

Paper-Based Blood Typing Device That Reports Patient's Blood Type "in Writing"***

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The British author, J. K. Rowling, presented a visionary idea in her novel "Harry Potter and the Chamber of Secrets" that one can interrogate a piece of paper for information and get unambiguous answers from the paper in writing. Here we report a study, following her vision, on using a low-cost bioactive paper device to perform ABO and rhesus (RhD) blood typing tests and obtain test results from the paper in writing. Paper text patterns are designed and printed to allow interactions between grouping antibodies and red blood cells. Composite text patterns consisting of the bioactive and non-bioactive sections are used to form the letters and symbols for the final display of the testing report. This paper-based blood typing device rapidly reports patient's blood type in unambiguous written text.

Patterned bioactive paper as a tool for biochemical analysis has become a platform for making low-cost and user-operated devices for diagnosis,^[1] point of care (POC),^[1–6] pathogen and biomarker detection,^[1,4,5] and food and drinking water quality testing.^[7] The potential of this platform to deliver affordable, rapid, and user-friendly diagnostic sensors for disease screening, healthcare and drinking water quality evaluation to the developing countries has become increasingly clear.^[1,4,8–10] Over the last five years, there has been an explosion of research in bioactive paper-based low-cost sensor fabrication,^[1,3,4,11–16] electronic transmission of colorimetric assays for real-time diagnosis,^[6,17] as well as new diagnostic and environmental applications using low-cost sensors.^[6,7,18–21] Colorimetric and electrochemical methods are the preferred analytical approaches for bioactive paper sensors, since these methods are proven to be effective in qualitative and semi-quantitative sensing and can be easily adapted onto paper.^[3,6,21,22] However, those studies also revealed certain limitations of those new concepts in their practical applications. Whilst a colorimetric or voltammetric

signal can report the test results, in most situations the results need to be interpreted by trained personnel. This is particularly true if an assay has multiple outcomes and requires careful examination in order for the diagnosis to be made. Therefore, among the many challenges in low-cost diagnostics, unambiguous reporting of the test results by the sensors to the users is critical. Where sensors are used in developing regions for large-scale disease screening, even if we can fabricate sensors that are robust enough to function under an unsupported field condition, misinterpretation of the assay results may still be a significant factor that can compromise the value of low-cost diagnostics.

Are there other means by which we can design and interrogate bioactive paper sensors for diagnostic results? An inspiration can be found in the movie adapted from J. K. Rowling's novel "Harry Potter and the Chamber of Secrets";^[23] Potter interrogated Tom Riddle's Diary by writing on a page of paper in the Diary "Do you know anything about the Chamber of Secrets?"; the paper responded with a "Yes" in writing, instead of with a color change. The artist's vision shows that non-conventional mechanisms of reporting assay results using paper-based sensors should be explored. To the authors' best knowledge, equipment-free bioactive paper-based diagnostic devices capable of reporting multiple conditions in written text from a single test is currently not available. In this study, we present a bioactive paper blood typing device that is capable of reporting ABO RhD blood types rapidly and in written text.

Correct typing of human blood is extremely important in blood transfusion and in events of medical emergency. The blood type of an individual is determined by the presence or absence of certain antigens on the surface of a red blood cell (RBC). In addition, antibodies existing in blood serum protect the body from incompatible and hostile antigens.^[24] The vast majority of techniques for ABO and RhD typing of blood to date have been based upon the principle of haemagglutination reactions between RBCs and antibodies. The absence of agglutination indicates no haemagglutination reaction.^[24] Recently low-cost bioactive paper^[19] and bioactive thread^[18,25] microfluidic sensors have been reported for human blood grouping applications. These devices are also based on the principle of haemagglutination reactions. Through observing the differences in wicking distances of the agglutinated red blood cells and the blood serum, an indication of haemagglutination reaction can be identified. However, paper- and thread-based technologies still require users who have the blood typing knowledge to interpret the result. For the blood typing devices to be more adaptable to conditions in developing countries, they must be able to report results unambiguously to users who may not have the

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knowledge to interpret the results based on the first principle. An easy way to bridge this application gap is to design paper-based devices that can report blood typing results to the users in written text.

