supporting Information

ABSTRACT: "Thread-based microfluidics" research has so far focused on utilizing and manipulating the wicking properties of threads to form controllable microfluidic channels. In this study we aim to understand the separation properties of threads, which are important to their microfluidic detection applications for blood analysis. Confocal microscopy was utilized to investigate the effect of the microscale surface morphologies of fibers on the thread's separation efficiency of red blood cells. We demonstrated the remarkably different separation properties of threads made using silk and cotton fibers. Thread separation properties dominate the clarity of blood typing assays of the ABO groups and some of their weak subgroups (A_x and A_3). The microfluidic thread-based analytical devices (μTADs) designed in this work were used to accurately type different blood samples, including 89 normal ABO and 6 weak A subgroups. By selecting thread with the right surface morphology, we were able to build μTADs capable of providing rapid and accurate typing of the weak blood groups with high clarity.



KEYWORDS: *thread, microfluidics, chromatographic separation, surface morphology, weak ABO blood typing*

1. INTRODUCTION

In recent time, thread has been employed as a low-cost substrate for the fabrication of microfluidic diagnostics.[1,2] Several studies have reported applications of qualitative immunoassays[3] and semiquantitative analyses of biomedical and environmental samples.[4−6] Other studies have explored ideas of building controllable flow valves, mixers, and thread networks to mitigate sample flow for more advanced thread-based microfluidic sensor applications.[7,8] These studies provide a good understanding of the wetting, liquid transport, as well as color display properties of threads,[9] which are essential for the engineering of functional microfluidic thread-based analytical devices (μTADs). Additionally, the use of μTADs for blood typing has been reported by our group.[10] Our study shows that threads made from suitable fibers, once treated with blood grouping antibodies, can be used as efficient diagnostic devices to provide normal ABO and RhD blood typing analysis.

The basic principle of using μTADs to perform blood typing assays is that when a blood sample is introduced onto a thread that has been treated with a blood grouping antibody, the antibody is dissolved into the plasma phase of the sample. If the RBCs of the sample carry the antigen corresponding to the antibody that the thread has been treated with, then the RBCs will agglutinate predominantly in the interfiber spaces of the thread. Conversely, if the RBCs do not carry the corresponding antigens to the antibodies introduced onto the thread, they will not agglutinate and remain as free cells, moving with the wicking plasma phase along the interfiber gap capillaries. This difference can be easily identified by the naked eye. In assay device design, however, blood typing applications not only require the threads to have desirable liquid transport properties but must also allow for the clear separation of agglutinated RBCs from the plasma phase to provide a final assay result of high clarity for visual identification.

Although using thread for blood typing has been validated for normal ABO and RhD groups,[10] the underlying transport mechanisms of RBCs in threads are still not fully understood. We observed that assays performed with threads made of different fibers can have surprisingly large differences in their level of clarity; these observations could not be explained by results we obtained from macroscopic assaying experiments. This highlights the importance of gaining a better understanding of the fundamental properties of fiber for thread-based sensor design.

Clinically, the ABO blood group system has a number of subgroups; in type A blood, there are subgroups A_1, A_2, A_3, A_x, etc. Among these, A_3 and A_x are considered weak subgroups. RBCs of weak groups carry a much smaller number of antigens, which leads to weak hemeagglutination reactions when they are exposed to group A antibodies.[11] That in turn will lead to the formation of comparatively small agglutinated RBC aggregates in a positive assay, which can be difficult to differentiate from a negative assay. Correct typing of weak ABO subgroups is therefore difficult to achieve, even in professional laboratories; success being heavily reliant on the availability of special equipment and experienced clinical staff.[12] These difficulties have been well-documented in immunological blood grouping research and in clinical practice.[12] Some alternate methods give

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the weak subgroups mixed field hemeagglutination, which could lead to the weak groups being mistyped as group O, A or B by using a device with insufficient power to clearly differentiate weakly agglutinated RBCs from free ones. This is dangerous both when individuals with weak ABO types receive blood, and when their blood is donated for transfusion into others. More worryingly, individuals of group A_x almost exclusively possess the anti-A_1 antibody in their blood serum. If, as a result of mistyping, a patient of group A_x receives transfusion from a group-A_1 individual, the risk of a hemolytic transfusion reaction is significant because the A_1 antigen is one of the strongest antigens and will have strong hemeagglutination reaction with A_1 antibodies.[13−15] Despite the seemingly small fraction of the population (1:5,100 or 0.02%) who possess these weak subgroups, it is important to consider that more than 107 million units of blood donations are collected each year, which means that ∼21 000 could be expected to contain blood of weak ABO subgroups, with the same proportion among the recipients of blood transfusions.[13−15]

