Fabrication of Paper Sensors for Practical Applications

Thesis in fulfillment of the requirement for the degree of
Doctor of Philosophy in Chemical Engineering

By

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March 2017
Dedicated to My Parents
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General Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Signature: ………………………

Print Name: Rong Cao

Date: 13-03-2017
Monash University

Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 3 original papers published in peer-reviewed journals and 1 submitted publication. The core theme of the thesis is the fabrication of paper sensors for practical applications. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Department of Chemical Engineering under the supervision of Prof. Wei Shen.

(The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.)

In the case of Chapter 2, 3 and 4, my contribution to the work involved the following:

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Polysaccharides as protectants for paper-based analytical devices with antibody

Initiation, key ideas, experimental works, analysis of results, writing up

1) Wendy Tian. Assisted in experimentation 5%
2) Wei Shen. Key ideas, paper reviewing, editing 15%

I have/ have not (circle that which applies) renumbered sections of published papers in order to generate a consistent presentation within the thesis.

Student signature: ___________________

Date: 13-03-2017

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student’s and co-authors’ contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature: ___________________

Date: 13-03-2017
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I would like to express my sincere gratitude and thanks to my supervisor Prof. Wei Shen for his constant guidance, encouragement, support and patience throughout my PhD candidature. Prof. Shen is the one of most impressive and passionate supervisors I have ever worked with. He provided me the opportunity to start the excellent and interesting PhD project. During my PhD candidature, he trained me to think critically and independently; encouraged me to try innovative ideas; taught me to improve my writing skills for publication in top journals and give attractive presentations; motivated me to do better than what I would expect from myself; supported me when I was in stress; inspired me to never be afraid of failure. He is always there to help me and guide me to enjoy both research and life, which will be treasured all my life. I really appreciate his supervision and enjoy working with him.

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ABSTRACT

Paper-based analytical devices have attracted wide interest and developed into a fast-growing new field in analytical chemistry. The potential of paper-based sensing platforms to enable rapid testing of analytes for environmental, food safety, and clinical applications in less-industrialized countries and remote regions has become increasingly clear. The success or failure of paper-based analytical devices will likely be determined by their use beyond laboratories. While significant attention has been given to some critical features of paper sensors such as sensitivity, specificity and multiplexed detection, there remain many other hurdles to be overcome to achieve successful real-life applications. One significant limitation with paper-based analytical devices is the lack of a simple and versatile method for the immobilization of biomolecules onto paper. A second one is the instability of biomolecules on paper during shipping and storage. In addition, many paper-based analytical devices do not entirely fulfill the requirements of being user-friendly, equipment-free or with minimum equipment. Therefore, the motivation of this PhD project is to fill some gaps between a prototype in the laboratory and a product at home for paper-based chemical and biological sensors. We hope that paper-based analytical devices will be used as ideal tools for point-of-care clinical diagnostics and environment monitoring in the future.

This thesis includes four parts, which emphasize on different steps in the fabrication of paper-based analytical devices. The first part focuses on biomolecule immobilization strategies for paper-based biosensing platforms. Since direct physical adsorption of biomolecules does not always promise reproducible results, a simple and versatile covalent binding approach capable of immobilizing a broad range of biomolecules is desirable. The second part concentrates on the storage and stabilization of paper-immobilized biomolecules by lyophilization, because a limitation to the implementation of paper sensors in the real world is the short shelf-life of many biorecognition reagents deposited on devices under ambient conditions. The third part further explores a simple and low-cost polymer modification method to prolong the shelf-life of paper sensors with immobilized biomolecules. The last part explores the
novel designs to ensure paper sensors with convenient usability and low cost. The feasibility of “a pen/stamp on paper with controllable wettability” has been investigated for the reduction of the fabrication cost of paper-based analytical devices.

The studies performed in this project enhance the power of paper-based analytical devices as a platform technology for practical applications. Inexpensive modification approaches used here can save time and cost in paper functionalization; the stability of protein-based paper sensors is also improved via a simple modification method. In addition, novel designs in sensor fabrication and in practical applications are proposed. The results of the studies show enormous potential for integration with future work, which will strongly drive the development of paper-based sensing devices for practical applications.
LIST OF PUBLICATIONS

Peer-Reviewed Journal Papers

The following published papers are included in the main body of this thesis as individual chapters. The sections of these published papers have been renumbered in order to generate a consistent presentation within the thesis. Papers in their published format are included as Appendix I in this thesis.


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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D</td>
<td>two-dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>three-dimensional</td>
</tr>
<tr>
<td>AgNPs</td>
<td>silver nanoplates</td>
</tr>
<tr>
<td>AKD</td>
<td>alkyl ketene dimer</td>
</tr>
<tr>
<td>Anti-A</td>
<td>anti-antigen A blood grouping antibodies</td>
</tr>
<tr>
<td>Anti-B</td>
<td>anti-antigen B blood grouping antibodies</td>
</tr>
<tr>
<td>Anti-D</td>
<td>anti-antigen D blood grouping antibodies</td>
</tr>
<tr>
<td>AP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>ARC</td>
<td>Australian Research Council</td>
</tr>
<tr>
<td>CPRG</td>
<td>chlorophenol red β-galactopyranoside</td>
</tr>
<tr>
<td>ASA</td>
<td>alkyl succinic anhydride</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolyl phosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CFP</td>
<td>cellulose filter paper</td>
</tr>
<tr>
<td>CMC</td>
<td>carboxymethyl cellulose</td>
</tr>
<tr>
<td>CSL</td>
<td>Commonwealth Serum Laboratories</td>
</tr>
<tr>
<td>DMG</td>
<td>dimethylglyoxime</td>
</tr>
<tr>
<td>DVS</td>
<td>divinyl sulfone</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assays</td>
</tr>
<tr>
<td>FFU</td>
<td>for further manufacturing use</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>GO</td>
<td>graphene oxide</td>
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<td>GMA</td>
<td>glycidyl methacrylate</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
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<tr>
<td>ICP-MS</td>
<td>inductively coupled plasma mass spectrometry</td>
</tr>
<tr>
<td>IgA</td>
<td>immunoglobulin A</td>
</tr>
<tr>
<td>IgD</td>
<td>immunoglobulin D</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
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<tr>
<td>IgE</td>
<td>immunoglobulin E</td>
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<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
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<td>immunoglobulin M</td>
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<tr>
<td>ISBT</td>
<td>international society of blood transfusion</td>
</tr>
<tr>
<td>LbL</td>
<td>layer-by-layer</td>
</tr>
<tr>
<td>LODs</td>
<td>limits of detection</td>
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<tr>
<td>LPAD</td>
<td>laminated paper-based analytical device</td>
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<tr>
<td>NaAlg</td>
<td>sodium alginate</td>
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<tr>
<td>NBT</td>
<td>nitro blue tetrazolium</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
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<tr>
<td>PAH</td>
<td>poly(allylamine hydrochloride)</td>
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<tr>
<td>PAN</td>
<td>1-(2’-pyridylazo)-2-naphthol</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>phosphate buffered saline with Tween-20</td>
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<tr>
<td>PIM</td>
<td>polymer inclusion membrane</td>
</tr>
<tr>
<td>PSS</td>
<td>poly(sodium 4-styrenesulfonate)</td>
</tr>
<tr>
<td>PVP</td>
<td>polyvinylpyrrolidone</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cell</td>
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<tr>
<td>RBCs</td>
<td>red blood cells</td>
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<tr>
<td>RhD</td>
<td>rhesus D</td>
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<td>SEM</td>
<td>scanning electron microscope</td>
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<td>TETRYL</td>
<td>2,4,6-trinitrophenylmethylnitramine</td>
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<tr>
<td>UA</td>
<td>uric acid</td>
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<tr>
<td>UVO</td>
<td>ultra-violet ozone treatment</td>
</tr>
<tr>
<td>WCA</td>
<td>water contact angle</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WDPT</td>
<td>Water Drop Penetration Time</td>
</tr>
<tr>
<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
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<tr>
<td>β-gal</td>
<td>β-galactosidase</td>
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## LIST OF NOMENCLATURE

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<thead>
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<th>Symbol</th>
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<td>cm</td>
<td>centimeter</td>
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<tr>
<td>cP</td>
<td>centipoise</td>
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<tr>
<td>Da</td>
<td>dalton</td>
</tr>
<tr>
<td>d</td>
<td>day</td>
</tr>
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<td>femtomole</td>
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<td>g</td>
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<td>h</td>
<td>water penetration height</td>
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<tr>
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<td>ng</td>
<td>nanogram</td>
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<td>nanometer</td>
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<tr>
<td>pH</td>
<td>Potential of Hydrogen</td>
</tr>
<tr>
<td>p I</td>
<td>Isoelectric Point</td>
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<tr>
<td>p Ka</td>
<td>logarithmic acid dissociation constant</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>r</td>
<td>pore radius</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>t</td>
<td>penetration time</td>
</tr>
<tr>
<td>µm</td>
<td>micrometer</td>
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<tr>
<td>µg</td>
<td>microgram</td>
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µL  microliter
°C  degree Celsius
Chapter 1

Introduction and Literature Review
Chapter 1

1.1 Introduction

Paper-based chemical and biological sensors have become a promising analytical platform for the provision of simple, low-cost and user-operated devices. The requirement for an affordable, abundant, portable, and disposable sensing substrate makes paper a research hotspot. In addition, paper that is made of cellulose fibres has many advantages for sensor-making, including a variety of surface modification strategies and power-free liquid transport via capillary action. As a result, these advantages enable paper-based chemosensors and biosensors to be of great use in developing countries and remote regions, and to be a powerful alternative to glass, silica and polymer-based sensors.

Great efforts have been devoted to the development of paper-based sensing platforms, but the performance of many paper-based analytical devices is not sufficiently reliable yet to allow commercial development. Paper sensors still have limitations, such as the lack of a simple and versatile immobilization method for the attachment of biomolecules to paper, and the instability of biomolecules on paper during shipping and storage. Moreover, paper-based analytical devices do not entirely fulfil the requirements of being user-friendly, equipment-free or with minimum equipment. The motivation of this PhD project is to fill some of the design and performance gaps between prototyping and production of paper-based chemical and biological sensors via low-cost modification chemistries and novel sensor design, which will enhance the practical capabilities of paper-based sensors.

Firstly, a simple and versatile covalent binding approach capable of immobilizing a broad range of biomolecules may be desirable for paper-based bioassays, especially for immunoassays. Direct physical adsorption of biomolecules does not always provide reproducible results since biomolecules are weakly bound to paper fibres and can be easily washed off. Some studies have revealed the potential of polysaccharide coatings for the simple functionalization of cellulose paper. Considering that amino and carboxyl groups are two chemical groups commonly used for bioconjugation, it is of great value to offer a detailed analysis of polysaccharide-modified cellulose paper for bioassays. Based on this idea, the first part of this project focuses on the polysaccharide modification strategies for a cellulose paper-based biosensing platform.
After immobilizing biomolecules on paper, the storage and stabilization of paper-immobilized biomolecules is also a significant factor that determines the performance of these bioassays. In other words, a limitation of paper sensors for real-life applications is the short shelf-life of many biorecognition reagents once deposited on devices and stored at room temperature. In particular, proteins are very sensitive, losing function with heating, dehydration, and shearing. There have been a few reports on the stability of paper-supported enzymes and Immunoglobulin G (IgG). However, there are few research reports available addressing the most desirable conditions for storage of Immunoglobulin M-immobilized (IgM-immobilized) paper, which is used for paper-based blood typing devices. This thesis provides evidence that the activity of IgM on cellulose paper can be protected by lyophilization; we also further explored the low-cost polymer modification strategies for the long-term storage of paper-based blood typing devices, and these comprise the second and third part of this project, respectively.

It is also of great importance to design paper-based analytical devices in a user-friendly and cost-effective way, which can fulfil the requirements that paper sensors should be user-friendly, equipment-free or with minimum equipment. The last part of this thesis explores the novel designs to ensure paper sensors with good usability and low cost. Specifically, the feasibility of “a pen/stamp on paper with controllable wettability” has been investigated to reduce the fabrication cost of paper-based analytical devices. Besides, the advantage of the new concept (i.e., text reporting) is demonstrated via typical colorimetric assays. With the help of text-based information, even untrained users can quickly and simply obtain test results at home and in the field.

In summary, the broad aim of the research reported in this thesis is to explore low-cost modification strategies, storage approaches and novel concept designs to enhance the power of paper-based analytical devices as a platform technology for practical applications. The inexpensive modification approaches used here can save time and cost in the step of paper functionalization; the stability of protein-based paper sensors is also improved via a simple modification method. In addition, the novel designs in sensor fabrication and in practical applications are discussed. Details of progress in the exploration of fabricating paper-based sensors for practical applications are provided in
Chapters 2–5. Throughout this thesis, the on-going effort to make paper sensors into real use is emphasized.

This chapter focuses on a comprehensive review of recent developments in paper-based chemical and biological sensors. Based on this review, the strengths, limitations and knowledge gaps in current paper-based analytical devices are identified. The first section (1.2) describes the basic concepts of paper-based bio/chemical sensors and highlights the developed approaches to sensor fabrication, sensing element immobilization and result reporting. Next, two promising applications (i.e., paper-based blood typing and environmental monitoring) are discussed. The following section (1.3) provides a literature review summary and outlines the current research limitations and knowledge gaps of paper-based sensing platforms. Section 1.4 lists the specific research aims of this research project on the fabrication of paper sensors for practical applications, while the last section (1.5) presents the thesis structure including brief information on each chapter.
1.2 Literature review

1.2.1 Introduction of paper-based sensing platforms

The utilization of paper as a substrate material in analytical testing has a long history dating back to the 17th century with the use of litmus paper. Early scientific reports include uric acid (UA) detection, cadmium testing, and sugar analysis [1-3]. Following the development of paper chromatography in the early 20th century, paper-based test strips began to appear [4-6]. Many paper test strips, such as pregnancy test strips, are still commercially available and are extensively used. These paper-based tests are simple and affordable, but they are not sufficient for the operation of multiplex, quantitative analyses. In 2007, Whitesides and co-workers described paper-based microfluidic devices that could be used to simultaneously detect multiple analytes in liquid samples [7]. Their work enabled paper sensors to perform multiplex and semi-quantitative analyses based on advanced fabrication and patterning techniques. This novel concept has led to the rise of the new research area of patterned paper-based analytical devices for chemical/biochemical applications. Due to its affordability, disposability and portability, the paper-based sensing platform is the subject of growing interest for the development of low-cost point-of-care diagnostics and environmental monitoring technologies for people living in limited-resource settings [8-10].

1.2.2 Paper-based sensors

1.2.2.1 Paper properties and choices

Paper is a thin material produced by filtering a dilute aqueous suspension (~1 wt%) of fibres, colloidal filler particles and soluble polymers. As the major component of many paper types, cellulose fibres are derived from wood, rags or grasses. A paper sheet is a versatile material with many applications such as writing, printing and packaging. In order to realize these functions, the paper usually needs to be treated with sizing to alter its physical properties during the paper-making process. Sizing deposits functionally hydrophobic materials on the surface of cellulose fibre to reduce its surface free energy, which increases the water-paper contact angle and lowers the rate of water penetration [11]. Although many types of hydrophobization agents can be used in paper sizing,
most modern papers are sized with alkyl succinic anhydride (ASA), alkyl ketene dimer (AKD) and rosin. To take AKD as an example, AKD sizing can provide paper with a high level of water resistance [12-14]. Figure 1 demonstrates the cellulose-coupling chemistry of AKD with the formation of β-keto ester linkage under heating; the hydrophobic tail of the molecule turns out from the surface, providing paper with hydrophobicity [15].

![Chemical structure](image)

**Figure 1.** The proposed reaction mechanisms of AKD with cellulose fibres (R₁ and R₂ = C₁₄H₂₉ to C₁₈H₃₇). AKD reacts with fibres to lower surface energy and water-penetration rates.

Different additives can be utilized to obtain the desirable final paper properties. However, many papers with additive modification are not suitable to be the substrate for analytical assays, because the critical properties including porosity, surface chemistry, and optical properties have been changed [15, 16]. From the perspective of paper-based analytical devices, filter paper and chromatography paper composed of almost pure cellulose are the most widely used substrates. For instance, the excellent liquid wicking ability of filter paper can facilitate the fabrication of paper-based sensors by printing or coating [17]. Compared with other filter paper types, the Whatman® cellulose filter paper range is particularly popular, due to its well-controlled wettability, porosity, particle retention and liquid flow rate. The following table (Table 1) shows the basic parameters, including air flow and typical thickness, of several standard Whatman® qualitative filter papers.
Table 1. The basic parameters of Whatman® Standard Filter Paper Grade 1 to Grade 6.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
<th>Basis weight (g/m²)</th>
<th>Nominal thickness (µm)</th>
<th>Particle retention in liquid (µm)</th>
<th>Filtration speed Herzberg (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Medium flow</td>
<td>87</td>
<td>180</td>
<td>11</td>
<td>150</td>
</tr>
<tr>
<td>2</td>
<td>Medium flow</td>
<td>97</td>
<td>190</td>
<td>8</td>
<td>240</td>
</tr>
<tr>
<td>3</td>
<td>Medium flow, thick</td>
<td>185</td>
<td>390</td>
<td>6</td>
<td>325</td>
</tr>
<tr>
<td>4</td>
<td>Very fast</td>
<td>92</td>
<td>210</td>
<td>25</td>
<td>37</td>
</tr>
<tr>
<td>5</td>
<td>Slow</td>
<td>100</td>
<td>200</td>
<td>2.5</td>
<td>1420</td>
</tr>
<tr>
<td>6</td>
<td>Medium to slow</td>
<td>100</td>
<td>180</td>
<td>3</td>
<td>715</td>
</tr>
</tbody>
</table>

Of these filter papers, Whatman® filter papers Grade 1 and Grade 4 have been widely used in recent years. However, filter papers do not always have the desired characteristics for paper-based sensors. Therefore, other types of paper or paper-like substrates have been explored in the design of paper-based sensing platforms. For example, hydrophobic nitrocellulose membranes with a high degree of non-specific binding to biomolecules have been used as the substrate in constructing bioactive paper [18-20]. Even Kleenex towel paper has been reported as a suitable substrate for paper-based blood-typing assays [21].

1.2.2.2 Sensing element immobilization

As the main part of a sensor, the recognition component is responsible for the identification of the presence and quantification of analytes via specific interactions with analytes by using specific receptor elements, such as small molecules, proteins, nucleic acids and tissues. Hence, the addition of these receptor elements (especially biomolecules) to paper becomes a key process after choosing the suitable paper substrate. Many studies have reported immobilization methods for the attachment of sensing elements onto paper substrates. To briefly cover these innovations, the immobilization strategies are divided into three categories: physical immobilization, chemical immobilization and other novel strategies.

1.2.2.2.1 Physical immobilization

Physical immobilization refers to the adherence of molecule probes to the paper substrate by electrostatic and van der Waals forces. Paper is typically composed of
cellulose fibres, and has abundant hydroxyl groups and low concentrations of carboxyl groups present on the fibre surface, which makes the paper hydrophilic with a negative charge [22]. Since capillary forces and the hydrophilic nature of cellulose promote rapid sorption, aqueous solutions containing small receptor molecules are particularly easy to introduce onto dry paper via dip coating, pipetting, and inkjet printing. Under certain circumstances, biomolecules (e.g., antibodies, enzymes, and nucleic acids) can be spotted or printed onto dry filter paper without denaturation. Typically, Whitesides et al. [23] reported enzyme-linked immunosorbent assays (ELISA) performed in a 96-microzone plate on paper. A 96-microzone paper plate with an array (12 × 8) of circular test zones was used for running multiple paper-based ELISA in parallel (Figure 2a). Figure 2b demonstrates that indirect paper-based ELISA in its simplest form consists of five steps: 1) antigen immobilization on paper, 2) blocking the paper to prevent nonspecific protein adsorption, 3) detecting the immobilized antigen with an enzyme-conjugated detection antibody, 4) washing away unbound antibody, and 5) adding a solution of a substrate for the enzyme. In their experiments, the antibodies were physically adsorbed to the paper and their results show no loss of biomolecules by elution with the washing buffer.

Figure 2. Prototype demonstration of indirect paper-based ELISA. (a) A 96-microzone paper plate for ELISA with the equivalent dimensions of a 96-well microtiter plate. Each circular test zone of hydrophilic paper is bounded by hydrophobic photoresist. (b) Schematic diagram of a typical paper-based indirect ELISA utilizing antigen/antibody recognition (AP: alkaline phosphatase; BCIP/NBT: 5-bromo-4-chloro-3-indolyl...
phosphate/nitro blue tetrazolium). (Adapted with permission from Ref. [23] Copyright 2010 John Wiley & Sons)

However, in most cases, biomolecules are not firmly immobilized on cellulose paper via only physical forces. As mentioned above, paper has a slightly anionic surface that can only absorb positively charged compounds. Halder et al. [24] found out that high-molecular weight DNA did not adsorb onto cellulose at pH 6 and 8, whereas adsorption was observed at pH 4. In respect of proteins, electrostatic interactions between cationic regions on proteins and anionic cellulose are important attractive forces for proteins to adsorb onto paper [25]. However, physical adsorption alone is not sufficiently effective for biomolecules with an overall negative charge, because they are less firmly bound and easily washed off. According to Jarujamrus' findings, direct adsorption of antibodies onto paper might not give reproducible results since about 40% of antibody molecules adsorbed onto cellulose paper could actually desorb from the fibres [26]. In addition, paper-like substrates such as nitrocellulose membranes are limited in their ability to bind to certain classes of biomolecules and are generally inefficient in the capture of nucleic acids and some other molecules [27]. In brief, the physical immobilization strategy often leads to ligand drift, signal distortion and loss of ligand bioactivity in multi-step bioassays [28]. Recent studies show, however, most disadvantages of physical immobilization can be significantly improved in terms of biomolecule adsorption stability and limit of detection by adopting covalent immobilization of biomolecules to paper [29].

**1.2.2.2 Chemical immobilization**

Since permanent immobilization of biomolecules on paper surfaces is crucial to the development of effective paper-based multi-step bioassays, a more versatile paper substrate capable of immobilizing a broader range of biomolecules is desirable. For chemical immobilization, the biomolecule is strongly fixed on the surface of the paper through covalent bonding. Pure cellulose offers few functional groups for direct conjugation. The hydroxyl groups in the cellulose backbone are not sufficiently reactive for specific reactions under mild conditions. The low surface concentrations of carboxyl groups from inadvertent oxidation of the C6 hydroxyls and the oxidizing end of cellulose chains are the only functional groups available on pure cellulose [30]. Many studies have reported the chemical modification of cellulosic materials for
further functionalization. For example, cellulose membrane discs modified with carbonyldiimidazole and 1-cyano-4-dimethylaminopyridinium tetrafluoroborate [31], cellulose beads activated by epichlorohydrin [32], photoreactive cellulose membrane prepared by the use of 1-fluoro-2-nitro-4-azidobenzene [33], and cellulose-based carriers with DEAE-, CM- or NH₂-ligands [34], have been demonstrated to covalently immobilize various molecules. More recently, significant attention has been directed to the chemical modification of cellulose paper in order to offer more surface functional groups. Isaad et al. [35] immobilized different azo dyes on paper by esterification of the -OH groups of cellulose with -COOH groups; the resultant paper-based chemosensor can perform the colorimetric sensing of cyanide anions in aqueous media (Figure 3a). Tyagi et al. [36] developed a method of graft copolymerization of glycidyl methacrylate (GMA) onto cellulose filter paper (CFP) via a free-radical initiating process based on ceric ammonium nitrate. The epoxy groups formed can directly react with amino, carboxyl and sulfhydryl groups to form stable covalent bonds with biomolecules (Figure 3b). Wang et al. [37] used periodate oxidation to produce aldehyde groups on paper for the covalent attachment of antibodies, which is a stable immobilization strategy for paper-based immunodevices (Figure 3c). Araújo et al. [38] used linking agent 1, 4-phenylenediisothiocyanate to immobilize single-stranded probe DNA onto filter paper for target hybridization via capillary action (Figure 3d). Yu et al. [27] employed divinyl sulfone (DVS) to activate cellulose paper and then covalently immobilize small molecules, proteins, and DNA onto the hydroxyl groups through nucleophilic addition (Figure 3e). Although these covalent bimolecular immobilization strategies have successfully demonstrated the potential for the chemical modification of cellulose fibres, they are not without their limitations, including the multiple steps, hazardous conditions, and compromised paper properties. Therefore, a simple and versatile strategy to immobilize a variety of biomolecules onto cellulose paper is worth exploring.
Other processes use a polymer, physically adsorbed onto paper, to covalently bind with biomolecules. The functionalization of paper using polymer adsorption is a non-destructive (soft) method for introducing new conjugation sites on cellulosic materials, which has drawn much interest from researchers. Filipponen et al. [39] coated paper with carboxymethyl cellulose (CMC) modified with azide or alkyne functionality. Paper with modified CMC coating can provide reactive sites for azide–alkyne cycloaddition click reactions, and is suitable for the immobilization of functional units with azide or alkyne groups. Xu et al. [40] detailed the fabrication of functional paper with coated heteropolysaccharide oxidated with galactose 6-oxidase, which can yield many aldehyde groups for further functionalization. Wang et al. [41] reported the application of chitosan as the surface modifier to fabricate paper plates for ELISA; their
work has demonstrated in part, the potential of natural polysaccharide coating on paper. Some studies of cellulose model surfaces (i.e., Langmuir–Schaeffer cellulose films) have reported that polysaccharides’ structural similarity to cellulose makes these macromolecules able to engage in hydrogen bonding interactions with cellulose, which may lead to irreversible polymer adsorption [42, 43]. These long-chain and flexible polysaccharides with multiple functional groups have exhibited the potential to act as surface modifiers for paper sensor fabrication.

1.2.2.3 Immobilization via novel strategies

For paper-based biosensors, one limitation of their implementation in the real world is the short shelf-life of many biorecognition elements such as enzymes, antibodies, DNA aptamers, and bacteriophages. Therefore, some novel immobilization strategies including bioink printing and entrapment have been developed for paper-based biosensing platforms. Immobilization using bioinks is the covalent attachment of biomolecules to carrier particles that can then be printed on paper. Namely, biocompatible nanoparticles with immobilized detection reagents are deposited on and incorporated within the paper substrate. Typically, Mei et al. [44] reported photoluminescent graphene oxide (GO) paper-like sensors for the ultra-sensitive visual bioassays of peptide, protein and DNA. GO bears numerous phenol hydroxyl and epoxy groups at the basal plane and carboxylic groups at the lateral edge, thus providing direct interaction with biological species and the flexibility of chemical modification, and can be used as a bioink to be printed onto the paper-like substrate (i.e., polyvinylidene fluoride microporous membrane) for bioassays (Figure 4a). Savolainen et al. [45] printed polymer microcapsules with enzymes on paper substrate, to produce a bioactive paper with high water-resistance and long enzyme lifetime. The use of bioinks is expected to produce a biosensor that retains biomolecule activity and gives reproducible results for a prolonged period of time. In addition, paper-based biosensors can be produced by entrapping biomolecules on paper. Brennan’s group reported solid-phase paper biosensors produced by inkjet printing with sol-gel entrapped acetylcholinesterase or β-galactosidase [46, 47] (Figure 4b). The paper biosensor with immobilized acetylcholinesterase was used for the detection of neurotoxins while the paper biosensor with β-galactosidase was for heavy metal detection. Both can be stored at 4 °C for at least 60 days. Alkasir et al. [48] demonstrated a new type of paper-based bioassay for the colorimetric detection of
phenolic compounds including phenol, bisphenol A, catechol and cresols. The sensor is based on a layer-by-layer (LbL) assembly approach formed by alternatively depositing layers of chitosan and alginate polyelectrolytes onto filter paper and physically entrapping the tyrosinase enzyme in between these layers (Figure 4c). Gu et al. [49] exploited a protein-agarose hydrogel-based paper assay to immobilize infrared fluorescent protein for the detection of Hg (II) in a portable and robust fashion. These examples demonstrate that the immobilization of biomolecules with the help of polysaccharides can produce paper-based biosensors with a prolonged shelf-life.

Figure 4. Novel immobilization strategies for paper-based assays. (a) Fluorescence “off-to-on” mechanism of GO paper-like sensors for biological assays. (Adapted with permission from Ref. [44] Copyright 2012 John Wiley & Sons) (b) Schematic illustration of solid-phase paper biosensors with sol-gel entrapped biomaterials. (Adapted with permission from Ref. [46] Copyright 2009 American Chemical Society) (c) Schematic representation of the colorimetric tyrosinase paper-based sensor with sequential LbL deposition of alternate layers of chitosan, tyrosinase, and alginate onto the filter paper. (Adapted with permission from Ref. [48] Copyright 2012 American Chemical Society)

1.2.2.3 Read-out systems for paper-based sensors

Like the recognition step, the transduction step is also important for paper-based sensors. Detection is also a key aspect of paper-based analytical device development. The challenge is to make the signal reporting simple and easy. In order to maintain simplicity, affordability and portability, techniques like optical methods are well suited for the read-out system. Commonly-reported techniques for paper-based assays include
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colorimetric, fluorescence, electrochemical analysis and so on. Of these techniques, colorimetric detection has been widely used in paper-based analytical devices, and can offer a “yes/no” answer or semi-quantitative result. The change in color of the assay can be observed and interpreted by the naked eye, which enables the colorimetric transduction suitable for rapid and practical applications. The semi-quantitative results are usually obtained with the help of an imaging tool combined with image processing software.

Traditionally, colorimetric analytical devices on paper are designed based on spot assays. The color change can be produced by many different phenomena; many chemical and enzymatic reactions can be employed for the development of colorimetric paper sensors. A number of research studies are available on colorimetric detection via simple chemical reactions, such as ketone detection [50, 51], metal ion measurement [52-56], nitrite analysis [51, 57], protein assays [58, 59], and trinitro aromatic explosive identification [60]. Furthermore, various analytes have been identified through enzymatic reactions including alanine aminotransferase [61, 62], AP [62, 63], aspartate aminotransferase [61], bacteria [64], glucose [7, 17, 51, 65-70], heavy metal ions [47], neurotoxins [46], pesticides [71], phenolic compounds [48], and proteins [23, 72, 73]. In addition, nanomaterial-based colorimetric analyses have been used for paper sensors. A typical example is the immunoassay with gold nanoparticle labels, because gold nanoparticles have long-term stability, easily controllable size distribution, and good compatibility with biological molecules [74, 75]. To date, modified nanoparticles have been utilised to colorimetrically detect DNA [76], heavy metal ions [77, 78], infectious diseases [79], proteins and small molecules [80-82] on paper. More recently, Duan et al. [83] have developed a nanozyme-based paper strip for rapid local diagnosis of Ebola. In ELISA analysis for the Ebola virus detection, Fe₃O₄ magnetic nanoparticles were used to replace enzymes. They work like enzymes but make the signal stronger, giving a clearer visual result. The nanozyme test can detect much smaller amounts of the virus, and is 100 times more sensitive than the colloidal gold-based immunochromatographic strip test.

Although the above-mentioned colorimetric assays are simple, they still require some form of instrumentation such as a camera or scanner for semi-quantitative detection, and this increases operating costs and limits field use. Recently, some groups have
demonstrated novel measurement techniques such as count-, distance-, and time-based detection to replace intensity-based colorimetry, which can eliminate the need for an external reader to obtain a semi-quantitative result. For instance, Cate et al. [84] reported a simple, distance-based measurement of glucose, nickel, and glutathione on paper. Colorimetric detection reagent is deposited along the flow channel and can precipitate or aggregate upon reaction with the analyte. When the analyte flows out of the circular reservoir, it reacts with the colorimetric reagent and then color develops along the channel until no analyte remains (Figure 5a). Quantification of the analyte is achieved by measuring color length. Lewis et al. [85] developed a time-based approach on paper for quantifying the level of Hg$^{2+}$ and Pb$^{2+}$ ions in water. Wax printing technology is used to define hydrophilic regions for the sample flowing. Time between reactions in the regions of “START” and “STOP” is measured, which is proportional to analyte concentration (Figure 5b). Jeong et al. [86] designed a paper-based analytical device that can provide the assay result simply by counting the number of colored bars at a fixed time. A three-dimensional (3D) microfluidic network is fabricated by double-sided wax printing and lamination, which allows the fluid to move laterally and vertically. After a sample solution flows through dot-shaped detection zones on both upper and lower layers, the concentration of the target analyte can be determined by counting the number of bars developing color (Figure 5c). These techniques are not only independent of lighting condition, but also obviate the need for an external reader to obtain a semi-quantitative result. These novel semi-quantitative approaches minimize the requirements for the readout of results on paper-based analytical devices, and render measurements on paper more portable and straightforward. They indicate future directions for the development of paper-based assays.
Figure 5. Schematic illustrations of novel techniques used for result read-out on paper-based sensors. (a) Distance-based measurement for paper-based analytical devices. (Adapted with permission from Ref. [84] Copyright 2013 Royal Society of Chemistry) (b) Time-based detection for Hg$^{2+}$ and Pb$^{2+}$ on paper. (Adapted with permission from Ref. [85] Copyright 2014 Royal Society of Chemistry) (c) Snapshot images of the digital assay after adding a sample solution with different concentrations of bovine serum albumin (BSA) or glucose. (Adapted with permission from Ref. [86] Copyright 2015 Royal Society of Chemistry)

1.2.2.4 Fabrication of paper-based sensors

In order to allow the commercial development of paper-based assays, it is of great importance to fabricate paper-based analytical devices integrating recognition and transduction. Many studies have presented new concepts and possibilities of paper-based analytical devices by using different channel patterning strategies. Figure 6 gives an overview of various channel fabrication techniques such as cutting, dipping, etching, photolithography, printing, spraying and stamping [87]. Generally speaking, two-dimensional (2D) paper-based analytical devices have been fabricated primarily based on two principles: the selective cutting and/or removal of paper to form physical boundaries, and defining hydrophobic zones within the paper itself. At present, the most recent and popular approach used in the fabrication of paper-based analytical devices is printing including wax printing [66, 88, 89] and inkjet printing [17, 90-93].
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For instance, Lu et al. [66] presented a simple and low-cost method to generate paper-based analytical devices using a commercially available wax printer and an oven. The printer prints patterns of solid wax on the surface of the paper directly, and the wax pattern is melted to fully penetrate the paper. The method generates stable hydrophilic areas that consist of wicking channels, fluid reservoirs and reaction zones via wax-based hydrophobic barriers. Horseradish peroxidase (HRP), glucose and BSA assays were chosen to demonstrate colorimetric detection of analytes to verify the performance of the wax-printed paper-based analytical device. Li et al. [92] introduced digital inkjet printing techniques for paper-based microfluidic devices. A heptane solution of a paper sizing agent, AKD, was used to replace the original ink in the cartridge and printed on the filter paper with digital patterns designed using a commercial inkjet printer. The hydrophilic channel resolution, cross-section and liquid penetration behaviour were studied. Based on sizing techniques on paper, multiple low-cost paper sensors for chemical and biochemical detection have been developed. For instance, Li et al. [57, 94] used the paper microfluidic sensor to perform quantitative assays of two important biological markers, NO$^2-$ and UA, by depositing the samples in the hydrophilic detection zones on the paper. The colorimetric reactions were recorded using a scanner and the color intensities were measured for the quantification of the biological markers in the samples.

Figure 6. Various approaches for creating 2D paper-based sensors. (a) Printing methods. (b) Mask-guided patterning methods. (c) Physical boundaries-based methods. Abbreviations: LPAD, laminated paper-based analytical device; UVO, ultra-violet
ozone treatment. (Adapted with permission from Ref. [87] Copyright 2016 Annual Reviews)

However, the fabrication and patterning techniques discussed above are not confined to 2D paper-based analytical devices, and they have been extended to 3D paper-based sensing systems. As a potential platform, they have received considerable attention in recent years due to faster fluid distribution ability, increased device functionality and enhanced multiple parallel assay operations. Adhesive- [68, 95-97], origami- [58, 98, 99], and wax printing-based techniques [86, 88, 100] for 3D paper-based analytical device fabrication have been reported. Martinez et al. [68] described a method for fabricating 3D microfluidic devices by stacking layers of 2D patterned paper using a double-sided adhesive tape. The holes in the tape were cut and filled with a paste made from cellulose powder and water, which enables fluids to wick from layer to layer. The resulting 3D device allows the running of parallel assays and the results of the assays are displayed on the back of the device. Instead of using a double-sided tape, Lewis et al. [96] improved the fabrication procedures for preparing 3D devices using adhesive spraying. This method requires no tape layers and less alignment and therefore dramatically simplifies the fabrication process (Figure 7a). Liu et al. [99] fabricated simple and functional microfluidic devices on paper using origami, the traditional Japanese art of paper folding. Instead of layer stacking, the entire 3D device can be produced on a single sheet of flat paper using SU-8 photolithography. Channels, reservoirs, and a frame were first patterned on a piece of paper, and then the paper was folded to allow contact of specific zones and channels. A two-analyte colorimetric assay of glucose and protein was carried out to demonstrate the applicability of the device to chemical analysis (Figure 7b). Li et al. [100] created 3D paper-based analytical devices using a wax-printing technique capable of controlling the penetration depth of melted wax in a single layer of cellulose paper (Figure 7c). As a proof-of-concept demonstration, the multiplexed enzymatic detection of three biomarkers (glucose, lactate, and UA) was demonstrated by an analytical device with three layers of channels from a paper substrate.
**Figure 7.** The fabrication of 3D devices on paper. (a) Schematic of the adhesive-spraying method for fabricating 3D paper-based analytical devices. (Adapted with permission from Ref. [96] Copyright 2012 Royal Society of Chemistry) (b) Schematic of the network used for detecting glucose and BSA on paper (left) and photograph of an
However, microchannel patterning to direct liquid flow on paper devices is not all for the fabrication of paper-based chemical and biological sensors. Flow control, multistep processing, separation and preconcentration of complex samples are also important features for paper-based analytical devices. Various methods have been developed to offer efficient control over fluid flow, especially regarding flow speed and direction. One of the first demonstrations of the control of complex fluid flow was reported by Martinez et al. in 2010 [101]. They fabricated 3D microfluidic paper-based analytical devices with an “on” button designed to direct the flow path. Pressing the button with a stylus or a ballpoint pen closes the gap between two vertically-aligned microfluidic channels, and allows fluids to flow through (Figure 8a). The technique of controlling the movement of fluid in paper-based channels endows the paper-based analytical device with programming ability. Several other groups have reported other simple ways to adjust fluidic transport, primarily by altering channel geometry. For example, Fu et al. [102, 103] have demonstrated that the flow rate can be controlled by varying the length and width of a channel, which is useful for the design and implementation of multistep sequences for sample pretreatment and analysis on paper. In addition, dissolvable sugar barriers have been explored for flow delays. A typical example is a dissolvable sugar bridge reported by Houghtaling et al [104]. Dissolvable bridges composed of mannose or trehalose can serve as “on/off” switches with a tunable range of total fluid volumes delivered. The fluid keeps flowing through the bridge until the bridge is eventually dissolved. By tuning several experimental parameters (e.g., cross-sectional area of the bridge, composition of the bridge, and choice of feeder material), the total volume of fluid passing by can be varied. Other methods to control fluid flow include razor cutting on paper [105], hollow channel for fast liquid transport [106], and depolymerization of hydrophobic materials [85, 107, 108].