The text-reporting blood typing device we present here is partly based on the principle of haemagglutination reactions. Its fabrication involves the use of hydrophobic–hydrophilic contrast to form text patterns and to use these text patterns as sites for RBC and antibody interactions. The hydrophobic–hydrophilic contrast will ensure the unambiguous legibility of the text pattern of agglutinated blood to be displayed. In our design the text patterns are made hydrophilic, and antibody solutions are introduced into the corresponding text patterns (e.g. Anti-A into text pattern “A”). In a blood typing assay a blood sample is introduced to all text patterns. To clearly identify the occurrence of haemagglutination reactions inside the hydrophilic text patterns, a saline-washing step is employed. If the antibody in a text pattern is not the corresponding antibody to the antigens carried by RBCs, there will be no haemagglutination reaction; the non-agglutinated RBCs can be easily washed out of the text pattern with the saline solution. Contrary to this, if RBCs have haemagglutination reaction through the antibody-antigen interaction, agglutinated RBC lumps will form inside the fiber matrix of the paper and cannot be washed out by the saline solution.^[26] Those text patterns occupied by the agglutinate RBC lumps therefore have unambiguous legibility with high resolution and contrast.

In devices for ABO RhD blood typing, three antibodies (Anti-A, Anti-B, and Anti-D) are used to display blood typing results by text. The interaction of each antibody with RBCs can have two possible outcomes, that is, “Agglutination” and “No agglutination”. The total number of blood types determinable by the three antibodies will be $2^3 = 8$. Taking an A+ blood sample, for example, the interaction of the A+ blood sample with the three antibodies can be expressed as $\overline{A}BD$, that is, A (agglutination with antibody A occurs), $\overline{B}$ (no agglutination with antibody B), and D (agglutination with antibody D occurs). Following this expression, all different blood types (column 3) determinable by these antibodies through the presence and absence of RBC agglutination (column 2) can be listed in Table 1.

If we use only haemagglutination reactions to design a text-reporting device for blood typing, many blood types cannot be unambiguously reported by text. This situation

includes two circumstances where blood types have no haemagglutination reactions with the grouping antigens, therefore no visually perceivable text patterns can be formed to make the text report. The first circumstance involves all Rh(–) blood types; the lack of interactions of RBCs with Anti-D results in no haemagglutination reaction (i.e. $\overline{D}$) and therefore cannot form visually perceivable text patterns. The second circumstance involves O-type blood. Since red cells of O-type blood do not carry A and B antigens, they do not have haemagglutination reactions with either Anti-A or Anti-B (i.e. $\overline{A}\overline{B}$). O-type blood cannot be reported in written text formed by haemagglutination reaction only. Table 1 (column 4) lists all five blood types that are associated with these two circumstances. Further design of the devices must overcome these obstacles.

In this study we present a design of a composite text symbol that can report both D and $\overline{D}$ unambiguously. This symbol takes the form of “+”; it consists of a permanent “–” printed using a non-bioactive water-insoluble ink and a bioactive “|” printed using Anti-D (Figure 1 a). In a blood test when a sample is introduced into “|”. If the sample carries D antigen, haemagglutination reaction will occur inside “|” and saline washing will not be able to remove the agglutinated RBCs out of pattern “|”. The composite text symbol will report “+”. On the other hand, if the sample does not carry the RhD antigen, then no haemagglutination reaction will occur. After saline washing, no agglutinated blood sample will be perceivable in “|”; the composite text symbol will report “–” (Figure 1 a). This design fulfills the requirement of using one composite pattern to unambiguously report RhD(+) and RhD(–) in text.