The key thread property that governs the clarity of blood analysis is its separation ability which, to date, has not been investigated in depth. Thread-based blood analysis takes advantage of the presence of interfiber gaps in thread, which provide the capillary driving force for liquid wicking.[5] Liquid samples can therefore be delivered to the designated locations in a microfluidic device for analyte identification or quantification.[5] However, a successful thread-based blood typing device requires a thread to provide both the capillary wicking driving force and separation power to differentiate agglutinated RBC lumps from free RBCs in the wicking serum phase. The interfiber channels in a thread are helically structured and run almost parallel to one another; they form relatively simple microfluidic systems for sample wicking. If a thread is used for the purpose of separating particles from a liquid phase, the fiber morphology may play an important role because it defines the wall of the capillary channels, which, in the case of blood typing, affect the transport behavior of the free and agglutinated RBCs.

To design low-cost, high-performance thread-based sensors for blood analysis, the selection of fibers with desirable properties requires careful consideration. In this study, silk and cotton threads with different morphologies,[16−19] have been investigated and employed for typing of ABO blood samples and its weak subgroups, A_3 and A_x, using a chromatographic elution method.[20,21] The influence of threads' morphological properties on their performance as a size-based RBC lump separator has also been investigated using confocal microscopy. The confocal microscopic examination provides an insight into the separation mechanism of the agglutinated RBCs from free ones on each thread. Furthermore, the optimal pH of the buffer solution and eluting time have been established with respect to differentiating A_x and A_3 subgroups from each other, and from normal A and O blood types. Finally, the blood typing results have been presented with barcode shaped symbols to provide a simple means for data interpretation by lay personnel without the requirement of special training.

2. EXPERIMENTAL SECTION

Materials, Reagents, and Blood Samples. Hydrophobic silk fibers and cotton threads were obtained from the School of Fashion and Textiles, RMIT University, Melbourne, Australia. Blood samples were received from the Red Cross, Australia, which were collected from adult donors. The groups of all samples were already confirmed by the Red Cross using a mainstream laboratory blood typing technology (Gel Card). The blood samples were stored at 4 °C in Vacutainer test tubes containing lithium-heparin anticoagulant and used within 10 days of collection. Blood typing antibodies were received from the ALBA Clone, UK; anti-A Clone (Z001), anti-D blend (Z041), anti-B FFMU, and anti-A,B (Z021); they were stored at 4 °C and used as received. A physiological saline solution (PSS) was prepared from analytical grade (AR) by dissolving 0.9 g of NaCl in 100 mL of water. Ammonia solution was used to adjust the pH of the buffer solution before elution. All reagents used for preparing buffer solutions were purchased from Sigma-Aldrich. Anhydrous D-glucose was obtained from AJAX Chemicals Ltd., Australia. Fluorescein isothiocyanate (FITC, isomer I) and anhydrous dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich and MERCK Chemicals Ltd., Australia, respectively. ID-CellStab red cell stabilization solution was obtained from BioRad, Australia. The confocal images were captured with a Nikon Ai1Rsi confocal microscope in the Melbourne Centre for Nanofabrication. Water used for all dilutions was purified with Milli-Q water.

Methods. *Choice of Thread.* To evaluate our hypothesis, that fiber morphology plays a significant role in a thread's separation abilities in blood typing assays, cotton and silk threads with differing surface morphologies[19] were selected as models for this study. We obtained the ready-to-use cotton thread, and made hand-spun silk thread following the cotton thread's constructional parameters (around 200 fibers and 5 twists per centimeter). The surface morphologies of the threads were examined using a Scanning Electron Microscope (SEM) (Figure 1). The threads were then treated in a vacuum plasma reactor (K1050X plasma asher (Quorum Emitech, UK)) for 60 s at an intensity of 50W to increase their wettability.[5]

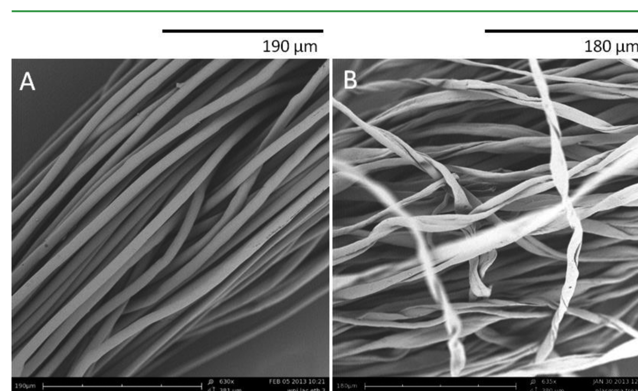

Figure 1. SEM images showing morphologies of (A) silk and (B) cotton threads.