Secondly, functionality such as multistep processing must be incorporated for the further exploration of the potential of paper-based analytical devices. Lutz et al. [109] designed 2D paper networks that use multiple converging fluid inlets to control the
arrival time of each fluid to a detection region. Apilux et al. [110] reported an automated sandwich-type ELISA with single-step sample application. The flow channel and device barrier patterns were created by the inkjet printing, which enabled direct control of the reagent flow time. Li et al. [111] designed novel paper-based magnetic valves that can count the time and turn a fluidic flow on or off. Time counting can be realised by a paper timing channel with an ionic resistor that can trigger a circuit to activate the electromagnet; the “on/off” switch for flow control is based on a magnetized paper cantilever driven by an electromagnet to connect or disconnect a paper channel (Figure 8b). Fridley et al. [112] demonstrated a multistep paper-based biosensor based on controlled rehydration of patterned reagent storage depots directly within the paper membrane. The controlled release of prestored reagents holds potential for sequential delivery for paper-based analytical devices. Based on these representative instances, it can be seen that multistep processing can be achieved primarily by adjusting the flow speed, using switches, or reagent prestorage.
Figure 8. Paper-based analytical devices with various functionalities. (a) Use of the fluidic de-multiplexer on paper. The “on” buttons can be compressed to direct fluid from a single inlet into any combination of eight outlets, before or after the fluid is added to the fluid inlet via a ballpoint pen. (Adapted with permission from Ref. [101] Copyright 2010 Royal Society of Chemistry) (b) A schematic diagram of a magnetic timing valve for fluid control in a paper-based analytical device. (Adapted with permission from Ref. [111] Copyright 2013 Royal Society of Chemistry) (c) Separation on paper using different concentrations of polyelectrolytes. Paper-based analytical device modified with different concentrations of poly(allylamine hydrochloride) (PAH) or poly(sodium 4-styrenesulfonate) (PSS) on each finger (top image), and fluorescein either forms a band or concentrates at the tip (bottom image). (Adapted with permission from Ref. [114] Copyright 2013 American Chemical Society) (d) Schematic illustration and operational principle of a paper-based device to separate single cell. (Adapted with permission from Ref. [115] Copyright 2015 Elsevier)

In addition, separation and preconcentration of complex samples are important features for paper-based analytical devices, since real-world samples are usually complex. The structure and surface chemistry of paper are usually used to enable sample separation and preconcentration. Osborn et al. [113] designed simple and low-cost paper networks with different geometries that can be used for dilution, hydrodynamic focusing, mixing and separation of samples. They further extended the potential of paper-analytical devices. Abbas et al. [114] reported that filter paper can be effectively used for separating and preconcentrating analytes by a simple cut and drop method. More specifically, complex samples are separated by a gradient of surface charge created by differential polyelectrolyte coating of the paper. The analytical device with a starlike shape enables preconcentration of the separated analytes at the tip of the paper star arms, because of the fast evaporation in a relatively small area (Figure 8c). Chen et al. [115] developed a paper-based device for the separation and cultivation of single
microalgae. Carbon-powder patterns were created on the filter paper by laser printing, and the resulting microwell pattern areas can retain only a single cell via varying diameter and depth. The paper device can also function as an incubator for microalgae cultivation (Figure 8d). Walsh et al. [116] designed a simple and cheap analytical device on paper, which can rapidly generate a gradient of chemoattractant on paper to examine cell migration response. Other methods for the separation and preconcentration of complex samples include isotachophoresis [117], separation of plasma from whole blood via red blood cell (RBC) agglutination [118] or by a blood separation membrane [119], and varying sample properties such as solubility or polarity in a given solvent [120].

1.2.3 Paper-based sensors for blood typing

There is strong interest in the development of point-of-care diagnostics in developing countries and resource-limited regions. Due to its affordability, simplicity, and portability, the paper-based sensing platform has been extensively explored for biomedical applications. As an important part of the effort, determining blood type on paper has been proposed and developed for places where easy, fast and low-cost tools are critically important.

1.2.3.1 Blood typing

Blood typing is the classification of blood based on the presence or absence of certain antigens on the surface of red blood cells (RBCs). When there are foreign antigens, the antibody present in the blood plasma protects the body from any perceived threat. A total of 35 human blood type systems are recognized, according to the International Society of Blood Transfusion (ISBT). Of these, the ABO and Rh blood group systems are of great importance in human-blood transfusion [121].

Landsteiner found that an antibody will not develop in the plasma unless the corresponding antigen is absent in the red cells of blood (Figure 9). In the ABO blood type system comprising A, B, AB, and O blood types, anti-A and anti-B antibodies are naturally present in the plasma if the corresponding antigens are absent [122]. For instance, people with type A have antibodies in the blood against type B. As the second most important blood type system after ABO, the Rh blood group system has 50 defined blood-group antigens. Of these, the five antigens D, C, c, E, and e are the most
important [123]. The Rh system classifies blood as Rh-positive or Rh-negative, usually based on the presence or absence of the D antigen. Transfusion reactions caused by mismatched ABO and Rh blood types can be serious and even fatal [124, 125]. Therefore, correct blood typing is extremely important for blood donations and transfusion, transplantation, and pregnancy health.

<table>
<thead>
<tr>
<th>Blood groups</th>
<th>Group A</th>
<th>Group B</th>
<th>Group AB</th>
<th>Group O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antigens on the RBCs</td>
<td>A</td>
<td>B</td>
<td>AB</td>
<td>O</td>
</tr>
<tr>
<td>Antigen A</td>
<td>Antigen B</td>
<td>Antigen A and B</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Antibodies in the plasma</td>
<td>Anti-B</td>
<td>Anti-A</td>
<td>None</td>
<td>Anti-A and Anti-B</td>
</tr>
</tbody>
</table>

**Figure 9.** The determination of ABO blood groups according to Landsteiner’s law.

Normally, there are two steps to typing blood groups in a clinical laboratory: forward grouping (detecting antigens on RBCs) and reverse typing (detecting antibodies in serum) [126]. A person’s blood type is confirmed by the agreement of these two tests. Forward and reverse typing are based on haemagglutination or agglutination. Haemagglutination is a specific form of agglutination of RBCs resulting from the formation of antigen-antibody complexes and the blood typing results are macroscopically visible. The traditional approaches to the identification of blood types in the laboratory are the slide test, tube technique and gel-based assay [126]. Of these, the tube technique and gel-based assay can be used for forward and reverse typing. These two techniques are sensitive and specific, but they are expensive, time consuming and require complicated steps, and are therefore not suitable for use in rural areas. The need to develop simple, low-cost and portable blood typing devices has led many to consider paper as a substrate material for blood typing.
1.2.3.2 Design and fabrication of paper-based sensors for blood typing

Khan et al. [127] found paper to be an alternative material for low-cost blood typing diagnostics. Because the transport of blood in paper substrate varies widely whether its red cells are agglutinated or not, the possibility of paper-based blood typing was investigated. The testing principle was based on the agglutination of RBCs in the presence of corresponding IgM antibodies. When droplets of blood were deposited onto paper strips pretreated with specific and nonspecific antibodies, significant differences in transport occurred. Agglutinated blood transports caused distinct RBC/blood plasma separation while no distinct separation was observed in paper channel with nonspecific antibodies. The differences in wicking and chromatography upon colloids stability/agglutination provide a novel method of blood testing (Figure 10a).

Al-Tamini et al. [128] developed a paper-based assay for rapid blood typing based on the different performances of agglutinated and non-agglutinated RBCs via elution. Blood samples were applied to paper treated with Anti-A, Anti-B, and Anti-D antibodies, respectively. After 30 s incubation, the reaction spots were eluted with 0.9% NaCl buffer. Agglutinated RBCs were fixed on the paper substrate, resulting in a high optical density of the spot while non-agglutinated RBCs could easily be eluted by the buffer and had faint or invisible blood spot. RBCs fixation on paper accurately detected blood groups (ABO and RhD) via elution in 100/100 blood samples including 4 weak AB and 4 weak RhD samples.

Li et al. [21] designed a text-reporting blood typing paper-based device based on three main steps: firstly, hydrophilic patterns ‘A’, ‘B’ and ‘|’ were created by AKD printing; secondly, three blood group IgM antibodies were introduced into the patterns by printing with an ink jet printer; lastly, blood samples were spotted into the pattern to react with antibodies and then washed. The antibody molecules were physically attached to the structure of the paper; they were therefore released from the structure after the introduction of blood samples. Specifically, with the addition of RBCs to the patterns with antibodies, if there is a haemagglutination reaction through the antibody-antigen interaction, agglutinated RBC lumps form inside the fibre matrix of the paper and cannot be easily washed out by the saline solution, while the non-agglutinated RBCs can be easily washed out of the text pattern with the saline solution (Figure 10b).
The bioactive paper text reporting blood typing device meets the basic requirements of sensitivity, specificity, and text legibility.

**Figure 10.** The determination of ABO blood groups followed by Landsteiner’s law. (a) Wicking of blood previously agglutinated from antigen-antibody interaction on paper: B+ blood with specific Anti-B (left image) and nonspecific Anti-A (right image). (Adapted with permission from Ref. [127] Copyright 2010 American Chemical Society) (b) Fabrication and testing procedures of the text-reporting blood-typing devices (top
image), and the actual tests of all eight ABO and RhD blood types (below image).
(Adapted with permission from Ref. [21] Copyright 2012 John Wiley & Sons)

It is noteworthy that all the above-mentioned studies focus on the development of paper-based forward grouping. However, reverse grouping is required to confirm the results of forward grouping before reporting a person's blood type. Therefore, more recently paper-based sensors for simultaneous forward and reverse blood grouping have been designed and fabricated. Noiphung et al. [129] proposed a new, paper-based analytical device that can perform three functions, including Rh and forward and reverse ABO blood typing within 10 min. Using wax printing and dipping techniques, a paper sensor with a forward (F) side and a reverse (R) side was obtained. On the F side, a diluted blood sample was used to flow through the antibody-immobilized channel. On the R side, an MF-1 membrane was used to trap RBCs and the plasma was separated by hydrophilic channels with A-cells and B-cells, respectively. The distance ratio of RBCs movement to plasma separation was employed as an indicator of agglutination for interpreting the blood type (Figure 11a). Subsequently, Songjaroen et al. [130] designed a paper-based device for simultaneous forward and reverse ABO blood group typing via barcode-like interpretation. The device comprised six parallel channels via wax printing on paper. For forward typing, the test channels were treated with Anti-A, -B and –A,B respectively, while the control channels were treated with the corresponding inactivated IgM antibodies. For reverse typing, LF1 blood separation membrane was used for the separation of plasma from whole blood, and 30% standard A-, B- and O-cells were added to the test channel, respectively. In the control channel, standard O-cells were used. The blood group was determined based on the haemagglutination of the corresponding antigen-antibody (Figure 11b). Compared with the previous work based on interpretation of the ratio of the length of RBCs and plasma movement, here the results were a barcode-like chart that was visually detected and easily combined with smartphone analysis.
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(a) Immobilized Antibody area

<table>
<thead>
<tr>
<th></th>
<th>Distance ratio</th>
<th>Interpretation</th>
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<tbody>
<tr>
<td></td>
<td>F-side</td>
<td>R-side**</td>
</tr>
<tr>
<td>1 = Anti-A</td>
<td>0.33</td>
<td>0.63</td>
</tr>
<tr>
<td>2 = Anti-D</td>
<td>0.25</td>
<td>-</td>
</tr>
<tr>
<td>3 = Anti-B</td>
<td>0.57</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>0.62</td>
<td>0.33</td>
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<tr>
<td></td>
<td>0.50</td>
<td>-</td>
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<tr>
<td></td>
<td>0.33</td>
<td>0.67</td>
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<td></td>
<td>0.67</td>
<td>0.13</td>
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<tr>
<td></td>
<td>0.50</td>
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<td></td>
<td>0.70</td>
<td>0.24</td>
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<tr>
<td></td>
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<td>-</td>
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<tr>
<td></td>
<td>0.33</td>
<td>0.53</td>
</tr>
</tbody>
</table>

(b) Forward grouping

- Sample zone
- Blood separation zone
- Agglutination pattern was read
- Diluted blood was applied on hydrophilic area
- Blood was diluted with NSS containing 1%Tween-20
- Standard cells were eluted with NSS containing 1%Tween-20
- Blood separation membrane was removed

Reverse grouping

- Area for standard cells
- Blood sample was added on sample zone
- Plasma was separated
- Standard cells were added on hydrophilic channels
1.2.3.3 Mechanism exploration of blood typing on paper

The difference in transportation behaviours between agglutinated blood and stable blood on paper provides a novel platform for blood typing. Therefore, a better designed paper sensor can be obtained if the mechanism of blood typing on paper is explored. To date, studies of the mechanism include the relationship between adsorption and desorption of antibodies, and RBC agglutination; the analysis of RBC agglutination on paper at the microscopic level; and the effect of paper structure on the visual clarity of blood typing.

Jarujamrus et al. [26] studied the mechanisms of RBC agglutination on antibody-loaded paper by semi-quantitative evaluation of the adsorption and desorption of antibody molecules on cellulose fibres. They found that the adsorbed antibody molecules alone on the paper matrix are not sufficient to immobilize RBCs into large agglutinated lumps. However, paper with both adsorbed and desorbed antibody molecules was able to agglutinate the RBCs more efficiently and provided reproducible test results. More specifically, the released antibody molecules can facilitate the bridging of RBCs in blood sample bulk to the adsorbed RBCs immobilized on the fibre surface by the adsorbed antibody molecules. They can also cause sufficiently large agglutinated lumps of RBCs. These lumps were not able to pass through the pores of the paper, and can be detected with the naked eye.

Li et al. [131] used confocal microscopy to study the transport and immobilization mechanism of human RBCs in a paper-based blood typing device. The morphology of the free and agglutinated RBCs labelled with fluorescein isothiocyanate (FITC) was different. The non-agglutinated RBCs did not undergo morphological change and were distributed rather uniformly in the spaces of the fibre network of filter paper while the
agglutinated RBCs were deformed and had an uneven distribution. The dimension of large agglutinated RBC lumps were similar to that of the interfibre pores inside the paper, and the entrapment and adhesion of those lumps happened mostly at the gaps and pores between fibres. Therefore, the lumps were hardly removed by the chromatographic elution (Figure 12). This study shows that the confocal approach can be a powerful tool for understanding the interaction between antibody molecules and RBCs on paper.

![Confocal images of non-agglutinated and agglutinated RBCs inside antibody-treated paper with different folds magnification.](image)

**Figure 12.** Confocal images of non-agglutinated and agglutinated RBCs inside antibody-treated paper with different folds magnification. (Adapted with permission from Ref. [131] Copyright 2013 Royal Society of Chemistry)

Su et al. [132] investigated the effect of paper structure on blood typing visualization. They chose commercial and experimental papers varying in fibre composition, basis weight, density and porosity to separate agglutinated RBCs from non-agglutinated RBCs. The separation performance was evaluated by optical density measurement of the blood spot. Results indicated that thin and porous papers provided the better performance of blood typing than thick and dense papers. The latter were inappropriate for blood typing as they tended to retain indiscriminately both agglutinated and non-aggregated RBCs. Li et al. [133] further investigated the effect of fibre type (i.e., softwood and hardwood) on the performance of paper-based blood typing sensors.
Different fibres led to different fibre network structures of the paper, which can influence the transport of RBCs in paper. Compared with long softwood fibre-based paper, short hardwood fibre-based paper with a low basis weight has a higher porosity and simpler pore structures, and has a simple internal pore structure that is well suited for the easy transport of RBCs. Their study showed that hardwood paper sheets with a basis weight of 20 g/m² allow for the easiest transport of RBCs, and thus have the best visual clarity of blood typing (Figure 13).

**Figure 13.** Blood typing tests on papers of different basis weights via lateral chromatographic elution (left image) or vertical flow-through (right image). (Adapted with permission from Ref. [133] Copyright 2014 American Chemical Society)

### 1.2.4 Paper-based sensors for environmental monitoring

In addition to paper-based blood typing devices, paper-based sensors for environmental monitoring are attractive because fast, inexpensive, and point-of-need analysis for pollutant detection is of great importance in resource-limited settings. With the rapid development of industries, heavy metal pollution in air, soil, and water has become a serious global problem, threatening human reproduction and health. Heavy metal ions in water are not biodegradable, and can cause serious damage to the human central nervous systems, kidneys, livers, bones, and teeth, when they enter the human body through the food chain [134-136]. Furthermore, human exposure to polluted air has been linked to a series of diseases and disorders including cancers of the lung, nose, and sinus cavity [137, 138]. In response to growing concerns, traditional techniques such as inductively coupled plasma mass spectrometry (ICP-MS) and atomic absorption/emission spectroscopy are widely used for environmental monitoring [139, 140]. However, these instrumental detection methods are expensive and, time-
consuming, and typically require professional operation. Currently, paper-based colorimetric devices for environmental monitoring have attracted wide attention.

One of the first examples of paper-based assays for heavy metals was a sensor with a dual electrochemical/colorimetric detection for simultaneously measuring Au$^{3+}$ and Fe$^{3+}$ in waste streams [141]. A colorimetric method based on the reaction of Fe$^{2+}$ with 1,10-phenanthroline was used for Fe determination. Since the colorimetric method is the easiest and most cost-effective when a qualitative or semi-quantitative analysis is sufficient, a growing number of colorimetric paper sensors for the determination of heavy metals have been developed. As demonstrated by Hossain et al. [47], a β-galactosidase (β-gal)-based colorimetric paper sensor has been designed for the rapid and sensitive measurement of multiple heavy metals in aqueous samples. The sensor comprises of three main parts: HB barrier (a hydrophobic barrier), CR zone (the circular region containing β-gal) and CG zone (the arm region containing chlorophenol red β-galactopyranoside (CPRG)). As the chromogenic substrate, CPRG can be hydrolyzed by β-gal to form a red-magenta colored product. Loss of the red-magenta color occurs when there are heavy metals in the liquid sample (Figure 14a). Feng et al. [56] developed a paper-based sensor array capable of discriminating eight different heavy metal ions (i.e., Hg$^{2+}$, Cd$^{2+}$, Pb$^{2+}$, Ag$^+$, Ni$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, and Co$^{2+}$), as shown in Figure 14b. Eight pyridylazo metal chelating compounds were chosen as heavy-metal ion indicators, containing 1-(2-Pyridylazo)-2-naphthol (P-1), 4-(2-Pyridylazo)resorcinol (P-2), 2-(3,5-Dibromo-2-pyridylazo)-5-(diethylamino) phenol (D-1), 1,3-Diamino-4-(5-bromo-2-pyridylazo) benzene (D-2), 2-(4-Diethylamino-2-hydroxyphenyl-azo)-5-bromopyrididine (D-3), 4-(5-Chloro-2-pyridylazo)-1,3-phenylenediamine (D-4), 4-(3,5-Dibromo-2-pyridylazo)-1,3-phenylenediamine (D-5), and 2-(5-Bromo-2-pyridylazo)-5-dimethylaminophenol (D-6). The cross-reactivity of the combination of all eight indicators provides the sensor the ability to discriminate between multiple heavy-metal ions. Wang et al. [142] reported a 3D paper-based analytical device for colorimetric determination of Cu$^{2+}$, Ni$^{2+}$, Cd$^{2+}$ and Cr$^{6+}$, using stacking layers of wax patterned paper and double-sided adhesive tape. Capillary force enables four sample streams to flow both vertically and laterally and to cross another layer without mixing. After the reactions with different types of reagents in different layers, chromogenic assays of the four heavy metal ions can be performed in different detection zones of the device (Figure 14c). The colorimetric analysis shows the
sensitivity to metal ions is \( \text{Cd}^{2+} \rangle \text{Cu}^{2+} \rangle \text{Ni}^{2+} \rangle \text{Cr}^{6+} \) and detection limits ranging from 0.19 to 0.35 ppm. In addition to the above-mentioned representative studies of paper-based multi-ion analysis, many other efforts have been dedicated to paper-based multiplexed detection of heavy metal ions.
Some groups have focused on the development of paper-based sensors for a specific heavy metal ion. Jayawardane et al. [55] demonstrated a paper-based sensor for the detection of Cu$^{2+}$, which is based on the use of a polymer inclusion membrane (PIM). In the PIM, 1-(2’-pyridylazo)-2-naphthol (PAN) is added to cause a colorimetric reaction with Cu$^{2+}$; the original yellow colour of the membrane changes to red/purple with the formation of the Cu$^{2+}$-PAN complex. By stacking 2, 3 or 4 hydrophilic zones, the sample volume can be increased and the sensitivity of the sensor can be enhanced with a limit of detection of 0.06 mg/L$^{-1}$ Cu$^{2+}$ in hot tap and wastewaters (Figure 15a). Apilux et al. [143] created a paper-based device modified with silver nanoplates
(AgNPs) for the simple and rapid determination of Hg$^{2+}$. The redox reaction between Hg$^{2+}$ and AgNPs can cause the disintegration of AgNPs into smaller particles, and the color change can therefore be monitored by the naked eye (Figure 15b). With digital camera imaging and software processing, a linear detection range of 5–75 ppm and a limit of detection of 0.12 ppm were reported. Rattanarat et al. [54] used a simple and low-cost microfluidic paper-based analytical device for quantifying levels of total Cr in airborne particulate matter. All soluble Cr can be oxidized to Cr$^{6+}$ by tetravalent cerium in the pretreatment zone. Then the as-obtained Cr$^{6+}$ reacts with 1,5-diphenylcarbazide to form a purple colored complex. A detection limit of 0.12 g was obtained, which is sufficient for monitoring occupational exposure to particulate Cr.

**Figure 15.** Paper-based analytical devices for a specific heavy metal ion detection. (a) Paper-based sensor with a PIM for the selective determination of Cu$^{2+}$. (Adapted with
Paper-based sensors have been also developed for non-metal environmental contaminants. For example, Jayawardane et al. [144] presented a low-cost disposable colorimetric paper-based analytical device for the determination of nitrite and nitrate. In the presence of a Zn microparticle-based reduction channel, the device is capable of detecting both nitrite and nitrate. The limits of detection (LODs) for nitrite and nitrate were 1.0 and 19 μM respectively, which are suitable for the on-site measurement of nitrite and nitrate in water samples. Alkasir et al. [48] demonstrated a new type of paper-based bioassay for the colorimetric detection of phenolic compounds including phenol, bisphenol A, catechol and cresols. Using a layer-by-layer (LbL) assembly approach, layers of chitosan and alginate polyelectrolytes were deposited onto filter paper and the tyrosinase enzyme was trapped between these layers. The enzymatically generated quinone imines for phenolics react with chitosan to produce colored compounds that can be detected with the naked eye. Pesenti et al. [60] developed a novel paper-based analytical device for the detection of three trinitro aromatic explosives (i.e., 1,3,5-trinitrobenzene (TNB), 2,4,6-trinitrophenylmethylnitramine (tetryl), and 2,4,6-trinitrotoluene (TNT)). Based on the Janowski reaction, potassium hydroxide deposited on the sensor causes a color change when explosives are present. The detection limits of TNB, TNT and tetryl were 7.5 ± 1.0 ng, 12.5 ± 2.0 ng and 15.0 ± 2.0 ng, respectively. Other environmental pollutants such as particulate matter in air, bacteria and pesticides have also been successfully detected based on colorimetric assay on paper.

For the most of the above-mentioned paper-based colorimetric sensing, an electronic reader for the analysis of the intensity of the output is required. Recently, some efforts have been made to design paper sensors with non-instrumented detection. For example, Cate et al. [84, 145] reported a simple, distance-based measurement of metal ions on paper. Colorimetric detection reagent is deposited along the flow channel and precipitates or aggregates upon reaction with the analyte. When the analyte flows out of the circular reservoir, it reacts with the colorimetric reagent and then colour develops along the channel until no analyte remains. Quantification of the analyte is achieved by
measuring color length (Figure 16a). Lewis et al. [85] reported a time-based approach on paper for quantifying the levels of Hg$^{2+}$ and Pb$^{2+}$ ions in water. Wax printing technology is used to define hydrophilic regions for the sample flow. The time between reactions in the regions of “START” and “STOP” is measured, which is proportional to analyte concentration (Figure 16b).

Figure 16. Novel quantitative methods used in colorimetric assays on paper. (a) Time-dependant response results for 15, 5, and 5 μg Fe, Ni, and Cu in the multi-channel sensor on paper. (Adapted with permission from Ref. [145] Copyright 2015 Royal Society of Chemistry) (b) Detailed composition of the time-based sensor on paper. (Adapted with permission from Ref. [85] Copyright 2014 Royal Society of Chemistry)
1.3 Review summary

From the literature review, it is clear that paper-based sensors have attracted wide interest and can be used for many applications such as blood typing and environmental monitoring. There are many reported methods of designing these paper-based analytical devices. However, there are still some drawbacks that limit the real life applications of paper-based sensors.

First of all, the literature review reveals the importance of a simple and versatile method for the biomolecule immobilization onto paper, and the potential of polysaccharide coatings on the functionalization of cellulose paper. Therefore, there is a need to offer detailed analysis about polysaccharide-modified cellulose paper as a substrate for paper-based bioassays.

Secondly, there have been some studies done on the protection of enzymes on paper substrate, including polymer microcapsules with enzymes, sol-gel entrapped enzymes, and LbL entrapped enzymes. However, there are few studies that focus on paper-immobilized antibody preservation. Thus it is of great value to explore a simple, low-cost and robust solution to the bioactivity protection of paper-immobilized antibody.

Thirdly, based on literature review, it is found that paper-based analytical devices usually can be achieved by using various techniques such as dipping, etching, photolithography and printing. These techniques rely on defining hydrophobic barriers within the paper. Also, many assays on paper need data analysis tools and other assistance to deliver the results to users, which limits the filed use. Therefore, many paper-based analytical devices do not entirely fulfil the requirements of being user-friendly, equipment-free or with minimum equipment. It is critical to develop a method to fabricate paper-based sensors, which can skip the preparation of hydrophobic barriers and provide the easy result interpretation.
1.4 Research aims

The overall aim of this research is to explore novel and non-conventional fabrication techniques and design concepts for paper-based sensors, in order to enhance their practicability in locations such as less-industrialized areas, rural regions, home care, field operations and emergency situations. The expected outcome of this research is that it will significantly widen the capabilities of paper-based sensors to meet the requirements of being user-friendly, equipment-free and delivered. The techniques and designs achieved in this research are expected to fill some gaps between prototyping and production of paper-based chemical and biological sensors.

Specifically, this thesis will focus on the following targets:

(1) To explore a simple and versatile covalent binding approach capable of immobilizing a broad range of biomolecules onto paper, obviating the requirement of multiple modification steps, toxic reagents and hazardous conditions in the process of covalent bimolecular immobilization.

(2) To study the stability of paper-immobilized antibody, and explore methods to prolong the shelf-life of paper sensors with immobilized antibody, providing stable and deliverable diagnostic devices to end-users.

(3) To explore novel low-cost fabrication techniques and sensing concepts of paper-based analytical devices, and to demonstrate functionality and feasibility of these sensors in diagnostic and environmental analysis.
Chapter 1

1.5 Thesis outline

Based on the research aim, a series of experimental studies have been conducted. The achieved research outcomes are outlined chapter by chapter as follows:

- **Chapter 2. A simple and low-cost approach for covalent biomolecule immobilization on paper**
  

  A simple and versatile covalent binding approach capable of immobilizing a broad range of biomolecules onto paper is developed in this chapter. We herein offered a detailed analysis of biomolecule immobilization strategies on paper through polysaccharide-coating chemistry. For this purpose, two typical polysaccharides (i.e., chitosan and sodium alginate) were exploited for modifying the paper substrate. The applicability of these modification strategies for biomolecule immobilization was demonstrated with enzyme immobilization studies. Interestingly, we found that even cellulose fibre-based paper without any modification could be easily utilized for the covalent conjugation of protein and serve as a useful tool for bioanalysis. Based on the results, we believe that the proposed method can provide a valuable perspective for covalent biomolecule immobilization on paper.

- **Chapter 3. A preliminary study on the stabilization of antibody immobilized on paper**
  
  *(Published paper: A preliminary study on the stabilization of blood typing antibodies sorbed into paper, Cellulose, 2014, 21: 717-727.)*

  To deliver robust and stable paper-based analytical devices to users, the stability of paper-immobilized antibody is investigated in this chapter. The primary blood typing antibodies (Anti-A, Anti-B and Anti-D IgM) were chosen as model proteins, because it is critical to develop a deliverable paper-based blood analysis device to end-users. Two strategies were explored. The first involved mixing additives such as polyvinylpyrrolidone, dextran and glycerol, with
antibodies before sorption onto paper. The second strategy relies on freeze-drying to stabilize antibodies on paper. The results show that freeze-dried antibodies sorbed on paper could be stored for a long period at ambient conditions without significantly loss of their activity. The thermal stability of antibodies on paper was also improved by freeze-drying. The lyophilization was proven to be a feasible method for the storage and stabilization of paper-immobilized antibodies.

• **Chapter 4. Polysaccharides as protectants for paper-based analytical devices with antibody**


This chapter further explores a simple and low-cost polysaccharide modification method to prolong the shelf-life of paper sensors with immobilized biomolecules, since the freeze-drying process is not a cost-effective way for the large-scale manufacturing of paper-based analytical devices with immobilized antibody. Our work shows that the use of polysaccharide additives is an effective approach to retain the activities of antibodies on paper. The detailed analysis of polysaccharide protection was investigated, which provides research insights that are generally applicable to the protection of other paper-immobilized biomolecules. The polysaccharide modification approach is of great use for the large scale development of a new generation of clinical and home-care blood testing devices.

• **Chapter 5. Precipitation assay meets low wettability on paper: a simple approach for fabricating patterned paper sensors**

*(Submitted paper: Precipitation assay meets low wettability on paper: a simple approach for fabricating patterned paper sensors.)*

This chapter demonstrates a novel fabrication concept of paper-based sensors by simply using “a pen/stamp on wettability-tuned paper”. To date, many studies have presented new designs and possibilities of paper-based analytical devices by using different channel patterning strategies, such as photolithographic techniques, and wax patterning methods. All these
approaches generate hydrophilic channels based on the defined hydrophobic barrier. Herein, pens and stamps containing reagents dispersed in a suitable solvent are used to enable rapid and accurate deposition of reagents onto paper sorbed with some polymers like bovine serum albumin. Without hydrophobic treatment, the patterned sensing components can remain in place on the paper substrate whose wettability is tuned via bovine serum albumin modification. After exposure to the analytes, the precipitates of sensing reactions are not delocalized, giving the simple and successful patterned signal readout. Besides, this chapter presents an effective extension of the text-reporting concept to environmental monitoring.

- **Chapter 6. Conclusions and future work**
This chapter summarizes the major contributions of this thesis and recommends future research approaches to building paper-based sensors for practical applications.
1.6 References

1. H. Schiff, Justus Liebig’s Annalen der Chemie, 1866, 140: 92-137.


Chapter 1


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Chapter 2

A simple and low-cost approach for covalent biomolecule immobilization on paper
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To develop a paper-based sensor, the first step is to effectively immobilize sensing elements onto the paper substrate. Unlike paper-based chemical sensors, some of paper-based biological sensors involve multiplexed assays such as lateral flow immunoassays and ELISA. This means that direct physical adsorption of biomolecules does not always provide reproducible results since biomolecules are weakly bound to paper fibres and can be easily washed off. Therefore, a simple and versatile covalent binding approach capable of immobilizing a broad range of biomolecules is desirable for paper-based multistep bioassays. This chapter presents a novel and low-cost approach to covalent biomolecule immobilization on paper. The results show that original paper can be utilized for the covalent conjugation of biomolecules via the use of residual carboxyl functional groups on paper (i.e., zero-step paper functionalization for covalent biomolecule immobilization). The work reported here provides a valuable perspective for the fabrication of paper-based biosensors.
Monash University

Declaration for Thesis Chapter 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

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<th>Nature of contribution</th>
<th>Extent of contribution (%)</th>
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<tr>
<td>Initiation, key ideas, experimental works, analysis of results, writing up</td>
<td>50</td>
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The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

<table>
<thead>
<tr>
<th>Name</th>
<th>Nature of contribution</th>
<th>Extent of contribution (%) for student co-authors only</th>
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<tr>
<td>Liyun Guan</td>
<td>Initiation, experimental works, analysis of results, writing up</td>
<td></td>
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<tr>
<td>Miaosi Li</td>
<td>Assisted in experimentation</td>
<td></td>
</tr>
<tr>
<td>Junfei Tian</td>
<td>Assisted in experimentation</td>
<td></td>
</tr>
<tr>
<td>Wei Shen</td>
<td>Key ideas, paper reviewing and editing</td>
<td>Supervisor</td>
</tr>
</tbody>
</table>

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

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*Note: Where the responsible author is not the candidate’s main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.
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A simple and low-cost approach for covalent biomolecule immobilization on paper

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2.1 ABSTRACT
As a potential platform for point-of-care clinical analyses and environment monitoring, paper-based biosensors have received considerable attention. In many cases, the conjugation of biomolecules onto paper surface is crucial for increasing the functionalities of paper-based biosensors. Until now, it is sometimes argued as in the literature that finding a surface chemistry for biomolecule covalent grafting to paper still remains a challenge. Here the study shows that at least to a certain extent some aspects of the argument involved are questionable, by demonstrating that paper without any modification can be utilized for the covalent conjugation of biomolecules and then serves as a tool for bioanalysis. Moreover, the detailed analysis of biomolecule immobilization strategies on paper through polysaccharide-coating chemistry has been offered as a contrast. The proposed method could provide a valuable perspective for paper-based biosensing platform.

2.2 KEYWORDS
Biomolecule immobilization, paper-based biosensors, polysaccharide coating, unmodified paper
2.3 INTRODUCTION

Biosensors as a diverse collection of bioanalytical devices for detecting and quantifying biomolecules, have been widely used in many areas, such as clinical and home-based diagnoses and environmental monitoring [1-3]. Typically, a biosensor consists of three main parts: a bio-recognition component, a signal transducing component and an output system. Among them, the bio-recognition component is responsible for the identification of the presence and quantification of analytes via specific interactions with analytes by using specific biological elements like proteins, nucleic acids and tissues [4,5]. Thus effective immobilization of these biological elements onto solid surfaces is an important step in biosensor fabrication. Considerable efforts have been devoted to endowing the supporting material surfaces with bio-recognition ability by attaching biomolecules covalently [6,7].

Nowadays, novel paper-based biosensors have emerged with potential as easy-to-use, rapid and inexpensive point-of-care devices [8,9]. Compared with commonly used substrate materials for biosensors, its advantages include low production cost, intrinsic water wicking ability and feasibility of patterning by printing technology [10,11]. So far, in paper-based biosensor design and fabrication the most used techniques for immobilizing biomolecules are based on physical adsorption. However, physical adsorption of biomolecules has an unavoidable weakness that it could not always promise reproducible results because biomolecules are weakly bound to paper fibres and could be easily washed off [12]. In order to significantly improve the performance of paper-based analytical devices for quantitative bioanalysis, effective and chemically reliable methods for immobilizing a broad range of biomolecules on paper sensors, mostly immobilization via covalent bonding, need to be explored.

To date, a variety of surface chemistries have been proposed to facilitate the covalent immobilization of biomolecules onto paper, such as divinyl sulfone chemistry, diazonium chemistry or polymer chemistry [12-14]. Among them, long-chain and flexible polysaccharides with multiple functional groups have exhibited the potential to act as surface modifiers for paper sensor fabrication. In particular, Hannes et al. used the Langmuir-Schaeffer cellulose film as a model to investigate polysaccharide adsorption for biomolecule conjugation, which could give instructions to paper modification via polysaccharide coating technique [15,16]. However, to the best of our
knowledge, the detailed study of using these polysaccharides for practical paper surface modification has not been assessed. Recently, Wang et al. reported the application of chitosan as the surface modifier to fabricate paper plates for paper-based enzyme-linked immunosorbent assays (ELISA); their work has demonstrated, partly, the potential of polysaccharide coating on paper [17].

Here comes the question, why bother doing paper functionalization? The reason is that paper is typically composed of cellulose fibres and hydroxyl groups in glucose are responsible for cellulose chemical activity [9]. Most published work took it for granted that hydroxyl groups need to be transformed to other functional groups to enable covalent immobilization of biomolecules by common bioconjugate techniques. However, it is neglected that paper has low concentrations of carboxyl groups, which could potentially facilitate the covalent immobilization of biomolecules and probably make paper ready for binding biomolecules covalently [9,18]. In other words, people may be distracted by the familiarity with cellulose and its derivatives; the functionalization strategies for paper they are looking for may not be necessary.

In this study, unmodified cellulose fibre-based paper was investigated for covalent biomolecule immobilization. An enzyme, alkaline phosphatase (AP) used as a model protein, was immobilized onto paper substrates. In a comparison study, two kinds of representative polysaccharides, chitosan and sodium alginate (NaAlg), were utilized for paper modification. They were deposited as surface modifiers on paper surfaces. The effects of these modification methods were evaluated with a colorimetric reaction of substrates corresponding to AP. To the best of our knowledge, the direct use of original paper for covalent linking of biomolecules has not been previously reported, that is, the surface activity of the residual carboxyl groups for immobilization has never been studied. Our results show that, despite of the low concentration, the residual carboxyl groups on unmodified paper can still provide a considerable capacity for AP immobilization. Furthermore, we also described the construction of a simple immunoassay on differently modified paper substrates. We hope that our results could make a contribution to future fabrication of paper-based biosensors.
Chapter 2

2.4 EXPERIMENTAL

2.4.1 Reagents and instrumentation

Whatman qualitative filter paper #1, cut into 10 mm × 10 mm pieces, was chosen as paper substrate throughout the work. In this study, all reagents and protein including bovine serum albumin (BSA) were purchased from Sigma-Aldrich. Glutaraldehyde, N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were used for chemically crosslinking. Medium molecular weight chitosan (200-800 cP, 1% in 1% acetic acid) and medium molecular weight NaAlg (≥2,000 cP, 2% in water) were chosen for the comparison study. 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) was used in conjunction with nitroblue tetrazolium (NBT) for the colorimetric detection of AP activity. Buffers and solutions, unless specially noted, were prepared with ultrapure water (18.2 MΩ cm, Milli-Q Gradient System, Millipore, USA).

The digital colorimetric images of the assays were generated by scanning the results using a desktop scanner (Epson Perfection 2450) set to color document scanning, 600 dpi resolution; the digital assay results were analysed in grayscale using ImageJ. In order to correct the influence of the paper background color to assay results, background grayscale value of paper with immobilized biomolecules before adding BCIP/NBT substrate was subtracted, unless otherwise noted. The color intensity values were obtained by taking the average of the quadruplicate readings, with the variation of values represented by error bars.

2.4.2 Preparation of unmodified paper with immobilized enzymes

2.4.2.1 Glutaraldehyde coupling

Typically, 20 µL of 3% glutaraldehyde in 0.01 M pH 7.4 phosphate buffered saline (PBS) was introduced to activate the original paper for 2 h in a wet box, and the paper was washed three times by adding 100 µL of the coupling buffer (1.5% (w/v) mannitol, 0.15% (v/v) glycerol and 0.01% (v/v) Tween 20) to the top surface of it, and then putting the bottom of the paper in contact with a piece of cotton wool to remove the excess buffer. Subsequently, 3 µL of an enzyme solution in the coupling buffer (100 µg mL⁻¹ AP) was spotted onto the freshly activated paper square and incubated in the wet box for 2 h. After that, the paper was washed with 100 µL of Tris-HCl buffer (50 mM,
pH 9.2, containing 0.1% (v/v) Tween 20) three times and allowed to dry for 30 min under ambient conditions. Finally, 9 µL of a colorimetric substrate solution for AP (i.e., BCIP/NBT) was added to the paper. After being incubated for 2 h, the paper square was scanned by the desktop scanner and the intensity of the color was measured using ImageJ.