For O-type blood samples, we again employ another composite text symbol to report both “O” and “non-O” types of blood samples. This composite text symbol takes the form of “⊗”; it consists of a permanent letter “O” printed using water-insoluble ink and a “×” printed using an equal-volume mixture of Anti-A and Anti-B (see Figure 1 b). In a blood test when a sample is introduced into “×”, the sample is mixed with Anti-A and Anti-B inside “×”. If the sample carries A-antigen or B-antigen, or both, then haemagglutination reaction will occur inside “×” and saline washing will not be able to remove the agglutinated RBCs out of the pattern. The composite text symbol will report “⊗”. On the other hand, if the sample does not carry A-antigen and B-antigen, then no haemagglutination reaction will occur inside “×”.

Table 1: Blood types that can be reported in text by specific antigen–antibody agglutination reactions before and after implementing our design solutions (see Figure 1).^[a]

No.	Agglutination	Blood types determined by RBC agglutination	RBC antigens unable to be expressed in text by RBC agglutination (and reasons)	RBC antigens unable to be expressed in text after implementing design solution 1	Text reporting after implementing design solutions 1 and 2
1	ABD	AB+			Enabled
2	ABD	A+			Enabled
3	$AB\overline{D}$	AB–	x ($\overline{D}$)		Enabled
4	$A\overline{B}\overline{D}$	A–	x ($\overline{D}$)		Enabled
5	$\overline{A}BD$	B+			Enabled
6	$\overline{A}BD$	O+	x ($\overline{A}, \overline{B}$)	x ($\overline{A}, \overline{B}$)	Enabled
7	$\overline{A}\overline{B}\overline{D}$	B–	x ($\overline{D}$)		Enabled
8	ABD	O–	x ($\overline{D}, \overline{A}, \overline{B}$)	x ($\overline{A}, \overline{B}$)	Enabled

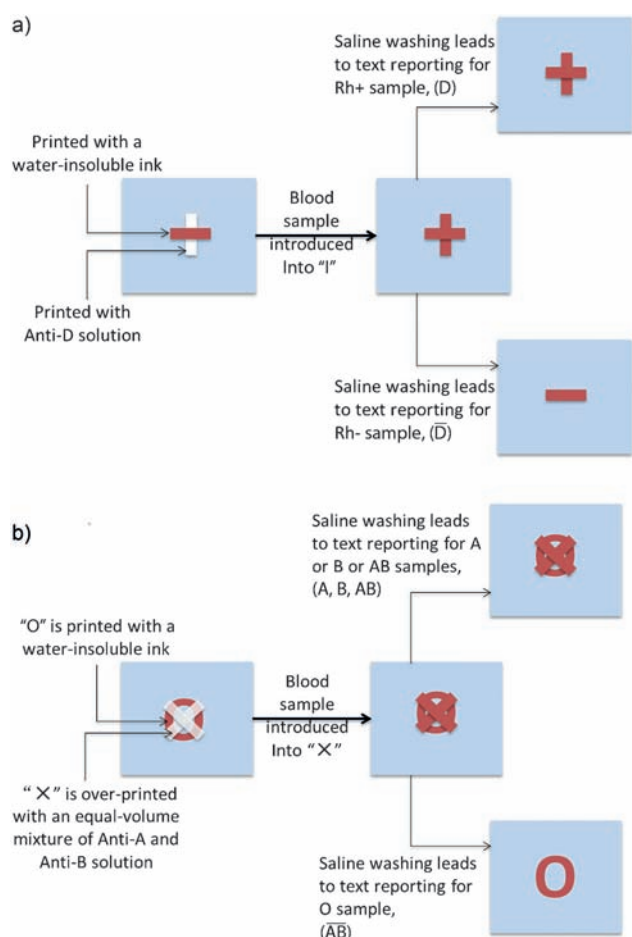


Figure 1. The two design solutions using composite text symbols to report blood test results in text. a) Design solution 1: The composite text symbol for reporting the presence of RhD takes the form of "+"; it consists of a permanent "-" printed using a non-bioactive water-insoluble ink and a "|" printed using Anti-D. b) Design solution 2: The composite text symbol for reporting "O" and "non-O" types of blood samples takes the form of "O"; it consists of a permanent letter "O" printed using non-bioactive water-insoluble ink and a "x" printed using an equal-volume mixture of Anti-A and Anti-B.