Device Fabrication and Blood Typing Method. Blood typing devices were fabricated by affixing cotton and silk threads to a sticky holder. These threads form "multi-elution columns" parallel to one another.[3,4] Prior to a blood typing test, 2 μL of a commercial blood typing antibody (anti-A, anti-B, anti-A,B, and anti-D on first, second, third, and fourth thread, respectively) was deposited on one end of each thread and allowed to dry at ambient temperature (∼23 °C) for 10 min. Half a microliter of whole blood (40% hematocrit)[11] was introduced on each thread at the same spot where the antibody was introduced, and allowed 40 s for RBCs and the antibody to interact. Half a microliter of blood sample can stain around 0.5−0.7 cm of the thread, a length that is readily visible to the naked eye and is enough to react with antibody and generate a definitive assay result. Errors due to micro pipetting can therefore be monitored, such that small variations within the above range do not affect the assay result. The lower ends of threads were then dipped into 100 μL of the elution buffer. The buffer acts as a chromatographic mobile phase that elutes the free (nonagglutinated) RBCs along the thread, whereas agglutinated RBC lumps become immobilized in the interfiber gaps of the thread.

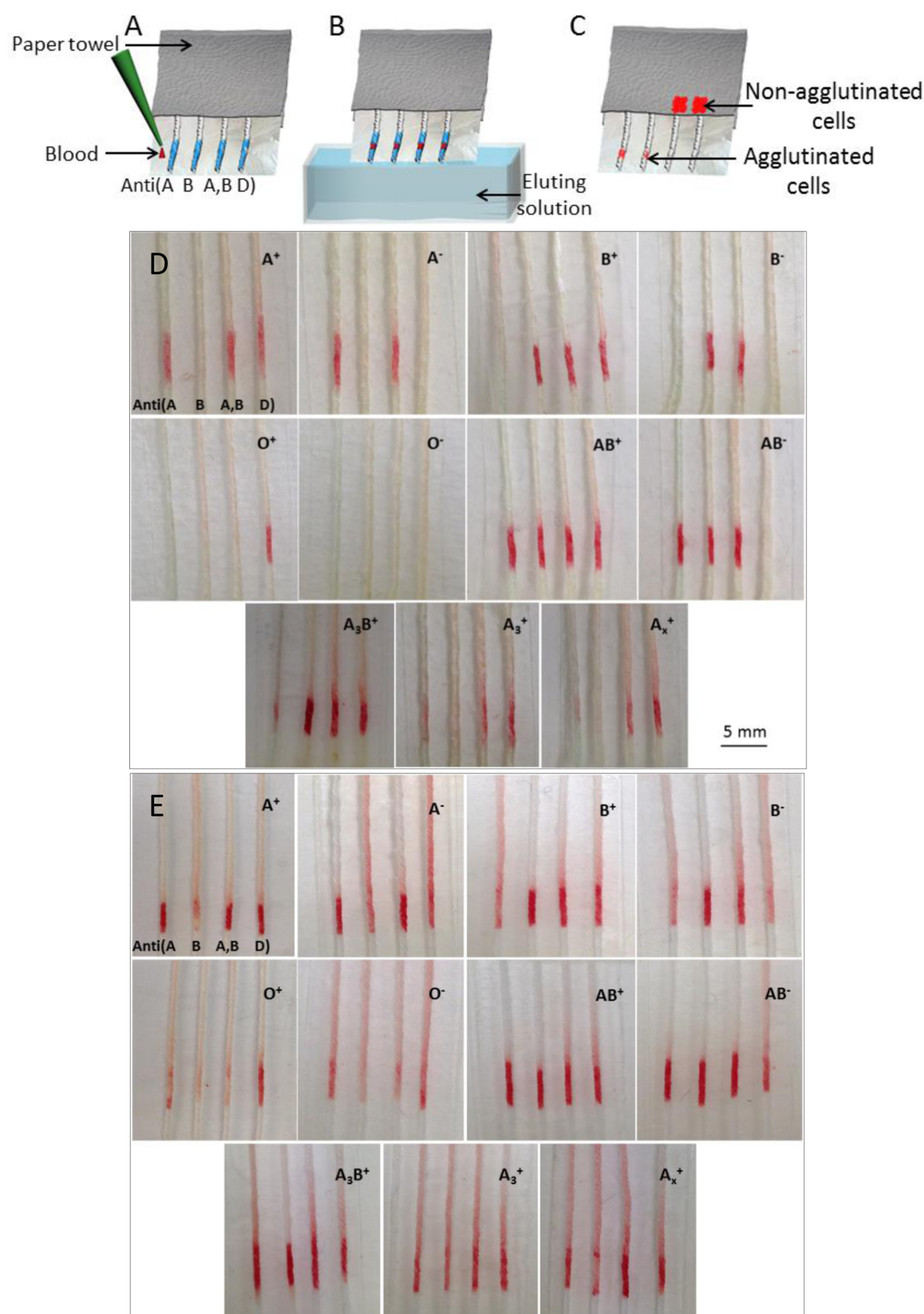

Figure 2. Schematic diagram showing (A−C) the blood typing method on thread. (A) Blood typing antibody (anti-A, anti-B, anti-A,B, and anti-D) and whole blood sample were deposited on each thread before (B) eluting with buffer. (C) Positive and negative results after elution. Images of ABO blood typing and its weak subgroups on (D) silk and (E) cotton threads. The threads for each test were pretreated with four different blood grouping antibodies; anti-A, anti-B, anti-A,B, and anti-D, respectively.