2.4.2.2 NHS/EDC coupling

Typically, 20 µL of a mixed solution (0.1 M NHS and 0.1 M EDC) was introduced to activate the original paper in the wet box for 15 min, and then this activation step was repeated once more. Subsequently, the paper square was washed with the coupling buffer (3 × 100 µL). Afterwards, 3 µL of a 100 µg mL⁻¹ AP solution in the coupling buffer was spotted onto the freshly activated paper square and incubated in the wet box for 2 h. The paper was then washed with Tris-HCl buffer (3 × 100 µL) and allowed to dry for 30 min under ambient conditions. Finally, BCIP/NBT was added to the paper; after 2 h, the image was recorded by the desktop scanner and then analysed.

2.4.3 Preparation of polysaccharide-coated paper with immobilized enzymes

In a typical experiment (Figure 1), 20 µL of chitosan solution (0.05%, w/v) in aqueous acetic acid solution or 20 µL of NaAlg solution (0.05%, w/v) was dropped onto one piece of paper. After being dried under ambient conditions overnight, polysaccharide-coated paper was ready for use. Enzyme could be covalently immobilized on glutaraldehyde activated chitosan-modified paper square or NaAlg-modified paper square activated by NHS/EDC. Based on the above-described procedures, a variety of reaction parameters like polysaccharide concentration were examined for polysaccharide coating.

2.4.4 Immobilized enzymes’ stability assays

Different paper substrates with immobilized AP (0.05% chitosan or 0.05% NaAlg for paper coating, AP concentration of 100 µg mL⁻¹ for immobilization) were stored at room temperature for various periods of time. Afterwards, the residual enzymatic activity of immobilized AP was evaluated by the color-producing enzymatic reaction as mentioned above.
2.4.5 Fabrication and processing of immunoassays

3 µL of 100 µg mL⁻¹ rabbit Immunoglobulin G (IgG) was immobilized on different paper substrates for 2 h. After washing with 1 mL of PBST (0.1% (v/v) Tween 20 in PBS), the paper squares were immersed in a blocking buffer (5% (w/v) BSA in PBS) for 30 min, followed by washing with 1 mL of PBST to remove the free BSA. An AP-conjugated goat anti-rabbit IgG solution (3µL, prepared by diluting the stock solution 1000 times in PBS) was added onto the test paper and allowed to incubate for 5 min. The test zones were then washed with 1 mL of PBST, and allowed to dry under ambient conditions for 30 min. Finally, 9 µL of a solution of BCIP/NBT was added to the paper, and after 30 min the results were recorded and analysed by ImageJ.

![Diagram](image.png)

Figure 1. Scheme of AP immobilization on polysaccharide-coated papers. (a) Coupling of AP to NaAlg-coated paper via NHS/EDC activation. (b) Coupling of AP to chitosan-coated paper via a Schiff base.

2.5 RESULTS AND DISCUSSION

2.5.1 Demonstration of paper-based bioassay format

In the current study, in order to simplify the experimental procedure, spotting assays were performed on paper squares. Specifically, the most widely utilized Whatman filter paper for bioassays was chosen as the substrate in our study. Then, after immobilizing a small amount of enzyme (3 µL) onto paper substrate, we would utilize 9 µL of enzyme substrate to wet test paper completely for better understanding the accurate distribution of enzyme. We tested the efficiency of immobilizing proteins and evaluated the conservation of protein biological activity on different types of paper via using solutions of AP as a model protein, because AP is considered very sensitive particularly when using colorimetric detection and also less stable than other popular alternatives such as the enzyme horseradish peroxidase (HRP).
Chapter 2

### 2.5.2 Covalent attachment of protein to unmodified paper

The amount of protein covalently immobilized on original filter paper could vary with different coupling chemistries (Figure 2). Glutaraldehyde-mediated coupling on filter paper is not efficient for enzyme immobilization. Apparently, there are no primary amine groups on paper for covalent bonding of glutaraldehyde (Table 1). Because of the lack of primary amine on filter paper, any further covalent attachment of enzyme through the glutaraldehyde coupling would not be possible.

**Table 1.** X-ray photoelectron spectroscopy (XPS) analysis of different papers.

<table>
<thead>
<tr>
<th>samples</th>
<th>original paper</th>
<th>chitosan-coated paper</th>
<th>washed chitosan-coated paper a)</th>
<th>NaAlg-coated paper</th>
<th>washed NaAlg-coated paper a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1+C2 (C-C, C-H)</td>
<td>0.063±0.002</td>
<td>0.069±0.003</td>
<td>0.125±0.000</td>
<td>0.067±0.000</td>
<td>0.092±0.002</td>
</tr>
<tr>
<td>C3 (C-N, C-O)</td>
<td>0.692±0.001</td>
<td>0.697±0.004</td>
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<td>0.696±0.005</td>
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<tr>
<td>C4 (C=O, O-C-O, N-C=O)</td>
<td>0.231±0.000</td>
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<td>0.021±0.001</td>
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<td>Si</td>
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a) Paper was washed thoroughly with plenty of PBS (2 × 200 µL) followed by ddH$_2$O (2 × 200 µL).

Surprisingly, NHS/EDC activation strategy displays different behaviour, depending on diverse crosslinking mechanisms. The results obtained from NHS/EDC activation are exactly opposite to those from glutaraldehyde activation method (Figure 2). Our data show successful enzyme immobilization on NHS/EDC-activated paper, which indicates the possibility that carboxyl groups on paper are sufficiently active to react with EDC and further with NHS to form the activated surface for protein immobilization. It is a
known fact that paper surface is slightly negative charged, which is partly caused by the
presence of carboxyl groups [9,18]. In the case of Whatman qualitative filter paper #1,
the carboxyl group content of cellulose is 8.0 µmol g⁻¹. To further evaluate this
possibility, we performed direct enzyme immobilization on filter paper without the
NHS/EDC activation. Once eliminating the activation step, paper surface would only
have a low concentration of carboxyl groups, and would not be able to acquire the more
reactive NHS ester groups from the NHS/EDC coupling chemistry; we therefore
expected insignificant enzyme immobilization on the unmodified paper. Colorimetric
measurement of paper samples treated with direct enzyme deposition followed by Tris-
HCl buffer rinsing shows insignificant colour change. This result supports our
reasoning that the native residual carboxyl groups on original paper surface may
provide a certain level of NHS/EDC coupling for biomolecule immobilization.

![Bar chart](image)

**Figure 2.** Detection of immobilized AP on differently treated unmodified paper:
glutaraldehyde coupling (left) and NHS/EDC coupling (right).

### 2.5.3 Covalent attachment of protein to polysaccharide-modified paper

Polysaccharide-modified paper was contrasted with activated original paper by
NHS/EDC for examining the performance of protein immobilization. The reason why
polysaccharide-coated paper was taken as a reference is that polysaccharides as an
important class of biopolymers have been successfully used as surface modifiers to
functionalize paper-based analytical devices. Meanwhile, there is a lack of detailed
analysis of polysaccharide modification strategies for paper-based biosensing platforms.
which can increase our knowledge about the chemistries of these bioconjugation systems and their bioassay performances.

Some studies on cellulose model surfaces (i.e., Langmuir-Schaeffer cellulose films) have reported that polysaccharides’ structural similarity to cellulose makes these macromolecules possible to engage in hydrogen bonding interactions with cellulose, which could lead to irreversible polymer adsorption [15,16]. Here, when it comes to practical cellulose fibre-based paper, XPS surface analysis was performed to verify paper functionalization via polysaccharide coating. As shown in the XPS elemental composition data (Table 1), nitrogen was not detected on unmodified paper. As for chitosan-coated paper, however, even after thorough washing with water, the similar appearance of a nitrogen signal in the XPS spectrum indicates the successful irreversible adsorption of chitosan on paper. Similarly, after the coating of NaAlg, sodium could be observed by XPS on NaAlg-coated paper, which demonstrates the rinsing–resistant adsorption of NaAlg. The results validate that polysaccharide has similar affinity with cellulose fibre–based paper just as it has with Langmuir-Schaeffer cellulose films.

2.5.3.1 AP immobilized on chitosan-modified paper

Chitosan-coated paper was exploited for enzyme immobilization. Two major mechanisms contributed to the biomolecule immobilization – electrostatically driven adsorption mechanism and a covalent coupling mechanism with Schiff bases. The abundant primary amine functional groups in chitosan enable the polymer to carry positive charges under acidic conditions, with a pKa of ~6.5 [19]. AP was used in this study as the model biomolecule with a pI range of 4.4-5.8, which allows chitosan and the enzyme to have electrostatic interactions.

In addition to the electrostatic interaction, chitosan activated by glutaraldehyde is able to immobilize biomolecules with amino groups through the Schiff reaction. Figure 3a shows that the trends of enzyme immobilization through physisorption and covalent coupling were similar, that is, the quantity of the immobilized protein increased with the increase of chitosan proportion used for paper surface treatment. The quantity of enzyme immobilization through covalent bonding was higher on paper that treated by low to medium concentration of chitosan solution (≤0.5%, w/v); but became almost the same as the chitosan concentration further increased to 1% (w/v). The results reveal
that even non-activated chitosan can immobilize the enzyme strongly enough to withstand rinsing by Tris-HCl buffer that could lead to the deprotonation of chitosan. Although the electrostatic charge of chitosan could be weakened, the hydrophobic interactions between chitosan and enzyme molecules could still contribute to enzyme physisorption [20].

From the perspective of paper bioassays based on popular colorimetric analysis, paper should ideally not acquire any background color after the surface modification with polysaccharides. However, as shown in Figure 3b, paper modified with chitosan solution concentration above 0.1% does acquire an unwanted level of background color after being activated by glutaraldehyde. It is possible that the adsorbed chitosan could go through a phase inversion in weak alkaline solution (PBS) and become conducive to form the interpenetrating polymer network, which might allow, although could not be confirmed, the subsequently introduced glutaraldehyde to bring about crosslinked chitosan [21]. In order to retain the high brightness of the paper and to achieve a practically significant covalent coupling ability to biomolecules, low concentrations of chitosan solution (not more than 0.05%) is more suitable for the fabrication of chitosan-coated paper biosensing platform, which is in some agreement with previous report by Wang et al., where they used 0.025% chitosan for paper modification [17]. In that case, the enzyme immobilization capacity of NHS/EDC-activated original paper was considerable, just slightly lower compared to that of chitosan-coated paper.
Figure 3. Detection of immobilized AP on chitosan-modified paper. (a) Colorimetric intensity of AP assay as a function of chitosan solution concentration. Insets: Images of colored product from AP assay on glutaraldehyde-activated paper (top row) or non-activated paper (bottom row). (b) Background intensity for chitosan-modified paper with immobilized AP before BCIP/NBT incubation. Insets: images of pairs of paper with immobilized AP with (right) or without glutaraldehyde activation (left).

2.5.3.2 AP immobilized on NaAlg-modified paper

As another commonly-utilized macromolecule, NaAlg has a large number of carboxyl groups distributed along the polymer backbone, which enables covalent immobilization of biomolecules via the well-understood NHS/EDC chemistry. Unlike the chitosan immobilization system, the amount of protein immobilized on NaAlg-modified paper varied with different methods (Figure 4a). In the case of NaAlg-modified paper with NHS/EDC activation, the amount of immobilized AP exhibited a low dependence on the concentration of the NaAlg solutions employed for surface treatment, which may be attributed to the fact that the residual carboxyl groups on unmodified paper can react with biomolecules bearing primary amine groups. The results show that NaAlg-coated paper has similar enzyme immobilization capacity as NHS/EDC-activated original paper.

It is observed in Figure 4a that the physisorption of AP on non-activated NaAlg paper was weak, particularly in the low NaAlg concentration range (below 0.1%). Electrostatic repulsion could be responsible, since both NaAlg and AP carry negative charges. The reason for the increased AP physisorption in higher NaAlg concentration, however, is unclear. Possibly, the increased NaAlg concentration that could change the pore size of paper to some extent made the adsorbed enzyme difficult to be washed off, which can affect the reproducibility of paper-based analytical device signal data. Unlike chitosan modification, NaAlg modification does not raise the background signal, which is a desirable performance for paper-based colorimetric bioassays (Figure 4b).
Figure 4. Detection of immobilized AP on NaAlg-modified paper. (a) Colorimetric intensity of AP assay as a function of NaAlg solution concentration. Insets: Images of colored product from AP assay on non-activated paper (top row) or NHS/EDC-activated paper (bottom row). (b) Background intensity for NaAlg-modified paper with immobilized AP before BCIP/NBT incubation. Insets: images of pairs of paper with immobilized AP with (right) or without NHS/EDC activation (left).

2.5.4 Comparison of immobilization methods

Since the high biomolecule immobilization capacity could improve the performance of bioassays, it is of importance to quantify the amount of biomolecules that could be immobilized on the substrates. Here, the covalent immobilization of AP on three paper samples with different treatments (i.e., 0.05% chitosan-modified paper activated by glutaraldehyde; 0.05% NaAlg-modified paper and original paper activated by NHS/EDC) was studied by spotting solutions of AP with concentrations varying from 0 to 1 mg·mL⁻¹. As expected, the quantity of immobilized enzyme increased as a function of AP concentration (Figure 5). The colorimetric analysis of these paper samples that had been modified by chitosan or NaAlg did not show significant difference from that of original paper treated with by NHS/EDC chemistry. The above results suggest that original paper has the potential to simplify the route of covalent biomolecule conjugation onto it. The long-term stability on various paper substrates was also investigated. Our results (shown in Figure 6) indicate that AP retained some activity over a period of at least one month when stored at room temperature. To some extent,
covalent coupling could help to maintain the active conformation of the biomolecules. Intuitively, covalent immobilization could reduce the likelihood of enzyme aggregation by conjugating them with supports to restrict their movement. Unfortunately, the results in Figure 6 demonstrate that paper modified with polysaccharides did not offer a significant advantage over the unmodified paper treated with NHS/EDC chemistry in preserving the bioactivity of AP. During storage, low-concentration chitosan and NaAlg may not offer effective protection for biomolecules.

**Figure 5.** Colorimetric assays for covalently immobilized AP on different types of paper squares.

**Figure 6.** Stability of immobilized AP on different paper squares with varying storage time.
2.5.5 Assessment of bioassays on different papers

In order to demonstrate the capability and versatility of different types of paper in conjugating biomolecules, we performed a simple-form paper-based immunoassay, that is, the interaction and recognition between rabbit IgG and AP-conjugated anti-rabbit IgG. As illustrated in Figure 7, for both original and NaAlg-modified paper, there is an obvious signal difference between NHS/EDC-activated and non-activated paper, in accord with the role played by carboxyl groups during the process of biomolecule immobilization. The relatively weak signal observed on the non-activated paper can be attributed to a low level of the physical adsorption of rabbit IgG onto the paper. However, in the case of chitosan-modified paper, even non-activated paper had a strong signal, which means that the physisorption of rabbit IgG could be resistant to the stringent washing steps. Perhaps it is of some value to verify the feasibility of utilizing unactivated chitosan-modified paper for bioassays in the future. Besides, it is worth noting that the activated original paper showed no significant difference with activated chitosan paper, which, once again, demonstrated the ability of NHS/EDC conjugation strategy to covalently immobilize biomolecules bearing amino groups onto the unmodified paper.

![Graph showing colorimetric intensity comparison between activated and non-activated papers](image)

**Figure 7.** Paper-based immunoassays for the detection of rabbit IgG immobilized on different paper substrates.
Chapter 2

2.6 CONCLUSIONS

On the grounds that popular covalent methods of attaching biomolecules to substrates for biosensing platforms include amine chemistry and carboxylic chemistry, this work provides detailed data about the application of chitosan and NaAlg on cellulose fibre-based paper modification, which could offer some valuable suggestions for other applications using bioactive paper. Besides, there is a common view that for covalent biomolecule immobilization, paper must be functionalized at first. However, our results indicate that efforts may not be required for the step of functionalization by using residual carboxyl functional groups on paper (i.e., zero-step paper functionalization for covalent biomolecule immobilization). The work reported here would provide a valuable perspective for the fabrication of paper-based biosensing platform.

2.7 ACKNOWLEDGEMENT

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2.8 REFERENCES


Chapter 3

A preliminary study on the stabilization of antibody immobilized on paper
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In the previous chapter, a simple and versatile covalent binding approach capable of immobilizing a broad range of biomolecules for paper-based biosensors was presented. After immobilizing biomolecules on paper, the storage and stabilization of paper-immobilized biomolecules is also a significant factor that determines the performance of these bioassays. Unlike common sensing elements in paper-based chemosensors, the sensing elements in paper-based biosensors, especially protein, are very sensitive, losing function with heating, dehydration, and shearing. To ensure quality of products, paper-based biosensors must have sufficient longevity to allow them to be delivered to end-users with a satisfactory level of bioactivity. Therefore, a preliminary investigation of the stability and stabilization of biomolecules immobilized on paper is reported in this chapter. Antibody was chosen as a model, and two antibody protection strategies (i.e., mixing additives with antibody and freeze-drying antibody) were performed. The results show that the freeze-dried antibody immobilized on paper can be stored for a long period of time at room temperature without significant loss of the bioactivity. In addition, the thermal stability of paper-immobilized antibody is also improved by the freeze-drying approach. The work reported in this chapter can serve as a guide for the future development of paper-based biosensing platforms.
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Monash University

Declaration for Thesis Chapter 3

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

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The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

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<td>Gil Garnier</td>
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<td>Wei Shen *</td>
<td>Key ideas, paper reviewing and editing</td>
<td>Supervisor</td>
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The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate’s and co-authors’ contributions to this work*.

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*Note: Where the responsible author is not the candidate’s main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.
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A preliminary study on the stabilization of antibody immobilized on paper

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3.1 ABSTRACT
This study preliminarily investigated the stability and stabilization of protein immobilized on paper. The primary blood typing antibodies (Anti-A, Anti-B and Anti-D IgM) were chosen as the model protein, and two protection strategies were proposed. The first involved mixing additives such as polyvinylpyrrolidone (PVP), dextran and glycerol, with antibody before immobilization onto paper. All the additives tested improved the antibody stability on paper, but their protection for storage at room temperature was limited. The second strategy relied on freeze-drying to stabilize paper-immobilized antibody. Freeze-dried antibody on paper can be stored for long periods at ambient conditions without significantly loss of the activity. The thermal stability of antibody on paper was also improved by freeze-drying. The results show that the freeze-drying is an effective approach to retain the activities of IgM blood group antibodies on paper. The work reported here can serve as a guide for the future development of paper-based biosensing platforms.

3.2 KEYWORDS
Paper-based diagnostic devices, blood typing, antibody activity, freeze-drying
3.3 INTRODUCTION

Paper-based bioanalytical devices have drawn much interest in recent years as a platform for low-cost analytical devices in diagnostic and environmental applications. Paper made of cellulose fibre is cheap and widely available – a suitable material for manufacturing basic analytical sensors on a large scale to meet the demand of diagnosis and disease screening in developing areas of the world. The rapid technical advancement and development of paper-based sensors can be attributed to the numerous advantages of the porous structure of paper and the surface chemistry of cellulose fibres. Historically, concepts of using patterned paper to fabricate multi-well assay plates [1] and paper microfluidic devices [2] have been explored from as early as 1937 and 1949. The first paper-based diagnostic sensor for semi-quantitative glucose assay in urine was demonstrated in 1956 [3]; this invention was further developed into immunological paper test devices whose functions were based on colorimetric and electrochemical mechanisms [4]. Since 2007, patterned paper diagnostics have been re-discovered and pursued by a number of research groups globally [5-10], initiated by the Whitesides’ group [11]. In the past years, research and innovation in bioactive paper diagnostics have created a number of low-cost conceptual devices that have potential for commercialization.

A critical requirement of all paper-based diagnostic devices is the longevity of their biochemical activity. These devices must have a sufficient stability to allow temporary storage and transportation in the supply chain. This is because the technological developments for paper-based diagnostics generally take place in developed countries, whereas a significant proportion of the users of basic diagnostics are living in developing countries. Therefore diagnostic devices must be able to retain their bioactivity for a sufficiently long period of time and withstand elevated temperature to allow the devices to be delivered to the users. This requirement has been defined, along with other six requirements for low-cost analytical devices designed for developing world, by the World Health Organization (WHO) as “ASSURED” [12]. Although significant progress in bioactive paper research has been made, practical obstacles to maintaining the bioactivity of paper-based devices must be overcome in order for the devices to function reliably under unsupported field conditions. To this end, engineering strategies need to be applied to provide simple and effective methods to enhance the time and temperature stability of paper-based biosensors.
In this study, the primary blood typing antibodies (Anti-A, Anti-B and Anti-D IgM) were chosen as the model protein to evaluate protein stabilization strategies on paper. As an important type of protein, antibody can denature via many pathways [13-15]. All antibody molecules are structurally similar; they comprise two different kinds of polypeptide chains – heavy and light. Each chain is divided into two regions – the variable (V) region and the constant (C) region. Additionally, antibodies are divided into five different classes (IgA, IgD, IgE, IgG and IgM) based on their C regions; among those classes, IgM is the least stable [16].

Our group has proved that the primary blood typing antibodies have potential applications for paper-based biosensing platform, by demonstrating a novel paper-based ABO and rhesus D (RhD) blood typing sensor that can report patients’ blood typing results in written text [8, 9]. Our text-reporting blood typing sensor was designed based on the antibody-specific direct agglutination of red blood cells (RBCs) by IgM group monoclonal antibodies [17]. Unlike other paper-based blood typing sensors where paper are used merely as a supporting substrate to display the occurrence or non-occurrence of haemagglutination, our design uses the fibre network of paper to differentiate the agglutinated red blood cell (RBC) aggregates from the non-agglutinated cells. Whilst this concept of reporting results in text demonstrates a high level of user-friendliness, the engineering and manufacturing aspects of the sensor must also be investigated to ensure that the longevity of antibodies in cellulose fibre networks meets the sensor design requirements. Herein we report results of a preliminary study on different approaches to extend longevity of ABO and RhD blood group antibodies in paper-based sensors stored under room temperature. These results will serve as a guide for the future development of paper-based biosensing platforms.

3.4 EXPERIMENTAL

3.4.1 Reagents and instrumentation

Antibodies against RBC antigens approved for human blood grouping were obtained from Alba Bioscience, Edinburgh, UK. They were IgM antibodies commercialized under the names of Albaclone® Anti-A, Anti-B, and Anti-D Optimum. Anti-A and Anti-B are a blue and a yellow solution respectively, while Anti-D is a clear solution. Phosphate buffer saline (PBS) sourced from Sigma Aldrich, Australia was used as diluent for all antibody solutions. All the antibodies were stored at 4 °C.
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Reagent RBCs Revercell™ (15% A1 and 15% B red blood cells) and Abtectcell™ III (3% R1R1, 3% R2R2 and 3% rr RBCs) were purchased from Commonwealth Serum Laboratories (CSL) Limited, Australia, stored at 4 °C and used within 30 days. They were concentrated by centrifugation to 40% before reacting with blood group antibodies.

Dextran (average molecular weight 35,000–45,000), polyvinylpyrrolidone (PVP, average molecular weight 40,000), and glycerol were obtained from Sigma Aldrich. Saline solution (0.9% NaCl, w/v) was prepared with Milli-Q water. Kleenex paper towel was cut into 1 cm × 1 cm squares that were used to absorb blood group antibody solutions for making blood typing sensor. A 2.5 L Freeze Drying system (FreeZone®, Labconco Corp., Kansas City, USA) was used to freeze-dry blood group antibodies immobilized on paper. An oven (Memmert, Australia) was used for heat treatment of freeze-dried antibodies on paper.

3.4.2 Preparation of paper-immobilized antibody with stabilizing additives

Anti-A, Anti-B and Anti-D antibody solutions, containing 6% dextran, 10% glycerol and 1% PVP respectively, were prepared by mixing the antibody and additive solutions to the required additive concentration; antibody concentration in the final solutions with different additives was kept constant. On each paper square, 10 μL antibody-additive mixture was introduced to form the antibody loaded paper. After hanging to dry in a fume hood for 2 min, the paper squares were stored in plastic petri dishes under room temperature. Paper squares without additives were also prepared as controls. This procedure of sample preparation creates series of antibody-loaded papers with different additives. Taking Anti-A as an example, the paper series contain Anti-A with (1) no additives; (2) 1% PVP; (3) 6% dextran and (4) 10% glycerol, which are referred to as N-A, P-A, D-A and G-A, respectively. Papers immobilized with Anti-B and Anti-D antibodies were denoted as N-B, P-B, D-B, G-B; N-D, P-D, D-D, G-D respectively, following the same rule as Anti-A loaded papers. The stability of all paper squares was tested after being stored for 0, 7, 14, 21, and 28 days under room temperature.
3.4.3 Freeze-drying of paper-immobilized antibody

3.4.3.1 Preparation of freeze-dried paper immobilized with antibody

20 µL of antibody solution (i.e., anti-A, anti-B or anti-D), was introduced onto each of the total of ninety paper squares with a micropipette. This quantity was greater than that for preparing additive-protected papers; this is because freeze-dried papers suffer from antibody loss due to the burst of air bubbles in antibody solutions during vacuuming. The paper squares were stored in groups of ten in sealable polypropylene sample tubes and frozen at −80 °C immediately for 30 mins. Then they were freeze dried at a shelf temperature of −40 °C and a chamber pressure of 60 mTorr for 18 h. After freeze drying, these paper squares were sealed in plastic bags and stored in a desiccator at room temperature for further tests. Freeze-dried paper squares loaded with Anti-A, Anti-B and Anti-D antibodies were referred to as FD-A, FD-B, and FD-D, respectively. The room temperature longevity of antibodies was tested.

3.4.3.2 Temperature treatment of paper-immobilized antibody

After freeze-drying, the paper squares loaded with antibodies were treated for 6 hours at 4 °C, 25 °C, 40 °C and 60 °C. The stabilities of FD-A, FD-B and FD-D were tested 30 min after temperature treatment.

3.4.4 Test of stability of paper-immobilized antibody

For each group of paper with immobilized antibody, 3 µL antigen-positive and 3 µL antigen-negative reagent blood cells were respectively introduced onto two antibody-loaded paper squares to study if agglutination of antigen-positive RBCs could be triggered by the corresponding antibody. Specifically, for Anti-A, A1 and B RBCs were used as antigen-positive and antigen-negative reagent blood cells respectively; for Anti-B, B and A1 cells were used as antigen-positive and antigen-negative reagent blood cells respectively; for Anti-D, R1R1 and rr cells were used as antigen-positive and antigen-negative reagent blood cells respectively. Thirty seconds was allowed for the antibodies in the paper to react with the RBCs. Then, three aliquots of 25 µL saline solution were introduced onto each testing paper, while blotting papers (drink coaster blotting, 280 g m⁻²) were used to remove liquids during the washing process.
3.4.5 Blood sample agglutination evaluation

To compare the activity of the IgM antibody on paper, both antigen-positive and antigen-negative reagent blood cells were respectively introduced onto two testing paper squares loaded with the same antibody. These paper squares were referred to as ‘+’ and ‘−’ testing papers. If the activity of the antibody is retained in paper, agglutination of the antigen-positive RBCs will occur; a clear colour difference between ‘+’ and ‘−’ papers will be observed. However, if the antibody in paper has lost its activity, the color difference between ‘+’ and ‘−’ papers is small. This is because the color difference is related to the intensity of RBC agglutination, which is governed by the antibody activity in paper. On the other hand, for the antigen-negative RBCs, no agglutination occurs in paper; the non-agglutinated RBCs can be washed out by the saline solution [9], leaving a much weaker trace of blood stain in the testing zone (Figure 1). The average grey value of the measured color intensity difference was used to evaluate the antibody activity in paper.

Figure 1. A schematic diagram showing the activity test of antibodies loaded in paper using the reported protocol of paper-based blood typing devices [18].

In obtaining the grayscale data, the test paper squares carrying the agglutination patterns were imaged with a desktop scanner (Epson Perfection 2450, color photo setting), then imported into Adobe Photoshop software. Images were further converted into grey mode (0-255) and the mean grey intensity of the reaction spot was obtained using the histogram function [19]. The ultimate relative intensity value was generated by subtracting the measured intensity of the antigen-positive reaction paper from the
intensity of the antigen-negative reaction control. Error bars (standard deviation) were obtained from three repeats of the test.

3.5 RESULTS AND DISCUSSION

3.5.1 Effect of additives on paper-immobilized antibody stability

The stability of antibodies in papers with and without additive was tested every 7 days for 28 days. Figure 2 shows the agglutination patterns of RBCs in papers with and without the pre-mixed additives on Day 0 (top row) and Day 28 (bottom row). The bottom row of Figure 2a shows that antibodies in papers not protected by additives (N-A, N-B and N-D) have lost the ability to agglutinate antigen-positive RBCs after 28 days of storage at room temperature. In contrast, antibodies in papers with the protection of additives retained a reasonable level of their activities after 28 days under the same storage condition and can effectively agglutinate the antigen-positive RBCs.

Figure 2. The specific (+) and non-specific (−) test results of paper squares loaded with antibodies on 0 day (top) and 28th day (bottom): (a) paper squares without additives (N-A), (b) paper squares with 1% PVP (P-A), (c) paper squares with 6% dextran (P-D), (d) paper squares with 10% glycerol (G-A). All samples were kept at room temperature in the dark. The dimensions of all the paper squares are 1 cm × 1 cm.

Figure 3 shows the color intensity measurement of agglutinated RBCs on papers carrying different antibodies as a function of storage time at room temperature. All
additives showed some degree of protection of the antibodies. Paper loaded with antibodies, but without additives, began to lose their ability to agglutinate RBCs after 7 days at room temperature (Figure 3a, b and c); after 14 days these papers had lost nearly all their bioactivities and were unable to agglutinate antigen-positive RBCs. At the same time it was observed that these papers developed a significant level of hydrophobicity. This phenomenon has been observed in an earlier study [18]. This result suggests that antibody molecules in paper may not have gained much protection from interactions with cellulose fibre at the molecular level. Although cellulose fibre surface has a large number of –OH groups, which are intuitively capable of interacting with antibody molecules, these –OH groups may not be sufficiently free to interact effectively with antibody molecules to provide protection. The dimension of the microfibres forming on the cellulose fibre surface may be too large and too rigid to protect antibody molecules.

Figure 3. Influence of additives on the stability of antibodies in paper. Papers loaded with (a) Anti-A, (b) Anti-B, (c) Anti-D were stored at room temperature in the dark. The error bars represent the standard deviations of triplicates.

The increased hydrophobicity of paper squares carrying unprotected antibodies can be explained as the result of protein dehydration and denaturing. As the antibody dries in paper, the capillary action will draw the antibody molecules towards one another. During this process the hydrogen bonding network around antibody molecules will alter, leading to irreversible aggregation of antibody molecules to form on fibre surfaces. At the same time the protective environment of water molecules surrounding the antibody molecules will change, leading to changes in the antibody molecular conformation. The increase in hydrophobicity is likely caused by the exposure of the hydrophobic sections of the protein. This explanation is supported by our further study (not presented) where antibody-loaded papers were kept wet; antibodies in wet papers
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retained their hydrogen bonding network unaltered and were able to retain their bioactivities for a much longer time. Polar groups of amino acid residues in antibody peptide chains, including $-\text{OH}$, $-\text{NH}_2$, $-\text{COOH}$ groups, are responsible for maintaining the stereo configuration of the antibody molecules through maintaining the hydrogen bonding network within and around the antibody. The antibody molecule is unique to the corresponding antigen and it must have the unique stereo configuration in order to approach the specific binding sites of the antigen and bind the antigen. Therefore, any alteration of the antibody hydrogen bonding network can alter the stereo configuration of its antigen binding sites or structure around the sites. This will lead to the loss of the antibody’s ability to specifically bind to its corresponding antigen. The abundant free water molecules in a wet paper provide an environment where hydrogen bonding network around antibody molecules changes much more slowly than on dry paper; wet paper therefore provides longer protection to antibodies than dry paper of 3 months.

For papers loaded with antibody-glycerol mixtures (G-A, G-B and G-D), measurable color density differences between the ‘+’ and ‘−’ assays were clearly observed after 28 days of storage at room temperature. This indicates that glycerol has a protective effect on antibodies, which reduces with time. The protective effect of glycerol on protein in aqueous systems has been reported [20]. The small molecular size of glycerol and its strong hydrogen bonding potency with water allow glycerol to be a part of the hydrogen bonding network formed dominantly by water molecules around antibody molecules. We have placed the antibody-glycerol solution mixture on glass slides to perform antibody aging tests (not presented). We found glycerol can provide effective protection to antibody molecules when used in an aqueous system. These results suggest that glycerol cannot provide long-term protection to paper-immobilized antibody. The most likely reason for this unexpected result lies in the extensive interactions of glycerol with cellulose fibres. Fabritius et al. [21] studied the penetration behaviour of glycerol into paper sheet using optical coherence. They found that glycerol molecules could rapidly penetrate into cellulose fibre wall, swelling fibres. The diffusion of glycerol into the fibre wall is detrimental to its antibody protection ability as it decreases its availability to antibody molecules. Our data therefore show a negative aspect of using a low molecular weight humectant as a protective reagent for antibodies when the antibodies are introduced to the surface of cellulose fibres, which also have natural and strong interactions with the additive molecules.
Polymeric additives have been used for antibody stabilization and serological applications. For example, PVP has been used as a blood plasma extender and has low level of toxicity [22] and dextran has also been used as an antibody stabilizer [23, 24]. Figure 3 shows that PVP and dextran provide greater stability to antibodies than glycerol. This is expected as both polymers used in this work have a molecule weight $> 3 \times 10^4$ Da. It is difficult for molecules of such size to penetrate into the fibre walls, therefore they are more available to effectively interact with antibody molecules and the water molecules surrounding them. Antibodies in paper protected by dextran demonstrate a higher color intensity of RBC agglutination from day 0 than that by PVP. However, this protective effect was similar over time, which can be seen from the near-parallel curves of the color intensity loss with time (Figure 3). Such difference is most likely caused by the physical properties of those additives on paper and not by biochemical properties, since this difference is consistently observed for all three antibodies from day 0. We believe that the higher concentration of dextran in the antibody mixture compared to that of PVP explains the difference. This dextran-antibody complex could cause stronger RBC agglutination to occur closer to the surface of the paper, leading to the stronger color intensity observed.

3.5.2 Stability of freeze-dried antibody immobilized on paper

Freeze-drying as a physical drying method is commonly used to provide long-term storage stability to proteins and pharmaceuticals [23-25]. In this study we focus on understanding the effectiveness of this method in protecting antibodies in a cellulose fibre network. Figure 4 shows the agglutination intensity on paper sensors. The agglutination of antigen-positive RBCs by the corresponding antibodies after long storage time shows that the protective effect of freeze-drying on antibodies is strong. The color intensity measurement presented in Figure 5 also confirms the visual assessment results in Figure 4. These results show that freeze-drying can preserve antibodies on paper more efficiently than small molecular weight and polymeric additives. The strong protective effect of freeze-drying on antibody in the cellulose fibre network is likely to be caused by the absence of capillary action between antibody molecules and the cellulose fibre surface. Before freeze-drying, the intermolecular distance between the antibody molecules in an aqueous solution is greater than the hydrogen bonding distance, because of the presence of water molecules. Since water is removed from the paper as vapour, the Laplace pressure, which is associated with the
liquid meniscus, is absent. It is likely that in the absence of the Laplace pressure the antibody molecules are not pressed towards one another, but remain in their natural position as the water is being gradually removed.

![Image](image1.png)

**Figure 4.** The specific (+) and non-specific (–) test results of freeze-dried paper squares loaded with antibodies after stored for (a) 0 day, (b) 3 days, (c) 7 days, (d) 14 days, (e) 28 days, and (f) 42 days. All samples were stored at room temperature in the dark. The dimensions of all the paper squares are 1 cm × 1 cm.

![Image](image2.png)

**Figure 5.** The stability of freeze-dried antibodies in paper squares. The error bars give the standard deviations of triplicates. All the freeze-dried paper squares loaded with antibodies were kept at room temperature in the dark.

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No additive was used in this study with freeze-drying to preserve the antibodies. Our results show that the presence of cellulose fibres does not adversely affect the protection of freeze-drying on the antibodies within the time of study. These results are also in agreement with recent studies reported for this type of paper-based sensors, where antibodies predominantly locate in interfibre pores and do not diffuse into the wall of the cellulose fibres [26, 27].

3.5.3 Thermal stability of freeze-dried antibody immobilized on paper

Freeze-dried papers loaded with antibodies were aged at different temperatures for 6 h to test the thermal stability. The evaluation of antibody stability was performed by measuring the grayscale intensity of RBC agglutination as mentioned above. Biomolecules like protein are prone to denature and become deactivated by exposure to extreme pH or heat. In this study, we found that unprotected Anti-A in an aqueous solution became irreversibly denatured when heated at 60 °C for 1 h while Anti-B lost its activity in just 30 min. Even though Anti-D has the strongest vitality, it was deactivated by heating at 60 °C for 6 h in an aqueous solution.

However, the thermal resistance of these freeze-dried antibodies in fibre network was found to be significantly improved. Our results show that the freeze-dried antibodies in paper retained most of their activities after incubation under 4 °C, 25 °C, 40 °C and 60 °C for 6 h (Figure 6). Figure 7 presents the measured color intensity of RBC agglutination on papers loaded with the antibodies incubated at different temperatures. The results show that freeze-drying of antibodies loaded in paper is capable of not only increasing the longevity of antibodies at room temperature, but also significantly increasing the thermal stability of the antibodies. The lack of aggregation of antibody molecules in freeze-dried papers is likely to also contribute to the thermal stability of the antibody molecules. The large distance between the freeze-dried antibody molecules reduces the possibility of hydrogen bonding between the non-agglutinated antibody molecules.
Figure 6. The specific (+) and non-specific (−) test results of freeze-dried antibodies in paper squares after 6 h of incubation at different temperatures, (a) 4 °C, (b) 25 °C, (c) 40 °C and (d) 60 °C. The dimensions of all the paper squares are 1 cm × 1 cm.

Figure 7. The thermal stability of freeze-dried antibodies in paper squares. The error bars represent the standard deviation of triplicates. All freeze-dried paper squares loaded with antibodies were incubated at different temperatures (4 °C, 25 °C, 40 °C and 60 °C) for 6 h, and were tested 30 min after incubation.

