A NEW PAPER-BASED MICROFLUIDIC DEVICE FOR SENSITIVE DETECTION OF NITRATE IN SEAWATER

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A NEW PAPER-BASED MICROFLUIDIC DEVICE FOR SENSITIVE DETECTION OF NITRATE IN SEAWATER

BY

AMER CHARBAJI

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN MECHANICAL ENGINEERING

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OF

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ABSTRACT

Nitrate is a naturally occurring nutrient that is part of the nitrogen cycle. Monitoring of this nutrient in water bodies ensures its safety for consumption, guards against water nutrient pollution, and provides oceanographers and marine scientists with data to better understand nitrogen cycling and other phenomena of interest. Currently, there are several techniques available for nitrate detection. However, they either lack the required sensitivity for use in the field, or are costly, time consuming, and require special handling of the water sample as it gets sent to a laboratory for analysis.

Paper-based technologies possess several advantages that can facilitate sensitive detection of nitrate in the field by providing an inexpensive, easy to use, portable, and disposable device. This study developed a system based on the use of a microfluidic paper-based device for detecting nitrate in seawater by incorporating different technologies to improve nitrate reduction and detection.

A new composite material improved nitrate reduction by 36% than what has been previously accomplished. This composite material was used in a novel paper-based device that utilized a folding detection zone architecture and immobilized detection reagent. This resulted in a limit of detection and quantification of nitrate of 0.53 ppm and 1.8 ppm, respectively. These results constitute over 40% enhancement from what has been previously realized for the detection of nitrate in water using paper-based technology. The results of this study also contributed to the field of paper-based technology by providing new designs, materials, insights and conclusions that further enhance and deepen the understanding of this technology.
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Dedication

To my parents, sisters, brother and good friends… Thank you for all your support throughout the years. I couldn’t have done it without you!
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Nitrate is a nutrient that naturally exists in the environment and is part of the nitrogen cycle [1]. Healthy ecosystems can keep the amounts of this nutrient in balance which allows plants to grow. However, due to human activity, mainly emanating from excessive use of fertilizers, industrial applications, sewage treatment plants and from many other sources, a large amount of nitrate ends up finding its way to water bodies which results in water nutrient pollution [2–4]. Also, nitrate is the most stable form of nitrogen in oxygenated environments and all other forms of nitrogen-containing compounds can become sources of nitrate in water [5, 6]. In many cases, this causes algal blooms that are unsightly and can raise numerous environmental and health concerns. Some algal blooms release toxins that will make humans sick if they ingest contaminated water or consume tainted aquatic animals. Drinking water with “elevated” concentrations of nitrate can result in several health issues with some being related to different types of cancer [7]. The United States Environmental Protection Agency (EPA) has set the maximum permissible concentration of nitrate in drinking water to be 10 parts per million (ppm) [8]. This concentration is measured as a nitrogen value [9]. The EPA also considers nitrate concentrations greater than 3 ppm as indicative of groundwater contamination and those greater than 1 ppm to indicate human activity. Therefore, continuously measuring the concentrations of nitrate in natural water bodies or in sources used for drinking water is of great importance to make sure that there isn’t
any alarming increase or buildup of its concentration beyond what is considered normal for that particular environment which may subsequently lead to eutrophication or other problems down the line. Additionally, oceanographers and marine scientists continuously measure surface water nitrate concentrations since it affects phytoplankton biomass, indicates bacterial denitrification, and is a metric for nitrogen cycling and reactive nitrogen transport in aquatic systems [10–14]. Therefore, having an inexpensive, portable and sensitive device, allows them to better choose the location from which they will collect their water samples to send back to the lab for further analysis.

There are several different techniques available to measure concentrations of nitrate in water [15]. However, the most sensitive technique requires the collection of a water sample and then sending it to a laboratory for analysis; which is costly, time consuming, and requires special handling of the water sample [16, 17]. Other techniques are available for analysis of results in the field; however, these techniques are not sensitive enough for detecting the concentrations of nitrate in nature. The simplest technique used for the detection of nitrate is the colorimetric dip strip; which changes color when the nutrient is present in the water sample. This technique requires the user to dip the strip in the water sample and then waiting for the color to form before comparing it to a color chart to find the concentration. A new detection technique based on the use of paper-based devices has been gaining popularity over the past several years [18–32]. While detection of nitrite was shown possible in the low parts per billion range, the detection of nitrate was multiple orders of magnitude greater than that [20–22, 24, 29], even though it uses the same detection principle. This is due to the fact that the
Griess assay, the most commonly used method for analyzing the concentrations of nitrate or nitrite, is specific to the detection of nitrite and can’t detect nitrate directly. This means that nitrate has to be reduced, or “converted”, to nitrite first before it can be detected by the Griess assay.

The overall objective of this dissertation was to develop a system based on the use of a microfluidic paper-based device for detecting nitrate in seawater. The developed system had to achieve an improved detection limit than what has been achieved by paper-based technologies to date for detecting nitrate in water. The system had also to retain the advantages of using microfluidic paper-based technology i.e. low cost, user friendly, portable and easily disposable. At the start of this project, only one paper-based device for detecting nitrate had been developed by Jayawardane et al. [29]. However, by the time this dissertation was submitted, and in addition to the system presented in this dissertation, four other paper-based devices for detecting nitrate in food and saliva samples had also been developed by other research groups [21, 22, 24, 33].

This project was accomplished by improving nitrate reduction and detection methodology. Different reducing agents have been considered and used for detecting nitrate. However, zinc remains the preferred reducer since it is non-toxic which allows the paper-based device to be easily discarded after use. Chapter 2 provides a literature review of the use of zinc in paper-based technology. Chapter 3 introduces and provides material characterization results of a new composite material called “Zinculose” that is made up of zinc and cellulose fibers. This material has zinc particles embedded within the matrix of the channel rather than having the zinc particles simply sitting on the surface of the reduction channel of the paper-based device. Use of Zinculose resulted in
an enhancement of nitrate conversion efficiency of 36% than what has been previously reported.

Chapter 4 focuses on the development of the paper-based device for nitrate detection. The main aim was to improve the “quality” and “quantity” of the color that forms in the detection zone since this improves the sensitivity of the device. Several different paper-based architectures were designed and tested with advantages and disadvantages observed and noted for each design. The device that incorporated a folding detection zone resulted in the most uniform color i.e. best “quality” of color. This architecture was then optimized to improve the signal intensity i.e. increase the “quantity” of color. The limits of detection and quantification of nitrate in water achieved by the paper-based device were 0.53 ppm and 1.8 ppm, respectively. These results constitute over 40% enhancement than what has been previously realized for the detection of nitrate in water using paper-based technology.

Chapter 5 investigates the use of vanadium (III) chloride as the reducing agent in a paper-based device for detecting nitrate. While vanadium (III) chloride has its own set of advantages such as the development of simple dip strips for educational purposes, Zinculose allows for signal amplification by letting more sample pass through the composite channel without the reducing material being washed away. Chapter 6 provides insight into the shelf life of the “G1” and “G2” pads used in the developed paper-based device. It also presents a practical system for measuring nitrate concentrations in the field. This is based on the use of a specifically designed lightbox that allows for a more sensitive measurement of nitrate in the field compared to naked
eye observation of the color that forms. Chapter 7 provides the conclusions and a list of recommendations for future work.

REFERENCES


CHAPTER 2

Literature Review of the Use of Zinc and Zinc Compounds in Paper-Based Microfluidic Devices

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Abstract

Zinc and its compounds, alloys and composites play an important role in the modern day world and find application in almost every aspect that can improve the quality of our lives. This ranges from supplements and pharmaceuticals that are meant to improve our health and wellbeing to additives meant to guard or reduce corrosion in metals. However, over the past several years, a new area of technology has been garnering a great deal of attention and has made use of zinc and its compounds. This is with reference to paper-based microfluidic technology that offers several ad-antages and that keeps expanding in the amount of applications it covers. In this paper, a review is offered for the applications that have used zinc or zinc compounds in paper-based microfluidic devices.

Introduction

Paper has been used for biological and chemical applications for over a century [1], [2]. However, over the past several years, a lot of research and resources have been dedicated to developing new paper-based devices, or improving their performance, for use in a wide range of applications. Paper-based devices have been used for biomedical applications [3], food safety [4], soil analysis [5], water analysis [6] and in many other miscellaneous applications [7]. Muller et al. [8] may have developed the first simple paper-based microfluidic device in 1949 [9–11]; however, it was the Whitesides group in 2007 [12] who gave a renewed view and push for the possible applications of this technology. The main advantages of paper-based microfluidic devices are that they are inexpensive, simple, portable and easy to use. However, the most significant ad-antage is that these devices depend on capillary action to flow the sample across the different
sections of the device without a need for a pump, which results in device miniaturization and cost savings. Paper-based microfluidic devices have adapted several of the conventional detection techniques such as colorimetric detection, electro-chemical detection, chemiluminescence, fluorescence, mass spectrometry and sur-face-enhanced Raman spectroscopy [13]. And since zinc and its compounds are utilized in a broad range of applications due to their inherent advantages, it was clear that they were to be used in paper-based microfluidic devices.

Zinc is an essential trace element for the human body since it plays an important role in maintaining cell health and functionality and is crucial for the function of more than 300 enzymes [14], [15]. The human body contains 2 to 3 grams of zinc and requires a daily intake of 10 to 15 mg [16]. Zinc also finds use in many different applications out-side of the human body mainly due to its abundance and nontoxicity [17]. Zinc and zinc oxide have been used in the rubber industry, pharmaceutical and cosmetic industries, textile industry, electronics industry, for enhancing filtration, in making batteries, and in many other miscellaneous applications [18–21]. Zinc oxide is an inorganic material with specific properties that is extremely produced after silicon dioxide and titanium dioxide [22]. Zinc oxide nanomaterial is stable, safe to human beings and has been categorized by the Food and Drug Administration in the United States as 'generally recognized as safe' [23]. It has been abundantly used in environmental and medical applications due to its nontoxicity, biosafety, biocompatibility and biodegradability [24]. Textiles have been functionalized by zinc oxide to impart favorable properties such as improved antimicrobial [25], super hydrophobicity [26], self-cleaning [27], thermal insulation [28], UV-absorption [29],
and flame retardancy [30]. The cotton fibers in paper-based devices have also been functionalized by zinc oxide to provide or enhance analytical or engineering purposes.

In this review, some of the paper-based microfluidic devices that have used zinc or zinc compounds will be highlighted with the different applications they were used in.

**Application of Zinc and Zinc Compounds in Paper-Based Devices**

**Biomedical**

Zinc and its oxide are biocompatible [31], [32] and have been used in several paper-based devices for different biomedical applications. Li et al. [33] used zinc oxide nanowires for the electrochemical detection of glucose in spiked phosphate-buffered saline and human serum, Figure 2-1. However, they anticipate that their device can be used to detect glucose in whole blood if a filtering membrane is added on top of their device’s reaction zone to filter out the blood cells. Ferreira et al. [34] used zinc microparticles as the reducing agent in a paper-based device to detect nitrate in human saliva.

![Figure 2-1 Paper-based device developed by Li et al. [33] that uses zinc oxide nanowires for the electrochemical detection of glucose.](image)
Detection of biomarkers using paper-based devices will help in making rapid and in-formed decisions whether further investigation is needed to determine the health status of an individual. Tiwari et al. [35] functionalized paper with zinc oxide nanorods to preconcentrate myoglobin which is a biomarker for heart disease. Guo et al. [36] developed a paper-based device integrated with zinc oxide nanowires that can detect three cardiac biomarkers, human heart-type fatty acid binding protein, cardiac troponin I, and myoglobin, simultaneously. Kong et al. [37] constructed a paper-based device utilizing zinc oxide nanoflowers for the sensitive detection of the biomolecules, L-glutamic acid and L-cysteine. Sun et al. [38] constructed an ultrasensitive electrochemical immunosensor using branched zinc oxide nanorods and porous zinc oxide spheres with gold nanoparticle composite for the detection α-fetoprotein which is a tumor marker used in the diagnosis of hepatocellular carcinoma. Sun et al. [39] used tetra-carboxyl naphthalocyanine zinc sensitized zinc oxide nanorods as photoactive materials in a paper-based photoelectrochemical immunosensor to detect the carcinoembryonic antigen. Liu et al. [40] constructed a paper-based device for the colorimetric determination of the carcinoembryonic antigen based on the intrinsic peroxidase activity of zinc ferrite (ZnFe2O4)-multiwalled carbon nanotubes. Wang et al. [41] constructed a hollow-channel paper based device with CdS quantum dots/reduced graphene oxide/zinc oxide nanorod arrays for the photoelectrochemical detection strategy of multi-component detection of multiple microRNAs.

Portable, rapid and sensitive detection of diseases, particularly infectious, would play a significant role in decreasing their spread. Li et al. [42] reported on the first micro-fluidic paper-based origami nanobiosensor that uses zinc oxide nanowires to improve
the biosensing performance of detecting the human immunodeficiency virus p24 antigen. Li et al. [43] also used zinc oxide nanowires directly grown on the working electrode of the paper-based device to enhance the detection of the p24 antigen used as a marker for the human immunodeficiency virus and showed that their device was capable of differentiating between concentrations of CR3022, an IgG antibody to the Covid-19 coronavirus. Narang et al. [44] developed a paper-based device incorporating zinc-silver nanoblooms that used cyclic voltammetry for the electrochemical detection of the herpes virus DNA. Kim [45] grew zinc oxide nanorods on cellulose paper and then added gold nanoparticles to these rods so as to enhance the Raman signal in detecting the presence of prenatal diseases and identifying the types of diseases from amniotic fluids.

Finally, it is very useful to have a simple and rapid method for detecting different drugs. Caglayan et al. [46] used a fluorescent coordinatively unsaturated metal complexes based on the zinc II ion to provide an easy-to-detect fluorescence response in the detection of the banned performance-enhancing drug Adrafinil. Narang et al. [47] developed a paper-based device for the detection methylenedioxymethamphetamine which is an addictive narcotic and a potential neurotoxic agent used as a recreational drug.

**Environmental Applications**

Paper-based microfluidic devices generally integrate a suitable detection method such as colorimetric, electrochemical or fluorescent techniques for quantitative analysis of nutrients in air, soil, or water [11]. Zinc and its compounds have been used in microfluidic paper-based devices for environmental applications to provide rapid,
inexpensive, and quantitative analysis. Nitrate is part of the nitrogen cycle but contributes to water nutrient pollution when present at elevated concentrations [48], [49]. Jayawardane et al. [50] and Charbaji et al. [51], Figure 2-2, used zinc microparticles as a reducing agent in paper-based devices meant for the detection of nitrate in water. The zinc microparticles provided the reduction of nitrate to nitrite needed for its detection by the Griess assay. While Jayawardane et al. created a reduction channel by depositing the zinc microparticles on the surface of the paper, Charbaji et al. used a newly developed composite material made up of cellulose and zinc microparticles, that they called Zinculose [52], Figure 2-3, to provide the required reduction step. Zhang et al. [53] used du-al-emission manganese-doped ZnS semiconductor nanocrystals on paper-based test strips for the visual detection of diethylphosphorothioate (organophosphate) residues.

Figure 2-2 Paper-based device developed by Charbaji et al. [51] for the detection of nitrate in water.
Another environmental application that made use of paper-based microfluidic devices was the detection of pentachlorophenol. Pentachlorophenol is a xenobiotic that enters the environment as a byproduct of various industrial processes and is a cause for great environmental concern. Sun et al. [54] developed a disposable paper-based microfluidic origami device with functionalized zinc oxide nanospheres to detect pentachlorophenol.

Paper-based devices were also used in the detection of gases. Gimenez et al. [55] developed a paper-based oxygen sensor that uses the absorption and desorption of oxygen from the zinc oxide crystals deposited and dried over graphite electrodes drawn on paper for detection. Koga et al. [56] deposited zinc oxide on paper using a two-step paper-making process to create a molecular sensor and they used nitrogen dioxide to test its sensing performance.

Food Safety

Monitoring the quality of agricultural and food products in order to ensure its safety is of utmost importance to safeguard the health and wellbeing of consumers.
Developing small-size field deployable sensing systems and combining that with the state-of-the-art communications techniques is a way to perform qualitative and quantitative multi-component analysis of food products to guarantee its safety [57]. Paper-based devices have many desirable advantages that make them ideal candidates for such an application and they have been demonstrated to be effective in testing the quality of food products [58]. Several paper-based devices utilizing zinc or its compounds have been developed to detect different analytes or toxins in food. Yukird et al. [59] deposited zinc oxide from a solution over 2 detection zones in a paper-based device. The first was a multiwall carbon nanotube electrode and the second was a laser desorption ionization mass spectrometric detection zone. This was meant to improve their sensitivity in detecting ‘bisphenol A’ which is chemical compound used in the production of food containers.

Chen et al. [60] developed a fluorescence paper-based sensor based on zinc 5, 10, 15, 20-tetra(4-pyridyl)-21H-23H-porphine (nano ZnTPyP) quenching CdTe quantum dots for the detection of three carbamate pesticides which are metolcarb, carbofuran, and carbaryl. Teepo et al. [61] and Ratnarathorn et al. [62], Figure 2-4, used zinc microparticles as the reducing agent in paper-based devices to detect the presence of nitrate in food samples.
Figure 2-4 Paper-based device developed by Teepo et al. [61] for the detection of nitrate in food samples. The reducing zone contained zinc dust.