The blood sample introduction spots were then captured using a digital camera (Apple iPhone 5, auto color photo setting without flash light; object distance of 10 cm).

Confocal Microscopy Study of RBCs in Thread. The mechanism of how the surface morphology of fibers interferes with the blood typing results on threads has been investigated using a fluorescent confocal microscope. Fluorescent labeling of RBCs for confocal microscopy was performed as previously described.[22] In short, 10 mg of FITC was dissolved in 250 μL of Dimethyl sulfoxide (DMSO) and then diluted with ID-CellStab solution to obtain a FITC concentration of 0.8 mg/mL. A 0.6 mL of this solution, mixed into 40 μL of D-glucose solution (10 mg/mL), was added to 0.4 mL of RBCs before incubating for 2.5 h, with mixing at a low centrifuge force of 100 g to prevent the RBCs from settling down. Afterward, stained RBCs were washed with PSS a total of 10 times to remove unbound FITC (with centrifugation at 1300 g for 3 min after each wash). These stained RBCs were resuspended to 40% hematocrit with ID-CellStab solution.

To ensure that the blood staining protocol does not significantly affect the activity of the RBC antigens, we performed blood typing assays using whole blood and stained RBCs from the same sample on thread. A 0.5 μl sample of whole and stained blood was introduced to antibody treated silk thread and then eluted using the elution buffer. The degree of agglutination was examined by capturing and transferring the images to Adobe Photoshop for color intensity analysis. As shown in Figure S1 in the Supporting Information, staining has caused slight changes in the RBC agglutination color density on thread. However, such changes are not significantly enough to affect the visual identification of the assay results in any way.