3.6 CONCLUSIONS

We show in this study that the longevity of blood group antibodies immobilized on paper is partially improved by the mixing of additives such as glycerol, PVP and dextran. Among them, low molecular weight additives that are capable of penetrating into the fibre wall provide a lower level of protection to antibodies than the high molecular weight polymers. This finding provides useful guidance to future paper biosensor design in that the interactions between additives and cellulose fibres must be considered in order to optimize the protective effect of the additives on biomolecules.
Furthermore, freeze-drying provides a much higher level of protection to antibodies than additives. The lack of antibody aggregations in freeze-dried paper reduces the intermolecular hydrogen bonding of antibody molecules; this allows them to retain their natural configuration and interact with the antigens on the RBC surfaces. The non-aggregation of freeze-dried antibodies in paper has also shown much higher thermal stability than the unprotected antibodies. Our preliminary study shows that freeze-drying is a feasible method for providing long-term protection to paper-immobilized biomolecules, whereas mixing additives with biomolecules needs to be further studied for applications where inexpensive long-term protection is required. It is expected that the outcome of this study could be used for other paper-based biosensors to satisfy the basic requirement of sensor longevity.

3.7 ACKNOWLEDGEMENT

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3.8 REFERENCES


Polysaccharides as protectants for paper-based analytical devices with antibody
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In the previous chapter, the preliminary study shows that freeze-drying technology is a feasible approach to retain protein activity on paper. Considering the requirement for instrumentation and costs, it is necessary to explore other cheap strategies for the conservation of protein activity on paper. In the previous work, the freeze-drying method was compared with additive mixing. The results show that the use of polymeric additives (e.g., dextran) may have the potential to provide long-term protection to paper-immobilized protein. Hence, in this chapter we explore a simple and low-cost approach (i.e., polysaccharide-based film encapsulation) to achieve stable antibodies on paper to improve bioassay performance. Our work focuses on exploring easy and versatile approaches to the protection of paper-immobilized antibodies as well as the interactions of protein and natural polysaccharides on the paper substrate. The results of this work provide an important basis for the further development of paper-based biosensors.
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Monash University

Declaration for Thesis Chapter 4

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

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<td>Initiation, key ideas, experimental works, analysis of results, writing up</td>
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The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

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<tr>
<td>Wendy Tian</td>
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<tr>
<td>Wei Shen *</td>
<td>Key ideas, paper reviewing and editing</td>
<td>Supervisor</td>
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The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

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*Note: Where the responsible author is not the candidate’s main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.
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Polysaccharides as protectants for paper-based analytical devices with antibody

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4.1 ABSTRACT

Paper with immobilized protein can provide a low-cost platform for diagnostics, but evidence is emerging that the instability of protein on paper during shipping and storage severely limits its commercial development. Of these, paper-immobilized antibody, though widely used, is in urgent need of improvement in the shelf life. Based on the preliminary study, herein we further provided a detailed investigation of a simple, low-cost and versatile approach for achieving stable antibody on paper to improve bioassay performance. Anti-Blood Group A IgM was chosen as the model antibody and its activity was tested via agglutination reactions. Representative polysaccharides were used as protectants and their ability to stabilize antibody under heat and desiccation stresses was investigated. It was found that the presence of dextran with high concentration markedly stabilized paper-immobilized IgM for at least 120 days. Our results indicate that dextran with good film-forming ability could be a protectant for paper-immobilized antibody.

4.2 KEYWORDS

Dextran, antibody immobilization, paper-based biosensor, polysaccharide protection
4.3 INTRODUCTION

Protein, as sensitive biological compounds, has an increased tendency to lose function under some external stress such as heat, radiation, desiccation and shearing [1-3]. It usually requires temperature-controlled supply chains to retain biological activity in the pharmaceutical and biotech industries, resulting in cost increase and use limitation [4,5]. Formulations for protein storage and stability have been developed; freeze-drying is the most common strategy to preserve protein in the form of dehydrated powders [6]. Such powders are typically used to extend the shelf life of products, but some types of protein still can be damaged during the process [7,8].

The development of many experimental approaches and applications in biological and chemical sciences has brought about an increasing requirement for the immobilization of protein onto solid substrates [9,10]. Protein immobilized on various supports also needs to be protected to perform the biological functions. Among them, paper has demonstrated excellent properties as a novel support, including low cost, portability and feasibility of patterning via inkjet printing [11,12]. Many studies reported the potential of protein-based paper biosensors as simple point-of-care devices, but at the same time highlighted the need for protein stabilization strategies [13,14].

As an important type of protein, antibody, also known as immunoglobulin, plays a crucial role in current bioactive paper-based diagnostics [15-17]. The storage and stabilization of paper-immobilized antibody is a basic concern to evaluate the performance of these bioassays. The work by Ramachandran et al. and the preliminary study in our group have shown that freeze-dried antibody immobilized on paper could be stored for a long period of time at room temperature without significant loss of the bioactivity [18,19]. However, the freeze-drying process is not a cost-effective way for the large-scale manufacturing of bioactive paper with immobilized antibody, which, in turn, makes itself not suitable to be a generic approach. Different from antibody protection, enzyme, as another commonly used type of protein for bioactive paper, has been successfully protected on paper by several easy and inexpensive approaches in the past few years. Alkasir et al. observed that tyrosinase enzyme, entrapped between chitosan and sodium alginate layers deposited onto filter paper, could show 92% residual activity after 260-day storage [20]. Zhang et al. reported that glucose oxidase and lactate oxidase could retain full activity over 6 months of storage on starch-coated
Chapter 4

paper [21]. Therefore, currently antibody is in urgent need of a simple, low-cost and robust solution of its bioactivity protection. Encouraged by the above-mentioned successful application of natural polysaccharides on paper-immobilized enzyme preservation, it is of great value to examine the compatibility of this cost-effective approach with paper-immobilized antibody. Meanwhile, although the studies have presented effective enzyme protection on paper via polysaccharides, there is a lack of detailed analysis about polysaccharide use, which could give more accurate instructions to the fabrication of bioactive paper.

Of the five classes of immunoglobulins (IgA, IgD, IgE, IgG and IgM), IgG and IgM are by far the most frequently used antibodies in bioassays, and they share a similar structure and function [22]. Considering IgM is more susceptible than IgG to denaturation, Anti-Blood Group A IgM with great use was chosen as a model antibody in our study. These agglutinating antibodies have been widely used to fabricate paper-based analytical devices for point-of-care diagnostics. Rapid blood typing can be performed on paper by using agglutinating antibodies to induce red blood cell (RBC) agglutination [23,24]. Besides, plasma separation based on RBC agglutination has been proved to be useful for designing paper-based bioassays for detecting plasma components like glucose [25]. Thus the long-term stability of antibody on paper is important for improving bioassay performance. Herein, various polysaccharide strategies of immobilizing IgM on paper were investigated, including polysaccharide covering, supporting and blending. The important factors involving polysaccharide modification and IgM immobilization were analysed by agglutination reactions. The results provide an easy and inexpensive path to prolong the shelf-life of antibody-based paper biosensors and serve as valuable references for the implementation of paper-based biosensing systems.

4.4 EXPERIMENTAL

4.4.1 Reagents and instrumentation

Low-molecular-weight chitosan (Cat. No. 448869, 20-300 cP, 1% in 1% acetic acid), low-molecular-weight sodium alginate (i.e., NaAlg, Cat. No. A0682, 4-12 cP, 1% in H₂O) and low-molecular-weight dextran (Cat. No. D1662, ~4 cP 10% in H₂O [26]) were purchased from Sigma-Aldrich. Anti-Blood Group A IgM (abbreviated as IgM in this study) for further manufacturing use (FFMU) purchased from Commonwealth
Serum Laboratories (CSL) Australia was used as the model antibody. Red blood cells (RBCs) from CSL RevercellTM (15% A1 and 15% B RBCs) were concentrated to 45% before use. Kleenex paper towel (Kimberly-Clark, Australia), as the paper substrate, was cut into 1 cm × 1 cm pieces to immobilize antibodies. All reagents were used without further purification and all solutions were prepared using ultrapure water (18 MΩ cm, Milli-Q Gradient System, Millipore).

Dark field images of papers were obtained by an Olympus BX60F microscope. Fluorescence images of fluorescein isothiocyanate labelled bovine serum albumin (FITC-BSA) and RBCs labelled with FITC were captured with a Nikon confocal microscope. SEM images were recorded with a FEI Nova NanoSEM™ scanning electron microscope. Water Drop Penetration Time (WDPT) test was carried out by a contact angle instrument (Dataphysics OCA230, Germany) as follows: A paper square was mounted onto the sample holder, and a 3 μL ultrapure water droplet was delivered onto the paper surface by a glass syringe. Then the penetration time of the water droplet through the paper was calculated from the video of the water drop penetration process. The test was repeated three times at room temperature. The images of agglutination assays (see below for details) were recorded by a desktop scanner and quantitative analysis was achieved using ImageJ software to invert and measure the grayscale intensity of blood spots. Error bars (standard deviation) were obtained from three repeats of the test.

4.4.2 Red blood cell agglutination assays

The interaction of IgM with RBCs can have two possible results: agglutination, referred as positive assay (+), and non-agglutination, referred as negative assay (-). Kleenex paper towel that has larger paper pore size than filter paper possesses an enhanced ability to elute non-agglutinated RBCs, although Kleenex paper towel and filter paper have comparable ability to fix agglutinated RBCs [27]. Thus Kleenex paper towel was chosen as the paper substrate in this study. The color intensity value can be obtained by subtracting the measured intensity of the negative from that of the positive in the grayscale mode (0–255). Successful testing should easily distinguish the positive assay from the negative one; that is, the positive assay should report a well-defined blood spot on paper while non-agglutinated RBCs from the negative assay can be easily washed out of the paper by 0.01 M pH 7.4 phosphate-buffered saline (PBS) (Figure 1a
and 1b). So a high color intensity value would be obtained. In order to perform the bioactivity evaluation of paper-immobilized IgM, both 3 μL 45% antigen-positive and antigen-negative reagent RBCs (i.e., A1 and B RBCs) were respectively introduced onto two testing paper squares immobilized with IgM, followed by 90 s incubation and then 2× 50 µL PBS washing. If the bioactivity of IgM is not retained on paper, the positive assay could not report a well-defined blood spot, but falsely show the result as a negative one (Figure 1c). Therefore, we would get a low color intensity value. The value of color intensity declined with the decrease of paper-immobilized IgM activity. Obviously, agglutination assays as an indicator provide a simple and semi-quantitative method for evaluating the bioactivity of paper-immobilized IgM.

![Figure 1](image_url)  
*Figure 1.* Schematic presentation of antibody activity test via agglutination assays on paper.

**4.4.3 Antibody immobilization on papers with different polysaccharide formulations**

*Polysaccharide covering:* 5 μL of IgM was spotted on the paper squares and dried for 30 min. Then 5 μL of chitosan solution in aqueous acetic acid with 0.9% NaCl, NaAlg solution in PBS and dextran solution in PBS were respectively applied to the paper squares, followed by 30 min drying at room temperature. With this method, IgM spotted on paper surface was covered by polysaccharides (Figure 2).
Polysaccharide supporting: 5 µL of chitosan, NaAlg and dextran solution were applied to the paper squares and allowed to dry for 30 min, respectively. Then 5 µL of IgM was spotted on each of the freshly prepared papers and dried for 30 min. With this method, IgM was spotted on a polysaccharide layer that supported the IgM and reduced the contact between IgM and paper (Figure 2).

Polysaccharide blending: 5 µL of IgM solution containing chitosan, NaAlg or dextran were respectively applied to the paper squares, which were then dried for 30 min. With this method, IgM was incorporated into the polysaccharide matrix (Figure 2). The activity of immobilized IgM was evaluated by the agglutination reactions as mentioned above.

Figure 2. Schematic presentation of antibody immobilization on paper via different polysaccharide strategies.

4.4.4 Thermal and storage stability studies of different papers with immobilized antibody

During heating studies, the freshly-prepared paper samples were aged in an oven at 60 °C for various incubation periods. For long-term stability studies, all paper samples were prepared as previously described, placed in pill containers under ambient conditions, and stored at room temperature in the dark. Residual bioactivity of paper-immobilized IgM was evaluated using agglutination tests.

4.5 RESULTS AND DISCUSSION

4.5.1 Concentration-dependent antibody bioactivity

The knowledge of paper-immobilized antibody stability can strongly help to reduce the cost while sustaining the effectiveness of paper bioassays. So we first investigated the impact of antibody concentration on the stability of paper-immobilized IgM when stored at room temperature in the dark. The serial dilution data show that IgM, although being diluted, retained its activity at day 0 (Figure 3). However, the loss of IgM activity was observed at all storage concentrations within 10 days. The original IgM (protein
concentration ranging from several mg mL\(^{-1}\) to tens of mg mL\(^{-1}\)) had the relatively low rate and extent of deactivation, while activity loss was comparatively obvious for diluted IgM. As shown in the inset image in Figure 3, there is no significant color difference between positive and negative results for paper samples with 50-fold and 100-fold diluted IgM, causing low intensity values. The results were consistent with previous reports that many proteins could display greater stability at higher concentration [28]. The obvious deactivation of IgM only after being stored for 10 d for the whole dilution-series suggests that proper storage is required for antibody stability on paper. However, the results (not shown) indicate that the dilution-series of IgM via dextran blending strategy retained its activity within 10 days. Thus it is of great value to perform a detailed analysis of polysaccharide protection for paper-immobilized IgM. Considering the commonly used concentration range of paper-immobilized protein (tens of to hundreds of \(\mu\)g mL\(^{-1}\)) and agglutination assay responses to the dilution-series of IgM, a 1:10 dilution of IgM was used throughout this study, unless specially noted.

**Figure 3.** Influence of concentration on the activity of paper-immobilized IgM when stored for 0 (■) and 10 days (□). The inset image shows the color difference between paper samples incubated with A1 RBCs (+) and B RBCs (−) respectively, after 10 d storage.

### 4.5.2 Polysaccharide modification and antibody immobilization

Inspired by successful biomolecule immobilization via layer-by-layer assembly technique for many applications [29,30], herein we proposed three simple types of
antibody immobilization strategies: covering, supporting and blending, which were classified based on the role played by polysaccharides during the process of antibody immobilization. Representative polysaccharides, i.e., cationic chitosan, anionic NaAlg and neutral dextran, were chosen for antibody immobilization. On one hand, cellulosic fibres have a porous structure (1-30 nm micropores) [31]. Due to the nanoporosity of cellulosic fibres, polysaccharides with molecular weights of several thousands could penetrate into the fibre wall, which may preclude the effective antibody-polysaccharide interactions. On the other hand, polysaccharides with high molecular weight cannot be applied onto paper easily and cannot provide effective protection for proteins via intermolecular interactions due to steric hindrance [32], so polysaccharides with relatively low molecular weights (up to tens of thousands) were utilized. Moreover, an increase of polysaccharide concentration would obviously increase the viscosity of the polysaccharide solution, resulting in the difficulty of sample loading onto paper. In practice, chitosan and NaAlg were used with the concentration of no more than 2% w/v while concentrated dextran solution (up to 40% w/v) was employed. We believe that the differences in the compositions and structures of polysaccharides determine the different operating ranges of concentration. Besides hydroxyl groups, amino groups in chitosan and carboxyl groups of NaAlg can also form hydrogen bonds with hydroxyl groups of polysaccharide chains respectively, which could cause the relatively high viscosity of polysaccharide solutions.

Dark field microscopy and SEM were used to characterize the nature of polysaccharide coatings on paper. Many polysaccharides were reported previously to have the good film-forming ability because of their intra and intermolecular hydrogen bonds [33]. High-concentration dextran (i.e., 40%), as shown in Figure 4, could supply enough polysaccharide to fill the pores and voids between the fibres, thereby developing a thin film on the paper surface. By contrast, chitosan and NaAlg at 2% concentration only interpenetrated the fibre network, generated evenly distributed coatings on surface of paper fibres and did not obviously change the surface porosity of paper. Then the morphology and distribution of immobilized antibody via different strategies was examined by using 1 mg mL\(^{-1}\) FITC-BSA as a model protein. Figure 4 shows that FITC-BSA via the blending strategy could be relatively well distributed on the paper. Also, FITC-BSA via the covering strategy and supporting strategy could be relatively well distributed on the paper (data not shown). It was found that proteins with different
immobilization strategies could penetrate almost throughout the paper network and were relatively homogeneous in the direction of lateral solution movement.

![Figure 4](image)

**Figure 4.** Imaging and characterization of paper samples. Upper row: dark field images of different papers. Middle row: SEM images of different papers. Lower row: confocal images of papers immobilized with FITC-BSA via the blending strategy. Insets: images of the edges of the solution flow. Paper without polysaccharide modification was chosen as the control.

### 4.5.3 Antibody performance on different papers

An ideal immobilization strategy should not do damage to the functionality of biomolecules, and therefore the effect of employing various polysaccharide formulations for IgM immobilization on paper was initially evaluated by performing RBC agglutination assays. All three chitosan-based immobilization methods, as shown in Figure 5, were proved to fail to perform effective agglutination assays. It was therefore not suitable to evaluate the protective power of chitosan to antibody molecules via agglutination assays. Such behavior is related to electrostatic interactions between the positively-charged protonated amine on chitosan chains and negatively-charged RBC membrane. When treated with chitosan, RBCs lost their typical
biconcave morphology and coalesced into a clot that could not be easily washed off paper sheet by PBS buffer (Figure 6). Thus no significant difference was observed between positive and negative testing, which resulted in the low intensity value.

**Figure 5.** Agglutination assays on different papers: paper-immobilized IgM with % w/v concentrations of polysaccharides (chitosan from 0.5 to 2, NaAlg from 0.5 to 2, dextran from 10 to 40); paper-immobilized IgM without polysaccharides as a control. Relative intensity was expressed as a percentage compared with the color intensity value of the control.

**Figure 6.** Confocal images of FITC-labelled RBCs on different papers: (a) control paper; (b) chitosan paper; (c) NaAlg paper; (d) dextran paper.

Unlike chitosan, different immobilization approaches using NaAlg or dextran display different results. Among the three strategies, the most effective RBC agglutination was observed via polysaccharide-blending immobilization strategy (Figure 5), and the color intensity was found in the increasing order of covering<supporting<blending strategies.
Despite a lack of electrostatic attraction like chitosan, NaAlg and dextran can still act as a physical shield to restrict RBC access to the active sites of IgM when choosing the covering method, which in turn indicates the limitation of polysaccharide-covering strategy as a generic approach for immobilizing biomolecules on paper because of potential interference with bioassays. However, the other two strategies enabled IgM to react with RBCs more efficiently than the control (paper-immobilized IgM without polysaccharides), as polysaccharides with certain viscosity could reduce the flow rate and facilitate the interaction between the immobilized IgM and RBCs. The latter strategy (blending) was better than the former (supporting) since blending could promise a sufficient contact between IgM and RBCs.

4.5.4 Paper-immobilized antibody storage

To evaluate the application potential of polysaccharide as paper-immobilized antibody stabilizer, we compared the stability of IgM under different conditions. As mentioned above, IgM that was deposited on paper without any polysaccharide protection denatured rapidly and lost its function. Figure 7 shows that IgM deposited on paper by the blending strategy in the presence of NaAlg or dextran has the improvement in shelf life. For polysaccharide solutions at the same concentration, the supporting strategy can provide less protection to IgM than the blending strategy during 2-month storage; that is to say, the signals from the supporting strategy decreased more rapidly than those from the blending strategy over 2 months of storage (data not shown). Irreversibility of polysaccharide adsorption to cellulose has been reported because of the similarity in their chemical structures [34]. Unlike the homogeneous blending, we believe that the supporting strategy makes polysaccharide unable to dissolve sufficiently to engage in the interactions with IgM effectively when adding IgM on polysaccharide-coated paper, which is not good for the protection of IgM on paper. The blending method, on the other hand, delivered a relatively satisfying signal within 2 months. Even with the low concentration, a detectable signal was still observed at day 60, indicating that some of antibody was still active (Figure 7). These results confirmed that polysaccharide could extend the shelf life of antibody deposited on paper.
Figure 7. IgM storage stability on different papers. (a) Paper-immobilized IgM via NaAlg blending. (b) Paper-immobilized IgM via dextran blending. Relative activity was expressed as the percentage of IgM activity that remained after storage in comparison to the activity at day 0 for each group.

Stability improvements via blending strategy became much stronger in the comparison of supporting and blending in the two polysaccharide systems; stabilization of antibodies blended with polysaccharide was proved to be dependent on polysaccharide concentration: color intensity increased with increasing concentration for NaAlg and dextran (Figure 7). During dehydration and storage, IgM blended with polysaccharides could bring about the matrixes with high viscosity and low molecular mobility. The entrapment of IgM in dried polysaccharides with an amorphous state prevents the unfolding of antibody molecules. As can be seen in Figure 7a, NaAlg, although only small amounts were used, still could provide a viscous environment for IgM preservation. In terms of dextran, higher concentration resulting in higher viscosity and more IgM-dextran interactions prevented IgM denaturation. Notably, when the dextran dosage in the blend was increased to 40%, about 85% initial potency of IgM was retained after 2 months of storage at room temperature (Figure 7b) and paper-immobilized IgM shows ~ 80% residual activity after 120-day storage (data not shown). These data indicate that the presence of dextran film is important for the improvement in stabilization of paper-immobilized IgM. As mentioned above, when 40% dextran solution resolidified into films upon drying, protein molecules were well distributed throughout the dextran film matrix. In this case, the entrapment of IgM into a biocompatible dextran film with good dispersion performance could effectively prevent IgM from going through conformational changes during storage.
Besides paper bioactivity, paper wettability, which determines the penetration of liquid samples or reagents into the paper sheet, is another important property that affects the performance of paper-based bioassay. We believe that a moderate level of wettability is favorable for bioactive paper, which may enhance the interaction between liquid samples or reagents and paper-immobilized biomolecules. In this study, antigens on the surfaces of RBCs and IgM in the polysaccharide layer require time to interact. This interaction is dependent upon the dissolution of polysaccharide matrices and mixing of RBCs with released IgM. A moderate wettability of bioactive paper could allow time for those events to occur. In contrast to this, very low paper wettability is not conducive to antigen-antibody interaction, since low wettability suggests that slow dissolution of polysaccharide might be encountered, which could cause incomplete or even no assay development because of insufficient paper wetting. As can be seen in Table 1, an increased storage time can reduce the wettability of papers with immobilized IgM, which can be attributed to the exposure of the hydrophobic regions of the antibody, promoting intermolecular interactions leading to aggregation. Since protein denaturation could cause an increase in the surface hydrophobicity, we therefore expected a significant improvement of paper wettability via 40% dextran blending that could offer efficient antibody protection. The data of the water drop penetration time supports our reasoning, and the paper with 40% dextran blending strategy has a moderate level of wettability after storage that could be useful for preforming paper-based bioassays. The high stability of IgM in the dextran film provided effective support for the retention of hydrophilicity of paper with immobilized IgM.

**Table 1.** Water drop penetration time (second) of paper samples with immobilized IgM in the absence of polysaccharides (as the control) or in the presence of polysaccharides via blending strategy.

<table>
<thead>
<tr>
<th>Time</th>
<th>Control</th>
<th>NaAlg 0.5%</th>
<th>NaAlg 1%</th>
<th>NaAlg 2%</th>
<th>Dextran 10%</th>
<th>Dextran 20%</th>
<th>Dextran 40%</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 d</td>
<td>6.780±0.728</td>
<td>31.13±3.004</td>
<td>36.39±3.872</td>
<td>52.56±6.111</td>
<td>41.01±6.040</td>
<td>56.00±12.994</td>
<td>17.58±4.626</td>
</tr>
<tr>
<td>60 d</td>
<td>15.29±1.972</td>
<td>46.14±5.68</td>
<td>76.72±10.52</td>
<td>98.66±9.003</td>
<td>54.19±5.327</td>
<td>70.61±5.004</td>
<td>30.18±7.013</td>
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Samples were stored at RT conditions. The data represent the mean ± the range.
4.5.5 Thermal Stability of Antibody Immobilized on Paper

Obviously, the limited refrigeration resources, particularly in developing countries, also promote demand for good thermostabilization of paper-based biosensors; that is, paper-immobilized biomolecules should be relatively stable under continuous hot weather exposure. Hence the heat stability of paper-immobilized IgM was evaluated after exposure to 60 °C for various heating times. As shown in Figure 8a, IgM in aqueous solution with and without polysaccharides lost its activity by heating at 60 °C for 1 h. Because the degradative processes of protein could be facilitated in the presence of water [35], different concentrations of NaAlg or dextran cannot offer enough protection for IgM molecules in aqueous solutions against heat denaturation.

Interestingly, paper-immobilized IgM displays different levels of stability when tested under acute heat stress conditions. Even in the absence of polysaccharides, only a moderate loss (~ 40%) of IgM activity was observed after an eight-hour heat treatment (Figure 8b). Considering the structural relevance of IgM and IgG, the results are in reasonable agreement with those obtained by Wang et al., where they reported paper-immobilized IgG was relatively stable at 80 °C and lower [16]. We think that the immobilization of antibody onto paper can reduce thermal movement, which in turn prevents antibody denaturation through aggregation. IgM on paper protected by polysaccharides shows similar profile of activity loss to IgM on paper without protection, but having a reduced magnitude. Polysaccharides therefore provide protection against thermal inactivation for paper-immobilized IgM. Both NaAlg and dextran can suppress the heat-induced inactivation of paper-immobilized IgM in a concentration-dependent manner. The highest recovery of IgM activity was observed in the presence of the highest concentration of NaAlg (2%) and dextran (40%); approx. 20% and 15% activity loss after 8-hour heating, respectively. Such protection could be related to the inhibition of structural alterations in antibody. Polysaccharides like NaAlg and dextran, having a relatively high glass transition temperature in the dry state, can inhibit molecular mobility and consequently enhance IgM thermostability on paper.

Pronounced stabilization of IgM in dextran film at the elevated temperature suggests this method could also provide sufficient thermal stability to paper-based biosensors. The concentration of dextran has less effect on thermal stability than on storage stability. We believe that it may be associated with the different rates of dehydration under different conditions. Dextran film provides an inexpensive and effective
protection environment without the use of vacuum and desiccant. Moreover, unlike chitosan and NaAlg, natural polymeric carbohydrates like dextran, having hydroxyl groups as the primary functional groups, is very hydrophilic and non-charged, which could be benign to typical bioassays [36, 37].

**Figure 8.** IgM thermal stability at 60 °C. (a) Paper-based agglutination assay of IgM solution after 1 h heating: IgM solution with or without polysaccharides (control) was heated and then dried on paper followed by the assay. (b) Paper-immobilized IgM thermal stability as a function of time. Paper-immobilized IgM without polysaccharides as a control. Relative activity was expressed as the percentage of IgM activity that remained after incubation in comparison to the activity at hour 0 for each group.

### 4.6 CONCLUSIONS

In this work, the application area of polysaccharide protection was extended and dextran was applied as an effective formulation component for enhancing stability of antibody attached to paper. Appropriate coating system (blending) could increase the shelf life of IgM compared with IgM deposited onto unmodified paper. The stabilization data presented here indicate that the formation of hydrophilic films in paper plays a vital role in protecting paper-immobilized antibody. Dextran reduced protein unfolding and aggregation at elevated temperatures, and also provided structural stability to IgM during storage. Since most studies emphasize the invention of paper-based bioassays, we believe that our simple and low-cost protection strategy will be of use for the large scale development of paper-based bioanalytical devices.
4.7 ACKNOWLEDGEMENT

The authors thank John Zhu at the Melbourne Centre for Nanofabrication (MCN) in the Victorian Node of the Australian National Fabrication Facility (ANFF) for confocal imaging and technical help. Postgraduate research scholarships from Monash Graduate Education and Faculty of Engineering are gratefully acknowledged.

4.8 REFERENCES


Chapter 5

Precipitation assay meets low wettability on paper:
a simple approach for fabricating patterned paper sensors
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While the previous chapters focus on important steps in the fabrication of paper-based analytical devices, this chapter return to the paper-based sensor itself, that is, the design of paper-based sensors as a whole. Here we report on a simple and effective approach to fabricate patterned paper sensors that perform precipitating assays. This obviates the need for hydrophobic barriers and provides the simplest signal readout with results presented as text. More specifically, pens and stamps filled with reagent inks are used to fabricate text-patterned chemical and biochemical sensors on bovine serum albumin (BSA)-modified paper with tuned wettability, without the requirement for the preparation of hydrophobic barriers. After exposure to the analytes, the precipitates of sensing reactions are not delocalized, giving a simple and successful text-patterned signal readout. We demonstrate the broad applicability of this technique by sensing typical chemical and biological analytes. In addition, text-patterned paper sensors allow for the multiplexed measurement of different analytes. These patterned paper sensors yield comparable accuracy and precision to those of assays performed on conventional paper-based analytical devices. This work provides an easy, low-cost and generic route for fabricating cost-effective and user-friendly paper-based analytical devices.
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Monash University

Declaration for Thesis Chapter 5

Declaration by candidate

In the case of Chapter 5, the nature and extent of my contribution to the work was the following:

<table>
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<th>Nature of contribution</th>
<th>Extent of contribution (%)</th>
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<tr>
<td>Initiation, key ideas, experimental works, analysis of results, writing up</td>
<td>90</td>
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The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

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<th>Name</th>
<th>Nature of contribution</th>
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<tbody>
<tr>
<td>Wei Shen *</td>
<td>Key ideas, paper reviewing and editing</td>
<td>Supervisor</td>
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The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

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*Note: Where the responsible author is not the candidate’s main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.
Precipitation assay meets low wettability on paper: a simple approach for fabricating patterned paper sensors

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5.1 ABSTRACT

The development of paper-based analytical platforms has brought a requirement on easily creating patterned paper sensors. This article describes a facile method for fabrication of patterned paper sensors using a combination of wettability-controlled paper and the precipitation assays between analytes and sensing elements. Different from common fabrication processes in which patterned channels are defined by creating hydrophobic barriers, herein the direct deposition of the patterns of sensing elements onto paper is performed by simple writing and stamping. Without hydrophobic treatment, the patterned sensing components can remain in place on the paper substrate whose wettability is tuned via BSA modification. After exposure to the analytes, the precipitates of sensing reactions are not delocalized, giving a simple and successful patterned signal readout. The results of typical colorimetric assays show that slow capillary flow and insoluble products together on paper provide an extremely easy route for fabricating multifunctional patterned paper sensors.

5.2 KEYWORDS

BSA modification, patterned paper sensor, precipitation assay, wettability control
5.3 INTRODUCTION

Due to its affordability, portability and disposability, paper has become a promising sensing substrate for the provision of simple, low-cost and easy-to-use analytical devices [1-3]. These paper-based chemical and biological sensors are of great use for point-of-care clinical analyses and environment monitoring in developing countries and remote regions [4, 5]. The most common signal transduction technique of paper-based sensors is colorimetric sensing because of its inherent simplicity and compatibility with daily use devices such as a smartphone or a tablet [6]. Traditionally, paper-based sensors for colorimetric detection are designed based on spot assays [7]. But currently some groups have demonstrated novel measurement techniques including count-, distance-, and text-based detection [8-10]. Among them, the concept of reporting results in text allows for the simplest signal readout with a high level of user-friendliness and minimum equipment [10]. Usually, the highly functional patterned paper sensors are fabricated by using various techniques including dipping [11], etching [12, 13], photolithography [14, 15], printing [16-18], spraying [19], stamping [20] and writing [21]. These techniques use their corresponding hydrophobic materials to define hydrophobic barriers for creating the microfluidic patterning of paper substrates. Of these methods, the low cost and simplicity of stamping and writing have made them attractive for rapid proof-of-concept studies, assay optimization, and practical applications in low-resource settings.

Recently, Carrasquilla et al. [22] reported a simple technique for creating a text-patterned aptamer-based biosensor on paper wherein concatemeric DNA aptamers as the sensing elements, different from the monomeric type, could easily remain in place even without hydrophobic barriers. Their study demonstrated that the rational selection of sensing elements may be an effective approach to simplify the fabrication of paper-based analytical devices (i.e., eliminating barrier-building steps). Since a receptor part and a transducer part are two basic functional units for chemical and biological sensors, instead of changing the sensing elements of the receptor part in a paper sensor, is it possible that complicated hydrophobic treatment can be skipped by regulating the transducer part? It is commonly found in nature that a stone can be still with a streamlet running around it. Also, we noticed that color-based detection is desired in many applications, and precipitating colorimetric assays have been abundantly employed to detect diverse analytes such as biological samples [9], heavy metals [23], and toxins
Inspired by that, we think that the resulting insoluble, colored products of precipitating colorimetric assays would probably be trapped within the cellulose network of the paper after adding the aqueous analyte. Therefore, the successful patterned signal readout could be obtained even without consumption of hydrophobic materials.

More specifically, in porous networks of paper the color of colorimetric assays is influenced by wicking behaviour, and it is well known that paper surface modification can alter the way liquid penetrates paper. Modified paper can exhibit the desired degree of resistance to the penetration of liquid while unmodified paper readily absorbs water and other fluids [25]. Hence we hypothesized that in modified paper with slow liquid spreading, capillary action would be insufficient to cause the global motion of sensing elements. Moreover, modified paper would offer position control and spatial confinement of assay regions with the help of the low solubility of colored products. Since many groups have used commercial filter paper in their studies of paper sensing and biosensing, here we chose Whatman™ Grade 1 chromatography paper to illustrate our approach where patterned paper chemosensors and biosensors were simply and fast fabricated.

In this paper, BSA was firstly used for paper modification because of its compatibility and varied applications in biochemical assays. Then further patterning on BSA-modified paper via stamping and writing was performed and evaluated. To demonstrate the potential of BSA-modified paper as the sensor substrate, two representative colorimetric assays, a horseradish peroxidase (HRP) assay and a nickel(II) assay, were examined in detail. In both cases, assay reagents were directly patterned on BSA-modified paper with a pen or a stamp, and the assay performances were investigated. In addition, we successfully constructed a multifunctional integrated sensor combining two functional assays on a paper substrate (i.e., copper(II) and nickel(II) assays). We believe that this study will make a contribution to the future fabrication of paper-based analytical devices.
5.4 EXPERIMENTAL

5.4.1 Reagents and instrumentation

Copper sulphate pentahydrate, nickel(II) chloride hexahydrate, dimethylglyoxime (DMG), sodium diethyldithiocarbamate trihydrate (DDTC), BSA, HRP, fluorescein isothiocyanate labelled bovine serum albumin (FITC-BSA), fluorescein isothiocyanate labelled anti-human IgG antibody produced in goat (FITC-IgG), and 3,3’,5,5’-tetramethylbenzidine (TMB) liquid substrate system (insoluble type) were purchased from Sigma-Aldrich. All reagents were used as received and all solutions were made using Milli-Q grade distilled deionized water (18 MΩ cm, Millipore). Whatman™ Grade 1 chromatography paper, Staples™ rollerball pens, and rubber stamps made using a photosensitive seal machine were exploited for fabricating sensors.

5.4.2 Preparation of BSA-modified paper

Chromatography paper was immersed in a solution of BSA for 10 min, and then dried under ambient conditions. After being heated in an oven at 45 °C for 24 h, the BSA-modified paper was ready for use. The surface morphology of paper samples was analyzed using a FEI Nova NanoSEM™ scanning electron microscope. BSA distribution on paper was examined using a Nikon confocal microscope. The wettability of the prepared papers was evaluated by water contact angle (WCA) measurements with a conventional sessile drop method, using a contact angle instrument (Dataphysics OCA230, Germany).

5.4.3 Pattern characterization on BSA-modified paper

For the rollerball pen, the ink chamber was thoroughly cleaned and then refilled with water containing food coloring or FITC-BSA. For the rubber stamp, the stamp pad was inked with colored water or FITC-BSA to enable even spreading of ink over the stamp. Different symbols were patterned on the paper surface by writing and stamping. The patterns on the paper were recorded using a scanner (Epson Perfection 2450) or an ultraviolet lamp (Spectroline, ENF-240C/F).

5.4.4 Contact patterns for sensing on paper

A typical chemosensor on paper was constructed via writing. A pen inked with 80 mM DMG in ethanol was used to write “N” letters on BSA-modified paper. Then 40 µL of
a nickel solution was added to the paper and allowed to react for 10 min. The images were recorded by the desktop scanner and then analyzed. A typical biosensor on paper was fabricated by stamping. A stamp inked with a solution of 250 ng mL⁻¹ HRP was used to imprint “HRP” letters on BSA-modified paper. Then a solution of TMB was applied to the paper and allowed to react for 10 min. The images were recorded by the scanner and then analyzed.

A multifunctional integrated sensor on paper for simultaneous determination of Cu(II) and Ni(II) was prepared via writing. For Ni(II) assays, sufficient Na₂S₂O₃ dissolved in Milli-Q water (40 mg mL⁻¹) was first introduced onto paper for masking the interference of Cu(II). After that, different geometries (i.e., “C” and “I” letters) containing respectively 80 mM DDTC and 80 mM DMG were engineered on BSA-modified paper. Then a solution containing 100 mg L⁻¹ Cu(II) and 100 mg L⁻¹ Ni(II) was applied to the paper and allowed to react for 10 min. The images were recorded by the scanner and then analyzed.

5.5 RESULTS AND DISCUSSIONS

5.5.1 Direct stamping and writing patterned paper sensors

Herein, text-patterned paper sensors are employed since they enable end users to quickly obtain clear and simple test results in the form of letters and symbols that appear on the paper substrate. Also, the ubiquitous office stationery such as a pen and a stamp is used for fabricating text-patterned paper sensors. In comparison to conventional techniques for manufacturing patterned paper sensors, our alternative approach with a hydrophobic barrier-free process provides simplicity and requires minor instrumentation. Figure 1a schematically illustrates our design wherein the colorimetric testing reagent as the sensing element is patterned onto the paper substrate by simply stamping and writing. Specifically, when the inked pen or stamp comes into contact with a BSA-modified paper sheet, the aqueous assay reagent flows into the sheet, where it impacts selected positions of the paper substrate to form a clear pattern since the wettability of the paper is controlled. After the analyte is introduced, the resulting product remains confined in the pattern shape due to its low solubility in water and the tuned wettability of paper. Obviously, there are two important components in the process: a paper sheet with controlled wettability and a precipitating assay. If they become compromised, the presence of liquid assay reagent and analyte
results in significant spreading by wicking, which finally brings about the failed signal readout (Figure 1b).

**Figure 1.** Schematic illustration of fabrication of patterned sensor on (a) BSA-modified paper and (b) chromatography paper.

### 5.5.2 BSA modification for paper with tailored wettability

To construct the patterned paper sensor, paper wettability was first tuned via BSA modification. As shown in Figure 2a, SEM images reveal that BSA modification did not have an obvious effect on the morphology of the initial chromatography paper. The unique porous structure of the cellulose paper was retained even after 5% BSA treatment. The insets in Figure 2a show that the resultant papers could still provide a white background after modification with BSA. Moreover, the flexibility and mechanical integrity of the papers were well preserved (data not shown), which make them potentially useful for the development of paper-based sensing platforms. Despite no apparent difference in paper morphology and macroporosity before and after BSA treatment, confocal fluorescence imaging of paper treated with FITC-BSA confirms the successful BSA modification. The paper shows the characteristic green fluorescence, and the fluorescence intensity gradually increased with increasing protein concentration (Figure 2b).
Figure 2. Imaging and characterization of paper samples. (a) SEM images of papers modified with different concentrations of BSA (scale bar: 50 µm). Insets: photographs of papers with 10 mm diameter. (b) Confocal images of papers modified with different concentrations of FITC-BSA (scale bar: 200 µm).