Chen et al. [63] established a high sensitivity paper-based fluorescent sensor to detect L-theanine in tea water which is one of the markers used to evaluate the sweetness and freshness of tea. Their device used CdTe quantum dots/corn carbon dots with nano tetra pyridel-porphine zinc to provide the quenching effect. Another paper-based fluorescent sensor utilizing CdTe quantum dots and spherical nano tetra pyridel-porphine zinc was developed by Chen et al. [64] for the identification and quantitative analysis of caffeine.

**Miscellaneous Applications**

Although the above sections highlighted microfluidic paper-based devices developed for biomedical, environmental and food safety applications, it’s important to mention that there are a lot of other applications that have used paper-based devices also utilizing zinc or its compounds. This section will refer to some of these paper-based devices used for miscellaneous applications. Song et al. [65] have provided a decent review of paper-based physical sensors utilizing zinc oxide nanostructures up to the year 2017. Therefore, this review looks at papers published from 2017 onwards for paper-based...
based physical sensors utilizing zinc compounds. Wang et al. [66] developed a one-axis piezoelectric accelerometer using zinc oxide nanowires that have been grown on paper, Figure 2-5. The fabrication of their device is inexpensive and doesn’t require sophisticated equipment. Dubourg et al. [67] developed a paper-based ultraviolet sensor utilizing zinc oxide. They used a laser post processing step to induced significant modifications to the surface morphology and structure of the zinc oxide film. This contributed to the super-hydrophobicity of the printed zinc oxide nanoparticles which reduced humidity interference while enhancing sensitivity for ultraviolet detection.

![Figure 2-5](image)

**Figure 2-5 (A) Schematic of the paper-based one-axis piezoelectric accelerometer developed by Wang et al. [66]. (B) Photograph of the paper-based device.**

Paper-based devices were also developed that could provide a flexible power-source. Purohit et al. [68] developed microfluidic galvanic and hybrid cells on a single layer of paper. The cell had zinc and copper powder that were painted on the paper to serve as the anode and cathode. Zhang et al. [69] developed a zinc-air battery with zinc foil pasted on titanium foil to act as the anode. Burrola et al. [70] presented an alkaline
nickel oxide hydroxide/zinc battery with a zinc anode cut from commercially available sheets.

An interesting paper-based device using zinc oxide was developed by Zhang et al. [71]. They used zinc oxide nanorods to fabricate flexible light emitting diodes on paper and used it as an excitation light source in a multiplexed photoelectrochemical immunosensor.

**Challenges and Future Trends**

While zinc, its oxide and compounds have been seeing increased use in paper-based microfluidic devices, their usage is expected to expand to encompass more applications. However, some challenges still need to be overcome to ensure repeatability in the preparation of zinc oxide nanoparticles to enable the surface modification of these particles with organic compounds [20]. Also, zinc oxide may need to be combined with other material such as metals, semiconductors, and nanocarbons to overcome its limitations such as fast recombination of photogenerated electron-hole pairs, photocorrosion and need for UV light for activation in photocatalytic applications [72].

The development of composite materials using zinc microparticles such as Zinculose [52] will help improve the performance of paper-based devices using zinc microparticles in different applications. As paper-based electrochemical devices start to play a bigger role in the paper-based platform technology, use of zinc oxide is expected to enhance the sensitivity and selectivity of these devices [73]. Zinc oxide have demonstrated effectiveness in biomedical fluorescence assays [74] and will be instrumental when paper-based devices are developed for fluorescence assays. It is
anticipated that the future direction is to develop more paper-based devices for photonic and electronic applications which will use zinc oxide nanoparticles since these particles are ideal for use in the field of photonics, nanoscale electronics and optoelectronics [75], [76].

Conclusions

Paper-based microfluidic technology is a field that is still developing with frequent new applications and advancements being achieved. Zinc, its oxide and compounds have been utilized in a lot of different applications using paper-based technology. This is the case since zinc, zinc oxide and zinc compounds are inexpensive, biocompatible, non-toxic, environmentally friendly, and have their distinctive physical and chemical properties. They have been widely utilized to improve the properties and performance of cellulose substrates in paper-based analytical devices. In this review, paper-based devices that have used zinc or zinc compounds in different applications were highlighted. These devices were developed for biomedical, environmental, food safety and for several miscellaneous applications. However, it is anticipated that more devices utilizing zinc, its oxide or compounds will be developed in the future. These devices will either improve the performance of the ones that have already been designed before or will be completely novel sensors for new applications.

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Conflict of Interest

The authors declare no conflict of interest.

References


CHAPTER 3

Zinculose: A New Fibrous Material with Embedded Zinc Particles

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Abstract

In this paper, we report a simple and inexpensive procedure to make a composite material of cellulose fibers with embedded zinc microparticles. This fibrous material is produced by sedimentation and is referred to as “Zinculose”. Zinculose increases the surface contact area between a sample fluid and zinc microparticles. The effect of different parameters including fiber content, zinc content, water volume, applied weight and its duration on the thickness of produced Zinculose were investigated. Results show that thickness depends on the amount of initial fiber and zinc while other parameters investigated had little to no effect. Measured porosity values for Zinculose ranged between 0.699 and 0.843. Characterization of flow in Zinculose exhibits a linear relationship between distance and the square root of time which is a distinctive feature of capillary driven flow in porous media. This is an important quality that allows Zinculose to be easily incorporated into any paper-based microfluidic device that requires a sample to flow and interact with zinc microparticles without disrupting the flow path between different sections of the device. An application is presented in which a strip of Zinculose is used to convert nitrate to nitrite. With the use of Zinculose in a paper-based microfluidic device, a conversion efficiency of 27% nitrate to nitrite was achieved. This presents a 36% enhancement over what has been previously published when zinc microparticles were not embedded within the fibers of the paper channel.

Introduction

Use of zinc has been gaining popularity over the past several years for the many advantages it possesses. The attractiveness of developing zinc-based systems stems from the abundance and nontoxicity of zinc which broadens its areas of application [1].
Zinc is used in the battery, cosmetic, electronics, pharmaceutical, photocatalytic and rubber industries as well as in many other miscellaneous applications [2,3]. Properties of zinc make it a preferred material of choice for different biological and chemical applications of which some have already been adapted for use in paper-based microfluidic devices for point of care diagnostics [4–7].

Cotton fibers have been functionalized with zinc in the form of zinc oxide for a multitude of reasons. For the most part, cotton fibers were functionalized with zinc oxide for its antimicrobial properties [8]. Other properties imparted on cotton fibers include UV protection [9], super-hydrophobicity [10], self-cleaning capability [11], thermal insulation [12], increased electrical conductivity [13] and flame retardancy [14]. Cotton fibers functionalized with zinc oxide were also used in light emitting diodes [15], in different sensor applications [16–19] and as anodes in lithium-ion batteries [20]. Different coating approaches can be utilized to functionalize cotton fibers with zinc oxide. These include pad-dry-cure, dip-coating, sonochemical coating, sol-gel, ultrasonic irradiation and atomic/molecular layer deposition [8]. All of these techniques require several processing steps and use of multiple chemicals and solvents. Functionalized cotton fibers retaining residues of the chemicals used in the functionalization process may not be suitable for use in certain chemical analyses if these residues interfere with anticipated reactions. Gimenez et. al [21] produced a composite material by compressing zinc oxide nanocrystals and cellulose powder under high pressure and without the use of any chemicals. But this material is made into solid pellets which is not suitable for use in paper-based microfluidic devices. Also, zinc and zinc oxide possess different properties, so it would be advantageous to produce a
composite material made up of cotton fibers and pure zinc particles without the use of chemicals.

In this paper, we present a simple and inexpensive procedure to make Zinculose without the use of any chemicals. Important parameters affecting material and flow characteristics of Zinculose are reported. An application is presented in which Zinculose is used in a paper-based microfluidic device for converting nitrate to nitrite. To the best of our knowledge, this is the first paper that details a simple and inexpensive approach for producing a composite material made up of cotton fibers and zinc microparticles by the process of sedimentation and without the use of chemicals.

Methods

Materials

The following materials were used in the production of Zinculose. Chromatography paper (GE Healthcare Whatman 1 - 3001878) was chosen because it is entirely made up of pure cotton fibers without any additives and is suitable for chemical applications. Blotting paper (GE Healthcare Whatman GB003 - 10547922) and zinc powder (99.3%, Fisher Chemical Zinc Certified Powder Z5-500) with a particle size less than 40μm as observed using the electron microscope. A plastic cloche (7.2 cm and 9.5 cm inner base diameter and height, Nicole a9952-1) with a 2 mm hole drilled at the top was used as the top part of the paper mold. A foam pot insert with a thickness of 50 mm (FloraCraft PI432GS) was used as the bottom part of the mold onto which the Zinculose mixture would precipitate onto. It fits 8 mm deep into the cloche with the remaining part left outside the cloche. The inside of the pot insert was removed from the bottom leaving 1 cm of foam around all edges. Holes were pierced throughout the

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top surface of the foam pot using a 1 mm round metal wire with a spacing of 4 mm between the holes.

The layout of the paper-based microfluidic device was designed using a vector graphics software (CorelDraw X6). A solid ink wax printer (Xerox ColorQube 8570) was used to print the design on Whatman 1 chromatography paper. Wax printing is the most popular technique used in fabricating paper-based microfluidic devices [22]. Devices were then cut out using a laser engraver (Epilog mini 40W) and placed in an oven at 120 °C for 3 minutes to form the hydrophobic platform of the device. Wax in paper creates a barrier to the flow of water [23]. Nitrate and Nitrite standard solutions (SPEX CertiPrep ASNO392X & ASNO292X) were diluted with ultrapure water and were used to measure the conversion efficiency of Zinculose. A cellulose fiber sample pad (Millipore CFSP203000) served as the sample port of the device while the nitrite test field of a commercial dip strip (CTL Scientific Supply Corp Quantofix 91313) served as its detection zone. A 0.0127 mm thick double sided tape (FLEXmount 0.5 mil SELECT DF071736) was used to bind the different sections of the microfluidic device.

**Fabrication Procedure**

To make Zinculose, a sheet of chromatography paper is folded and torn into pieces and placed in the plastic mixing cup of a commercial grade blender (Oster Smash Blend 14). 250 ml of deionized (DI) water is added to the cup and mixed on high for 5 to 6 minutes until there are no more fiber clumps observed in the mixture. This step ensures separation of individual cellulose fibers from the matrix making up the chromatography sheet. The cup is then placed on a magnetic stirrer (Fisher Scientific Isotemp) and a small amount of zinc powder is weighed using a microbalance (Fisher Scientific
ALF64) and added to the mixture. Teflon encapsulated stirrers (1 and 2 inch) are added to the mixture and stirred at 350 RPM for 25 minutes. While the mixture is being stirred, a sheet of chromatography paper is cut into 4 equal parts (12.5 × 12.5 cm), 3 of which are placed in a tray filled with DI water to soak for at least 15 minutes. 2 of these wet sheets are then stacked on one another and on the top surface of the foam pot. The Zinculose mixture is then transferred to the cloche with the 2 mm hole covered with tape (3M 600 Transparent Scotch Tape). The magnetic stirrers are removed using a pair of forceps and the bottom part of the mold is secured to the upper part. The mold is then flipped over and placed on a water collecting tray, the tape covering the 2 mm hole is removed and the water is allowed to drain out for 30 minutes, Figure 3-1 (a).

Figure 3-1 (a) Zinculose mixture precipitates onto the foam as water drains out (b) Exposed surface of Zinculose after the water is allowed to drain out (c) Zinculose membrane after air drying in room conditions for 12 hours (d) Zinculose strips used in analysis

Afterwards, the cloche is removed and the remaining wet chromatography sheet being soaked in DI water is placed on the surface of the precipitated Zinculose, Figure 3-1 (b). A 10 × 12 cm blotting paper is then added on top of the wet chromatography paper. This blotting paper gets replaced by another dry one as soon as water reaches all its edges. The bottom chromatography papers are then carefully peeled off the foam pot
insert and the arrangement is flipped over and laid on a flat surface with the blotting paper at the bottom and the peeled chromatography papers on top. A 10 × 12 cm blotting paper is then added on top of these chromatography papers. A 15 cm petri dish is placed on top of the arrangement and a 0.5 kg weight is added to the petri dish for 25 minutes. The weight, petri dish and blotting paper are then removed and the 2 chromatography papers are slowly and carefully peeled off the Zinculose starting at an edge and moving at a very sharp angle. The arrangement is then flipped over and the remaining sheet of chromatography paper is peeled off. The Zinculose sheet is allowed to air dry under room conditions for at least 12 hours, Figure 3-1 (c), before being cut into 1 cm strips using a guillotine paper cutter and placed in a box where relative humidity is maintained under 30%. All test strips were allowed to equilibrate with the ambient for at least 1 hour under room conditions before any analysis was performed, Figure 3-1 (d).

**Results and Discussion**

**Characterization**

The uniformity and coverage of zinc particles in a sheet of Zinculose was observed using a SIGMA VP Field Emission-Scanning Electron Microscope (SEM) with an energy dispersive X-ray spectroscopy detector (EDS). Figure 3-2 shows a standard Whatman Grade 1 chromatography paper (left) and a Zinculose sheet (right) at different magnifications using the SEM. These images show that the manufacturing process didn’t destroy individual cellulose fibers and that zinc microparticles are spread throughout these fibers.
Corrosion of pure zinc in deionized water varies between 15 and 150 μm/year [24]. During the manufacturing process of Zinculose, zinc microparticles are submerged in deionized water for less than 2 hours and then allowed to air dry. To see if there’s a significant change on zinc microparticles due to the manufacturing procedure, a sample of these particle was submerged in deionized water for 2 hours and then allowed to air dry for 24 hours before being examined and compared to a sample which is fresh out of the box. SEM images show that there’s no difference in the surface morphology of the zinc microparticles, Figure A1-1. X-ray diffraction measurements for these samples in addition to a sheet of Zinculose were carried out using a Rigaku Ultima IV X-ray
differential diffractometer. Data was acquired with a CuKα x-ray source operated at 40 kV and a current of 40 mA. Samples were measured in continuous scan mode between 5 and 90° with a scan rate of 2 degrees per minute and an angular resolution (2θ) of 0.1 deg. The intensity peaks were compared to those attained by Lupan et. al for Zn/ZnO microspheres [25]. Results show that there isn’t a significant difference in the various “Zn” peaks for both zinc samples, Figures A1-2 to 4. Also, the “ZnO” peaks have much less intensity magnitudes compared to the “Zn” peaks. This means that the zinc microparticles did not oxidize any further due to their exposure to the manufacturing process and that they are still made up of pure zinc with a thin oxide layer. The intensity magnitudes of the various peaks for the Zinculose sheet were less than those for the zinc samples. This change in the magnitude of intensities is due to the variation in zinc amount between Zinculose and that of the zinc samples. However, the relative intensities between the various “Zn” and “ZnO” peaks are similar to those of the zinc samples. Further oxidation of zinc is therefore not a concern in this process and zinc is still the dominant phase even after a storage period of 6 months.

**Effect of Various Production Parameters on Thickness of Produced Zinculose**

Thickness is a property that is always specified for papers used in analytical applications. It is also a design parameter for paper-based microfluidic devices. Thickness can affect the volume of sample required for an assay in addition to its sensitivity [26]. Therefore, it’s of importance to understand the effect of different production parameters on the thickness of produced Zinculose. Different parameters in Zinculose production were investigated for the setup we are using. The parameters
examined were the amount of fiber content, zinc content, volume of water, applied weight and its duration. To measure the thickness of produced Zinculose, sheets were cut into 1 cm wide strips, which gave 5 to 6 such strips for each sheet. Thickness was then measured at the middle of each strip and 1 cm away from each edge for a total of 3 measurements per strip and 15 measurements per sheet. 3 sheets were used in the analysis of most parameters.

**Fiber Content**

There is a correlation between the weight and area of Whatman 1 chromatography paper, Figure A1-6. This relationship is referred to as the basis weight (grammage) and has the units of mass divided by area. Therefore, fiber content can be either specified by the weight of chromatography paper used to make Zinculose or by its area. We found early on that it was relatively easier to cut a chromatography paper into a required size than to keep modifying strips to achieve a required weight. Therefore, we changed the amount of fiber used in the produced Zinculose by varying the area of chromatography paper used in our production setup.