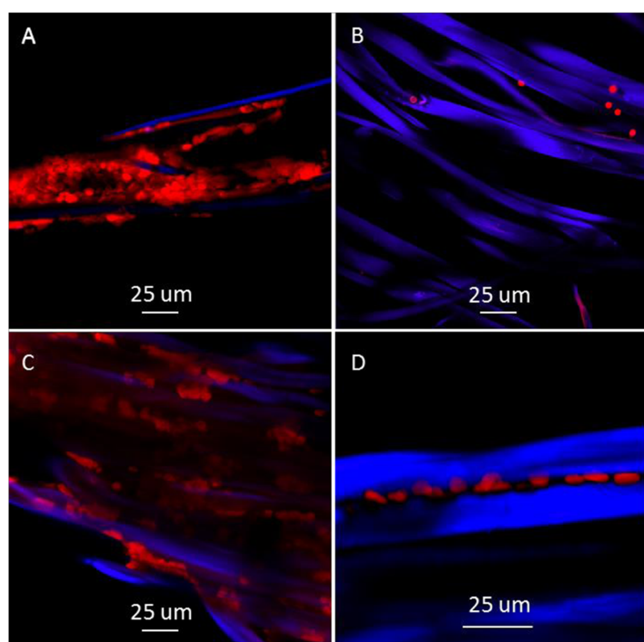

Figure 3. Confocal images of (A, C) agglutinated and (B, D) free RBCs on silk and cotton threads after buffer elution for 60 s. Agglutinated RBCs are immobilized in the interfiber gaps of (A) silk and (C) cotton threads. (B) Free RBCs are almost thoroughly removed from silk thread by buffer elution. (D) On cotton thread; however, RBCs are stuck in intrafiber gaps, creating a permanent red stain.

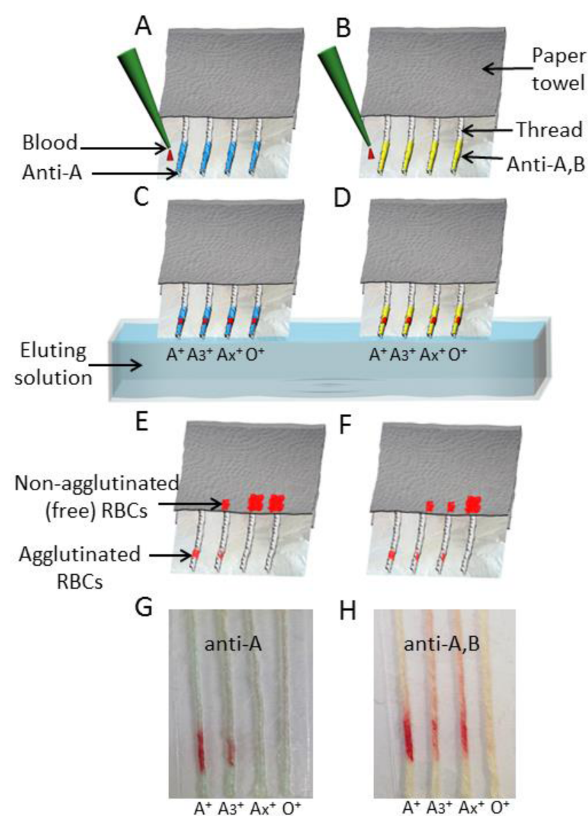

Figure 4. Schematic diagram showing the identification of weak A subgroups on silk thread using two blood grouping antibodies: (A, C, E, G) with anti-A, and (B, D, F, H) with anti-A,B. The assay procedures are described as follows: (A, B) Introducing blood on the antibody treated thread. (C, D) Devices before elution. (E, F) Devices after elution. (G, H) Final assay results. As shown, the degree of each hemeagglutination reaction for A_x, A_3, A, and O is different.

The conventional glass slide technique was also performed (see Figure S2 in the Supporting Information). Images captured using the confocal microscope demonstrated that stained RBCs still formed aggregates as expected in the presence of antigen-specific antibody (see Figure S2A in the Supporting Information), but shows no agglutination when the antibody present is nonspecific (see Figure S2B in the Supporting Information).

Effects of Buffer Elution Ph and Elution Time on Blood Typing. To improve the blood typing results on thread, we compared the color intensities (measured as the mean intensity of red color in RGB format in Adobe Photoshop 5.5) of the blood spots after elution with buffer solutions of different pH values for different durations of time. The buffer solutions were prepared by diluting PSS with 0.1 mM of ammonia solution to obtain serial elution buffers with different pH values (8, 9, and 10). The buffer solutions were then used to elute the blood spots for different durations of time (10, 30, 60, 90, and 120 s). The best condition was selected and used to identify the blood typing tests. In all assays, a 0.5 μL of blood sample was introduced onto the antibody treated thread, incubated for 40 s before being eluted with a buffer solution.

Identification of Weak A Subgroups. Because anti-A could not reliably identify weak A subgroups such as A_x and A_3, we decided to use both anti-A and anti-A,B to build the blood typing devices.[23] Two sets of threads were treated using anti-A and anti-A,B. Half a microlitre of whole blood was introduced onto each thread and left to incubate for 40 s. Thread was then eluted with a buffer solution for 60 s. The degree of the A_x and A_3 reactions with anti-A and anti-A,B has been used to differentiate them from each other and from the normal A and O blood types. The blood typing results were evaluated by the naked eye to determine the highest degree of agglutination.