After saline washing, the composite text symbol will report "O" (Figure 1b). This design fulfills the requirement of using one composite pattern to unambiguously report "O" and "non-O" types of blood samples in text. The three blue dots in the printed device are for positioning purpose.

Negative text patterns consisting of letters "A" and "B" and two text symbols, "x" and "|" were created electronically. A Canon ink jet printer was used to print a 2% (w/v) heptane solution of an alkenyl ketene dimer (AKD)^[3,4] on the paper sample, forming the negative patterns of letters and symbols (see Figure S1 in the Supporting Information). Printing of negative patterns of letters and symbols ensures that these patterns remain hydrophilic and are surrounded by hydrophobic areas. The hydrophilic patterns are, however, invisible. After printing of the hydrophilic patterns, 2.5 μ L of Anti-A, clone 10090; Anti-B, clone 10091; and Anti-D, clone 20093 were introduced into the hydrophilic patterns "A", "B", and "|", respectively, by ink jet printing or writing with a pen

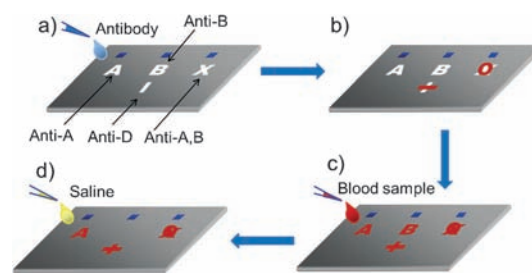


Figure 2. Fabrication and testing procedures of the text-reporting blood-typing devices. Negative patterns of letters and symbols are printed using a heptane solution of an alkenyl ketene dimer; letters and symbols remain hydrophilic and are surrounded by hydrophobic areas (see Figure S1 in the Supporting Information). a) Anti-A and Anti-B are introduced into the corresponding letters. An equal-volume mixture of Anti-A and Anti-B is introduced into "x", and Anti-D is introduced into "|". b) Letter "O" and symbol "-" are printed over "x" and "-", respectively, using a non-bioactive and water-insoluble ink. c) A blood sample is introduced in the device for blood typing test. d) After washing each pattern with $2 \times 50 \mu\text{L}$ of saline solution, the blood typing result is reported by the device in text.

(Figure 2a). Two and a half microlitres of an equal-volume mixture of Anti-A and Anti-B were introduced into the pattern "x" (Figure 2a). Then letter "O" was printed using a water-insoluble ink over the hydrophilic and invisible "x" symbol; symbol "-" was also printed with a water-insoluble ink over the hydrophilic and invisible "|" symbol as shown in Figure 2b. The strong hydrophilic–hydrophobic contrast ensures a high resolution of the text patterns formed by antibody solutions. After drying under ambient conditions the device is ready for use. Detailed fabrication steps can be found in the Supporting Information.

In a blood typing assay, 2.5 μ L of blood sample were introduced into each of the antibody-loaded patterns (i.e. A, B, x, and |) with a micropipette (Figure 2c). Twenty seconds were allowed for the antibodies in the letter and symbol patterns to react with the antigens carried by the RBCs. After 20 seconds, two aliquots of 50 μ L saline solution were introduced into each of the letter and symbol patterns. In cases where haemagglutination reactions occur in certain text or symbol patterns, the agglutinated blood sample could not be washed out of those patterns. The agglutinated RBCs formed legible text patterns that report the occurrence of the specific haemagglutination reaction. On the other hand, if the haemagglutination reaction does not occur, the non-agglutinated blood can be easily washed out of the patterns through the vertical flow, leaving no visible trace of the blood sample (Figure 2d). The legible letter and symbol patterns, in combination, form the text report of the blood type of the sample. Figure 3a shows the expected reports for the eight blood types by the device; Figure 3b shows a photo of the actual blood type tests of all eight blood types.