Figure 3-3 shows the thickness of produced Zinculose as a function of fiber content over the range of 35 to 280 cm². Zinculose sheets produced with a 35 cm² initial fiber content were thin and flimsy and could tear easily. Zinculose sheets produced with a 280 cm² initial fiber content were too thick for use in a microfluidic device and were very difficult to handle especially when cutting into strips. We found that the acceptable range of initial fiber content for our production setup was anywhere from 50 cm² to 140 cm². We produced Zinculose sheets with a 70 cm² initial fiber content since it was good in terms of cutting, handling and use in paper-based microfluidic devices.
Figure 3-3 Average thickness of produced Zinculose sheets as a function of the initial area of chromatography paper used (n=45 and the error bars show the standard deviation)

Zinc Content

During Zinculose production, the mixture precipitates on the top surface of the foam pot insert. The area of the produced Zinculose sheet is therefore always constant and equals 41 cm$^2$. The amount of zinc initially added to the mixture determines the basis concentration of zinc in the produced Zinculose. For example, adding 82 mg of zinc powder to the mixture produces a Zinculose sheet with a zinc content of 2 mg/cm$^2$. Zinculose with a zinc content of 0 mg/cm$^2$ means that no powder was added to the mixture although all other steps in the production process are followed and remain the
same including the magnetic stirring of the mixture. Twelve zinc concentrations were tested and thickness measurements show a linear dependence of thickness on zinc content, Figure 3-4.

![Graph showing linear dependence of thickness on zinc content](image)

**Figure 3-4 Average thickness of produced Zinculose sheets as a function of zinc content (n=15 and the error bars show the standard deviation)**

**Water Volume**

The effect of water volume used during the production process on the thickness of produced Zinculose was studied. The range of volume tested was dictated by the capacity of our production setup. The cloche used in our setup can hold a maximum of 275 ml of water and the blender cup would need a minimum of 150 ml of water for the mixture to be in range of the blades. Four volumes were tested which were 150, 200,
250 and 275 ml. The range of water volume tested didn’t seem to have an effect on the thickness of produced Zinculose, Figure A1-7.

**Applied Weight**

Adding a weight during the drying process was meant to aid in removing as much retained water as possible before letting the Zinculose film air dry. It also served as a means to ensure a smooth surface of uniform thickness throughout the produced Zinculose sheet. Four different values of weights were applied, 0, 0.5, 1 and 2 kg. The variation in thickness of the produced Zinculose sheets was within the error range of the results and was negligible when compared to the effect that fiber content has on thickness, Figure A1-8.

**Duration of Applied Weight**

Four weight application times were chosen which were 0, 25, 50 and 75 minutes. Results show no effect on the thickness of produced Zinculose, Figure A1-9. The 0-minute test duration had a higher variation than the other data points in the set which reinforces the understanding that applying a weight during the drying process would provide a more uniform thickness throughout the produced Zinculose.

**Thickness Approximation**

The following regression model was developed to approximate Zinculose thickness based on the amount of fiber and zinc used

\[
\text{Thickness} = 0.006573 \times \text{Fiber Content} + 0.006725 \times \text{Zinc Content} + 0.000046 \times \text{Fiber Content} \times \text{Zinc Content}
\]
Figure 3-5 shows thickness measurements of Zinculose as a function of fiber and zinc contents. The figure also shows the thickness calculated using the thickness approximation model in solid lines. As can be seen in the figure, the coefficient of determination ($R^2$ value) is greater than 0.95. This is a strong indicator of how appropriate the proposed model is in predicting the thickness of Zinculose.

Figure 3-5 Points on the graph are for the average measured thickness of produced Zinculose sheets as a function of fiber and zinc content. Solid lines show an approximation of thickness using the model ($n \geq 45$ for the measured points and the error bars show the standard deviation)
Reproducibility of Zinculose Production

The reproducibility of the process used to make Zinculose was investigated by measuring the thickness of 16 Zinculose sheets produced using the same conditions. Sheets 1 through 8 were produced by one operator, sheets 9 through 15 were produced by a second operator and sheet 16 was produced by a third operator. Thickness measurements show that there are some variations between the different sheets, Figure A1-10. However, Tukey pairwise comparison of the thickness shows that there’s not a single sheet that is significantly different than all of the other sheets, Figure A1-11. Hence the process currently employed can be considered highly reproducible and the variation can be attributed to operator error. It’s also worthy to mention that variations in thickness between these 16 sheets are negligible when compared to the effect that fiber content has on the thickness of produced Zinculose.

Porosity Measurements

Porosity of paper can be either calculated or measured. Porosity can be calculated by following the definition of porosity which is the volume of voids per the total volume of material [27,28]. This method is easy to use and appropriate when the density of fibers making up the material is known. This calculation becomes difficult and more involved for composite materials whose fiber density would first need to be calculated. Measurement techniques yield acceptable results that can be informative when making comparisons. Different techniques have been used to measure porosity of paper such as mercury porosimetry, analysis of absorbed water volume and electric current measurements [29–31].
Porosity values of different Zinculose sheets were measured following an approach similar to that mentioned by Park et al [31]. Zinculose sheets were cut into 2 cm × 1 cm strips and then held from a corner on the short edge using a tweezer and slowly lowered to let the opposite edge touch the surface of ultrapure water without dunking or dipping the strip in water. Water would then flow up the strip by capillarity and fill up the void volume. The strip is removed from touching the surface of water 3 seconds after the water front line reaches the top end. The volume of absorbed water is then measured and porosity is calculated by dividing the volume of absorbed water by the volume of the strip. Whatman 1 chromatography paper was used as a reference material to validate this method since its porosity values have been published before. Porosity measurements of 3 chromatography sheets gave values of 0.714, 0.709 and 0.668 with standard deviations of 0.058, 0.027 and 0.029 respectively (n ≥ 20). These measured values are close to the 0.707 value calculated by Castro et al. [28] and the 0.678 value given by Walji et al. [32] with an error that is less than 6%. This error is deemed acceptable considering that error between calculated porosity values and those measured by mercury porosimetry can be up to 8% [27].

This method was used to measure the porosity of 9 sheets of Zinculose produced using the same manufacturing conditions. 4 strips of each sheet were used and the porosity values ranged from 0.699 to 0.843 with a standard deviation of 0.016 and 0.039 respectively. This variation in porosity values can be attributed to the manual process currently used to produce Zinculose. All of the different Zinculose production conditions mentioned above gave porosities that were within this range of values. A more uniform and controlled porosity value is expected to be achieved if sheet formers
suites for research purposes are utilized or if the Zinculose manufacturing process becomes more automated.

**Zinculose Flow Characteristics**

Flow in porous material due to capillarity and imbibition has been the subject of study for many years and different models have been developed to characterize it [33–35]. Although these models were not necessarily developed to study flow in paper, most of these models have been adapted for such a purpose. The most commonly used model in paper microfluidics is the Lucas-Washburn (L-W) model due to its simplicity [28]. This model states that the distance covered by the fluid in a porous material is proportional to the square root of time. The Lucas-Washburn equation has the form:

\[ x(t) = \sqrt{\frac{\gamma rt \cos \theta}{2\mu}} \]

Where \( \gamma \) and \( \mu \) are the surface tension and dynamic viscosity which are properties of the fluid. \( r \) is the capillary radius which is a property of the porous material and \( \theta \) is the contact angle which is a property of the surface interaction between the fluid and the material system. This model was originally developed to characterize flow in glass capillary tubes in which the radius term “\( r \)” is well defined. Adapting this model to characterize flow in paper substrates left the definition more ambiguous since paper is a matrix of interlayered fibers without a distinct capillary size. To overcome this, and to keep with the simplicity of the model, researchers usually define an average, or an effective, pore radius for the paper substrate which is determined empirically. Another
difficulty when applying this model is assigning a value for the contact angle that forms between the fluid and the cellulose fibers in paper. Different values have been proposed for the contact angle forming between water and cotton fibers and it ranged in value between 0° and 41.5° [27,36]. Other models have also been developed or had the L-W model modified to include other effects and considerations such as gravity, evaporation, swelling of the fibers due to flow, and swelling due to the hygroscopic nature of fibers [28,37].

To run the flow characterization tests on Zinculose, sheets were cut into 1 cm wide strips. The strips were then attached to a holder that had a scale marked on both sides. The holder was made from a 30 cm × 8 cm backing card (DCN Dx’s backing card) which was cut using a laser engraver from a design created on CorelDraw X6. A 100 ml Pyrex beaker was filled with 20 ml of ultrapure water and the sample holder was then slowly lowered into the beaker. An 8-megapixel video camera with 30 frames per seconds capability was used during the flow test. The recordings were replayed using a media player (Avidemux) to determine the position of the center of the fluid front line with respect to time.

Flow results show that strips with higher zinc concentrations had slower flows, Figure A1-12. This result is expected since zinc oxide, present on the surface of the zinc microparticles, is hydrophobic [38,39] and strips with higher zinc concentrations meant that the fluid front had a higher probability of arriving at a location on the fiber occupied by a zinc microparticle. Also, strips with a higher porosity value had a faster flow rate than Zinculose strips with a lower porosity value, Figure A1-13. Higher porosity values can be attributed to either the presence of more pores in the material or to larger pore
radii. These results are in agreement with those obtained by Bohm et. al showing faster flow rates for paper with higher porosity [40]. These flow tests obey the L-W model when considering the effect of contact angle and pore radius on the flow rate. They also exhibit a linear relationship between distance and the square root of time with a coefficient of determination $R^2$ larger than 0.99. This indicates a strictly capillary driven viscous flow which is an important property for microfluidic paper [40].

**Application: Improving Nitrate Conversion Efficiency**

Spectrophotometric techniques based on the Griess reaction are the most commonly used methods for the detection of nitrate and nitrite in biological, environmental and food samples [41]. The Griess reaction was first described in 1864 and demonstrated suitable for the detection of nitrite in 1879 [42]. Griess reagents were successfully implemented in paper-based microfluidic devices for the detection of nitrite in biological [43], environmental [44] and food samples [45]. The Griess reaction is specific for nitrite only and so, nitrate has to be reduced to nitrite first before being detected [46]. While cadmium is the most commonly used agent to reduce nitrate to nitrite [46], zinc microparticles gave similar reducing results and were successfully used in a paper-based microfluidic device for the detection of nitrate [44]. The reported device utilized a reduction chamber which contained zinc microparticles deposited from a water suspension. Three problems may arise when using this deposition technique. The first is the repeatability of depositing the same amount of zinc microparticles in the reduction chamber. Zinc microparticles rapidly precipitate from suspension in water, so pipetting the same amount of zinc becomes a challenge. The second is that zinc particles aren’t spread throughout the entire volume of the reduction chamber; they are simply
lying on the surface. Even their distribution on the surface may not be uniform due to the coffee ring effect [47]. This results in a non-ideal mixing with the sample which leads to a 20% reduction efficiency of nitrate to nitrite. The third issue is that these zinc microparticles are not held in place and are swept into the detection zone by the flowing sample in any 2D device configuration.

To overcome these drawbacks, we used Zinculose as the reduction chamber in a paper-based microfluidic device for the detection of nitrate shown in Figure 3-6 below.

**Figure 3-6 Detection zone is folded over the Zinculose strip for 1 second after a certain reduction time**

Operation of this device is very simple. A water sample is first pipetted onto the sample pad and then the detection zone is folded over the Zinculose strip for 1 second after a certain reduction time. The device is then scanned using a desktop scanner (Canon TS6020) at a resolution of 600 DPI. Table 3-1 shows the range and optimum values of the parameters used in the proposed paper-based microfluidic device. The color formed in the detection zone is quantified by its red, green and blue intensity values measured using ImageJ software, Figure A1-14. The green intensity value is then divided by the sum of the corresponding red and blue intensity values for each
measurement in order to capture the entire color information. Green intensity was chosen since it shows the highest difference in value with respect to change in nitrite concentration. This can be attributed to the fact that the highest absorbance of light in the Griess reaction occurs at a wavelength that corresponds to that of the visible green light [48,49].

**Table 3-1 Range of parameters tested for the proposed paper-based microfluidic device**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Range Tested</th>
<th>Optimum Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Volume (μL)</td>
<td>75 - 115</td>
<td>95</td>
</tr>
<tr>
<td>Time before folding (min)</td>
<td>1 - 13</td>
<td>11</td>
</tr>
<tr>
<td>Time before scanning (min)</td>
<td>1 - 7</td>
<td>4</td>
</tr>
</tbody>
</table>

An exponential decay calibration curve was first established for nitrite over the concentration range 0, 2.5, 5, 7.5, 10, 15, 20, 25, 30, 40, 60, 80 and 100 ppm, Figure A1-15. A 100 ppm nitrate solution was then used in devices with Zincculose strips of different zinc concentrations. The resulting red, green and blue intensity values were measured using ImageJ and used to calculate the conversion efficiency from the calibration curve. Table 3-2 shows the conversion efficiency results of nitrate to nitrite for different zinc concentrations. Zincculose with a zinc concentration of 16 mg/cm² gave the best conversion efficiency of 27%, which is 36% higher than the value reported by
Jayawardane et al. [44]. Figure 3-7 shows a sample of the results obtained to measure the conversion efficiency of nitrate to nitrite using the paper-based microfluidic device.

**Table 3-2 Conversion Efficiency of Zinculose (n=3, standard deviation is after the plus-minus sign)**

<table>
<thead>
<tr>
<th>Zinc Content (mg/cm²)</th>
<th>Conversion Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-0.12 ± 0.15</td>
</tr>
<tr>
<td>2</td>
<td>7.58 ± 0.31</td>
</tr>
<tr>
<td>4</td>
<td>14.45 ± 3.79</td>
</tr>
<tr>
<td>6</td>
<td>16.41 ± 2.36</td>
</tr>
<tr>
<td>8</td>
<td>15.10 ± 1.43</td>
</tr>
<tr>
<td>10</td>
<td>16.42 ± 1.32</td>
</tr>
<tr>
<td>12</td>
<td>22.44 ± 1.03</td>
</tr>
<tr>
<td>14</td>
<td>22.44 ± 2.32</td>
</tr>
<tr>
<td>16</td>
<td>27.25 ± 1.77</td>
</tr>
<tr>
<td>18</td>
<td>24.50 ± 5.02</td>
</tr>
</tbody>
</table>
Table 1: Conversion efficiency of nitrate to nitrite with different zinc concentrations

<table>
<thead>
<tr>
<th>Concentration (mg/cm²)</th>
<th>Conversion Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>24.98 ± 1.86</td>
</tr>
<tr>
<td>16</td>
<td>23.33 ± 1.62</td>
</tr>
</tbody>
</table>

Figure 3-7 Paper-based microfluidic devices used to measure conversion efficiency of nitrate to nitrite. The 3 devices on the left have Zincholose with zinc concentration of 0 mg/cm² while the ones on the right have a zinc concentration of 16 mg/cm².

Visual inspection of all detection zones after folding unto Zincholose strips didn’t reveal any signs of zinc particles. Seven paper-based microfluidic devices were constructed with strips of Zincholose of concentration 16 mg/cm² and operated as mentioned earlier. These detection zones were then allowed to air dry for few hours before being inspected using an SEM with an EDS detector. Multiple areas on the detection zones were analyzed and a very few number of particles were observed, Figure A1-16. Analysis of these particles using EDS revealed that they were zinc particles, Figure A1-17. Since the number of these particles were very few along the entire detection zone and since they didn’t affect conversion results as can be seen by the
relatively small standard deviation, we can consider that zinc particles are immobilized by the fibrils in Zinculose for all practical purposes.

**Conclusions**

A new composite material of cellulose fibers with embedded zinc microparticles was developed. The effect of different parameters during the manufacturing process was investigated and a procedure to produce the material in a consistent and reproducible manner is outlined in this paper. Thickness of produced Zinculose exhibits a dependence on the initial fiber and zinc content. A simple method was utilized to measure porosity with errors that are comparable to those given by the more sophisticated mercury porisometry method. Flow characteristics reveal a linear relationship between distance and the square root of time with zinc content as a parameter which is indicative of a strictly capillary driven viscous flow. Using Zinculose in a paper-based microfluidic device resulted in a 36% increase in the conversion efficiency of nitrate to nitrite compared to published results in which zinc was not embedded within the fibers. While we present an application for Zinculose in a paper-based microfluidic device for the detection of nitrate, this material can also be used in other paper-based microfluidic devices that require a fluidic sample to flow and interact with zinc microparticles along the path of flow.

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Supplementary Material

A supplementary material document accompanies this paper found in Appendix I.

References


CHAPTER 4

A New Paper-Based Microfluidic Device for Improved Detection of Nitrate in Water

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Abstract

In this paper, we report a simple and inexpensive paper-based microfluidic device for detecting nitrate in water. This device incorporates two recent developments in paper-based technology suitable for nitrate detection and has an optimized microfluidic design. The first technical advancement employed is an innovative fibrous composite material made up of cotton fibers and zinc microparticles that can be incorporated in paper-based devices and results in better nitrate reduction. The second is a detection zone with an immobilized reagent that allows the passage of a larger sample volume. Different acids were tested—citric and phosphoric acids gave better results than hydrochloric acid since this acid evaporates completely without leaving any residue behind on paper. Different microfluidic designs that utilize various fluid control technologies were investigated and a design with a folding detection zone was chosen and optimized to improve the uniformity of the signal produced. The optimized design allowed the device to achieve a limit of detection and quantification of 0.53 ppm and 1.8 ppm, respectively, for nitrate in water. This accounted for more than a 40% improvement on what has been previously realized for the detection of nitrate in water using paper-based technology.