Generating Barcode-Shaped Symbols. An easy method for interpreting blood typing results is beneficial for the successful application of microfluidic thread-based blood typing devices. Herein, in order to provide a simple interpretation, a set of barcode shaped symbols have been designed and then matched to each blood type. The symbols include white, black, and gray lines resembling the negative, positive and weak reactions of RBCs with different blood typing antibodies, respectively.

3. RESULTS AND DISCUSSION

Effect of Morphological Structure on Separation Properties of Thread. Morphological structures of silk (Figure 1A) and cotton (Figure 1B) threads were investigated using SEM. The silk fiber surface is smooth with no intrafiber gaps or lobes.[19] Thread constructed with silk has regular and continuous microfluidic channels formed by interfiber gaps.[19] The regular channels provide a possibility for the separation of agglutinated and free RBCs through silk thread during elution. In comparison, cotton thread is made of hollow, ribbonlike, cellulose fibers with intrafiber gaps on their surface.[19] The intrafiber gaps in cotton thread provide rather irregular microfluidic channels that trap or mechanically immobilize RBCs within the thread. This makes cotton thread an unsuitable substrate for blood typing purpose.

Normal ABO and Its Weak Subgroups Blood Typing Using Different Threads. The blood samples were introduced onto antibody treated threads and eluted with buffer solution of pH = 9 for 60 s (Figure 2A–C). Specific antibody–antigen reactions produced agglutinated RBCs including blood type A with anti-A and anti-A,B, blood type B with anti-B and anti-A,B and blood samples of type RhD^+ with anti-D.[20] After elution, a clear separation of agglutinated and free RBCs by silk thread could be observed. This is because