To investigate the effect of BSA modification on the wetting behaviour of paper substrates, the water contact angle and the kinetics of the penetration of the droplet into the paper network were measured. As illustrated in Figure 3a, both untreated paper and paper treated with 0.1% BSA absorbed water instantly (< 2 seconds). At higher BSA concentrations (not less than 0.5%), the papers displayed a similar contact angle at ~130° after the droplet was placed for 2 seconds. On further investigation of the water drop penetration time of paper samples, it was found that the wettability of modified papers gradually decreased along with increased BSA concentration (data not shown). The slowest water wetting behaviour was observed for paper treated with 5% BSA; water could not completely spread on the paper surface in 3 minutes. These results can be attributed to the heat denaturation of BSA. Heat treatment enables protein to unfold, exposing the hydrophobic groups and thus causing an increase in the surface hydrophobicity. For patterned paper sensors, we believe that the penetration of aqueous samples should be relatively slow that may enhance their interaction with paper-despoisted assay reagents, but not too slow to result in incomplete or even no assay development due to inadequate paper wetting. Thus paper with tunable hydrophilicity, using BSA concentration as the controlling parameter, is favorable for sensing.
applications. In addition, Figure 3b clearly demonstrates that the wetting of untreated and treated paper by water is related to the performance of ink scribing. Herein, dyed water (i.e., food coloring mixed in water) was used for ink scribing of straight lines. Optical micrographs show that the feature width of drawn lines decreases with increasing hydrophobicity: untreated paper = 1058 ± 56 μm, 0.1% BSA-sized paper = 567 ± 36 μm, 0.5% BSA-sized paper = 516 ± 31 μm, 1% BSA-sized paper = 503 ± 17 μm, and 5% BSA-sized paper = 477 ± 32 μm (each width measured for 5 distinct features). The lower the wettability becomes, the thinner the line will be. The images reveal that the hydrophobicity of the engineered paper surface serves to focus the deposition of food coloring onto a smaller area, thus avoiding permeating and migration. (i.e., uneven and fuzzy lines).

\[
\begin{array}{c|c|c|c|c|c}
0\% \text{BSA} & 0.1\% \text{BSA} & 0.5\% \text{BSA} & 1\% \text{BSA} & 5\% \text{BSA} \\
\hline
\text{Absorbed} & \text{Absorbed} & & & \\
\end{array}
\]

**Figure 3.** (a) Images of droplets (3 μL water) 2 seconds after being placed on a series of papers coated with BSA. (b) Optical micrographs of food coloring drawn on the paper substrates by pen (scale bar: 500 μm).

### 5.5.3 Characterization of patterns on BSA-modified paper

Since a series of BSA modification shows differences with regard to drawn lines on paper, pattern fidelity and resolution were further tested on BSA-modified paper. Contact patterns were obtained by ink wicking through the dried paper sheet, when moving the pen across the paper or pressing the stamp against the paper. Considering that aqueous solutions containing small-molecule chemicals or biomolecules are widely
used to deposit assay reagents on paper, patterning of dye ink (i.e., food coloring mixed in water) and FITC-labelled IgG was investigated on paper substrates.

For dye ink patterns, Figure 4 demonstrates that different physical responses of paper substrates to wetting affected the pattern features differently. In terms of pen patterning, the absorption of ink on unmodified paper often causes a number of pattern quality problems. As stated above, clunky and fuzzy lines were generated on the paper without BSA modification; they finally led to blurred and uneven text. However, it is not an issue on BSA-modified paper that can restrict ink penetration. Significant decrease of ink spreading with well-defined patterns was, for example, observed on 0.5% BSA-modified paper. Even with a high concentration of BSA (5%), the ink can still be transferred from the reservoir to the paper as the pen moves, creating patterns of dye ink. Consistent with pen patterning, stamp patterning has a similar performance. Position control and spatial confinement of stamp patterns can be obtained on BSA-modified paper. Then FITC-IgG was used as a model bioink for patterning on paper substrates. In accordance with the previous results, BSA-modified paper facilitated more precise deposition of biomolecules compared with untreated paper (Figure 5). Due to loss in resolution with the capillary-driven flow of bioink during construction of the contact patterns, it is different to fabricate millimetre-sized patterned sensors with high fidelity on untreated paper. All the results indicate that the intended dimension of patterns could mainly be determined by the tip size of the pen or be set by the template size of the stamp because of the large restriction of ink diffusion into paper sheets with not less than 0.5% BSA modification. In other words, clear text-based patterns can be created with high resolution (mm order) on BSA-modified paper.

![Figure 4](image)

**Figure 4.** Images of dye ink patterns via writing (upper row) and stamping (lower row) on different papers with % w/v concentrations of BSA modification (scale bar: 5 mm).
Figure 5. Fluorescent images of FITC-IgG patterns via writing (left column) and stamping (right column) on (a) chromatography paper and (b) 0.5% BSA-modified paper (scale bar: 3 mm).

5.5.4 Patterned sensors on BSA-modified paper

5.5.4.1 Stamped paper sensor for biosensing

In order to demonstrate the feasibility of our design for biosensing, a HRP-TMB system (insoluble type) was chosen as the model, because it is widely used in many biological assays. In the presence of HRP, the TMB liquid substrate system develops a dark blue, insoluble reaction product. In a typical spot test, only 5 femtomol of HRP (100 ng mL\(^{-1}\)) can be detected by the naked eye on 0.5% BSA-modified paper, while a large amount of HRP (10\(^4\) ng mL\(^{-1}\), 500 femtomol) is required to obtain a significant color change on untreated paper (Figure 6a). Obviously, BSA-modified paper has a relatively slow wicking rate, and thus provides highly-concentrated HRP/TMB across the paper network, which causes an increase in the reaction rate. It is worth noting that the insoluble visible precipitation was confined in a smaller area on the paper sized by a higher concentration of BSA (refer to the inset in Figure 6a). The colored product did not migrate from the spot where HRP was added, although the volume of TMB was larger than that of HRP (data not shown). Since the letter patterned readout allows for the simplest signal reporting to non-professional users in written text, an HRP colorimetric assay was further performed by stamping the text “HRP” on the paper. As expected, the insoluble precipitation can be localized at the patterned paper area when treated with TMB, generating a colored “HRP” with an intensity related to the amount of BSA used (Figure 6b). In addition, a complicated butterfly pattern was created,
which was composed of two parts: a crayon-drawn body and HRP-patterned wings. A clearer image was obtained on BSA-modified paper.

**Figure 6.** (a) Analytical curves for HRP on different papers via spotting assay. Inset: colorimetric responses to 100 ng mL\(^{-1}\) of HRP on different papers. (b) Images of stamped HRP assay on papers with a bottom-up BSA increase from 0% – 1% (250 ng mL\(^{-1}\) of HRP). Scale bar: 5 mm.

### 5.5.4.2 Written paper sensor for chemosensing

In addition to biochemical analyses, chemical sensing on paper also has many uses in food safety and environmental monitoring. For the present study, the determination of heavy metal ions (Ni\(^{2+}\)) was chosen to show the practicability of our design. Detection of Ni\(^{2+}\) in an aqueous solution can be performed based on the precipitation of nickel(II) with DMG, which can produce a poorly soluble and pinkish red precipitate (i.e., Ni(DMGH)\(_2\)). The detection of nickel(II) in aqueous solutions at the ppm level was first performed on paper using a spotting assay. As shown in Figure 7a, serial dilutions of Ni\(^{2+}\) solution were analyzed on 1% BSA-modified paper and the analytical curve exhibited a good linear behaviour for a concentration range of 0 – 10 ppm. Despite the lack of hydrophobic barriers and the small volume of the aqueous Ni\(^{2+}\) solution (3 µL), a visual reading of the color dot could be accomplished with the naked eye more easily on BSA-modified paper than on untreated paper (see the inset in Figure 7a). Similarly, nickel(II) colorimetric assay was also performed by text reporting (Figure 7b). DMG
was written on the paper using the letter “N”, with a crayon-written letter “I” on the right. When presented with Ni^{2+}, the insoluble Ni-DMG complex was formed and delivered the entire text “NI” to the users. Specially, ethanol was used to dissolve DMG, which means that DMG written on paper cannot be confined to a small area to improve the pattern quality and clarity. However, the written assay still ensures sufficient legibility of the text pattern to be displayed even without hydrophobic barriers. The insoluble Ni-DMG complex can be localized at the written pattern area on BSA-modified paper, generating a colored “NI” with an intensity that is proportional to the amount of Ni^{2+} present. The written assay can provide a Ni^{2+} concentration-dependent response similar to that of the spotting assay. DMG on a paper substrate would remain in place when present with Ni^{2+}, since the insoluble colored Ni-DMG complex could be substantially hindered by the cellulose matrix. A discernible color change was observed, even when the concentration of nickel ions was as low as 1 ppm, which is useful for warning of drinking water contamination. Currently, a wide variety of indicators have been developed to produce metal-indicator complexes with low solubility for colorimetric detection of heavy metal ions, and our design may therefore have great potential for the fabrication of paper-based chemosensors.

**Figure 7.** (a) Analytical curve for nickel (II) via a spotting assay on 1% BSA-modified paper. Inset: colorimetric responses to Ni^{2+} of different concentrations on papers. (b)
Images of written Ni-patterned colorimetric assay with a bottom-up Ni\(^{2+}\) increase (0 ppm, 1 ppm, 2.5 ppm, 5 ppm, 10 ppm, 20 ppm). Scale bar: 5 mm.

5.5.5 Patterned paper sensor for multidetection

The capability of complex functionality offered by our design was also tested by creating a multiplexing assay for simultaneously monitoring Cu\(^{2+}\) and Ni\(^{2+}\). Since the assay results presented as text allow for the simplest signal readout, multiplexing was obtained via written letters “C” and “I” on 1% BSA-modified paper, which denoted DDTC (an indicator for Cu\(^{2+}\)) and DMG (an indicator for Ni\(^{2+}\)), respectively. As mention previously, the crayon-written letters were indicative of letter-shaped assay regions. As shown in Figure 8, visually perceivable letter patterns (i.e., yellow-brown colored “C” and pinkish-red colored “I”) can be formed to make the successful text reporting, when applying a sample containing Cu\(^{2+}\) and Ni\(^{2+}\). The results clearly demonstrate that potentially different analytes can be detected at the same time, because the resulting precipitates could remain in the predetermined areas on BSA-modified paper. From what has been discussed above, we believe that patterned paper sensors are able to perform multiplexed analysis without cross-talk among discrete sensing regions.

**Figure 8.** Images of text-patterned paper sensor for monitoring Cu(II) and Ni(II) before and after addition of the sample containing 100 ppm Cu(II) and 100 ppm Ni(II). Letter “C”: Cu-DDTC complex; letter “I”: Ni-DMG complex. Letters not containing “C” and “I” were written using crayons.

5.6 CONCLUSIONS

In summary, our work has discussed the combination of precipitating assays with wettability-tuned paper as a novel solution to the fast fabrication of patterned paper sensors. Not dependent on hydrophobic barriers to create defined patterns on paper.
substrates, the strategy renders paper-based sensors more portable and less resource sensitive. We have shown that the design can function not only via spot-based quantitative detection, but also provide patterned paper sensors with customized needs including text reporting and multiplexed detection. The successful stamped HRP assay and written Ni assay indicate the generality of the approach. Also, simultaneous detection of copper(II) and nickel(II) proves the capability of complex functionality of patterned paper sensors. Given the fact that many precipitation reactions have been developed and are now available for the identification of wide-ranging targets, we believe that the approach proposed here offers a valuable perspective for developing paper-based analytical devices.

5.7 ACKNOWLEDGMENT
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5.8 REFERENCES


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Chapter 6

Conclusions and Future Work
This thesis presents a series of studies that address key challenges that restrict the commercial development of paper-based analytical devices. These studies significantly enhance the affordable, deliverable, equipment-free and user-friendly features of paper-based analytical devices. In a series of trials of blood typing and environmental sensing, the proposed strategies have been shown to be effective ways to enhance the practical capabilities of paper-based analytical devices, and to be highly desirable for professionals or non-professional users.

One significant breakthrough of this thesis is zero-step paper functionalization for covalent biomolecule immobilization, which can reduce the manufacturing costs. As the first step in paper-based biosensor fabrication, effective immobilization of biological elements on paper surfaces has received much attention. To date, many surface modification techniques have been proposed to facilitate the covalent immobilization of biomolecules onto paper, which can promise reproducible results for paper-based multiple-step assays. The work in this study indicates that the step of functionalization can be avoided by using residual carboxyl functional groups on paper. This facile and versatile method avoids the multiple modification steps, toxic chemicals and hazardous conditions in paper functionalization.

Obviously, a paper-based analytical device with immobilized biomolecules requires a sufficient shelf life to allow shipping and storage, and this is a major concern for its commercial development. Considering that paper-immobilized antibody is in urgent need of improved shelf life, the second distinctive aspect of this PhD project is to enhance the longevity of paper-based analytical devices with antibody. To take the paper-based blood typing device as an example, the work has shown that the activity of antibody on paper can be protected by freeze-drying, and the polysaccharide-based film encapsulation strategy proposed in this thesis effectively addresses the longevity issue. It enables paper-based analytical devices to be deliverable to users without a costly cold chain; the transportation and storage costs of paper-based analytical devices can be also reduced. In addition, the survival mechanism of antibody in the cellulose fibre network of paper sensors is presented in this thesis. Such fundamental studies have great value in improving the longevity of paper-based analytical devices, and can contribute to the development of practical and effective devices.
Currently, the popular techniques to fabricate paper-based sensors involve localizing adsorbed sensing elements using hydrophobic barriers by photolithography, etching, plasma treatment, flexographic or screen-printing methods. As another achievement in this thesis, a simple approach to the fabrication of patterned paper sensors without the need of hydrophobic barriers is presented. More specifically, pens or stamps loaded with reagents dispersed in a suitable solvent have been developed to enable rapid and accurate deposition of reagents onto paper with pre-deposited polymers like BSA. Using this method, paper-based sensors are ready for analytical applications without the need for hydrophobic barriers. The technique involves simply writing or stamping on a paper sheet, which defines the test areas. When aqueous samples are added to the device, the test areas defined by pen/stamp and pre-deposited polymers become available to react with analytes in the sample. Colorimetric assays conducted on devices prepared by this “pen/stamp on paper” technique have comparable accuracy and precision to assays conducted on conventional devices prepared by popular channel patterning strategies on paper.

Throughout this project, the continued effort to make paper-based analytical devices suitable for real-life use is emphasized. The novel techniques and ideas explored in this thesis provide effective solutions to the serious concerns with existing paper-based sensing applications, including affordability, deliverability, minimum equipment, and user-friendliness. These concepts and methods hold enormous potential for integration with future work in paper-based sensing, which will strongly drive the practical application of paper-based analytical devices for point-of-care diagnostics, and on-site environmental sensing. Based on the research covered by this thesis, the following future studies are recommended:

(1) Investigation of the feasibility of polysaccharide protection for paper-based ELISA, which is usually an antibody-based multistep assay. Combined with the study in this thesis, a complete low-cost protection system for paper-based biological sensors may be established, and the shelf life for these paper sensors can be obtained;

(2) Design of novel paper sensors for environmental monitoring that have the enhanced sensitivity and stability of metal ion indicators on paper, and generate some data from the field test using these paper sensors;
(3) Exploration of new methods to fabricate patterned paper sensors without hydrophobic barriers, and expansion of the text-reporting concept to many other biochemical and biomedical applications on paper;

(4) Combination of paper with telemedicine-based platforms, such as smartphones, tablets, and other portable or wearable sensors, as the assay data can be rapidly converted to a digital image or a discrete number by the use of telemedicine, which provides an excellent solution for point-of-care testing and real-time analysis.

The ultimate goal of our research is to take paper-based analytical devices from prototype to production, in order to contribute to the environment and health in developing countries. End-users and patients will benefit greatly if paper-based analytical devices can be successfully commercialized based on our efforts.
Appendix I

Published First and Co-Authored Papers Included
in the Main Body of This Thesis
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A zero-step functionalization on paper-based biosensing platform for covalent biomolecule immobilization

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ABSTRACT

As a potential platform for point-of-care clinical analyses and environment monitoring, paper biosensors have received considerable attention. In many cases, the conjugation of biomolecules onto paper surface is crucial for increasing the functionalities of paper-based bioanalytical devices. Until now, it is sometimes argued as in the literature that finding a surface chemistry for biomolecule covalent grafting to paper still remains a challenge. Here the study shows that at least to a certain extent some aspects of the argument involved is questionable, by demonstrating that paper without any modification could be utilized for the covalent conjugation of enzymes and serves as a tool for bioanalysis. Moreover, the detailed analysis of biomolecule immobilization strategies on paper through polysaccharide-coating chemistry has been offered as a contrast. We believe that the proposed method could provide a valuable perspective for paper-based biosensors.

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1. Introduction

Biosensors as a diverse collection of bioanalytical devices for detecting and quantifying biomolecules, have been widely used in many areas, such as clinical and home-based diagnoses and environmental monitoring [1–3]. Typically, a biosensor consists of three main parts: a bio-recognition component, a signal transducing component and an output system. Among them, the bio-recognition component is responsible for the identification of the presence and quantification of analytes via specific interactions with analytes by using specific biological elements like proteins, nucleic acids and tissues [4,5]. Thus effective immobilization of these biological elements onto solid surfaces is an important step in biosensor fabrication. Considerable efforts have been devoted to endowing the supporting material surfaces with bio-recognition ability by attaching biomolecules covalently [6,7].

Nowadays, novel paper-based biosensors have emerged with potential as easy-to-use, rapid and inexpensive point-of-care devices [8,9]. Compared with commonly used substrate materials for biosensors, its advantages include low production cost, intrinsic water wicking ability and feasibility of patterning by printing technology [10,11]. So far, in paper-based sensor and paper-based ELISA plate design and fabrication the most used techniques for immobilizing biomolecules are based on physical adsorption. However, physical adsorption of biomolecules has an unavoidable weakness that it could not always promise reproducible results because biomolecules are weakly bound to paper fibers and could be easily washed off [12]. In order to significantly improve the performance of paper-based devices for quantitative bioanalysis, effective and chemically reliable methods for immobilizing a broad range of biomolecules on paper sensors, mostly immobilization via covalent bonding, need to be explored.

To date, a variety of surface chemistries have been proposed to facilitate the covalent immobilization of biomolecules onto paper, such as divinyl sulfone chemistry, diazonium chemistry or polymer chemistry [12–14]. Among them, long-chain and flexible polysaccharides with multiple functional groups have exhibited the potential to act as surface modifiers for paper sensor fabrication. In particular, Orelma et al. used the Langmuir–Schaeffer cellulose film as a model to investigate polysaccharide adsorption for biomolecule conjugation, which could give instructions to paper modification via polysaccharide coating technique [15,16]. However, to the best of our knowledge, the detailed study of using these polysaccharides for practical paper surface modification has not been assessed. Recently, Wang et al. reported the application of chitosan as the surface modifier to fabricate paper plates for paper-based ELISA; their work has demonstrated, partly, the potential of polysaccharide coating on paper [17].

Here comes the question, why bother doing paper functionalization? The reason is that paper is typically composed of cellulose fibers and hydroxyl groups in glucose are responsible for cellulose chemical activity

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[9]. Most published work took it for granted that hydroxyl groups need to be transformed to other functional groups to enable covalent immobilization of biomolecules by common bioconjugate techniques. However, it is neglected that paper have low concentrations of carboxyl groups, which could potentially facilitate the covalent immobilization of biomolecules and probably make paper ready for binding biomolecules covalently [9,18]. In other words, people may be distracted by the familiarity with cellulose and its derivatives; the functionalization strategies for paper they are looking for may not necessary.

In this study, unmodified cellulose fiber-based paper was investigated for covalent immobilization. An enzyme, alkaline phosphatase (AP) used as a model protein, was immobilized onto paper substrates. In a comparison study, two kinds of representative polysaccharides, chitosan and sodium alginate (NaAlg), were utilized for paper modification. They were deposited as surface modifiers on paper surfaces. The effects of these modification methods were evaluated with a colorimetric reaction of substrates corresponding to AP. To the best of our knowledge, the direct use of original paper for covalent linking of biomolecules has not been previously reported, that is, the surface activity of the residual carboxyl groups for immobilization has never been studied. Our results show that, despite of the low concentration, the residual carboxyl groups on unmodified paper can still provide a considerable capacity for AP immobilization. Furthermore, we also described the construction of a simple immunoassay on differently modified paper substrates. We hope that our results could make a contribution to future fabrication of paper-based bioanalytical devices.

2. Experimental

2.1. Materials and instruments

Whatman qualitative filter paper #1, cut into 10 mm × 10 mm pieces, was chosen as paper substrate throughout the work. All reagents and proteins used in this study were purchased from Sigma–Aldrich. Glutaraldehyde, N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were used for chemically crosslinking. Medium molecular weight chitosan (200–800 cP, 1% in 1% acetic acid) and medium molecular weight sodium alginate (≥2000 cP, 2% in water) were chosen for the comparison study. 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) was used in conjunction with nitroblue tetrazolium (NBT) for the colorimetric detection of AP activity. Buffers and solutions, unless specially noted, were prepared with ultrapure water (18.2 MΩ cm, Mill-Q Gradient System, Millipore, USA).

The digital colorimetric images of the assays were generated by scanning the results using a desktop scanner (Epson Perfection 2450) set to color document scanning, 600 dpi resolution; the digital assay results were analyzed in grayscale using ImageJ. In order to correct the influence of the paper background color to assay results, background grayscale value of biomolecule modified paper without adding BCIP/NBT substrate was subtracted, unless otherwise noted. The color intensity values were obtained by taking the average of the quadruplicate readings, with the variation of values represented by error bars.

2.2. Preparation of unmodified paper with immobilized enzymes

2.2.1. Glutaraldehyde coupling

Typically, 20 μL of 3% glutaraldehyde in 0.01 M pH 7.4 phosphate buffered saline (PBS) was introduced to activate the original paper for 2 h in a wet box, and the paper was washed three times by adding 100 μL of the coupling buffer (1.5% (w/v) mannitol, 0.15% (v/v) glycerol and 0.01% (v/v) Tween 20) to the top surface of it, and then putting the bottom of the paper in contact with a piece of cotton wool to remove the excess buffer. Subsequently, 3 μL of an enzyme solution in the coupling buffer (100 μg mL⁻¹ AP) was spotted onto the freshly activated paper square and incubated in the wet box for 2 h. After that, the paper was washed with 100 μL of Tris–HCl buffer (50 mM, pH 9.2, containing 0.1% (v/v) Tween 20) three times and allowed to dry for 30 min under ambient conditions. Finally, 9 μL of a colorimetric substrate solution for AP (i.e., BCIP/NBT) was added to the paper. After being incubated for 2 h, the paper square was scanned by the desktop scanner and the intensity of the color was measured using ImageJ.

2.2.2. NHS/EDC coupling

Typically, 20 μL of a mixed solution (0.1 M NHS and 0.1 M EDC) was introduced to activate the original paper in the wet box for 15 min, and then this activation step was repeated once more. Subsequently, the paper square was washed with the coupling buffer (3 × 100 μL). Afterwards, 3 μL of a 100 μg mL⁻¹ AP solution in the coupling buffer was spotted onto the freshly activated paper square and incubated in the wet box for 2 h. The paper was then washed with Tris–HCl buffer (3 × 100 μL) and allowed to dry for 30 min under ambient conditions. Finally, BCIP/NBT was added to the paper; after 2 h, the image was recorded by the desktop scanner and then analyzed.

2.3. Preparation of polysaccharide-coated paper with immobilized enzymes

In a typical experiment (Fig. 1), 20 μL of chitosan solution (0.05%, w/v) in aqueous acetic acid solution or 20 μL of NaAlg solution (0.05%, w/v) was dropped onto one piece of paper. After being dried under ambient conditions overnight, polysaccharide-coated paper was ready for use. Enzyme could be covalently immobilized on glutaraldehyde activated chitosan-modified paper square or NaAlg-modified paper square activated by NHS/EDC. Based on the above-described procedures, a variety of reaction parameters like polysaccharide concentration were examined for polysaccharide coating.

2.4. Immobilized enzymes’ stability assays

Different paper substrates with immobilized AP (0.05% chitosan or 0.05% NaAlg for paper coating, AP concentration of 100 μg mL⁻¹ for immobilization) were stored at room temperature for various periods of time. Afterwards, the residual enzymatic activity of immobilized AP was evaluated by the color-producing enzymatic reaction as mentioned above.

2.5. Fabrication and processing of immunoassays

Rabbit IgG (3 μL, 100 μg mL⁻¹) was immobilized on different paper substrates for 2 h. After washing with 1 mL of PBST (0.1% (v/v) Tween-20 in PBS), the paper squares were immersed in a blocking buffer (5% (w/v) BSA in PBS) for 30 min, followed by washing with 1 mL of PBST to remove the free BSA. An AP-conjugated goat anti-rabbit IgG solution (3 μL, prepared by diluting the stock solution 1000 times in PBS) was added onto the test paper and allowed to incubate for 5 min. The test zones were then washed with 1 mL of PBST, and allowed to dry under ambient conditions for 30 min. Finally, 9 μL of a solution of BCIP/NBT was added to the paper, and after 30 min the results were recorded and analyzed by ImageJ.

3. Results and discussion

3.1. Demonstration of the paper-based bioassay format

In the current study, in order to simplify the experimental procedure, spotting assays were performed on paper squares. Specifically, the most widely utilized Whatman filter paper for bioassays was chosen as the substrate in our study. Then, after immobilizing a small amount of enzyme (3 μL) onto paper substrate, we would utilize 9 μL of enzyme
substrate to wet test paper completely for better understanding the accurate distribution of enzyme. We tested the efficiency of immobilizing proteins and evaluated the conservation of protein biological activity on different types of paper via using solutions of AP as a model protein, because AP is considered very sensitive particularly when using colorimetric detection and also less stable than other popular alternatives such as the enzyme horseradish peroxidase (HRP).

3.2. Covalent attachment of protein to unmodified paper

The amount of protein covalently immobilized on original filter paper could vary with different coupling chemistries (Fig. 2). Glutaraldehyde-mediated coupling on filter paper is not efficient for enzyme immobilization. Apparently, there are no primary amine groups on paper for covalent bonding of glutaraldehyde (Table S1, Supporting information). Because of the lack of primary amine on filter paper, any further covalent attachment of enzyme through the glutaraldehyde coupling would not be possible.

Surprisingly, NHS/EDC activation strategy displays different behavior, depending on diverse crosslinking mechanisms. The results obtained from NHS/EDC activation are exactly opposite to those from glutaraldehyde activation method (Fig. 2). Our data show successful enzyme immobilization on NHS/EDC-activated paper, which indicates the possibility that carboxyl groups on paper are sufficiently active to react with EDC and further with NHS to form the activated surface for protein immobilization. It is a known fact that paper surface is slightly negative charged, which is partly caused by the presence of carboxyl groups [9, 18]. To further evaluate this possibility, we performed direct enzyme immobilization on filter paper without the NHS/EDC activation. Once eliminating the activation step, paper surface would only have a low concentration of carboxyl groups, and would not be able to acquire the more reactive NHS ester groups from the NHS/EDC coupling chemistry; we therefore expected insignificant enzyme immobilization on the unmodified paper. Colorimetric measurement of paper samples treated with direct enzyme deposition followed by Tris–HCl buffer rinsing shows insignificant color change. This result supports our reasoning that the native residual carboxyl groups on original paper surface may provide a certain level of NHS/EDC coupling for biomolecule immobilization.

3.3. Covalent attachment of protein to polysaccharide-modified paper

Polysaccharide-modified paper was contrasted with activated original paper by NHS/EDC for examining the performance of protein immobilization. The reason why polysaccharide-coated paper was taken as a reference is that polysaccharides as an important class of biopolymers have been successfully used as surface modifiers to functionalize paper-based analytical devices. Meanwhile, there is a lack of detailed analysis of polysaccharide modification strategies for paper-based biosensing platform, which could increase our knowledge about the chemistries of these bioconjugation systems and their bioassay performances.

Some studies on cellulose model surfaces (i.e., Langmuir–Schaeffer cellulose films) have reported that polysaccharides' structural similarity to cellulose makes these macromolecules possible to engage in hydrogen bonding interactions with cellulose, which could lead to irreversible polymer adsorption [15,16]. Here, when it comes to practical cellulose fiber-based paper, XPS surface analysis was performed to verify paper functionalization via polysaccharide coating. As shown in the XPS elemental composition data (Table S1, Supporting information), nitrogen was not detected on unmodified paper. As for chitosan-coated paper, however, even after thorough washing with water, the similar appearance of a nitrogen signal in the XPS spectrum indicates the successful irreversible adsorption of chitosan on paper. Similarly, after the coating of NaAlg, sodium could be observed by XPS on NaAlg-coated paper, which demonstrates the rinsing-resistant adsorption of NaAlg. The results validate that polysaccharide has similar affinity with cellulose fiber-based paper just as it has with Langmuir–Schaeffer cellulose films.

3.3.1. AP immobilized on chitosan-modified paper

Chitosan-coated paper was exploited for enzyme immobilization. Two major mechanisms contributed to the biomolecule immobilization – electrostatically driven adsorption mechanism and a covalent
coupling mechanism with Schiff bases. The abundant primary amine functional groups in chitosan enable the polymer to carry positive charges under acidic conditions, with a pKa of ~6.5 [19]. AP was used in this study as the model biomolecule with a pI range of 4.4–5.8, which allows chitosan and the enzyme to have electrostatic interactions.

In addition to the electrostatic interaction, chitosan activated by glutaraldehyde is able to immobilize biomolecules with amino groups through the Schiff reaction. Fig. 3A shows that the trends of enzyme immobilization through physisorption and covalent coupling were similar, that is, the quantity of the immobilized protein increased with the increase of chitosan proportion used for paper surface treatment. The quantity of enzyme immobilization through covalent bonding was higher on paper treated by low to medium concentration of chitosan solution (<0.5%, w/v); but became almost the same as the chitosan concentration further increased to 1% (w/v). The results reveal that even non-activated chitosan can immobilize the enzyme strongly enough to withstand rinsing by Tris–HCl buffer that could lead to the deprotonation of chitosan. Although the electrostatic charge of chitosan could be weakened, the hydrophobic interactions between chitosan and enzyme molecules could still contribute to enzyme physisorption [20].

From the perspective of paper bioassays based on popular colorimetric analysis, paper should ideally not acquire any background color after the surface modification with polysaccharides. However, as shown in Fig. 3B, paper modified with chitosan solution concentration above 0.1% does acquire an unwanted level of background color after being activated by glutaraldehyde. It is possible that the adsorbed chitosan could go through a phase inversion in weak alkaline solution (PBS) and become conducive to form the interpenetrating polymer network, which might allow, although could not be confirmed, the subsequently introduced glutaraldehyde to bring about crosslinked chitosan [21]. In order to retain the high brightness of the paper and to achieve a practically significant covalent coupling ability to biomolecules, low concentrations of chitosan solution (not more than 0.05%) is more suitable for the fabrication of chitosan-coated paper biosensing platform, which is in some agreement with previous report by Wang et al., where they used 0.025% chitosan for paper modification [17]. In that case, the enzyme immobilization capacity of NHS/EDC-activated original paper was considerable, just slightly lower compared to that of chitosan-coated paper.

3.3.2. AP immobilized on NaAlg-modified paper

As another commonly-utilized macromolecule, NaAlg has a large number of carboxyl groups distributed along the polymer backbone, which enables covalent immobilization of biomolecules via the well-understood NHS/EDC chemistry. Unlike the chitosan immobilization system, the amount of protein immobilized on NaAlg-modified paper varied with different methods (Fig. 4A). In the case of NaAlg-modified paper with NHS/EDC activation, the amount of immobilized AP exhibited a low dependence on the concentration of the NaAlg solutions employed for surface treatment, which may be attributed to the fact that the residual carboxyl groups on unmodified paper can react with biomolecules bearing primary amine groups. The results show that NaAlg-coated paper has similar enzyme immobilization capacity as NHS/EDC-activated original paper.

It is observed in Fig. 4A that the physisorption of AP on non-activated NaAlg paper was weak, particularly in the low NaAlg concentration range (below 0.1%). Electrostatic repulsion could be responsible, since both NaAlg and AP carry negative charges. The reason for the increased AP physisorption in higher NaAlg concentration, however, is unclear. Possibly, the increased NaAlg concentration that could change the pore size of paper to some extent made the adsorbed enzyme difficult to be washed off, which can affect the reproducibility of paper-based analytical device signal data. Unlike chitosan modification, NaAlg modification does not raise the background signal, which is a desirable performance for paper-based colorimetric bioassays (Fig. 4B).

3.4. Comparison of immobilization methods

Since the high biomolecule immobilization capacity could improve the performance of bioassays, it is of importance to quantify the amount of biomolecules that could be immobilized on the substrates. Here, the covalent immobilization of AP on three paper samples with different treatments (i.e., 0.05% chitosan-modified paper activated by glutaraldehyde; 0.05% NaAlg-modified paper and original paper activated by NHS/
EDC) was studied by spotting solutions of AP with concentrations varying from 0 to 1 mg mL\(^{-1}\). As expected, the quantity of immobilized enzyme increased as a function of AP concentration (Fig. 5). The colorimetric analysis of these paper samples that had been modified by chitosan or NaAlg did not show significant difference from that of original paper treated with NHS/EDC chemistry. The above results suggest that original paper has the potential to simplify the route of covalent biomolecule conjugation onto it. The long-term stability on various paper substrates was also investigated. Our results (shown in Fig. 6) indicate that AP retained some activity over a period of at least one month when stored at room temperature. To some extent, covalent coupling could help to maintain the active conformation of the biomolecules. Intuitively, covalent immobilization could reduce the likelihood of enzyme aggregation by conjugating them with supports to restrict their movement. Unfortunately, the results in Fig. 6 demonstrate that paper modified with polysaccharides did not offer a significant advantage over the unmodified paper treated with NHS/EDC chemistry in preserving the bioactivity of AP. During storage, low-concentration chitosan and NaAlg may not offer effective protection for biomolecules.

3.5. Assessment of bioassays on different papers

In order to demonstrate the capability and versatility of different types of paper in conjugating biomolecules, we performed a simple-form paper-based immunoassay, that is, the interaction and recognition between rabbit IgG and AP-conjugated anti-rabbit IgG. As illustrated in Fig. 7, for both original and NaAlg-modified paper, there is an obvious signal difference between NHS/EDC-activated and non-activated paper, in accord with the role played by carboxyl groups during the process of biomolecule immobilization. The relatively weak signal observed on the non-activated paper can be attributed to a low level of the physical adsorption of rabbit IgG onto the paper. However, in the case of chitosan-modified paper, even non-activated paper had a strong signal, which means that the physiosorption of rabbit IgG could be resistant to the stringent washing steps. Perhaps it is of some value to verify the feasibility of utilizing unactivated chitosan-modified paper for bioassays in the future. Besides, it is worth noting that the activated original paper showed no significant difference with activated chitosan paper, which, once again, demonstrated the ability of NHS/EDC conjugation strategy to covalently immobilize biomolecules bearing amino groups onto the unmodified paper.

4. Conclusions

On the grounds that popular covalent methods of attaching biomolecules to substrates for biosensing platforms include amine chemistry and carboxylic chemistry, this work provides detailed data about the application of chitosan and NaAlg on cellulose fiber-based paper modification, which could offer some valuable suggestions for other applications using bioactive paper. Besides, there is a common view that for covalent biomolecule immobilization, paper must be functionalized at first. However, our results indicate that efforts may not be required for the step of functionalization by using residual carboxyl functional groups on paper (i.e., zero-step paper functionalization for covalent biomolecule immobilization). We believe that the work reported here would provide a...
valuable perspective for the fabrication of paper-based biosensing platform.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.sbsr.2015.09.002.

References

A preliminary study on the stabilization of blood typing antibodies sorbed into paper

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Abstract This study investigated the stability of the primary blood typing antibodies (Anti-A, Anti-B and Anti-D IgM) on paper. This knowledge is critical to manufacture a new type of paper-based blood typing device where blood group antibodies must be kept active on paper for extended periods. Two strategies were explored. The first involved mixing additives such as polyvinylpyrrolidone (PVP), dextran and glycerol, with antibodies before sorption onto paper. While all the additives tested improved the antibody stability on paper, their protection for storage at room temperature was limited; dextran provided the longest protection, followed by PVP and then glycerol. The second strategy relied on freeze-drying to stabilize the antibodies in paper. Freeze dried antibodies sorbed into paper could be stored for long periods at ambient conditions without significantly loss of their activity. The thermal stability of antibodies in paper was also improved by freeze-drying. Our work shows that the use of additives and freeze-drying are effective approaches to retain the activities of IgM blood group antibodies on paper. These approaches will be further explored for the large scale development of a new generation of clinical and home-care blood testing devices.

Keywords Paper-based diagnostic devices · Blood typing · Antibody activity · Freeze drying

Introduction

Bioactive paper-based analytical devices have drawn much interest in recent years as a platform for low-cost analytical devices in diagnostic and environmental applications. Paper made of cellulose fibre is cheap and widely available; it is a suitable material for manufacturing basic analytical sensors on a large scale to meet the demand of diagnosis and disease screening in developing areas of the world. The rapid technical advancement of the paper-based sensor research and development can be attributed to the numerous advantages of the porous structure of paper and the surface chemistry of cellulose fibres. Historically, concepts of using patterned paper to fabricate multi-well assay plates (Yagoda 1937) and paper microfluidic devices (Müller and Clegg 1949) have been explored from as early as 1937 and 1949. The first paper-based diagnostic sensor for semi-quantitative glucose assay in urine was demonstrated in 1956 (Comer 1956); this invention was further developed into immunological paper test devices whose functions were based on colorimetric and electrochemical mechanisms (Dungchai et al. 2001).
Since 2007, patterned paper diagnostics have been re-discovered and pursued by a number of research groups globally (Wang et al. 2010; Kauffman et al. 2012; Li et al. 2010; Li X et al. 2012; Li M et al. 2012; Khan et al. 2010), initiated by the Whitesides Group (Martinez et al. 2007). In the past 7 years, research and innovation in bioactive paper diagnostics have created a number of low-cost conceptual devices that have potential for commercialization.

A critical requirement of all paper-based diagnostic devices is the longevity of their biochemical activity. These devices must have a sufficient stability to allow temporary storage and transportation in the supply chain. This is because the technological developments for paper-based diagnostics generally take place in developed countries, whereas a significant proportion of the users of basic diagnostics are living in developing countries. Therefore diagnostic devices must be able to retain their bioactivity for a sufficiently long period of time and withstand elevated temperature to allow the devices to be delivered to the users. This requirement has been defined, along with other six requirements for low-cost analytical devices designed for developing world, by the World Health Organization (WHO) as “ASSURED” (Mabey et al. 2004). Although significant progress in bioactive paper research has been made, practical obstacles to maintaining the bioactivity of the paper devices must be overcome in order for the devices to function reliably under unsupported field conditions. To this end, engineering strategies need to be applied to provide effective and simple methods to enhance the time and temperature stability of bioactive paper-based sensors.

Our group has initiated a novel paper-based ABO and rhesus (RhD) blood typing sensor that can report patients’ blood typing results in written text (Li X et al. 2012; Li M et al. 2012). Unlike other paper-based blood typing sensors where paper are used merely as a supporting substrate to display the occurrence or non-occurrence of haemagglutination, our design uses the fibre network of paper to differentiate the agglutinated red blood cell (RBC) aggregates from the non-agglutinated cells. Whilst this concept of reporting results in text demonstrates a high level of user-friendliness, the engineering and manufacturing aspects of the sensor must also be investigated to ensure that the longevity of antibodies in cellulose fibre networks meets the sensor design requirements.