Introduction

Paper-based microfluidic technology has seen a great deal of advancements over the past several years due to the growing interest in the many advantages they provide, most notably, their low cost, portability, deployability, ease of use, and disposability. Most recent advancements in paper-based technology were for water analysis [1–3], biomedical applications [4–6], food safety analysis [7,8], soil analysis [9], and in many
other applications [10]. Although paper has been used for biological and chemical applications for over a century with the simple use of litmus paper, paper chromatography, and dried blood cells [11,12], only recently have more complex systems (lab-on-paper) been developed and achieved on paper-based devices. The first simple paper-based microfluidic device may be attributed to that mentioned by Muller et al. in 1949 [13–15], but it was the Whitesides group [16] who showed the promise and endless possibilities of this technology. Paper-based devices make use of capillary action to flow fluids through paper without the use of a pump. This removes the need for an external power source to drive the fluid and results in product miniaturization and major cost savings.

Paper-based microfluidic devices are made up of multiple sections that serve different purposes. The simpler devices generally have a sample port, onto which the sample fluid is loaded; transport channels, which connect the different sections of the device; re-action zones, at which the sample fluid reacts or mixes with dry or wet reagents; and a detection zone, at which a signal is formed that can be either qualitative in nature or can be measured quantitatively. The majority of paper-based devices utilize colorimetric reactions to produce a quantifiable signal [17]. Plenty of papers have already been published on these devices, including various fabrication methods and sensing techniques [18–20].

While a large number of applications have been described and implemented using paper-based microfluidic devices, opportunities for improving their performance still exist due to ongoing advancements in the field of paper-based technology. An example is the performance of a paper-based microfluidic device for the detection of nitrate.
Nitrate is a naturally occurring ion that is part of the nitrogen cycle [21]. It is also an essential nutrient needed for plant growth but plays a significant role in water nutrient pollution when present at elevated concentrations [22]. Nitrate in water emanates from several different sources with large quantities coming from fertilizer or manure runoff, atmospheric deposition, agricultural sources, septic tanks, and wastewater treatment plants. Since nitrate is the most stable form of nitrogen in oxygenated environments, all other forms of nitro-gen-containing compounds in water can also become sources for dissolved nitrate [23,24]. Drinking water with high levels of nitrate increases the risk of developing colorectal cancer, thyroid disease, and central nervous system birth defects [25]. The United States Environmental Protection Agency (U.S. EPA) has set the maximum contaminant level of nitrate in drinking water as 10 ppm; however, concentrations greater than 3 ppm indicate contamination of the groundwater and those greater than 1 ppm indicate human activity [26]. As monitoring the quality of surface, ground, and drinking water has become a major concern in present times [27], measuring nitrate levels in water for environmental protection purposes and to ensure its safety and suitability for consumption has become even more pressing. There are several detection techniques currently in use to measure nitrate levels in water [28]; however, these conventional methods require costly instruments and time consuming analysis [29]. They also require special sample handling and preparation, which requires trained personnel [30]. Therefore, microfluidic technology for water quality analysis, which includes paper-based technology, has been growing as it provides several advantages including rapid and economical detection techniques [31,32]. Thus far, only five paper-based microfluidic devices have been developed for the detection of nitrate.
These devices use the Griess assay for the colorimetric detection of nitrate in water [33], food samples [34–36], and human saliva [37]. Since the Griess assay is specific to nitrite, nitrate molecules have to be first reduced to nitrite before undergoing the reaction. The majority of these devices used zinc microparticles to achieve this reduction. However, there have been two recent developments in paper-based microfluidic technology. The first is a new composite material, which we developed in [38], which increases the reduction efficiency of nitrate to nitrite. The second is a functionalized paper, one that has an immobilized Griess reagent [39], which allows the passage of a larger volume of sample over the detection zone. Moreover, device architecture plays a major role in paper-based microfluidics. Primarily, proper control of fluid flow through the different sections of the device is of great importance to achieve the required reduction or reaction times. The design chosen for the device impacts the quality and uniformity of the signal developed in the detection zone. This largely affects the performance of the device and the limits of detection attained.

In this work, we follow an engineering approach by incorporating these two latest innovations in a new paper-based microfluidic device to improve nitrate detection in water samples. The final optimized architecture employs a folding design that allows for a more uniform color to develop in the detection zone. Results show an enhancement of over 40% in the detection and quantification limits of nitrate in water compared to what has been previously achieved using paper-based technology. It is worth mentioning that different paper-based microfluidic designs were initially developed and tested before and after employing the two innovations. These designs are briefly discussed in the Supplementary Information document, which gives some of the
advantages and disadvantages observed for each design. This should aid other researchers when developing paper-based devices for any application of interest.

Materials and Methods

The Griess Assay

The Griess reaction was first described in 1864 and demonstrated suitable for the detection of nitrite in 1879 [40]. It is the most commonly used spectrophotometric method for quantifying concentrations of nitrate and nitrite [41,42]. The Griess assay involves 2 reaction steps that take place under acidic conditions. In this reaction, nitrite molecules have to first react with sulfanilamide to form diazonium ions (Equation (1) below). These ions then react with naphthyl ethylene diamine (NED) molecules to produce a visible azo dye, which is pinkish red in color (Equation (2)). Therefore, nitrate has to be reduced to nitrite first before being detected by the Griess assay. There are different reducing agents that can be used to reduce nitrate to nitrite. The most commonly used reducing agents are cadmium, copperized cadmium, zinc, nitrate reductase, hydrazine sulfate, titanium (III) chloride, or vanadium (III) [28,31]. Cadmium reduction is the leading method used for nitrate detection; however, more researchers are using zinc since it is a less toxic reductant and not as harmful to humans or the environment as cadmium [43,44]. Moreover, it is worth mentioning that Jayawardane et al. [33] obtained similar results for nitrate detection when using cadmium or zinc in a paper-based microfluidic device. Therefore, we used zinc to reduce nitrate to nitrite (Equation (3)).
Sulfanilamide + NO₂⁻ → \textit{acid} \text{ Diazonium salt} \hspace{1cm} (1)

Diazonium salt + N − (1naphthyl)ethylenediamine \textit{acid} → Pinkish − red azo dye \hspace{1cm} (2)

\text{NO}_3^- + 2\text{H}^+ + 2\text{e}^- \xrightarrow{\text{zinc}} \text{NO}_2^- + \text{H}_2\text{O} \hspace{1cm} (3)

**A New Composite Material That Improves Nitrate Conversion Efficiency**

Zinculose, a recently developed composite material, is made up of cotton fibers with zinc microparticles embedded within the matrix of the material. It allows for a greater contact area between nitrate molecules and the zinc microparticles since these particles are directly present along the flow path of the sample and not simply sitting on the surface of the paper, Figure 4-1. Zinculose can be produced by a simple and inexpensive procedure. Different parameters that go into the production of Zinculose were discussed in detail in a previous article [38]. Previously, researchers used pipetting [33–35] or placing paper in a suspension [37] to deposit zinc microparticles on paper. The main drawback of these methods is the lack of reproducibly depositing the same amount of zinc microparticles in the device as these particles rapidly settle down in any water suspension. Ferreira et al. [37] tried to overcome this drawback by weighing the disks being loaded with zinc before and after suspension; however, this is a very labor-intensive and time-consuming process as drying the disks takes 30 min. Another drawback is the availability of zinc microparticles to interact with the sample. The deposited zinc microparticles are left sitting on the surface of the material, which results in a non-ideal mixing with the sample that is flowing within the paper matrix. A third drawback is that these zinc microparticles are not bound to the paper and are free to flow into the detection zone, which can be detrimental to the performance of any 2D
paper-based device. Use of Zinculose in paper-based devices overcomes these drawbacks.

![Figure 4-1 A scanning electron microscope image of a Zinculose strip at 500×](image)

**Immobile Griess Reagent**

Through a collaborative research effort between a chemistry and a mechanical engineering team funded by the National Science Foundation (NSF) to develop new paper-based devices for improving the detection of nutrients in water [45], the researchers developed multiple paper-based devices and tested them for the detection of nitrate using the Griess assay. However, the signal formed in the detection zone was non-uniform and showed a color gradient as the color was free to move with the flow of sample. Therefore, we decided that immobilizing the detection reagent would capture the color formed and would allow the flow of more sample over the detection zone, which should improve the performance of the device. This was recently successfully achieved by functionalizing one of the two reagents used in the Griess assay on paper, which resulted in an improvement in the detection limit of nitrite [39]. Functionalizing
the detection zone is an equipment free technique to concentrate the analyte of interest in a certain region by flowing a volume of sample that exceeds what is necessary to satisfy the hydrophilic zone [46]. This results in a very drastic improvement in the sensitivity of the device by improving the limit of detection and limit of quantification [47]. Incorporating a functionalized detection zone with an immobilized reagent to concentrate the analyte improved the detection limit of the device with the folding architecture by allowing the flow of a larger sample (Figure A2-35).

**Device Architecture**

Several architectures were employed in the development of this device. Specifically, various fluidic valve strategies were initially explored in an attempt to control nitrate reduction time and improve its efficiency. Each of the different designs tested had its own set of advantages and disadvantages, which are mentioned in the Supplementary Information. Most of these architectures were used before the implementation of Zinculose and the functionalized detection zone. A lateral flow strip incorporating these two advancements was also developed (Figure A2-26); however, signal quality was an issue as there were streaking lines of color and a very distinctive color gradient over the detection zone. This was the case because of the color dispersion associated with horizontal flow [37]. Therefore, a folding device architecture was adopted, Figure 4-2. This allowed for a precise control mechanism for nitrate reduction and offered a very uniform color formation in the detection zone. This architecture was further optimized to improve the limits of detection and quantification of nitrate.
Figure 4-2 Device uses a simple mechanical folding architecture where the detection zone folds over the G1 pad. This provides the required delay step for nitrate to reduce to nitrite and allows for a uniform color formation in the detection zone. The G1 pad was either a strip of Zinculose for nitrate detection or chromatography paper (CHR1) for nitrite detection.

Device Preparation, Operation, and Analysis Procedure

The microfluidic device was designed using a vector graphics software (CorelDraw X6) and printed on chromatography paper (CHR1-GE Healthcare Whatman 1-3001878) using a solid ink wax printer (Xerox ColorQube 8570). This paper grade was used since it is made up of pure cotton cellulose fibers without any additives and is suitable for chemical applications. The device was then cut out using a laser engraver (Epilog mini 40W) and placed in an oven at 120 °C for 3 min to melt the printed wax and form hydrophobic surfaces. A guillotine cutter was used to cut sample pads from cellulose strips (Millipore CFSP203000). The waste pad was made up of 3 layers of chromatography paper, 10 × 20 mm in size. The different components of the microfluidic device were joined using a double-sided tape (0.0127 mm in thickness—
FLEXmount 0.5 mil SELECT DF071736). A 5 × 7 mm double-sided tape was used in between the detection zone (8 × 10 mm) and the waste pad and also in-between the 3 layers of the waste pad to ensure continuity of fluid transfer. The components of the device and their dimensions are given in Figure 4-3.

![Image of the device and its components](image1)

(a) Dimensions of the device and its components; (b) components of the paper-based microfluidic device

Ninety-five microliter of sample is pipetted into the sample pad. The sample then flows to the G1 pad and nitrate molecules interact with the zinc microparticles and reduce to nitrite molecules. These nitrite molecules then react with the sulfanilamide to form diazonium ions. After an 11 min reduction time, the detection zone is folded over the G1 pad so that the diazonium ions can flow and react with the immobilized NED to form the colored azo dye, Figure 4-4a. A 1.25-inch paper binder clip is used to keep the device folded for 10 min to increase color formation and intensity in the detection zone. The binder clip is then removed, and the device is scanned using a desktop scanner (Canon TS6020) at a resolution of 600 DPI. Figure 4-4b shows the color analysis zone used in ImageJ, version 1.52a.
Figure 4-4 (a) The detection zone is folded over the G1 pad for 10 min. (b) Color analysis zone used in ImageJ to quantify the color intensity; this area is 105 by 175 pixels, which is approximately 4.5 by 7.5 mm

Reagents

The following reagents were used to collect the data for the results in this paper. Sulfanilamide (98%, Alfa Aesar-A1300136), citric acid (≥99%, Alfa Aesar-A103950B), sodium nitrate (≥99.5%, Honeywell Fluka-31440), sodium nitrite (≥99%, Honeywell Fluka-31443), and ASTM Type 1 deionized water (resistivity > 18 MΩ/cm, LabChem-LC267405). A real seawater sample from the Sargasso Sea region known for its low nutrient content [2,39,48] was used to see if the ions usually found in seawater have any effect on the performance of the device. This seawater sample was filtered through a 0.2 µm filter to remove any organic matter prior to its use. Zinculose strips were prepared using the procedure outlined in [38]. In short, cotton fibers from
chromatography paper (CHR1-GE Healthcare Whatman 1-3001878) and zinc powder (99.3%, Fisher Chemical Zinc Certified Powder Z5-500) are mixed together in defined quantities and under controlled conditions to form a slurry that is then precipitated to produce a composite sheet. This sheet is then allowed to dry under room conditions and then cut into strips of required dimensions. The detection zones were cut from obtained cellulose strips with immobilized N-(1-naphthyl)ethylenediamine (NED) [39]. All aqueous solutions were prepared using deionized water on the day of testing. The reagent solution used in the G1 pad was prepared with 50 mM sulfanilamide and 330 mM citric acid; these concentrations were successfully used in [33, 49–52]. The device fabricated for detecting nitrate used a strip of Zinculose in the G1 pad whereas the device meant for detecting nitrite used a strip of chromatography paper instead. Chromatography paper or Zinculose strips were immersed in the reagent solution for 2 min to fully saturate before being allowed to air dry in room conditions for 2 h. The detection zone strips were immersed in a solution of 330 mM citric acid for 2 min before being allowed to air dry in room conditions for 2 h since this showed improved results (Figure A2-33). This result is in line with the requirements for the 2 reactions of the Griess assay, i.e., nitrite reacting with sulfanilamide and diazonium ions reacting with NED, to take place under acidic conditions [49]. Use of other acids such as hydrochloric, sulfuric, and phosphoric acids in the paper-based device was also investigated. However, hydrochloric acid completely evaporates without leaving any residue behind on paper so as to reproduce the required acidic conditions when the paper is rewet. This is in line with the results obtained by Cardoso et al. [53], who achieved a better detection result when the hydrochloric acid was added to the paper-based device after the addition
of the sample. Sulfuric acid with the 0.5, 1, 2.2, 4.5, 6.6, and 8.8 M concentrations were
tested. The 0.5 and 1 M concentrations made the chromatography paper very brittle and
difficult to cut into size and fit into the paper-based device, whereas the higher
concentrations of sulfuric acid tested did not dry on paper and so were impractical for
use in the paper-based device. Citric acid gave better results than phosphoric acid and
was therefore used.

**Testing Range and Limits of Detection and Quantification**

The concentrations tested were obtained by diluting a 1000 ppm solution freshly
prepared on the day of testing by dissolving the required amount of nitrate or nitrite salt
in deionized water. The following concentrations of 0, 0.01, 0.025, 0.05, 0.075, 0.1,
0.25, 0.5, 0.75, 1, 2.5, 5, 7.5, 10, 15, 25, and 50 ppm nitrate or nitrite were tested in
deionized and seawater. Three samples per each concentration were tested in a
completely randomized testing order. A MATLAB code was developed to fit the data
to an exponential decay function of the form $y = a \times \exp (-x/b) + c$ similar to the function
used in [39] since we are using the detection zones they provided. The symbolic toolbox
in MATLAB was used to calculate the limit of detection (LOD) and limit of
quantification (LOQ) by finding the analyte concentration corresponding to the intensity
values $y_{LOD}$ and $y_{LOQ}$ on the calibration curve using the following equations [54]:

For LOD: $y_{LOD} = \bar{y}_B - 3 \sigma_B$  \hspace{1cm} (4)

For LOQ: $y_{LOQ} = \bar{y}_B - 10 \sigma_B$  \hspace{1cm} (5)

where $\bar{y}_B$ is the mean color response of the blank and $\sigma_B$ is its respective standard
deviation.
Results and Discussion

Signal Uniformity

The lateral flow strip architecture, Figure 4-5, that was previously developed in this work revealed that color first starts forming at random points of contact on the overlap between the G1 pad and the detection zone with the immobilized NED. We termed these sites as “seeding points” since color would preferentially continue to develop and become darker at these locations as more sample flowed through, with this being why color streaking and non-uniformity were observed in the detection zone (Figure A2-29). To overcome this drawback, we moved to a folding architecture design that allowed the detection zone to fold over the G1 pad, thus allowing the seeding points to be uniformly spread across a larger area on the detection zone, which resulted in a substantial enhancement in the uniformity of the signal obtained (Figure A2-30). Therefore, the area of the detection zone that directly overlaps the G1 pad was analyzed using ImageJ to quantify the color formed. Flood coating the detection zone with citric acid and allowing it to air dry improved the performance of the device (Figure A2-33). This is in agreement with the requirement of having the two reactions of the Griess assay take place under acidic conditions [49]. Moreover, as mentioned in Section 2.3, flowing more sample over the detection zone is an equipment-free method to enhance results. Therefore, we added a waste pad underneath the detection zone to permit the flow of more sample over it. This allowed concentrating the analyte in the detection zone and resulted in an improvement in the limit of detection and quantification and the production of reproducible results (relative standard deviation = 5.2%, n = 8).
Optimization of Device Parameters

To keep the number of experiments to run reasonable and manageable, we optimized the current platform using the traditional one-factor-at-a-time approach. First, the sample volume was optimized. Afterwards, the reduction time required to provide the darkest signal was then selected. Different zinc content in Zinculose were tested, and finally, the color development time to provide the darkest signal was chosen. Table 4-1 shows the testing range and optimum values of the different parameters investigated for the proposed paper-based device. The optimum value was selected as the testing condition that gave the lowest intensity value (darkest color formed) (Figures A2-36 to 39). ImageJ assigns a value of 255 to the absolute white while it assigns a value of 0 to the absolute black. All other colors can be reproduced by a combination of the red, green, and blue components. The darker the color, the lower these values are. Oppositely, the lighter the color, the higher the values of the red, green, and blue components are. That is why the value of the sensor response (color intensity) decreases with increasing the concentration of analyte since the color becomes darker. The green component of the color shows the largest range of value with respect to change in the nitrate or nitrite concentration because the color formed is pinkish red in color. This means that the color mostly absorbed is the green component [37]. The red and blue
components also show a difference in value, but the range of their change is not as large as that of the green. Previous researchers have also chosen the green intensity for their analysis of nitrate or nitrite [33,37–39]. Therefore, we also utilized green in our analysis. However, we normalized this green value by the summation of the red and blue components of the signal so as to capture the entire information of the color produced in the detection zone.