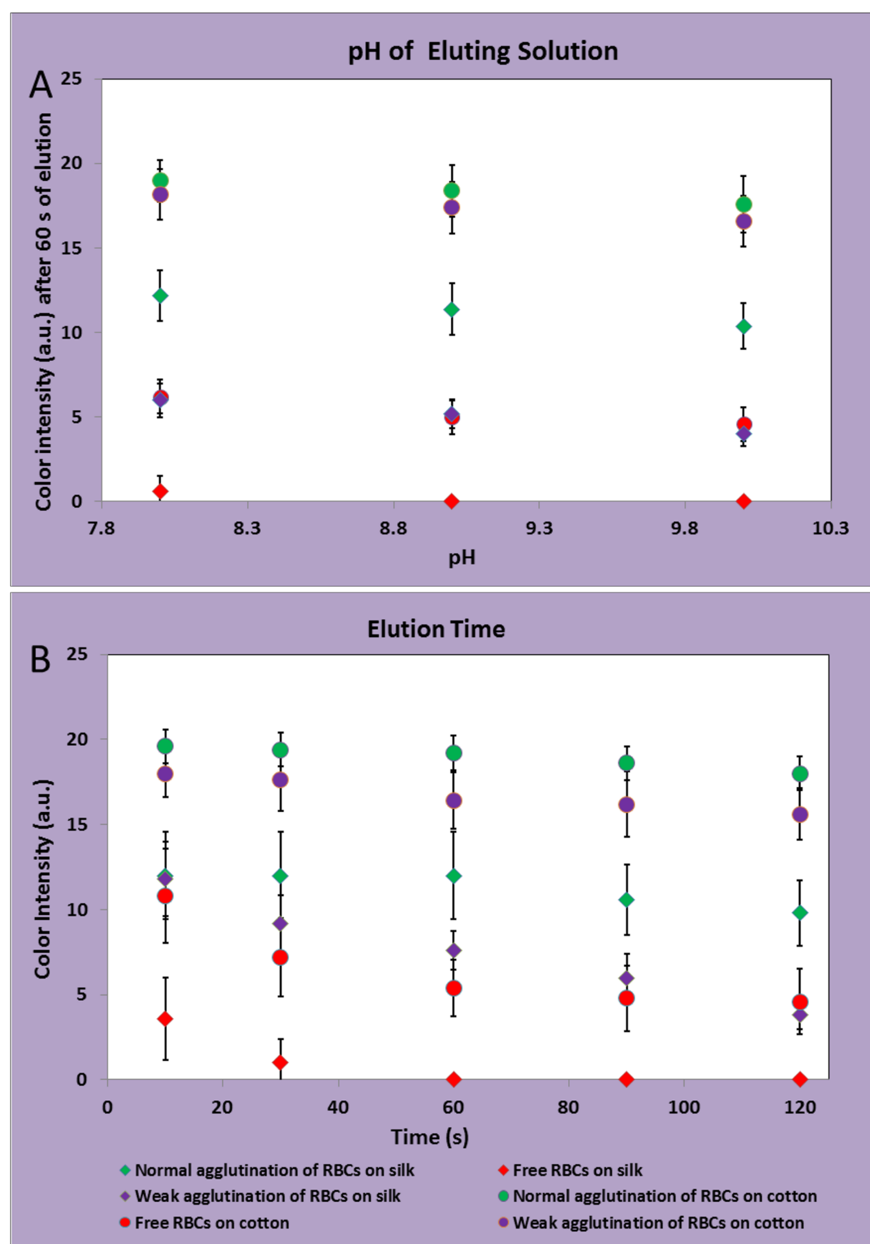

Figure 5. Improvement of blood typing assay on silk and cotton threads using the elution method. (A) Samples were eluted using buffer solution of different pH values for 60 s. (B) Samples were eluted using a buffer (pH 9) for different durations.

that interfiber channels in silk thread provide an efficient separation of the agglutinated RBCs from the free ones, making silk a suitable substrate for blood typing (Figures 2D and 3A, B).

However, in cotton thread, the free RBCs became stuck within the intrafiber gaps; making it difficult for cotton thread to differentiate the agglutinated and free RBCs (Figures 2E and 3C, D). Free RBCs are able to fold and enter into pores of an average diameter of ~3 μm.[24] In addition, RBCs can travel horizontally or perpendicularly with respect to the fiber surface during elution and become entrapped in intrafiber gaps of different sizes. Figure 3D shows disclike RBCs lodged into a narrow intrafiber gap on the surface of a cotton fiber. This situation can get worse in weak ABO blood typing, as mixed field agglutination reaction always occurs and a very clear separation of small agglutinated lumps from free RBCs is required. In such circumstances, the differentiation of normal and weak reactions is almost impossible. Figure 3D also shows that material affinity interactions between the cotton fiber surface and RBC are weak, because no free RBCs could be observed on the smooth cotton fiber surface. Therefore, mechanical entrapment of RBCs by intrafiber gaps is the major mechanism responsible for the low clarity of blood typing assay.

Effect of Different Antibodies in Weak A Subgroup Blood Typing. Through using the elution method to investigate the potencies of specific antibodies for weak A subgroups, we found a suitable surface for detection of A_x and A_3. As is shown in Figure 4, the RBCs of A_3 samples agglutinated on silk threads pretreated with anti-A and anti-A,B, but more weakly than normal A. A_x samples, however, agglutinated more strongly on the thread with deposited anti-A,B rather than anti-A. The use of two antibodies in our design is suitable for A_x and A_3 blood typing purposes and can be used as a key for differentiating A_3 and A_x from each other and from normal A and O groups.

Table 1. Summary of the Efficacy of Blood Typing of normal ABO and Its Weak Subgroups for Blood Samples from 95 Donors Using Silk Thread[a]

blood type	RBCs immobilization with anti-A	RBC immobilization with anti-B	RBC immobilization with anti-A,B	RBC immobilization with anti-D
A^+ (n = 24)	24/24	0/24	24/24	24/24
A^- (n = 9)	9/9	0/9	9/9	0/9
B^+ (n = 6)	0/6	6/6	6/6	6/6
B^- (n = 5)	0/5	5/5	5/5	0/5
AB^+ (n = 3)	3/3	3/3	3/3	3/3
AB^- (n = 2)	2/2	2/2	2/2	0/2
O^+ (n = 25)	0/25	0/25	0/25	25/25
O^- (n = 15)	0/15	0/15	0/15	0/15
A_3^+ (n = 1)	1/1	0/1	1/1	1/1
A_3B^+ (n = 3)	3/3	3/3	3/3	3/3
A_x^+ (n = 2)	0/2	0/2	2/2	2/2

[a]Table is adapted with permission from ref [20]. Copyright 2011 American Chemical Society.