Although antibodies are generally more stable than some other proteins because of their relatively rigid three-dimensional structures, they can denature via a variety of pathways (Paborji et al. 1994; Chu et al. 2005; Wang et al. 2007). All antibody molecules are structurally similar; they comprise two different kinds of polypeptide chains—heavy and light. Each chain is divided into two regions—the variable (V) region and the constant (C) region. Additionally, antibodies are divided into five different classes (IgM, IgG, IgA, IgD and IgE) based on their C regions; they are denoted as μ, γ, α, δ and ε, respectively. Among those classes, IgM antibodies are the least stable (Janeway et al. 2001).

Blood group antibodies belong to either the IgM or IgG class, although some can be IgA. Since an IgM antibody molecule possesses five IgG units, it has 10 potential epitope binding sites. Whereas an IgG antibody possesses a single IgG unit and has only two epitope binding sites. Apart from differences in binding sites, the molecular sizes of IgM and IgG are also different, being 30 and 14 nm respectively (Daniels and Bromilow 2007). In clinical blood typing assays IgM antibodies are used to directly agglutinate antigen-positive red cells, whereas IgG antibodies usually need to be used together with anti-human globulin or enzymes to enhance agglutination (Daniels and Bromilow 2007). Our text-reporting blood typing sensor (Li X et al. 2012; Li M et al. 2012) was designed based on the antibody-specific direct agglutination of RBCs by IgM group monoclonal antibodies. The IgM antibodies are sorbed into the cellulose fibre network of paper, where antibody molecules are in contact with cellulose fibre and microfibrils. The fibre network provides a different environment for the sorbed antibody molecules and their stability in such an environment has not been studied. Herein we report results of a preliminary study on different approaches to extend longevity of ABO and RhD blood group antibodies in paper-based sensors stored under room temperature. These results will serve as a guide for the future non-accelerated aging studies to be carried out.

**Experimental details**

**Reagents and apparatus**

Antibodies against RBC antigens approved for human blood grouping were obtained from Alba Bioscience,
Edinburgh, UK. They were IgM antibodies commercialized under the names of Albaclone® Anti-A, Anti-B, and Anti-D Optimum. Anti-A and Anti-B are a blue and a yellow solution, respectively, whilst Anti-D is a clear solution. Phosphate buffer saline (PBS) sourced from Sigma Aldrich, Australia was used as diluent for all antibody solutions. All the antibodies were stored at 4 °C.

Reagent red blood cells RevercellTM (15 % A1 and 15 % B red blood cells) and AbtectcellTM III (3 % R1R1, 3 % R2R2 and 3 % rr red blood cells) were purchased from CSL Limited, Australia, stored at 4 °C and used within 30 days. They were concentrated by centrifugation to 40 % before reacting with blood group antibodies.

Dextran (average molecular weight 35,000–45,000), polyvinylpyrrolidone (PVP, average molecular weight 40,000), and glycerol were obtained from Sigma Aldrich. Saline solution (0.9 % (w/v) NaCl) was prepared with MilliQ water and AR grade NaCl from Sigma Aldrich.

Kleenex paper towel was cut into 1 cm × 1 cm squares which were used to absorb blood group antibody solutions for making blood typing sensor. A 2.5 L Freeze Drying system (FreeZone®, Labconco Corp., Kansas City, USA) was used to freeze dry blood group antibodies sorbed into paper. An oven (Memmert, Australia) was used for heat treatment of freeze-dried antibodies for temperature stability tests.

Preparation of antibodies with stabilizing additives sorbed in paper

Anti-A, Anti-B and Anti-D antibody solutions containing 1 % PVP, 6 % dextran and 10 % glycerol respectively were prepared by mixing the antibody and additive solutions to the required additive concentration; antibody concentration in the final solutions with different additives was kept constant. On each paper square 10 μL antibody-additive mixture was introduced to form the antibody loaded paper. After hanging to dry in a fume hood for 2 min until antibodies fully sorbed, the paper squares were stored in plastic petri dishes under room temperature. Paper squares without additives were also prepared as controls. This procedure of sample preparation creates series of antibody-loaded papers with different additives. Taking Anti-A as an example, the paper series contain Anti-A with (1) no additives; (2) 1 % PVP; (3) 6 % dextran and (4) 10 % glycerol, which are referred to as N–A, P–A, D–A and G–A, respectively. Papers sorbed with Anti-B and Anti-D antibodies were denoted as N–B, P–B, D–B, G–B; N–D, P–D, D–D, G–D, respectively, following the same rule as Anti-A loaded papers. The stability of all paper squares was tested after being stored for 0, 7, 14, 21, and 28 days under room temperature.

Freeze drying of antibody sorbed in paper

Preparation of freeze-dried papers sorbed with antibodies

Twenty microlitres of antibody solution (i.e., anti-A, anti-B or anti-D) was introduced onto each of the total of ninety paper squares with a micropipette. This quantity was greater than that for preparing additives protected papers; this is because freeze-dried papers suffer from antibody loss due to the burst of air bubbles in antibody solutions during vacuuming. The paper squares were stored in groups of ten in sealable polypropylene sample tubes and frozen at −80 °C immediately for 30 min. Then they were freeze dried at a shelf temperature of −40 °C and a chamber pressure of 60 mTorr for 18 h. After freeze drying, these paper squares were sealed in plastic bags and stored in a desiccator at room temperature for further tests. Freeze-dried paper squares loaded with Anti-A, Anti-B and Anti-D antibodies were referred to as FD-A, FD-B, and FD-D, respectively. The room temperature longevity of antibodies was tested.

Temperature treatment of antibodies sorbed in paper

After freeze-drying, the paper squares loaded with antibodies were treated for 6 h at 4, 25, 40 and 60 °C. The stabilities of FD-A, FD-B and FD-D were tested 30 min after temperature treatment.

Test of stability of antibodies sorbed in paper

For each group of paper sorbed with antibodies, 3 μL antigen-positive and 3 μL antigen-negative reagent blood cells were respectively introduced onto two similarly antibody-loaded paper squares to study if agglutination of antigen-positive red blood cells could be triggered by the corresponding antibody. Specifically, for Anti-A, A1 and B red blood cells were used.
as antigen-positive and antigen-negative agent cells respectively; for Anti-B, B and A1 cells were used as antigen-positive and antigen-negative agent cells respectively; for Anti-D, R1R1 and rr cells were used as antigen-positive and antigen-negative agents respectively. Thirty-seconds was allowed for the antibodies in the paper to react with the red blood cells. Then, three aliquots of 25 μL saline solution were introduced onto each testing paper, while blotting papers (drink coaster blotting, 280 g/m²) were used to remove liquids during the washing process.

Blood sample agglutination evaluation

To compare the activity of the IgM antibodies sorbed into paper, both antigen-positive and antigen-negative reagent blood cells were respectively introduced onto two testing paper squares loaded with the same antibody. These paper squares were referred to as ‘+’ and ‘−’ testing papers. If the activity of the antibody is retained in paper, agglutination of the antigen-positive red blood cells will occur; a clear colour difference between ‘+’ and ‘−’ papers will be observed. However, if the antibody in paper has lost its activity, the colour difference between ‘+’ and ‘−’ papers is small. This is because that the colour difference is related to the intensity of red blood cell agglutination, which is governed by the antibody activity in paper. On the other hand, for the antigen-negative red blood cells, no agglutination occurs in paper; the non-agglutinated red blood cells can be washed out by saline solution (Li M et al. 2012), leaving a much weaker trace of blood stain in the testing zone (Fig. 1). The average grey value of the measured colour intensity difference was used to evaluate the antibody activity in paper.

In obtaining the gray scale data, the test paper squares carrying the agglutination patterns were imaged with a desktop scanner (Epson Perfection 2450, colour photo setting), then imported into Adobe Photoshop software. Images were further converted into grey mode (0–255) and the mean grey intensity of the reaction spot was obtained using the histogram function (Tian et al. 2011). The ultimate relative intensity value was generated by subtracting the measured intensity of the antigen-positive reaction paper from the intensity of the antigen-negative reaction control. Error bars (standard deviation) were obtained from three repeats of the test.

Results

Effect of additives on paper-sorbed antibody stability

The stability of antibodies in papers with and without additive was tested every 7 days for 28 days. Figure 2 shows the agglutination patterns of red blood cells in papers with and without the pre-mixed additives on Day 0 (top row) and Day 28 (bottom row). The bottom row of Fig. 2a shows that antibodies in papers not protected by additives (N–A, N–B and N–D) have lost
the ability to agglutinate antigen-positive red blood cells after 28 days of storage at room temperature. In contrast, antibodies in papers with the protection of additives retained a reasonable level of their activities after 28 days under the same storage condition and can effectively agglutinate the antigen-positive red blood cells.

Figure 3 shows the colour intensity measurements of agglutinated red blood cells on papers carrying different antibodies as a function of storage time at room temperature. All additives showed some degree of protection of the antibodies. Paper loaded with antibodies, but without additives, began to lose their ability to agglutinate red blood cells after 7 days at room temperature (Fig. 3a, b, c); after 14 days these papers had lost nearly all their bioactivities and were unable to agglutinate antigen-positive red blood cells. At the same time it was observed that these papers developed a significant level of hydrophobicity. This phenomenon has been observed in an earlier study (Tian et al. 2012). This result suggests that antibody molecules sorbed in paper may not have gained much protection from interactions with cellulose fibre at the molecular level. Although cellulose fibre surface has a large number of –OH groups, which are intuitively capable of interacting with antibody molecules, these –OH groups may not be sufficiently free to interact effectively with antibody molecules to provide protection. The dimension of the microfibers forming the cellulose fibre surface may be too large and too rigid to protect antibody molecules from forming undesirable aggregation.

The increased hydrophobicity of paper squares carrying unprotected antibodies can be explained as the result of protein dehydration and denaturing. As the antibody dries in paper, the capillary action will draw the antibody molecules towards one another. During this process the hydrogen bonding network around antibody molecules will alter, leading to irreversible aggregation of antibody molecules to form on fibre surfaces. At the same time the protective environment of water molecules surrounding the antibody molecules will change, leading to changes in the antibody molecular conformation. The increase in hydrophobicity is likely caused by the exposure of the hydrophobic sections of the protein. This explanation is supported by our further study (not presented) where antibody-loaded papers were kept wet; antibodies in wet papers retained their hydrogen bonding network unaltered and were able to retain their bioactivities for a much longer time. Polar groups of amino acid residues in antibody peptide chains,
including –OH, –NH₂, –COOH groups, are responsible for maintaining the stereo configuration of the antibody molecules through maintaining the hydrogen bonding network within and around the antibody. The antibody molecule is unique to the corresponding antigen and it must have the unique stereo configuration in order to approach the specific binding sites of the antigen and bind the antigen. Therefore any alteration of the antibody hydrogen bonding network can alter the stereo configuration of its antigen binding sites or structure around the sites. This will lead to the loss of the antibody’s ability to specifically bind to its corresponding antigen. The abundant free water molecules in a wet paper provide an environment where hydrogen bonding network around antibody molecules changes much more slowly than on dry paper; wet paper therefore provides longer protection to antibodies than dry paper of 3 months.

For papers loaded with antibody–glycerol mixtures (G–A, G–B and G–D), measurable colour density differences between the ‘+’ and ‘−’ assays were clearly observed after 28 days of storage at room temperature. This indicates that glycerol has a protective effect to antibodies, but reduces with time. The protective effect of glycerol on protein in aqueous systems has been reported (Vagenende et al. 2009). The small molecules size of glycerol and its strong hydrogen bonding potency with water allow glycerol to be a part of the hydrogen bonding network formed dominantly by water molecules around antibody molecules. We have placed the antibody–glycerol solution mixture on glass slides to perform antibody aging tests (not presented). We found glycerol can provide effective protection to antibody molecules when used in an aqueous system. These results suggest that glycerol cannot provide long-term protection to antibodies in paper.

Polymeric additives have been used for antibody stabilization and serological applications. For example, PVP has been used as a blood plasma extender and has low level of toxicity (Gombotz et al. 1994) and Dextran has also been used as an antibody stabilizer (Chang et al. 2005a, b). Figure 3a–c showed that PVP and dextran provide greater stability to antibodies than glycerol. Antibodies in paper protected by dextran demonstrated a higher colour intensity of red blood cell agglutination from Day 0 than that by PVP. However, this protective effect was similar over time, which can be seen from the near-parallel curves of the colour intensity loss with time (Fig. 3a–c). Such difference is most likely caused by the physical properties of those...
additives on paper and not by biochemical properties, since this difference is consistently observed for all three antibodies from Day 0. We believe the higher concentration of dextran in the antibody mixture compared to that of PVP explains this difference. This dextran-antibody complex could cause stronger red blood cell agglutination to occur closer to the surface of the paper, leading to the stronger colour intensity observed.

Stability of freeze-dried antibodies sorbed in paper

Freeze drying as a physical drying method is commonly used to provide long-term storage stability to proteins and pharmaceuticals (Blakeley et al. 1990; Chang et al. 2005a, b). In this study we focus on understanding the effectiveness of this method in protecting antibodies in a cellulose fibre network. Figure 4 shows the agglutination intensity on paper sensors. The agglutination of antigen-positive red blood cells by the corresponding antibodies after long storage time shows that the protective effect of freeze drying on antibodies is strong. The colour intensity measurement presented in Fig. 5 also confirms the visual assessment results in Fig. 4. These results show that freeze-drying can preserve antibodies on paper more efficiently than small molecular weight and polymeric additives.

No additive was used in this study with freeze-drying to preserve the antibodies. Our results show that the presence of cellulose fibres does not adversely affect the protection of freeze-drying on the antibodies within the time of study. These results are also in agreement with recent studies reported for this type of paper-based sensors, where antibodies predominantly locate in interfibre pores and do not diffuse into the wall of the cellulose fibres (Jarujamrus et al. 2012; Li et al. 2013).
Thermal stability of antibodies sorbed in freeze-dried papers

Freeze-dried papers loaded with antibodies were aged at different temperatures for 6 h to test the thermal stability. The evaluation of antibody stability was performed by measuring the gray scale intensity of red blood cell agglutination following the procedure outlined in section Blood sample agglutination evaluation.

Biomolecules, such as proteins and nucleic acids, are prone to denature and become deactivated by exposure to extreme pH or heat. In this study, we found that unprotected Anti-A in an aqueous solution became irreversibly denatured when heated at 60 °C for 1 h while Anti-B lost its activity in just 30 min. Even though Anti-D had the strongest vitality, it was deactivated by heating at 60 °C for 6 h in an aqueous solution. However, the thermal resistance of these freeze-dried antibodies in fibre network was found to be significantly improved. Our results showed that the freeze-dried antibodies in paper retained most of their activities after incubation under 4, 25, 40 and 60 °C for 6 h (Fig. 6). Figure 7 presents the measured colour intensity of red blood cell agglutination on papers loaded with the antibodies incubated at different temperatures. The results show that freeze-drying of antibodies loaded in paper is capable of not only increasing the longevity of antibodies at room temperature, but also significantly increasing the thermal stability of the antibodies. The cellulose fibre network...
Discussion

Stabilization of antibody molecules involves the prevention of the formation of intermolecular aggregation as well as alteration of certain intra-molecular conformation, which may destroy or block the access of the antigens to the specific recognition sites of the antibodies. The blood group antibodies in general are relatively stable in suitably buffered aqueous media; commercial grouping antibodies usually last for 2 years when stored at the recommended condition of 2–8 °C (ALBAClonal Monoclonal ABO and RhD reagent, Alba bioscience). In the fabrication of paper-based blood typing device, drying of the antibody is required. As water evaporates, the concentration of antibody molecules in the solution increases. It is possible that in the final drying stage the capillary force draws the antibody molecules together. The size of IgM antibody molecules is around 30 nm (Daniels and Bromilow 2007); capillary action of the aqueous media is able to draw the molecules together, or to draw them against the cellulose fibres. In papermaking the capillary force is responsible for drawing cellulose fibres towards one another into the hydrogen bonding range during paper drying; this process enables the ultimate formation of hydrogen bonding between fibres which gives paper its tensile strength. The capillary action in the drying of antibodies in paper could also draw antibody molecules within the hydrogen bonding distance; antibody aggregations so closely packed could lead to the dissociation and reformation of inter- and intra-molecular hydrogen bonds.

The water molecules associated to the antibody molecules play an important role in the stability of the antibodies. Small humectant molecules, such as glycerol, have been used for stabilizing protein molecules during drying (Crowe et al. 1993a, b). In this study, however, we show that glycerol has a limited stabilization effect on the antibodies in the presence of cellulose fibres. The most likely reason for this unexpected result lies in the extensive interactions of glycerol with cellulose fibres. Fabritius and Myllylä (2006) studied the penetration behaviour of glycerol into paper sheet using optical coherence. They found that glycerol molecules could rapidly penetrate into cellulose fibre wall, swelling fibres. The diffusion of glycerol into the fibre wall is detrimental to its antibody protection ability as it decreases its availability to antibody molecules. Our result therefore shows a negative aspect of using a low molecular weight humectant as a protective reagent for antibodies when the antibodies are introduced to the surface of cellulose fibres, which also have natural and strong interactions with the additive molecules. To support this explanation, we repeated the glycerol protection experiment on a glass slide (result not shown); since glycerol cannot diffuse into glass, its protective effect on antibodies lasted much longer than on paper. This observation provides guidance for developing strategies of antibody protection in cellulose fibre networks—interactions of stabilizing reagents with the cellulose fibres must be considered.

The use of polymers as additives to protect the activities of antibodies is more effective. This is expected as both polymers used in this work have a molecule weight $>3 \times 10^4$ Da. It is difficult for molecules of such sizes to penetrate into the fibre walls (Solberg and Wegberg 2003), therefore they are more available to effectively interact with antibody molecules and the water molecules surrounding them.

The strong protective effect of freeze-drying on the antibodies in the cellulose fibre network is likely to be caused by the absence of capillary action between antibody molecules and the cellulose fibre surface. Before freeze-drying, the intermolecular distance between the antibody molecules in an aqueous solution is greater than the hydrogen bonding distance, because of the presence of water molecules. Since water is removed from the paper as vapour, the Laplace pressure, which is associated with the liquid meniscus, is absent. It is likely that in the absence of the Laplace pressure the antibody molecules are not pressed towards one another, but remain in their natural position as the water is being gradually removed.

A similar phenomenon can be seen in the preparation of low density cellulose fibre networks using the freeze drying method; the removal of water from a cellulose fibre suspension in the absence of the Laplace pressure results in the formation of a fluffy fibre network of very low density. The large voids in such fibre networks keep the distance between individual fibres greater than the hydrogen bonding.
distance. Likewise, freeze-dried antibody in paper has the advantage of having much less aggregation, which allows the antibodies to retain their potency to interact with antigen sites on red blood cell surfaces. It is therefore understandable that the presence of a cellulose fibre network does not have obviously adverse effect the freeze-drying of antibodies in paper.

The lack of aggregation of antibody molecules in freeze-dried papers is likely to also contribute to the thermal stability of the antibody molecules. The large distance between the freeze-dried antibody molecules reduces the possibility of hydrogen bonding between the non-agglutinated antibody molecules.

Conclusion

We show in this study that the longevity of blood group antibodies sorbed into paper (Anti-A, -B and -D) is improved by the mixing of additives such as glycerol, PVP and dextran. Among them, low molecular weight additives that are capable of penetrating into the fibre wall provide a lower level of protection to antibodies than the high molecular weight polymers. This finding provides useful guidance to future paper sensor design in that the interactions between additives and cellulose fibres must be considered in order to optimize the protective effect of the additives on antibody molecules. Furthermore, freeze-drying provides a much higher level of protection to antibodies than additives. The lack of antibody aggregations in freeze-dried paper reduces the inter-molecular hydrogen bonding of antibody molecules; this allows them to retain their natural configuration and interact with the antigens on the red blood cell surfaces. The non-aggregation of freeze-dried antibodies in paper has also shown much higher thermal stability than the unprotected antibodies. Cellulose fibre networks do not show noticeable adverse effects to the freeze-drying of antibodies in paper. Our results show that freeze-drying is a suitable method for providing long-term protection to antibodies in paper, whereas additives may be used for applications where shorter-term protection is required. It is expected that the outcome of this study could be used for other paper-based biological sensors to satisfy the basic requirement of sensor longevity.

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Polysaccharides as protectants for paper-based analytical devices with antibody

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**Abstract**

Paper with immobilized protein can provide a low-cost platform for diagnostics, but evidence is emerging that the instability of protein on paper during shipping and storage severely limits its commercial development. Of these, paper-immobilized antibody, though widely used, is in urgent need of improvement in the shelf life. Herein we provided a detailed investigation of a simple and versatile approach for achieving stable antibody on paper to improve bioassay performance. Anti-Blood Group A IgM was chosen as the model antibody and its activity was tested via agglutination reactions. Representative polysaccharides (i.e., chitosan, sodium alginate and dextran) were used as protectants and their ability to stabilize antibody under heat and desiccation stresses was investigated. It was found that the presence of dextran with high concentration markedly stabilized paper-immobilized IgM for at least 120 days. Our results indicate that dextran with good film-forming ability could be a protectant for paper-immobilized antibody.

1. Introduction

Protein, as sensitive biological compounds, has an increased tendency to lose function under some external stress such as heat, radiation, desiccation and shearing [1–3]. It usually requires temperature-controlled supply chains to retain biological activity in the pharmaceutical and biotech industries, resulting in cost increase and use limitation [4,5]. Formulations for protein storage and stability have been developed; freeze-drying is the most common strategy to preserve protein in the form of dehydrated powders [6]. Such powders are typically used to extend the shelf life of products, but some types of protein still can be damaged during the process [7,8].

The development of many experimental approaches and applications in biological and chemical sciences has brought about an increasing requirement for the immobilization of protein onto solid substrates [9,10]. Protein immobilized on various supports also needs to be protected to perform the biological functions. Among them, paper has demonstrated excellent properties as a novel support, including low cost, portability and feasibility of patterning via inkjet printing [11,12]. Many studies reported the potential of protein-based paper biosensors as simple point-of-care devices, but at the same time highlighted the need for protein stabilization strategies [13,14].

As an important type of protein, antibody, also known as immuno-globulin, plays a crucial role in current bioactive paper-based diagnostics [15–17]. The storage and stabilization of paper-immobilized antibody is a basic concern to evaluate the performance of these bioassays. The work by Ramachandran et al. and the preliminary study in our group have shown that freeze-dried antibody immobilized on paper could be stored for a long period of time at room temperature without significant loss of the bioactivity [18,19]. However, the freeze-drying process is not a cost-effective way for the large-scale manufacturing of bioactive paper with immobilized antibody, which, in turn, makes itself not suitable to be a generic approach. Different from antibody protection, enzyme, as another commonly used type of protein for bioactive paper, has been successfully protected on paper by several easy and inexpensive approaches in the past few years. Alkasir et al. observed that tyrosinase enzyme, entrapped between chitosan and sodium alginate layers deposited onto filter paper, could show 92% residual activity after 260-day storage [20]. Zhang et al. reported that glucose oxidase and lactate oxidase could retain full activity over 6 months of storage on starch-coated paper [21]. Therefore, currently antibody is in urgent need of a simple, low-cost and robust solution of its bioactivity protection. Encouraged by the above-mentioned successful application of natural polysaccharides on paper-immobilized enzyme preservation, it is of great value to examine the compatibility of this cost-effective approach with paper-immobilized antibody. Meanwhile, although the studies have presented effective enzyme protection on paper via polysaccharides, there is a lack of...
detailed analysis about polysaccharide use, which could give more accurate instructions to the fabrication of bioactive paper.

Of the five classes of immunoglobulins (IgA, IgD, IgE, IgG and IgM), IgG and IgM are by far the most frequently used antibodies in bioassays, and they share a similar structure and function [22]. Considering IgM is more susceptible than IgG to denaturation, Anti-Blood Group A IgM with great use was chosen as a model antibody in our study. These agglutinating antibodies have been widely used to fabricate paper-based analytical devices for point-of-care diagnostics. Rapid blood typing can be performed on paper by using agglutinating antibodies to induce red blood cell (RBC) agglutination [23,24]. Besides, plasma separation based on RBC agglutination has been proved to be useful for designing paper-based bioassays for detecting plasma components like glucose [25]. Thus the long-term stability of antibody on paper is important for improving bioassay performance. Herein, various polysaccharide strategies of immobilizing IgM on paper were investigated, including polysaccharide covering, supporting and blending. The important factors involving polysaccharide modification and IgM immobilization were analyzed by agglutination reactions. The results provide an easy and inexpensive path to prolong the shelf-life of antibody-based paper biosensors and serve as valuable references for the implementation of paper-based biosensing systems.

2. Materials and methods

2.1. Reagents

Low-molecular-weight chitosan (Cat. No. 448869, 20–300 cP, 1% in 1% acetic acid), low-molecular-weight sodium alginate (i.e., NaAlg, Cat. No. A0682, 4–12 cP, 1% in H₂O) and low-molecular-weight dextran (Cat. No. D1662, ~4 cP 10% in H₂O [26]) were purchased from Sigma-Aldrich. Anti-Blood Group A IgM (abbreviated as IgM in this study) for further manufacturing use (FFMU) purchased from Commonwealth Serum Laboratories (CSL), Australia was used as the model antibody. Red blood cells (RBCs) from CSL Revercell™ (15% A1 and 15% B RBCs) were concentrated to 45% before use. Kleenex paper towel (Kimberly-Clark, Australia), as the paper substrate, was cut into 1 cm×1 cm pieces to immobilize antibodies. All reagents were used without further purification and all solutions were prepared using ultrapure water (18 MΩ cm, Mill-Q Gradient System, Millipore).

2.2. Instrumentation

Dark field images of papers were obtained by an Olympus BX60F microscope. Fluorescence images of fluorescein isothiocyanate labelled bovine serum albumin (FITC-BSA) and RBCs labelled with FITC were captured with a Nikon confocal microscope. SEM images were recorded with a FEI Nova NanoSEM™ scanning electron microscope. Water Drop Penetration Time (WDPT) test was carried out by a contact angle instrument (Dataphysics OCA230, Germany) as follows: A paper square was mounted onto the sample holder, and a 3 µL ultrapure water droplet was delivered onto the paper surface by a glass syringe. Then the penetration time of the water droplet through the paper was calculated from the video of the water drop penetration process. The test was repeated three times at room temperature. The images of agglutination assays (see below for details) were recorded by a desktop scanner and quantitative analysis was achieved using ImageJ software to invert and measure the grayscale intensity of blood spots. Error bars (standard deviation) were obtained from three repeats of the test.

2.3. Red blood cell agglutination assays

The interaction of IgM with RBCs can have two possible results: agglutination, referred as positive assay (+), and non-agglutination, referred as negative assay (-). Kleenex paper towel that has larger paper pore size than filter paper possesses an enhanced ability to elute non-agglutinated RBCs, although Kleenex paper towel and filter paper have comparable ability to fix agglutinated RBCs [27]. Thus Kleenex paper towel was chosen as the paper substrate in this study. The color intensity value could be obtained by subtracting the measured intensity of the negative from that of the positive in the grayscale mode (0−255). Successful testing should easily distinguish the positive assay from the negative one; that is, the positive assay should report a well-defined blood spot on paper while non-agglutinated RBCs from the negative assay can be easily washed out of the paper by 0.01 M pH 7.4 phosphate-buffered saline (PBS) (Fig. 1A and B). So a high color intensity value would be obtained. In order to perform the bioactivity evaluation of paper-immobilized IgM, both 3 µL 45% antigen-positive and antigen-negative reagent RBCs (i.e., A1 and B RBCs) were respectively introduced onto two testing paper squares immobilized with IgM, followed by 90 s incubation and then 2×50 µL PBS washing.
If the bioactivity of IgM is not retained on paper, the positive assay could not report a well-defined blood spot, but falsely show the result as a negative one (Fig. 1C). Therefore, we would get a low color intensity value. The value of color intensity declined with the decrease of paper-immobilized IgM activity. Obviously, agglutination assays as an indicator provide a simple and semi-quantitative method for evaluating the bioactivity of paper-immobilized IgM.

2.4. Antibody immobilization on papers with different polysaccharide formulations

Polysaccharide covering: 5 µL of IgM was spotted on the paper squares and dried for 30 min. Then 5 µL of chitosan solution in aqueous acetic acid with 0.9% NaCl, NaAlg solution in PBS and dextran solution in PBS were respectively applied to the paper squares, followed by 30 min drying at room temperature. With this method, IgM spotted on paper surface was covered by polysaccharides (Fig. 2).

Polysaccharide supporting: 5 µL of chitosan, NaAlg and dextran solution were applied to the paper squares and allowed to dry for 30 min, respectively. Then 5 µL of IgM was spotted on each of the freshly prepared papers and dried for 30 min. With this method, IgM was spotted on a polysaccharide layer that supported the IgM and reduced the contact between IgM and paper (Fig. 2).

Polysaccharide blending: 5 µL of IgM solution containing chitosan, NaAlg or dextran were respectively applied to the paper squares, which were then dried for 30 min. With this method, IgM was incorporated into the polysaccharide matrix (Fig. 2). The activity of immobilized IgM was evaluated by the agglutination reactions as mentioned above.

2.5. Thermal and storage stability studies of different papers with immobilized antibody

During heating studies, the freshly-prepared paper samples were aged in an oven at 60 °C for various incubation periods. For long-term stability studies, all paper samples were prepared as previously described, placed in pill containers under ambient conditions, and stored at room temperature in the dark. Residual bioactivity of paper-immobilized IgM was evaluated using agglutination tests.

3. Results and discussion

3.1. Concentration-dependent Antibody Bioactivity

The knowledge of paper-immobilized antibody stability can strongly help to reduce the cost while sustaining the effectiveness of paper bioassays. So we first investigated the impact of antibody concentration on the stability of paper-immobilized IgM when stored at room temperature in the dark. The serial dilution data show that IgM, although being diluted, retained its activity at day 0 (Fig. 3). However, the loss of IgM activity was observed at all storage concentrations within 10 days. The original IgM (protein concentration ranging from several mg/mL to tens of mg/mL) had the relatively low rate and extent of deactivation, while activity loss was comparatively obvious for diluted IgM. As shown in the inset image in Fig. 3, there is no significant color difference between positive and negative results for paper samples with 50-fold and 100-fold diluted IgM, causing low intensity values. The results were consistent with previous reports that many proteins could display greater stability at higher concentration [28]. The obvious deactivation of IgM only after being stored for 10 d for the whole dilution-series suggests proper storage demands antibody stability on paper. However, Fig. S1 shows that the dilution-series of IgM via dextran blending strategy retained its activity within 10 days. Thus it is of great value to perform a detailed analysis of polysaccharide protection for paper-immobilized IgM. Considering the commonly used concentration range of paper-immobilized protein (tens of to hundreds of µg/mL) and agglutination assay responses to the dilution-series of IgM, a 1:10 dilution of IgM was used throughout this study, unless specially noted.

3.2. Polysaccharide modification and antibody immobilization

Inspired by successful biomolecule immobilization via layer-by-layer assembly technique for many applications [29,30], herein we proposed three simple types of antibody immobilization strategies: covering, supporting and blending, which were classified based on the role played by polysaccharides during the process of antibody immobilization. Representative polysaccharides, i.e., cationic chitosan, anionic NaAlg and neutral dextran, were chosen for antibody immobilization. On one hand, cellulose fibers have a porous structure (1–30 nm micropores) [31]. Due to the nanoporosity of cellulose fibers, polysaccharides with molecular weights of several thousands could penetrate into the fiber wall, which may preclude the effective antibody-polysaccharide interactions. On the other hand, polysaccharides with high molecular weight cannot be applied onto paper easily and cannot provide effective protection for proteins via intermolecular interactions due to steric hindrance [32], so polysaccharides with relatively low molecular weights (up to tens of thousands) were utilized. Moreover, an increase of polysaccharide concentration would obviously increase the viscosity of the polysaccharide solution, resulting in the difficulty of sample loading onto paper. In practice, chitosan and NaAlg were used with the concentration of no more than 2% w/v while concentrated dextran solution (up to 40% w/v) was employed. We believe that the differences in the compositions and structures of polysaccharides determine the different operating ranges of concentration. Besides hydroxyl groups, amino groups in chitosan and carboxyl groups of NaAlg can also form hydrogen bonds with hydroxyl groups of polysaccharide chains respectively, which could cause the relatively high viscosity of polysaccharide solutions.

Dark field microscopy and SEM were used to characterize the
nature of polysaccharide coatings on paper. Many polysaccharides were reported previously to have the good film-forming ability because of their intra and intermolecular hydrogen bonds [33]. High-concentration dextran (i.e., 40%), as shown in Fig. 4, could supply enough polysaccharide to fill the pores and voids between the fibers, thereby developing a thin film on the paper surface. By contrast, chitosan and NaAlg at 2% concentration only interpenetrated the fiber network, generated evenly distributed coatings on surface of paper fibers and did not obviously change the surface porosity of paper. Then the morphology and distribution of immobilized antibody via different strategies was examined by using 1 mg/mL FITC-BSA as a model protein. Fig. 4 and Fig. S2 show that FITC-BSA could be relatively well distributed on the paper. Compared with the inset images, it was found that proteins with different immobilization strategies could penetrate almost throughout the paper network and were relatively homogeneous in the direction of lateral solution movement.

3.3. Antibody performance on different papers

An ideal immobilization strategy should not do damage to the functionality of biomolecules, and therefore the effect of employing various polysaccharide formulations for IgM immobilization on paper was initially evaluated by performing RBC agglutination assays. All three chitosan-based immobilization methods, as shown in Fig. 5, were proved to fail to perform effective agglutination assays. It was therefore not suitable to evaluate the protective power of chitosan to antibody molecules via agglutination assays. Such behaviour is related to electrostatic interactions between the positively-charged protonated amine on chitosan chains and negatively-charged RBC membrane. When treated with chitosan, RBCs lost their typical biconcave morphology and coalesced into a clot that could not be easily washed off paper sheet by PBS buffer (Fig. S3). Thus no significant difference was observed between positive and negative testing, which resulted in the low intensity value.
Unlike chitosan, different immobilization approaches using NaAlg or dextran display different results. Among the three strategies, the most effective RBC agglutination was observed via polysaccharide-blending immobilization strategy (Fig. 5), and the color intensity was found in the increasing order of covering < supporting < blending strategies. Despite a lack of electrostatic attraction like chitosan, NaAlg and dextran can still act as a physical shield to restrict RBC access to the active sites of IgM when choosing the covering method, which in turn indicates the limitation of polysaccharide-covering strategy as a generic approach for immobilizing biomolecules on paper because of potential interference with bioassays. However, the other two strategies enabled IgM to react with RBCs more efficiently than the control (paper-immobilized IgM without polysaccharides), as polysaccharides with certain viscosity could reduce the flow rate and facilitate the interaction between the immobilized IgM and RBCs. The latter strategy (blending) was better than the former (supporting) since blending could promise a sufficient contact between IgM and RBCs.

3.4. Paper-immobilized antibody storage

To evaluate the application potential of polysaccharide as paper-immobilized antibody stabilizer, we compared the stability of IgM under different conditions. As mentioned above, IgM that was deposited on paper without any polysaccharide protection denatured rapidly and lost its function (Fig. S4). Fig. 6 and Fig. S5 show that IgM deposited on paper by the supporting and blending strategy in the presence of NaAlg or dextran has the improvement in shelf life. For polysaccharide solutions at the same concentration, the signals from the supporting strategy decreased more rapidly than those from the blending strategy during 2-month storage. Irreversibility of polysaccharide adsorption to cellulose has been reported because of the similarity in their chemical structures [34]. Unlike the homogeneous blending, we believe that the supporting strategy makes polysaccharide unable to dissolve sufficiently to engage in the interactions with IgM effectively when adding IgM on polysaccharide-coated paper, which is not good for the protection of IgM on paper. The blending method, on the other hand, delivered a relatively satisfying signal within 2 months. Even with the low concentration, a detectable signal was still observed at day 60, indicating that some of antibody was still active (Fig. 6). These results confirmed that polysaccharide could extend the shelf life of antibody deposited on paper.

Stability improvements via blending strategy became much stronger in the comparison of supporting and blending in the two polysaccharide systems; stabilization of antibodies blended with polysaccharide was proved to be dependent on polysaccharide concentration: color intensity increased with increasing concentration for NaAlg and dextran (Fig. 6). During dehydration and storage, IgM blended with polysaccharides could bring about the matrixes with high viscosity and low molecular mobility. The entrapment of IgM in dried polysaccharides with an amorphous state prevents the unfolding of antibody molecules. As can be seen in Fig. 6A, NaAlg, although only small amounts were used, still could provide a viscous environment for IgM preservation. In terms of dextran, higher concentration resulting in higher viscosity and more IgM-dextran interactions prevented IgM denaturation. Notably, when the dextran dosage in the blend was increased to 40%, about 85% initial potency of IgM was retained after 2 months of storage at room temperature (Fig. 6B) and paper-immobilized IgM shows ~ 80% residual activity after 120-day storage (Fig. S6). These data indicate that the presence of dextran film is important for the improvement in stabilization of paper-immobilized IgM. As mentioned above, when 40% dextran solution resolidified into films upon drying, protein molecules were well distributed throughout the dextran film matrix. In this case, the entrapment of IgM into a biocompatible dextran film with good dispersion performance could effectively prevent IgM from going through conformational changes during storage.

Besides paper bioactivity, paper wettability, which determines the penetration of liquid samples or reagents into the paper sheet, is another important property that affects the performance of paper-based bioassay. We believe that a moderate level of wettability is favorable for bioactive paper, which may enhance the interaction between liquid samples or reagents and paper-immobilized biomolecules. In this study, antigens on the surfaces of RBCs and IgM in the polysaccharide layer require time to interact. This interaction is dependent upon the dissolution of polysaccharide matrices and mixing of RBCs with released IgM. A moderate wettability of bioactive paper could allow time for those events to occur. In contrast to this, very low paper wettability is not conducive to antigen-antibody interaction, since low wettability suggests that slow dissolution of polysaccharide might be encountered, which could cause incomplete or even no assay development because of insufficient paper wetting. As can be seen in Table S1, an increased storage time can reduce the wettability of papers with immobilized IgM, which can be attributed to the exposure of the hydrophobic regions of the antibody, promoting intermolecular interactions leading to aggregation. Since protein denaturation could cause an increase in the surface hydrophobicity, we therefore expected a significant improvement of paper wettability via 40% dextran blending that could offer efficient antibody protection. The data of the water drop penetration time supports our reasoning, and the paper with 40% dextran blending strategy has a moderate level of wettability after storage that could be useful for preforming paper-based bioassays. The high stability of IgM in the dextran film provided effective support for the retention of hydrophilicity of paper with immobilized IgM.