**Table 4-1 Parameters investigated for the proposed paper-based device and their optimum values**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Range Tested</th>
<th>Optimum Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample volume (µL)</td>
<td>80–100</td>
<td>95</td>
</tr>
<tr>
<td>Reduction time (minutes)</td>
<td>10–15</td>
<td>11</td>
</tr>
<tr>
<td>Zinc content (mg/cm²)</td>
<td>0–30</td>
<td>20</td>
</tr>
<tr>
<td>Color development time (minutes)</td>
<td>1–15</td>
<td>10</td>
</tr>
</tbody>
</table>

**Testing in Deionized Water**

Figure 4-6 shows the calibration curves developed for the detection of nitrate and nitrite in deionized water using the device. The limit of detection and quantification for nitrate are 0.533 ppm and 1.765 ppm, respectively, whereas the limit of detection and quantification for nitrite are 0.018 ppm and 0.061 ppm, respectively. A color chart showing the evolution of color formation as a function of concentration is provided for semi-quantitative analysis, Figure 4-7. There was a slight background signal in the
nitrate detection for the 0 ppm condition. This was not observed in the detection zone of the nitrite device, even though they use the same immobilized reagents. Although the zinc microparticle assay used in making Zinculose is of high purity (99.3%), there is also 0.001% “nitrogen compounds” in this zinc assay. Since nitrate is the most stable form of nitrogen in oxygenated environments [23,24], it is possible that this slight background signal comes from the nitrogen compounds accompanying the zinc microparticles. However, this signal was found to be very faint and did not significantly affect the results.
Figure 4-6 (a) An exponential decay calibration curve in the form \( y = a \times \exp(-x/b) + c \), where \( a = 0.2059 \), \( b = 7.794 \), and \( c = 0.3047 \) was established for nitrite in deionized water. (b) An exponential decay calibration curve in the form \( y = a \times \exp(-x/b) + c \), where \( a = 0.1299 \), \( b = 18.65 \), and \( c = 0.3638 \) was established for nitrate in deionized water. The error bars represent the standard deviation.
Figure 4-7 A color chart showing the evolution of color formation with increase in concentration

Testing in Sargasso Seawater

Figure 4-8 shows the calibration curves developed for the detection of nitrate and nitrite in Sargasso seawater using the device. The limit of detection and quantification for nitrate are 1.951 ppm and 5.135 ppm, respectively, whereas the limit of detection and quantification for nitrite are 0.025 ppm and 0.310 ppm, respectively.
Figure 4-8 (a) An exponential decay calibration curve in the form \( y = a \times \exp\left(-\frac{x}{b}\right) + c \), where \( a = 0.1164 \), \( b = 18.85 \), and \( c = 0.3819 \) was established for nitrite in
Sargasso seawater. (b) An exponential decay calibration curve in the form \( y = a \times \exp\left(-\frac{x}{b}\right) + c \), where \( a = 0.1164 \), \( b = 18.85 \), and \( c = 0.3819 \) was established for nitrate in Sargasso seawater. The error bars represent the standard deviation.

**Comparison of Results**

Table 4-2 provides a comparison between the testing conditions and results achieved using the paper-based device developed in this work and those attained with paper-based devices fabricated previously. The limits of detection and quantification achieved were 55% and 41%, respectively, better than what has been previously achieved for the detection of nitrate in water using a paper-based device. This improvement can be attributed to the architecture utilized in addition to incorporating two new innovative materials in this device. The performance of the device developed in this work is also better than the other paper-based devices designed for the detection of nitrate in all other media except the ones designed by Ratnarathorn et al. [35] and Thongkam et al. [36]. Moreover, the results may be considered more accurate as the testing range encompasses the calculated limits of detection and quantification [39]. It is worth mentioning that while the limit of detection of the device developed in this work is higher than that achieved by the devices created in [35,36]—the range of application of the device developed in this work is larger, 0.01 to 50 ppm vs. 0.4 to 20 ppm and 0.5 to 40 ppm, respectively. Since the device developed in this study uses the same chemistry (zinc and Griess assay) as the devices previously developed for the detection of nitrate in food and saliva samples, we believe that this platform will also be applicable for these more complex matrices.
Table 4-2 Comparison of results from this work with previous paper-based devices for detecting nitrate

<table>
<thead>
<tr>
<th>Reference</th>
<th>Nutrient</th>
<th>Media</th>
<th>Sample Volume (µL)</th>
<th>Testing Time (min)</th>
<th>Testing Range (ppm)</th>
<th>LOD (ppm)</th>
<th>LOQ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>This work</td>
<td>Nitrite</td>
<td>Water</td>
<td>95</td>
<td>21</td>
<td>0.01–50</td>
<td>0.018</td>
<td>0.061</td>
</tr>
<tr>
<td></td>
<td>Nitrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.533</td>
<td>1.765</td>
</tr>
<tr>
<td>[33]</td>
<td>Nitrite</td>
<td>Water</td>
<td>20</td>
<td>4.5–8.5</td>
<td></td>
<td>0.46–6.9</td>
<td>0.046</td>
</tr>
<tr>
<td></td>
<td>Nitrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.1–62</td>
<td>1.178</td>
</tr>
<tr>
<td>[34]</td>
<td>Nitrite</td>
<td>Food sample</td>
<td>80</td>
<td>12</td>
<td>2–10</td>
<td>1.2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Nitrate</td>
<td></td>
<td></td>
<td></td>
<td>10–50</td>
<td>3.6</td>
<td>12</td>
</tr>
<tr>
<td>[35]</td>
<td>Nitrite</td>
<td>Food sample</td>
<td>25</td>
<td>10</td>
<td>0.4–20</td>
<td>0.4</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Nitrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.1</td>
<td>1.2</td>
</tr>
<tr>
<td>[36]</td>
<td>Nitrite</td>
<td>Food sample</td>
<td>20</td>
<td>5</td>
<td>0.1–40</td>
<td>0.4</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>Nitrate</td>
<td></td>
<td></td>
<td></td>
<td>0.4–20</td>
<td>0.4</td>
<td>1.4</td>
</tr>
<tr>
<td>[37]</td>
<td>Nitrite</td>
<td>Saliva</td>
<td>15</td>
<td>20–120</td>
<td>0.23–11.5</td>
<td>0.002</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td>Nitrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>12.4–74.4</td>
<td>4.96</td>
</tr>
</tbody>
</table>
Device Portability, Longevity, and Commercialization

The developed paper-based device is very portable since it is only few centimeters in size. It is also very user-friendly and easy to use. The device can be easily incorporated into a portable imaging platform that allows the user to easily and reproducibly fold the device, Figure 4-9, and then analyze results in the field similar to what has been developed in [55–57]. These platforms are suitable for use in the field as they do not require any external power supply and they directly interface with a smartphone to provide instantaneous quantitative results. Although this study used a paper binder clip to keep the device folded for 10 min, the data show that there was no statistically significant difference between the results obtained by the binder clips and those obtained by the 3D holder (two-sample t-test at the 95% confidence level; \( p = 0.4 \) and \( DF = 6 \)).

Figure 4-9 A 3D-printed holder that allows for a reproducible folding of the paper-based device. This holder can be integrated into a portable imaging platform that can be used in the field.
Device longevity depends on the stability of the different components making up the device. The zinc microparticles in Zinculose maintain their crystalline structure for over 6 months [38]. The detection zones with immobilized NED that have been flood coated with citric acid and stored in a desiccant box at room temperature, away from light and with a relative humidity less than 30% for 1 month, gave almost identical results to detection zones that have been freshly flood coated with citric acid on the day of testing (two-sample t-test at the 95% confidence level; p = 0.987 and DF = 4). Sulfanilamide oxidizes and changes color in a matter of days. This color degradation of sulfanilamide can be slowed down and the shelf life of the device improved by storing in nitrogen or under vacuum, away from light and under cold temperatures below 4 °C [33,35,37,39,58]. However, a chemistry approach to prohibit the oxidation and degradation of sulfanilamide needs to be further examined to aid the above engineering approaches. This would be similar to the case of commercial dip strips that utilize the Griess assay and have a shelf life of a couple of years when kept in their box under normal room conditions without necessitating sophisticated storage requirements.

These paper-based devices are relatively small in size and do not consume a large amount of material or use hazardous chemicals, making them environmentally friendly. The cost of each device is in the order of several U.S. cents only.

**Conclusions**

In this study, we followed an engineering approach to develop a highly sensitive paper-based device for the detection of nitrate in water. Several device architectures utilizing different valve alternatives were initially designed and tested. A simple paper-based design with a folding architecture was adopted. Folding the detection zone over
the reagent pad improved the quality and uniformity of the signal developed in the
detection zone as well as the detection limit. The device also incorporated two
advancements in the field of paper-based technology—a new composite material
improved the conversion efficiency of nitrate while the immobilized reagent allowed
for more sample to flow through the detection zone. The limits of detection and
quantification for the proposed nitrate device were 0.53 ppm and 1.8 ppm, respectively,
in water. This represents 55% and 41%, improvement, respectively, than what has been
previously achieved for the detection of nitrate in water using a paper-based device.
Future work will include improving the shelf life of the device by enhancing the stability
of the sulfanilamide by prohibiting its oxidation. Work will also include developing a
suitable lightbox for use in the field. Additionally, analysis of nutrients in food samples
is a very interesting area for further research.

Supplementary Material

A supplementary material document accompanies this paper found in Appendix II.

Author Contributions

Conceptualization, A.C., C.A., and M.F.; methodology, A.C.; software, A.C. and
H.H.-B.; validation, A.C.; formal analysis, A.C. and H.H.-B.; investigation, A.C.;
resources, C.A. and M.F.; data curation, A.C., C.A., and M.F.; writing—original draft
preparation, A.C.; writing—review and editing, A.C., H.H.-B., C.A., and M.F.;
visualization, A.C.; supervision, C.A. and M.F.; project administration, C.A. and M.F.;
funding acquisition, C.A. and M.F. All authors have read and agreed to the published
version of the manuscript.
Funding

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Informed Consent Statement

Not applicable

Data Availability Statement

Data is contained within the article or supplementary material. Additional data not presented in this article is available on request from the corresponding author.

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References


CHAPTER 5

Colorimetric Determination of Nitrate after Reduction to Nitrite in a Paper-Based Dip Strip

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Abstract

Paper-based microfluidic technology is a relatively new field of research that provides low-cost platforms and sensors for point-of-care diagnostics. While the majority of research in this field has been for biomedical applications, more and more paper-based devices and platforms are being designed and developed for environmental applications such as water quality monitoring and assessment. One such application is the detection of nitrate in water samples. Colorimetric detection of nitrate by paper-based devices using the Griess assay requires the reduction of nitrate to nitrite before undergoing the reaction. In this paper, we measured the performance of a paper-based dip strip for detecting nitrate and nitrite by calculating its limit of detection and limit of quantification. We also calculated the reduction efficiency of vanadium (III) chloride in the dip strip for detecting nitrate. Our results show that the reduction time of nitrate via vanadium (III) chloride is much longer than that when using zinc microparticles. Our results also show that the performance of the dip strip using vanadium (III) chloride for nitrate detection is not as good as more intricate paper-based devices that have a separate reaction zone with zinc microparticles. The limits of detection and quantification calculated were 3.352 and 7.437 ppm and the nitrate reduction efficiency varied over the range of nitrate concentrations tested.

Introduction

Paper-based microfluidic technology has been gaining a lot of attention over the past several years for the many advantages it provides. Most importantly, paper-based microfluidic technology allows the development of low cost, portable and easy-to-use devices and sensors that can be easily disposed of. These devices can also provide
qualitative or quantitative results and data at the point of care without the need for specialized equipment or power sources. Several paper-based devices have been developed for various applications such as for water analysis [1–4], biomedical applications [5, 6], food analysis [7–10], soil analysis [11] and many other miscellaneous applications [12–15]. The field of paper-based microfluidics is expected to continue garnering greater attention as more applications are sought after or the performance improved for the ones already developed [16].

Paper-based devices are generally made up of several different sections that serve different purposes. While more complex devices may include valves and actuators to manipulate fluids and perform multistep reactions [17, 18], simpler devices generally include a sample port, transport channels, reactions zones and a detection zone [19]. The majority of paper-based devices use colorimetric detection since it is the simplest technique to produce a quantifiable signal [20, 21]. Properties of the material used in paper-based devices influence assay performance and have a substantial impact on the development of paper-based sensors [22]. Therefore, proper material selection and optimization is critical to enhancing the performance of assays in paper-based devices [23]. This is usually an iterative and an ongoing process to learn and adapt different advancements in the field of paper-based technology to check for the possibility of improving the output and performance of paper-based sensors. An example is the selection of a suitable reducing agent to be used in a paper-based device meant for detecting nitrate in water.

Nitrate is part of the nitrogen cycle [24] and is an essential nutrient needed for plant growth; however, it plays a significant role in water nutrient pollution when
present in excessive amounts [25, 26]. Nitrate is also the most stable form of nitrogen in oxygenated systems and all other forms of nitrogen containing compounds can become a source for it [27, 28]. Ingesting nitrate has been linked to colorectal cancer, thyroid disease, and central nervous system birth defects [29]. Therefore, it is important to measure nitrate levels in water for environmental monitoring purposes and to ensure its safety for consumption. Different techniques are readily available to measure nitrate concentrations in water but are either costly, time consuming or may require trained personnel [30, 31]. Several paper-based sensors have been developed for the rapid and inexpensive detection of nitrate in water, food and human saliva and their limits of detection (LOD) and limits of quantification (LOQ) are given in Table 5-1.

**Table 5-1 Performance of paper-based sensors developed for detecting nitrate in different media**

<table>
<thead>
<tr>
<th>Reference</th>
<th>Media</th>
<th>LOD (ppm)</th>
<th>LOQ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[32]</td>
<td>Water</td>
<td>0.533</td>
<td>1.765</td>
</tr>
<tr>
<td>[33]</td>
<td>Water</td>
<td>1.178</td>
<td>2.976</td>
</tr>
<tr>
<td>[9]</td>
<td>Food Sample</td>
<td>3.6</td>
<td>12</td>
</tr>
<tr>
<td>[34]</td>
<td>Food Sample</td>
<td>0.4</td>
<td>NA</td>
</tr>
<tr>
<td>[35]</td>
<td>Food Sample</td>
<td>0.4</td>
<td>1.4</td>
</tr>
<tr>
<td>[36]</td>
<td>Human Saliva</td>
<td>4.96</td>
<td>16.74</td>
</tr>
</tbody>
</table>

NA, not available.

All of the paper-based devices developed thus far for measuring nitrate levels have used the Griess assay for detection since it is the most commonly used spectrophotometric method for quantifying concentrations of nitrate and nitrite [37, 38]. However, this assay is specific to nitrite molecules and therefore, nitrate molecules have to be reduced to nitrite first before detection. There are several different reducing agents that can reduce nitrate to nitrite such as cadmium, copperized cadmium, zinc, nitrate
Reducase, irradiation by ultraviolet light, hydrazine sulfate, titanium (III) chloride, vanadium (III), hydroxylamine, tin chloride or ascorbic acid [36, 39, 40]. Some of these reducing agents are not suitable for use in paper-based devices while others have been tested and used in this type of sensors.