Effects of Elution Buffer pH and Elution Time on Blood Typing. To obtain unambiguous results of blood typing, buffer solutions with different pH values (8, 9, and 10) have been used to elute RBCs from antibody treated silk and cotton threads for 60 s. Figure 5A shows that the main effect determining the assay clarity is the fiber type (or fiber surface morphology), which is in consistence with the results in Figure 3 and the above discussion. pH, however, has a secondary effect to the assay clarity on silk. Figure 5A shows that as the pH increases to 9 and higher, the negative assays on silk become clean (color density = 0), this increases the assay clarity on silk thread. The most likely reason is that silk fiber and free RBCs carry weak acidic groups which dissociate more strongly at high pH, this causes stronger charge repulsion between the silk fiber and the RBCs, allowing free RBCs to be eluted away from silk fiber surfaces more thoroughly.

With regard to eluting time, a buffer solution (pH = 9) has been used to elute the RBCs from threads for different durations (10, 30, 60, 90, and 120 s). As shown in Figure 5B, the optimal elution time is around 60 s. Under this condition, free RBCs of negative assays could be removed from the silk thread completely, while in positive assays of weak blood groups, the small agglutinated RBC clusters were not significantly dissociated. This condition therefore produced weak blood group assays of the highest clarity. Elution time longer than 60 s produced slightly weaker color density for positive assays of weak blood groups, which is most likely caused by the dissociation of small agglutinated RBC clusters of the weak groups. Ninety five blood samples of different groups were assayed with 60 s elution time using the silk thread, the results (Table 1) were in full agreement with the assays performed by the Red Cross Australia. In all cases, the specific antibody−antigen reactions produced the immobilized hemagglutinated RBCs on silk thread surface; no nonspecific reaction between antibodies and RBCs were observed. Interestingly, silk thread can also report unambiguous positive assays for normal and weak blood groups if elution times longer than 60 s are used. This is because that the clarity of silk thread assays are high, all negative assays have zero color density, making the color densities of positive assays to stand out clearly.

Barcode Shaped Symbols. To have an easy blood typing result interpretation, we provided a set of printed patterns similar to barcodes to confirm the result of each blood test by visual matching. In Table 2, the black and gray lines refer to the strong and weak agglutination reactions between antibody and RBCs, respectively. The white lines however represent negative agglutination reactions between the blood samples with antibody. Only one symbol is used in each case to show the blood type (Figure 2D and Table 2), which can be useful for easy result interpretation, especially in the region with a lack of trained personnel.

Table 2. Barcode Shaped Symbols Provided for Easy Interpretation of Blood Typing Results[a]

Blood group	Symbol
A^+ (n = 24)	
A^- (n = 9)	
B^+ (n = 6)	
B^- (n = 5)	
AB^+ (n = 3)	
AB^- (n = 2)	
O^+ (n = 25)	
O^- (n = 15)	
A_3^+ (n = 1)	
A_3B^+ (n = 3)	
A_x^+ (n = 2)	

[a]The white, black, and grey symbols refer to the negative, positive, and weak antibody−antigen reactions, respectively.

4. CONCLUSIONS

In this study, the effect of the surface morphologies of silk and cotton fibers on the separation properties of threads made from these fibers has been investigated for the application of blood typing based on the principal of chromatographic elution. Blood typing devices made of silk thread deliver significantly higher assay clarity than those made of cotton thread. SEM study shows that cotton fibers have irregular intrafiber gaps of several microns in width, whereas silk fibers have no intrafiber gaps. This surface morphological difference between silk and

cotton fibers significantly affect the migration behavior of free RBCs in these threads. Confocal microscopic investigations showed that intrafiber gaps on cotton fiber surfaces can mechanically trap free RBCs; cotton thread therefore provides poor separation power to differentiate the agglutinated RBCs from the free ones. This makes blood typing assays using cotton thread very low clarity and unsuitable for visual identification. In contrast, silk fibers, having a smooth surface do not trap RBCs; silk threads therefore provide much more efficient separation power for differentiating free RBCs from agglutinated ones. The high clarity blood typing assays by silk threads are well-suited for visual identification. This study provides new insights into the selection of the right threads for building sensors with the desired performance.

The degree of RBC agglutination with different antibodies on silk thread has also been used as a strategy to differentiate six samples of the weak A blood types from one another and from the normal ABO red blood cells. The ability to differentiate different weak ABO groups from the normal ABO groups is a very important, as it will reduce transfusion mistakes for weak ABO group patients. Silk thread delivers results for both weak and normal ABO groups that can be easily interpreted by the naked eye with the aid of a matching chart.

■ ASSOCIATED CONTENT

Supporting Information

Comparison of whole blood samples and stained red blood cells (RBCs) in blood typing assays on thread; Confocal images of FITC stained red blood cells on glass slide. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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