![Fig. 6. IgM storage stability on different papers. (A) Paper-immobilized IgM via NaAlg blending. (B) Paper-immobilized IgM via dextran blending. Relative activity was expressed as the percentage of IgM activity that remained after storage in comparison to the activity at day 0 for each group.](image-url)
in the presence of the highest concentration of NaAlg (2%) and dextran (40%); approx. 20% and 15% activity loss after 8 h heating, respectively. Such protection could be related to the inhibition of structural alterations in antibody. Polysaccharides like NaAlg and dextran, having a relatively high glass transition temperature in the dry state, can inhibit molecular mobility and consequently enhance IgM thermal-stability on paper (Fig. S7). Pronounced stabilization of IgM in dextran film at the elevated temperature suggests this method could also provide sufficient thermal stability to paper-based biosensors. The concentration of dextran has less effect on thermal stability than on storage stability. We believe that it may be associated with the different rates of dehydration under different conditions. Dextran film provides an inexpensive and effective protection environment without the use of vacuum and desiccant. Moreover, unlike chitosan and NaAlg, natural polymeric carbohydrates like dextran, having hydroxyl groups as the primary functional groups, is very hydrophilic and non-charged, which could be benign to typical bioassays [36,37].

4. Conclusions

In this work, the application area of polysaccharide protection was extended and dextran was applied as an effective formulation component for enhancing stability of antibody attached to paper. Appropriate coating system (blending) could increase the shelf life of IgM compared with IgM deposited onto unmodified paper. The stabilization data presented here indicate that the formation of hydrophilic films in paper plays a vital role in protecting paper-immobilized antibody. Dextran reduced protein unfolding and aggregation at elevated temperatures, and also provided structural stability to IgM during storage. Since most studies emphasize the invention of paper-based bioassays, we believe that our results of this study will be of use for improving process, distribution and use of paper bioassays without the costly cold chain.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2016.12.079.

References

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Appendix II

Published Co-Authored Papers Not Included in the Main Body of This Thesis
Barcode-Like Paper Sensor for Smartphone Diagnostics: An Application of Blood Typing

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Supporting Information

ABSTRACT: This study introduced a barcode-like design into a paper-based blood typing device by integrating with smartphone-based technology. The concept of presenting a paper-based blood typing assay in a barcode-like pattern significantly enhanced the adaptability of the assay to the smartphone technology. The fabrication of this device involved the use of a printing technique to define hydrophilic bar channels which were, respectively, treated with Anti-A, -B, and -D antibodies. These channels were then used to perform blood typing assays by introducing a blood sample. Blood type can be visually identified from eluting lengths in bar channels. A smartphone-based analytical application was designed to read the bar channels, analogous to scanning a barcode, interpret this information, and then report results to users. The proposed paper-based blood typing device is rapidly read by smartphones and easy for the user to operate. We envisage that the adaptation of paper-based devices to the widely accepted smartphone technology will increase the capability of paper-based diagnostics with rapid assay result interpretation, data storage, and transmission.

Paper-based analytical devices (PADs) have generated great interest in the field of medical diagnostics in recent years because they demonstrate great potential to develop affordable, rapid, and practical diagnostic sensors for resource-limited areas.1−5 Numerous research works have been reported, covering methods of paper sensor fabrication,6−7 analyte-specific assays,8 and various result-reporting ideas, including colorimetric,9 fluorescent,10 electroluminescent,11 and electrochemical,12 as well as text-reporting.13 Among the analyte-specific assays, PADs were demonstrated to be able to provide semiquantitative to quantitative analysis.14−16 Recently, PADs, investigated as a class of field-based, equipment-free blood typing devices, have achieved the same accuracy but have been proven to be much more rapid than the mainstream blood typing technologies.13,17,18 These developments show that current research has produced a wide range of new concepts for making paper-based devices a new platform that does not require hospital and laboratory support. Among these concepts, several have been extensively developed and have almost reached commercialization.19,20

Most PADs research focuses on the development of stand-alone devices that function under nonlaboratory conditions.20−22 While in this direction many innovative ideas have been explored and reported, it should be noted that PADs will be more successful if they can be adapted to existing technologies that are ubiquitous in modern society. From the early days of PADs research, it was proposed that camera phones can significantly enhance low-cost diagnostics by connecting the users who perform the tests with professionals who receive the results and provide medical instructions.23,24

The extremely high acceptance of smartphone technology by the human population in both developed and developing countries makes this technology a valuable infrastructure for any other technology to build upon.25 The adaptation of PADs to smartphone technology, particularly through software innovation, will significantly enhance the power of PADs as a platform technology for diagnostic applications through providing simple analytical tools, increasing coverage and connection between users and professionals, and allowing data sorting, storage, and management for local and temporary medical facilities in remote medical emergencies and disaster response missions.

In this study, we applied a barcode reading concept to adapt paper-based blood typing assays to smartphone technology. Blood typing is a routine clinical assay in surgical procedures, medical emergencies, and blood transfusions, as well as in blood banking.26 Our group has reported a series of studies on the fabrication of new types of PADs and thread-based analytical devices for blood typing.27−29 These studies have demonstrated new stand-alone blood typing devices that can be operated by users without the need of any supporting instrument. While the stand-alone devices promise a superior platform to the mainstream hospital-based technologies in...
providing health care to home-based users and users living in impoverished areas, adaptation to smartphones through software innovation will provide further advantages to paper-based blood typing technology in digital data sorting, storage, and transmission. It is anticipated that the use of software will be an effective means of reducing human error in data sorting and storage. In this work, we developed a simple software for smartphones and used it for paper-based blood typing assays.

**EXPERIMENTAL SECTION**

**Reagents and Apparatus.** Antibodies against RBC antigens approved for human blood grouping were obtained from the Commonwealth Serum Laboratory (CSL), Australia. They were IgM antibodies commercialized under the names of Epilclone Anti-A, Anti-B, and Anti-D (IgM) FFMU Concentrate. All of them are transparent solutions. Phosphate-buffered physiological salt solution (PBS, pH 7.4), sourced from Sigma-Aldrich, Australia, was used as the diluent for all antibody solutions. Alginic acid sodium salt (NaAlg) was acquired from Sigma-Aldrich, Australia. Anti-A and Anti-B were diluted to 1/5 with PBS solution for use in our study. All antibodies were stored at 4 °C.

Blood samples acquired from adult volunteers of a known blood group were provided by Red Cross, Australia. Samples were stored in Vacutainer tubes containing heparin, citrate, and EDTA and refrigerated at 4 °C; they were used within 10 days of collection. Reagent red blood cells Revercell (15% A1, 15% B red blood cells) and Abtectcell III (3% R1R1, 3% R2R2, and 3% rR red blood cells) were purchased from CSL Limited, Australia, stored at 4 °C and used within 30 days of delivery. All blood samples, including reagent blood cells, were diluted to 15% with PBS for performing blood typing assays in our printed barcode paper devices. Ultrapure water (≥18.2 MΩ) purified by a Milli-Q System (Millipore, Bedford, MA, USA) was used for the preparation of all solutions. Kleenex paper towels (Kimibly-Clark, Australia) with a basis weight of 34 g/m² and an apparent thickness of 140 μm were purchased from a local supermarket.

**Bar Pattern Design for Blood Typing.** Alkyl ketene dimer (Preci900, Hercules Australia Pty. Ltd.) was used as the cellulose hydrophobization reagent. Analytical-grade n-heptane (Sigma-Aldrich, Australia) was used as the solvent for the dimers. The Kleenex paper, cut into A4 size, was selected as the substrate to fabricate the bar pattern paper-based blood typing devices. The fabrication procedure involves using a reconstituted commercial desktop ink jet printer (Canon Pixma ip4500) to print computer-generated patterns onto the paper with an ink solution of alkyl ketene dimer–heptane (3%, v/v) according to previous works.30 The printed paper was heated in an oven at 105 °C for 1 h to allow the hydrophobicity of the printed area on paper to fully develop. In this work, the device was fabricated with three separate channel patterns, which were labeled with the printed letters “A,” “B,” and “D.” Each pattern consisted of a bar channel (0.3 cm × 5 cm), a buffer rinsing zone (r = 0.2 cm), and a bridging channel linking these two (Figure 1). The channel patterns were hydrophobic and used for the assaying application.

**Preparation of Antibody-Loaded Bar Channels.** Before loading antibodies, the bar channels were treated with NaAlg for the purpose of enhancing antibody loading efficiency. NaAlg solutions with concentrations of 0%, 0.01%, 0.05%, and 0.1% (w/v) were prepared using Millipore-purified water. The patterned paper devices were divided into four groups; the channels of each group were treated with 10 μL of each of the four NaAlg solutions, respectively. All the papers modified with NaAlg solutions were dried for 30 min at room temperature.

All channels marked with “A” (channel “A”) in all four groups of NaAlg-treated papers were treated with Anti-A; 10 μL of diluted Anti-A antibodies were uniformly deposited into the entire channel length. Likewise, for channels “B” and “D”, 10 μL of Anti-B and 10 μL of Anti-D antibodies were deposited into the “B” and “D” channels of all papers, respectively. All the paper devices were dried for 1 h at room temperature.

Covalent antibody immobilization involves two steps: activating the NaAlg-modified paper surface using EDC-NHS chemistry and then conjugating it with the antibody molecules. In the paper surface activation step, 20 μL of an equal-volume mixture of 20 mg/mL EDC and 12 mg/mL NHS solutions was uniformly deposited into each channel and the activation reaction was allowed 30 min for completion in an environment with a controlled high humidity. After activation, each channel was rinsed with 60 μL of H2O three times. For the conjugation of antibodies, all of the “A” bar channels were uniformly deposited with 10 μL of diluted Anti-A antibodies and allowed 1 h for conjugation at 50% humidity. Similarly, bar channels “B” and “D” of all groups were deposited with 10 μL of Anti-B and 10 μL of Anti-D, respectively, under the same conditions, for conjugation reactions. At the end of the conjugation reactions, each channel was rinsed three times using 60 μL of PBS and then allowed to dry for 1 h at room temperature.

**Water Drop Penetration Time (WDPT) Measurement.** To evaluate the influence of paper surface modification on paper wettability, a water drop penetration time test was adopted. The untreated and antibody-conjugated Kleenex paper towel was cut into 1 cm × 1 cm squares for water penetration time testing. A contact angle instrument (Data-physics OCA230, Germany) was used to take video of the water drop penetration process. The experimental procedure was as follows: a piece of a paper square was horizontally fixed to the measurement platform; a 3 μL water droplet was delivered onto the paper surface by a syringe; the drop landing
and penetration processes were recorded at a frame speed of 56 frames/s; the final penetration time was calculated by the system software from the video data. Three measurements were taken for each paper sample.

**Blood Typing Using the Barcode-Like Paper Device.** A volume of 3 μL of prediluted 15% blood samples was introduced into each channel via its sampling site indicated by the red line (Figure 1); red blood cells were permitted to react with the antibody in the channel for 30 s. Then, a volume of 10 μL of PBS buffer solution was introduced onto the rinsing zone and allowed to elute through the channel by capillary wicking for 1 min. The testing assay was then scanned and interpreted by smartphone-based technology.

**Smartphone-Based Analysis Design.** The mobile software designed for carrying out the preset analytical procedures (app) was developed on the Android platform. The software was coded with Java using ADT (Android development tools), and the functions of the smartphone were controlled through APIs (application programming interfaces). The app can be supported by Android version 2.2 and above. The app was designed to read the information in each bar channel along its length, since the blood penetration along the channel gives the most distinguishable difference between a positive and negative result. In this study, a Google Nexus 5 smartphone (Google Android version 4.4) was used to read the final blood typing results.

**RESULTS AND DISCUSSION**

**The Sensor Design Concept.** Paper-based analytical devices have been designed for ABO/RhD blood grouping based on the principle of hemagglutination reaction between RBCs and antibodies. When a blood sample wicks into the fiber network of paper treated with the corresponding antibody, hemagglutination will occur within the fiber network which will lead to the formation of large lumps that lock inside the fiber network and cannot be eluted out. Therefore, when hemagglutination occurs in the bar channels of our blood typing device (Figure 1), buffer elution will not elute the agglutinated RBC lumps along the channel, leaving a short bar of blood stain with a strong color. The deep red color came from the aggregated red blood cells. In contrast to this, when RBCs contact a noncorresponding antibody inside the fiber network, no hemagglutination will occur. Free RBCs can be eluted along the channel by PBS solution, forming a diluted bar of much greater length along the channel (Figure 1). For the purpose of interpreting an assay result, if we can distinguish a "short" bar from a "long" bar in a channel, it will be possible to identify whether hemagglutination has occurred in this channel or not. Therefore, making sure the "short" and "long" information are distinguishable is an important consideration when designing this sensor.

However, the length difference between agglutinated (positive, "P") and nonagglutinated (negative, "N") RBCs was not significant enough to be clearly identified (Figure S-1a, Supporting Information), since the limited volume of blood samples cannot provide enough driving force for non-agglutinated RBCs to continue penetrating forward. Therefore, we introduced PBS buffer solutions as eluting buffer into channels to supply a driving force for free RBCs to continue to move forward. The RBCs eluting length difference between positive and negative tests was distinct: agglutinated RBCs formed a short eluting length in the bar channel, while nonagglutinated RBCs led to a much longer eluting length (Figure S-1b, Supporting Information). Such difference makes this sensor viable for accurate identification by smartphone-based analysis. In the final design of this blood typing assay (Figure 1), three separate patterns labeled with "A", "B", and "D" were designed for the identification of A, B, and D antigens, respectively, to determine ABO and RhD blood types.

**Sensor Development.** On the basis of the above sensor design concept, a smartphone-based app was designed to read the RBC elution length in a channel. To ensure unambiguous results identification using this design idea, different physicochemical channel modifications have been considered. Emphasis is given to the design of the bar channels to provide the negative and positive tests with a significant sample eluting length difference for easy and fast identification by the software. This relies on detailed consideration of channel surface treatment to enhance the antibody sorption and wetting ability of paper. For this reason, different paper surface modification methods have been investigated to obtain the maximum eluting length difference between positive and negative tests.

**Physical or Chemical Immobilization.** Physical and chemical methods for antibody immobilization were investigated so that the method capable of producing a larger difference between the positive and negative assays could be selected. The chemistry applied here is the EDC/NHS activating agent pair which is commonly used for the coupling of primary amines with carboxyl groups to yield amide bonds. Since there are sparse carboxyl groups on the paper, NaAlg was used to increase carboxyl groups on paper because of its great biocompatibility and the retention of NaAlg has been proven to be satisfactory. However, the antibody-treated channels with chemical coupling have failed to retain a sufficient amount of antibody on the paper surface for performing a blood typing assay. Also, this approach causes a decrease of paper wettability as shown by the water drop penetration test (WDPT) (Table S-1, Supporting Information). These undesirable outcomes make the chemical coupling approach unsuitable, since poor paper wettability prevents the blood sample from wicking into the fiber network and contacting antibody molecules. In contrast to this, antibodies physically immobilized on paper can be freely released from the fiber matrix into the blood samples to effectively collide and react with RBCs to allow hemagglutination reactions to occur.

**NaAlg Concentration.** Among the systems that were considered above, physical immobilization was judged to be the only appropriate method to provide a suitable surface modification for the sensor design. To optimize the physical immobilization of NaAlg, the bar channels were first modified with 0% (w/v) NaAlg (Figure S-2,1, Supporting Information), 0.01% (w/v) NaAlg (Figure S-2,2, Supporting Information), 0.05% (w/v) NaAlg (Figure S-2,3, Supporting Information), and 0.1% (w/v) NaAlg (Figure S-2,4, Supporting Information), followed by treatment with Anti-A antibodies (Figure S-2a, Supporting Information), Anti-B antibodies (Figure S-2b, Supporting Information), and Anti-D antibodies (Figure S-2c, Supporting Information). Both positive (Figure S-2, "P", Supporting Information) and negative (Figure S-2, "N", Supporting Information) assays were performed by, respectively, introducing antigen-positive and -negative RBCs in bar channels modified with different concentrations of NaAlg and the corresponding antibodies. As shown in Figure S-2, Supporting Information, all positive assays had shorter bar lengths than the negative reactions. Although antigen-positive and -negative blood samples could be distinguished among all
the NaAlg modified channels, the modification that led to the greatest difference was selected for more accurate identification. Therefore, 0.05% NaAlg was chosen for the final sensor design since the length difference was the greatest with this concentration (Figure 2).

![Figure 2](image.png)

**Figure 2.** Eluting length difference in “A,” “B,” and “D” reaction bar channels modified with different concentrations of NaAlg. Bar channels based on untreated paper were used as controls (No modification by NaAlg). “A,” “B,” and “D” channels were, respectively, loaded with Anti-A, Anti-B, and Anti-D antibodies. The error bars represent the standard deviation of five tests.

**Sensor Validation.** The final paper-based blood typing device was designed under optimum conditions based on the above results. Figure 3 shows a photo of the actual tests of all eight ABO/RhD blood types by the optimized barcode-like paper-based blood typing device.

![Figure 3](image.png)

**Figure 3.** Actual assays of all eight ABO/RhD blood types by the optimized barcode-like paper-based blood typing device.

smartphone-based analysis software (app) was developed. The basic principle of the identification process is as follows: If a short RBC eluting length appears in one channel, it will be encoded as “1” by the app; on the contrary, if a long eluting length occurs in the channel, it will be encoded as “0”. On the basis of this design, all eight blood types are encoded as “AB+/1 1 1”, “AB−−/1 1 0 0”, “A+/1 0 1”, “A−−/1 0 0 0”, “B+/0 1 1”, “B−−/0 1 0 0”, “O+/0 0 1”, and “O−−/0 0 0 0”. The code messages will be presented from the “A”, “B”, and then “D” channel for further interpretation. Therefore, for instance, when acquiring code message “1 1 0”, the app will interpret it as blood type “AB−−”.

To quantitatively assess whether one channel message is “1” or “0”, references with eluting lengths of positive and negative assays of this channel will be required for comparison purposes. The app designed in this study has preloaded references which were obtained from statistical analyses based on a large amount of known blood samples; therefore, after acquiring the eluting lengths of the target blood assay, the app will compare the lengths with the references, transfer the length data to code messages, and then interpret the code messages into the corresponding blood type.

The basic statistical analysis principles used to obtain references are as follows: (1) assume both the eluting length values of negative ($L_0$) and positive ($L_+$) reactions in one channel, respectively, follow normal distributions: $L_0 \sim N(\mu_0, \sigma_0^2)$ with unknown mean $\mu_0$ and unknown variance $\sigma_0^2$ and $L_+ \sim N(\mu_+, \sigma_+^2)$ with unknown mean $\mu_+$ and unknown variance $\sigma_+^2$; (2) on the basis of data measured from negative reactions (sample size is $Q$) in this channel, get the sample mean $\bar{L}_0$ and sample standard deviation $s_0$. Since the true value of standard deviation $\sigma_0$ is unknown, the distribution of the sample mean $\bar{L}_0$ follows the $t$ distribution ($t_0$) with mean $\mu_0$ and standard deviation ($s_0/(Q)^{1/2}$); (3) on the basis of data measured from positive reactions (sample size is $Q$) in this channel, get the sample mean $\bar{L}_+$ and sample standard deviation $s_+$. Similarly, since the true value of standard deviation $\sigma_+$ is unknown, the distribution of the sample mean $\bar{L}_+$ follows the $t$ distribution ($t_+$) with mean $\mu_+$ and standard deviation ($s_+/(Q)^{1/2}$); (4) two $t$ distributions ($t_0$ and $t_+$) obtained from step (2) and step (3) are taken as the references for testing whether the length value is “0” or “1”. For instance, two basic steps will be run by this app for determining the code message of the “A” channel: (1) Get the average value of eluting length in the “A” channel of target sample; (2) At a significance level of 0.01, if
the value only falls into the confidence intervals for the mean $\mu_0$ based on $t_0$ distribution of “A” channels, get message “0”; if the value merely falls into the confidence intervals for the mean $\mu_1$ based on $t_1$ distribution of “A” channels, get message “1”; if the value falls into the confidence intervals of both $t_0$ and $t_1$ distribution, the one whose sample mean value is closer to the tested value will be chosen; otherwise, do not report the result.

This app was tested on a Google Nexus 5 smartphone for identifying blood assays (Figure 5) in this study. The blood type of the target assays will be displayed and reported with text on the screen. For simple operation of this app, three scanning channels, which were indicated by three red solid lines, have been designed for, respectively, scanning three scanning channels, which were indicated by three red lines of the app match with the “A”, “B”, and “D” bar reaction channels of the paper-based device. Figure 6 shows that the snapshots of all eight ABO/RhD blood group types were successfully text-reported by this Android app. This barcode-like design makes smartphone-based analysis easier and more reliable through reading the length information, which is less affected by environmental factors (such as light source, temperature, etc.) compared to other forms of signals such as colorimetric and electric.

Validation for Blood Typing. A total of 98 blood samples were assayed using this barcode-like blood typing device. All samples were also assayed in the pathological laboratory of Red Cross Australia using the mainstream blood typing technologies. These samples contain all the 8 blood types in the ABO blood typing system. All results agree with those reported by the Red Cross Australia. All results are shown in Table S-2, Supporting Information.

CONCLUSIONS

In this study, a barcode reading concept has been applied for the first time in designing paper-based devices by adapting to smartphone-based technology. This sensor design has been used for the application of blood typing. Users can obtain all eight ABO/RhD blood types as text messages on the screen of the smartphone, without the need of further interpretation. The specific barcode-like design of the paper-based device makes smartphone-based analysis more reliable by reading the bar length information. In addition, by utilizing smartphone technology, test results from paper sensors can be automatically saved in e-form and transferred conveniently between users and professionals. It is expected that paper-based technology with smartphone diagnostics will be greatly expanded and applied as a diagnostic platform for medical and environmental applications.

ASSOCIATED CONTENT

Supporting Information

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Notes

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A low-cost forward and reverse blood typing device—a blood sample is all you need to perform an assay†

Miaosi Li, Junfei Tian, Rong Cao, Liyun Guan and Wei Shen*

For all user-operated blood typing devices in today’s market, including those designed by us in our previous research, a buffer-activation or buffer-washing step is required. The buffer-activation step, as is employed in some commercial blood typing devices, involves dissolving the antibodies deposited in the assaying zones of the device before the introduction of a blood sample for an assay. The buffer-washing step involves washing the blood sample in the assay zone in the end of the assay for result reporting. While all these devices work well, the activation or washing step does reduce the adaptability of those devices to resource-poor areas and under emergent circumstances. In this study, we designed a new device to perform forward and reverse blood typing assays without the buffer-activation or buffer-washing. Low-cost plastic slides were patterned to form channels containing dried grouping antibodies. Blood typing assays can be performed by simply placing a few microlitres of a blood sample into the channels and then tilting the slide. The sample flows along the channel under gravity, dissolving dried antibody and then spreading into a film, unveiling the reaction of red blood cells (RBCs) and antibodies. This device enables easy visual identification of the agglutinated and non-agglutinated RBCs in typically 1 minute. Both forward and reverse blood typing assays can be performed using this device. To optimize the device design, the antibody dissolution profile, assay sensitivity, and device longevity were investigated in this work.

Introduction

In today’s world there is an increasing need for affordable healthcare devices which would enable many conventional diagnostic assays to be carried out from home. The home-based and patient-operated assays, if made reliable and rapid, can significantly alleviate the pressure on hospitals and pathological laboratories in developed countries. At the same time, these technologies also carry the hope to minimize the impact of disease outbreaks and to increase the drinking water safety in impoverished areas. This is because the centralized laboratories and hospitals taken for granted in the cities of the developed world are absent in remote and impoverished areas. In the past few years, novel diagnostic devices built on low-cost substrates such as paper, thread, and plastic and glass slides have demonstrated the possibility for such a hope to become reality. These innovations showed the potential impact of low-cost analytical technologies on future human health and environmental care. Among those innovations, a series of blood typing diagnostics based on paper and thread platforms have been developed. The paper- and thread-based devices have significant advantages over the current laboratory- and hospital-based technologies due to their high adaptability to unsupported field conditions, user-friendliness and assaying speed.

Blood typing is a routine clinical test, but also a test of paramount importance for avoiding fatal haemolytic transfusion reactions (HTRs) during surgeries, clinical emergencies and blood transfusions. Equally important, since the world annual blood donations are around 75 million units, routine and rigorous sorting of blood types must be performed in large numbers and speedily. These healthcare and clinical requirements urge the continuous development of accurate, user-friendly and low-cost blood typing technologies.

For ABO and Rh blood groups most clinical techniques are based on the visual observation of haemagglutination reactions, although advanced gene-sequencing technology providing precise determination of blood type through DNA analysis is now available. Upon the contact of RBCs with an antibody, the absence of RBC agglutination indicates that there is no haemagglutination reaction; this observation confirms that there are no corresponding antigens on the RBC surface to the grouping antibody. Conversely, if agglutination is observed, it confirms that corresponding antigens to the antibody are present on the RBC surface.
Furthermore, for the ABO blood system Landsteiner’s rule applies, which states that, for an individual, if an antigen is present on the surface of his RBCs, the corresponding antibody will be absent from his blood plasma. Instead, the reciprocal antibody will be present in the plasma or serum. For example, an individual of blood type A has A antigen on his RBCs and antibody B in his serum. The normal blood typing assay, also known as the forward blood typing assay, uses blood grouping antibodies to identify the specific antigens on RBCs. Conversely, there is another blood typing assay which determines the antibodies in the serum by using the reagent RBCs with known antigens. This blood typing assay is known as reverse blood typing. Since a reversed blood typing assay determines the interactions between the reagent RBCs and the antibodies in a patient’s serum, it also relies on the observation of RBC agglutination. Details to explain the forward and reverse blood typing can be found in Fig. S1 and S2 in the ESL.

In many countries, both forward and reverse blood typing are required to confirm the patient’s blood type before a blood transfusion or transplantation is allowed to proceed. The laws that determine blood types by forward and reverse blood typing are shown in Table 1.

Table 1  Blood type confirmation by forward and reverse blood typing methods

<table>
<thead>
<tr>
<th>Reaction of antibodies with blood</th>
<th>Reaction of reagent cells with serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-A</td>
<td>Anti-B</td>
</tr>
<tr>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
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<td>–</td>
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</tbody>
</table>

In order to further improve the adaptability of paper-based devices to non-laboratory conditions, future designs will need to explore new concepts that can significantly reduce the effort of the user required to perform the blood typing assay. In this work we present a new concept of a blood typing device which does not require the user to apply the saline buffer for activating or washing the device in order to perform an assay. This concept relies on the dissolution of blood grouping antibodies deposited on a non-absorbing substrate by a blood sample, and the subsequent thinning of the sample into a film for blood typing result identification. In the fabrication of the device, channels are formed to guide the blood sample flow. Furthermore, we investigated two factors that affect the performance and sensitivity of the device: the thickness of the blood sample film and the antibody dissolution behaviour. A chromatographic elution method was designed to provide a semi-quantitative estimation of the antibody dissolution profile from the device surface. The antibody longevity on the plastic substrate was studied for one month under ambient laboratory conditions. Apart from performing the general forward blood typing assays, we have also demonstrated the use of our new device to perform reverse blood typing. Presently, reverse blood typing can only be performed in central laboratories and hospitals. This study is the first one to demonstrate reverse blood typing using a low-cost device. We believe that our device concept will allow forward and reverse blood typing to be combined into one user-friendly device.

**Experimental**

**Materials**

Plastic slides were purchased from 3M (3M Visual Systems Division, USA). Blood samples were sourced from Red Cross Australia, Sydney. They were stored at 4 °C and used within 7 days of collection. All the antibodies were purchased from ALBA Bioscience, Edinburgh, UK.

The red blood cells required for reverse blood typing, including 15% A1 cells, 15% B cells and 3% C1 cells, were obtained from CSL, Australia; they were concentrated to 45% hematocrit level (the average human whole blood) by centrifugation, stored at 4 °C and used within 30 days. The 0.9% (w/v) NaCl saline solution and the phosphate-buffered saline (PBS) were prepared with AR grade NaCl (Univar) and phosphate (Aldrich), using MilliQ water. Glycercol and Tween 20 were purchased from Aldrich. Surface treatment of the plastic slides was carried out using a plasma reactor (K1050X plasma asher (QuorumEmitech, UK)).
Methods

Blood typing procedure. A blood typing device for use in impoverished regions must allow for direct visual identification of test results; the device should function with a minimum effort from the user and without the need for any equipment. Following these requirements, we explored a new device design concept which provides direct and rapid visual identification of the test result, while minimizing the effort from the user to perform the test. It focuses on eliminating the saline washing step. Fig. 1 describes the advantage of forming a blood sample film on an antibody-treated plastic slide for blood typing assays and the device design. Fig. 1(a) and (c) show the result of adding a drop of blood sample into a drop of antibody solution on a plastic slide. Although agglutination of RBCs by the corresponding antibodies had occurred, it could not be visually observed. However, if the slide is tilted to allow the drop to flow under gravity and form a thin film, the user can immediately identify the agglutination of the RBCs by an antibody. Fig. 1(b) and (d) show the flowing blood sample and the grouping antibodies on a tilted plastic slide, clearly showing agglutinated and non-agglutinated RBCs, respectively. In order to reduce the users’ efforts to perform the assay, blood grouping antibodies were coated onto the plastic slide so that users are not required to administer antibodies for forward blood typing. This device design principle requires the rapid dissolution of a sufficient quantity of grouping antibodies by the blood sample; therefore, the antibody dissolution profile must be verified experimentally.

Plastic slides were first treated with plasma at an intensity of 50 W for 1 minute; a water-resistant pen was then used to draw the boundaries to demarcate the sample flow channels. Three microlitres of antibodies (anti-A, anti-B and anti-D) were dropped respectively on top of the three channels designated to these antibodies, and the slide was tilted to allow antibodies to flow through the channel under gravity. After the antibodies covered the entire length of the channels and were completely dried, 3 μL of blood sample was dropped from the top of each channel, following the same procedure. Typically, it takes 30 seconds for the blood sample to flow through the entire channel. Antibody-specific agglutination of RBCs can be identified immediately as the blood sample flows through the channels treated with antibodies and forms films. A schematic protocol of the test is shown in Fig. 2.

Quantification of antibody dissolution rates from plastic slides. Since the working principle of the plastic slide device relies on the rapid dissolution of the antibodies deposited on the slide, it is necessary to characterize the dissolution rates of all three antibodies employed for the device design. To do this, PBS buffer was used to perform a controlled dissolution study of all antibodies from the plastic slide surface. Fig. 3 shows the experimental procedure: channels on a plastic substrate were pre-treated with antibody and allowed to dry under ambient conditions. 10 μL PBS was used to flow through each channel to dissolve the antibody; the contact time was controlled at 30 s. The flow-through PBS solutions containing the dissolved antibodies were collected using Kleenex paper towel; the solutions wetted the paper towel and formed circular wetting zones as shown in Fig. 3.

In order to quantify the dissolved antibodies, antibody concentrations of the collected PBS washing solution were analyzed. We designed the quantification step as follows: a blood sample carrying the corresponding antigen to an antibody was introduced into the zone of the collected PBS washing solution on paper towel. After 30 seconds of incubation time under ambient condition, the edge of the paper was immersed

Fig. 1 Demonstration of a blood typing assay of an A+ blood sample on a plastic slide substrate by: (a) placing a drop of blood sample on a drop of anti-A; (b) tilting the substrate to allow the blood sample and anti-A solution to spread into a film, immediately revealing the positive assay result; (c) a drop of A+ blood sample placed on a drop of anti-B; (d) tilting the substrate to reveal the negative assay result.

Fig. 2 The designed procedure for blood typing on a plastic slide.
into a chromatography tank containing PBS for elution (Fig. 3), following the method we reported previously. If the antibody concentration is high enough, the RBCs of the blood sample will agglutinate, forming a blood stain with a strong colour which cannot be eluted away by PBS. The elution pattern was scanned into a computer and then converted to a monocolour mode (grey scale mode) with the ImageJ software. The optical density of the grey scale image of the blood spot was determined using the software. The grey scale is digitized into 256 steps, which represent different tones from dark to bright in an ascending manner, with 0 being the darkest and 255 being the brightest tone. The optical density of the blood spot therefore provides a simple and semi-quantitative method for determining the antibody dissolution behaviour. An antibody dissolution standard calibration curve can be established by determining the optical densities of agglutinated blood spots by a series of step dilutions of an antibody.

Results and discussion

There are in total eight different blood types in the ABO and RhD blood groups; all of them can be clearly identified by the plastic slide method (Fig. 4). After a blood sample was introduced into the channels, it takes typically up to 1 minute for 3 μL of blood sample to flow through the entire length of the channel. During this process, antibody-specific agglutination of RBCs forms large lumps, which become clearly identifiable when the blood sample flows halfway through the channels, making the assay time with the plastic slide shorter than 1 minute.

Antibody dissolution from the plastic slides

Fig. 5(a) shows the chromatographs of the agglutinated type A blood sample by a serially diluted anti-A; the dilution was made from 1 (original anti-A) to 512 fold. The serial dilution data show that the anti-A retained its activity after being diluted 128 fold. Further dilution, however, weakens the antibody activity, causing weak RBC agglutination. Fig. 5(b) presents the anti-A dilution curve of the blood spot colour density against the dilution factor. Since the concentration of the commercial antibody was unknown, the dilution factor was used as the relative antibody concentration. Fig. 5(c) shows the result of a serial dissolution of anti-A from the plastic slide; the anti-A standard solution was gradually diluted into a series of concentrations and dropped onto the paper towel, followed by the introduction of reagent red blood cell A onto each antibody spot. Then the chromatographic elution method was applied to the paper towel and the colour intensity of each blood spot was tested for building the standard curve of anti-A dilution behaviour, as shown in Fig. 5(a) and (b). The standard curve in Fig. 5(b) shows that a significant loss of anti-A activity to A antigen on the RBC surface by visual evaluation occurred only

![Fig. 3 Schematics of the procedure for studying the antibody dissolution behaviour from the plastic slide. This procedure involves an antibody dissolution step by PBS, followed by quantifying the concentration of the antibodies washed off the plastic slide.](image-url)
when it was diluted to 1/128 of its original concentration. This result suggests that the dissolving rate of anti-A is slow, and the following phenomenon provided reasoning. The standard curve in Fig. 5(b) can be fitted to a logarithmic formula (formula (1)) to establish the relationship of the colour density and the relative concentration of the antibody. This standard curve provides a way to quantify the antibody that was washed off the plastic substrate by the saline solution. Since the precise original antibody concentrations were unknown, we could assume that they were $C_A$, $C_B$, and $C_D$, and measure the concentration changes caused by the saline dissolution. Through measuring the colour density of each blood spot in Fig. 5(c), the relative concentration of anti-A released from the substrate after each saline wash can be calculated; the results are shown in Table 2. Anti-A deposited on the plastic substrate dissolved only 11.1% by the first wash, the remaining anti-A on the substrate still retained sufficient bioactivity for blood typing after another three such washes.

$$\text{Optical density A} = 26.5 - 14.6 \times \ln(f_A - 5.42 \times 10^{-4});$$

where $f_A$ is the dilution factor of anti-A; $C_A$ is the concentration of anti-A of each dilution.

Following the same procedure for quantifying anti-A, the dissolution behaviour of antibodies B and D has also been quantified. The calibration curve of anti-B dilution showed that anti-B lost its activity after a dilution of 1/16, indicating its weaker activity compared with anti-A (see Fig. S3a in the ESI†). The calibration curve was fitted with formula (2) (Fig. S3b†), which quantitatively showed that the concentration of anti-B in the first saline wash was 23.1% of its original concentration $C_B$ (Table 2). The more efficient dissolution of anti-B by saline solution than of anti-A confirms that anti-B can be dissolved more easily from the plastic slide, therefore anti-B weakened more rapidly than anti-A with the number of washes by saline (Fig. S3c†). As shown in Fig. S1c† anti-B could sustain three washes and the residual anti-B on the plastic substrate still had sufficient activities for unambiguous blood typing. However, as for anti-D, the dissolution was even more efficient (Fig. S4a and b†), our measurement showed that 92.0% of its original concentration was dissolved (formula (3) and Table 2) and removed from the plastic substrate in the first saline wash; antibody D lost its activity at 1/8 dilution (Fig. S4c†).

$$\text{Optical density B} = 41.4 - 17.2 \times \ln(f_B - 0.02); \quad f_B = \frac{C_B}{C_A} \quad (2)$$

$$\text{Optical Density D} = 81.1 - 10.7 \times \ln(f_D - 0.03); \quad f_D = \frac{C_D}{C_D} \quad (3)$$

where $f_B$ and $f_D$ are the dilution factors of anti-B and anti-D; $C_B$ and $C_D$ are the concentrations of anti-B and anti-D after each dilution.

According to the definition of reflective optical density used in the printing industry, the reflective optical density is defined as a logarithmic ratio of the reflected radiation from a printed grey tone on paper to the reflected radiation from the unprinted paper. This is usually presented in the form of logarithm based to 10, but can be easily converted to the form of natural logarithm:

$$D = -\ln \left( \frac{I}{I_0} \right) \quad (4)$$

where $D$ is the reflective optical density, usually measured with a reflective densitometer in the printing industry, $I$ and $I_0$ are the reflective radiation intensity from a printed grey tone and from unprinted paper, respectively. In this study grey tones generated by the agglutinated blood spot on paper loaded with different amounts of antibodies create a similar concept for the tones to be quantified by reflective optical density.

Blood spot optical density data (Fig. 5, S3b and c†) can also be correlated with concentrations of corresponding antibody (or dilution) data by logarithm functions, which suggests that optical density data can be correlated with the concentration ratios of the dissolved antibodies. Such correlations have been experimentally given in eqn (1)–(3) and are expected to provide semi-quantitative results for antibody dissolution evaluation.

### Sensitivity

The sensitivity of any blood typing device must be investigated for typing blood samples with low concentrations of RBCs. This requirement is essential, as clinically the RBC concentration from blood samples of anaemia patients could be more than 50% lower than those from a healthy patient. Since almost all commonly used blood typing assays rely on the development of large agglutinated RBC lumps, those methods can be less sensitive to samples with low RBC concentrations.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Number of dissolution washes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X1</td>
</tr>
<tr>
<td>Anti-A</td>
<td>Colour density</td>
</tr>
<tr>
<td></td>
<td>Relative concentration</td>
</tr>
<tr>
<td>Anti-B</td>
<td>Colour density</td>
</tr>
<tr>
<td></td>
<td>Relative concentration</td>
</tr>
<tr>
<td>Anti-D</td>
<td>Colour density</td>
</tr>
<tr>
<td></td>
<td>Relative concentration</td>
</tr>
</tbody>
</table>
The sensitivity study of the plastic slide method was conducted by identifying the agglutination patterns of serially diluted reagent RBCs that carry known antigens. The original RBC samples used for testing were the red cells A1, B and C1. To prepare samples with low RBC concentrations, the suspension medium of the reagent red cells was first removed by centrifugation and then the red cells were diluted to haematocrit of 45% with PBS; this RBC concentration simulates the blood RBC concentration of a healthy individual. Low RBC concentration samples were prepared by diluting this sample by factors of 75%, 50% and 25% with PBS. The diluted blood samples were then used for the sensitivity tests of the device and results are shown in Fig. 6. All positive tests of diluted blood samples A, B and D can still be clearly identified via RBC agglutination, even though they were diluted to 25%. These results confirm that the plastic slide blood typing device is able to deliver the equivalent sensitivity of a high performance blood typing device. The film forming process of the blood sample provides a simple way to enhance the sensitivity of blood typing using the plastic slide method.

**Antibody longevity on the plastic slide**

It was found that the original antibodies gradually lost their activity within 10 days of deposition on plastic slides and allowed to dry. This is because the dried antibodies dehydrated after long exposure to air. As a result, the surface of the channel became more hydrophobic and this significantly reduces the speed of the blood flow in the channel. Fig. 7 shows the hydrophobic development of the slide surface with time. To solve this problem, glycerol and Tween 20 were chosen as additives to prevent antibody dehydration and to increase the channel surface wettability. Glycerol has been used as a traditional additive for protecting biomolecules from denaturing. Its high humectant effect attracts water molecules and prevents biomolecules from dehydration; such a protective effect is most likely related to the presence of 3 hydroxyl groups in the glycerol molecule and its small molecular size. Apart from attracting water molecules, glycerol may also provide direct hydrogen-bonding, like many sugar molecules, to stabilize the biomolecule. The addition of Tween 20 as a biologically compatible surfactant is intended to enhance the wettability of the deposited antibody layer on the plastic slide after ageing. Additives with a series of different proportions of glycerol and Tween 20 were mixed with the antibodies to form solutions to treat the channels of the device; the wettability of the channels was tested at different storage times by measurement of the flow length of blood samples in the channels in 30 seconds; results are presented in Table 3. Our results show that antibody solutions containing 20% glycerol, or 10% glycerol and 0.1% Tween 20 enhanced the channel wettability; testing after 30 days of storage showed that the device’s wettability was unchanged and all antibodies still retained their activity. We chose 10% glycerol and 0.1% Tween 20 as the preferred additive formulation to perform further experiments. Fig. 8 shows the blood test results obtained after 45 days of storage under ambient temperature and open to air; all the antibodies were still active and gave accurate blood typing within 30 seconds (Fig. 8).

![Fig. 6 Sensitivity tests of the plastic slide method by dilution of reagent red blood cell carrying known antigens: (a) A1 cells in the anti-A treated channel; (b) B cells in the anti-B treated channel; (c) C1 cell in the anti-D treated channel. The negative control channel was treated with BSA only.](Image)

![Fig. 7 Lifetime detection for blood group A interaction with the anti-A treated device for different periods.](Image)

<table>
<thead>
<tr>
<th>Additive</th>
<th>Blood sample flow distance (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure antibody</td>
<td>1 hour 2 days 10 days 30 days</td>
</tr>
<tr>
<td>Tween 20 (0.1%)</td>
<td>21 13 8 4</td>
</tr>
<tr>
<td>Tween 20 (0.5%)</td>
<td>25 14 3 3</td>
</tr>
<tr>
<td>Tween 20 (1%)</td>
<td>25 12 3 3</td>
</tr>
<tr>
<td>Glycerol (1%)</td>
<td>20 8 3 3</td>
</tr>
<tr>
<td>Glycerol (5%)</td>
<td>25 15 8 4</td>
</tr>
<tr>
<td>Glycerol (10%)</td>
<td>25 17 12 6</td>
</tr>
<tr>
<td>Glycerol (10% + Tween 20 0.1%)</td>
<td>25 25 25 25</td>
</tr>
<tr>
<td>Glycerol (20%)</td>
<td>25 25 25 25</td>
</tr>
</tbody>
</table>

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Published on 15 December 2014. Downloaded by Monash University on 21/05/2015 05:21:43.
Reverse blood typing using the plastic slide assay

The reverse blood typing assays were also performed using the plastic slide method. Patients’ blood serum was first separated from the whole blood sample, which can be prepared by the traditional centrifugation or the new low-cost POC methods on membrane or paper. Then the serum samples were dropped into the channels on the plastic slide and allowed to spread throughout the channel. Reagent red blood cells A and B were then pipetted into separate serum-coated channels. By allowing the reagent RBCs to spread in the channel and form a film, the agglutinated RBCs can be clearly identified by the naked eye without any aid (Fig. 9).

Proposed mechanism of RBC agglutination on the plastic slide

The use of film formation as a sensitive method for blood typing takes advantage of the following two processes: firstly, the spreading of blood samples over the antibody coated channel surface provides a large contact area between the blood sample and antibody. The large contact area promotes interactions between the RBCs in the blood sample and the antibodies on the plastic slide; agglutination of RBCs by their corresponding antibodies can therefore occur quickly. Secondly, the relatively slow spreading of blood samples in an antibody treated channel is likely to give sufficient time for the blood sample and antibody to interact and incubate. This, combined with the small thickness of the blood sample film, tends to lead to the formation of sheet-like agglutination lumps, which are very easy to visually identify. The plastic slide device thus offers a sensitive means for rapidly performing both forward and reverse blood typing assays.

Conclusions

In this work we designed and demonstrated a new user-friendly blood typing device. The device was fabricated by patterning the plastic slide with channels treated with different blood grouping antibodies; this design requires the user to simply introduce the blood sample into the channels to complete the blood typing assay, without the need for buffer washing or stirring operations. This device functions by letting the blood sample spread under gravity over a surface treated with blood grouping antibodies. The agglutinated RBC lumps can be rapidly and clearly differentiated from the non-agglutinated RBCs, providing unambiguous visual identification of the positive and negative blood typing assays. This method provides a user-friendly design concept that requires minimum effort from the user to perform an assay to determine the blood type of a patient within 1 minute.

The film-forming principle of this method provides a high level of sensitivity to identify the blood types of samples with low RBC concentrations. Preliminary investigation of the longevity of the device was also conducted; a mixture of glycerol and Tween 20 was chosen as an effective additive mixture to maintain the bioactivity of the antibodies on the device for the testing period of 30 days.

Acknowledgements

This work is supported by the Australian Research Council (ARC). Funding received from ARC through grant numbers DP1094179 and LP110200973 is gratefully acknowledged. The authors thank Haemokinesis for its support through ARC Linkage Project, and Mr Hansen Shen for proof-reading the manuscript. Ms Miaosi Li also thanks the Monash University Research and Graduate School and the Faculty of Engineering for postgraduate research scholarships.
Notes and references

Surface Modification of Cellulose Paper for Quantum Dot-based Sensing Applications

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Cellulose paper specimens with and without surface modification were compared in order to study their physicochemical compatibility with quantum dots (QDs) for biochemical sensing applications. Silane and chitosan modification methods were applied. The distribution of QDs deposited on untreated paper and papers modified with silane and chitosan were investigated in order to understand the interaction between QDs and fibre. Modified papers were shown to significantly reduce the undesirable redistribution of QDs during paper drying. The retention ability and thermal resistance of QDs to the loss of fluorescence on modified papers were also studied for the purpose of determining the most suitable paper surface modification for developing QD-Paper-based analytical devices (QD-PADs). Furthermore, chitosan-modified paper was used to design QD-PADs to quantify glucose concentration in aqueous samples; the quenching effect of the enzymatic product on the fluorescent emission of QDs was used as the indicator system. The change of fluorescence of QDs was measured by a simple in-house constructed fluorescence imaging system. The detection limit of glucose was 5 mg/dL, which is comparable with other reported paper sensors for detection of glucose.

Keywords: Cellulose Paper; Surface Modification; Quantum Dots; Paper-based Analytical Devices; Glucose Sensing

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INTRODUCTION

In recent years cellulose paper has attracted the interest of those working in the field of developing paper-based analytical devices (PADs) for diagnostics applications (Balu et al. 2009; He et al. 2013). Made of cheap and widely available cellulose fibre (Hubbe et al. 2008), paper is a suitable material for manufacturing analytical sensors on a large scale to meet the demand of diagnostics from human populations living in developing regions. The rapid advancement of PADs research can be attributed to the unique physicochemical properties of paper including: compatibility with biological samples (Tan et al. 2012); easy surface modification to immobilize proteins, DNA, and other molecules (Xiao and Huang 2011; Araujo et al. 2012); and controllable wettability for transporting fluids (Tian et al. 2010). Historically, concepts of using paper for chemical assay tests have been explored from as early as 1937 (Yagoda 1937). Since 2007, paper-based diagnostics have been rediscovered and pursued by many research groups globally; a trend initiated by the Whitesides Group (Martinez et al. 2007).
During the development of PADs, several techniques have been reported for qualitative or semi-quantitative sensing applications, such as colorimetric (Xiao et al., 2013), electrochemical (Dungchai et al., 2009), chemiluminescent (Liu et al., 2013), and fluorescent methods (Ma et al., 2012). At the same time, a variety of sensor probes, whose properties change when they interact with analytes, have been adapted to these methods and used to indicate the functions of paper sensors (Li et al., 2012; Liu et al., 2013). The performances of probes applied in PADs are extremely important for obtaining accurate assay results. Quantum dots (QDs) have been considered to have great potential as fluorescence probes for biosensing (Hai et al., 2013; Chen et al., 2013). In particular, the comparable dimensions of QDs and biomolecules, such as proteins and DNA, enable the preparation of biocompatible QD-bioconjugates with special recognition functions (Li et al., 2011). Their excellent properties such as photo-stability and broad absorption spectra, together with high surface activity, make QDs advantageous probes in the development of biochemical sensors (Li et al., 2011).

Recently, a few studies have been published about the combination of QD probes with cellulose paper for sensing applications (Niu et al., 2011; Yuan et al., 2012; Noor et al., 2013). Yuan et al. reported the use of polymer QD-enzyme hybrids on PADs to detect the presence of a substrate with corresponding enzymes (Yuan et al., 2012). Noor et al. proposed a paper-based solid phase nucleic acid hybridization assay using immobilized QDs as donors in fluorescence resonance energy transfer (Noor et al., 2013). The QDs and QD conjugates were immobilized onto the surface of paper through imidazole ligands modification chemistry. These works successfully demonstrated applications of PADs using QDs as sensing probes. In this study, we further investigated the physicochemical compatibility between cellulose paper and QDs. Different surface modifications of cellulose paper were studied to evaluate their effects on QD particles distribution, retention ability, and thermal stability for sensing applications. A simple fluorescence imaging system has been constructed in-house in order to obtain the fluorescence characteristics of the QDs on cellulose paper. Furthermore, QD-Paper-based analytical devices (QD-PADs) based on these studies have been successfully developed for glucose detection with fluorescence intensity change of QD probes.

**EXPERIMENTAL**

**Reagents and Apparatus**

Chitosan (medium molecular weight) and 3-aminopropyltrimethoxysilane (APTMS, >97%) were purchased from Sigma Aldrich. Phosphate buffer solution (PBS) tablets and Tween 20 were obtained from Sigma Aldrich. Tween-20 (0.5%, v/v) was spiked into 0.01 mol/L pH 7.4 PBS solutions as wash buffer (PBST). Ultra-pure water (>18.0 MΩ) was obtained from a Milli-Q integral water purification system. All reagents were used without further purification. Carboxyl group functionalized CdS$_x$Se$_{1-x}$/ZnS alloyed quantum dots (QDs) ($\lambda_{em} = 630$ nm) were acquired from Sigma Aldrich.

Whatman No.1 filter paper (diameter = 20 mm) was obtained from Sigma Aldrich; this grade of filter paper is made of pure cellulose fibres. The paper was cut into different sizes for further use. K1050X Plasma Asher (Quorum Emitech, U.K.) was used for plasma treatment. The vacuum level for the treatment was 0.6 mbar and the paper was placed in the center of the chamber for 30 s of treatment.
Surface Modification of Cellulose Paper

Silane Modification

The preparation procedures for silane modified paper can be summarized as follows (Li et al. 2010; Koga et al. 2011; Jin et al. 2011, 2012): a volume of 3 mL 2.5% (v/v) 3-aminopropyltrimethoxysilane (APTMS) was prepared using 80% (v/v) ethanol solvent, and thus was hydrolyzed to form reactive silanol groups. Then 20 pieces of paper squares (1 cm × 1 cm), after plasma treatment, were immersed in the resulting solution for 2 h, followed by thorough washing with ethanol. Subsequently, the solvent was evaporated in the fume hood at room temperature for 10 min. The obtained paper squares were thermally treated at 110 °C for 3 h.

Chitosan Modification

To prepare chitosan modified paper, a volume of 10 mL 0.1% (w/v) chitosan was freshly prepared using a weak acid solution. Then 20 µL of chitosan solution were uniformly deposited on 1 cm × 1 cm paper squares, and then dried at room temperature for 1 h. Subsequently, each paper square was washed three times with 20 µL of ultrapure water, and then dried at room temperature. The chitosan retention was reported to be strong, due to the opposite charges of the chitosan and cellulose with the former being cationic and the latter being anionic (Wang et al. 2012; Xiao et al. 2013).

Preparation and Characterization of QDs on Cellulose Paper

Fluorescence and SEM characterization

Papers with APTMS- and chitosan-modification were prepared following the above procedures in Section “Surface Modification of Cellulose Paper”. Untreated filter paper, APTMS- and chitosan-modified filter papers were cut into 1 cm × 1 cm squares for further QDs compatibility studies. Then 4 µL of 0.02 mg/mL QDs were dropped into the center of each paper square and allowed to dry at room temperature for 20 min.

For the purpose of collecting the fluorescence signals of QDs on the paper, a simple fluorescent imaging system (Fig. 1) was constructed in-house to capture the fluorescence signals.

Fig. 1. Schematic diagram of the fluorescence imaging system assembled in-house.

The in-house built fluorescent imaging system was comprised of a 365 nm emission UV-lamp, a sample stage, and a digital camera. The UV-lamp, used as an excitation light source, was placed at the bottom of a dark chamber and the sample stage was fixed above
the UV-lamp. After turning on the light source, the UV light passed through the optically transparent sample stage and excited the fluorescence of the samples. The fluorescence signals emitted from the samples were captured by the camera right on the top of the sample stage. Before each fluorescence test, the light source was turned on and warmed up for 15 min to allow the stabilization of the radiation intensity.

The cellulose papers with and without QDs were further investigated by a MECM Nova NanoSEM scanning electron microscope (SEM) in order to observe the morphology of cellulose paper and the distribution of QDs on the cellulosic structure of paper. A volume of 4 µL 1 mg/mL, instead of 0.02 mg/mL QDs, was dropped onto each paper square for preparing samples for SEM study.

Retention tests by washes with aqueous solution

The retention ability of QDs on APTMS- and chitosan-modified cellulose papers was also investigated following the procedures below: three aliquots of 40 µL 0.5% PBST solution were introduced onto each testing paper, while standard blotting papers (drink coaster blotting, 280 g/m²) were used to remove liquid during the washing process. The fluorescence of QDs on paper squares before and after washing were measured using the above in-house built fluorescence imaging system.

The quantitative analyses were carried out following the description in Section “The Quantitative Analysis Technique”.

Temperature treatment of QDs on cellulose paper

Immediately after preparation, three groups of paper squares loaded with QDs were treated at temperatures of 4 °C, 25 °C, and 45 °C. The fluorescence signals of these paper squares were respectively measured after 0 h, 24 h, 36 h, and 60 h of treatment. The quantitative analyses were performed following the procedures described in Section “The Quantitative Analysis Technique”.

Development of QD-PADs for Glucose Detection

Chitosan-modified paper squares (0.5 cm × 0.5 cm) were employed to make QD-PADs for detecting glucose. Five hundred microliters of QD suspension (0.04 mg/mL) was mixed with 500 µL of glucose oxidase (100 U/mL). A volume of 2 µL of the above suspension was dropped onto the center of each chitosan-modified paper square and allowed to dry for 15 min under room temperature (Gill et al. 2008). Then several 1.2 µL volumes of glucose solution, with concentrations of 0, 5, 10, 20, 50, 100, 150, and 200 mg/dL, were respectively introduced onto each paper square, and then allowed to react for 15 min under a controlled high humidity.

The fluorescence images of all the paper squares were captured by the in-house built fluorescence imaging system. Quantitative analyses were performed following the descriptions in Section “The Quantitative Analysis Technique”.

The Quantitative Analysis Technique

The test paper squares with QDs fluorescence images were imported into Adobe Photoshop. The mean red intensity of the reaction spot was obtained using the histogram function of the software. Error bars (standard deviation) were obtained from five repeats of all the tests.
RESULTS AND DISCUSSION

Characterization of QDs on Cellulose Paper

Fluorescence characterization

The fluorescence emission performance of QDs on cellulose paper, with and without modification, was investigated using the above system (Fig. 1). Figure 2 shows the fluorescence images of untreated paper (Fig. 2, b1), APTMS- (Fig. 2, b2), and chitosan-modified paper (Fig. 2, b3). The blue color in these images was the background UV light source, and the red color is the fluorescence of QDs. In Fig. 2, b1, an extremely weak red color spread around the edge of the paper surface and could be easily observed by the naked eye. This was because QD nanoparticles dried on the untreated cellulose paper tend to migrate with the solvent phase (PBS solutions). In contrast to this, clear red fluorescence signals of QDs were captured in the center of both APTMS- (Fig. 2, b2) and chitosan-modified papers (Fig. 2, b3), which indicates that QDs were well restricted within the center range of modified papers. Whatman #1 filter paper used in this study was made of pure cellulose fibres with no additives such as strengthening or whitening agents. The basic structure of untreated paper is composed of cellulose fibers that are firmly bond together through hydrogen bonding (Sahin and Arslan 2008). This kind of paper has inactive, slightly anionic surface with a low, negative, surface-charge density (Wang et al. 2012). In this situation, negative-charged COOH—QDs particles have a weak tendency to strongly attach to the paper surface by the physical immobilization method. Therefore, when QDs particles were dropped onto the untreated cellulose paper, QDs will be carried by the mobile phase (PBS solution) wicking front and spread to the edge of the paper. However, the surface chemistry property of paper will change after modification processes due to the introduction of active functional groups, which enhance the charge interaction between QDs particles and the paper surface. Hence, QD particles will be left behind from the solvent phase wicking front and immobilized in the center area of paper (Fig. 2, b2 and b3).

Fig. 2. a) Possible schemes of the QDs captured by modified cellulose paper, b) Fluorescence images of QDs on cellulose paper (1) without modification, (2) with APTMS-modification, (3) with chitosan modification.
It was observed that the average size of the formed colored QD spots on APTMS-modified paper was larger than that on chitosan-modified paper. Additionally, the size of QDs on APTMS-modified papers was less uniform than that on chitosan-modified papers, and thus the reproducibility of QD spot sizes on APTMS-modified papers was not as consistent (not presented). The APTMS modification method in this study applied a silane coupling technique through the reaction between silanol groups (Si—OH) of APTMS and groups (mainly hydroxyl groups C—OH) of cellulose paper. The relatively low reactivity of existing groups (mainly C—OH groups) on paper will greatly affect the amount of introduced groups with ATPMS treatment processes (Pelton 2009). However, as a physical modification approach, the chitosan modification method can be applied reproducibly with uniform coating on the surface of paper. As a result, the outsourcing active groups in the cellulosic network of APTMS-modified paper are less densely distributed compared to that of chitosan-modified paper. Therefore, QD particles have a stronger interaction with chitosan modified paper; this difference is the main reason for the difference of QD spots between the two modified papers.

**SEM Characterization**

The porous fibre network structures of papers remained after modification with APTMS and chitosan (Fig. 3). Figure 3 shows the SEM images of untreated papers, papers with APTMS-modification, and papers with chitosan-modification loaded with QDs. QD particles show different distributions on non-modified and modified papers (Fig. 3). Abundant QD particles were observed around the edges of untreated paper (Fig. 3, a2) while very a small quantity of particles could be observed around the center of the paper (Fig. 3, a1). However, the distribution of QDs was the opposite in the cases of APTMS- and chitosan-modified papers; most QD particles gathered around the center of the modified papers (Fig. 3, b1 and c1). The results are consistent with the above fluorescence characterization studies, indicating that surface modification can result in immobilization of QD particles to any point of interest on paper (e.g. to the center area of the paper square in this study).

In addition, particles tend to attach to the cellulose fibres rather than stay in pores of fibre network (Fig. 3, b1 and c1), suggesting that most of the active groups of APTMS and chitosan have been modified onto the fibres. Furthermore, QD particles have been found to be more closely packed on chitosan-modified paper (Fig. 3, c1) than that on APTMS-modified paper (Fig. 3, b2). The results illustrate that the charge interaction between QD particles and a chitosan-modified fibre surface was stronger than their interaction with a fibre surface modified with APTMS. The former captured more QD particles at points of interest.

QD particle aggregates were observed on modified paper. Their sizes were greater than separate QD particles (about 6 nm), and they lose the ability to produce fluorescence signals (Liu et al. 2012). One reason for the formation of large particles could be that a part of the original aqueous QD suspension had started to form aggregates, which has been observed and reported previously (Noh et al. 2010). Another reason, which is more likely, is that the high concentration of QD suspension applied to the paper surface for SEM tests had aggregated during the drying process.

Retention of QDs on Modified Cellulose Paper

The retention ability of QD probes on a paper substrate is critical to the performance of QD-PADs. This is because when an analyte solution is introduced onto the detection point of the sensor, QD probes with weak bonding to fibres could be eluted away from the detection point, leading to signal distortion in the detection zone. To study the retention of QDs on the two modified papers, 0.5% PBST solutions were applied to wash modified papers loaded with QDs. The more QD particles retained after washing means the stronger interaction between QDs and paper. In this study, the red intensity measured from the QD spots is the fluorescence intensity of QDs. Figure 4 shows the fluorescence intensity changes of modified papers with QDs after washing. The fluorescence intensity decreased by 26.5% on APTMS-modified paper (Fig. 4, left), while for QDs on chitosan-modified paper (Fig. 4, right), the intensity dropped by 18.2%. This result indicates that the retention ability of QDs on chitosan-modified paper was greater than that on APTMS-modified
paper, which was further evidence that the interaction between QDs and chitosan-modified paper is stronger.

**Fig. 4.** The fluorescence intensity of QDs on APTMS-modified (left) and chitosan-modified (right) cellulose paper before and after washing with PBST buffer solutions

### Thermal Stability of QDs on Modified Paper

Since thermal stability is a very important performance indicator of PADs for sensor applications (Guan *et al.* 2014), both APTMS- and chitosan-modified papers loaded with QDs were aged at different temperatures to test their thermal stability. Thermal stability was evaluated by measuring the red intensities of QDs spots formed in the center of the paper before and after aging. Figure 5 shows little intensity change of QDs bounded to both modified papers after aging at 4 °C; in contrast to this, a significant decrease in fluorescent intensity was observed when the papers were treated at 45 °C. Intensity decrease of QD spots on papers also occurred with a treatment temperature of 25 °C, however, it was less than that with the treatment temperature of 45 °C. The results indicate that QDs fixed in a cellulosic network could remain relatively stable at low temperatures, but a rise of temperature will lead to partial loss of fluorescence. Moreover, the results showed that the intensity decrease of QDs on APTMS-modified paper (Fig. 5a) was much greater than that on chitosan-modified paper (Fig. 5b) after aging at 45 °C. It is therefore most likely that the temperature-driven fluorescence loss of QDs on chitosan-modified paper is slower than that on APTMS-modified paper because of the stronger interaction between QD particles and the former paper.

**Fig. 5.** The fluorescence intensity of QDs on (a) APTMS-modified and (b) Chitosan-modified cellulose paper after at 4 °C, 25°C, and 45 °C temperature treatment with different time
Development of QD-PADs for Glucose Detection

To design practical QD-PADs, the primary consideration is immobilizing QDs in the detection zones of the paper to reduce QD migration. However, QDs deposited on untreated cellulose filter paper will migrate with the solvent and mostly gather around the edge of the paper. These migrated QDs were no longer suitable for any sensor applications. Yuan et al. (2012) attempted to solve this problem by encapsulating QDs with oppositely charged polymer. The present study shows that suitably modified papers could well solve the migration problem of QDs. Therefore in this study, chitosan-modified paper was selected to fabricate QD-PADs for demonstrating sensor application.

QDs have been reported as a sensitive probe for hydrogen peroxide, and further used in the detection of glucose with glucose oxidase as the catalyst to produce hydrogen peroxide (Wu et al. 2010). The possible mechanism that stimulates the fluorescence quenching of QDs is the oxidation of S²⁻ surface states, which presumably yields Zn²⁺ surface traps for the electrons (Gill et al. 2008). Figure 6a shows the fluorescence quenching effect of the QDs on the chitosan-modified paper by different concentrations of glucose. The calibration curve (Fig. 6a) represents the quenching effect of glucose yields

$$\frac{I_0}{I} = 1.139 + 0.0069C_{\text{glucose}} (r^2 = 0.95),$$

where $I_0$ is the mean red intensity of QD-PADs when the concentration of glucose is 0, and $I$ is the measured mean red intensity for the target sample. The detection limit of glucose is 5 mg/dL and the detection linear range is between 20 mg/dL and 200 mg/dL. Compared with the previous methods utilizing the glucose oxidase and QDs-polymer co-immobilization method in normal filter paper (Yuan et al. 2012), a lower detection limit of glucose was obtained in this study. Also in comparison to the solution-based method, this study largely reduces the usage of QDs and other reagents.

![Fig. 6.](image)

Fig. 6. a) Response of QD-PADs using chitosan-modified paper to different concentrations of glucose 0, 5, 10, 20, 50, 100, 150, 200 mg/dL (from 1 to 8); b) corresponding calibration curve.
CONCLUSIONS

1. This study investigated the physicochemical compatibility between QD particles and cellulose papers for the purpose of developing reproducible QD-PADs. The migration problem of QDs moving with the solvent phase in the fibre network structure of paper was greatly reduced by modifying the surface of cellulose. The retention ability and thermal stability of QD probes on paper have been studied as critical performance indicators of QD-PADs.

2. QD-PADs using chitosan-modified paper was demonstrated for glucose detection and the detection limit is comparable with reported paper-based glucose sensors.

3. This work provides an important framework for the development of QD-PADs and it is expected that QD-PADs can lay a foundation for further developing practical and stable sensor for diagnostic and analytical applications.

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“Periodic-Table-Style” Paper Device for Monitoring Heavy Metals in Water

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Supporting Information

ABSTRACT: If a paper-based analytical device (μ-PAD) could be made by printing indicators for detection of heavy metals in chemical symbols of the metals in a style of the periodic table of elements, it could be possible for such μ-PAD to report the presence and the safety level of heavy metal ions in water simultaneously and by text message. This device would be able to provide easy solutions to field-based monitoring of heavy metals in industrial wastewater discharges and in irrigating and drinking water. Text-reporting could promptly inform even nonprofessional users of the water quality. This work presents a proof of concept study of this idea. Cu(II), Ni(II), and Cr(VI) were chosen to demonstrate the feasibility, specificity, and reliability of paper-based text-reporting devices for monitoring heavy metals in water.

Water contamination is one of the most serious environmental problems facing the world today. The increasing human activities and rapid industrializations are introducing a huge amount of pollutants into the environment, which will undoubtedly contaminate natural water bodies and put human health and ecosystems at risk. Among various pollutants, heavy metals are always one of big concerns due to their severe toxicities so that they have been included in “Blacklist” by the United States Environmental Protection Agency (U.S. EPA). When these heavy metals enter into the human body, they could easily bind to vital cellular components and accumulate in organisms, resulting in a series of diseases and disorders (e.g., cancers, osteomalacia, kidney malfunction, etc.). As the first step of water pollution prevention, accurate and rapid monitoring of the heavy metals is vital. Ideally monitoring methods are expected to identify point sources of pollutants and the variation of nonpoint sources of pollutants in the environment.

Several methods are widely used for identification and quantification of heavy metals, including colorimetry, atomic absorption spectroscopy (AAS), inductively coupled plasma atomic emission spectroscopy (ICP-AES), inductively coupled plasma mass spectroscopy (ICPMS), and so on. Although these methods are capable of achieving highly sensitive detection, identification and quantification of heavy metals, they always require the support of well-equipped laboratories and skilled operators, which can be very expensive. Therefore, alternative and inexpensive methods are in demand for field-based or on-site water monitoring by personnel with limited training or even unskilled home-users.

Patterned paper, as a platform technology for making low-cost and user-operated analytical devices, offers a possibility to construct new heavy metal sensors that meet the above-mentioned desirable features. Patterned paper has been used to make sensing devices for disease screening, point of care (POC), pathogen and biomarker detection, and food and water quality testing. In environment monitoring, research works using paper-based microfluidic devices have, to date, demonstrated the possibility of providing quantification of heavy metals, including Cu, Cd, Hg, Pb, Cr(VI), Ni, etc. These works provide a new approach to monitor heavy metals in water in situations where designated laboratories are not available. However, the assay procedures and result interpretation (involving colorimetric or electrochemical signals) cannot be easily followed and understood by untrained or home users without professional assistance. Besides the high sensor sensitivity and specificity, high portability, rapid detection, and easy result interpretation are highly desirable performance features for new sensing devices. Most recently, researchers have demonstrated that paper-based sensors can be designed to communicate assay results with users via text message, removing errors in assay result interpretation.

One imaginative way to significantly improve the performance of a future paper-based sensor in detecting, quantifying, and reporting heavy metals in water could be to print the chemical symbols of heavy metals with their corresponding and specific indicator systems in a format of the periodic table of elements. Testing of heavy metals in water would simply involve dipping the paper sensor in water, the sensor could then reveal the testing results if the concentrations of heavy metals are higher than legislated standards by showing the chemical symbols of the heavy metals present in water. Such a chemically responsive periodic-table-style paper sensor could be designed to not only provide qualitative answers of what is the
water but also provide quantitative (i.e., safe-to-use) information on the heavy metals in water.

Following this idea, we demonstrate the feasibility of paper-based heavy metal sensors, capable of reporting text messages to users if heavy metal concentrations in water reach a legislated unsafe level. We “print” indicator systems for target heavy metals on a patterned paper in chemical symbols of the target metals. This concept can promptly inform even nonprofessional users with clear and unambiguous written warnings against unsafe water. We also demonstrate another use of the same indicator systems to quantify heavy metal concentrations in water. This will enable environmental workers to obtain reliable semiquantitative heavy metal analysis under nonlaboratory conditions and at low-cost.

An ideal paper-based heavy metal sensor for text-reporting has to meet the following performance requirements. First of all, the indicator must be sensitive and form the specific complex with the metal ion to be detected and shows a distinctive color. Second, the indicator system as well as the indicator–metal complex must not be easily leachable into water. Third, the nondetection area of the sensor must be water-repellent. On the basis of these requirements, we chose Cu(II), Cr(VI), and Ni(II) as model heavy metals to demonstrate our idea. The indicator systems for these heavy metals meet the above requirements reasonably well.

Chromatography no. 1 paper was employed to pattern the chemical symbols of Cu, Cr, and Ni by means of generating hydrophilic and hydrophobic boundaries on paper. Computer-generated chemical symbols were first printed on office paper and then soaked in molten wax. After wax treatment, the office paper was allowed to cool down to room temperature. The letters on the wax-loaded office paper was then cut out, forming the cut-out pattern. This office paper was then put on to a piece of chromatography paper and heated with an electric iron, and the wax was transferred to the chromatography paper to form hydrophilic symbols of Cu, Cr, and Ni, surrounded by wax.24 The so-patterned chromatography paper was the final patterned substrate for further construction of the analytical devices. Figure 1 shows the process of fabrication of the patterned chromatography paper (Supporting Information contains further details). Indicator systems were then added into the chemical symbols of the metals to make the device active.

Cu(II) is one of the most common heavy metals in water, and according to the Australian drinking water guidelines for fresh and marine water quality, Cu concentration in drinking water must not be higher than 1 mg/L.25 Also, the Australian and New Zealand guidelines for fresh and marine water quality set the standard of Cu(II) in water for irrigation and livestock drinking between 0.2 mg/L (long-term use) and 5 mg/L (short-term use).26 To fabricate a text-reporting Cu(II) sensor, a 10 μL aliquot of hydroxylamine (0.1 g/mL) in acetic buffer (6.3 M, pH 4.3) was first added into the letter channel. In total, 50 mg of bathocuproine was dissolved in 1 mL of chloroform as the indicator for Cu, and 40 mg PEG 400 was also added into this solution to prevent the detection channel from becoming hydrophobic.26 Before the addition of each solution, the paper device was air-dried for 10 min. Cu(II) forms Cu–bathocuproine complex which has an orange to brown color; the complex is not highly soluble in water.

In a Cu(II) assay, the formation of an orange to brown Cu–bathocuproine complex became visible after 10 μL aliquot of water sample containing higher than 0.8 mg/mL Cu(II) was dropped onto the device, which displays the “Cu” symbol (Figure 2a). Otherwise, if Cu(II) concentration is below 0.8 mg/L, the symbol remains colorless. The use of 10 μL of sample is that it just fills the symbol. The color of the symbol became brighter as the copper concentration increases. This appearance of the “Cu” symbol on the device informs users that Cu(II) concentration is higher than the drinking water standard. In practical use, the device can be simply dipped into a water sample to “read out” the result.

Hexavalent chromium, Cr(VI), is considered as a highly toxic heavy metal ion. A water system contaminated by Cr(VI) can be severely harmful to the environment and humans. For the chromium assay on the paper device, 1,5-diphenylcarbazide (DPC) was dissolved into 50% (v/v) acetone to make the 1 mg/mL solution. The solution was introduced into the “Cr” symbol on paper, followed by an addition of 1% H2SO4. Under the acidic conditions, a magenta to purple complex will form when a 10 μL of Cr(VI) solution is added into the “Cr” symbol. Like the Cu(II) assay, the Cr(VI) assay showed a purple symbol of “Cr” when the Cr(VI) concentration exceeded 0.5 mg/L. By the Australian and New Zealand guidelines for fresh and marine water quality, the trigger value of Cr(VI) in the water resources for purpose of irrigation, livestock drinking and other industries is between 0.1 mg/L (long-term use) and 1 mg/L (short-term use),26 the appearance of “Cr” on the paper (Figure 2b) warns the users against long-term use of the water for their industrial activities.

Figure 1. Schematic diagram of the fabrication of patterns of heavy metal chemical symbols on chromatographic paper.

Figure 2. Colorimetric assays showing heavy metal ions of different concentrations: (a) Cu(II), (b) Cr(VI), and (c) Ni(II).
Nickel is another heavy metal which could enter into the water system through mining, manufacturing activities, and through leaching from e-wastes. For a Ni(II) assay, a 10 μL aliquot of dimethylglyoxime (DMG) dissolved in an ethanol solution (80 mM) was first introduced into the “Ni” symbol, followed by an addition of 10 μL of a solution of NaF and Na₂S₂O₃ dissolved in Milli-Q water (20 and 80 mg/mL, respectively). The latter solution was used for masking the interference of Cu(II) and naturally existing Fe(III) in water. The Ni-DMG complex has a stable pink-magenta color. When 50 μL of water sample containing ≥0.5 mg/L Ni(II) was introduced into the device, the symbol “Ni” became visible. Since the standard of Ni in the Australian and New Zealand guidelines for fresh and marine water quality is 0.2 mg/L (long-term use) or 2 mg/L (short-term use), the appearance of the “Ni” symbol warns the users that the quality of the water is unsafe for long-term purpose (Figure 2c).

To examine the interference tolerance of the paper assays, each metal ion, with and without the presence of interfering ions, was assayed on paper and compared. For this purpose, validation of quantitative information obtained from paper was first carried out using a colorimetric method which is described in detail in the Supporting Information. Briefly, paper was cut into 1 cm × 1 cm squares; a series of a heavy metal solution of known concentrations, including blanks, were prepared. Assays of these solutions were performed on paper squares and then scanned to obtain color assay images. Assay images were then analyzed using computer software to determine the reflective color density to establish calibration curves for quantification of target heavy metal ion in water. The calibration curves were then used to determine the paper devices’ interference tolerance, the results were then compared with those obtained using an ICP-AES. Specifically, for examining interference tolerance of Cu assay on paper, sample solutions containing Cu(II) as the target ion and higher concentrations of Ni(II), Cr(VI), Fe(III), and Zn(II) as interfering ions were prepared. Similar procedures were used to evaluate the interference tolerance of Cr(VI) and Ni(II) assays. Our results show that interfering ions of 10 times the concentration of the target ions do not affect the assay results (Figure 3a−c). Interesting also, assays showed no color change to samples that contained only interfering ions but no target ions (Figure 3d). Furthermore, other interfering metal ions such as Mn(II) and Co(II) which have similar atomic structure to the target ions as well as Na(I), K(I), Ca(II), and Mg(II), which can exist in high concentrations from some water sources, have also been studied. Results showed that no significant interference could be observed (Figure S4 in the Supporting Information). The interference tolerance ratio of different ions when determining 1 mg/L Cu(II), Cr(VI), and Ni(II) were presented in Table 1.

Pseudoenvironmental samples were prepared to demonstrate the applicability of the paper device for practical water quality monitoring. Tap water from our laboratory was spiked with two different known levels of Cu(II), Cr(VI), and Ni(II) and was then used for determination of the three heavy metals. On the one hand, the target ion concentrations were measured using paper squares (see the Supporting Information); images of the paper assays were collected to obtain the color intensity information using the software; and the concentration of each heavy metal was calculated by means of the best-fitting equation of the corresponding calibration curve (Figure S3 in the Supporting Information). Here paper squares were used to obtain error bar information; text-reporting devices were also used, and results obtained were in good agreement with the paper squares. On the other hand, ICP-AES was used to measure the same samples to compare results with the paper-based assays. Figure 4 shows the determination of Cu(II), Ni(II), and Cr(VI) in tap water with multiple ions by paper-
based assays and ICP-AES with 5 parallel tests, respectively. The paper-based assays show a mean error within 10% against results obtained by ICP-AES. The recovery rate of the spiked water samples obtained using our paper device and ICP-AES were compared (Table S1 in the Supporting Information), indicating the reliability of the paper assay as a rapid and user-friendly semiquantitative assay for environmental monitoring.

Coming back to the periodic table sensor design concept, we fabricated the three chemical symbols on one paper device. We show in Figure 5 that an assay could be performed by simply dipping the device into a water sample and instantaneously obtain the result as a text message. However, we also noticed that while Cu(II) and Ni(II) form water insoluble chelates with their corresponding indicators and remain stable on paper, the color product 1,5-diphenylcarbazone-Cr(III) or Cr(III)−DPCA complex is water-soluble and could not remain on paper stably. Thus, the Cu and Ni symbols were fabricated on the lower section of the device, whereas the Cr symbol was on the upper section (Figure 5a,b). The Cr(VI) assay requires a separate sample addition process to stop the leaching of the chelate into water (Figure 5c,d).

The stability of the three ion-indicator complexes has also been investigated: the Cu(II)−bathocuproine and Ni(II)−DMG complexes showed high stability; colors of these complexes showed no obvious fading after more than 6 months under the ambient laboratory conditions. The Cr(III)−DPCA complex faded significantly after 24 h in the same environment. The different stability of these ion−indicator complexes are related to the stability of the indicators: bathocuproine and DMG are stable compounds, while DPC is light-sensitive. The Cu(II)−bathocuproine and Ni(II)−Jacqillet complexes showed no obvious fading after more than 6 months under the ambient laboratory conditions. The Cr(III)−DPCA complex requires further improvement of its stability.

Protection of the DPC indicator and Cr(III)−DPCA complex against light exposure is necessary. It is possible that by significantly reducing the leaching of indicators and the metal ion−indicator complexes through further research, the concept of paper-based text-reporting sensor can be expanded to analyzing other heavy metal ions. It is also expected that an expansion of text-reporting paper sensors beyond this application may be possible in the future, not only for detection of other heavy metal ions but also for a wide range of chemical/biochemical analysis and sensing.

**ASSOCIATED CONTENT**

 Supporting Information

Additional information as noted in text. 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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