Nitrate reductase, irradiation by ultraviolet and hydrazine require lengthy reduction times [41] which may not be suitable for paper-based sensors due to concerns of sample evaporation. Titanium (III) chloride is violet in color and absorbs light in the same range as the azo dye product of the Griess assay [41]. Ferreira et al. [36] tested tin chloride, hydroxylamine, ascorbic acid and zinc microparticles. They used zinc microparticles in their paper-based nitrate sensor since the other agents tested did not extensively reduce nitrate to nitrite. Experimental results by Jayawardane et al. [33] showed that cadmium and zinc microparticles produced similar results for nitrate reduction in their paper-based device. They opted for zinc microparticles due to the higher toxicity of cadmium. Thongkam et al. [35] developed a very simple paper-based device for measuring nitrate and nitrite concentrations in food samples and they used vanadium (III) chloride to reduce nitrate before detection.

We had previously developed a sensitive paper-based nitrate sensor by testing different device architectures and optimizing the different components of the device [32]. The final device adopted a folding architecture with part of the detection chemistry immobilized at the detection zone. This improved the quality and uniformity of the signal developed. The device also incorporated a new composite material made-up of zinc microparticles and cellulose fibers to enhance nitrate reduction. A nitrate conversion efficiency of 27% was achieved using this new composite material called...
Zinculose [42]. However, the results obtained by Thongkam et al. [35] for nitrate detection in food samples by using vanadium (III) chloride as a reducing agent are very promising. In this paper, we measure the performance of a dip strip using vanadium (III) chloride for reducing nitrate by calculating its limits of detection and quantifications. We also calculate the nitrate reduction efficiency of vanadium (III) chloride and compare the results to those obtained when using Zinculose.

Methods

Thongkam et al. [35] studied the effect of the different parameters on nitrate detection. They tested different concentrations of sulfanilic acid and N-(1-Naphthyl)ethylenediamine dihydrochloride used in the Griess assay for detection. They also examined the effect of different concentrations of vanadium (III) chloride and reaction times on the intensity of the color produced in the detection zone. In this paper, we use the optimum concentrations they have found when preparing the reagents to be used in our experiments.

Materials

The items below were used in preparing and running the experiments presented in this paper. Whatman grade 1 filter paper (GE Healthcare Whatman 1-1001824), backing cards (DCN Dx MIBA-050), sulfanilamide (98%, Alfa Aesar-A1300136), N-(1-Naphthyl)ethylenediamine dihydrochloride (Alfa Aesar-J6321414), hydrochloric acid (Fisher Chemical-A142-212), sodium nitrate (≥99.5%, Honeywell Fluka-31440), sodium nitrite (≥99%, Honeywell Fluka-31443), and ASTM Type 1 deionized water (resistivity > 18 MΩ/cm, LabChem-LC267405).
Methods

Strips 1 × 8 cm were cut out from a 30 × 8 cm backing card using a guillotine paper cutter. Three circles, 6 mm in diameter each, were punched out using a tissue biopsy from the Whatman filter paper and stuck onto the backing card, Figures 5-1 and A3-1.

![Diagram of dip strip with detection zone](image)

Figure 5-1 (a) Schematic showing the components and dimensions of the dip strip used. (b) The yellow circle shows the color analysis zone used in ImageJ to quantify the color intensity of one of the detection zones; the diameter of the circle is about 125 pixels, which is approximately 5.3 mm.

Nitrate and nitrite solutions at concentrations of 1000 ppm were freshly prepared on the day of testing by dissolving the required amount of nitrate or nitrite salt in deionized water. These solutions were then diluted using deionized water into the following concentrations 0.5, 1, 2.5, 5, 10, 20 and 40 ppm. We followed the procedure outlined by Thongkam et al. [35] in preparing the detection chemistry for nitrate and nitrite. For nitrite detection, the solution was called reagent “A” and consisted of equal parts (1:1 ratio) volume of sulfanilic acid and NED solution. For nitrate detection, the solution used was called reagent “B” and consisted of equal parts (1:1:1 ratio) volume
of the above sulfanilic acid, NED solution and the reducing reagent solution. The sulfanilic acid used in reagents “A” and “B” was prepared by dissolving 0.1 g of sulfanilamide in 100 mL of 2 mol L\(^{-1}\) hydrochloric acid. The NED solution used in reagents “A” and “B” was prepared by dissolving 0.1 g of N-(1-Naphthyl)ethylenediamine dihydrochloride in 100 mL of deionized water. The reducing reagent solution used in reagent “B” was prepared by dissolving 3 g of vanadium (III) chloride in 100 mL of 6 mol.L\(^{-1}\) hydrochloric acid. 2 μL of reagent A or B was pipetted onto each circle and allowed to air-dry for at least 30 min, Figure A3-2. Each dip strip was then submerged into the appropriate nitrate or nitrite solution for 1 s, shaken to remove excess fluid and then scanned using a desktop scanner (Canon TS6020) at a resolution of 600 DPI. The nitrate dip strips were scanned after 10 min and the nitrite dip strips were scanned after 5 min following the optimized scan times previously found by Thongkam et al. [35]. The detection zones were analyzed using ImageJ in RGB mode similar to how they analyzed their results. We have previously shown that the green component of the measured color intensity shows the largest difference in value over the concentration of nitrate or nitrite for paper-based devices using the Griess assay [43]. Therefore, the data for the different color intensities were provided in the supplementary file, Tables A3-1 to 4. A MATLAB code was used to fit the data to an exponential decay function of the form \(y = a \times \exp (-x/b) + c\), and the symbolic toolbox was used to calculate the limits of detection and quantification. The limits of detection and quantification were obtained by finding the analyte concentrations corresponding to \(y_{LOD}\) or \(y_{LOQ}\) on the calibration curves developed. \(y_{LOD}\) or \(y_{LOQ}\) were calculated using the following equations [44]:
\[ y_{LOD} = \bar{y}_B - 3 \sigma_B \]
\[ y_{LOQ} = \bar{y}_B - 10 \sigma_B \]

Where \( \bar{y}_B \) corresponds to the mean color intensity of the blank solution (0 ppm) and \( \sigma_B \) is its respective standard deviation.

**Results and Discussion**

The detection zones of the nitrate dip strips showed little to no color change after 10 min, Figure A3-3, but color started to form after a much longer wait time, so the dip strips were scanned after 1 h as well, Figure A3-4. The following section shows the results obtained for the nitrate and nitrite dip strips.

**Nitrate and Nitrite Analysis**

Figure 5-2 shows the calibration curves developed for the detection of nitrate in deionized water after a reaction time of 10 min and 1 h. The limits of detection and quantification for nitrate after 10 min are 37.03 and 121 ppm, respectively. The limits of detection and quantification for nitrate after 1 h are 3.352 and 7.437 ppm, respectively.

Figure 5-3 shows the calibration curves developed for the detection of nitrite in deionized water after a reaction time of 5 min and 1 h. The limits of detection and quantification for nitrite after 5 min are 0.522 and 0.854 ppm, respectively. The limits of detection and quantification for nitrite after 1 h are 0.889 and 1.823 ppm, respectively.
Figure 5-2 An exponential decay calibration curve in the form $y = a \times \exp\left(-x/b\right) + c$, where $a = 2741$, $b = 41,430$, and $c = -2492$ was established for nitrate after a
reaction time of 10 minutes. (b) An exponential decay calibration curve in the form
\[ y = a \times \exp \left( -\frac{x}{b} \right) + c \]
where \( a = 80,230 \), \( b = 147,700 \), and \( c = -79,980 \) was established for nitrate after a reaction time of 1 hour. The error bars represent the standard deviation.
(b) An exponential decay calibration curve in the form \( y = a \times \exp \left( -\frac{x}{b} \right) + c \), where \( a = 54.94 \), \( b = 14.57 \), and \( c = 197.4 \) was established for nitrite after a reaction time of 5 minutes. (b) An exponential decay calibration curve in the form \( y = a \times \exp \left( -\frac{x}{b} \right) + c \), where \( a = 45.56 \), \( b = 15.65 \), and \( c = 205.2 \) was established for nitrite after a reaction time of 1 hour. The error bars represent the standard deviation.

**Reduction Efficiency**

The reduction efficiency of vanadium (III) chloride was calculated using the data obtained in the above experiments used to calculate the LOD and LOQ for nitrate and nitrite. First the results obtained from the nitrite experiment after 1 h were used to establish the calibration curve using the method outlined in methods section. Then the results obtained from the nitrate experiment after 1 h were used to calculate the intersection of the measured result with the calibration established for nitrite using the
symbolic toolbox. Table 5-2 gives the nitrate conversion efficiency calculated. As can be seen from the table, the conversion efficiency varies between almost 0% to 27%.

**Table 5-2 Calculated nitrate conversion efficiency.**

<table>
<thead>
<tr>
<th>Nitrate Concentration (ppm)</th>
<th>Concentration Calculated (ppm)</th>
<th>Reduction Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>0.098</td>
<td>19.54 ± 0.80</td>
</tr>
<tr>
<td>1</td>
<td>0.006</td>
<td>0.61 ± 0.23</td>
</tr>
<tr>
<td>2.5</td>
<td>0.300</td>
<td>12 ± 0.05</td>
</tr>
<tr>
<td>5</td>
<td>0.524</td>
<td>10.48 ± 0.08</td>
</tr>
<tr>
<td>10</td>
<td>1.086</td>
<td>10.86 ± 0.01</td>
</tr>
<tr>
<td>20</td>
<td>3.296</td>
<td>16.48 ± 0.03</td>
</tr>
<tr>
<td>40</td>
<td>10.896</td>
<td>27.24 ± 0.01</td>
</tr>
</tbody>
</table>

1This concentration is normalized by subtracting the intensity calculated for 0 ppm from all other concentrations.

**Discussion**

The limits of detection and quantification obtained for nitrate and nitrite in our analysis were much higher than those obtained by commercial dip strips using the Griess assay. This can be attributed to one or more of the following reasons: using the RGB mode in data analysis, not depositing enough reagent volume for reaction or using hydrochloric acid since it evaporates completely without producing acidic conditions.
when rewet. The reaction with the Griess assay should take place under acidic conditions [45].

A maximum reduction efficiency of 27% was obtained by vanadium (III) chloride. This is similar to the reduction efficiency obtained by Zincholose (27%). However, this reduction efficiency was only obtained for a high nitrate concentration of 40 ppm while lower concentrations resulted in a much lower reduction efficiency. This raises the question of repeatability and uniformity of vanadium (III) chloride nitrate reduction when used in paper-based devices.

Each of the two reducing agents, zinc microparticles and vanadium (III) chloride, has its own set of advantages and should be used in specific applications with an appropriate device design. Zincholose is a composite material that can be incorporated into any paper-based device. The zinc microparticles in Zincholose are held in place by the matrix, which allows the passage of more sample volume through the material and the reduction of more molecules as they pass through it. This allows for signal amplification as more molecules become available to be captured and detected. However, vanadium (III) chloride is not immobilized and would wash away in any lateral flow paper-based device design. Nitrate reduction using vanadium (III) chloride takes much longer than that by zinc microparticles. That is why commercial dip strips generally use zinc microparticles in the detection zone to reduce nitrate to nitrite before detection, Figure A3-5. Vanadium (III) chloride allows for the development of simple dip strips since the reducing reagent can be mixed with the detection chemistry and easily deposited in the detection zone. However, the limits of detection and
quantification achieved by dip strips utilizing vanadium (III) chloride are not as good as those obtained in more intricate designs using zinc microparticles.

Conclusions

Paper-based microfluidic technology is a relatively new field of research that is gaining a lot of attention and is producing a lot of innovation. In this paper, we measured the performance of a dip strip utilizing vanadium (III) chloride to reduce nitrate before detection. We observed that vanadium (III) chloride has some drawbacks that make it impractical for use in paper-based devices meant for detecting nitrate. These include long reduction times required and low limits of detection and quantification obtained. Therefore, we recommend using zinc microparticles as the reducing agent for nitrate detection in paper-based devices. Future work will include developing a suitable lightbox, similar to [46], that emits green light for measuring nitrate and nitrite concentrations using paper-based devices utilizing the Griess assay in the field.

Supplementary Material

A supplementary material document accompanies this paper found in Appendix III.

Author Contributions

administration, C.A. and M.F.; funding acquisition, C.A. and M.F. All authors have read and agreed to the published version of the manuscript.

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Not applicable

**Informed Consent Statement**

Not applicable

**Data Availability Statement**

Data is contained within the article or supplementary material. Additional data not presented in this article is available on request from the corresponding author.

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References


CHAPTER 6

A Practical System for the Quantitative Determination of Nitrate and Nitrite in the Field

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Abstract

As paper-based devices have been gaining interest over the past several years, so has the color analysis techniques broadly utilized for measuring signal intensity in colorimetric sensors. Therefore, it is critical to properly analyze the colored signal that forms so as to gain as much information as possible and properly characterize the concentration of the analyte. Of particular interest is paper-based devices for measuring the concentrations of nitrate and nitrite using the Griess assay. Most of the paper-based devices utilizing the Griess reaction have used a desktop scanner to capture the signal formed and a computer image processing software to digitize the signal and quantify it. Digitizing the colorimetric signal left room for the operator to choose the color channel to be used in measuring the concentration of the analyte of interest. In this paper, we provide a comparison between the response of the different color channels that can be used with their corresponding limits of detection and quantification for the signal produced on a commercially available dip strip and a paper-based device utilizing the Griess assay for detection. Our analysis shows that the green channel in the red, green and blue color breakdown provides the largest difference in signal over concentration of nitrate or nitrite which makes it easier to differentiate between concentrations and yields the best limits of detection and quantification. Also, drying a solution of sulfanilamide and citric acid on chromatography paper and storing under cold conditions (≤ 4 °C) showed minimal change in color over 100 days compared to storing under room conditions. Lastly, we are implementing green LEDs (λ ≈ 540 nm) on a lightbox that can be remotely controlled via a cellphone for in-the-field analysis of paper-based devices utilizing the Griess assay for detecting nitrate and nitrite.
Introduction

Over the past several years, a lot of research and resources have been dedicated to developing new paper-based devices, or improving their performance, for use in a wide range of applications. Paper-based devices have been used for biomedical applications [1], food safety [2], soil analysis [3], water analysis [4] and in many other miscellaneous applications [5]. The main advantages of these devices is that they are inexpensive, simple, portable and easy to use. However, the most significant advantage is that these devices depend on capillary action to flow the sample across the different sections of the device without a need for a pump, which results in device miniaturization and cost saving. Different sensing techniques have been applied in paper-based devices to measure the resulting signal intensity at the detection zone; however, the majority of these devices use a colorimetric reaction to produce the signal [6]. This colorimetric signal can then be captured and analyzed using an image processing software to quantify it [7]. This raises the question on how to properly analyze the colorimetric signal for a certain reaction since the resulting color signal is digitized into different color channels that need to be further processed by the operator to provide characteristic information such as limits of detection and quantification. An example would be the Griess assay utilized on paper-based devices for detecting nitrate or nitrite.

The Griess assay is the most commonly used spectrophotometric method for measuring the concentrations of nitrate and nitrite [8,9]. It involves a diazotization reaction of nitrite and then a coupling reaction to produce a colored azo dye. Although there are several variations of this assay, the most frequently used assay for quantitative analyses employs sulfanilamide and N-(1-naphthyl)ethylenediamine as reagents [10].
that undergo reactions 1 and 2 described below. The Griess assay has been successfully implemented on paper-based devices for measuring nutrient concentrations in biological, environmental and food samples. While some researchers have recently analyzed the colorimetric response of the Griess assay on their paper-based devices using an in-house developed cellphone application [11], a portable light box [12] or by photographing the detection zone [13], the majority of researchers have used desktop scanners and a computer image processing software to quantify the results. However, the choice for the color channel to be used in the analysis was different amongst these researchers. The color channels used were green or normalized green [14–18], red [19], magenta [20] and average or normalized grayscale [21–26].

\[
\text{Sulfanilamide} + \text{NO}_2^{-} \xrightarrow{\text{acid}} \text{Diazonium salt} \tag{1}
\]
\[
\text{Diazonium salt} + \text{N–(1naphthyl)ethylenediamine} \xrightarrow{\text{acid}} \text{Pinkish–red azo dye} \tag{2}
\]

In this paper, we measure the colorimetric response of a commercial dip strip and a paper-based device that utilize the Griess assay for detection. We then provide the different color intensities quantified using an image processing software and show that the green channel of the red, green and blue color breakdown shows the largest difference in intensity over the range of concentration tested. We also show that drying a solution of sulfanilamide and citric acid on chromatography paper and storing under cold conditions (≤ 4 °C) shows minimal change in color compared to that stored under room conditions. We are also implementing LEDs that emit green light with a wavelength \( \lambda \approx 540 \) nm in a lightbox, similar to the one previously developed at our lab,
which is remotely controlled via cellphone for in-the-field analysis of paper-based devices utilizing the Griess assay for nitrate and nitrite detection.