The sensitivity of the bioactive paper text-reporting device was evaluated by testing blood samples (A+ and B+) diluted with saline solution (see Figure S4 in Supporting Information). This method is used to evaluate the sensitivity of blood typing assays for samples from patients with anaemia conditions; these samples have a decreased RBC color

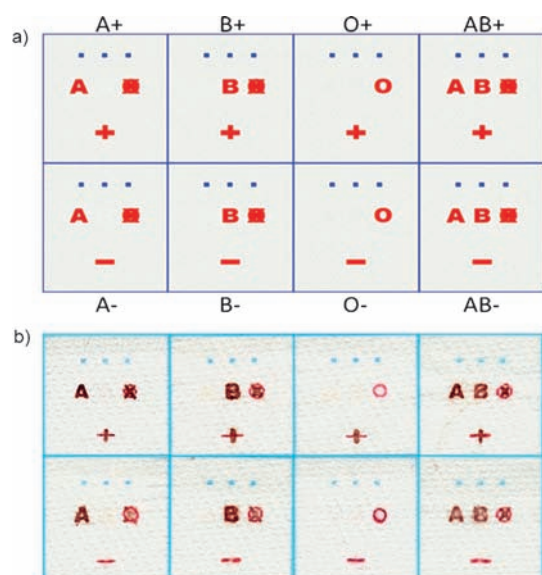


Figure 3. a) A schematic of the expected text patterns reported by the blood type device fabricated based on our design. The corresponding blood types are given to assist the reader. b) The actual tests of all eight ABO RhD blood types. The device size is 25 mm×25 mm.

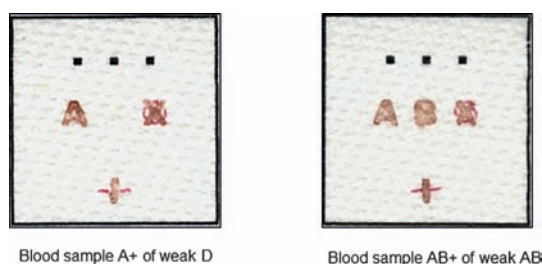


Figure 4. The text reports of weak blood samples using the paper-based text-reporting devices.

intensity. The bioactive paper text-reporting device can confidently identify A and B antigens on RBCs after the blood samples were diluted by a factor of four and D antigens by a factor of two. This level of sensitivity is sufficient, since dilution by a factor of two is considered an adequate threshold for anaemia samples (see the Supporting Information).

A total of 99 samples were tested using the text-reporting device; this sample set contained all eight blood types. All test results using the bioactive paper text-reporting devices matched with the results obtained by the pathological laboratory using the gel card technology. Details of the tested samples are presented in Table 1 in the Supporting Information. Among the samples, two weak samples, a weak AB and a weak D, were confirmed by the pathological laboratory following the gel card protocol. These weak samples were tested using the bioactive paper text-reporting device. The device is able to make clear identification of the blood types of these weak samples and the results match with the confirmation made by the pathological laboratory (see the Supporting Information). Figure 4 shows the test results of the two weak samples by bioactive paper text-reporting

device. The ability of the bioactive paper text-reporting device to confidently identify weak samples is also supported by our recent validation study of paper-based assays for rapid blood typing.^[27]

We show in this study that this bioactive paper text-reporting blood typing device meets the basic requirements of sensitivity, specificity, and text legibility. It is capable of reporting all blood types in the ABO RhD system. This reporting method significantly increases the ability of the device to report the results unambiguously to non-professional users. It is expected that an expansion of text-reporting bioactive paper sensors beyond the ABO RhD blood typing application may be possible. The concept of text-reporting using paper-based devices for low-cost diagnostic applications will be valuable for making affordable, sensitive, specific, rapid, equipment-free, and user-friendly (ASSURED^[10]) devices for developing countries.

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