Materials and Methods

Material and Reagent Preparation

The nitrite solutions were prepared on the day of testing by dissolving a certain amount of sodium nitrite (≥99%, Honeywell Fluka - 31443) in ASTM Type 1 deionized (DI) water (resistivity > 18 MΩ/cm, LabChem - LC267405) to create a 1000 ppm standard solution. This standard was then diluted using the DI water to create 0.01, 0.025, 0.05, 0.075, 0.1, 0.25, 0.5, 0.75, 1, 2.5, 5, 7.5, 10, 15, 25 and 50 ppm nitrite solutions. A commercial dip strip (CTL Scientific Supply Corp Quantofix 91313) was used in this experiment. The folding paper-based device used was made up of chromatography paper (CHR1 - GE Healthcare Whatman 1 - 3001878) that had been wax printed using a solid ink printer (Xerox ColorQube 8570). The paper-based device, the process of preparing and testing it was documented in [15]. The G1 pad was prepared by flood coating a strip of chromatography paper or Zinculose in a solution of 50 mM sulfanilamide (98%, Alfa Aesar - A1300136) and 330 mM citric acid (≥99%, Alfa Aesar - A103950B) for 2 minutes and then allowing it to dry under room conditions for at least 2 hours. The G2 pad was prepared by flood coating a cellulose strip with immobilized N-(1naphthyl)ethylenediamine (NED) [18] with 330 mM citric acid for 2 minutes and then allowing it dry under room conditions for at least 2 hours.

Operation and Analysis Procedure

Three samples were tested for each concentration by dipping one commercial strip in the nitrite solution and holding for one second with the testing order being completely
randomized. Color was allowed to form in the detection zone before it was captured using a desktop scanner (Canon TS6020) at a resolution of 600 DPI. The scanned images were then processed using ImageJ to quantify the color formed. A MATLAB code was used to provide a calibration curve by fitting the data to an exponential decay function of the form $y = a \cdot \exp(-x/b) + c$. The code also utilized the symbolic toolbox to calculate the limits of detection (LOD) and quantification (LOQ). These values are obtained by retrieving the nitrate or nitrite concentrations that correspond to the color intensity value $y_{LOD}$ and $y_{LOQ}$ on the calibration curves using the following equations [27]:

$$y_{LOD} = \bar{y}_B - 3 \sigma_B$$

$$y_{LOQ} = \bar{y}_B - 10 \sigma_B$$

Where $\bar{y}_B$ is the mean color response of the blank solution (0 ppm nitrite) and $\sigma_B$ is its corresponding standard deviation.

**Results and Discussion**

**Signal and Acquisition Time**

Following the manufacturer’s instructions on operation of the commercial strips, one strip was dipped in the sample solution for 1 second, shaken, and then the color was allowed to form. For color capture time, a quick test at two nitrite concentrations, 25 ppm and 50 ppm, was run. The time at which the darkest signal is obtained was chosen, this corresponds to the lowest intensity value on the graph. The image processing software used in the experiments was ImageJ which is available for free download. ImageJ assigns a value that varies between 0 and 255 to three distinct color channels; the red, blue and green channels. All other colors can be obtained by a combination of
these colors with the absolute white color having a value of 255 for each of these 
channels while the absolute black color having a value of 0 for each. Therefore, as the 
color becomes darker, the intensity value becomes lower. ImageJ also provides 
weighted and unweighted grayscale values from these three color channels. The 
unweighted grayscale is the arithmetic mean of the three color channels while the 
weighted grayscale value is computed by using a multiplier of 0.299, 0.587 and 0.114 
to each of the red, green and blue channels, respectively [28].

Figure 6-1 shows the intensity value for the different colors channels provided by 
ImageJ. As can be seen in the figure, the intensity is almost constant in value or becomes 
darker for up to 3 minutes and then the value starts increasing. So the images for the 
commercial dip strips were all captured at 3 minutes.

![Graph showing intensity over time for different color channels](image)
Figure 6-1 Intensity value development for the different color channels provided by ImageJ. The image on the left is for a nitrite concentration of 25 ppm while the image on the right is for a nitrite concentration of 50 ppm. n=3 and the error bars represent the standard deviation.

Color Response

3 samples for each of the concentrations 0, 0.01, 0.025, 0.05, 0.075, 0.1, 0.25, 0.5, 0.75, 1, 2.5, 5, 7.5, 10, 15, 25 and 50 ppm nitrite solutions were tested in a completely randomized order. Figure 6-2 below shows the signal response for each of the color channels in addition to the calibration curve fits.
Figure 6-2 Color response for the different color channels as a function of nitrite concentration i.e. (a) unweighted, (b) weighted, (c) red, (d) green (e) and blue. n=3 and the error bars represent the standard deviation.

As can be seen from Figure 6-2 and Table 6-1, the green color channel shows the largest difference in value over the entire concentration of nitrite being tested and gives the best coefficient of determination, LOD and LOQ values. This may have to do with the fact that the color being formed is pinkish-red in color which means that the component of color mostly being absorbed is the green one [15,16]. Also, the peak absorbance value for the Griess assay has been reported to be 540 nm [8] which corresponds to that of green visible light. Since the green color component gave the highest difference in value over the range of nitrite tested, it will be used for the analysis. Normalizing the green component with the remaining red, blue or both color components is an approach that can be used for data transformations [29].
Table 6-1 The coefficient of determination ($R^2$) for the different calibration curves formed and the limits of detection and quantification for the different color channels

<table>
<thead>
<tr>
<th>Color Channel</th>
<th>$R^2$</th>
<th>LOD (ppm)</th>
<th>LOQ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unweighted</td>
<td>0.9765</td>
<td>3.431</td>
<td>21.34</td>
</tr>
<tr>
<td>Weighted</td>
<td>0.9854</td>
<td>2.436</td>
<td>12.9</td>
</tr>
<tr>
<td>Red</td>
<td>0.849</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Green</td>
<td>0.9917</td>
<td>1.721</td>
<td>8.064</td>
</tr>
<tr>
<td>Blue</td>
<td>0.9591</td>
<td>6.353</td>
<td>44.9</td>
</tr>
</tbody>
</table>

Paper-based Device Utilizing the Griess Assay

This section provides the signal response for a paper-based device utilizing the Griess assay for the detection of nitrate and nitrite. This is the same folding paper-based device documented in [15] and the data was acquired following the same methodology mentioned in that paper. Figure 6-3 and Table 6-2 show the colorimetric signal response and sensitivity for the different color channels for the folding paper-based that detects nitrate.
Figure 6-3 Color response for the different color channels as a function of nitrate concentration for the folding paper-based device (a) red, (b) green and (c) blue. n=3 and the error bars represent the standard deviation.

Table 6-2 The coefficient of determination ($R^2$) for the different calibration curves formed and the limits of detection and quantification for the different colors for the folding paper-based device that measures nitrate

<table>
<thead>
<tr>
<th>Color Channel</th>
<th>$R^2$</th>
<th>LOD (ppm)</th>
<th>LOQ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red</td>
<td>0.9633</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Green</td>
<td>0.9954</td>
<td>1.527</td>
<td>4.85</td>
</tr>
<tr>
<td>Blue</td>
<td>0.9939</td>
<td>3.007</td>
<td>9.415</td>
</tr>
</tbody>
</table>
Figure 6-4 and Table 6-3 show the colorimetric signal response and sensitivity for the different color channels for the folding paper-based that detects nitrite.
Figure 6-4 Color response for the different color channels as a function of nitrite concentration for the folding paper-based device (a) red, (b) green and (c) blue. n=3 and the error bars represent the standard deviation.

Table 6-3 The coefficient of determination ($R^2$) for the different calibration curves formed and the limits of detection and quantification for the different colors for the folding paper-based device that measures nitrite

<table>
<thead>
<tr>
<th>Color Channel</th>
<th>$R^2$</th>
<th>LOD (ppm)</th>
<th>LOQ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red</td>
<td>0.9757</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Green</td>
<td>0.9948</td>
<td>0.1669</td>
<td>0.635</td>
</tr>
<tr>
<td>Blue</td>
<td>0.9976</td>
<td>0.8798</td>
<td>3.288</td>
</tr>
</tbody>
</table>
As can be seen from the above figures, the green component shows the largest difference in value over the range of nitrate and nitrite concentrations tested and yields the best limits of detection and quantification.

**G1 and G2 Pads Under Different Storage Conditions**

G1 and G2 pads were stored in a freezer (≤ -20 °C), fridge (≤ 4 °C) and under room conditions. These pads were scanned using a desktop scanner (Canon TS6020) at a resolution of 600 DPI and the images were processed using ImageJ to quantify the change in color over 100 days.
(b)

(c)
Figure 6-5 Color intensity for the G1 and G2 pads over a 100 day period in different storage conditions. (a) G1 pad stored in a freezer (≤ -20 °C), (b) G1 pad stored in a fridge (≤ 4 °C) and (c) G1 pad stored under room conditions. (d) G2 pad stored in a freezer (≤ -20 °C), (e) G2 pad stored in a fridge (≤ 4 °C) and (f) G2 pad stored under room conditions.

Figure 6-5 shows that the G1 pad stored under room conditions showed a large change in color as can be observed by eyesight because of its distinctive color degradation. The other strips didn’t show any color degradation and this can also be observed in the graphs since the color lines remain almost flat over the 100-day period. Most interesting is the result for G1 pad stored in a fridge (≤ 4 °C) since this pad didn’t show any visible color degradation. This may be due to the fact that the sulfanilamide and citric acid solution were dried on chromatography paper. This chromatography paper is made up of pure cellulose fibers with no additives whatsoever. This makes it
possible to store paper-based devices utilizing this G1 pad in a fridge over a prolonged period of time without worry of degradation of the sulfanilamide.

**Lightbox for in-the-field Analysis of Paper-Based Devices**

While desktop scanners provide a simple and inexpensive method for capturing the colorimetric signal in paper-based devices, these scanners are not practical for in-the-field use. Instead, a lightbox, Figure 6-6, similar to the one previously created for in-the-field analysis of phosphate [4] will be used. However, since the peak absorbance of the Griess assay occurs in the green region of the visible light ($\lambda \approx 540$ nm), the lightbox will be modified to include LEDs that emit green light ($\lambda \approx 540$ nm). The lightbox will be remotely controlled via a cellphone and will allow for in-the-field analysis of paper-based devices utilizing the Griess assay.

![Colorimetry](image)

**Figure 6-6 Proposed lightbox for in-the-field analysis of paper-based devices utilizing the Griess assay**
Conclusions

Many researchers have used a desktop scanner and a computer image processing software to capture the colorimetric signal in paper-based devices utilizing the Griess assay. However, the choice of which color channel to be used in the analysis has been kept up to the operator’s own discretion. In this paper, the colorimetric response of a commercially available dip strip in addition to that of paper-based devices utilizing the Griess assay was provided. It was observed that the green component of the color has the largest difference in value over the concentration range tested and yields the best limits of detection and quantification. It was also shown that drying a solution of sulfanilamide and citric acid on chromatography paper doesn’t show any color degradation for 100 days when stored in a fridge (≤ 4 °C). Finally, a lightbox that emits green light (λ ≈ 540 nm) for in-the-field analysis of paper-based devices utilizing the Griess assay will be used for quantitative analysis of these devices in the field.

Acknowledgments

The authors would like to acknowledge the support from Rhode Island EPSCoR which is funded by the National Science Foundation under Award #OIA-1655221. The authors would also like to acknowledge the students, research scientists and visiting scholars at the Microfluidics Laboratory at the University of Rhode Island for their help and support.
References


for quantitative colorimetric assays relying on light reflectance principle,”
*Electrophoresis*, vol. 35, no. 8, pp. 1152–1159, Apr. 2014.


CHAPTER 7

CONCLUSIONS AND FUTURE WORK

In this work, we developed a new composite material for nitrate reduction in paper-based devices called “Zinculose”, a new paper-based device utilizing a folding detection zone architecture for nitrate detection, and proposed a system for nitrate detection in the field.

Zinculose is a composite material made up of cellulose fibers with embedded zinc microparticles that can be produced by a simple and inexpensive sedimentation procedure. Zinculose increases the surface contact area between zinc microparticles and a sample fluid as it flows through the material. This allowed for a 36% enhancement in nitrate conversion efficiency over what has been previously published when zinc microparticles were not embedded within the fibers of the paper channel. A paper-based device with a folding detection zone was developed for the sensitive detection of nitrate in water. This device was the culmination of several earlier designs that utilized different valve technologies to control fluid flow for optimizing nitrate reduction. The device also incorporated Zinculose and an immobilized detection reagent so as to amplify the signal as more sample is allowed to flow through the detection zone without washing away the detection chemistry. The limits of detection and quantification of nitrate in water achieved by this paper-based device were 0.53 ppm and 1.8 ppm, respectively. These results constitute over 40% enhancement than what has been previously realized for the detection of nitrate in water using paper-based technology. A portable light box utilizing
green light is proposed for detecting nitrate in the field. This allows for a more sensitive measurement of nitrate concentrations in the field compared to naked eye observation. In general, this study contributes to the field of paper-based technology by providing new designs, materials, insights and conclusions that further enhance and deepen the understanding of this technology.

This work showed that hydrochloric acid evaporates completely without leaving any residue behind when dried on paper. Therefore, recommended future work is to optimize paper-based devices that have been developed with reagents utilizing hydrochloric acid for regulating or providing acidic conditions. Additionally, specific lightboxes can be developed that emit light at the peak of absorbance of the reaction product of interest to provide enhanced detection results in the field. Use of chromatography and storing in the fridge showed that the color of the pad with dried sulfanilamide didn’t change over a 100-day period. However, a chemistry approach is recommended to further stabilize sulfanilamide so that the device can be used in the field without the need for specific storage conditions. Data processing is also another interesting field of research for enhancing detection using paper-based technology. It may be possible to process the image of the color that develops in the detection zone by “summing” the pixels that show color and “discarding” the pixels that don’t show any color or show a color value below a certain threshold.
APPENDIX I

Supplementary Material for Zinculose: A New Fibrous Material with Embedded Zinc Particles

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Figure A1-1 shows magnified images (2000x) of zinc microparticles. The image on the left is for a sample of zinc microparticles that is fresh out of the box. The image on the right is for a sample of zinc microparticles that was submerged in deionized water for 2 hours and then allowed to air dry for 24 hours.

Figure A1-1 Magnified images (2000x) of zinc microparticles

Figure A1-2 shows the X-ray diffraction pattern of a sample of fresh out of the box zinc powder. Figure A1-3 shows the X-ray diffraction pattern of a zinc powder sample that was submerged in deionized water for 2 hours and then allowed to air dry for 24 hours. Figure A1-4 shows the X-ray diffraction pattern of a Zinculose sheet (16 mg/cm²) that has been stored for 6 months in a chamber whose relative humidity is maintained less than 30%. Figure A1-5 shows the X-ray diffraction pattern of the zinc samples in addition to the Zinculose sheet.
Figure A1-2 X-ray diffraction pattern of a sample of fresh out of the box zinc powder.

Figure A1-3 X-ray diffraction pattern of a zinc powder sample that was submerged in deionized water for 2 hours and then allowed to air dry for 24 hours.
Figure A1-4 X-ray diffraction pattern of a Zinculose sheet (16 mg/cm$^2$) that has been stored for 6 months in a chamber whose relative humidity is maintained less than 30%.

Figure A1-5 X-ray diffraction pattern of the zinc samples in addition to the Zinculose sheet.
Figure A1-6 shows a very strong correlation between the area and weight of Whatman 1 chromatography paper. Figure A1-7 shows the effect of water volume on thickness of produced Zinculose. Figure A1-8 shows the effect of applied weight on the thickness of produced Zinculose. Figure A1-9 shows the effect of duration of applied weight on thickness of produced Zinculose. Figure A1-10 shows the reproducibility of the Zinculose production process.

Figure A1-6 Average weight of chromatography paper as a function of its area (n≥3 and the error bars show the standard deviation)
Figure A1-7 Average thickness of produced Zinculose sheets as a function of water volume used during the production process (n=45 and the error bars show the standard deviation)

Figure A1-8 Average thickness of produced Zinculose sheets as a function of applied weight (n=45 and the error bars show the standard deviation)
Figure A1-9 Average thickness of produced Zinculose sheets as a function of the duration of applied weight (n=45 and the error bars show the standard deviation)

Figure A1-10 Average thickness of produced Zinculose vs. sheet number (n=15 and the error bars show the standard deviation)
Figure A1-11 shows the Tukey pairwise comparison of the thickness which shows that there’s not a single sheet that is significantly different than all of the other sheets despite being produced by 3 different operators.

**Comparisons for Thickness**

**Tukey Pairwise Comparisons: Sheet**

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_Means that do not share a letter are significantly different._

Figure A1-11 Tukey pairwise comparison of the thickness for the 16 sheets produced by 3 different operators for the same test conditions (n=15)
Figure A1-12 shows the average distance covered by ultrapure water as a function of time and zinc content. This figure is the average of 3 tests with 3 samples per test for each zinc concentration; points lying outside 2 standard deviations of the mean (capturing 95% of the data) were discarded. The linear fits are $y = 0.5242x$ ($R^2 = 0.9986$), $y = 0.5014x$ ($R^2 = 0.9980$), $y = 0.4908x$ ($R^2 = 0.9985$) and $y = 0.4133x$ ($R^2 = 0.9989$) for Zinculose with 0, 2, 6 and 12 mg/cm$^2$ zinc content respectively.

Figure A1-12 Average distance covered by water as a function of time for Zinculose with different zinc content ($n\geq8$ and the error bars show the standard deviation)
Figure A1-13 shows the average distance covered by ultrapure water as a function of time and porosity. The linear fits are $y = 0.4118x$ ($R^2 = 0.9969$), $y = 0.3412x$ ($R^2 = 0.9966$), $y = 0.4084x$ ($R^2 = 0.9956$) and $y = 0.5542x$ ($R^2 = 0.9979$) for Zinculose runs 1, 2, 3 and 4 with porosity values 0.729, 0.699, 0.73, 0.795 and standard deviations 0.016, 0.016, 0.04, 0.005 respectively. Zinculose runs 1 and 3 had almost an identical value for porosity and their flow rate profile was almost overlapping.

Figure A1-13 Average distance covered by water as a function of time for Zinculose with different porosities (n=3 and the error bars show the standard deviation)
Figure A1-14 shows the color analysis zone.

![Figure A1-14](image)

Figure A1-14 A scanned image of a paper-based microfluidic device being analyzed by Image J with the yellow square showing the zone used for color analysis of all devices used in this study.

Figure A1-15 An exponential decay calibration curve in the form $y = a \cdot exp(-x/b) + c$ where $a = 0.2421$, $b = 22.11$ and $c = 0.2431$ was established for nitrite. This curve was used to measure the conversion efficiency of nitrate to nitrite. (n=3 and the error bars show the standard deviation)
Table A1-1 RGB intensity values used to produce the nitrite calibration curve. Test order was randomized.

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Table A1-2 RGB intensity values used to measure the conversion efficiency of nitrate to nitrite. Test order was randomized.

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Figure A1-16 shows a zinc microparticle observed using an electron scanning microscope. Figure A1-17 shows EDS mapping on the observed zinc microparticle.

**Figure A1-16** A zinc microparticle observed using an electronic scanning microscope
Figure A1-17 EDS mapping of the zinc microparticle.
APPENDIX II

Supplementary Material for A New Paper-Based Microfluidic Device for Improved Detection of Nitrate in Water

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Paper-based microfluidic device – R1:

The first paper-based microfluidic device (R1) designed had a wax valve printed on filter paper (Whatman grade 41). This wax valve acts as a delay valve to allow the nitrate sample to interact with the zinc microparticles in the reduction chamber and reduce to nitrite before it is allowed to flow through to the detection zone. Ahead of the wax valve there’s a dried 2µL spot of PBST [1]. However, in [1], the material used for the platform was nitrocellulose (NC) and not filter paper. The valve actuation time was more reproducible and consistent in NC than in the case of filter paper, Figure A2-6 below. Nitrocellulose changed the color of the detection zone even in the case of a blank sample (0 ppm nitrite or nitrate) and therefore was not used in the device and replaced by filter paper.

Figure A2-1 Paper-based microfluidic device - R1
Figure A2-2 Paper-based microfluidic device architecture - R1

Figure A2-3 Components of R1
Figure A2-4 Dimensions of R1

Table A2-1 The parameters tested using paper-based microfluidic device

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate sample concentration (ppm)</td>
<td>100</td>
</tr>
<tr>
<td>Sample volume (µL)</td>
<td>25</td>
</tr>
<tr>
<td>Mass of zinc deposited per reduction chamber (mg)</td>
<td>~ 2</td>
</tr>
<tr>
<td>Number of Reduction Chambers per device</td>
<td>3</td>
</tr>
<tr>
<td>Deposited volume of Griess Reagent (µL)</td>
<td>4</td>
</tr>
<tr>
<td>Griess Reagent drying time (min)</td>
<td>20</td>
</tr>
<tr>
<td>Scanning Time (min)</td>
<td>10-40</td>
</tr>
<tr>
<td>Advantages</td>
<td>Disadvantages</td>
</tr>
<tr>
<td>------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Autonomous and easy to use: User has to only pipette the sample</td>
<td>The valve actuation time varies and is not exactly identical since it depends on the randomness in the paper. Humidity conditions played a major role on the repeatability of activation of the valve</td>
</tr>
<tr>
<td>Design is modular: The user can add as many reduction chambers as they please to improve conversion efficiency of nitrate</td>
<td>The wax valve is an actual barrier and makes the flow rate much slower when it is activated</td>
</tr>
<tr>
<td>-</td>
<td>The donut shaped double-sided tape in between the reduction chambers and the device is very tricky and takes a lot of time to peel</td>
</tr>
<tr>
<td>-</td>
<td>Pipetting the same amount of zinc in the reduction chamber is very difficult since zinc microparticles precipitate from the mixture</td>
</tr>
</tbody>
</table>
- Color is not uniform in the detection zone. The fluid reaches the detection zone from the center and flows outward which creates a color gradient in the detection zone

**Paper-based microfluidic device – R2:**

The revised design of the paper-based microfluidic device (R2) also had a wax valve printed on filter paper. The design is very similar to R1. However, it is more simplified by having less components to prepare and thus making it easier and faster to fabricate. The set of advantages and disadvantages is almost identical to that of R1. The only difference is that the double sided tape is easier to peel and stick onto a larger area.

![Figure A2-5 Paper-based microfluidic device – R2](image)
Figure A2-6 Components of R2

Figure A2-7 Dimensions of R2
Figure A2-8 Fluidic testing of the wax valve. The device on the left has PBST before the wax valve and so the valve “opens” after a certain reduction time and allows the fluid to flow into the detection zone. The device on the right doesn’t have any PBST before the valve and so the wax valve “holds” the fluid and doesn’t allow it to pass into the detection zone
Figure A2-9 Comparison of R1 & R2

Figure A2-10 Average time it takes for a 0.25 mm wax valve to “open” and allow fluid to flow according to paper type. The paper types used in this test were 1-
Nitrocellulose HF09004XSS, 2-Nitrocellulose HF09002XSS and 3-Whatman filter paper grade 41. The error bars represent the standard deviation for four trials

**Paper-based microfluidic device – R3:**

Since the wax valve was not giving reproducible results on filter paper and since we couldn’t use nitrocellulose as part of the platform then we had to modify the design to include a mechanical valve which was a bridge that connected the reduction chamber to the detection zone. The new microfluidic design (R3) included a folding bridge that would connect the different components of the device.

![Figure A2-11 Paper-based microfluidic device – R3](image)
Figure A2-12 Design R3 uses a folding bridge to connect the different components of the device. It has 3 stacked reduction chambers.

Figure A2-13 Dimensions of R3
Table A2-3 Advantages and disadvantages of the R3 design

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controlled actuation time. User will fold the bridge after a</td>
<td>It’s not autonomous and requires the user to fold the bridge.</td>
</tr>
<tr>
<td>certain reduction time</td>
<td></td>
</tr>
<tr>
<td>Design is easy to fabricate and easy to use</td>
<td>The bridge may not fold in the same location every single time</td>
</tr>
<tr>
<td></td>
<td>which may affect the results</td>
</tr>
<tr>
<td>-</td>
<td>Pipetting the same amount of zinc in the reduction chamber is</td>
</tr>
<tr>
<td></td>
<td>still a challenge</td>
</tr>
<tr>
<td>-</td>
<td>Color formed in the detection zone is not uniform</td>
</tr>
</tbody>
</table>

**Paper-based microfluidic device – R4:**

The R3 design was slightly modified to R4 to include 1 reduction chamber instead of 3. This made it easier to fabricate more devices in a shorter period of time. The connecting bridge was also made narrower so that it would be easier for the user to fold the bridge close to the connecting channel which would give more reproducible results. The set of advantages and disadvantages is identical to that of R3.
14 Paper-based microfluidic device – R4

Figure A2-15 Design R4 is a simplified version of R3. It also uses a folding bridge to connect the different components of the device. However, it only uses 1 reduction chamber that can hold more zinc.
Figure A2-16 Dimensions of R4

Figure A2-17 Fluidic testing of R4 with colored water. Color is not uniform in the detection zone.
Paper-based microfluidic device – R5:

The user error in folding the bridge prompted us to redesign the device to include a different valve mechanism. We wanted to keep the device simple and easy to use without requiring any external power source. So we decided to use a compressed sponge in design R5 to act as the valve actuation mechanism similar to [2] for autonomous operation of the device.

Figure A2-18 Paper-based microfluidic device – R5
Figure A2-19 Fluidic testing of R5 with colored water

Figure A2-20 Testing of R5 with a nitrate sample. Color was formed in the fluidic channel before the detection zone. There was a color gradient in the fluidic channel and no color was observed in the detection zone.
The sponge would sometimes get stuck in the shaft of the housing as it was expanding. This caused the device to have a non-repeatable activation time. The design was slightly modified to make the sponge square instead of being circular. However, this issue was still a problem that gave non-consistent activation times. The fluid transfer rate between the activation channel and the sponge played a very important role. We tried filter paper and glass fiber. We found that glass fiber was better than filter paper in transferring the fluid into the sponge for it to expand.

Figure A2-21 Paper-based microfluidic device architecture – R5
Figure A2-22 Components of R5

Figure A2-23 R5 with a square sponge. The hydrophobic disk is filter paper that had wax printed using the solid ink wax printer and then melted to create a hydrophobic surface. Whereas the hydrophilic disk is pure filter paper.
Table A2-4 Advantages and disadvantages of the R5 design

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autonomous operation. User has to only pipette the sample at the start and the sponge valve will provide the required time delay for nitrate reduction.</td>
<td>The sponge didn’t expand completely vertical on every run. It bent in some cases while it was expanding and got stuck in the shaft of the housing and therefore couldn’t connect the channels. In other situations, it took longer to expand. So the activation time was difficult to control.</td>
</tr>
<tr>
<td>Design is easy to fabricate and easy to use.</td>
<td>The sponge required a large volume of fluid to expand. The fluid transfer rate between a paper activation channel and the sponge was too slow. The activation channel was replaced with glass fiber.</td>
</tr>
<tr>
<td>-</td>
<td>Pipetting the same amount of zinc in the reduction chamber remains a challenge.</td>
</tr>
</tbody>
</table>
Paper-based microfluidic device – R6:

From the previous designs, it was clear that color formation was a critical issue since the color was not uniform and in a lot of cases formed in the fluidic connecting channel before the detection zone. Also, pipetting the same amount of zinc in the reduction chamber was very labor intensive and time consuming. Therefore, we implemented 2 new innovations in microfluidic paper-based technology in R6.

Figure A2-24 Paper-based microfluidic device – R6
Figure A2-25 R6 design. Griess 1 is sulfanilamide and citric acid since the detection zone had immobilized NED.

Figure A2-26 R6 with Zencilose that have 2 different zinc contents.
Table A2-5 Advantages and disadvantages of the R6 design

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very easy to fabricate. Fabrication is done by preparing a card that has</td>
<td>The color formed in the detection zone was not</td>
</tr>
<tr>
<td>all the different components, <strong>Error! Reference source not found.</strong> The</td>
<td>uniform</td>
</tr>
<tr>
<td>card is then cut using a guillotine cutter into several lateral flow</td>
<td></td>
</tr>
<tr>
<td>strips</td>
<td></td>
</tr>
<tr>
<td>Very easy to use. The user just needs to dip the sample pad in the</td>
<td>The lateral flow strip was too long</td>
</tr>
<tr>
<td>solution for few seconds</td>
<td></td>
</tr>
<tr>
<td>Zinculose provided the reduction capability to reduce nitrate to nitrite.</td>
<td></td>
</tr>
<tr>
<td>There was no longer a need to prepare the reduction chamber for each</td>
<td></td>
</tr>
<tr>
<td>device individually</td>
<td></td>
</tr>
</tbody>
</table>

**Paper-based microfluidic device – R7:**

R7 is almost identical to the architecture of R6. However, it was shorter and there were separate regions for G1 (sulfanilamide and citric acid) and the detection zone that
was functionalized with immobilized NED. This increased the longevity of the device as placing G1 solution on the immobilized NED turned the color of the paper slightly pinkish as it dried. The advantages and disadvantages for R7 are the same as those stated for R6.

Figure A2-27 A fabricated card that has all the components for the lateral flow strip. Strips are cut out of this card using a guillotine cutter
Figure A2.28 Paper-based microfluidic device – R7

Figure A2.29 Non-uniformity of color formed in the detection zone. We observed that color starts forming at random points along the overlap between the G1 pad and the detection zone with the immobilized NED. We termed these points as
“seeding points” as color preferentially continues to develop and becomes darker at these locations as more sample flows through. That is why color streaking and non-uniformity is observed in the detection zone.

**Paper-based microfluidic device – R8:**

Due to the non-uniformity in the color formed in the later flow architecture and the color gradient that forms in the detection zone, we decided to use a folding device design to produce a more uniform color in the detection zone.

![Figure A2-30](image)

**Figure A2-30** The initial design with a folding detection zone architecture. The color produced in the detection zone is very uniform.
Figure A2-31 Paper-based microfluidic device – R8

Figure A2-32 An exponential decay calibration curve in the form $y = a \cdot \exp(-x/b) + c$ where $a = 0.184$, $b = 18.43$ and $c = 0.3162$ was established for nitrite in deionized
water. The error bars represent the standard deviation. The limit of detection and quantification were 0.227 ppm and 0.361 ppm respectively

The Griess assay involves two reactions that take place under acidic conditions [3]. So we flood coated the detection zones that were functionalized (immobilized NED) with citric acid 330 mM for 2 minutes and allowed to air dry before using in the device. This improved the performance of the device by lowering the limits of detection and quantification.

![Exponential decay calibration curve](image)

**Figure A2-33** An exponential decay calibration curve in the form \( y = a \cdot \exp(-x/b) + c \) where \( a = 0.202 \), \( b = 17.44 \) and \( c = 0.2981 \) was established for nitrite in deionized water. The error bars represent the standard deviation. The limit of detection and quantification were 0.191 ppm and 0.259 ppm respectively
**Paper-based microfluidic device – R9:**

The signal formed in the detection zone of R8 is very uniform and there’s no color gradient similar to what was observed in the detection zone of R7. However, R7 allows the passage of a larger volume of sample of the detection zone. Passing a larger volume of sample over a detection zone with immobilized reagent is known to concentrate the analyte of interest [4] and improve the limits of detection and quantification [5]. Therefore, R8 was modified to include a waste pad that would allow the passage of a larger volume of sample over the detection zone.

![Figure A2-34 Paper-based microfluidic device – R9](image)
An exponential decay calibration curve in the form $y = a \cdot \exp\left(-\frac{x}{b}\right) + c$ where $a = 0.2059$, $b = 7.794$ and $c = 0.3047$ was established for nitrite in deionized water. The error bars represent the standard deviation. The limit of detection and quantification were 0.018 ppm and 0.061 ppm respectively.

**Optimization of the different parameters for device operation:**

**Sample Volume:**
Figure A2-36 Signal vs. sample volume. The error bars represent the standard deviation for three trials

Reduction Time:

Figure A2-37 Signal vs. reduction time. The error bars represent the standard deviation for three trials

Zinc Content:
Figure A2-38 Signal vs. zinc content. The error bars represent the standard deviation for three trials

Color Development Time:

Figure A2-39 Signal vs. color development time. The error bars represent the standard deviation for three trials
Figure A2-40 Color formed in the detection zone vs. nitrate or nitrite concentration.

References:


APPENDIX III

Supplementary Material for Colorimetric Determination of Nitrate after Reduction to Nitrite in a Paper-Based Dip Strip

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Figure A3-1 shows a dip strip used in our experiments.

![Dip strip used in experiments](image)

**Figure A3-1 Dip strip used in experiments**

Figure A3-2 shows the dip strips after the solutions are dried on the detection zones. Solutions “A” and “B” are prepared following the procedure outlined by Thongkam et al. [1].

![Dip strip used in experiments after the solutions are dried on the detection zones](image)

**Figure A3-2 Dip strip used in experiments after the solutions are dried on the detection zones**
Figure A3-3 Color formed in the detection zone vs. nitrate or nitrite concentrations after several minutes

Figure A3-4 Color formed in the detection zone vs. nitrate or nitrite concentrations after 1 hour
Table A3-1 ImageJ analysis of nitrate detection zones after 10 minutes. Test order was randomized

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<tr>
<th>Standard</th>
<th>Nitrate Concentration (ppm)</th>
<th>Run Order</th>
<th>Unweighted Intensity Value (a.u.)</th>
<th>Weighted Intensity Value (a.u.)</th>
<th>RGB Intensity Value (a.u.)</th>
<th>Red Intensity Value (a.u.)</th>
<th>Green Intensity Value (a.u.)</th>
<th>Blue Intensity Value (a.u.)</th>
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Table A2-2 ImageJ analysis of nitrate detection zones after 1 hour. Test order was randomized.
Table A3.3 ImageJ analysis of nitrite detection zones after 5 minutes. Test order was randomized.

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<th>Standard</th>
<th>Nitrite Concentration (ppm)</th>
<th>Run Order</th>
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<th>Weighted Intensity Value (a.u.)</th>
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<th>Red Intensity Value (a.u.)</th>
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Table A3-4 ImageJ analysis of nitrite detection zones after 1 hour. Test order was randomized

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Figure A3-5 Zinc microparticles observed using an electron scanning microscope with EDS analysis in the nitrate test fields of commercial dip strips (a) Quantofix 91313 (b) Quantofix 91351

References: