

Development of Three-Dimensional Electrochemical and Colorimetric Paper-Based Sensors

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Abstract

Point-of-care (POC) diagnostic technologies hold significant promise for addressing healthcare challenges, particularly in resource-limited settings. Microfluidic paper-based analytical devices (μ PADs) have emerged as a versatile platform due to their cost-effectiveness, portability, and ease of fabrication. However, challenges related to usability, reliability, and scalability persist, challenging a more widespread adoption of this technology. This thesis investigates the development and optimization of three-dimensional microfluidic paper-based devices for colorimetric and electrochemical detection, emphasizing design considerations to improve usability, reproducibility, and manufacturability.

Chapter 1 outlines the current challenges in healthcare diagnostics, emphasizing the unmet needs in developing economies and the critical role of POC technologies. Existing μ PAD modalities and their advantages are discussed, alongside the potential for signal modality advancements to enhance device performance. Chapter 2 explores the integration of three-dimensionality into paper-based colorimetric devices, addressing issues of signal clarity through innovative linear-format designs that improve color interpretation. This approach reduces variability in user interpretation and enables more reliable quantitative outputs. Chapter 3 explores the potential to further control the microenvironment within which the linear colorimetric readout forms. This approach studies the influence of pH, paper buffer capacity, and nanoparticle impregnation on improving the interfacial signal characteristics such as width, intensity, and localization.

In Chapter 4, the development of three-dimensional electrochemical PADs (ePADs) is presented. A novel electrode configuration addresses critical challenges in interelectrode distance consistency and manufacturability, enabling interchangeable designs and enhanced reproducibility of electrochemical signals. Chapter 5 expands upon this work, introducing areas of improvements through developing multiplexed and standalone iterations of the previously presented three-dimensional ePADs. The multiplexed devices incorporate compartmentalization strategies to minimize signal interference and crosstalk, demonstrating potential for robust multi-analyte POC diagnostics. The standalone iteration of the 3D ePAD shows a more sustainable fabrication of the device, without depending on wax printing to guide the sample flow. Collectively, these insights pave the way for scalable and versatile POC diagnostic platforms capable of addressing diverse healthcare needs. This thesis aims to provide a roadmap for advancing the applicability of three-dimensional μ PADs, bridging the gap between academic prototypes and real-world solutions.

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Publications and Work in Preparation

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Chapter 1

Introduction: Microfluidic Paper-Based Devices for Point-of-Care Diagnostics

1.1.1 Healthcare challenges in Developing Countries

Healthcare systems in developing countries are at the nexus of multifaceted challenges and opportunities, navigating the intricate landscape of limited resources, diverse health needs, and evolving global health dynamics. The current state of healthcare in developing countries is influenced by multiple factors, including the healthcare infrastructure, the availability and accessibility of services, the capacity and distribution of healthcare professionals, disease prevention strategies, and the critical aspects of financial sustainability and resource allocation.^{1,2} For instance, insufficient funding affects infrastructure development, workforce capacity, and the procurement of essential medical supplies. Grappling with these constraints, developing countries encounter a substantial challenge in providing comprehensive and accessible healthcare services. This situation, which lead to the spread of multiple diseases in developing countries, necessitates the urgency of addressing these health disparities.^{3,4}

The lack of well-established healthcare infrastructure can take the form of insufficient hospitals, clinics, and medical facilities, which collectively hinders the delivery of timely and quality healthcare services. Particularly, rural areas face the strongest infrastructural deficits, leading to inequalities in healthcare access between urban and remote regions.⁵ In addition, developing countries often experience shortages in healthcare professionals, including doctors, nurses, and allied health workers. Inadequate workforce capacity not only hampers the delivery of healthcare but also places a strain on the existing healthcare staff, leading to burnout and compromised quality of care.^{2,6} Infectious diseases, maternal and child health issues, and the increasing burden of non-communicable diseases compound the health challenges in developing countries. These spreading conditions

place additional burdens on healthcare systems, requiring targeted interventions and effective health policies.^{7–9}



Figure 1.1. Diagnostic pathway and challenges to POC testing in South Africa. Reprinted with permission from ref [10]. Copyright 2018 Springer Nature.

Developing countries face mounting global health challenges, with pandemics like COVID-19 exposing the critical vulnerabilities of under-resourced healthcare systems (**Figure 1.1**).¹⁰ These crises highlight the urgent need for strategic policy reforms, strengthened international cooperation, and sustained investments in infrastructure and workforce capacity. Addressing these challenges requires building resilient healthcare systems capable of responding to both current and emerging health threats.¹¹ However, achieving this resilience demands innovative approaches that prioritize equity, accessibility, and efficiency in healthcare delivery.

To bridge these gaps, developing countries have begun adopting diverse strategies, such as integrating community-based healthcare models, expanding telehealth services, and employing sustainable financing mechanisms. Coupled with international collaborations and aid programs, these approaches have laid the foundation for transformative change.² Augmenting these strategies, point-of-care (POC) device innovations could stand out as a practical and cost-effective solution. By leveraging technology and implementing evidence-based policies, POC devices offer the potential to revolutionize diagnostics and healthcare delivery in resource-limited settings, ultimately ensuring equitable access and improving population health outcomes.^{12,13}

1.1.2 The need for point-of-care devices in developing countries

One of the primary challenges faced by developing countries is the disproportionate burden of both communicable and non-communicable diseases. While infectious diseases continue to exact a heavy toll, the rising popularity of chronic conditions adds complexity to healthcare delivery.¹⁴ Diabetes, for instance, is becoming progressively prevalent in low- and middle-income countries, with complications often going undiagnosed due to limited access to glucose and HbA1c testing.¹ Similarly, anemia, often linked to iron deficiency, affects a considerable portion of the population in these regions, particularly women and children.¹⁵ Additionally, exposure to pesticides can lead to organophosphate poisoning, which is characterized by acetylcholinesterase (AChE) inhibition, posing a significant health risk.¹⁶ In this context, the need for rapid and accurate diagnostic tools becomes paramount. Traditional diagnostic methods often rely on centralized laboratories, which can lead to delays in diagnosis and treatment initiation, increasing the chances of developing worsened complications. Furthermore, the cost and

complexity of conventional diagnostic equipment make it impractical for deployment in resource-constrained settings, creating a major gap in healthcare delivery.¹⁷

Point-of-care (POC) diagnostic sensors have emerged as a potential solution to address these challenges. These tools could offer the promise of rapid, on-site testing which can improve patient outcomes and reduce the burden on overwhelmed healthcare systems.¹⁸

POC diagnostics can enable healthcare workers to make quicker informed decisions, potentially reducing the need for referrals to distant healthcare facilities. It can also allow for a closer routine monitoring of chronic conditions, giving a chance for a sooner intervention before condition deterioration.¹⁹ By bringing diagnostic capabilities closer to the patient, POC devices can help overcome some of the infrastructural and logistical barriers that hinder effective healthcare delivery in developing countries. Nevertheless, for POC diagnostics to be effective in these settings, they must be designed with consideration for the specific constraints and needs of resource-limited environments, including factors such as cost, ease of use, robustness, manufacturing feasibility, and independence from complex infrastructure.²⁰

1.2 Point-of-care devices for disease detection

1.2.1 Milestones in point-of-care device development

POC devices are systems that can be used by untrained personnel, either by self-testing or a clinical personnel whose training is not in clinical sciences, to provide diagnostic information at the patients' site.^{21,22} POC technology is one of the fastest growing fields of medicine over the decade, gaining global recognition as diagnostic tools. This recognition is owing to the increased convenience the POC systems continue to provide,

in addition to their accurate and rapid result generation, which could lead to earlier disease diagnoses.²³ The enhanced extraction, miniaturization, microfluidics, and data analysis technologies utilized by POC devices have resulted in making them a growing alternative to laboratory-based testing. Nonetheless, there are a lot of areas of improvement in terms of POC accuracy and sensitivity, to gain more overall confidence in the diagnostic decisions based on their measurements.²⁴

While POC devices for has been gaining increasing recognition recently, the first recorded biomarker detection was dated over 3000 years ago and found on papyrus of ancient Egyptians.²⁵ It reported a primary pregnancy test in which a potential gravida was to urinate over seeds of barley and wheat. It was claimed that growing one of the seeds would indicate the sex of the expected child, while growing none would indicate non-pregnancy.²⁶ It was challenging to replicate the same experiment in modern days, due to urine toxicity, which caused researchers to either dialyze or dilute tested urine samples. However, one study achieved a 70% sensitivity and 100% specificity 5 days after germination.^{27,28} The foundations of modern POC test, on the other hand, were laid by Edmonds in the second half of the last century, being the first to detect glucose in urine and blood by employing dried chemical reagent strips.^{23,29} This start was followed up with remarkable POC technology development, which included emerging methods like immunochromatographic assays and biosensor technologies.^{30,31} Over time, these methods have led to the globalized rapid POC development to be used for immediate evidence-based diagnosis, therapeutic decision making, and patient status monitoring, especially with chronic diseases.³²

1.2.2 Ideal features for the perfect point-of-care testing device

The World Health Organization (WHO) first established guidelines for POC devices in 2006, initially focusing on devices intended for detecting sexually transmitted diseases.³³ These guidelines introduced the ASSURED criteria, stipulating that POC devices should have the ideal features of being: affordable, sensitive, specific, user-friendly, rapid and robust, equipment free, and deliverable to end-users. Since these guidelines were introduced around two decades ago, they have been revisited more recently in order to incorporate the globalized new technologies which have recently emerged. An update to these guidelines was introduced in 2019 recommending POC devices to also feature real-time connectivity for digital healthcare and ease of sample collection.³⁴ This recent update resulted in the “REASSURED” guidelines for point-of-care tests. Despite these comprehensive guidelines, the critical aspect of usability—defined as the capacity for non-professional users to obtain reliable and interpretable results—has received insufficient attention in POC device development. These criteria, while establishing standards for effective point-of-care measurements, present unique engineering challenges for device developers, necessitating solutions that balance technical sophistication with operational simplicity.³⁵

1.2.3 Challenges of POC testing

As the POC technology continues growing, the industry is yet to address several challenges to enhance POC performance. Unlike laboratory-based testing, reagents included in POC testing could eventually cost more since they are packed for unit-use test formats.^{32,36} Matching the definition of POC testing, the wide spread of POC devices led to their use by non-medical users or clinical staff with limited laboratory testing

training. While the central laboratory personnel are trained for handling different specimens to ensure high quality results, in-home POC tests might receive less operational attention, increasing the false result probability.³⁶ In addition, the portability of POC devices raises storage conditions issue such that the test reagents might be affected by temperature, humidity, or light. Storage conditions can also affect POC tests that require an additional measurement device, such as glucometers, which require protection from adverse conditions to ensure accuracy.^{37–39} Not only for storage, but the exposure to ambient conditions can pose a challenge also for POC device transportation, especially during supplying POC devices from urban or industrial areas to remote and less developed regions.⁴⁰ The microfluidics industry, however, can offer a solution to reach those larger demographics in remote areas.³² By leveraging low-cost, lightweight, and robust substrates, μ PADs can enhance stability during storage and transport while simplifying the testing procedure. These features could make μ PADs more suitable for use in remote and resource-limited settings.^{41,42}

1.3 Microfluidic paper-based analytical devices

The microfluidics technology involves the manipulation of minute fluid volumes through microscale channels and, eventually, devices, which allows for a precise control of small volume samples.⁴³ This miniaturized fluid control offers advantages such as enhanced reagent mixing, sample conservation, rapidity, and flexibility which resulted in higher processing efficiency.⁴⁴ Fundamentally, the principles of microfluidics technology stem from the fluidic behavior at the micrometer scale, in which devices such as micropumps, micropipes, and microvalves are employed to manipulate and/or regulate the fluid flow.⁴⁵ This microfluidic technology has been employed for a wide range of applications. For

instance, in therapeutics and medical diagnostics, microfluidics are employed for disease biomarker detection,⁴⁵ personalized medicine,⁴⁶ and drug screening.⁴⁷ Based on their advantageous features, employing microfluidics in these applications could eventually facilitate potential to meet the demands of commercialization and large-scale experiments.³²

Like traditional microfluidic devices, microfluidic paper-based analytical devices (μ PADs) are detection devices that support reactions in the micron scale. Instead of engraving the fluidic conduits, the sample flow path is defined through patterning a paper substrate to create hydrophilic and hydrophobic channels.⁴⁸ While modern microfluidics originated in the second half of the last century, the μ PADs concept was first introduced in 1902 when Dieterich used wax to create a hydrophobic barrier on a filter paper substrate.⁴⁹ Over the century, subsequent advancement followed that patent,^{50–54} including a proposal by Manz et al for a miniaturized total chemical analysis system (TAS) which further revolutionized the field by integrating multiple laboratory functions on a single chip.⁵⁵ Over a decade later, innovative microfluidic devices fabricated from chromatography paper with hydrophobic barriers were developed for colorimetric analysis of glucose and protein using artificial urine samples (**Figure 1.2 A–C**).⁵⁶ These devices utilized capillary action to direct the sample fluid to chemical reagents embedded within the paper. Upon rehydration, the colorimetric reactions occurred such that their results could be visually inspected and interpreted. Following that, a myriad of studies have showcased inventive μ PADs capable of fluid transport, reagent mixing, and executing complex biochemical assays (**Figure 1.2D**).^{48,57} These operations eliminate the need for external pumps or power sources for fluid movement. Often, such advancements are inspired by developments in polymer

microfluidics or traditional laboratory flow-based methods.⁵⁸ These studies underscored the potential of paper-based microfluidics to offer a simpler and more cost-effective alternative which could better meet the high demand at the POC.

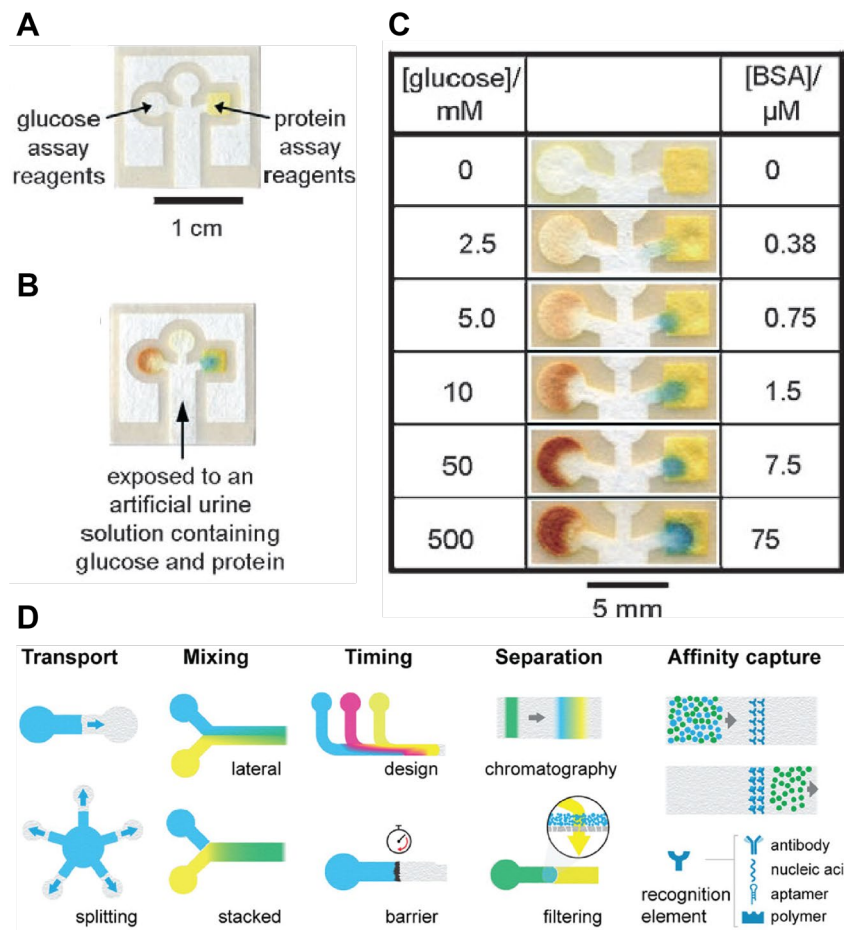


Figure 1.2. Evolution of Microfluidic Paper-Based Analytical Devices (μPADs). (A–C) Early μPAD designs utilizing chromatography paper with hydrophobic barriers for colorimetric detection of glucose and protein in artificial urine, demonstrating the concept of reagent rehydration and capillary-driven fluid transport. Reprinted with permission from ref [56]. Copyright 2007 John Wiley and Sons. (D) Modern μPADs showcasing advanced designs capable of fluid transport, reagent mixing, and complex biochemical assays without requiring external pumps or power sources. Reprinted with permission from ref [57]. Copyright 2024 John Wiley and Sons.

Using paper as a substrate provides μPADs with many advantages as compared to conventional glass- or polymer-based microfluidic devices. μPADs offer advantages such as cost-effectiveness, biodegradability, and minimal ecological footprint since paper is an

abundant substrate source.^{59,60} Through simple patterning of paper sheets, the geometries of hydrophilic areas could be used to guide the sample flow driven by capillary action.⁵⁷ These paper-based microchannel networks can be fabricated using multiple techniques, such as photolithography, inkjet printing, wax printing, wax spraying, and thermal-induced wax infiltration.⁶¹ With no need for external driving equipment, e.g. pumps, this control enables a myriad of applications such as sample pretreatment,⁶² separation,⁶³ and sensing.⁶⁴ Depending on factors like clear operation instructions and the signal modality integrated with the paper-based device, paper-based devices show a promising prospect to enhance user friendliness of POC testing. Notably, μ PADs have been reported to meet the ASSURED standard proposed by the WHO described earlier.⁶⁵ According to this standard, μ PADs could offer affordability with limited resources, user-friendliness, ease of accessibility, and ability to be powered by batteries.

1.3.1 Substrate material properties for μ PAD fabrication

The selection of materials for fabricating μ PADs is critical to their performance, requiring consideration of mechanical strength, physicochemical properties, transparency, and ease of fabrication.⁶¹ The selected paper must exhibit sufficient mechanical durability to maintain structural integrity and resist deformation or disintegration when exposed to liquid media, i.e. samples.⁴⁸ Upon sample introduction, it is important to maintain a controlled liquid flow within well-defined detection zones, necessitating an optimal hydrophilic pathway while avoiding leakage. Surface modifications, such as silanization, renders the paper hydrophobic, must account for changes in hydrophilic properties, as they directly influence fluid dynamics.^{66,67} The paper material should also be chemically inert, avoiding any interaction with the analytes or stored reagents used in the assay. For

instance, Storing acidic reagents on paper substrates is impractical, as they can degrade or etch the paper over time. The extent of this degradation depends on factors such as the acid's pH, concentration, and the physicochemical properties of the paper.⁶⁸ Lastly, the inherent irregularity in the paper's fibrous structure could hinder result consistency, necessitating surface engineering to ensure signal uniformity and mitigate issues like the "coffee ring effect".⁶⁹ Proper management of these factors is vital for ensuring consistency and accuracy to use papers for analytical applications.

The μ PADs development has notably advanced in recent years, using various paper substrates for different applications.^{70,71} Among these, filter and chromatography papers have been extensively employed due to their unique structural properties. Filter and chromatography papers are primarily composed of fibers with microholes on their surface. Despite allowing for liquid diffusion, this paper structural feature filters larger solid particles. The pore size of different filter papers has a direct influence on their specific surface areas and capillary action-induced liquid diffusion properties.⁵⁹ While accurate pore size information is not always available for commercial filter paper grades, techniques like mercury intrusion porosimetry (MIP), Brunauer–Emmett–Teller (BET) gas adsorption, and microcomputed tomography (μ CT) can be used to characterize the paper pore structure.⁷² In addition, retention size, which indicates the largest particle size a paper can filter, is commonly available and could serve as a useful approximation or a crude surrogate for the paper's pore size. Given these considerations, the tested filter paper should be carefully selected, as its pore size characteristics directly influence fluid dynamics.

Whatman No.1 filter paper, composed of approximately 98% α -cellulose, is widely used in μ PAD research due to its uniform thickness and consistent capillary action.^{56,73,74} In addition to traditional filter papers, conductive carbon fiber and metal fiber papers have enabled advances in electrochemical sensing.⁷⁵ With its staggered fibers, conductive carbon fiber paper reduces electron transport distance and increases surface area, facilitating the detection of ionic analytes.⁷⁶ Similarly, conductive carbon inks and thin metal layers integrated into μ PADs have advanced applications in detecting compounds and overcoming challenges like super-Nernst responses in heavy metal detection.⁷⁷ These material innovations underscore the versatility of μ PADs across diverse analytical and diagnostic platforms.

1.4 2D vs 3D designs

Since first published,⁵⁶ two-dimensional (2D) μ PADs represent a significant advancement in point-of-care diagnostics. These coplanar devices are relatively simple to fabricate and operate, with all assay steps occurring on a single paper layer. The versatility of 2D μ PADs has led to their widespread adoption in literature, employing multiple signal modalities, as will be discussed in the following sections.^{61,78} The design of 2D μ PADs can be further categorized based on their geometry, functional components, or signal modality.⁴⁸ While 2D μ PADs offer numerous advantages, they also face certain limitations. Signal retention can be problematic due to lateral diffusion within the paper, potentially leading to reduced sensitivity and spatial resolution. Additionally, the confined nature of the 2D format may restrict the signal strength and dynamic range of some assays.⁷⁹ These challenges have motivated the development of three-dimensional μ PAD designs, which aim to address these limitations and offer enhanced versatility and accuracy in signal readout.

Compared to 2D devices, 3D μ PADs can offer more intricate designs and strategies. This 3D μ PADs capability is enabled through the combined vertical and lateral fluid flow through multiple layers of paper and interactions with different reagents.⁸⁰ These designs could involve constructing multilayered fluidic flow channels, incorporating channel microvalves, and/or folding paper substrates in an origami-based fashion.^{81–83} In these designs, using the vertical flow devices employs gravity-driven flow, which could improve sample processing and reduce analysis time. Additionally, vertical flow could afford incorporating multiple functional layers, each serving a specific purpose such as sample filtration,⁶³ reagent storage,⁸⁴ or detection.⁸⁵ Origami-inspired designs enable creating complex fluidic networks through simple folding operations, allowing for feasible control over sample flow and, hence, reaction timing. This approach has been useful in developing multiplexed assays.^{83,86} Given the structure variability that they support, 3D μ PADs are a valuable design option to advance the detection technology at the POC.

1.5 Signal modalities in μ PADs

In recent years, there have been notable advancements in colorimetric,⁸⁷ electrochemical,⁸⁸ fluorescence,⁸⁹ chemiluminescence,⁹⁰ among other signal modalities within microfluidic technology. Each approach provides a certain detection effectiveness which varies with respect to the involved assay, the sample matrix complexity, and the analyte physicochemical properties. In line with the focus of this work, the following section will provide discuss both colorimetric and electrochemical detection methods.

1.5.1 Colorimetric Detection

Colorimetry is one of the most common detection techniques integrated with the μ PAD at the point-of-care.^{91–93} In this technique, the presence of the analyte is associated with a visual color change, resulting from a single or a series of chemical reactions. The liquids undergoing this, or these, chemical reaction(s) are driven to the reaction area by capillary action – one of the main advantages of paper substrates. The extent of the change in that resulting color is proportional to the concentration of the measured analyte.⁸⁷ Though this color change is frequently detectable by naked eye, analytical software tools, such as ImageJ,⁹⁴ can be employed for more precise measurements. At the core of creating a reliable colorimetric sensor lies the selection of colorant. Multiple classes of colorimetric reagents have been incorporated with μ PADs including inorganic nanoparticles⁸⁹ and organic chromophores.⁹⁵ Besides colorant selection, the choice of paper substrate can also influence the desired signal output, depending on the physicochemical properties of the analyte of interest (**Figure 1.3**).⁶³

Relying only on visual detection to get, at least, a qualitative result makes colorimetry a promising detection technique to integrate with μ pads. This is because it adds to the cost-effectiveness and simplicity of the device operation. However, this detection method can fall short on providing a stable color for extended period of time. This is because the colored product formed at the reaction zone usually diffuses, causing variability in the color formed, e.g. coffee-ring effect.^{96,97} This variability could influence the user

interpretation of the result which would influence the accuracy of the diagnostic decision made accordingly.

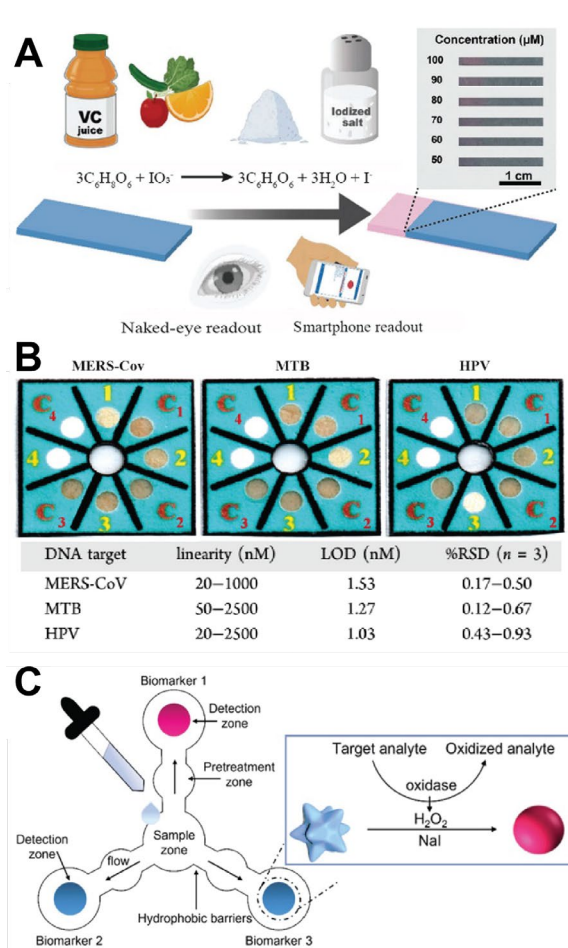


Figure 1.3. Microfluidic paper-based analytical devices (μPADs) with colorimetric reporter. (A) POC μPADs for detections of iodate and ascorbic acid, representative color change in presence of different concentration of ascorbic acid was shown. Reprinted with permission from ref [91]. Copyright 2023 John Wiley and Sons. (B) Paper-based colorimetric DNA sensor for detecting viral or bacterial oligonucleotide, the color change, concentration linearity and LOD for detecting MERS-Cov, MTB and HPV were presented. Reprinted with permission from ref [92]. Copyright 2017 American Chemical Society. (C) Simultaneous detection of three salivary biomarkers using monolithic paper-based device. Reprinted with permission from ref [93]. Copyright 2021 MDPI.

1.5.2 Electrochemical Detection

While colorimetry is the most commonly integrated technique with μ PADs for detection applications, it does not always provide low limits of detection for target analytes. Alternatively, electrochemical detection could provide more sensitive results that are more adequate for quantitative measurements.⁵⁷ In electrochemical assays, the concentration of the target analyte is correlated to a change in the number of electrons at the working electrode surface. That change in is typically related to either losing or gaining electrons by the interacting ions, denoted as electrochemical oxidation or reduction, respectively. To keep the neutrality of an electrochemical system, both the oxidation and reduction reactions are inseparable, since the gaining electrons by one ion necessitates that another ion in the reaction medium will lose these electrons. The redox process refers to the reduction and oxidation reactions which simultaneously take place in an electrochemical reaction.^{88,98} This redox process generates a current, in amperometric detection, or a potential change, in potentiometric detection, that is measured by the μ PAD.⁹⁹ For amperometric measurements, the relationship between the analyte concentration and the current is governed by Faraday's laws of electrolysis,¹⁰⁰ which correlate the amount of substance reacted at the electrode to the electric charge passed through the system. In chronoamperometric measurements, this relationship is further described by Cottrell's equation,¹⁰¹ which relates the diffusion-controlled current to the analyte concentration over time.

$$i(t) = \frac{n F A c \sqrt{D}}{\sqrt{(\pi t)}}$$

where $i(t)$ is current at time t (A), n is number of electrons transferred, F is Faraday's constant (96485 C/mol), A is electrode area (cm^2), c is bulk concentration of the analyte (mol/cm^3), D is diffusion coefficient of the analyte (cm^2/s), and t is time (s).

For potentiometric measurements, however, the relationship between the analyte concentration and the electrochemical signal is governed by the Nernst equation,¹⁰² which describes the potential of an electrochemical cell as a function of the concentration of the analyte.

$$E = E^o - \frac{RT}{nF} \ln Q$$

where E is the electrode potential under non-standard conditions (V), E^o is the standard electrode potential (V), R is the universal gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$), T is the temperature in kelvins (K), and Q is the reaction quotient, which is the ratio of the activities (or concentrations) of products to reactants. Besides amperometry,¹⁰³ potentiometry,¹⁰⁴ and voltammetry,¹⁰⁵ other electrochemical techniques that have been used for μPAD sensing applications include conductometry, electrochemical impedance, and coulometry, each dependent on specific electrical characteristics.⁷⁰

The electrochemical system involves a standard three-electrode setup; namely a working electrode, a counter electrode, and a reference electrode. The working electrode (WE) is where the electrochemical reaction of interest occurs. It is responsible for the oxidation or reduction of the analyte. The potential of the working electrode is controlled relative to the reference electrode, and the resulting current is measured. The material of the working electrode is chosen based on the specific application and the nature of the analyte, with carbon and gold being among the most used materials. Counter electrode (CE), also known

as the auxiliary electrode, completes the electrical circuit by allowing current to flow between itself and the working electrode. It balances the current observed at the working electrode, ensuring that the electrochemical reaction can proceed. To avoid interference with the reactions occurring at the working electrode, the counter electrode is typically made of an inert material, such as platinum or carbon. Reference electrode (RE) provides a stable

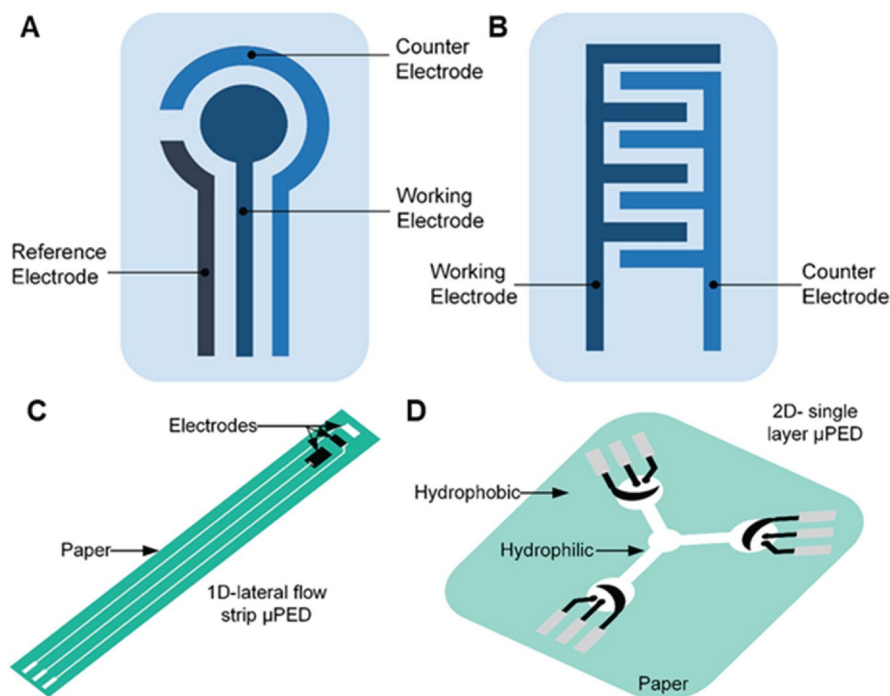


Figure 1.4. Patterns with optimal surface area for different electrochemical paper-based analytical devices (ePADs). (A) the three-electrode system and (B) the two-electrode system. (C) 1D lateral flow strip with a three-electrode system; (D) 2D single layer μ PADs with three-electrode system. Reprinted with permission from ref [108]. Copyright 2023 Elsevier.

and known potential against which the working electrode's potential is measured. While it does not participate in the electrochemical reaction, the reference electrode ensures an accurate and reproducible measurement by maintaining a constant potential. In a conventional electrochemical cell, common reference electrodes include the silver/silver chloride (Ag/AgCl) electrode and the saturated calomel electrode (SCE).^{106,107}

The above-described three-electrode system is often used for electrochemical sensing technology, especially with techniques like chronoamperometry (CA) (i-t curve), open circuit potential (OCP), voltammetry, and electrochemical impedance spectroscopy (EIS).¹⁰⁸ On the other hand, two-electrode systems, which utilize only working and counter electrodes, are used for potentiometry, OCP, and some EIS measurements (**Figure 1.4**).¹⁰⁹ For use at the POC, electrochemical μ PADs have been designed 2D or 3D, as previously described. Running these electrochemical μ PADs require a potentiostat to apply a current and/or potential on the electrochemical system and measure the output signal, based on the applied technique. The majority of potentiostat employed for electrochemical analysis are costly and lab-binding, demanding operation by skilled professionals. While this option might not suit a limited-resource setting use, various portable alternatives have been developed to be used at the point-of-care. Typical examples include CheapStat,¹¹⁰ Dstat,¹¹¹ SweepStat,¹¹² NFC-enabled potentiostat,¹¹³ and Universal Wireless Electrochemical Detector (UWED).¹¹⁴ Nonetheless, some of these devices still require computers for operation, which might not always be available at the point-of-care. Alternatively, smartphone-integrated electrochemical sensing analogs can stand out a programmable platform for wearable and portable applications.¹¹⁵

1.6 Challenges/practical considerations in signal formation and interpretation

1.6.1 Colorimetric μ PADs

To develop impactful point-of-care technologies, device prototypes must balance analytical performance and compatibility with field-based constraints. Ideally, signal measurement should be accurate enough to detect target analytes, yet simple enough

for untrained users to operate and interpret. Among the various signal modalities, colorimetric detection is the most widely used.^{87,89} Although colorimetric devices that provide visual readouts are often deemed simple, field trial operators have reported challenges in interpreting results, particularly among inexperienced users—a concern that typically diminishes with practice.¹¹⁶ However, forming these colorimetric signals can require tedious operational steps, such as precise liquid handling, manipulation of the device architecture, and the use of external equipment like UV sources, incubators, or smartphone cameras.^{117,118}

Colorimetric signals are often utilized for qualitative (i.e., on/off) measurements that can be visually inspected for the presence or absence of a color change.^{119,120} For semi-quantitative or quantitative assessments, readout zones may be compared to printed reference guides or designed for distance-based measurements.¹²¹ Smartphone integration has also become a common approach for analyzing signal intensity.¹¹⁸ The first paper-based microfluidic devices,⁵⁶ as well as many subsequent prototypes,^{117,122,123} primarily employed chemistries that formed soluble colorimetric reaction products within the patterned paper zones (e.g., for glucose or albumin detection). However, unlike simple litmus paper or urine dipstick tests, colorimetric products in μ PADs often exhibit non-uniform distribution within the test zone.⁸⁴ This issue can arise from unoptimized storage conditions (e.g., the “coffee-ring effect” from dried reagents) or imbalances in product diffusion and fluid flow (i.e., wicking effects).⁶⁹

Within circular test zones, such heterogeneous signals, can lead to interpretation errors, especially when compared against printed reference guides that assume uniform color distribution. Addressing these challenges requires careful consideration of signal

formation dynamics and fluid flow behavior to enhance the reliability and usability of colorimetric μ PADs.

1.6.2 Electrochemical μ PADs

Fabricating electrodes on paper is challenging due to its inherently porous and rough texture. Existing fabrication methods largely stem from printed electronics and microfabrication, though some techniques are specifically designed for electrode deposition on paper.¹²⁴ Several criteria should be taken into consideration when evaluating which of these methods is more practical for electrode fabrication. These criteria include, but not limited to, electroanalytical sensing performance, accessibility of fabrication equipment, scalability for high-throughput manufacturing, tunability of the deposition method, compatibility with various paper substrates, adaptability to design changes for rapid prototyping and ecological impact of each fabrication method.⁵⁷ Using these criteria, multiple techniques have been recently compared to evaluate their effectiveness for practical incorporation of electrodes on paper substrates (**Figure 1.5**). These techniques include screen printing,¹²⁵ inkjet printing,¹²⁶ metal sputtering,¹²⁷ pencil drawing,¹²⁸ and laser pyrolysis.¹²⁹ While each method has its own strengths and weaknesses, no single approach is universally ideal. However, screen printing, inkjet printing, and laser pyrolysis could be more suitable for high-throughput manufacturing. Conversely, metal sputtering and pencil drawing are more niche techniques, each with distinct advantages in terms of accessibility and electrode deposition resolution.⁵⁷ Eventually, the ideal choice should depend on the specific application and intended usage environment.

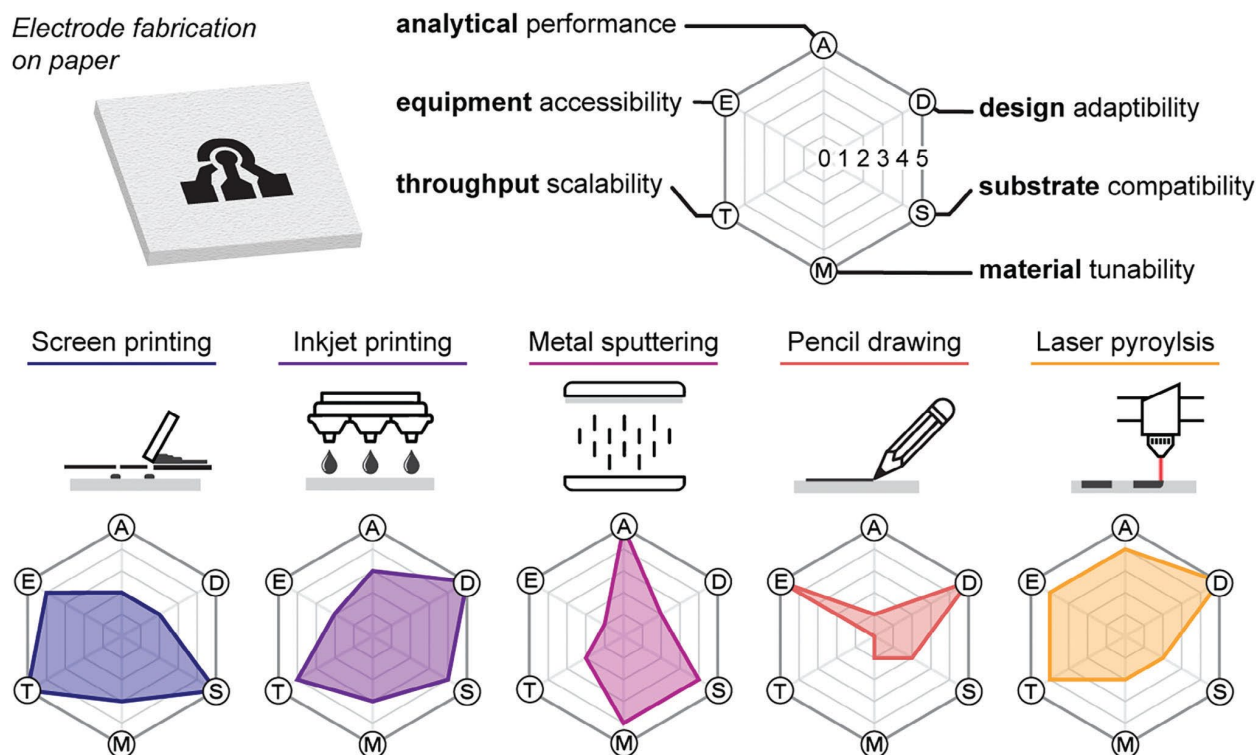


Figure 1.5. Comparison of the most popular and emerging technologies for electrode fabrication on paper. The criteria are qualitatively assigned to each fabrication method and presented in radar charts for each fabrication technique (bottom). Reprinted with permission from ref [57]. Copyright 2024 John Wiley and Sons.

While ePADs reported in the literature hold great promise for point-of-care applications, the manufacturing process can face several challenges. A primary concern is maintaining a minimal and consistent distance between the working and reference electrodes during electrode incorporation or deposition onto the paper substrate.¹³⁰ This consistency is crucial because the distance between the WE and RE directly affects the solution resistance (R_s), also known as ohmic resistance or Type 1 resistance, of the electrochemical cell.^{131,132} This type of resistance arises from the ionic conductivity of the electrolyte between these electrodes and influences the accuracy of potential /measurements. A smaller WE-RE distance helps to minimize this uncompensated solution resistance, resulting in higher signal currents and greater sensitivity in detection applications. While electrochemical workstations may attempt to compensate for some of

this resistance, full compensation is not always achievable, making a minimal WE-RE distance essential for optimal performance.^{133–136}

Achieving minimal interelectrode distance consistently during fabrication poses some practical challenges, particularly when using simpler manufacturing techniques. While advanced methods like laser micromachining¹³⁷ or photolithography¹³⁸ can achieve precise electrode placement, they are costly and may not be suitable for low-cost POC devices. Simpler techniques, such as screen printing or stencil printing, however, often struggle to maintain precise and uniform electrode spacing. For instance, in the first reported ePAD paper,¹³⁰ Henry and coworkers specified an interelectrode distance of 0.15 mm between the working and reference electrodes. Nevertheless, visual analysis of the fabricated devices revealed inconsistencies in maintaining this distance, highlighting the challenge of achieving precise electrode positioning even in early designs. While the early studies utilized coplanar,^{139,140} electrode configurations, more research has explored three-dimensional ePAD designs to address these challenges, aiming to improve consistency in electrode spacing and reduce solution resistance more effectively.

For instance, many origami-inspired devices have been reported in which one cell electrode is deposited on one layer while the other two electrode are co-deposited on a different layer (**Figure 1.6**).^{141–144} While this approach offers more flexible manufacturing, the manual foldable nature of these device designs could probably introduce variability in layer alignment and uneven distribution of contact pressure between the electrodes. During assembly, slight misalignments or differences in how tightly the layers are folded could create air gaps or inconsistent contact between the electrode layers, which might lead to variations in electrical properties and signal reliability. Since the origami-inspired

folding does not firmly secure the layers in the same manner every time, some electrode areas may experience better contact with (i) electrolyte and/or (ii) other electrode, through electrolyte, than others.¹⁴³ These inconsistencies can affect the reproducibility of electrochemical measurements, making it challenging to standardize the manufacturing process for large-scale production.

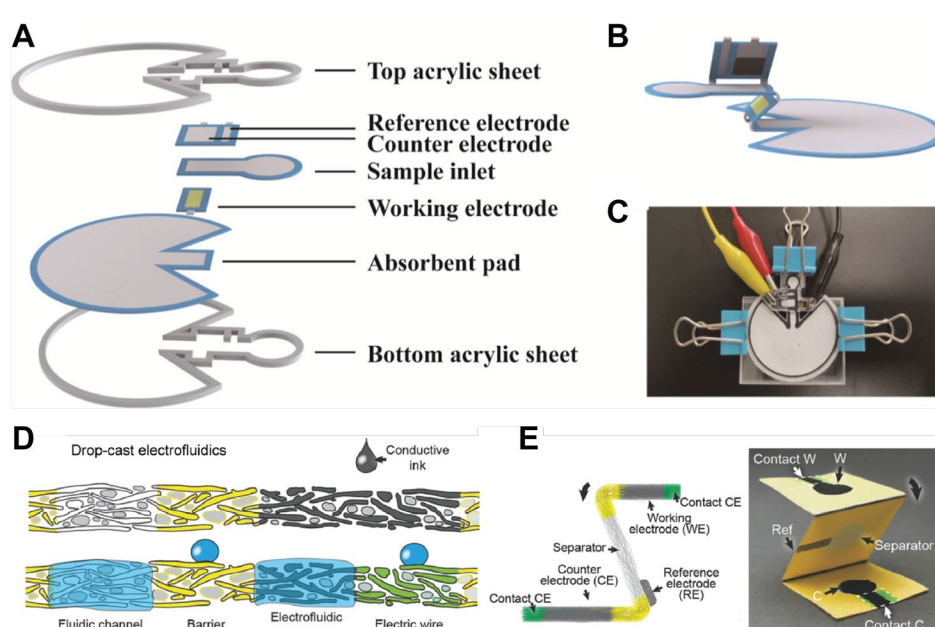


Figure 1.6. Three-dimensional electrochemical paper-based analytical devices (ePADs). (A-C) detailed and compact structures for origami inspired device with quasi-steady flow. Reprinted with permission from ref [143]. Copyright 2024 American Chemical Society. (D-E) foldable ePAD with electrodes fabricated through a combination of wax printing and drop-cast conductive inks. Reprinted with permission from ref [144]. Copyright 2024 John Wiley and Sons.

While the paper porous structure and hydrophobic patterning enable flexibility, these features can also lead to challenges in achieving consistent electrical and fluidic performance. For instance, the ink-based electrodes may not always uniformly penetrate the paper substrate, resulting in variations in conductivity and response times. Additionally, folding and handling the devices could cause mechanical stress on the

electrode layers, potentially leading to cracking or delamination, which would impact the long-term durability and reliability of the devices. The literature on ePADs often lacks comprehensive reporting on failure rates, which is a critical oversight given the various factors that can contribute to signal instability. Overcoming these issues would likely require refining the fabrication techniques and materials to better withstand mechanical stress while maintaining the device's low-cost advantage. For instance, addressing the air gap introduction limitation would require more robust mechanical securing methods or adhesive strategies to maintain consistent electrode positioning. However, this modification should be carefully considered, to avoid increasing the complexity and cost of the device assembly process.

1.7 Conclusions

Indeed, the development of 3D μ PADs has introduced a new level of functionality and complexity to the field. By utilizing intricate manipulations and designs, these devices have increasing potential to be highly valuable for POC technology. While controlling device fabrication and signal clarity remain a challenge, overcoming these issues is critical to increase the device output reliability and usability. Throughout my doctoral research, I have worked toward developing multiple designs to fabricate and test dimensional paper-based microfluidic devices. These designs enable more versatile fabrication of device prototypes that would offer measurement of different classes of analytes. These measurements are supported using colorimetric and electrochemical signal modalities.

The following chapters detail the development of signals at converging liquid fronts to create line-format readouts of soluble assay products in three-dimensional paper-based devices (Chapter 2),⁶⁴ further development of these linear readouts for enhanced signal intensity and localization (Chapter 3), a new device design strategy to create an electrochemical paper-based analytical device with controlled interelectrode distance and interchangeable electrode configuration (Chapter 4), and enhanced iterations for this electrochemical device that supports multiplexity and free-standing (Chapter 5).

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Chapter 2

Generating signals at converging liquid fronts to create line-format readouts of soluble assay products in three-dimensional paper-based devices

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2.1 Introduction

Lateral flow test (LFT) strips have achieved widespread success in performing diagnostic tests at the point of care (POC) because their common architecture supports the development of a variety of immunoassay formats, utilizes scalable manufacturing processes to facilitate production and dissemination, and enables the interpretation of assay results by visual inspection. Nonetheless, the lateral flow device architecture largely limits testing to analytes that can be captured by immobilized molecular recognition agents (e.g., antibodies¹ or nucleic acids²). Also, LFTs predominantly offer qualitative (i.e., presence or absence of a test line) or semi-quantitative determination of one highly concentrated biomarker.^{3,4} This compromised signal resolution, in addition to their inability to control the sample flow rate, which could lead to incomplete reagent mixing time, would inherently restrict how broadly LFTs can be applied as a platform for POC measurements.⁵ Furthermore, LFTs often have a limited capacity for multiplexing – commonly testing for the presence of a single analyte or two.⁶ This limitation might restrict the applicability of LFTs at the POC in case the presence of multiple biomarkers needs to be simultaneously detected.⁷

As an alternative to LFTs, paper-based microfluidic devices or microfluidic paper analytical devices (μ PAD) have drawn recent appeal due to their potential to support a broader test menu than LFTs (e.g., cell counts, nucleic acid amplification, immunoassays),^{8–13} compatibility with a wide range of assay readout methods (e.g., colorimetric, fluorescent, electrochemical, chemiluminescent),^{14–18} ability to support device complexity (e.g., evaporative concentration, origami or “pop-up”, fluid control

valves)^{8,19–21} and utilize creative options for generating a user interface (e.g., wicking distance, timed readout, zones filled, and text appearance).^{22–26}

The first paper-based microfluidic device, and similarly many subsequent prototypes, demonstrated the detection of analytes (e.g., glucose and albumin) using chemistries that formed soluble colorimetric reaction products within the void volume of patterned paper zones.¹⁵ However, unlike litmus paper or urine dipstick tests, colorimetric products in μ PADs can have a non-uniform distribution within the test zone, which is caused either by unoptimized storage conditions (e.g., “coffee-ring effect” from dried reagents) or issues with balancing product diffusion or net fluid flow (i.e., by wicking).^{27–29} Heterogeneous signals in test zones, regardless of whether devices are designed to be operated in two- or three-dimensional configurations, can result in interpretation errors, particularly when printed read guides are presented with colors that are uniform in hue and spatial position (**Figure 2.1**).³⁰

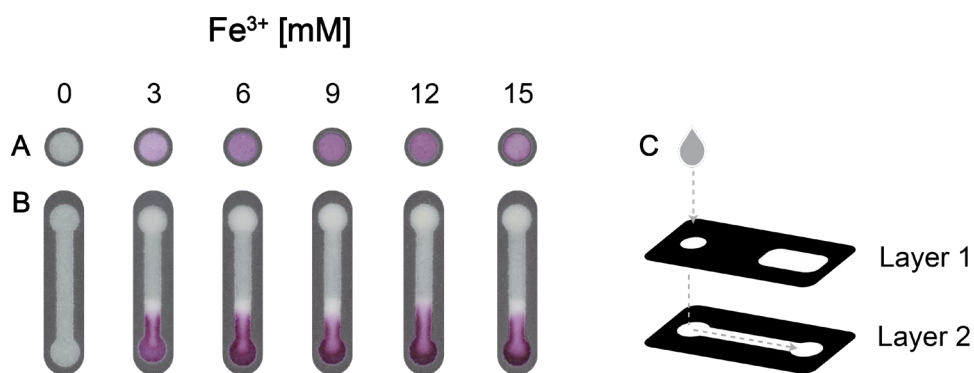


Figure 2.1. Comparison of signal formation for a colorimetric assay for Fe(III) in different paper device geometries treated with Ferrozine and ascorbic acid. (A) Zones patterned in single layers of paper produce a purple reaction product that is difficult to differentiate at different concentrations of Fe(III) and with a non-monotonic increase in intensity. (B) Channels positioned at the ends of simple three-dimensional devices (C) produce non-uniform distributions of color whose intensities and positions make qualitative and quantitative interpretations difficult to perform. Reproduced with permission from Abdullah, I.H., Wilson, D.J., Mora, A.C., Parker, R.W. and Mace, C.R., **2023**. Generating signals at converging liquid fronts to create line-format readouts of soluble assay products in three-dimensional paper-based devices. *Lab on a Chip*, 23(18), pp.4010-4018.

A variety of strategies have been developed to address challenges related to the position and distribution of colorimetric signals in patterned paper zones. For assay formats that require molecular recognition, incorporation of nylon¹³ or nitrocellulose³¹ membranes into device designs can support reagent immobilization by physisorption and capture of analytes under continuous flow. Alternatively, the surfaces of cellulosic paper fibers can be functionalized chemically^{32–34} or biochemically (e.g., via engineered proteins)^{35,36} to support the immobilization of reagents.^{37,38} Detection reagents can also be crosslinked throughout the open pore structure of a paper substrate to create an immobilized network of materials that would otherwise diffuse throughout the paper architecture.^{39,40}

Using origami-style vertical flow assays is also an alternative approach to show improved colorimetric signal.⁴¹ Image analysis of the colorimetric signal using smartphones coupled with advanced algorithms can also enhance signal interpretation and quantification.⁴² While these approaches provide assay developers with a variety of device design options, they each require target analytes to be captured or reagent-specific functionalization strategies to restrict the formation of a colorimetric signal to a defined location. Signal formation in a defined location can also be limited by the user's ability to precisely fold the origami device layers. In case of using smartphone-based signal interpretation, ensuring proper ambient lighting is necessary to avoid compromised color analysis precision.

To generate spatially-controlled, colorimetric signals without the requirements for molecular recognition or reagent immobilization, we developed a new class of paper-based microfluidic devices that form and confine colorimetric signals to the interfaces of converging liquid fronts (**Figure 2.2A**). The fluidic network patterned into the device splits a single sample inlet into separate and ultimately opposing channels. Analytes can undergo partial reactions with unique reagents stored in each channel. The products of these partial reactions are directed towards a common test zone. Others have shown paper devices designed for the sequential, timed delivery of reagents from multiple channels that are directed towards a test zone.^{43,44} In these devices, flow is continuous as long as sufficient volume is available to saturate the paper. Because paper-based devices are inherently self-metering,⁸ if a single channel is split and recombined, net fluid flow stops when the advancing fluid fronts meet. At this interface between fronts, where reactions between previously separated reagents are now possible and controlled by

diffusion, assay products can be isolated without affinity-based capture and lead to the formation of colorimetric signals that are confined to the contour of the interface.

Using this device design strategy, we demonstrate the detection of multiple classes of analytes—ions, small molecules, and enzymes—by producing line-format signals like those provided by LFTs but resulting from soluble reaction products. We demonstrate our approach by developing assays for iron (III), acetylcholinesterase, and lactate. Additionally, we demonstrate the scalability of this method by increasing the number of fluid fronts that converge in a common detection zone to create multiplexed assays: two converging channels create one interface (i.e., singleplex) while three or four converging channels create three or four interfaces, respectively (i.e., multiplex). We demonstrate the ability to create multiple lines in common zone by combining test and control assays for both iron (III) and acetylcholinesterase. Overall, these devices provide a new method of generating user-friendly signal formats for analytes that are currently incompatible with molecular recognition-style assay architectures.

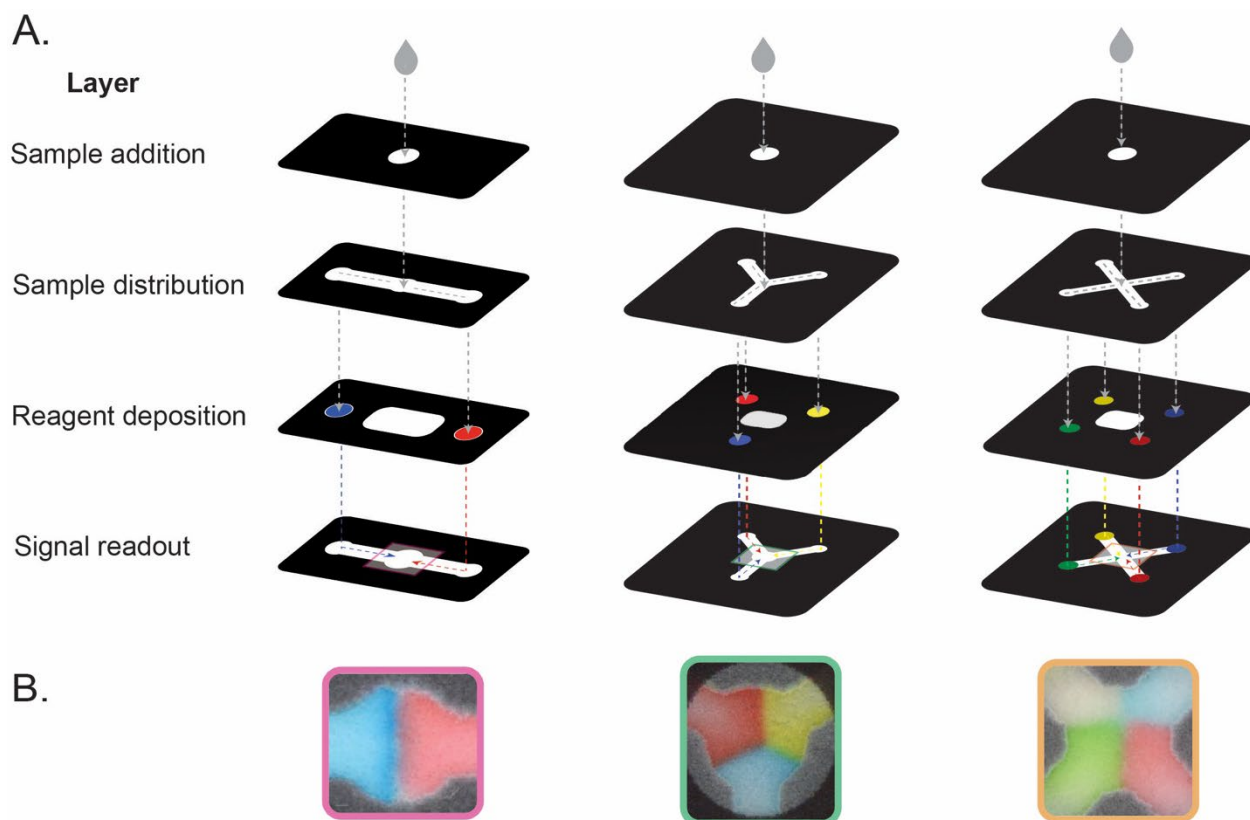


Figure 2.2. Schematics of three-dimensional paper-based devices designed to form single and multiple liquid interfaces in a single detection zone. (A) Unique device geometries can be designed to form one-line, three-line, and four-line interface patterns. Dashed lines indicate fluid flow within and between layers. Upon application of a sample, fluid is distributed to opposite sides of the device to rehydrate stored reagents. After rehydration, solutions are directed toward a central detection zone where they meet to form a liquid interface. (B) In these examples, colored dyes were used to demonstrate interfacial signal development. The detection zone is approximately 5 mm in diameter. Reproduced with permission from Abdullah, I.H., Wilson, D.J., Mora, A.C., Parker, R.W. and Mace, C.R., **2023**. Generating signals at converging liquid fronts to create line-format readouts of soluble assay products in three-dimensional paper-based devices. *Lab on a Chip*, 23(18),pp.4010-4018.

2.2 Device design considerations

The features patterned into the paper device autonomously control how a sample is split into opposing channels and recombined to form interfaces. The properties and quantities of reagents stored within paper layers determine rehydration times and the rates at which they wick through the device and toward the ultimate detection zone.^{44,45} For assays that require reagents with different chemical properties (e.g., solubility, rehydration rate, or interactions with the surrounding paper matrix) to be stored on opposite sides of the device, a number of device architecture variables may be adjusted to provide timed convergence of liquid fronts at the desired location.^{14,46} These variables include the geometries of patterned paper layer features, such as hydrophilic channels and the readout zone, and proper layer alignment, see **Table 2.1** and **Table 2.2**.^{47,48}

To ensure a proper layer alignment, we used an in-house developed a layer alignment pin tool and protocol to position device features reproducibly and ensure the interface position is only dependent on synchronized delivery of fluid fronts to the detection zone.⁴⁹ This approach consistently provides interfaces at the center of device detection zones (**Figure 2.3A**). In the assays described below, a symmetrical channel design reproducibly afforded interfaces in the center of the test zone (**Figure 2.3B**).

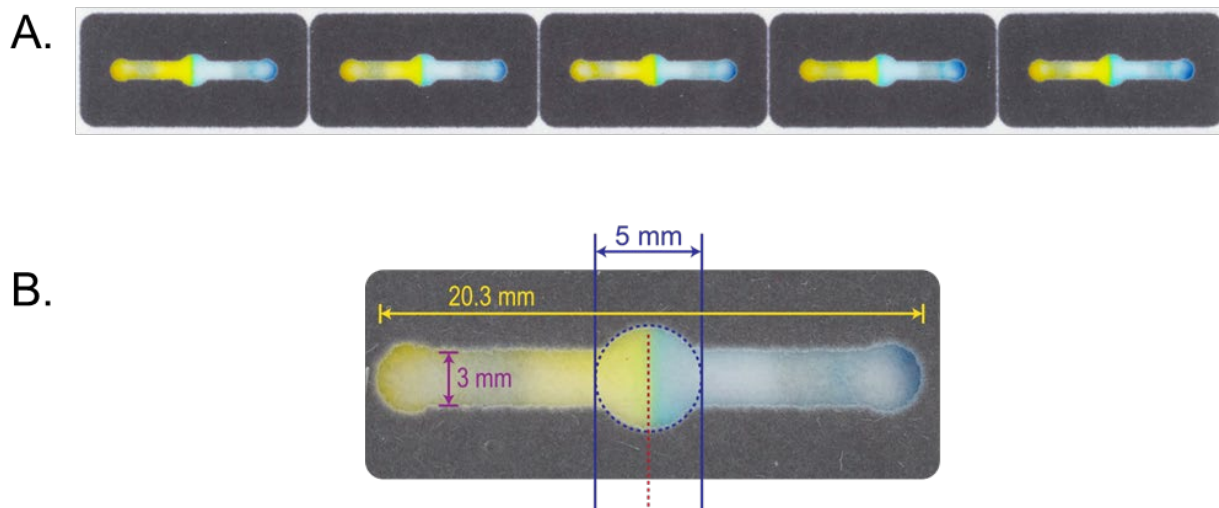


Figure 2.3. Devices assembled using the in-house-developed layer alignment tool. (A) The fabrication approach consistently provides interface formation within the center zone of the detection layer for devices that contain equivalent amounts of stored dyes. (B) Schematic of the device channel layer containing the circular readout zone where assay interfaces are formed. Measurements of channel length (yellow), channel width (magenta), and zone width (blue) are provided, as is a means (red dashed line) to identify the absolute center of the readout zone that could be reached if flow from both channel inlets were perfectly timed. Reproduced with permission from Abdullah, I.H., Wilson, D.J., Mora, A.C., Parker, R.W. and Mace, C.R., **2023**. Generating signals at converging liquid fronts to create line-format readouts of soluble assay products in three-dimensional paper-based devices. *Lab on a Chip*, 23(18), pp.4010-4018.

In addition, to improve signal contrast, we decided to pattern a white hydrophilic area in the layer preceding the readout zone layer – the reagent storage layer. The centered white area enhances the colorimetric signal visibility by creating a wet background that is 60 (± 1) a.u. brighter on a greyscale intensity. A sheet of transparent laminate was added below the bottom layer to seal the device, prevent evaporation, and still visualize the result (**Figure 2.4**).

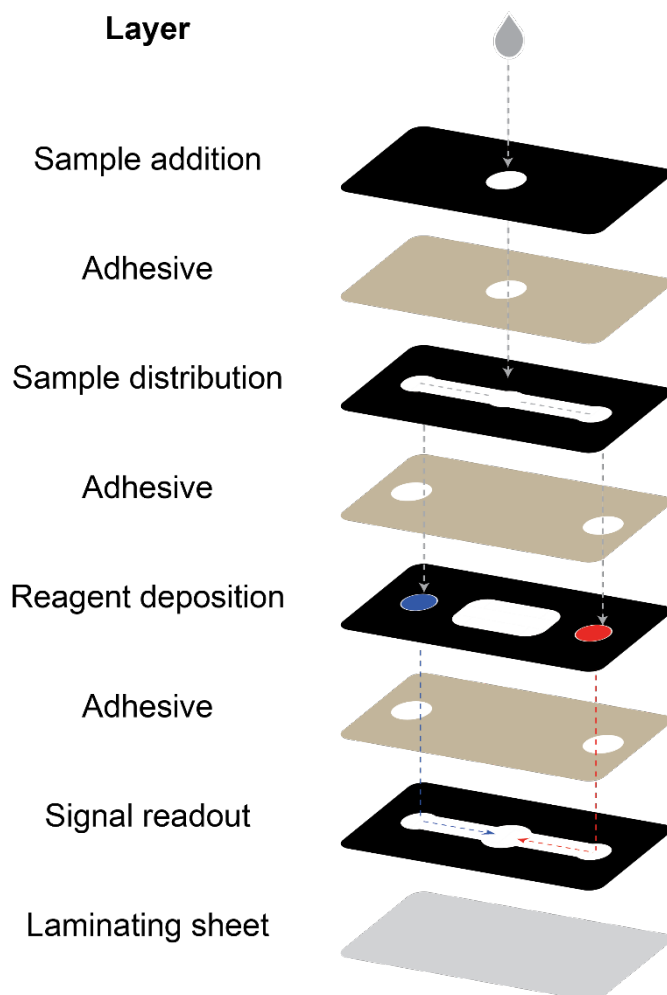


Figure 2.4. Full device schematic. The four wax-printed layers are backed with adhesive layers and stacked. The final wax printed layer is backed with a laminate sheet to assemble a full device. Reproduced with permission from Abdullah, I.H., Wilson, D.J., Mora, A.C., Parker, R.W. and Mace, C.R., **2023**. Generating signals at converging liquid fronts to create line-format readouts of soluble assay products in three-dimensional paper-based devices. *Lab on a Chip*, 23(18), pp.4010-4018.

2.2.1 Accuracy and precision of interface position

Forming a linear colorimetric signal in a three-dimensional paper-based device can be influenced by multiple factors. The factors studied include hydrophilic channel geometry, readout zone geometries, paper grade, and sample volume. We investigated the influence of these factors on position, width, and intensity of the colored linear signal formed at the interface.

2.2.1.1 Influence of channel geometry

We studied the impact that the channel length and width and readout zone shape have on the positioning of formed interfaces. We added 1 μL of yellow and blue food coloring on opposite sides of the reagent storage layer of our full, four-layer device. We dried all reagents for 7 minutes at 65 °C, then added 30 μL of DI H_2O to initiate flow. We scanned devices after 3 minutes and used Line and Plot Profile tools in ImageJ to measure the positions of formed interfaces within the readout zone. If the interface formed off-center to the left, measurements were assigned a negative value, while positive values were assigned to interfaces formed off-center to the right; interfaces perfectly in the center of the readout zone have a measured offset of 0 mm.

We measured the position within the readout zone where interfaces formed using devices that varied in channel width (2, 3, and 4 mm) and channel arm length (7, 10, and 13 mm). These data are compiled in (**Table 2.1**). We observed that channel width had minimal impact on the accuracy of the interface position, with 3-mm channels having modestly better precision than channels that were narrower or wider. Channel arm length, however, did have a substantial effect on either the accuracy or precision of the interface position,

where 10-mm channel arm length had the best performance. According to that, we selected 3 x 10 mm channels for all further experiments.

Table 2.1. Accuracy and precision of interface position as a function of channel width and arm length (n=10). Reproduced with permission from Abdullah, I.H., Wilson, D.J., Mora, A.C., Parker, R.W. and Mace, C.R., **2023**. Generating signals at converging liquid fronts to create line-format readouts of soluble assay products in three-dimensional paper-based devices. *Lab on a Chip*, 23(18), pp.4010-4018.

	Dimensions (mm)	Offset distance (mm)	CV%
Channel width	2	-0.2 ± 0.5	21
	3	-0.3 ± 0.3	13
	4	0.3 ± 0.5	17
Channel arm length	7	1.8 ± 0.7	17
	10	-0.3 ± 0.3	13
	13	-0.2 ± 1.1	50

2.2.1.2 Influence of readout zone geometry

Using the same experimental conditions described for the channel geometry section, we also varied the shape of the readout zone. In this study, we compared square, circular, and straight zone geometries where interfaces would form (**Table 2.2**). All three shapes formed interfaces with similar accuracies and precisions, and we selected circular test zones for all further experiments.

Table 2.2. Accuracy and precision of interface position as a function of readout zone geometry (n=10). Reproduced with permission from Abdullah, I.H., Wilson, D.J., Mora, A.C., Parker, R.W. and Mace, C.R., **2023**. Generating signals at converging liquid fronts to create line-format readouts of soluble assay products in three-dimensional paper-based devices. *Lab on a Chip*, 23(18), pp.4010-4018.

Zone shape	Image	Offset distance (mm)	CV%
Square		0.1 ± 0.4	12
Circle		-0.3 ± 0.3	13
Straight		0.0 ± 1.0	11

2.2.1.3 Influence of paper grade

We have begun preliminary evaluations of the choice of paper grade on the formation of interfaces. We still do not fully understand the roles that the material properties of paper play in assay performance, since our findings are too preliminary. However, as can be seen below, **Table 2.3**, properties such as particle retention size, a crude surrogate for pore size, do not significantly influence the position of where the interface is formed within a test zone. Alternatively, Ahlstrom 54 paper grade showed the lowest variation with respect to the interface formation position. It is unclear if this observation is only related to the retention size of Ahlstrom 54, 10 μm , especially that Whatman 1 paper grade showed the highest variation, CV = 59%, while it has a similar retention size, 11 μm . Further investigation is needed to understand if these findings are correlated with the pulp processing conditions exercised by each manufacturer such as pH and chemical treatment.

Table 2.3. Accuracy and precision of interface position as a function of paper grade (n=5).

Paper grade	Particle retention size (μm)	Offset distance (mm)	CV%
Whatman 4	20–25	-0.6 ± 0.5	26
Ahlstrom 55	15	-0.3 ± 0.4	20
Whatman 1	11	-1.3 ± 0.7	59
Ahlstrom 54	10	-0.4 ± 0.1	4
Ahlstrom 613	6	-0.8 ± 0.8	47
Ahlstrom 601	2.5	-0.5 ± 0.5	27

2.2.1.4 Influence of sample volume

We used an Fe(III) assay to investigate the effect of applied sample volume on the absolute position, width, and color intensity of interfaces formed in our paper-based devices. We found that the minimum volume required to rehydrate stored reagents and drive liquid fronts to converge in the readout zone was 14 μL . However, it would be desirable to determine the range of volumes that were suitable for assays to be performed reproducibly and not just at the minimum where any errors in sample application could lead to a device failure. We performed assays with sample volumes between 20–100 μL and measured offset distances of the formed interface, the width of the interface 7 minutes after formation of color, and the intensity of the red channel in the scanned image, **Table 2.4**. While all volumes above 20 μL had similar offset distances and channel widths, only volumes above 30 μL had reached a plateau in the intensity of the interface signal and no benefit was observed to using larger volumes. We therefore chose to use 30 μL sample volumes to develop all singleplex assays.

Table 2.4. Sample volume influence on signal positioning, width, and intensity (n=5). Reproduced with permission from Abdullah, I.H., Wilson, D.J., Mora, A.C., Parker, R.W. and Mace, C.R., **2023**. Generating signals at converging liquid fronts to create line-format readouts of soluble assay products in three-dimensional paper-based devices. *Lab on a Chip*, 23(18), pp.4010-4018.

Sample volume (μL)	Offset distance (mm)	CV%	Line width (mm)	CV%	Red channel intensity (a.u.)	CV%
20	-0.3 ± 0.5	23	0.9 ± 0.1	10	32.5 ± 7.9	24
30	-0.2 ± 0.1	3	1.1 ± 0.1	13	46.6 ± 4.1	9
40	-0.3 ± 0.3	14	0.9 ± 0.1	13	47.4 ± 6.3	13
50	-1.0 ± 0.4	29	1.0 ± 0.1	6	47.5 ± 3.6	8
75	-0.6 ± 0.4	20	0.9 ± 0.1	7	43.9 ± 5.4	12
100	0.4 ± 0.5	16	0.9 ± 0.1	8	44.0 ± 7.1	16

2.3 Analyte detection strategy

These paper devices perform assays through multistep or partial-step chemical reactions that are carried out in opposing channels but from a single sample input. The resulting colorimetric signal only forms when the advancing liquid fronts converge, and reagents and intermediate products can react by diffusing across the formed interface. In the single-analyte detection format, reagents are stored within the device such that one channel contains analyte-specific reagents, and the other channel contains a product-specific indicator. Upon introduction of the sample, a partial reaction occurs in the channel containing analyte-specific reagents. As the two liquid fronts converge, the assay is completed by reaction between the formed product and the product-specific indicator. Thus, a colorimetric interfacial signal develops in the readout zone and the signal is confined in the interface contour due to reaction specificity and timed transport of solutions.

2.4 Quantifying interface signal

We performed signal analysis using ImageJ.⁵⁰ To analyze signal intensity at the interface, we either (i) used the “Plot Profile” tool and analyzed the pixel intensity across the circular signal readout region or (ii) defined the interfacial signal using the “Rectangle” tool and then performed “RGB Measure” analysis using the available plugin. The time required to generate visible signals can vary based on the grade of paper used to fabricate the device, **Table 2.5**. Additionally, we investigated the effect of applied sample volume on the absolute position, width, and color intensity of interfaces formed in our paper-based devices. As previously shown in **Table 2.4**, we found that only volumes above 30 μL reach a plateau in the intensity of the interface signal, with no benefit observed using larger volumes. We used Whatman 4 for all layers of our paper-based device and observed that signal formation starts at approximately 2 minutes.

Table 2.5. Time needed to form interfaces as a function of paper grade using an Fe(III) assay. Measurements of time are the mean of 5 replicates. Reproduced with permission from Abdullah, I.H., Wilson, D.J., Mora, A.C., Parker, R.W. and Mace, C.R., **2023**. Generating signals at converging liquid fronts to create line-format readouts of soluble assay products in three-dimensional paper-based devices. *Lab on a Chip*, 23(18), pp.4010-4018.

Paper grade	Ahlstrom				Whatman	
	54	55	601	613	1	4
Retention size (μm)	10	15	2.5	6	11	20–25
Signal formation time (min)	2–3	2	4	3–4	3	2

Product generation accumulates over time and causes an increase in signal intensity, until saturating at approximately 7 minutes after the sample was added (**Figure 2.5A**). Additionally, the gradual increase in signal intensity causes nearly linear broadening of the width of the formed interface over time, which eventually compromises signal intensity.

Therefore, we determined the ideal signal read time is between 7–10 minutes, with the cut off time for signal interpretation to be 20 minutes. Prior to the cut off time, the signal intensity is at a maximum value and can be used for qualitative detection, which we demonstrate with Fe(III) ions (**Figure 2.5B**). After the cut off time, the signal intensity diminishes over time due to diffusion of chromogenic products away from the interface.

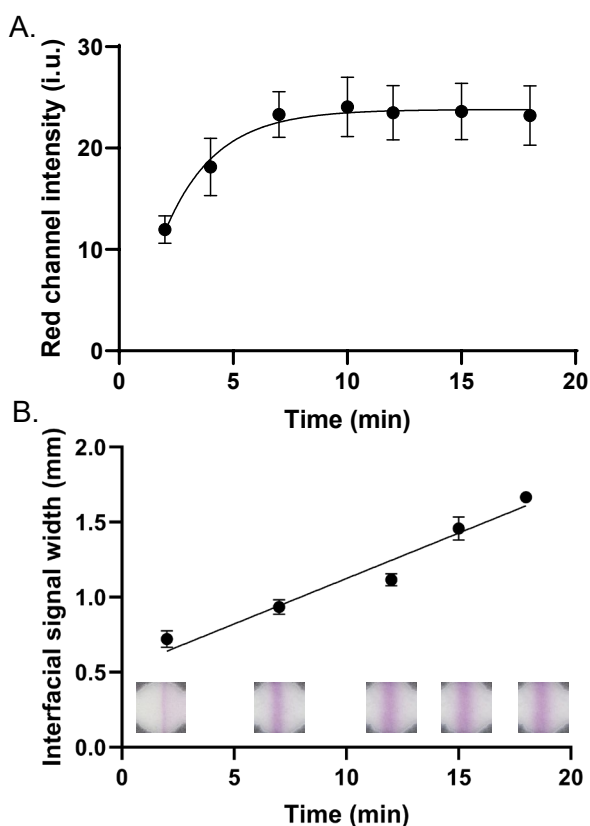


Figure 2.5. Results of experiments to evaluate the effect of reaction time on the intensity (A) and width (B) of assay interfaces for the colorimetric detection of Fe(III). When opposing wicking fronts converge in the test zone, assay reagents (a reduced Fe(II) products and Ferrozone, a specific indicator for Fe(II)) can react by diffusing across the interface formed when flow stops. We used (A) a saturation growth model to fit the relationship between the signal intensity (quantified via red channel for the purple reaction product) and time, and (B) linear regression to fit the relationship between the width of the colored interface and time. Error bars represent standard error of the mean for $n = 5$ measurements. Detection zones are approximately 5 mm in diameter. Reproduced with permission from Abdullah, I.H., Wilson, D.J., Mora, A.C., Parker, R.W. and Mace, C.R., **2023**. Generating signals at converging liquid fronts to create line-format readouts of soluble assay products in three-dimensional paper-based devices. *Lab on a Chip*, 23(18), pp.4010-4018.

2.5 Iron (Fe (III)) assay

For the representative ion assay, we selected Fe(III). Iron homeostasis is vital to multiple biological processes (e.g., oxygen transport and short-term oxygen storage) and the concentration of iron can be used as a diagnostic marker for anemia.⁵¹ We chose Fe(III) as a model analyte to highlight two key functions of our device: (i) the separation of reduction and chelation reactions and (ii) the spatial confinement of low molecular weight products. To detect Fe(III), we stored ascorbic acid (a reducing agent) and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid (Ferrozine; a colorimetric indicator of Fe(II))⁵² in opposite channels of the reagent storage layer (**Figure 2.6A**). When a sample is added to the device, it is split uniformly into each channel where it rehydrates and can react with these stored reagents. If Fe(III) is present in the sample, it will be reduced to Fe(II) in the channel containing ascorbic acid, while no reactions will occur in the channel containing Ferrozine. When the two wicking fronts converge at the detection zone on the bottom layer of the device, reduced Fe(II) is chelated by Ferrozine and forms a purple signal at the formed interface (**Figure 2.6B, Figure 2.7**).

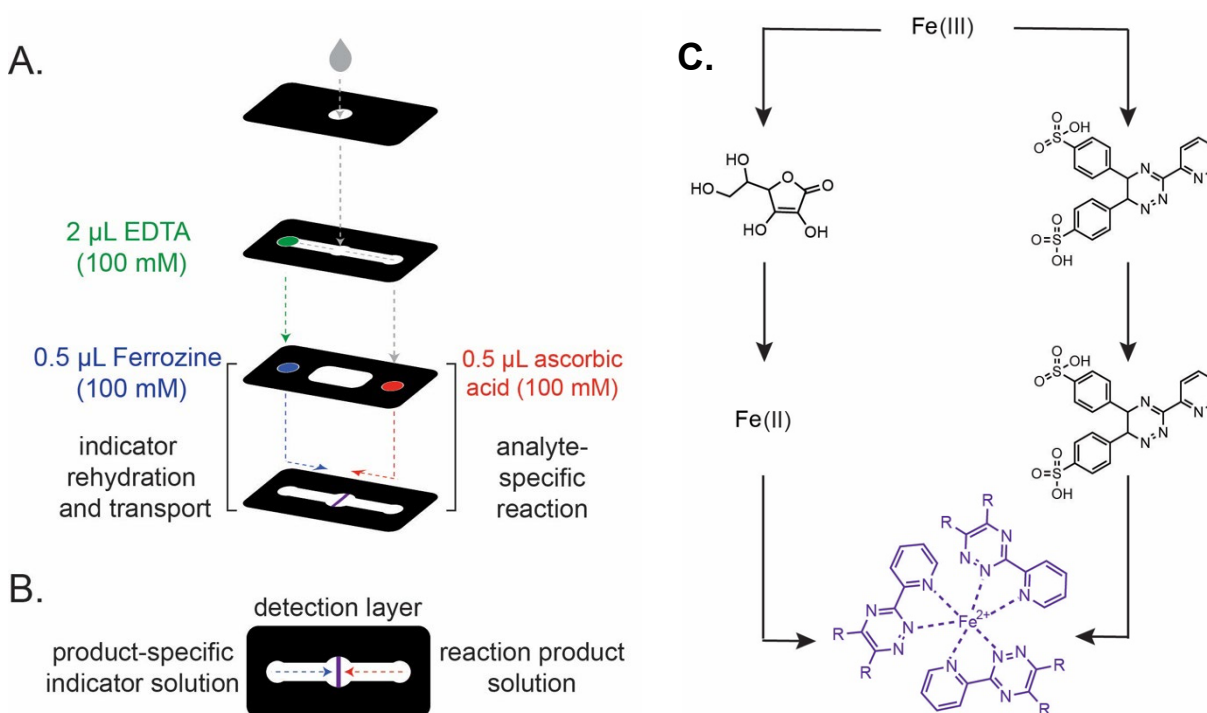


Figure 2.6. Fe(III) assay design strategy. (A) Schematic of fluid flow in the multilayer device. The sample is distributed to separate channels containing partial reagents responsible for the complete reaction. On one side of the device, Fe(III) is reduced to Fe(II) by ascorbic acid. On the other side of the device, the Ferrozine colorimetric indicator for the reaction product is rehydrated and transported to the detection zone. (B) When solution fronts converge in the detection layer, diffusion across a formed liquid interface facilitates the formation of colorimetric signals. (C) Chemical reaction scheme for the Fe(III) detection assay. Upon the addition, a sample of Fe(III) is split to distribute to opposite sides of the device and rehydrate stored reagents. In one device channel, the Fe(III) sample is reduced by ascorbic acid to produce Fe(II). In the other channel, the sample does not react with Ferrozine and solely aids in transporting the reagent to the next layer. Both liquid fronts (i.e., Fe(II) and Ferrozine) are directed toward a central detection zone where Fe(II) is chelated by Ferrozine to generate a purple product. Reproduced with permission from Abdullah, I.H., Wilson, D.J., Mora, A.C., Parker, R.W. and Mace, C.R., **2023**. Generating signals at converging liquid fronts to create line-format readouts of soluble assay products in three-dimensional paper-based devices. *Lab on a Chip*, 23(18), pp.4010-4018.

The addition of samples containing Fe(III) resulted in interfaces with colors whose intensities correlated linearly with concentration between 5.8–13.0 mM and had an LOD of 2.9 mM (**Figure 2.7A**). We chose this range of concentrations as it corresponds to the

biologically relevant concentration of total iron that could be liberated from hemoglobin.⁵³

While quantitative analysis is useful to characterize assay performance as changes are made to reagent conditions or device design, ultimately, we envision the application of this approach to be similar to how LFTs operate—using the formation (or absence) of a line as a qualitative indicator of the presence (or absence) of an analyte (**Figure 2.7B**).

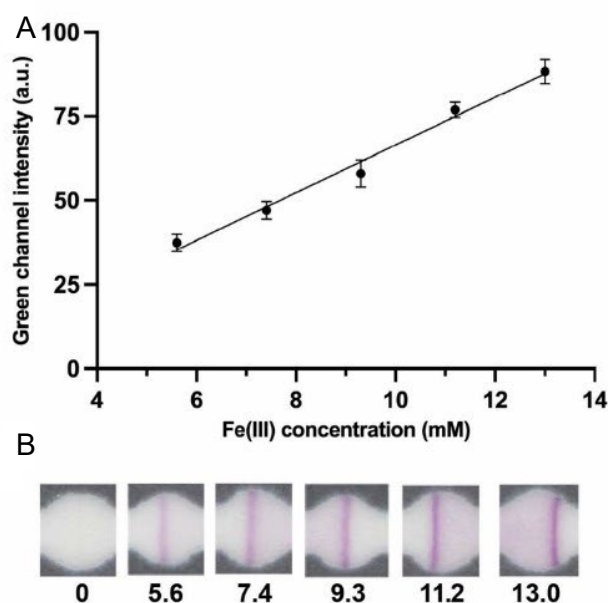


Figure 2.7. Calibration curve for quantitative detection of Fe(III). (A) A linear relationship is established between Fe(III) concentration and the green channel intensity. Error bars represent standard error of the mean for $n = 5$ measurements. (B) Scans of detection zone with interfaces formed at different concentrations. Detection zones are approximately 5 mm in diameter. Reproduced with permission from Abdullah, I.H., Wilson, D.J., Mora, A.C., Parker, R.W. and Mace, C.R., **2023**. Generating signals at converging liquid fronts to create line-format readouts of soluble assay products in three-dimensional paper-based devices. *Lab on a Chip*, 23(18), pp.4010-4018.

2.5.1 Minimizing background noise

In some experiments, we observed a noticeable background signal in the channel containing Ferrozine that scaled with the concentration of added Fe(III) (**Figure 2.8**). The presence of this background color could negatively impact the performance of the Fe(III) assay (i.e., reduced specificity and increased limit of detection).^{54,55} This color could be caused by metal impurities in the paper itself or the potential for Fe(III) to be an interferent in the specific detection of Fe(II) by Ferrozine. We minimized the background signal by incorporating ethylenediaminetetraacetic acid (EDTA) in the Ferrozine side of the sample distribution layer.^{56,57}

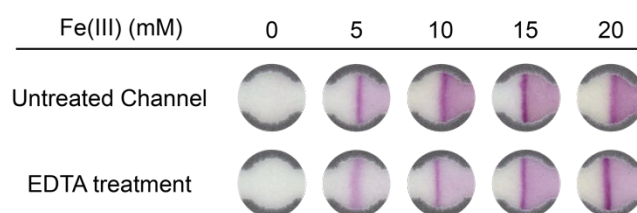


Figure 2.8. Detection of Fe(III) at liquid interfaces in paper-based microfluidic devices. Equimolar amounts of ascorbic acid and Ferrozine stored in opposite sides of the device enabled detection of millimolar concentrations of Fe(III), but the advancing front of the channel containing Ferrozine contributed a substantial amount of background signal. To diminish the presence of this undesired color that could mask the formation of positive signals, we treated the reagent distribution channel with EDTA. Reproduced with permission from Abdullah, I.H., Wilson, D.J., Mora, A.C., Parker, R.W. and Mace, C.R., **2023**. Generating signals at converging liquid fronts to create line-format readouts of soluble assay products in three-dimensional paper-based devices. *Lab on a Chip*, 23(18), pp.4010-4018.

2.5.1.1 Influence of Ferrozine concentration

We tested the effect of different Ferrozine concentrations on the signal to background ratio (**Table 2.6**). As the concentration of Ferrozine increased, the intensities of both the signal formed at the readout zone and the background increased. The increase in

background intensity did not negatively impact the signal to background ratio, which increased proportionally with the concentration of Ferrozine.

Table 2.6. Signal-to-background ratio (S:B) resulting from using different concentrations of Ferrozine in Fe(III) assay in the presence and absence of 100 mM EDTA. Measurements are the mean of 5 replicates. Reproduced with permission from Abdullah, I.H., Wilson, D.J., Mora, A.C., Parker, R.W. and Mace, C.R., **2023**. Generating signals at converging liquid fronts to create line-format readouts of soluble assay products in three-dimensional paper-based devices. *Lab on a Chip*, 23(18), pp.4010-4018.

S:B	[Ferrozine] (mM)			
	10	25	50	100
without EDTA	0.80	1.12	1.59	1.64
with EDTA	1.12	1.45	1.61	1.87

2.5.1.2 Influence of EDTA concentration

We also tested the effect of different EDTA concentrations on the signal to background ratio (**Table 2.7**). The increase in signal to background ratio in the presence of EDTA was higher than that without using EDTA, which further suggests that the incorporation of EDTA provided superior.

Table 2.7. Signal-to-background ratio (S:B) for assays for 100 mM Fe(III) using different concentrations of EDTA. Measurements are the mean of 5 replicates. Reproduced with permission from Abdullah, I.H., Wilson, D.J., Mora, A.C., Parker, R.W. and Mace, C.R., **2023**. Generating signals at converging liquid fronts to create line-format readouts of soluble assay products in three-dimensional paper-based devices. *Lab on a Chip*, 23(18), pp.4010-4018.

[EDTA] (mM)	0	10	25	50	75	100	200	300	400	500
S:B	1.32	1.41	1.44	1.53	1.57	1.81	2.00	1.71	1.70	1.77

2.6 Acetylcholinesterase (AChE) assay

2.6.1 Deice layer treatment

For the representative enzyme assay, we selected AChE. AChE hydrolyzes the neurotransmitter acetylcholine into choline and acetic acid to terminate neurotransmission at the cholinergic synapse. Inhibition of AChE by organophosphates, heavy metals, or chemical warfare agents can block acetylcholine transmission and lead to organ failure and death.⁵⁸ We chose AChE as a model analyte to evaluate how interfaces were formed when reactions were governed by two time-dependent processes: (i) the activity of the enzyme (i.e., the analyte) and (ii) diffusion and mixing of reactants across the interface. To detect AChE, we stored acetylthiocholine chloride and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) on opposite sides of the reagent storage layer (**Figure 2.9A**).

When AChE is present in the added sample, it reacts with acetylthiocholine to produce acetic acid and thiocholine (**Figure 2.9B**). At the interface, the liberated sulfhydryl group on the thiocholine interacts with DTNB to generate 2-nitro-5-thiobenzoic acid and form a yellow signal that we could quantify with image analysis. As expected for an enzymatic assay, the signal intensities saturated over time. Similar to the Fe(III) assay, signal from AChE assay took approximately two minutes to start forming and increased in width and intensity over time. To ensure color saturation, we used the scans taken 10 minutes after sample addition to analyze the signal intensity formed at the interface.

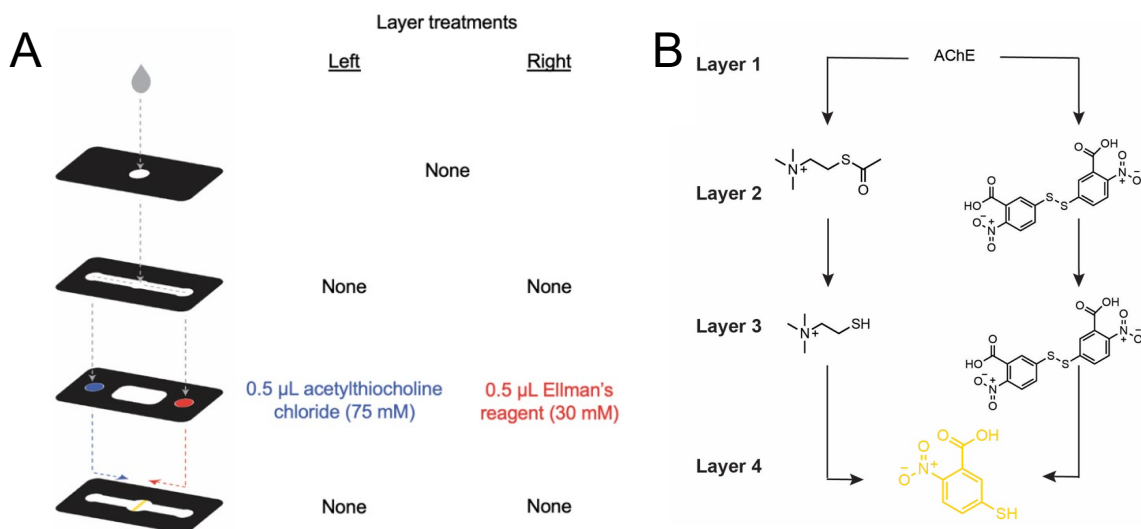


Figure 2.9. Acetylcholinesterase (AChE) assay design strategy. (A) Device layer treatments for the AChE assay. The reagent storage layer (device layer 3) is the only layer treated with reagents for this assay. One zone is treated with 0.5 μ L acetylthiocholine chloride (75 mM) and the other zone is treated with 0.5 μ L of Ellman's reagent (30 mM). The dotted line indicates fluid flow throughout the device where sample addition allows fluid to distribute to opposite sides of the device and rehydrate stored reagents. After rehydration, solutions are directed toward a central detection zone where they meet to form a yellow liquid interface. The detection zone is approximately 5 mm in diameter. (B) Simplified schematic for the assay chemistry that focuses on the reactions that occur in each layer and arm of the three-dimensional paper-based device, which leads to the specific formation of color (yellow) at the interface formed between combined fluid fronts. Reproduced with permission from Abdullah, I.H., Wilson, D.J., Mora, A.C., Parker, R.W. and Mace, C.R., **2023**. Generating signals at converging liquid fronts to create line-format readouts of soluble assay products in three-dimensional paper-based devices. *Lab on a Chip*, 23(18), pp.4010-4018.

2.6.2 Calibration curve for acetylcholinesterase detection

To analyze the results, we performed nonlinear regression of the data using a saturation growth model for concentrations of AChE ranging from 2–30 U/mL (**Figure 2.10A**). From the fit, we determined that there was a nearly linear relationship between the concentration of AChE and measured signal between 2–10 U/mL, and signals at higher concentrations were saturated and could be detected but not quantified (**Figure 2.10B**). While the physiological range of AChE in plasma is greater than 8 U/mL,⁵⁹ exposure to dangerous chemical agents (e.g., organophosphates) results in a reduction of AChE

activity into the linear range of this assay. While it can be hard to clearly detect yellow lines on white backgrounds with the naked eye, especially at lower analyte concentrations, this AChE assay remains useful to provide a visual reference for qualitative detection (i.e., “yes or no” presence of analyte). Given the versatility of our device design, it is possible to substitute assay reagents (i.e., other colorimetric substrates)⁵² for AChE to enhance signal contrast.

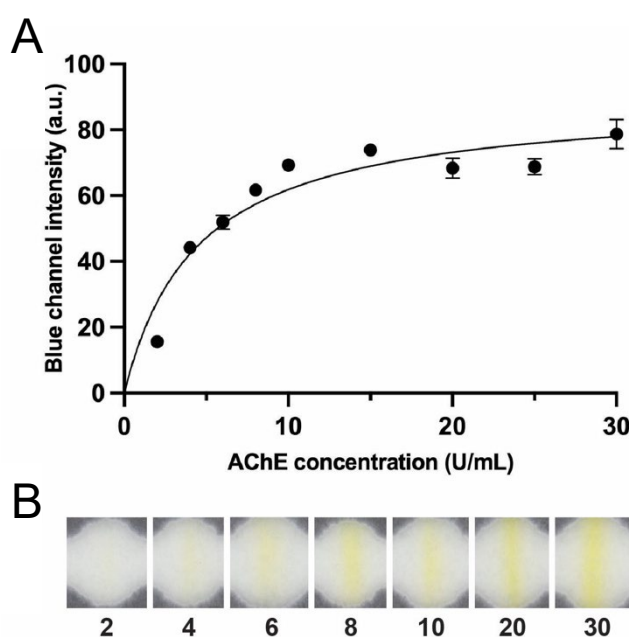


Figure 2.10. Acetylcholinesterase (AChE) detection using interfacial colorimetric signals. (A) Calibration curve for quantitative detection of AChE. A saturation growth model was applied for non-linear regression of the measured signal as a function of AChE concentration. Error bars represent standard error of the mean for $n = 5$ measurements. (B) Scans of detection zones with interfaces formed at different concentrations. Detection zones are approximately 5 mm in diameter. Reproduced with permission from Abdullah, I.H., Wilson, D.J., Mora, A.C., Parker, R.W. and Mace, C.R., **2023**. Generating signals at converging liquid fronts to create line-format readouts of soluble assay products in three-dimensional paper-based devices. *Lab on a Chip*, 23(18), pp.4010-4018.

2.7 Lactate assay

2.7.1 Device layer treatment

For the representative small molecule assay, we selected lactate. Lactate is an intermediate in the metabolic anaerobic carbohydrate pathway whose concentration in sweat changes with exercise. While exercising, undesired lactate accumulation in the active muscle can manifest as fatigue, soreness, and pain.⁶⁰ Also, sweat lactate can provide information about insufficient oxidative metabolism and pressure ischemia.⁶¹ We chose lactate as a model analyte to demonstrate how the device can accommodate multistep reactions. To detect lactate, we stored (i) nicotinamide adenine dinucleotide (NAD⁺) and lactate dehydrogenase (LDH) and (ii) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) on opposite sides of the second layer of the device (**Figure 2.11**). Then on the third layer, we again stored NAD⁺/LDH below the first treated layer and phenazine methosulfate (PMS) below MTT (**Figure 2.11**).

When a sample containing lactate is introduced and split by the sample distribution layer, the following reactions occur: (i) LDH converts lactate to pyruvate and (ii) transfers the liberated hydride ion to the co-stored NAD⁺ to produce NADH. In the other channel, both MTT and PMS are simply rehydrated. When the split sample converges at the interface, PMS mediates an electron transfer from NADH to reduce MTT and produce a purple-colored formazan product. For stable reagent storage and optimal performance, we tested different configurations of device layers, layer treatments, and drying conditions (i.e., light and temperature). From initial trials, we determined it is necessary to spatially separate PMS and MTT for reagent storage stability and to perform all manufacturing

steps in the dark at room temperature. The addition of hydrazine buffer on the fourth layer of the device was to neutralize the co-produced pyruvate (**Figure 2.11**).⁶²

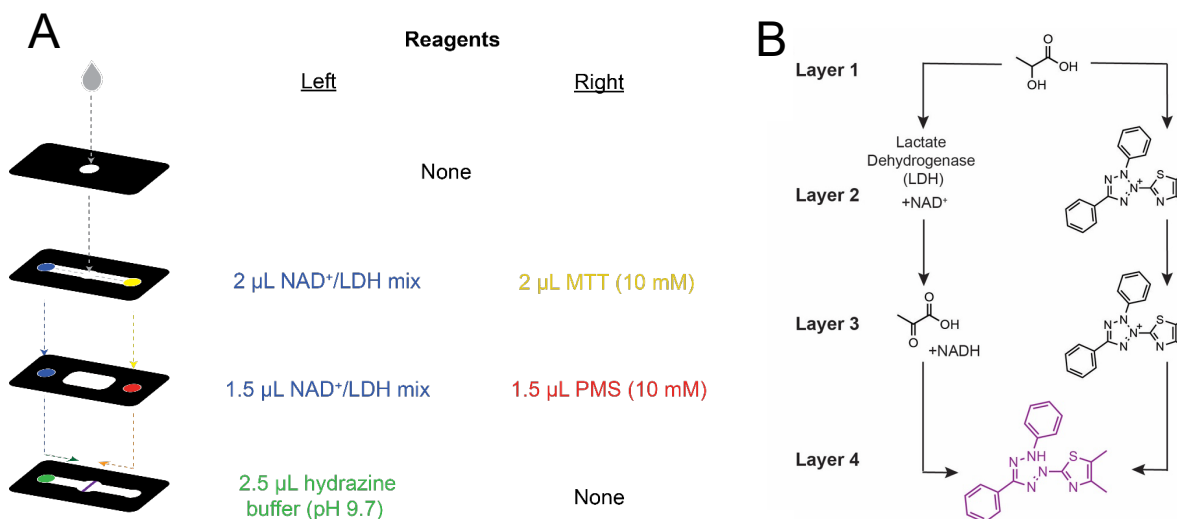


Figure 2.11. Lactate assay design strategy. (A) Device layer treatments for the lactate assay. The sample addition layer (device layer 1) is not treated with any reagents. On the sample distribution layer (device layer 2), the left side of the device is treated with 2 μL NAD^+ (30 mM) and lactate dehydrogenase (LDH, 1 mg/mL) and the right side of the device is treated with 2 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 10 mM). On the reagent storage layer (device layer 3), the left side of the device is treated with 1.5 μL NAD^+ (30 mM) and Lactate dehydrogenase (LDH, 1 mg/mL), and the right side of the device is treated with 1.5 μL phenazine methosulfate (PMS, 10 mM). Finally, on the interface formation layer (device layer 4), the left side of the device is treated with 2.5 μL of hydrazine buffer (pH 9.7) and the right side of the device is left untreated. The dotted line indicates fluid flow throughout the device where sample addition allows fluid to distribute to opposite sides of the device and rehydrate stored reagents. After rehydration, solutions are directed toward a central detection zone where they meet to form a purple liquid interface. The detection zone is approximately 5 mm in diameter. (B) Simplified schematic for the assay chemistry that focuses on the reactions that occur in each layer and arm of the three-dimensional paper-based device, which leads to the specific formation of color (blue/purple) at the interface formed between combined fluid fronts. Reproduced with permission from Abdullah, I.H., Wilson, D.J., Mora, A.C., Parker, R.W. and Mace, C.R., **2023**. Generating signals at converging liquid fronts to create line-format readouts of soluble assay products in three-dimensional paper-based devices. *Lab on a Chip*, 23(18), pp.4010-4018.

2.7.2 Calibration curve for lactate detection

For the lactate assay, the detected concentration range was 2–50 mM (**Figure 2.12A**). Unlike Fe(III) and AChE assays, we analyzed lactate assay results approximately 4 minutes after sample addition to avoid increased background signal over time. The high noise is likely due to both MTT and PMS reagents causing a yellow background color. Since the assay involves enzymatic reactions, we analyzed the results by nonlinear regression using a growth and saturation model. The data showed a nearly linear correlation between signal intensity (green channel) at concentrations of lactate less than 20 mM. At higher concentrations of lactate, the signal saturated and resulted in detectable but not quantifiable signal (**Figure 2.12B**). The nearly linear range of measurement is useful to quantify lactate in sweat, which decreases from 31 mM at the start of exercising to 12 mM at the point of exhaustion.⁶¹

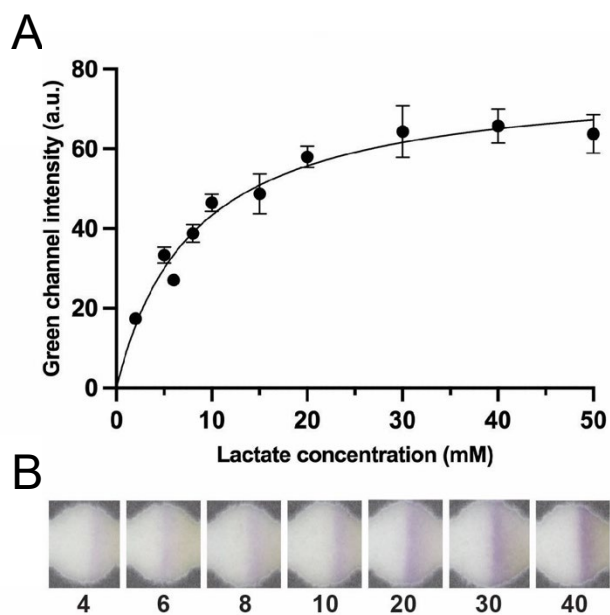


Figure 2.12. Lactate detection using interfacial colorimetric signals. (A) Resulting calibration curve for quantitative detection of lactate. A saturation growth model was applied for non-linear regression of the measured signal as a function of lactate concentration. Error bars represent standard error of the mean for $n = 5$ measurements. (B) Scans of detection zone with interfaces formed at different concentrations. Detection zones are approximately 5 mm in diameter. Reproduced with permission from Abdullah, I.H., Wilson, D.J., Mora, A.C., Parker, R.W. and Mace, C.R., **2023**. Generating signals at converging liquid fronts to create line-format readouts of soluble assay products in three-dimensional paper-based devices. *Lab on a Chip*, 23(18), pp.4010-4018.

2.8 Multiplexed interfacial color formation

2.8.1 Significance of multiplexing detection

In **Figure 2.2**, we demonstrated that it was possible to design a device such that multiple, independent channels (i.e., 3–4) could converge in a common zone to create multiple interfaces. While this preliminary experiment was performed with dyes stored in the device and rehydrated only with water, the results showed several useful features that were analogous to the singleplex format: (i) equal channel lengths and design symmetry could control the position of interfaces in a common zone, (ii) mixing only occurs across interfaces formed from advancing fronts, and (iii) net flow stops when the readout zone is filled to allow reactions to be confined only to formed interfaces. As a result, we evaluated the potential to create multiplexed assays using the interface device design by combining two of the aforementioned singleplex assays.

2.8.2 Multiplexed device layer treatments

Taking inspiration from a standard LFT, which comprises a single test line and single control line, we aimed to demonstrate the ability of our devices to support multiple test and control lines in the same read zone. We chose to develop an assay for Fe(III) and AChE, which would produce four interfacial signals: one test line and one control line for each respective analyte. **Figure 2.13** depicts the conditions that introduce assay reagents into their required spatial locations (layer and channel). One major difference from the singleplex assays is that control interfaces require the storage and eventual introduction of the partial reaction products formed in the test interfaces—namely, ferrous iron (FeCl_2) and a free thiol (cysteine)—to react with stored indicators. After all four channels wick

through to the final layer, the four resulting liquid fronts converge at the detection zone and form four interfacial signals.

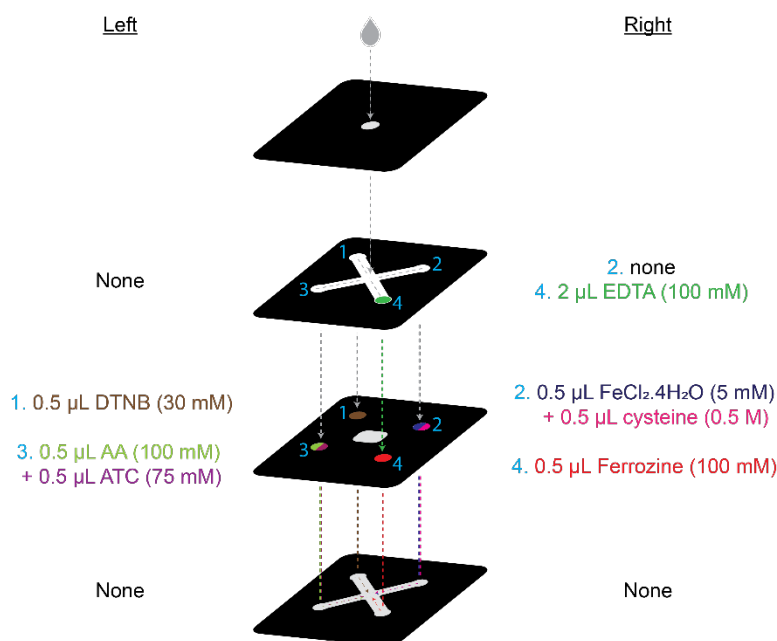


Figure 2.13. Device layer treatments for the formation of assays outputs at multiple interface (Fe(III), AChE, and their respective controls). The sample addition layer (device layer 1) is not treated with any reagents. On the sample distribution layer (device layer 2), zones 1 to 3 are not treated with any reagents and zone 4 is treated with 2 μL of EDTA (100 mM). On the reagent storage layer (device layer 3), zone 1 is treated with 0.5 μL cysteine, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent, 30 mM), zone 2 is treated with 0.5 μL $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (5 mM) and 0.5 μL cysteine (0.5 M), zone 3 is treated with 0.5 μL ascorbic acid (AA, 100 mM) and 0.5 μL acetylthiocholine (ATC, 75 mM), and zone 4 is treated with 0.5 μL of Ferrozine (100 mM). Finally, the interface formation layer (device layer 4) is not treated with any reagents. The dotted line indicates fluid flow throughout the device where sample addition allows fluid to distribute to opposite sides of the device and rehydrate stored reagents. After rehydration, solutions are directed toward a central detection zone. The detection zone is approximately 5 mm in diameter. Reproduced with permission from Abdullah, I.H., Wilson, D.J., Mora, A.C., Parker, R.W. and Mace, C.R., **2023**. Generating signals at converging liquid fronts to create line-format readouts of soluble assay products in three-dimensional paper-based devices. *Lab on a Chip*, 23(18), pp.4010-4018.

At the detection zone, a yellow interface formed at position 1 indicates the presence of AChE, a yellow interface formed at position 2 indicates the corresponding control line for AChE, a purple interface formed at position 3 indicates the presence of control line for Fe(III), and finally, a purple interface formed at position 4 indicates the presence of Fe(III). The resulting outputs for all possible combinations of analytes (positive and negative for each, with corresponding controls) are shown in **Figure 2.14**.

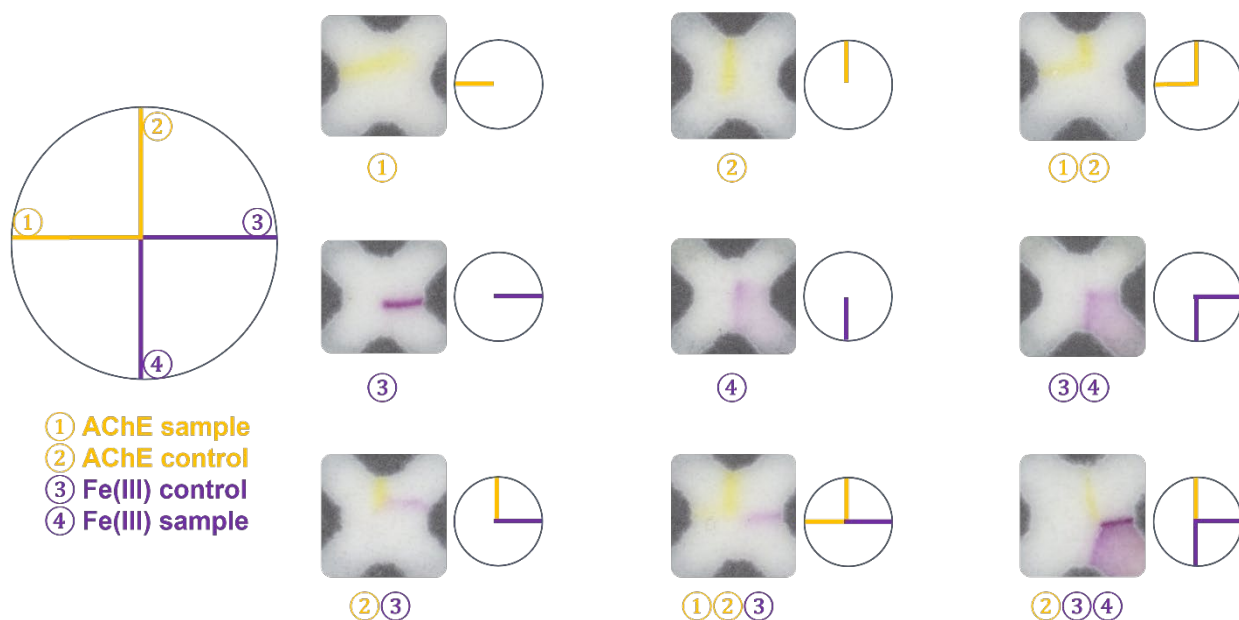


Figure 2.14. Demonstration of multiplexed assays in a common detection zone due to the convergence of multiple fluid fronts. Scans of detection zones showing interface patterns formed from combinations of tests and positive controls for Fe(III) and acetylcholinesterase (AChE) assays. Lines can be formed along all four coordinate positions according to the analyte and/or control they represent. The far left circle depicts a map of where signals will be formed for tests and controls of Fe(III) and AChE assays. Images of detection zones are accompanied by an individual map of the lines expected to be formed by a given experiment. Detection zones are approximately 5 mm in diameter. Reproduced with permission from Abdullah, I.H., Wilson, D.J., Mora, A.C., Parker, R.W. and Mace, C.R., **2023**. Generating signals at converging liquid fronts to create line-format readouts of soluble assay products in three-dimensional paper-based devices. *Lab on a Chip*, 23(18), pp.4010-4018.

2.9 Conclusion and future directions

In this chapter, we reported a new class of paper-based microfluidic devices that uses converging liquid fronts to constrain the location where assay chemistries are performed within test zones. Like the ubiquitous lateral flow test, which serves as an inspiration for the readout format of these devices, the shape of the colorimetric assay product is a line. We made this design decision to facilitate how assay results are interpreted,⁶³ either qualitatively or quantitatively. Unlike lateral flow tests, however, these paper devices do not rely on molecular recognition or affinity capture to immobilize analytes of interest or colorimetric reagents. Instead, by converging and halting two opposing wicking fronts, the location of soluble reaction products can be defined.

While we show the potential for this device format to support colorimetric assays for Fe(III), acetylcholinesterase, and lactate, we believe that similar applications are possible for other analytes represented by these classes (i.e., ions, enzymes, and small molecules). Although the dynamic ranges of these acetylcholinesterase and lactate assays are physiologically relevant, the limit of detection of Fe(III)—appropriate for total iron bound to hemoglobin (ca. mM)—is higher than required to measure ferric iron in serum⁶⁴ or drinking water (ca. μM).⁶⁵ We expect that optimizing assay conditions, inclusive of choices of indicator, paper treatment, pH, and grade of paper, may enable assays for iron in these micromolar regimes. Nonetheless, the Fe(III) assay successfully demonstrates the potential of μPADs to localize products of colorimetric reactions with ions to line-format interfaces for ease of readout.

In addition to singleplex assays, we further demonstrate that multiplexed assays—where multiple fluid fronts carrying distinct reaction products and/or indicators—can be supported by this approach to form interfaces in paper test zones. Unlike in singleplex assays, where efforts to optimize assay conditions and formulate reagents for storage within the device have relatively limited variables, the creation of multiplexed assays requires deliberate attention to reagent compatibility and cross-reactivity to limit false positives and negatives. Even so, the opportunity to integrate positive controls, which are frequently missing from assays developed for paper-based microfluidic devices, would address a major requirement for point-of-care tests. Beyond their use as procedural controls, we anticipate that the intensities of these lines may have the potential to be used as visual comparators to estimate the concentration of the analyte in the sample. In total, the unique design of these devices has the potential to simplify the user experience and interpretation of critical results at the point-of-use, while broadening the analytical capabilities and ultimate utility of paper-based microfluidic sensors.

2.10 Materials and Methods

2.10.1 Reagents and Device Materials

We purchased Whatman chromatography paper grade 4 (GE Healthcare Life Sciences) from Amazon. We purchased Ahlstrom chromatography paper grade 54 and 55 from Laboratory Sales & Services LLC. We purchased Ahlstrom chromatography paper grade 601 and 613 from Thomas Scientific. We purchased Flexmount Select DF051521 (permanent adhesive double-faced liner) from FLEXcon. We purchased Fellowes laminating sheets and Grass Green food color from Amazon. We purchased Allura Red AC from TCI. We purchased Trypan Blue from Invitrogen. We purchased Iron(III) chloride hexahydrate, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid (Ferrozine), nicotinamide adenine dinucleotide (NAD⁺), and phenazine methosulfate (PMS) from Acros Organics. We purchased ethylenediaminetetraacetic acid disodium salt dihydrate, acetylthiocholine chloride, L-lactate dehydrogenase, Acetylcholinesterase (AChE) from *Electrophorus electricus* (electric eel), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), glycine, and hydrazine sulfate from Sigma Aldrich. We purchased iron(II) chloride tetrahydrate, L-ascorbic acid, DL-cysteine, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent), and sodium L-lactate from Alfa Aesar. We purchased Tris buffer from VWR. We purchased 10X phosphate buffered saline (PBS) from Fisher Scientific. We purchased ASTM Type I water from Ricca Chemical Company. We used an EPSON Perfection V600 Photo scanner to acquire images of devices and completed assays.

2.10.2 Solution preparation

For the Fe(III) assay, we prepared solutions of Ferrozine, L-ascorbic acid, and EDTA in Type I water. To test the effect of changing the EDTA concentration on the S:B produced by the assay, we prepared different concentrations of EDTA (10–500 mM). To test the effect of changing the Ferrozine concentration on the S:B produced by the assay, we prepared different concentrations of Ferrozine (10–100 mM). For the calibration curve, we prepared different concentrations of $\text{FeCl}_3 \bullet 6\text{H}_2\text{O}$ (5.6–13 mM). For device layer treatments, we deposited 2 μL of 100 mM EDTA on one side of the sample distribution layer (device layer 2). We then deposited 0.5 μL of Ferrozine (100 mM) on the same side as the EDTA treatment and ascorbic acid (100 mM) on the opposite side of the reagent storage layer (device layer 3). To dry deposited reagents, we incubated the device layers in the oven for 20 minutes at 65 °C.

For the acetylcholinesterase (AChE) assay, we prepared fresh samples of different concentrations of AChE (2–30 U/mL) in 50 mM Tris buffer (pH 8) daily. We prepared 75 mM of acetylthiocholine chloride and 30 mM of Ellman's reagent (DTNB) in 100 mM PBS (pH 7.4). For device layer treatments, we deposited 0.5 μL of acetylthiocholine chloride and DTNB on opposite sides of the reagent storage layer (device layer 3). To dry deposited reagents, we incubated the device layers in the oven for 20 minutes at 65 °C.

For the lactate assay, we prepared a NAD^+ /LDH solution by mixing 45 μL of NAD^+ (30 mM) and 7.5 μL of lactate dehydrogenase (LDH, 1 mg/mL) in 97.5 μL of Type I water. For this mixture, we freshly prepared both NAD^+ and LDH solutions using DI water. We prepared 10 mM solutions of PMS and MTT in Type I water. We prepared glycine-hydrazine buffer by dissolving a mixture of hydrazine sulfate, glycine, and EDTA (disodium

dihydrate) in Type I water. The hydrazine buffer was stored in an amber bottle at 4 °C until use. The final buffer concentrations of hydrazine sulfate, glycine, and EDTA were 500 mM, 850 mM, and 15 mM respectively. The final pH of the hydrazine buffer was 9.7. To prepare the device, we deposited 2 μ L of the NAD⁺/LDH mixture and 10 mM of MTT on opposite sides of the sample distribution layer (device layer 2). On the reagent storage layer (device layer 3), we deposited 1.5 μ L of the NAD⁺/LDH mixture in the corresponding zone to the previous addition on the second layer. On the other side of the reagent storage layer (device layer 3), we deposited 1.5 μ L of 10 mM PMS. On the interface formation layer (device layer 4), we deposited 2.5 μ L of glycine-hydrazine buffer (pH 9.7) on the side that would be under the NAD⁺/LDH mixture treatments. We dried all treated paper layers in the dark room at room temperature for one hour.

For the multiplexed assays, we prepared all the reagents using the same concentrations and solvents as the singleplex assays. For the positive controls, we prepared a 5 mM solution of FeCl₂ • 4H₂O in Type I water and a 500 mM solution of cysteine in ethanol.

2.10.3 Iron assays in standard geometries of paper-based devices.

Similar to the conditions used to detect Fe(III) in the devices where readouts are performed at interfaces (e.g., **Figure 2.6**), we fabricated and treated two alternative device designs where assays are conducted in zones that are more standard to paper-based microfluidics: in single zones and at the end of a channel. Single-layer devices comprise circular zones with a diameter of approximately 5 mm (**Figure 2.1A**). We treated each zone with 0.5 μ L of both 100 mM ascorbic acid and 100 mM Ferrozine. Channel-based devices are simple three-dimensional devices that comprise a circular sample inlet

before terminating in a channel where readout is performed (**Figure 2.1B–C**). We treated the sample inlet with 2 μL of 100 mM ascorbic acid, while the end of the channel received 2 μL of 100 mM Ferrozine. Channel length is approximately 10 mm and circular zones have a diameter of approximately 5 mm. For these experiments, devices received 14 μL of Fe(III) at different concentrations. All layer treatments were dried for 7 minutes at 65 °C prior to assembly and use.

2.10.4 Fabrication of paper devices

We used Adobe Illustrator to design the device layers and then printed the hydrophobic wax barriers using a Xerox ColorQube 8570 wax printer. We cut all adhesive layers using a Boss laser cutter (BOSS-LS1630). We used a Promo Heat CS-15 T-shirt press (45 seconds at 138 °C) to melt the wax onto the chromatography paper to form hydrophobic barriers. Using a custom acrylic alignment jig, we laid the adhesive onto the chromatography paper and compressed both layers together. Next, we sent the chromatography paper attached to the adhesive through a laminator (Trulam) to remove any air bubbles trapped between the layers. The device layers were then treated with their respective assay reagents. After reagent treatment, we assembled the device layers using the custom acrylic alignment jig. Finally, we cut a strip of a Fellowes laminating sheet, adhered it under the readout layer of the device, then compressed with the laminator once again. The laminating sheet serves as a layer that prevents evaporation of water from the product solution formed.

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Chapter 3

**Possible areas of improvement for the interfacial colorimetric signals
of three-dimensional μ PADs**

3.1 Introduction

Microfluidic paper-based analytical devices (μ PADs) have gained significant traction for point-of-care (POC) diagnostics due to their low cost, ease of use, and compatibility with various detection modalities, including colorimetry.¹ Despite the progress in this field, achieving stable and reproducible colorimetric signals remains a key challenge. The reliance on colorimetric detection often limits the sensitivity and stability of these devices due to several inherent issues, including inconsistent signal intensity, heterogeneous color formation, and rapid signal degradation over time.²⁻⁴ Colorimetric assays in μ PADs often suffer from non-uniform signal formation. This can arise due to factors such as unoptimized reagent storage, the "coffee-ring effect," and uncontrolled wicking dynamics. The distribution of colorimetric products in the readout zones can be uneven, leading to compromised signal clarity which could complicate interpretation.^{4,5} Additionally, the chemical environment within the paper matrix can also influence colorimetric reactions. Factors such as pH, ionic strength, and the presence of interfering substances may alter the color intensity and stability of the signal, making the readout unreliable for quantitative applications.⁶⁻⁸

Three-dimensional paper-based devices (3D μ PADs) that form at interfacial liquid fronts could offer a partial solution for these challenges. For instance, 3D μ PADs that form interfacial colorimetric signals readouts mitigate the coffee rings effect. Consequently, signal intensity avoids variability across these signal formation interfaces.⁹ However, other challenges, such as temporal signal formation, persist even with 3D μ PADs demanding further optimization to improve device usability and performance.

For interfacial colorimetric signals, geometry-based challenges are mitigated. In our recent work, presented in chapter 2, the formation of linear readouts at converging liquid fronts has shown promise for improving signal localization and user interpretation. Typically, the linear readout format of interfacial signals avoids interpretation issues related to circular spot formats, such as coffee-ring effect.⁹ However, the stability of these interfacial signals is limited. The signals are often only stable for a short duration (~20 minutes) before losing intensity due to product diffusion and reagent depletion at the interface. Resolving this issue of temporal signal stability can broaden the application of interfacial assays in diagnostic settings, allowing semi-permanent signal formation. Recent literature has reported on various strategies aimed at stabilizing colorimetric signals, such as using hydrophilic surface treatments⁷ and reagent immobilization techniques,¹⁰ yet these methods do not fully address the dynamic environment at the liquid interface.

Several approaches have been investigated to enhance colorimetric signal intensity and stability in PADs. One area of progress involves modulating the chemical properties of the paper substrate. The pH of the paper matrix, for instance, plays a role in determining the formation and intensity of the colorimetric product. Colorimetric indicators often exhibit pH-dependent absorption properties, leading to shifts in color intensity based on the local pH. Recent studies have explored pre-treating paper with buffer salts to control the pH environment during assay development.^{11–13} However, the inherent buffer capacity of the paper itself could probably alter the expected pH, affecting the reproducibility of the colorimetric readout. This suggests that a deeper understanding of the interaction

between the paper's buffer capacity and the stored reagents is necessary to control the local chemical environment effectively.

Another area of research is to impregnate paper substrates with nanomaterials. Incorporating nanoparticles, such as silica^{14,15} or metal nanoparticles,^{16,17} into the paper matrix has been shown to enhance the localization of colorimetric signals and increase signal intensity. Nanoparticles can serve as catalytic sites that concentrate the reaction products or as structural elements that modulate fluid flow, thereby modifying the diffusion rate of the colorimetric product away from the detection zone. Recent studies have demonstrated that silica nanoparticle impregnation improves signal stability by reducing reagent mobility and enhancing the interaction between the analyte and the colorimetric reagent.¹⁴

Considering these advancements, this chapter aims to build on the previous work with 3D μ PADs. This work investigates some factors that might influence colorimetric signal stability and intensity such as: (i) storing pH modifiers, (ii) the buffer capacity of different paper types, and (iii) nanoparticle impregnation on signal localization and stability. By studying the impact of the paper's pH and its buffer capacity, we attempt to control the local chemical environment more precisely. Typically, drying down buffer salts within the reagent storage zones or employing different paper types, which entails varying buffer capacities, would alter assay environment. Thus, we hypothesize that accounting for the paper's inherent buffering properties and controlling the overall assay pH on the paper substrate is crucial for achieving consistent colorimetric signals.

To address the issues of signal localization and temporal stability, we will explore the impregnation of the paper matrix with silica nanoparticles. Nanoparticles are expected to

enhance signal intensity by increasing the surface area for reactions and stabilizing the reagent distribution. Moreover, the structural properties of nanoparticles may help restrict the lateral diffusion of colorimetric products, thereby maintaining the integrity of the interfacial signal over a longer duration. We hypothesize that this approach will result in improved signal-to-noise ratios and prolonged signal stability.

3.2 Device design considerations

3.2.1 Buffer capacity studies

3.2.1.1 Paper buffer capacity

We designed this preliminary experiment to evaluate the buffer capacity of various paper types. Equal quantities of paper scraps, 0.5 g, from different grades were immersed in equal volumes of deionized (DI) water. The aim was to determine whether differences in buffer capacity exist between papers based on their manufacturing processes, which are largely proprietary and not readily available online or in manufacturer documentation. The paper grades tested included Whatman Grade 1, Whatman Grade 4, Ahlstrom Grade 54, Ahlstrom Grade 55, Ahlstrom Grade 601, and Ahlstrom Grade 613. Each paper type was placed in a separate glass vial containing equal aliquots of DI water, 10 mL. The initial pH of the DI water was recorded before immersion. After immersing the papers, the vials were stored in a cool, dry place, and the pH of the water was measured after 48 hours and again after 7 days. Any observed pH changes were attributed to the buffer capacity of the corresponding paper type.

3.2.1.2 Iron (Fe (III)) assay

We chose Fe(III) as a model assay for pH and paper type studies due to its relative simplicity as compared to enzymatic assays tested in the previous chapter, acetylcholine esterase and lactate assays. These enzymatic assays are too sensitive to pH changes, unlike Fe(III) which involves reagents, Ferrozine and ascorbic acid, that are stable over a wide range of pH values.^{18,19} To detect Fe(III), we stored ascorbic acid (a reducing agent) and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid (Ferrozine; a colorimetric indicator of Fe(II)) in opposite channels of the reagent storage layer (**Figure 3.1A**). When a sample is added to the device, it is split uniformly into each channel where it rehydrates and can react with these stored reagents. If Fe(III) is present in the sample, it will be reduced to Fe(II) in the channel containing ascorbic acid, while no reactions will occur in the channel containing Ferrozine. When the two wicking fronts converge at the detection zone on the bottom layer of the device, reduced Fe(II) is chelated by Ferrozine and forms a purple signal at the formed interface (**Figure 3.1B**).⁹

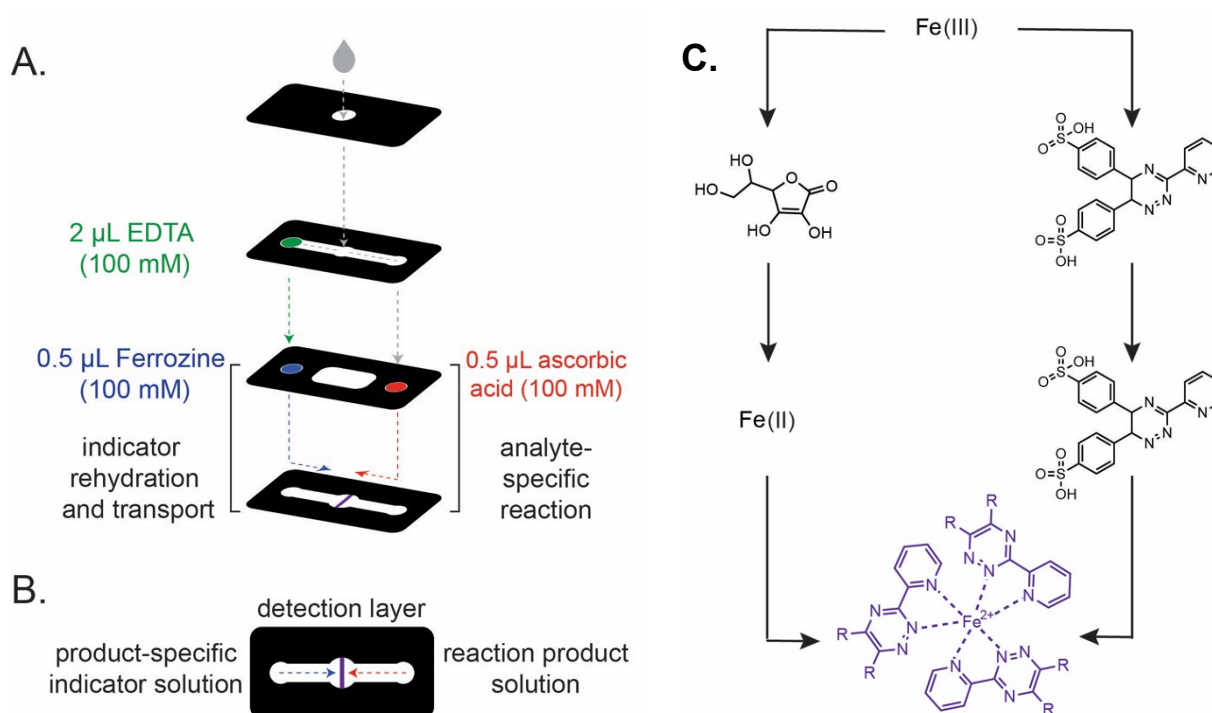


Figure 3.1. Fe(III) assay design strategy. (A) Schematic of fluid flow in the multilayer device. The sample is distributed to separate channels containing partial reagents responsible for the complete reaction. On one side of the device, Fe(III) is reduced to Fe(II) by ascorbic acid. On the other side of the device, the Ferrozine colorimetric indicator for the reaction product is rehydrated and transported to the detection zone. (B) When solution fronts converge in the detection layer, diffusion across a formed liquid interface facilitates the formation of colorimetric signals. (C) Chemical reaction scheme for the Fe(III) detection assay. Upon the addition, a sample of Fe(III) is split to distribute to opposite sides of the device and rehydrate stored reagents. In one device channel, the Fe(III) sample is reduced by ascorbic acid to produce Fe(II). In the other channel, the sample does not react with Ferrozine and solely aids in transporting the reagent to the next layer. Both liquid fronts (i.e., Fe(II) and Ferrozine) are directed toward a central detection zone where Fe(II) is chelated by Ferrozine to generate a purple product. Reproduced with permission from Abdullah, I.H., Wilson, D.J., Mora, A.C., Parker, R.W. and Mace, C.R., **2023**. Generating signals at converging liquid fronts to create line-format readouts of soluble assay products in three-dimensional paper-based devices. *Lab on a Chip*, 23(18), pp.4010-4018.

3.2.1.3 Buffer compositions and pH

For our buffer composition and pH experiments, we tested multiple buffer compositions and pH values. The tested buffers are citrate, citrate-phosphate, and acetate buffers. The citrate buffer (CSC) is composed citric acid and sodium citrate mixture, the citric-

phosphate buffer is composed of citric acid and disodium phosphate (CP), and the acetate buffer (AcO) is composed of acetic acid and sodium acetate. Both CSC and CP buffers are tested to cover the range of 3 – 6 pH values. AcO buffer, however, typically have a pH range of about 3.6 to 5.6. Thus, instead of pH 3 and 6, we employed pH 3.6 and 5.6 for AcO buffer. These buffers are tested because they cover a range of chelation strengths to the Fe(III) ions which might influence the signal formation in various ways. Obviously, pH values and buffers that support higher pH values are not tested since the Fe(III) reducing agent is ascorbic acid. Ascorbic acid loses its effectiveness as a reducing agent for Fe(III) ions with pH values higher than 6.8.

3.2.1.4 Buffer deposition method

It's important to notice here that these buffer deposition methods are considered like an added pH to the reaction medium. We can not make a claim that the pH of the added buffer is the actual pH value at the interface where the reaction is happening. We tested different methods to incorporate the pH buffer in the reaction medium. Typically we either (i) dissolved EDTA in the tested buffer, (ii) tested all reaction reagents (i.e. edta, ascorbic acid, and Ferrozine), or (iii) we only stored the buffer on the second layer instead of the EDTA as a complexation agent. The control experiment in this testing is the use of reagent deposited as depicted in **Figure 3.1A** such that all reagents are dissolved in DI water including EDTA whose pH value is 4.5.

3.2.1.5 pH multivariate analysis

We aimed to get a more comprehensive influence of three factors: pH, buffer composition, and buffer capacity of different paper grades. To achieve this aim, we conducted multiple heatmap studies to combine all three variables. We tested these different

combinations against high and low Fe(III) concentrations (1 and 10 mM) and evaluated the resulting signal and noise ratio of each combination. We then used the combinations that produced the highest signal-to-noise ratio to create calibration curves using both low and high Fe(III) concentration ranges. This experiment aimed to both increase the assay sensitivity and extend the dynamic range of the assay using the interfacial signals in our 3D μ PADs.

We performed a multivariate study to investigate the collective effect of paper type, buffer composition, and buffer pH. We recorded the signal intensity output as a function of the studied variables. Among these variables, paper type represents the change in both paper manufacturing process and retention size. Paper manufacturing represents the change in the processing conditions that the pulp is exposed to during the manufacturing process. Among these conditions, chemical treatment is one of the main factors that might influence the colorimetric signal intensity. Since different paper grades are prepared by a different manufacturing process, they are expected to differ in chemical treatment.²⁰ Paper grade also represents change in retention size, which can be considered a crude representation of pore size influence on the paper performance in colorimetric assays. As shown previously in chapter 2, this change in retention size is correlated with a change in signal characteristics, such as interface formation time and width (**Tables 2.3 and 2.5**).

In all tested devices, we used the buffer as a complexation agent instead of EDTA, since this alteration showed enhanced signal intensity. Devices were fabricated according to the method described in the previous chapter, except that the tested buffers are used instead of deposited EDTA treatment on the second layer (**Figure 3.1A**).

3.2.2 Paper treatment with SiO₂ nanoparticles

Similar to the buffer influence studies, we used the Fe(III) assay as a model experiment to evaluate the influence of paper impregnation with SiO₂ nanoparticles. We used the device treatment described in **Figure 3.1A**, except that citrate buffer (CS), pH 4, is used as a complexation agent instead of EDTA. We chose to proceed with this modification based on the results we obtained from the previous buffer studies, in which CS buffer (pH 4) produced the highest signal-to-noise ratio when using low Fe(III) concentration samples. All SiO₂ nanoparticle suspensions were only deposited on the fourth paper layer, since it is the layer where the colored product, i.e. signal, is formed. In all experiments, the blank represents an assay run on paper layers that are not treated with SiO₂ nanoparticles at all.

3.2.2.1 SiO₂ nanoparticle deposition

To optimize the treatment method for paper layers, we deposited 10 μ L of SiO₂ nanoparticles suspension on the W4 paper sheets in multiple ways. We performed this experiment using SiO₂ particles with 0.2, 1, and 3 μ m sphere size. Specifically, we applied the entire suspension volume either (i) on the front side of the paper layer, (ii) on the back side, or (iii) divided equally, with 5 μ L on each side. We recorded the signal intensity associated with using each of these deposition methods as a criterion for comparison.

3.2.2.2 SiO₂ suspension volume

To optimize the nanoparticle suspension volume for paper layers, we studied the influence of SiO₂ suspension volume by using a range of 10 – 50 μ L treatments for Whatman 4

paper grade. We tested the intensity resulting from using the different suspension volumes of different sizes of SiO₂ spheres (200 nm, 1 μm, 3 μm).

3.2.2.3 SiO₂ nanoparticles concentration

We studied the influence of deposited 3 μm SiO₂ nanoparticles concentration on both the width and intensity of the interfacial signal produced by using our 3D μPAD design for detection of 1 mM Fe(III). We recorded the change in both signal aspects 10 and 15 minutes after sample introduction to the device.

3.2.2.4 SiO₂ nanoparticle multivariate analysis

We used the optimized conditions of suspension deposition method, volume, and concentration to perform a multiple variable analysis study. The study involved varying both SiO₂ nanoparticle size and paper grades of different retention sizes. The SiO₂ nanoparticle sizes tested are 20, 80, 120, 200, 1000, and 3000 nm. The paper grades tested are Whatman Grade 4, Whatman Grade 1, and Ahlstrom Grade 601, with the retention sizes of 20–25, 11, and 2.5, respectively. The studied output was the onset signal width (minimum starting width), signal intensity, and the change in both outputs over time.

3.3 Results and discussion

3.3.1 Buffer capacity studies

3.3.1.1 Paper buffer capacity

The results summarized in **Table 3.1** indicate that all paper types caused an increase in the pH of the DI water, initial value of 6.93. This change reflects the buffering effect of the paper substrates. Among the tested paper types, Ahlstrom 54 and Ahlstrom 55 exhibited the highest pH values at both time points, reaching 7.58 and 7.57 after 2 days and slightly decreasing after 7 days. These results suggest that these papers have a higher inherent buffer capacity or release more alkaline compounds compared to other paper types. This observation suggests the more alkaline conditions Ahlstrom 54 and 55 grades might be treated with during paper processing. Conversely, Ahlstrom 613 showed the smallest pH increase, stabilizing at 7.28 after 2 days and slightly decreasing after 7 days, indicating a relatively lower buffering effect.

The Whatman papers (Grades 1 and 4) exhibited intermediate pH values, with slight decreases over time. For instance, Whatman Grade 1 showed a pH of 7.41 after 2 days, which reduced marginally to 7.37 after 7 days, suggesting a relatively stable buffering effect. These subtle changes over time might indicate minimal leaching or interactions affecting the DI water. Overall, the observed variations in pH among the paper types reflect differences in their manufacturing processes and chemical compositions, which influence their buffering capacities. Papers such as Ahlstrom 54 and 55, which demonstrated higher pH shifts, may be more suitable for applications requiring an elevated pH environment, while papers like Ahlstrom 613 could be favored for more

neutral conditions. Given these imperial findings, further studies could elucidate the specific components responsible for these buffering effects and their implications for microfluidic paper-based device performance.

Table 3.1. Changes in pH of DI water over time after immersion of different paper types. Initial DI water pH = 6.93.

Paper type	48 h	7 days
Whatman G4	7.35	7.33
Whatman G1	7.41	7.37
Ahlstrom 55	7.57	7.46
Ahlstrom 54	7.58	7.47
Ahlstrom 613	7.28	7.25
Ahlstrom 601	7.37	7.42

3.3.1.2 Influence of pH and buffer on signal intensity

With all pHs, we found that using either the citrate (CSC) or citrate-phosphate (CP) buffer as a complexation reagent, instead of EDTA, produced signals of higher intensities (**Figure 3.2**). This could be because these buffers, as weak chelators, might be less likely to sequester Fe(III) as strongly as EDTA. Consequently, this weaker chelation might allow more Fe(III) to be available for reduction to Fe(II) by ascorbic acid, which eventually reacts with Ferrozine increase the chromophore concentration at the interface. With that, we found that the buffer of pH 3 and pH 4 for CSC and CP buffers, respectively, produced the highest intensity among other pH values. Conversely, increasing the pH of each buffer showed a slight decrease in signal intensity. This inverse correlation between pH and signal intensity was observed with all tested buffer incorporation methods. This could be

attributed to that ascorbic acid reducing agent activity is pH-dependent such that the higher the pH, the lower the reduction rate.¹⁹

As compared to using the buffer on its own as a chelator, we found that dissolving either all reaction reagents or only EDTA in the buffer produced signals of lower intensities. For CSC buffer, both buffer incorporation methods (all reagents buffered and only EDTA buffered) resulted in similar signal intensities. For CP buffer, using the buffer on its own as a chelator still resulted in highest signal intensity. However, we found that when we dissolved all reaction reagents in this CP buffer that the produced signal was consistently lowest, as compared to other buffer incorporation methods. Dissolving EDTA in buffer with different pH values resulted in higher signal intensities than the unbuffered EDTA reagent, but it was still lower than that of buffer only treatment. For both tested buffers, same signal intensity trend was observed across pH values in which lower values resulted in higher signal intensity. We selected this pH range since ascorbic acid is not an effective reducing agent for Fe(III) at higher pH values.

In general, we observed that using the buffer as a chelator would produce signals of higher intensities than that of using EDTA (original pH 4.5). These results were observed with both buffer types, CSC and CP, and across all tested pH values, 3–6. while both buffers showed a similar signal intensity at pH 3, CP buffer showed a slightly higher signal intensity than that of CSC with higher pH values, 4–6. The CSC buffer showed a slight decrease in signal intensity as the pH increased though. This increase in signal intensity using citrate-phosphate buffer could possibly suggest that combination of citrate and phosphate can offer better pH buffering capacity over a broader range.^{21,22} The citrate-phosphate buffer might also suggest a synergistic effect to better facilitate the reduction

of Fe(III) and improve the formation of the Fe(II)-Ferrozine complex. While citrate can act as a chelating agent, phosphate might help maintain a consistent pH.^{23,24} Eventually, this enhanced pH stability suggests a more stable environment for the reduction of Fe(III) to Fe(II) by ascorbic acid and subsequent reaction with Ferrozine.

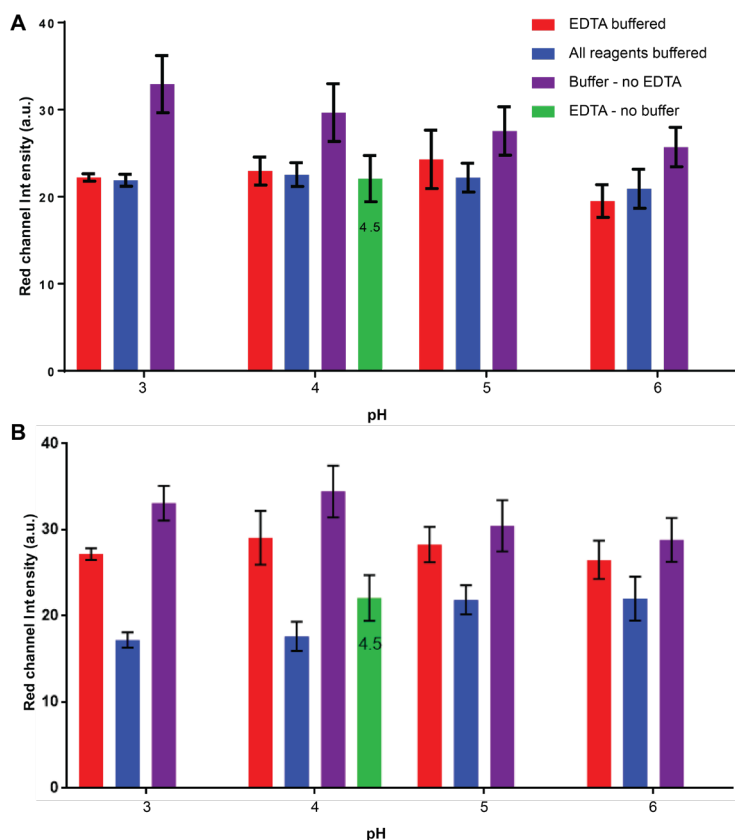


Figure 3.2. Effect of pH and buffer composition on signal intensity in Fe(III) detection assay. (A) Signal intensity results using citrate (CSC) buffer as a complexation reagent at varying pH levels. (B) Signal intensity results using citrate-phosphate (CP) buffer under similar conditions. Both buffers demonstrated higher intensity compared to EDTA, producing higher signal intensities. (N = 5)

3.3.1.3 pH multivariate analysis

3.3.1.3.1 Low Fe(III) concentration

From the perspective of paper type, we observed that Ahlstrom 55 consistently produced the lowest signal intensities across all buffer compositions and pH values tested, with an

average red channel intensity of 14.9 a.u. Similarly, Ahlstrom 601 exhibited the second lowest signal intensity, with an average red channel intensity of 19.7 a.u., except when citrate-phosphate (CP) buffer at pH 5 was used as a complexation agent. These findings may be attributed to the proprietary processing conditions of the papers, which are not disclosed by the manufacturer. Interestingly, the low signal intensities do not appear to correlate with the retention size of the paper, as Ahlstrom 55 and Ahlstrom 601 have significantly different retention sizes of 15 μm and 2.5 μm , respectively.

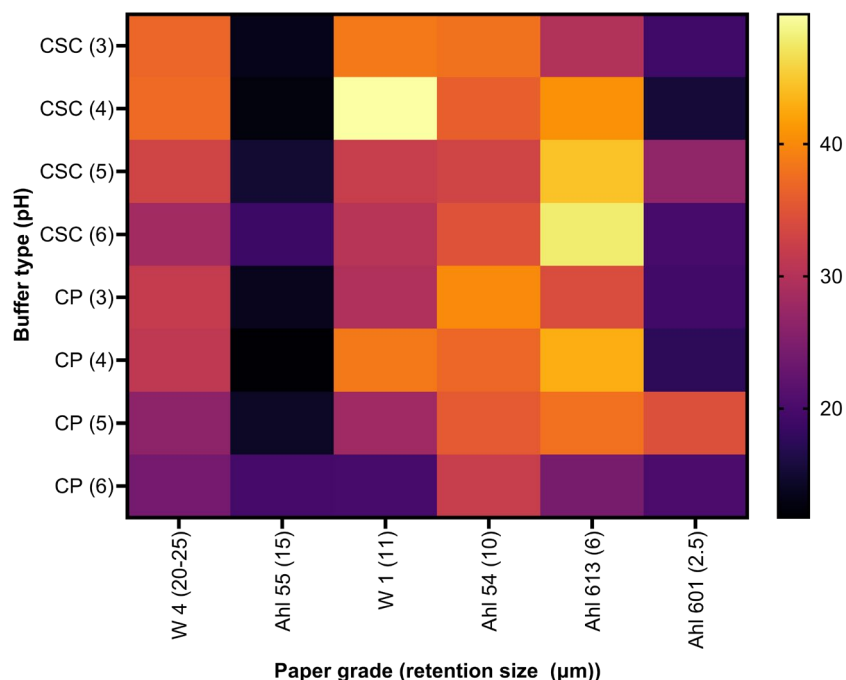


Figure 3.3. Heatmap representation of signal intensity as a function of paper type, buffer composition, and pH in Fe(III) detection assay, sample is 1 mM Fe(III). The heatmap illustrates the average red channel intensity values (a.u.) for various combinations of paper types, buffer compositions, and pH levels. Paper types are denoted as W for Whatman (Grades 4 and 1, with retention sizes of 25 μm and 11 μm , respectively) and Ahl for Ahlstrom (Grades 55, 54, 601, and 613, with retention sizes of 15 μm , 10 μm , 2.5 μm , and 6 μm , respectively). The y-axis represents the buffer type and pH, where CSC is citrate buffer and CP is citrate-phosphate buffer, with their corresponding pH values indicated in parentheses. (N = 5).

Contrary to this trend, paper types with retention sizes between these two values demonstrated higher signal intensities. Ahlstrom 613 produced the highest average red channel intensity of 37.7 a.u., suggesting that factors other than retention size—such as surface chemistry or porosity—might play a significant role in signal formation. The conditions yielding the highest signal intensities are summarized in **Table 3.2**.

While we tried running these experiments using acetate buffer too, no interface signals were detected at this low Fe(III) concentration. This could be attributed to the fact that acetate buffers do not typically support pH values less than 3.6. This lower limit value does not align with the fact that Fe(III) reduction by ascorbic acid is at its highest rate at 3 – 4 pH range.

Table 3.2. Signal intensities for selected paper grades and buffer conditions in Fe(III) detection assay, sample is 1 mM Fe(III). (N = 5).

Paper grade	Buffer type	pH	Average red channel intensity (a.u.) $\pm$ SEM
Whatman 1	CSC	4	49.8 $\pm$ 1.5
Ahlstrom 613	CSC	6	47.8 $\pm$ 2.0
Ahlstrom 613	CSC	5	44.4 $\pm$ 1.6

Using these findings, we decided to test a Whatman Grade 1 paper, treated with CSC pH 4, the conditions that produced highest signal, against samples of different Fe(III) concentrations. The study resulted in 2 calibration curves that covered a dynamic range of 0.1 – 1 mM and 1 – 10 mM (**Figure 3.4**). Running the Fe(III) assay with the device manufactured with conditions of paper type, buffer composition and pH, resulted in an LOD of 0.13 mM. This LOD is more than one order of magnitude lower than that obtained by the conditions reported in the previous chapter – Whatman 4 paper grade with 100

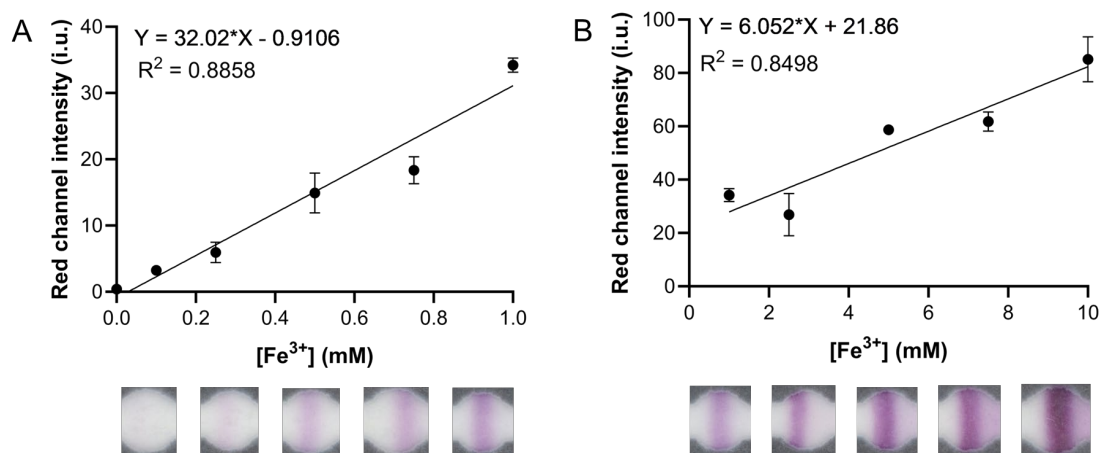


Figure 3.4. Calibration curves for Fe(III) detection using optimized device conditions. (A) Calibration curve covering the lower concentration range (0.1–1 mM) and (B) calibration curve covering the higher concentration range (1–10 mM) for Fe(III) detection using Whatman Grade 1 paper treated with CSC buffer at pH 4. Error bars represent the standard error of the mean (N = 5). Insets show the corresponding colorimetric signals for each Fe(III) concentration, highlighting the influence of optimized manufacturing variables, including paper type, buffer composition, and pH, on assay performance.

mM EDTA as a complexation agent (original pH = 4.5). These results highlight the influence of controlling the variables involved in the device manufacturing process. In the case of this device modification, these variables include the paper retention size, the reaction complexation compound, the assay pH.^{21–23}

3.3.1.3.2 High Fe(III) concentration

We tested the same combinations of paper grades, buffer compositions, and pH values using relatively higher Fe(III) concentration samples; 10 mM. We chose to repeat this study using that high concentration to evaluate if we can gain more control over the signal output to enlarge the upper concentration window of detection. Interestingly, the paper type that showed that highest signals with 1 mM Fe(III) samples, A613, did not show interface signals with most buffer treatments when 10 mM Fe(III) samples were tested,

Figure 3.5. This observation could hypothetically refer to a different interaction between

high Fe(III) concentrations and the employed buffer components, potentially causing interference or precipitation that hindered signal detection.²⁵

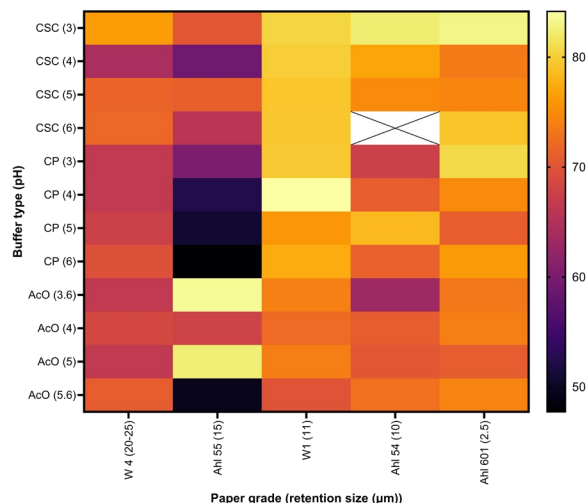


Figure 3.5. Heatmap representation of signal intensity as a function of paper type, buffer composition, and pH in Fe(III) detection assay, sample is 10 mM Fe(III). The heatmap illustrates the average red channel intensity values (a.u.) for various combinations of paper types, buffer compositions, and pH levels. Paper types are denoted as W for Whatman (Grades 4 and 1, with retention sizes of 25 μm and 11 μm , respectively) and Ahl for Ahlstrom (Grades 55, 54, and 601, with retention sizes of 15 μm , 10 μm , and 2.5 μm , respectively). The y-axis represents the buffer type and pH, where CSC is citrate buffer, CP is citrate-phosphate buffer, and AcO is acetate buffer with their corresponding pH values indicated in parentheses. (N = 5).

On average, Whatman Grade 1 showed the highest signal intensity in this study, with average red channel intensity of 77.1 a.u. While A601 paper type showed the second lowest signal intensity with 1 mM Fe(III) samples, it showed the second highest intensity with 10 mM Fe(III) samples. Regardless of the sample concentration, these findings further suggest that paper retention size is not the only factor that affects the signal intensity. However, and similar to low Fe(III) concentration samples, Ahlstrom 55 paper grade still showed the lowest signal intensity with the high Fe(III) concentration samples. This observation suggests that the chemical and physical properties of Ahlstrom 55, such as porosity and adsorption characteristics, might not be conducive to the Fe(III) assay.

This paper grade might have lower binding affinity for Fe(III) or Fe(II), affecting signal generation. The conditions that showed the highest signal intensities are in the order of described in **Table 3.3**.

Table 3.3. Signal intensities for selected paper grades and buffer conditions in Fe(III) detection assay, sample is 10 mM Fe(III). (N = 5).

Paper grade	Buffer type	pH	Average red channel intensity (a.u.) $\pm$ SEM
Whatman 1	CP	4	84.2 $\pm$ 9.3
Ahlstrom 601	CSC	3	83.0 $\pm$ 3.0
Ahlstrom 55	CSC	5	71.0 $\pm$ 11.3

Similar to the signal intensity analysis described above, we studied the correlation between the paper type, buffer composition, and pH variables and the noise associated with each combination (data not shown). From both signal and noise studies, we created a heatmap to visualize the relationship between these variable combinations and the signal-to-noise ratio.

Combining both data from signal and noise analyses, we obtained another graph that shows the signal-to-noise ratio for all tested combinations between paper grade, buffer composition, and pH, **Figure 3.6**. In general, Ahlstrom 601 and Whatman Grade 1 showed the highest S:N among other paper types, average S:N is 2.42 and 2.39, respectively. Collectively, the conditions that showed the highest signal-to-noise ratios are in the order of described in **Table 3.4**. On the other hand, Whatman Grade 4 and Ahlstrom 54 resulted in the lowest signal-to-noise ratio when high Fe(III) concentration samples were used.

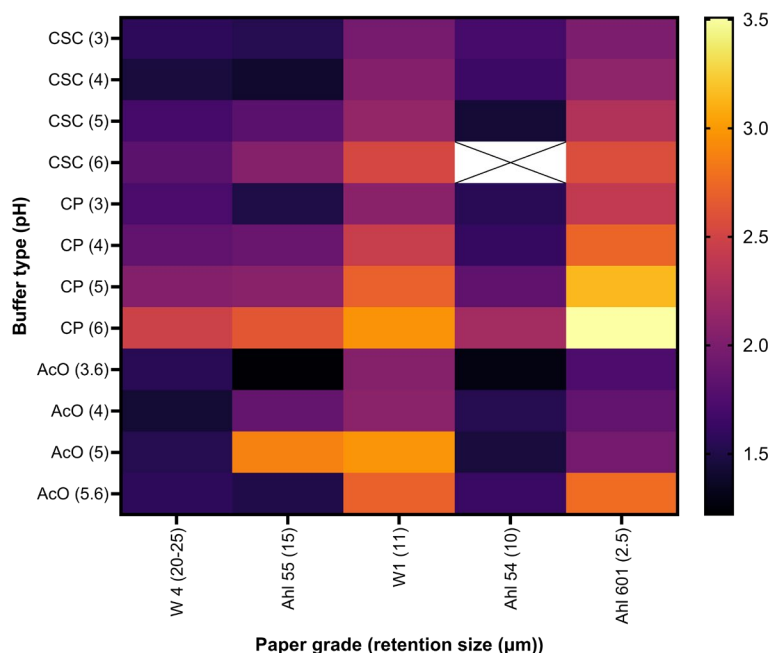


Figure 3.6. Heatmap representation of signal-to-noise as a function of paper type, buffer composition, and pH in Fe(III) detection assay, sample is 10 mM Fe(III). Paper types are denoted as W for Whatman (Grades 4 and 1, with retention sizes of 25 μm and 11 μm , respectively) and Ahl for Ahlstrom (Grades 55, 54, and 601, with retention sizes of 15 μm , 10 μm , and 2.5 μm , respectively). The y-axis represents the buffer type and pH, where CSC is citrate buffer, CP is citrate-phosphate buffer, and AcO is acetate buffer with their corresponding pH values indicated in parentheses. (N = 5).

From a buffer composition point of view, CP buffer type showed the highest average signal-to-noise ratio across studied paper grades. These results are in line with the findings shared earlier in which CP buffer showed highest signal intensity when high Fe(III) samples were applied. These results further suggest that the combination of citrate and phosphate can offer better pH buffering capacity over a broader sample concentration range.

Table 3.4. Average signal-to-noise ratios (S:N) for selected paper grades and buffer conditions in Fe(III) detection assay, sample is 10 mM Fe(III). (N = 5).

Paper grade	Buffer type	pH	Average S:N
Ahlstrom 601	CP	6	3.51
Ahlstrom 601	CP	5	3.14
Whatman 1	CP	6	2.96

Since it generated the highest signal-to-noise ratio, we decided to test Ahstrom 601, CP, and pH 6 experimental conditions against samples of different high Fe(III) concentrations. In this study, we tested the Ahlstrom 601-based devices with a 10 – 100 mM range of Fe(III) concentrations. such high Fe(III) concentrations are not typically relevant for biological or medical diagnostics, where the Fe(III) levels are generally much lower. However, evaluating these high concentrations can be instrumental in extending the dynamic range of our device. This broader range could make the device more versatile, allowing it to detect Fe(III) in a wider array of applications, such as industrial processes, environmental monitoring, and potentially other high-iron-content contexts. Despite analyzing the produced intensities using a number of color channels, none resulted in a correlation between the color intensity and the corresponding Fe(III) concentration, **Figure 3.7**. This lack of correlation might be due to a saturation effect. At high Fe(III) concentrations, the system could reach a saturation point where additional Fe(III) ions do not produce a proportional increase in signal intensity. This could lead to a plateau effect, preventing clear differentiation between varying concentrations.

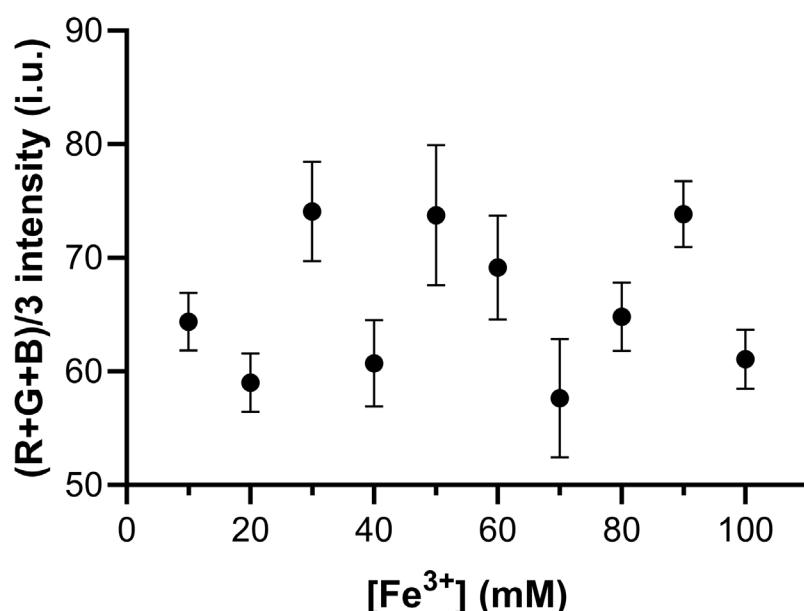


Figure 3.7. Signal intensity analysis of Ahlstrom 601-based devices for high Fe(III) Concentrations. Despite the high signal-to-noise ratio achieved under these conditions, no correlation was observed between Fe(III) concentration (10–100 mM) and colorimetric signal intensity for devices fabricated using Ahlstrom 601 paper, CP buffer, and pH 6. Error bars represent the standard error of the mean (N = 5).

3.3.2 Paper treatment with SiO₂ nanoparticles

3.3.2.1 SiO₂ suspension deposition

This experiment aims to evaluate which suspension deposition method is more efficient in signal intensification. We applied the entire suspension volume either (i) on the front side of the paper layer, (ii) on the back side, or (iii) divided equally, with 5 μ L on each side. These suspensions were only deposited on the fourth paper layer, since it is the signal readout layer. The conditions used in the Fe(III) assay of this study are the ones that showed the enhanced signal intensity in the previous section: Whatman Grade 4, CP buffer, and pH 4. Using signal intensity as the evaluation criterion, we observed that only paper layers treated on both sides displayed higher signal intensities, as compared to the

SiO₂ untreated papers, **Figure 3.8**. This enhancement in signal intensity could be attributed to the improved distribution of SiO₂ particles, which likely leads to better deposition within the void volumes of the paper pores.¹⁴ Across various SiO₂ particle sizes, we found that papers treated with 200 nm SiO₂ nanoparticles exhibited higher increase in signal intensity, compared to 1 and 3 μ m SiO₂ treated papers. To evaluate whether there is a relationship between the SiO₂ particle size and the paper retention size or not, we expanded our study to cover a larger range of paper types and SiO₂ particle sizes.

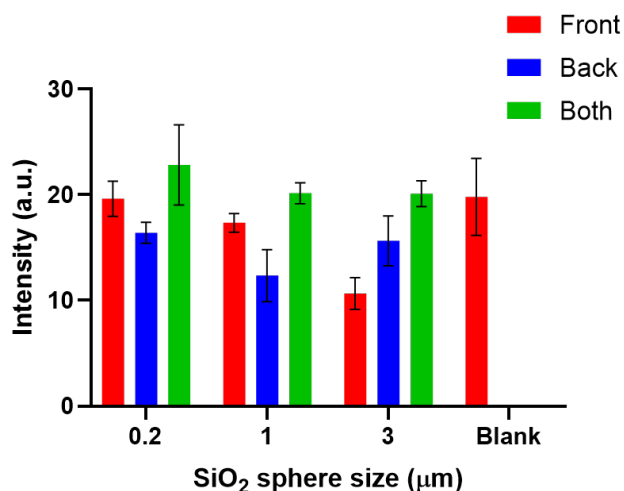


Figure 3.8. Effect of suspension deposition methods and SiO₂ particle size on signal intensity. Signal intensities for Fe(III) detection using different suspension deposition methods: front side only, back side only, and both sides. Error bars indicate standard error of the mean (N = 5).

3.3.2.2 SiO₂ suspension volume

We studied the influence of SiO₂ suspension volume by using a range of 10 – 50 µL treatments for Whatman 4 paper grade. We tested the intensity resulting from using the different suspension volumes of different sizes of SiO₂ spheres (0.2 µm, 1 µm, 3 µm), **Figure 3.9**. For the 0.2 µm SiO₂ spheres, we observed an increase of the intensity as the deposited volume of suspension increased, with the highest intensity observed at 50 µL. While the 3 µm SiO₂ spheres also showed an intensity increase with the deposited volume, the increase is less pronounced compared to the smaller sphere size, 0.2 µm. On the other hand, the highest intensity for 1 µm SiO₂ spheres is observed at 10 µL, not 50 µL. This indicates that, unlike the other sphere sizes, the signal intensity decreases with increasing volume for the 1 µm spheres. This deviation from the trend seen with the 0.2 µm and 3 µm spheres suggests that there might be an optimal volume for achieving the highest signal intensity with the 1 µm SiO₂ spheres. Overall, the study suggests that both the SiO₂ particle size and their suspension volume could play a role in determining the signal intensity, with the highest intensities generally correlated with larger volumes of suspension, particularly for smaller SiO₂ particles.

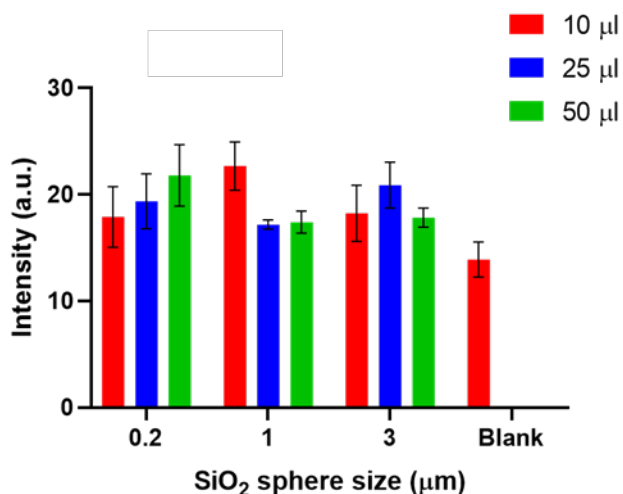


Figure 3.9. Effect of SiO₂ suspension volume and particle size on signal intensity. Error bars represent the standard error of the mean (N = 5).

3.3.2.3 SiO₂ nanoparticles concentration

We studied the influence of deposited 3 μm SiO₂ nanoparticles concentration on both the width and intensity of the interfacial signal produced by using our 3D μPAD design for detection of 1mM Fe(III) (**Figure 3.10**). We recorded the change in both signal aspects 10 and 15 minutes after sample introduction to the device. As for the colorimetric signal width, all SiO₂ treatments showed wider signals at the interface between the two converging liquid fronts. Like untreated papers, all treatments showed an increase in signal width as a function of time. Besides, the findings suggest a decline in the interfacial width as the suspension concentration increases. As for the colorimetric signal intensity, the measurements indicate that all SiO₂ paper treatments resulted in higher intensity as compared to untreated devices. This intensity enhancement could be attributed to the SiO₂ nanoparticles which likely occupy the void volumes in the paper pores, leading to a more localized colorimetric response. This is consistent with findings from previous studies on nanoparticle stability and deposition techniques.^{26,27}

Additionally, the untreated devices showed a slight decrease in signal intensity 15 minutes after sample introduction, compared to the signal recorded five minutes before. Nonetheless, between the signal recorded 10 and 15 minutes after sample introduction, both 0.5% and 10% SiO₂ nanoparticle treated devices showed unchanged and a slight increase in the signal intensity, respectively. In addition, among treated papers, we observed that the increase in signal intensity decreased with increasing the concentration of the SiO₂ suspension. This trend suggests that impregnating paper layers with lower concentrations of SiO₂ nanoparticles could improve the detection sensitivity of the Fe(III) assay.

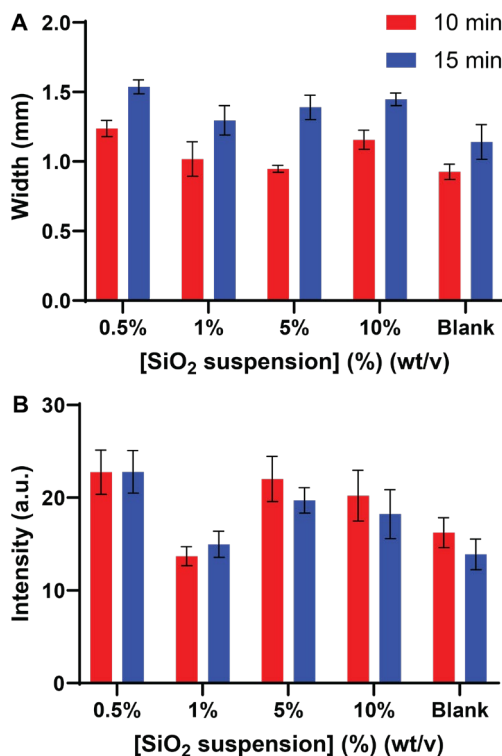


Figure 3.10. Effect of SiO₂ suspension concentration on interfacial signal width and intensity. (A) Signal width and (B) intensity for 3 μ m SiO₂ nanoparticle-treated papers at varying suspension concentrations (0.5%, 1%, 5%, and 10% w/v) measured at 10 and 15 minutes after sample introduction. Error bars indicate standard error of the mean (N = 5).

Figure 3.11A describes the relationship between the retention size (expressed by the 3 paper types plotted in the x axis) and the SiO₂ nanoparticle treatment of different sphere sizes (expressed by the 6 sphere sizes + the blank (untreated) on the y axis). These combinations between paper types and SiO₂ sphere size are tested for the minimum width of the interfacial signal they show as the two opposing liquid fronts converge. We observed that Whatman Grad 4, which has the largest retention size among tested papers, gives the widest average interfacial signal among tested papers. These results suggest that larger pore sizes provide more void space to accommodate reacting species. Therefore, the increased amount of the formed products is represented in the form of wider interfacial signals.²⁸ To this regard, **Figure 3.11B**, however, describes how the width of the formed interfacial signal increases/widens over time (mm per minute). While W4 showed the widest starting interface width, it showed the slowest rate of signal widening over time. Interestingly, Ahlstrom 601, which has the smallest pore size among the tested paper types, showed the fastest signal widening rate. This could probably be owing to its smallest pore size which restricts the movement of reactants at the readout layer. The presence of unreacted species at the readout layer could lead to a better chance to react at the interface, since they are in excess.²⁹ Specific studies might be necessary for each reaction to evaluate the compromise between the thickness of the initial interfacial signal and the rate of its widening over time. Such studies hold the promise to potentially lead to the formation of a quasi-permanent colorimetric signal.

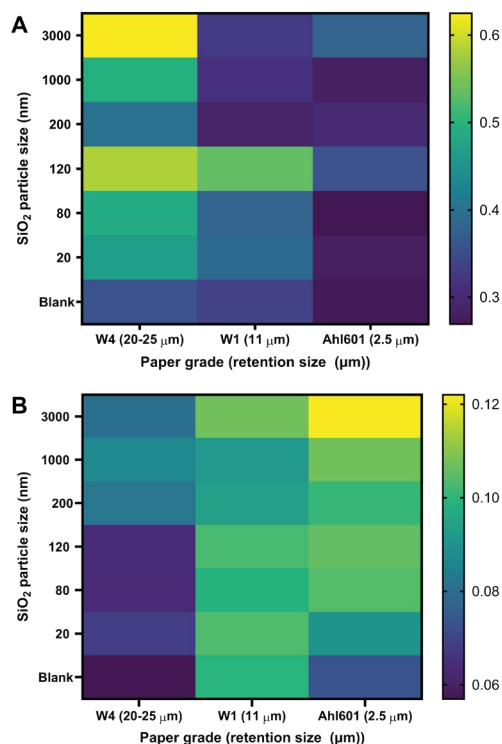


Figure 3.11. Effect of paper retention size and SiO₂ nanoparticle treatment on interfacial signal width and widening rate. (A) Heatmap illustrating the minimum width of the interfacial signal as a function of paper grade retention size (Whatman Grade 4, Whatman Grade 1, and Ahlstrom 601) and SiO₂ sphere size (20–3000 nm, including untreated blank). (B) Heatmap depicting the rate of interfacial signal widening (mm per minute) over time. (N = 3).

3.4 Conclusions

In this chapter, we explored multiple modifications to the 3D μ PAD design introduced in Chapter 2. We aimed to identify strategies for improving assay performance, particularly in terms of sensitivity and signal stability. These modifications focused on the interaction between chemical and physical properties within the device, including buffer capacity, pH, paper pore size, and silica nanoparticle impregnation.

We began by investigating the influence of buffer capacity, pH, and paper type on the Fe(III) assay. Our multivariate study tested combinations of citrate and citrate-phosphate buffers across various pH values and paper grades, examining their effects on signal intensity and noise. This approach provided insights on how specific conditions, such as using citrate-phosphate buffer at pH 4 with Whatman Grade 1 paper, enhanced assay sensitivity and produced higher signal-to-noise ratios. These improvements suggest that both the chemical environment created by the buffer and the intrinsic properties of the paper play a role in modulating assay performance.

However, these findings remained empirical and did not establish clear predictive relationships between the tested variables and assay outcomes. While it is evident that factors like pH and buffer composition significantly impact signal formation, the complex interaction between these variables and the paper buffer capacity limits the ability to design experiments based on a theoretical model. Instead, performance optimization relied on iterative experimentation.

In the second part of this chapter, we examined the effects of paper pore size and silica nanoparticle impregnation on interfacial signal intensity, width, and stability. By

systematically varying nanoparticle suspension volume, sphere size, and concentration, we aimed to study their influence on signal behavior. The results demonstrated that smaller nanoparticle sizes, such as 200 nm, and lower suspension concentrations generally yielded higher signal intensities. Additionally, the deposition location, particularly treating both sides of the paper, further enhanced signal clarity.

Despite these insights, the relationship between nanoparticle properties, paper retention size, and signal dynamics remained largely empirical. While wider pore sizes were associated with broader interfacial signals, the rate of signal widening over time depended on complex interactions between nanoparticle distribution and paper structure. These results underscore the potential for optimizing signal output through nanoparticle treatment but do not provide a predictive framework for experiment design.

In summary, this chapter underscores that assay performance in 3D μ PADs is influenced by multiple factors, including pH, buffer composition, paper grade, and nanoparticle impregnation. Each of these parameters affects signal intensity, width, and response to analyte concentration. However, the relationships between these factors are inherently complex and do not lend themselves to straightforward modeling or predictive design. As such, future efforts should strive for a deeper mechanistic understanding of how these variables interact to influence assay outcomes. By continuing to refine and test these modifications, we can incrementally improve the reliability and utility of 3D μ PADs in diagnostic applications.

3.5 Materials and methods

3.5.1 Reagents and Device Materials

We purchased Whatman chromatography paper grade 4 and 1 (GE Healthcare Life Sciences) from Amazon. We purchased Ahlstrom chromatography paper grade 54 and 55 from Laboratory Sales & Services LLC. We purchased Ahlstrom chromatography paper grade 601 and 613 from Thomas Scientific. We purchased Flexmount Select DF051521 (permanent adhesive double-faced liner) from FLEXcon. We purchased Fellowes laminating sheets from Amazon. We purchased Iron(III) chloride hexahydrate, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid (Ferrozine) from Acros Organics. We purchased ethylenediaminetetraacetic acid disodium salt dihydrate, acetylthiocholine chloride from Sigma Aldrich. We purchased iron(III) chloride hexahydrate from Alfa Aesar. We purchased 20, 80, 120, 200, 1000, and 3000 nm Silica Nanospheres from Nanocomposix. We purchased ASTM Type I water from Ricca Chemical Company. We used an EPSON Perfection V600 Photo scanner to acquire images of devices and completed assays.

3.5.2 Solution preparation

We prepared all Fe(III) assay solutions as described in Chapter 2. We prepared different dilutions of SiO₂ nanoparticles using DI water as a solvent.

3.5.3 Fabrication of paper devices

We fabricated all devices as described in Chapter 2. For SiO₂ deposition, we impregnated Layer 4 of devices with 10 μ L of suspension and dried it at 65 °C for 20 min. We then used the treated layer for further steps of device fabrication as described in Chapter 2.

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Chapter 4

Electrochemical paper-based analytical devices with three-dimensional electrode configuration

4.1 Introduction

Electrochemical detection is a powerful technique for the analysis of various compounds, especially those with electroactive properties.¹ Electrochemical techniques exhibit several advantages such as low signal-to-noise ratio and unique analyte redox potential, which led to improved sensitivity and selectivity.^{2,3} Accordingly, and combined with their portability and short analysis time, electrochemical sensors have been widely utilized.⁴ Screen-printed electrodes (SPEs) and electrochemical paper-based analytical devices (ePADs) have contributed to advance many fields, including electrochemical biosensing.⁵ These devices have been utilized a range of applications including clinical measurements,^{6,7} food analysis,⁸ and environmental monitoring⁹ due to their rapid supply of sensitive and accurate analysis using minimal sample volumes.¹⁰

With these considerable advantages, one of the main shortcomings of these devices is that they are limited to flatbeds.¹¹ Because all device electrodes are coplanar, this limits the versatility of these devices with respect to the substrates that they can be used with, i.e. they are incompatible to use with nonplanar substrates.^{12,13} Additionally, ePAD fabrication can pose a challenge, especially when complicated and/or expensive techniques are involved to create hydrophobic patterns.^{14,15} While minimal interelectrode distance in the ePAD is a critical factor for its electrochemical conductivity, this distancing condition can be challenging to maintain when using simple techniques such as screen printing. This is owing to the physical properties of paper substrates, such as roughness and porosity, which could influence the interelectrode distance consistency among devices during the manufacturing process.^{16,17}

Three-dimensional fabrication of paper-based devices has been recently reported in literature as their structure offers new capabilities to devices. For instance, devices can use three-dimensionality to collect or process samples and/or offer multiplexed detection.^{18–20} However, in these devices, cell electrodes, or at least two of them, still share the same paper layer, which does not provide a comprehensive solution for the interelectrode distance challenge. Also, the origami style of these devices implies that their layers are usually attached through folding, which does not provide strong interlayer adhesion nor consistent control over interelectrode distance due to the possibility of air gap incorporation.^{8,21} The fluctuating distances between device layers might influence the repeatability of their electrochemical performance leading to compromised usability.

To this end, in this chapter we show a novel 3D ePAD design which could be manufactured with the three-cell electrode deposited on unique layers instead of a common reaction zone. This design allows for more controllable interelectrode distances as they are defined by thickness of paper grades and adhesive layers used to bind the device layers. In this manner, studying the influence of changing the paper thickness can provide insights on the relationship between interelectrode distance and the correlated electrochemical signal in ePADs. Because electrodes are deposited on separate layers, the design supports electrode configuration interchangeability simply through swapping metal clamps connected to electrode pads. The design also ensures easier manufacturability since each electrode is stencil printed on a separate layer. Additionally, the design supports controlled studying of the influence of not only the interelectrode distance, but also the thickness of the electrode hosting layers, which is insightful in determining the practical considerations needed for optimized device performance.

4.2 Device design considerations

4.2.1 Three-dimensional device architecture

The three-dimensional device architecture comprises three electrodes (working, counter, and reference) that are stencil printed on separate layers of paper (**Figure 4.1**). We designed our device to separate the cell electrodes to allow control over electrode positioning to minimize the effect of uncompensated resistance between electrodes and also modify the formal order of each electrode (working/counter/reference vs. counter/working/reference).

We stencil printed each electrode over a hydrophilic rectangle that was patterned by wax printing on each electrode-hosting layer. We stencil printed the middle and lower electrodes, layers 3 and 5, to be wider than their hosting hydrophilic rectangle (**Figure 4.2**). The extra electrode width serves to be a contact area with the electrode pad. This additional contact area is essential to separate the Ag/AgCl pads from the electrolytic pathway in the flow layers.

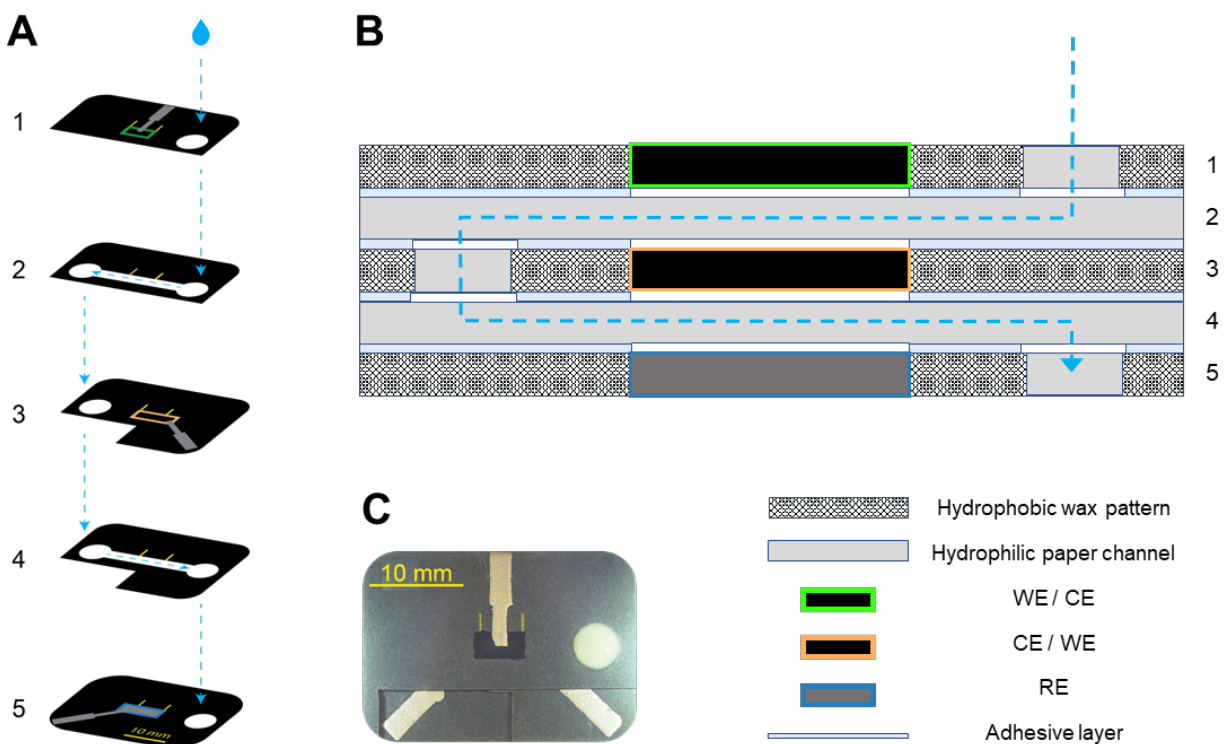


Figure 4.1. A schematic of the 3D ePAD design constructed with which can be fabricated using paper grades of different thicknesses. (A) Unique device geometry allows versatile definition of electrode configuration and separation distance between electrode surfaces equal to only paper + adhesive layers thickness. Based on the desired electrode configuration, the 3 electrode pads of the device are connected to their corresponding potentiostat metal clamps. Numbers to the left represent the order of paper-based device layers. Layers 1, 3, and 5 host working electrode (WE), counter electrode (CE), and reference electrode (RE), respectively. Layers 2 and 4 serve as flow layers between each two consecutive electrode-hosting layers. (B) Compact cross-sectional illustration of 3D ePAD layers. Light blue dashed lines indicate fluid flow through and between layers. Upon sample introduction, fluid flows in a serpentine fashion to create an electrolytic connection among the three-electrode paper-based electrochemical cell. Discontinuation in adhesive layers refers to laser cut holes created to direct sample flow within the device and allow electrolytic connection between electrodes. (C) Image of an actual TFN-based device where sample of 1 mM $K_3[Fe(CN)_6]$ in 100 mM KCl is introduced to the device from the hydrophilic circular zone in the top layer.

The undesirable contact between the Ag/AgCl pad and the electrolyte could lead to Ag/AgCl oxidation, which takes the form of a tarnish color to the electrode pad (**Figure 4.3A and 4.3B**). This uncontrolled electrode pad oxidation could compromise the electrical conductivity of the pad, resulting in less consistent signal output (**Figure 4.3C**). While the excess width of the middle electrode goes to one direction, right in this device design, it goes the opposite direction for the lower electrode to allow for pad connection to minimize chances of contact between clamps and leave room for more pads when multiplexing. The need for an excess electrode area does not exist for the top electrode since the electrode pad is not in contact with the electrolytic flow. Unlike middle and lower flow pads, we did not observe any color tarnishing to the silver color of this electrode pad.

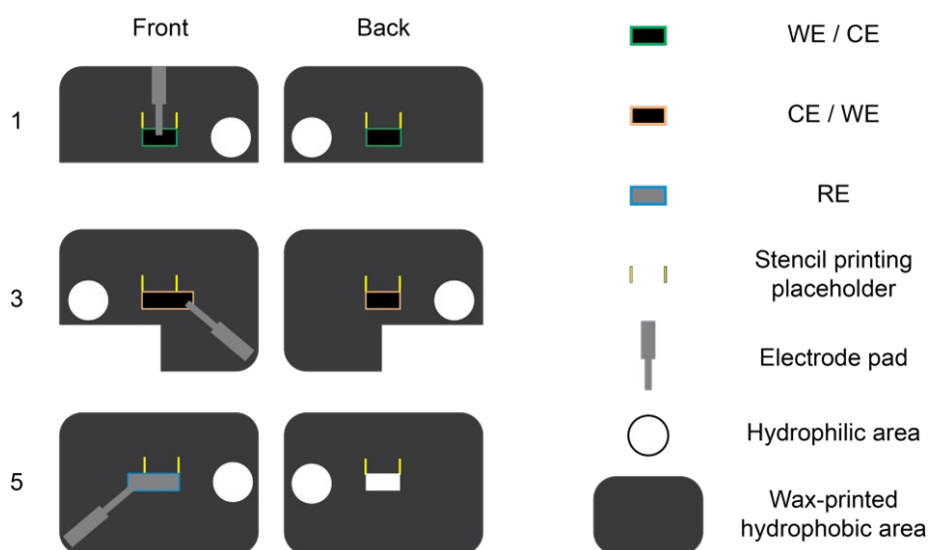


Figure 4.2. A schematic of the face and back of each electrode-hosting layer in the 3D ePAD design. The top and middle electrodes are double-sided to ensure carbon ink penetration across the paper layer thickness. The Ag/AgCl RE, however, is top-sided printed since electrolyte does not pass beneath its hosting layer. The electrode areas are wider in the middle and lowest layers to allow for a separate connection space with the Ag/AgCl electrode pads. The extra electrode width serves to be a contact area with the electrode pad, which is essential to separate the Ag/AgCl pads from the electrolytic pathway in the flow layers.

To each printed electrode, we stencil printed Ag/AgCl pads to connect the cell electrodes to their corresponding metal clamps from the electrochemical workstation (**Figure 4.1A**). Each electrode is positioned to match the center of the hydrophilic flow channel(s) above and/or underneath. Each electrode-hosting layer includes a circular hydrophilic zone to the right or the left of the electrode to direct the sample flow. In this way, every electrode-hosting layer of the device is a mirror image to the subsequent one, yet also uniquely addressable, thus simplifying manufacturing while also permitting flexibility in device design. Upon wicking, the circular hydrophilic zone to the right of the RE indicates the

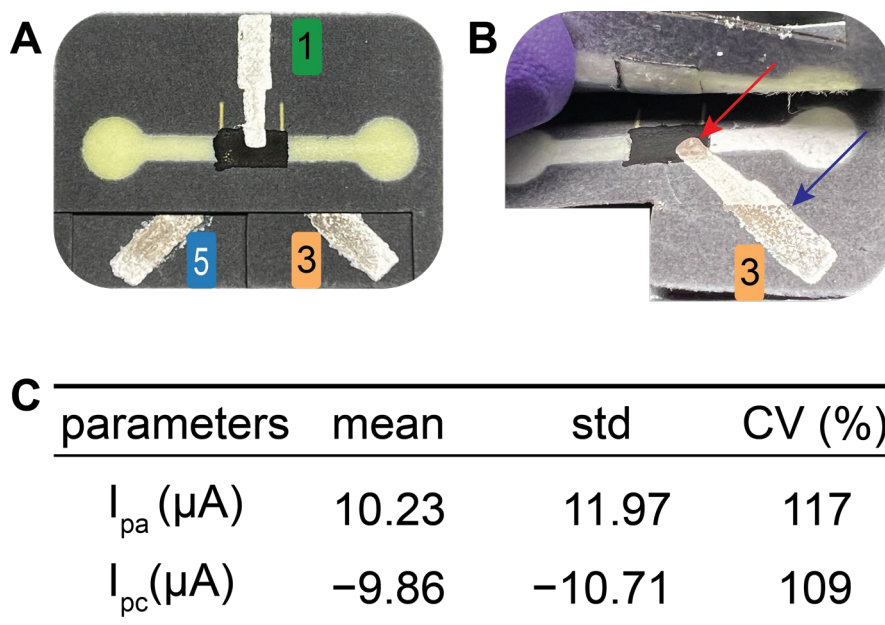


Figure 4.3. Color tarnishing of middle and lower electrode pads. (A) Top-view of the 3D ePAD chip, with electrode pads connected to middle and lower electrodes, layers 3 and 5, respectively, experiencing color tarnishing. This color change does not happen to top electrode pad, layer 1. (B) The color tarnishing occurs to the part of the electrode pad exposed to electrolytic path, red arrow, and/or air, blue arrow. The device design showed in these images is a previous iteration in which the electrode is centered on a lateral channel and sample is added from the circular zone to the right. (C) Table shows the variability of produced current values of anodic (I_{pa}) and cathodic peaks (I_{pc}). All device layers are A601-based, using WCR electrode configuration. Samples is 100 μL of 10 mM $K_3[Fe(CN)_6]$ in 1 M KCl and CV experiments were performed using scan rates of 100 mV/s. (N = 20).

device filling such that all device layers hydrophilic channels/zones have been wetted by the sample added. Device layers are affixed to each other by sheets of patterned, double-sided adhesives (**Figure 4.4**). The adhesive patterns are designed to allow electrolytic connection between each two subsequent electrode surfaces. As a result, two electrodes are separated from one another by a distance equal to the combined thickness of the paper and adhesive used.

The overall fluidic pathway in the device is serpentine: the sample wets the device inlet, wicks vertically to the first flow layer, layer 2, travels down the flow layer laterally to the outlet, then vertically two layers below, layer 3 and 4, where it changes directions. Patterned holes in the adhesive film separating channels on subsequent layers permit two electrodes to be wetted by the sample simultaneously, allowing electrolytic connection between each two facing electrode surfaces (**Figure 4.1B**). This process repeats until the sample reaches the circular zone of the bottom layer of the device. Adhesive films in between layers of paper ensure that leaks or contamination do not occur.

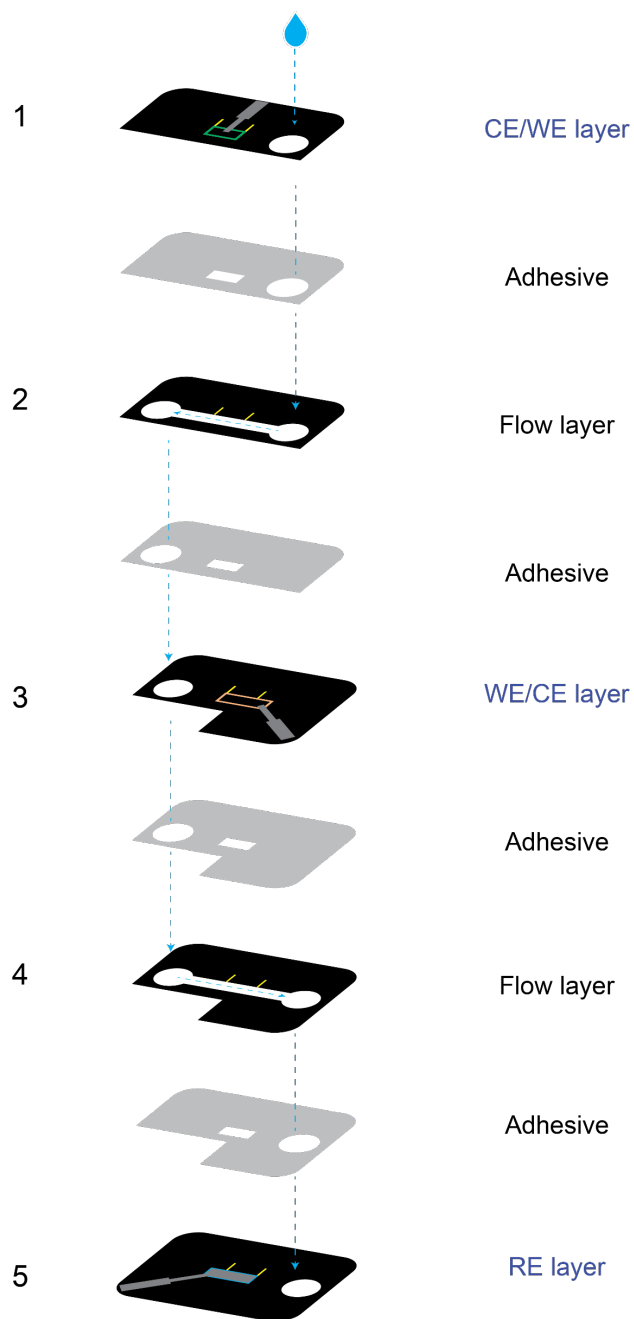


Figure 4.4. A detailed schematic of the 3D ePAD layers constructed with double-sided printed electrodes. Numbers to the left represent the order of paper-based device layers. Blue colored layer labels indicate the electrode-hosting paper layers. The schematic highlights the geometries of the laser cut adhesive holes between paper layers. These adhesive layers support permeant adhesion on both faces to ensure device layers integrity.

4.2.2 Influence of ink penetration

Stencil printed carbon or Ag/AgCl inks don't reside solely on the surface of the paper layer—they penetrate a depth into the paper that is challenging to control reproducibly and can result in an obstruction of sample flow. We stencil printed carbon ink on both sides of the layers hosting the top and middle electrodes, layers 1 and 3. The double-sided printing allows for a more direct electrolytic contact with electrodes. That is, the lower surface of the top electrode and both the upper and lower surfaces of the middle electrodes are in contact with the electrolyte passing through the flow layers (**Figure 4.5A**).

We decided not to print carbon electrodes on one side because printing carbon ink only on the upper surface of the electrode-hosting layer caused interrupted electrolytic contact, since the printed electrode renders the remaining midsection of the paper more hydrophobic. Thus, if only the upper midsection is stencil printed, the lower midsection will not exhibit full contact with the electrolyte in the flow layer underneath. In addition, the top-sided printing of the electrode increases the interelectrode distance by an amount equal to the lower midsection of the paper layer hosting the single-sided electrode (**Figure 4.5B**). This additional lower midsection would increase the distance between each two consecutive electrodes and, of course, the upper and lower electrodes. When electrodes are double-sided printed, the overall distance between the upper and lower electrodes would comprise (i) hydrophilic section (flow layer), (ii) electrolyte-filled adhesive thickness, and (iii) conductive electrode paste-printed paper (electrode-hosting layer). When electrodes are only top-sided printed, however, that distance would comprise the above mentioned elements two differences: (1) increased distance between upper and lower

electrodes, and (2) less conductive thickness overall. Both differences are due to partial conductive ink penetration (**Figure 4.5**). While we would not have this issue if we only printed the lower midsection of the top electrode-hosting layer with ink, this option would not allow feasible access to the electrode pad according to the multilayered device structure. Hence, we printed both sides of the top-layer electrode although the upper midsection of this electrode is not in direct contact with the electrolyte path – to allow for easier access between the electrode pad and the corresponding metal clamp connected to the electrochemical workstation.

We fabricated our device using two different grades of paper: TFN and Ahlstrom 601. TFN was selected as an example for thick papers (ca. 460 μm), Ahlstrom 601 (A601; ca. 270 μm) was selected as an example for medium thickness papers, while Kimtech Kimwipes (KW; ca. 70 μm) was selected as an example for thin papers.

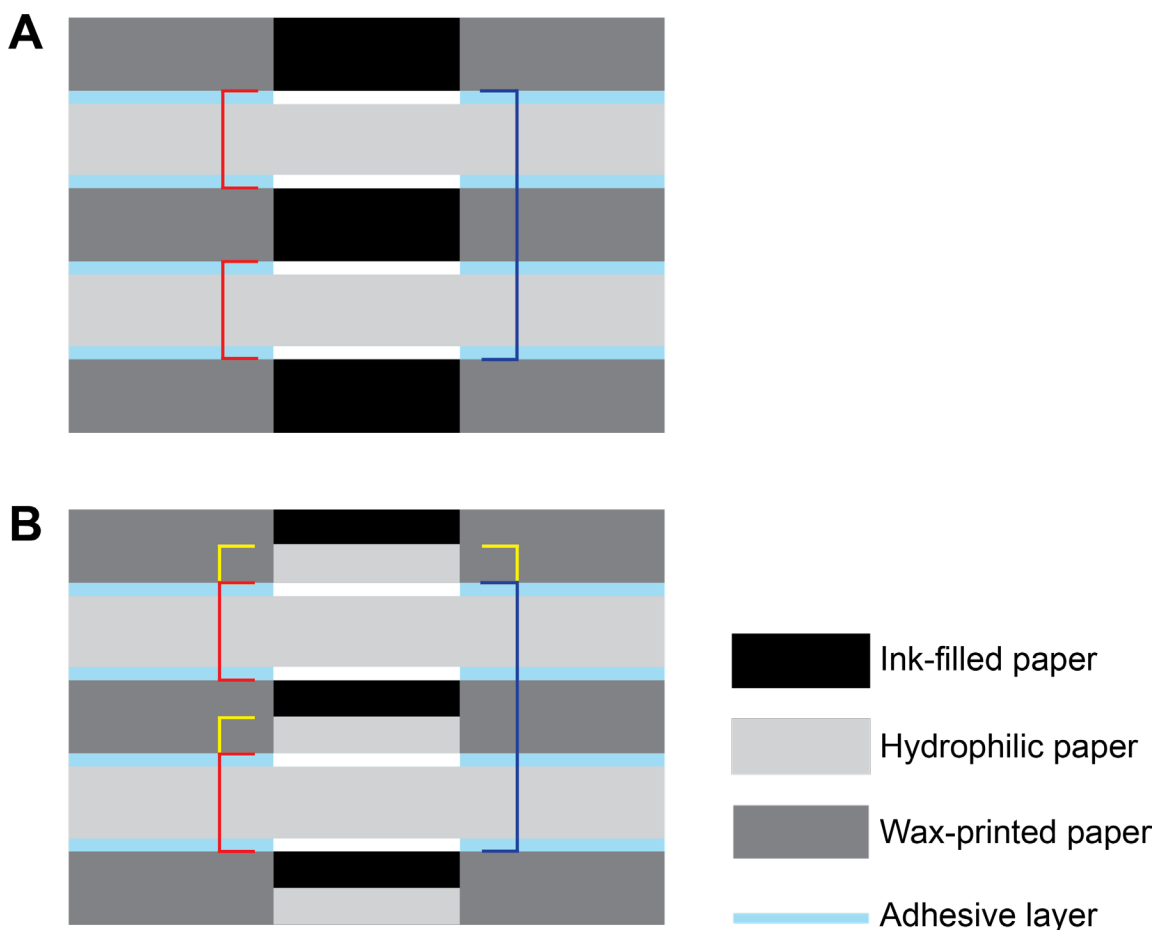


Figure 4.5. A schematic of cross-sectional view of the 3D ePAD layers, constructed using (A) double-sided printed and (B) top-sided printed electrodes. To the left of electrodes, brackets represent the distance between facing surfaces of each two consecutive electrodes. Red brackets refer to the combined distance created by flow layer plus adhesive sheet thicknesses. Yellow brackets, in B, represent the additional thickness added by the lower midsection of the paper when electrode ink is only top-sided printed. To the right of electrodes, brackets represent the distance between the bottom surface and the top surface of the upper and lower electrodes, respectively. For A, this distance components, in blue, include hydrophilic flow layers, adhesive sheets, and conductive carbon ink. For B, this distance is combined with the lower midsection of the paper containing the top-sided printed electrodes, in yellow. Discontinuation in adhesive layers refers to laser cut holes created to allow electrolytic connection between electrodes.

4.2.3 Interchangeability of electrode configurations

The design of our 3D-ePAD offers the configuration of printed electrodes to be interchangeable—where individual, electrode-patterned layers can play different roles in a measurement—simply by repositioning the metal clamps connecting electrode pads to the potentiostat. That is, when no WE modification is required, without needing to alter how a device is fabricated, assembled, or treated, 3D-ePADs can adopt an arrangement of electrodes that are ordered (i) working/counter/reference (WCR) or (ii) counter/working/reference (CWR). The final electrode is always the reference because of two reasons. First, changing RE order will not affect WE-RE distance when compared to CWR and WCR configurations. WE-RE distance is important because it is proportional to the uncompensated resistance of the electrochemical system. If we move the RE to the middle position, layer 3, the WE-RE distance will be the same as in the CWR configuration. If we move the RE to the top position, layer 1, we will only invert the order of the electrodes in the WCR and CWR configurations, depending on WE and CE orders in their respective configurations, without changing the inter-electrode distance. Second, we didn't reconfigure RE as a WE nor CE because they have different chemical compositions – RE is Ag/AgCl-based while WE and CE are carbon-based.

This configuration interchangeability is not supported by most of the ePAD designs reported in literature so far where all electrodes are coplanar.^{22–24} Many of these designs mainly focus on minimizing their WE-RE distance more than their WE-CE distance.^{16,25} This is because minimizing WE-RE is associated with lowering the uncompensated resistance between these two electrodes, while the potentiostat automatically compensates for the resistance arising from WE-CE distancing.^{26,27} Although these

device designs supports sensitive performance, their coplanar electrode configuration limits their versatility.¹³ In fact, interchanging electrode configuration between counter electrode and working electrode in conventional coplanar ePAD designs might be impractical because of their relatively large WE-CE distance. While the WE-CE resistance is mitigated by the potentiostat to some extent, aka iR compensation, the associated effects of increased resistance might not be eliminated. For instance, higher resistance in the electrochemical system could still introduce additional noise to the measurement, compromising the sensitivity and precision of the system. In addition, while WE and RE have minimal separation distance in many coplanar ePAD designs, they cannot be interchanged since they have different chemical composition. Commonly, the WE is made of carbon-based ink while RE is made of Ag/AgCl-based ink.^{22,25}

Additionally, the surface area of CE in conventional ePADs is much higher than that of WE.^{28,29} The reason for this enlarged surface area is to avoid limiting current transfer in the paper-based electrochemical circuit by the CE surface area.¹⁶ However, the considerable variation in electrode surface area poses a challenge to changing the electrode configuration. This is because if the CE were to be used as the WE, or vice versa, the CE surface area would be much smaller than that of WE, which would limit the current transfer in the circuit.

Controlling flow layer thicknesses and/or modifying electrode configuration in 3D ePADs can result in considerable change in the magnitude of a measured electrochemical current. This variation in signal output is attributed to the change in distancing between device electrodes, which contributes to the overall resistance of the device.²⁶ These differences may ultimately impact the performance or even applicability of a measurement

based on electrode configuration. We chose to evaluate these variables (i.e., device construction dictated by paper thickness and overall electrode configuration) using different electrochemical techniques: (i) electrochemical activity using cyclic voltammetry against potassium ferricyanide ($\text{K}_3[\text{Fe}(\text{CN})_6]$), (ii) chronoamperometry to detect glucose, and (iii) square wave anodic stripping voltammetry (SWASV) to detect $\text{Pb}(\text{II})$.

4.3 Results and Discussion

4.3.1 Influence of ink penetration

We tested the influence of ink penetration on both sample flow and signal output using different devices (**Figure 4.6**). We tested this influence using 3D ePADs made of different paper layer thicknesses; namely KW, A601, and TFN. The initial device design used to evaluate sample flow consisted of 3 electrode hosting layers without flow layers (**Figure 4.7B**). We used this design to test ink penetration when ink was stencil printed only on one side (top side) of the paper (**Figure 4.7**). In this design, each electrode is positioned in the center of a hydrophilic channel that also begins and ends with circular zones. These zones are in contact through patterned holes in the adhesive sheets binding layers together to create the final serpentine sample flow. We tested these TFN-based devices using WCR electrode configuration and linear sweep voltammetry (LSV) technique against samples of 1 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$ in 0.1 M KCl.

	Paper grade	Schematic	Actual	Ink penetration (%) [CI 95%]	Flow under electrode
A	KW			90.0 [83.2 – 96.7]	0 / 8
	A601			71.3 [60.5 – 82.2]	2 / 8
	TFN			61.4 [58.3 – 64.5]	8 / 8
B	KW			95.1 [91.7 – 98.4]	–
	A601			89.4 [81.3 – 97.6]	–
	TFN			93.2 [86.3 – 100.1]	–

Figure 4.6. Cross sectional schematic and images of the ink penetration associated with printing (A) top and (B) both sides of different papers with carbon ink. While images are not to scale, the heights of black rectangles in the schematic illustrations reflect the ink penetration percentage in their corresponding paper types. KW, A601, and TFN represent thin, medium, and thick papers. Although pristine carbon ink is used to stencil print electrodes on KW and TFN papers, 80% ink concentration is used to deposit electrodes on A601 papers to ensure deeper ink penetration. Ratios to the right represent the number of times the sample successfully flowed under the electrode in its hosting layer. These numbers are not applicable for double-sided printed electrode since sample flow is completely interrupted and a separate flow layer is introduced.

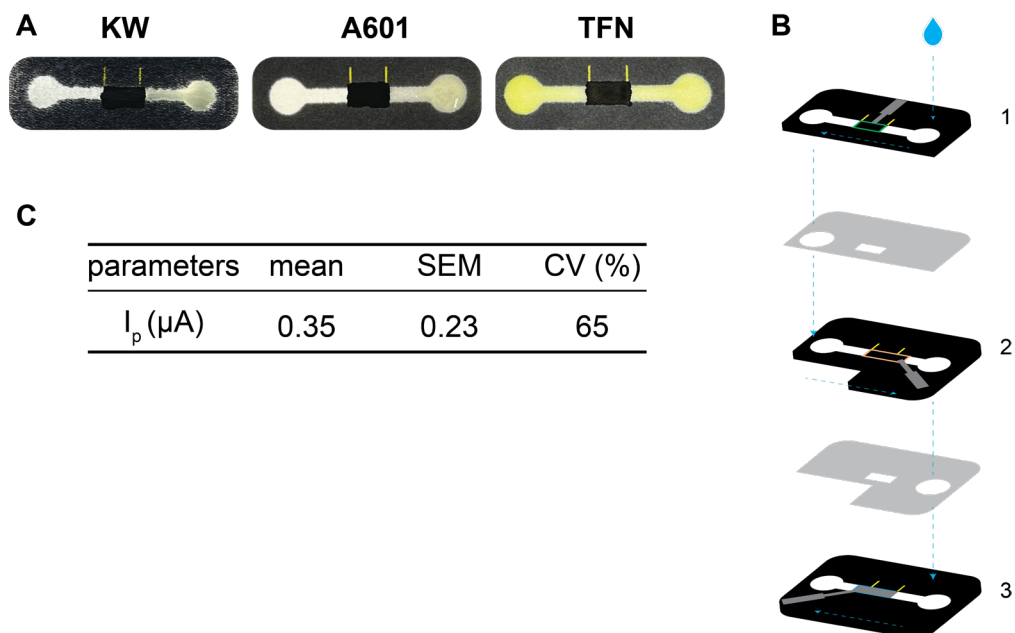


Figure 4.7. Sample flow under electrodes deposited on papers of different thicknesses. (A) Images of sample flow after being introduced to paper layers from the circular zone to the right of each electrode, wicking towards the left-hand side. While flow was interrupted when relatively thin papers were used, typically KW and A601, sample flow proceeded underneath TFN thick papers. (B) Schematic of the 3-electrode-hosting-layer device used to evaluate the top-sided printed TFN-based devices. Dashed blue lines indicate the lateral and vertical fluid flow across device layers. (C) Table shows the variability in peak current produced by running linear sweep voltammetry (LSV) using the top-sided printed TFN-based devices. For LSV, we used WCR electrode configuration and 100 μL samples of 10 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$ in 1 M KCl. (N = 4).

For 3D-ePADs made from TFN, the flow of an applied sample was not hindered by top-side printed electrodes. Printed ink penetrated approximately 61.4% [95% CI: 58.3 – 64.5] of the TFN paper thickness, which allowed for sample flow in the lower midsection underneath the printed ink (**Figure 4.8**). Despite the successful sample flow and the relatively simpler design, the current measured from the linear sweep

voltammograms showed high variability (CoV = 65% for peak current). The compromised reproducibility of this design could be attributed to the inconsistent interfacial contact between the sample (electrolyte) and the electrode surface. This inconsistency might be related to the large variability in ink penetration across the electrode width.

Schematic cross section



Ink concentration (% wt/wt)	Actual cross sections	Ink penetration (%) [CI 95%]
100		63.4 [54.5 – 72.3]
90		58.9 [49.8 – 68.0]
80		89.4 [81.3 – 97.6]
70		69.0 [61.6 – 76.4]

Figure 4.8. Cross sectional schematic and actual images of the ink penetration associated with printing both faces of A601 papers with carbon ink. Different ink concentrations, diluted with EtOH, of carbon ink produced penetration of variable depths. The ink penetration % represents the combined penetrations from both faces of the paper layer.

3D-ePADs made from A601 and KW, however, suffered from impeded or no sample flow as electrode inks blocked up to 100% of paper thickness. Ink penetration consumes 90.0% [95% CI: 83.2 – 96.7] and 71.3% [95% CI: 60.5 – 82.2] of KW and A601 thicknesses, respectively. We did not run LSV measurements using devices made of either of these paper types due to the interrupted sample flow – no electrolytic connection

among electrodes. Alternatively, we printed the carbon electrode ink on both sides for devices made of all paper types (TFN, A601, and KW). The double-sided printing aims to eliminate the ink penetration variability across the paper thickness, providing more consistent electrode access to sample flow. While we utilized double-sided printing only for carbon ink forming top and middle electrodes, we continued to stencil print Ag/AgCl ink forming RE, lower electrode, on one side of the paper, top side. This is because only one side of the RE surface is needed to ensure consistent contact with electrolyte, since the sample only wicks the flow layer above the RE.

As double-sided printing is expected to obstruct the flow in the electrode-hosting layer, due to deeper ink penetration, we tested this printing style with the 5-layered device described earlier. In these 5-layered devices, all 3 electrode-hosting and 2 flow layers were made of the same paper type.

While double sided printing on TFN electrode-hosting layers resulted in 93.2% [95% CI: 86.3 – 100.1] ink penetration, A601 showed less collective ink penetration across the paper thickness using pristine carbon ink, 63.4% [95% CI: 54.5 – 72.3]. As a result, we tested several solvent dilutions and found that 80% carbon ink concentration (wt/wt) provided the deepest ink penetration, 89.4% [95% CI: 81.3 – 97.6] (**Figure 4.8**). We used this ink dilution for carbon ink printing over all A601 paper sheets. We did not dilute the Ag/AgCl ink for any device, including A601-based, since it is only printed on one side, top side, of the lowest layer, layer 5. As previously mentioned, top-sided printing of KW resulted in 100% ink penetration. However, to use KW-based devices for our 5-layered device, we double-sided printed the electrode. The KW-based devices made of

double-sided printed electrodes showed less LSV peak current variance than top-sided printed devices (**Figure 4.9**).

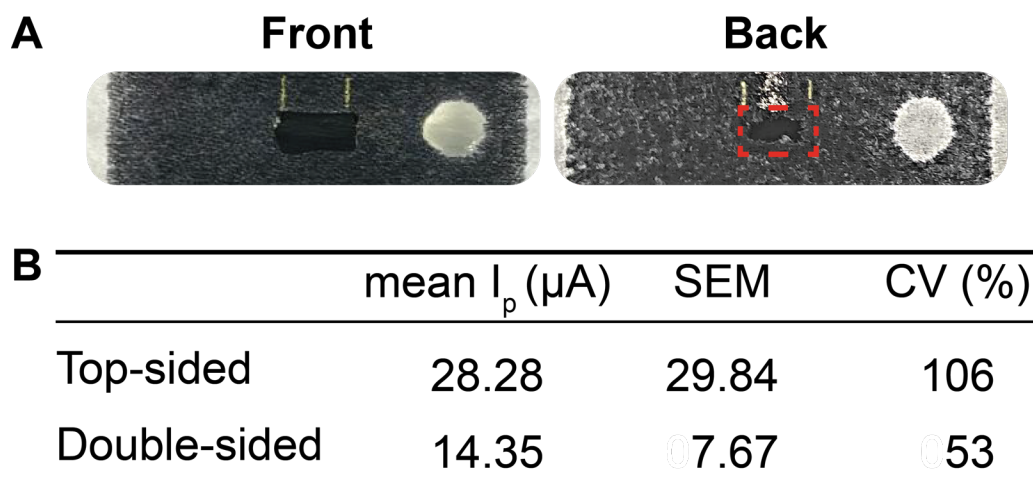


Figure 4.9. Printing top or both sides of KW paper grade. (A) Front and back sides of KW paper layer when hosting only top-sided printed electrodes. Dashed red rectangle highlights the irregularity of the ink filled area, since printed wax does not stay in shape after melting over KW paper layers. (B) Table shows the variability in LSV peak current and potential produced using top-sided and double-sided printed KW-based devices. For LSV, we used WCR electrode configuration and 100 μ L samples of 1 mM $K_3[Fe(CN)_6]$ in 100 mM KCl. (N = 4).

The mean LSV peak current varied across devices with respect to the paper thickness forming the layers of each device. For instance, the mean LSV peak current increased with decreasing the paper thickness when KW was used as a flow layer. Although the TFN-based devices showed the lowest current ($1.4 (\pm 92 \%) \mu$ A) among other paper types, this current output is more than three times higher than that obtained from single-sided printed TFN-based devices prepared from 3-layered devices (**Figure S5**). This increase in current suggests that double-sided printing could provide closer contact between the electrode surface and the electrode. Additionally, we hypothesize that the current output variability, both the mean values and their associated CoV%, among double-sided ink devices could be related to the thickness of both (i) the electrode-hosting layer, which probably influences the amount of ink deposited in the paper, and (ii) the flow

layer, which defines the interelectrode thickness. To test this hypothesis, we extended the LSV measurements by employing devices constructed from a matrix of various paper thickness combinations.

4.3.2 Influence of interelectrode thickness

Due to the versatility that our 3D ePAD design provides, we studied the impact of changing the paper thickness of the electrode-hosting and flow layers on the signal output. In this study, we examined if changing the flow layer thickness can be correlated with the interelectrode distance, which is associated with the overall device resistance.^{26,27,30} We evaluated the variability in peak current and potential by employing a combinatorial matrix of paper thicknesses. In this matrix, each paper type was utilized as the electrode-hosting layer and tested against flow layers of varying thicknesses, and vice versa. For all combinations, we tested both WCR and CWR electrode configurations using linear sweep voltammetry against samples of 1 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$ in 0.1 M KCl.

Interestingly, the measured currents differed substantially with respect to configuration and paper grade for all paper thickness combinations (**Figure 4.10**). In the WCR configuration, absolute values of currents ranged approximately 0.3–14.0 μA . Conversely, absolute values of currents in the CWR configuration were ranged approximately 2.5–68.2 μA in which many combinations demonstrated higher than one order of magnitude difference in electrochemical current. We believe this result could be attributed to the electrode arrangement of CWR format in which WE is in the middle vertical position between CE (top) and RE (bottom). This configuration allows minimal WE–CE and WE–RE distance, unlike WCR format that allows for only minimal WE–CE

distance while that distance is doubled between WE (top layer) and RE (lowest layer). As distance between WE and RE increases, uncompensated resistance of the overall cell increases, which leads to lower current.^{16,26}

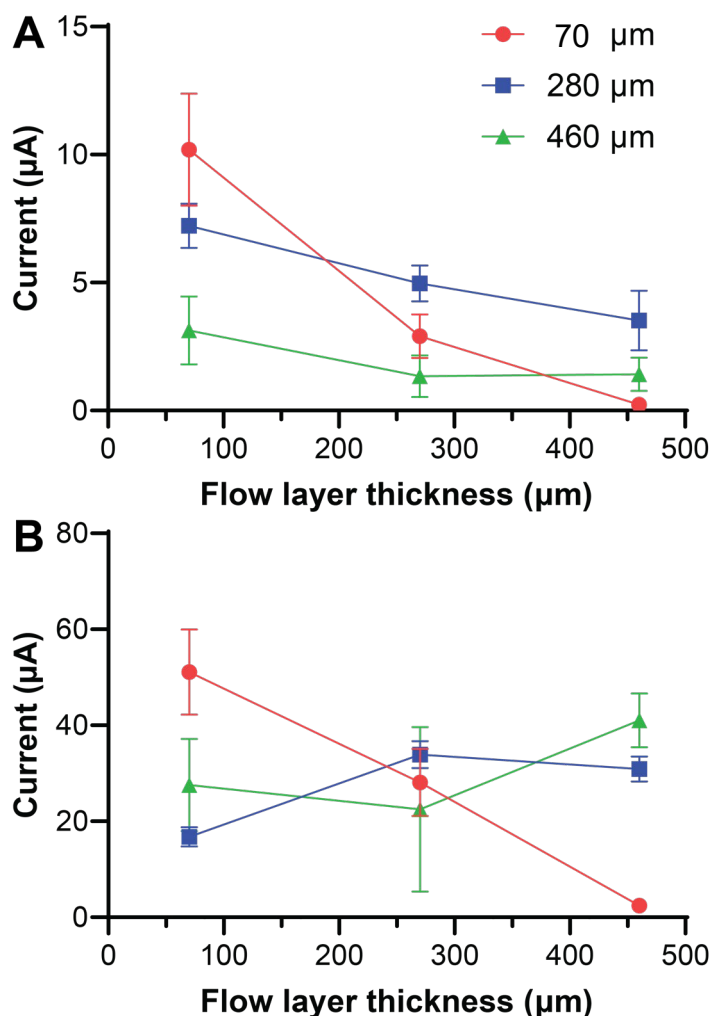


Figure 4.10. The relationship between the thicknesses of electrode-hosting and the flow layers based on the resulting LSV current. For all devices, both (A) WCR and (B) CWR electrode configurations are tested. Devices are fabricated using combinations of electrode-hosting and flow paper layers, using KW (70 μm), A601 (270 μm), and TFN (460 μm) paper grades to represent different thicknesses. Different electrode-hosting layers are represented in red, blue, and green based on their thicknesses. For every combination, the same paper type is used for all electrode-hosting layers, and another consistent type is used for all flow layers. Devices are tested with LSV using 100 μL samples of 1 mM $K_3[Fe(CN)_6]$ in 100 mM KCl. Error bars represent the standard error of the mean. (N = 4).

For all paper thickness combinations, all WCR configurations showed a correlation between the current magnitude and the flow layer thickness. For devices with the same electrode-hosting layer, thicker flow layers resulted in lower current response, which is in agreement with the fact that larger interelectrode distances increase the overall device resistance.³¹ Furthermore, the thinner electrode-hosting layers exhibited higher current responses. This trend was consistently observed across all KW flow layers and in both WCR and CWR configurations. The increased current density could possibly be attributed to faster redox species diffusion at the electrode surface demonstrated by thinner electrode-hosting paper layer.³² This trend shown by KW flow layers fails to persist with thicker papers, namely, A601 and TFN, for both WCR and CWR electrode configurations. This deviation from the trend might indicate that the increased resistance resulting from the greater interelectrode distance substantially contributes to the overall system resistance. It could also be related to porosity influence, which is not consistent across different paper types, since change in thickness is not necessarily the only factor affecting the current. Changing porosity, for instance, could impact the amount of electrolyte the hydrophilic area can accommodate, which changes the density of charge carriers, i.e. ions, in the space between electrodes.¹⁷ Collectively, these effects could probably outweigh the enhanced conductivity due to the improved diffusion of redox species at the electrode surface.

Using KW as electrode-hosting layers resulted in relatively higher CoV % than corresponding A601-based devices, up to three-fold more variability. This observation

might be related to the lower mechanical stability of KW as compared to thicker paper types

While TFN layers provided the highest mechanical stability for their devices, most devices that incorporated TFN layers demonstrated the lowest reproducibility. This case is applicable whether TFN is purposed as an electrode-hosting or a flow layer. Except for all TFN-based CWR devices, all TFN electrode-hosting devices showed CoV % that ranged between 35 % to 61 %. While the increased interelectrode distance may partially explain the observed TFN behavior, further investigation is required to fully understand the factors contributing to this electrochemical response in TFN-containing matrices. Inversely, electrode-hosting A601 layers showed the lowest CoV % that ranged from 8 % to 14 % except when TFN paper was used as flow layers. As a result, we chose to demonstrate the electrochemical characterization and utility of our device design using CWR configuration of all A601-based 3D ePADs.

4.3.3 Electrochemical characterization and utilization of 3D ePAD

To serve as a model for our device design, we selected the CWR electrode configuration of the device such that all electrode-hosting and flow layers are made of A601. Although all KW devices showed higher current response (**Figure 4.10**) we decided to use all A601 for manufacturability and repeatability reasons. Typically, the thin KW layers pose a challenge during the ink stencil printing and layer assembly due to their delicacy. The relatively more robust A601 layers, however, exhibit easier handling during device making while keeping subsequent electrode surfaces closer to one another, as compared to TFN, which results in higher current response. In addition, the low CoV % demonstrated by

A601 electrode-hosting layers suggests that it is a candidate for a reproducible electrochemical signal.

4.3.3.1 Cyclic voltammetry

We used $K_3[Fe(CN)_6]$ as a model for a redox-active compound to evaluate the electrochemical activity of our 3D-ePAD. By adding 75 μ L samples of 1 mM $K_3[Fe(CN)_6]$ in 0.1 M KCl, we ran cyclic voltammetry measurements using scan rates of 100 – 500 mV/s. Over the range of scan rates, we obtained the anticipated linear relationship between the square root of CV scan rate applied and the resulting current values for the CWR all-A601 3D-ePADs (**Figure 4.11**). This correlation confirms the consistent electrochemical behavior of the proposed 3D ePAD design and validates our theoretical predictions.³³ As described by the Randles-Sevcik equation, the obtained linearity suggests that the device operates within a diffusion-controlled regime, which is important for its intended future analytical applications at the POC. This diffusion-controlled regime represents the mass transfer of potassium ions in the electrode plating phase of the electrochemical reaction.³⁴ This predictable electrochemical response of the 3D ePAD also suggests its suitability for quantitative analysis.

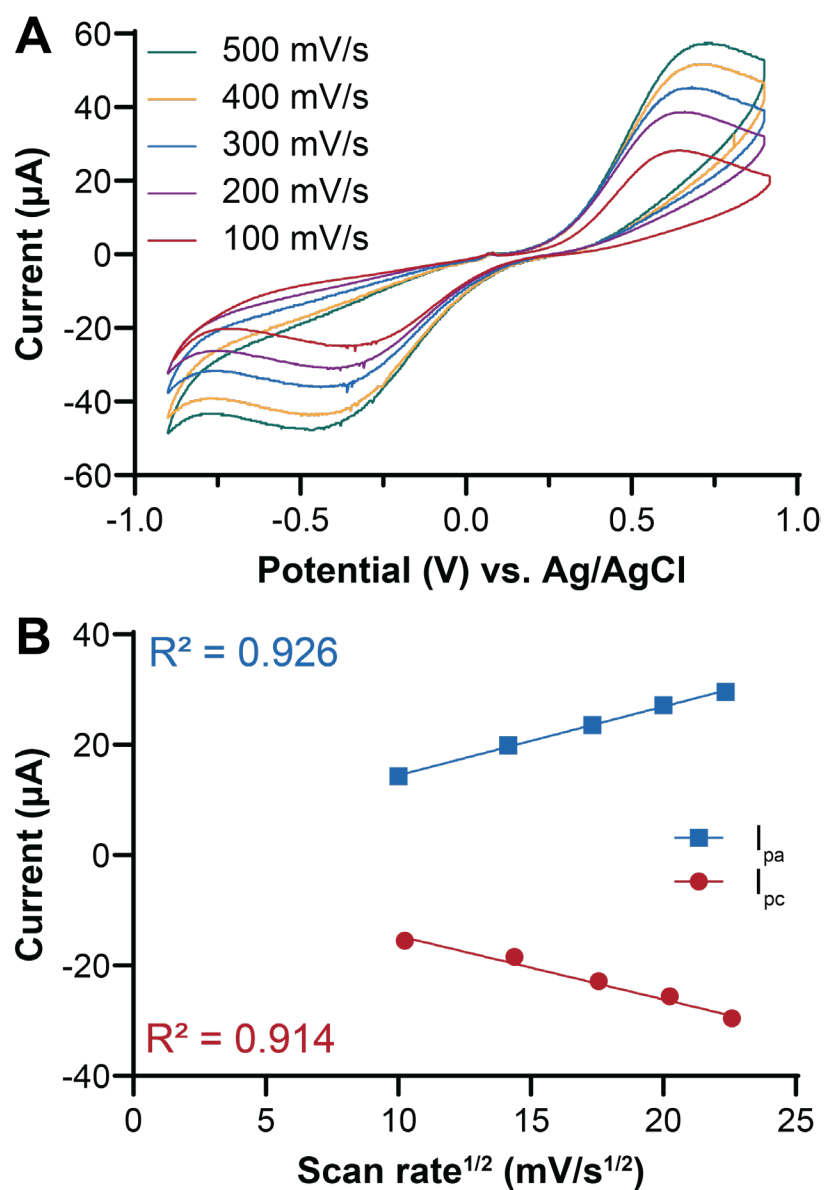


Figure 4.11. Cyclic voltammetry using all A601-based 3D ePAD with CWR electrode configuration. (A) Cyclic voltammograms produced by introducing samples of 100 μL of 1 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$ in 100 mM KCl and performed CV experiments using scan rates of 100 – 500 mV/s. (B) Linear relationships between square root of scan rates and their measured anodic (blue squares) and cathodic (red circles) peak currents. Error bars represent the standard error of the mean. (N = 4).

For all scan rates between 100 – 500 mV, the ratio between the anodic and cathodic current was close to unity ($i_{pa}/i_{pc} \sim 1$) (**Table 4.1**). The produced peak shapes reveal a Nernstian electrochemical reaction whose rate is governed by electroactive species diffusion to the WE surface.³⁵

Table 4.1. Peak current analysis of CV data generated by all A601-based 3D ePAD using a CWR electrode configuration

Scan rate (mV/s)	100	200	300	400	500
i_{pa}/i_{pc}	1.03	0.90	0.92	0.95	0.99

Expectedly, the peak-to-peak separation potential (ΔE_p) increased with increasing the CV scan rate (**Table 4.2**). As scan rates increase, the electrochemical system is allowed less time to reach equilibrium at each potential point. This decreased time results in larger separation between the potential values at which oxidation and reduction of the electroactive species happen, aka polarization overpotential.²²

Table 4.2. Peak-to-peak separation analysis of CV data generated by all A601-based 3D ePAD using a CWR electrode configuration

Scan rate (mV/s)	100	200	300	400	500
ΔE_p (V)	0.91	0.95	0.99	1.04	1.07

Theoretically, at 298 K, this polarization overpotential is 59 mV per n, where n is the number of electrons involved in the charge transfer process.³⁶ So, for the redox-active couple $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$, where the number of transferred electrons $n = 1$, the expected overpotential value is 59 mV. In our 3D ePAD, the increased polarization overpotential could be attributed to the paper substrate in which adsorption profile and

capillary action can lead to uneven distribution of redox active species at the electrode surface, suggesting a quasi-reversible electrochemical system.³⁷

As a frame of reference, we also recorded CV for SPE to which we can compare the peak potential values obtained by our 3D ePAD CWR electrode configuration (**Figure 4.12**).

The SPE peak-to-peak separation potentials resulted from cyclic voltammograms at different scan rates were closer than that of the proposed 3D ePAD, indicating faster charge transfer kinetics at the surface of SPE working electrode.³⁸

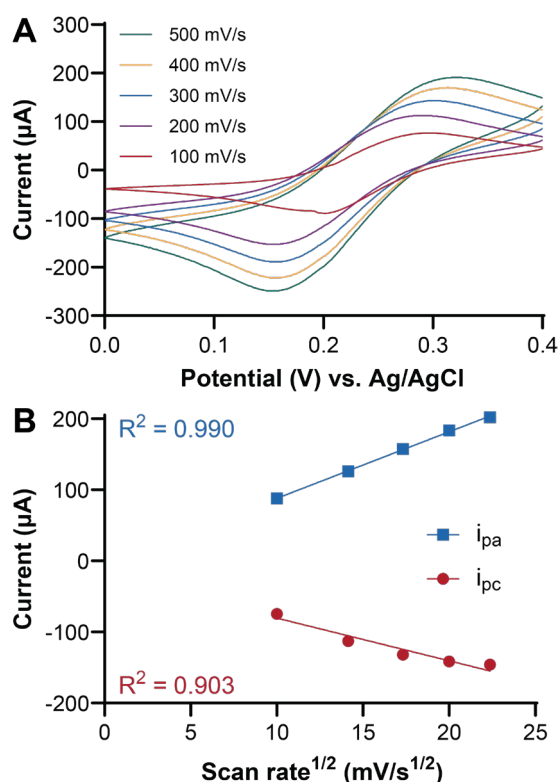


Figure 4.12. cyclic voltammetry using SPEs. (A) Cyclic voltammograms produced by SPE devices. The sample is 10 μL of 10 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$ in 1M KCl and performed CV experiments using scan rates of 100 – 500 mV/s. (B) Linear relationships between square root of scan rates and their measured anodic (blue squares) and cathodic (red circles) peak currents. Error bars represent the standard error of the mean. (N = 4).

4.3.3.2 Chronoamperometric detection of glucose

Glucose detection is crucial for clinical medicine and food industry, especially in diabetes diagnostic applications.³⁹ Enzymatic paper-based electrochemical sensors are a promising tool for glucose detection at the POC in which sensitivity, specificity, low cost, and fast response are important application factors.⁴⁰ To test the ability of our device design to perform enzymatic electrochemical detection, we chose to measure glucose by using chronoamperometry technique such that glucose oxidase was deposited in multiple locations in the flow layers. We performed this detection using the CWR electrode configuration in which all device layers were made of A601 paper grade. For all measurements, we found that the high charging current decays to a steady state Faradaic current within 40 s after applying step potential of 500 mV (**Figure 4.13**). Accordingly, we determined the steady state current as the average current value measured between 40 – 50 s after applying the step potential.

For the tested electrode configuration and paper grade, we found that the glucose concentration is proportional to the Faradaic current detected, as per Cottrell equation.³⁵ As shown in **Figure 4.13**, the linear calibration plot is described by the equation $y = 0.6369x + 2.570$ where x is the glucose concentration and y is the corresponding Faradaic current response. All proposed 3D ePAD iterations showed linear relationships between glucose concentrations and their corresponding Faradaic current. While the normal level of glucose in whole blood is 3.5 – 5.3 mM and 2.5 – 5.3 in plasma, the LOD for glucose detection using the proposed 3D ePAD was 1.75 mM ($N = 10$).⁴¹ The LOD exhibited by this device pave the way for utilizing the device design to detect glucose levels in other biological matrices such as urine, tears, and saliva.⁴²

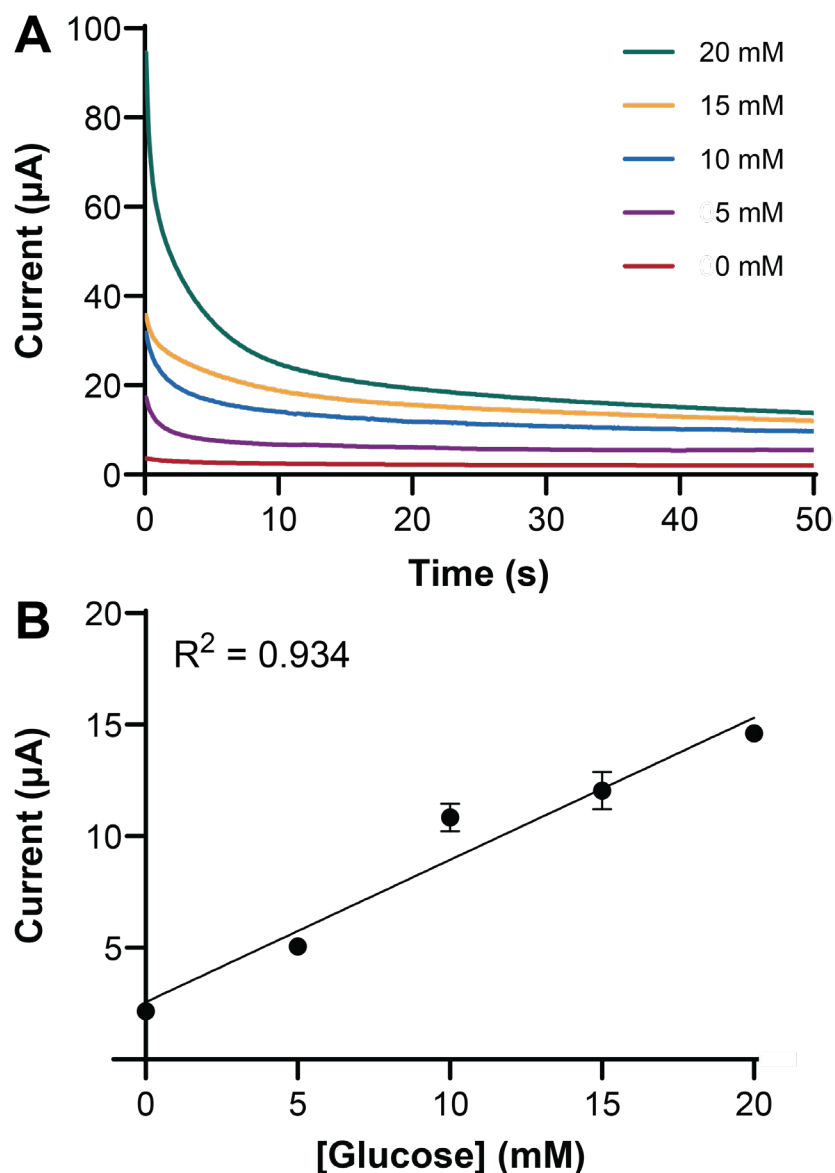


Figure 4.13. Chronoamperometric detection of glucose using all A601-based 3D ePAD with CWR electrode configuration. (A) Chronoamperometry plots for detecting 0 – 20 mM of glucose concentration ranges over a period of 50 s. (B) The resulting calibration curve shows a linear relationship between the glucose concentration (X) and the average resulting current in the duration between 40 s and 50 s (Y). This relationship is described by the equation $Y (\mu\text{A}) = 0.6369 \cdot X (\text{mM}) + 2.570$. Sample is 100 μL of varying glucose concentrations in DI water solutions. The applied a step potential is 500 mV. Error bars represent the standard error of the mean. (N = 3).

4.3.3.3 Anodic stripping voltammetric analysis of Pb(II)

Lead (Pb(II)) is one of the most potent water pollutant heavy metal ions, posing a serious threat to public health and global ecosystem.⁴³ Using paper-based electrochemical sensors for Pb(II) detection can be a powerful tool to utilize, especially in resource-limited regions where a fast selective sensor is needed while minimal access to sophisticated lab equipment is available.⁴⁴ To test the ability of our device design to perform a trace-level detection of ionic species, we chose to measure a wide range of Pb(II) concentrations using square-wave anodic stripping voltammetry (SWASV) technique. We chose SWASV for Pb(II) detection because it produces high signal-to-noise ratio, reducing the background noise resulting from the charging current during the potential scan.⁴⁵ Without any modification of the WE surface, we performed Pb(II) detection using the CWR electrode configuration in which all device layers were made of A601 paper grade.

We tested CWR electrode configurations against Pb(II) concentration range of 10 ppb – 1000 ppb. Because of that wide concentration range, the correlation between the generated current signal and Pb(II) concentration fitted a nonlinear three-parameter logistic model (**Figure 4.14**). Owing to their higher sensitivity, the signals generated by CWR A601 devices the tested covered a Pb(II) concentration range with an LOD of 4.4 ppb. The detection range exhibited by CWR configuration covers the permissible limit of Pb(II) concentration in fresh water.⁴⁶ The sensitivity of this device, being only a result of electrode configuration, signifies the usability of the configuration interchangeability offered by the device design. Since the devices are tested using only commercial unmodified carbon ink, the analytical performance of the proposed design is expected to

increase if the working electrode surface is chemically modified to capture different analytes.

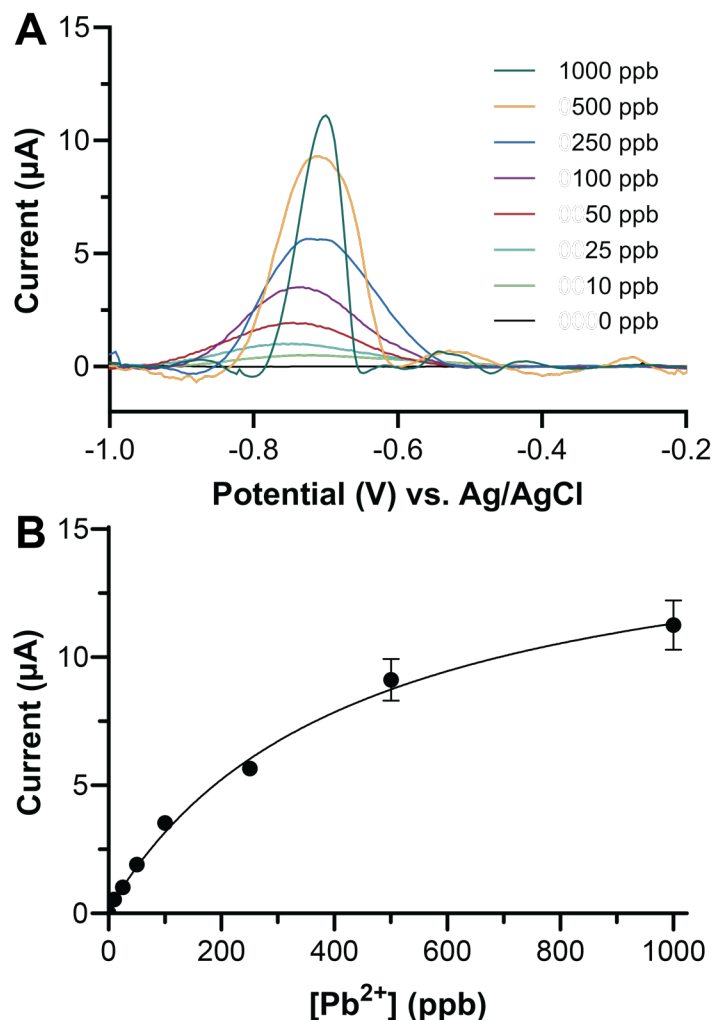


Figure 4.14. SWASV for detection of Pb(II) using 3D ePAD. (A) SWASV plots for Pb(II) detection using CWR configurations of A601-based paper layers. (B) Calibration curve of SWASV detection of Pb(II). Data are fit to a 3-parameter logistic (3PL) model with the following parameters: bottom is 0.1375, top is 16.09, and EC_{50} is 426.9. For all devices, we added 100 μL of Pb(II) concentrations in the range of 0 – 1000 ppb, which are dissolved in 0.1 M acetate buffer solutions (pH = 5). Deposition potential is -1.3 V and deposition time is 600 s. Error bars represent the standard error of the mean. (N = 4).

4.4 Materials and Methods

Reagents and Device Materials

We purchased Kimtech 34133 Kimwipes, 1-Ply, from Amazon. We purchased Alhstrom chromatography paper grade 601 from Thomas Scientific. We purchased Munktell TFN paper from Laboratory Sales and Services (Somerville, NJ). We purchased Flexmount Select DF051521 (permanent adhesive double-faced liner) from FLEXcon. We purchased 1/4" clear acrylic sheets from McMaster-Carr. We purchased Avery laminating sheets from Amazon. We purchased Elikliv 7" digital microscope with IPS screen - 1300X from Amazon. We purchased E3178 carbon ink and E2414 AG/AGCL ink from Ercon Inc. We purchased Glucose Oxidase from *Aspergillus niger* from Sigma. We purchased Lead(II) nitrate, 99+% from Thermo Scientific Chemicals. We purchased Samsill Economy Transparent Printer Sheets from Amazon. We purchased potassium ferricyanide from Acros Organics. We purchased carbon screen printed electrodes (WE-D-3mm-carbon / pseudo RE-Ag/AgCl / CE-carbon) from Zensor R&D (Taiwan). We purchased potassium chloride [4 M] saturated with silver chloride from Ricca Chemical Company. We purchased glacial acetic acid and sodium acetate anhydrous from Fisher Scientific. We purchased 99.5% anhydrous ethanol from Thermo Fisher. We purchased ASTM Type I water from Ricca Chemical Company. We used CH Instrunment 650E workstation to run all electrochemical measurements.

Solution preparation

For cyclic voltammetry and linear sweep voltammetry measurements, we prepared solutions of 10 and 1 mM potassium ferricyanide in 1 and 0.1 M potassium chloride solution, respectively. Potassium chloride solution is diluted from the 4 M solution from

Ricca Chemical Company. We prepared 0.1 M acetate buffer solution (pH 5) by mixing amounts of acetic acid and sodium acetate solutions and adjusting the pH to 5 using drops of 1 M hydrochloric acid and sodium hydroxide solutions. We daily prepared fresh samples of different concentrations of lead (II) nitrate solutions (1 ppb – 10 ppm) in acetate buffer (pH 5) by serial dilution.

For glucose assay, we deposited 3.25 μ L of 250 U/mL glucose oxidase in 600 mM potassium ferricyanide in 1 M potassium chloride solution on each flow layer of the A601-based device. The treated paper layers are dried for 20 min at 4 $^{\circ}$ C in dark over a silica gel bed. Sample is 150 μ L of different glucose concentrations. The increased sample volume aims to mitigate the sample evaporation effect.

Electrode deposition on paper

Electrodes were stencil printed using both carbon and Ag/AgCl inks. For KW and TFN paper types, ink was deposited as received. For carbon ink deposited on A601, 80 % (wt/wt) ink was used after dilution with 99.5% anhydrous ethanol. Dilution was done by mechanical mixing until a homogeneous paste was formed. We stencil printed Ag/AgCl ink to deposit all electrode pads. To dry deposited ink, we incubated their hosting device layers in the oven for 20 minutes at 65 $^{\circ}$ C.

Fabrication of paper devices

We used Adobe Illustrator to design the device layers and then printed the hydrophobic wax barriers using a Xerox ColorQube 8570 wax printer. We cut all adhesive, stencil, and paper layers using a Boss laser cutter (BOSS-LS1630). We used VWR® Forced Air Microbiological Incubator for drying conductive ink on paper sheets. We used Elikliv 7" digital microscope to image paper cross sections for ink penetration studies. We used a

Promo Heat CS-15 T-shirt press to melt the wax onto the chromatography paper to form hydrophobic barriers. For A601 and TFN-based devices, wax melting conditions were 45 seconds at 138 °C. For KW papers, wax patterns were created by transfer printing in which wax melting was done for only 5 seconds at 138 °C. Using a custom acrylic alignment jig, we built the device layers bottom up, starting with the RE-hosting layer. Using the jig, we laid the device layers and compressed them as layers compiled up. Next, we compressed the assembled device layers in the unheated T-shirt press to remove any air bubbles trapped between the layers.

4.5 Conclusions and future work

In this chapter, we introduced and characterized a novel 3D electrochemical paper-based analytical device that utilizes a three-dimensional electrode configuration to address limitations in traditional coplanar ePAD designs. The device architecture, which features individually layered electrodes separated by paper and adhesive films, allowed precise control over interelectrode distances and facilitated the study of their impact on electrochemical performance. The modular device design also enabled interchangeable electrode configurations, which provided flexibility for various analytical setups without requiring additional device fabrication.

Through systematic experiments, we demonstrated that interelectrode distance, dictated and controlled by the thickness of the mediating layers, extensively impacts the device resistance and, hence, current output. These results emphasized the importance of optimizing both material selection and layer construction to achieve reproducible and sensitive electrochemical signals. Moreover, the design versatility in adopting different electrode configurations can be employed to minimize the cell uncompensated resistance, enhancing device performance across multiple electrochemical techniques.

Besides these advancements, the findings also highlighted the potential for further improvement in device functionality and manufacturability. Two promising directions are proposed for future development, both of which are explored in detail in Chapter 5. First, the potential for multiplexing the 3D ePAD will be examined to enable simultaneous detection of multiple analytes within a single device. This multiplexed version aims to maintain facile manufacturability and high specificity and sensitivity for each analyte

paving the way for comprehensive point-of-care diagnostics in resource-limited settings. Second, a standalone iteration of the 3D ePAD will be explored to eliminate reliance on wax printing, which could pose a challenge related to scalability and hydrophobic barrier integrity. The modular nature of this standalone design will aim to allow for selective component replacement and reusability, making it a sustainable alternative for analytical applications. These proposed iterations not only extend the applicability of the 3D ePAD platform but also align with broader goals in diagnostic innovation: to create accessible, reliable, and sustainable solutions for diverse electrochemical sensing needs.

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Chapter 5

Possible areas of improvement for three-dimensional electrochemical μ PADs

5.1 Multiplexed three-dimensional ePADs for POC applications

5.1.1 Introduction

Recently, multiplexed ePADs have been reported as an important advancement in POC diagnostics, offering significant improvements in efficiency, cost-effectiveness, and diagnostic capabilities.¹ The ability to simultaneously detect multiple analytes on a single device addresses the growing demand for comprehensive and rapid medical testing, particularly in resource-limited settings. This approach not only reduces the sample volume required and the overall testing time but also minimizes the potential for human error associated with multiple separate tests. Furthermore, multiplexed ePADs align with the trend towards personalized medicine, enabling the analysis of multiple biomarkers to provide a more holistic view of a patient's health status.^{2,3}

The importance of multiplexing in ePADs extends beyond clinical applications, impacting fields such as environmental monitoring,⁴ food safety,⁵ and biodefense.⁶ By integrating multiple sensing elements on a single paper-based platform, the analytical performance of ePADs has exhibited a noticeable enhancement while maintaining their inherent advantages of simplicity, portability, and low cost.⁷ These ePAD multiplexing features, in addition to the accessibility of paper-based devices, has the potential to revolutionize on-site testing paradigms, enabling rapid decision making in various scenarios where traditional laboratory analysis is impractical or time consuming.

Recent advances in developing multiplexed ePADs have focused on innovative device designs and fabrication techniques to overcome the challenges of integrating multiple electrochemical sensors on a paper substrate. One significant development has been the introduction of three-dimensional paper microfluidic devices, which utilize multiple layers to segregate different sensing zones while maintaining a compact form factor. This approach has enabled the simultaneous detection of different analytes, demonstrating the scalability of multiplexed ePADs.^{8,9} Another notable advancement is the use of inkjet printing and screen-printing technologies to precisely deposit electrode materials and reagents, allowing for high-resolution patterning and improved reproducibility in device fabrication.^{10–12}

Despite these advancements, multiplexed ePADs face several limitations that researchers are actively addressing. One primary challenge is maintaining sensitivity and specificity for each analyte in a complex sample matrix, as the presence of multiple sensing elements can lead to cross-reactivity and interference.¹³ To mitigate this, recent studies have explored the use of nanomaterials and selective capture agents to enhance sensor performance and specificity.^{14–16} Also, the integration of microfluidic structures within paper-based devices has improved sample handling and distribution, addressing issues related to non-uniform flow and analyte diffusion in multiplexed systems.¹⁷

Signal interference remains a major probability for compromised selectivity in published ePADs, particularly in multiplexed systems where multiple electrochemical reactions occur in close proximity.^{1,18} This interference can manifest as crosstalk between adjacent sensing zones, leading to reduced accuracy and reliability of measurements. Recent studies have addressed this issue through various strategies, including the optimization of electrode geometries and spacing,^{18,19} the implementation of isolation barriers between sensing zones,²⁰ and the development of advanced signal processing algorithms to deconvolute overlapping electrochemical signals.²¹ These strategies can offer considerable separation of reaction zones which ensures adequate sample analysis. However, the quality of this analysis is assumed as long as later reagent diffusion does not disrupt measurements – a cause that is not often reported in scientific literature.

Additionally, some of these strategies might introduce additional fabrication complexity. Signal processing and algorithmic approaches offer the advantage of being implementable on existing hardware without physical modifications and can adapt to various interference patterns, though they often require significant computational resources and extensive training data sets to achieve reliable performance. Thus, despite these efforts, the challenge of minimizing signal interference while maintaining device simplicity and cost-effectiveness continues to be an active area of research in the field of multiplexed ePADs. Coplanar multiplexed ePADs have shown many advantages, especially during the device manufacturing process. The device coplanarity features all sensing elements and electrodes on a single plane, simplifying the fabrication processes and reducing material costs.^{1,22} However, coplanar ePADs could display some limitations,

particularly in terms of managing interference between closely spaced electrodes. Similar to their singleplexed counterparts, maintaining the interelectrode distance is also another challenge in the manufacturing of coplanar multiplexed 3DePADs.^{23,24} The proximity of sensing elements in this coplanar arrangement can intensify diffusional interference, signifying the need for a controlled electrode spacing and sensing zone isolation. To this end, this chapter will introduce several advancements that I have led towards versatile manufacturability of multiplexed electrochemical paper-based devices. The multiplexed 3D ePAD designs support controlled minimal interelectrode distance, while maintaining each electrode hosting layer uniquely addressable for facile fabrication. In addition to multiplexity, I will focus the second section of this chapter on describing a standalone version of our 3D ePAD device.

5.1.2 Device design considerations

5.1.2.1 Initial multiplexed 3D ePAD design: Electrode position evaluation

In the first iteration of our multiplexed 3D ePAD design, we evaluated its capability for simultaneous glucose detection using two adjacent working electrodes. The objective of this experiment was to determine whether the current output from both electrodes would be consistent. Consistent current from both working electrodes would indicate uniform electrochemical behavior, regardless of the physical position of the working electrode in its hosting layer. For this test, we employed A601 paper layers and positioned both working electrodes on the center of the layer. The working electrode-hosting layer is positioned in the middle of the other two electrode-hosting layers, CE and RE, forming a CWR configuration (**Figure 5.1**). The electrode pads of the working electrodes were

aligned in the same direction, while the counter electrode (CE) and reference electrode (RE) pad orientations remained unchanged from the singleplexed design.

We treated the upper flow layer, Layer 2, with 16 μL 250 U/mL glucose oxidase in 200 mM potassium ferricyanide in 1 M potassium chloride. Evaluating the obtained current from both electrodes serves as a metric to check if there is an influence from the electrode position, right or left in the electrode hosting layer, on the electrochemical connection among cell electrodes. For all tested multiplexed iterations of our 3D ePAD, both CE and RE were wider in dimensions to be in contact with the full width of both working electrodes. Also, the wider CE aims to minimize the chances of limiting the current transfer in the circuit. This modification could enhance circuit stability and reduce the risk of performance variability between the electrodes.

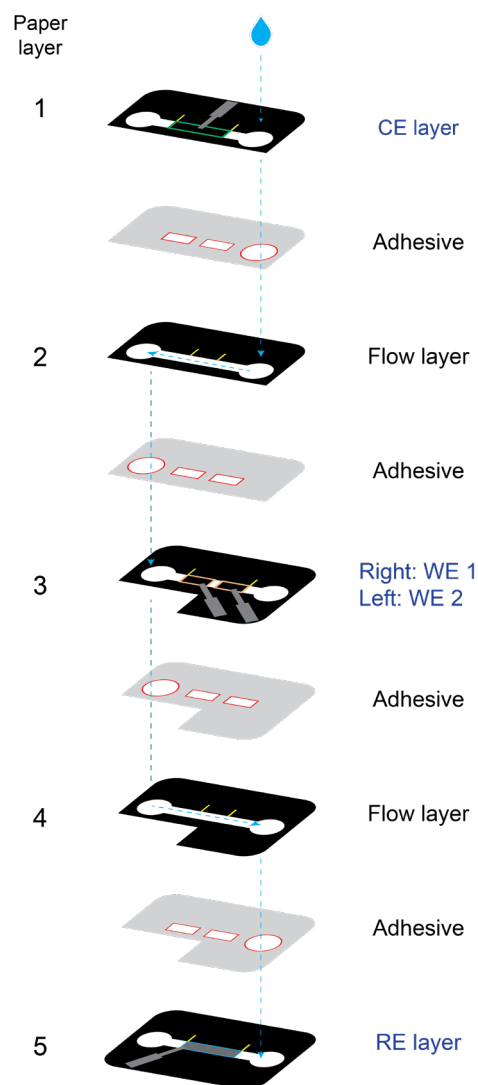


Figure 5.1. A detailed schematic of the first iteration of the multiplexed 3D ePAD. The layers are displayed in assembly order from bottom to top, with blue labels indicating the electrode-hosting layers (CE, 2 WE's, and RE). The design features two distinct working electrodes (WE 1 on the right, WE 2 on the left) deposited on the middle electrode-hosting layer. Working electrodes are positioned to create a CWR configuration. While CE and RE are wider to cover both working electrodes, their pads orientation remains consistent with the singleplexed device design. The laser-cut adhesive holes, red outlines, allow for fluid passage between layers while maintaining device integrity. Light blue dashed lines indicate fluid flow through and between layers.

5.1.2.2 Compartmentalized multiplexed 3D ePAD: Dual input design

To address issues of signal interference and reproducibility associated with close proximity of the working electrodes, we developed a novel compartmentalized version of the multiplexed 3D ePAD. This design strategically separates the two working electrodes using a hydrophobic wax-printed barrier, creating isolated compartments for each working electrode (**Figure 5.2**). For a CWR configuration, the compartmentalized design still maintains consistent interelectrode distances to both the upper and lower electrodes – counter and reference electrodes, respectively. By segregating the working electrodes into distinct compartments, this design minimizes the risk of cross-talk and signal interference, even when both assays rely on similar detection chemistries, such as the redox cycling of a mediator like potassium ferricyanide.

This compartmentalization enhances the versatility of the device, allowing two parallel assays to be conducted simultaneously. The concurrent reagent separation and rehydration aims to perform both assays without compromising their selectivity or the accuracy of current signal measured from each working electrode. In all compartmentalized multiplexed devices, we used carbon ink not only to print counter and both working electrodes, but also to print pseudo reference electrodes. Carbon-based pseudo-reference electrodes provide improved chemical stability, especially when exposed to varying sample matrices or redox environments, reducing the risk of leaching or contamination.

The fluidic architecture of the compartmentalized device design employs a comb-like sample flow structure (**Figure 5.3**). Upon sample introduction, the fluid wicks across each layer from one side of the device until it encounters the hydrophobic barrier at the center. This barrier diverts the flow towards the lower layers of the same compartment, preventing cross-contamination between the two sides. This comb-like flow pattern ensures efficient sample distribution within each compartment while maintaining chemical isolation between the assays conducted on either side of the device.

In this iteration, we designed the working electrode pads to be stencil-printed in opposite directions. This mirror-image configuration divides the device symmetrically into two superimposable halves, reducing the likelihood of signal variability due to asymmetry. This design feature mitigates potential discrepancies in sample volume and analyte distribution between the two compartments, which could otherwise result in differences in reagent concentration and, consequently, signal output.

We initially tested this compartmentalized multiplexed device using a dual-input sample introduction layer (Layer 1), where samples are added separately to each side of the device. This dual-sample capability allows for independent parallel analysis of two distinct analytes, enhancing the functionality of the device for multi-analyte detection. Additionally, the sample flow layer (Layer 2) can be treated with different reagents specific to the target analytes, enabling tailored detection chemistries for simultaneous analysis.

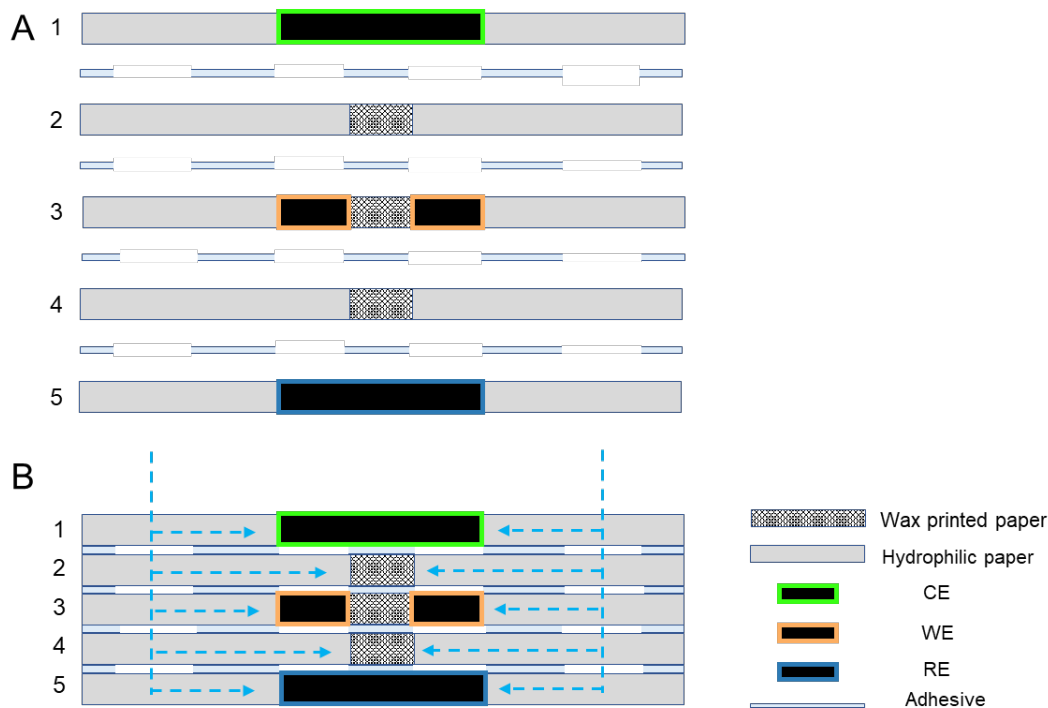


Figure 5.3. Cross-sectional illustration of the dual-input compartmentalized iteration of the multiplexed 3D ePAD. (A) Detailed and (B) compact illustrations show the layer configuration and fluidic architecture. Light blue dashed lines in (B) indicate fluid flow through and between layers. After their introduction to layer 1, dual samples wick their respective compartments within lower layers. Upon sample introduction, fluid flows in both sides, following a comb-like pattern to create an electrolytic connection among the three-electrode paper-based electrochemical cell while preventing interference across assays conducted in parallel on each working electrode. Discontinuation in adhesive layers refers to laser cut holes created to direct sample flow within the device and allow electrolytic connection between electrodes.

5.1.2.3 Compartmentalized multiplexed 3D ePAD: Single input design

To further enhance the versatility of the compartmentalized 3D ePAD and address sample volume limitations, we developed a single-input iteration of this multiplexed device (**Figure 5.4**). This version introduces a separate sample introduction/distribution layer, Layer 1. This layer aims to facilitate simultaneous filling of both compartments using a single sample containing the analytes of interest. The compartmentalized structure ensures even sample distribution and flow across both sides of the device, while allowing for rehydration of the dried recognition elements stored within each compartment (**Figure 5.5**).

This single-input design of this multiplexed 3D ePAD offers several advantages. First, it reduces the required sample volume, which is particularly beneficial in applications where obtaining sufficient biological sample volume is challenging, such as in tear or sweat analysis. Second, it minimizes the risk of device irreproducibility by reducing the number of user interactions with the device—requiring only a single sample introduction instead of two. Third, by utilizing a single sample that contains both analytes, the device demonstrates improved analyte specificity. Each analyte interacts selectively with its respective recognition element. For instance, glucose reacts only with the glucose oxidase enzyme on one side of the device, while lactate reacts with lactate oxidase on the other side. This selective interaction minimizes cross-reactivity and interference, reinforcing the capability of the device to perform accurate multiplexed detection. This iteration further underscores the robustness of the device architecture, offering a streamlined approach to multiplexed detection while maintaining the chemical isolation between parallel assays within the same sample matrix.

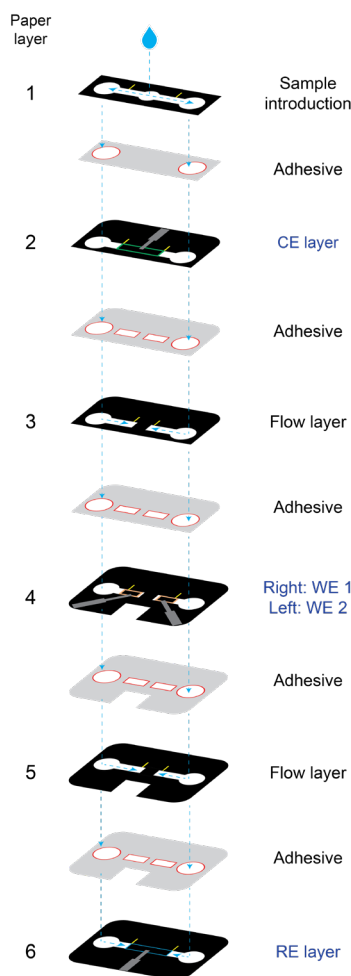


Figure 5.4. A detailed schematic of the single-input compartmentalized iteration of the multiplexed 3D ePAD. The device layers are displayed in assembly order from bottom to top, with blue labels indicating the electrode-hosting layers (CE, two WEs, and RE). This design features distinct sample introduction points for each working electrode (right: WE 1, left: WE 2), enabling independent filling of each compartment. The flow paths ensure that each sample remains isolated within its respective compartment, preventing cross-contamination. Laser-cut adhesive holes, outlined in red, facilitate fluid passage between layers while maintaining electrolytic contact and overall layer integrity. The wider CE and RE pads ensure full contact with both working electrodes, enhancing signal stability. The mirror-image configuration of the working electrode pads minimizes asymmetry, contributing to more uniform signal output between the two electrodes. This setup allows for simultaneous yet separate detection of different analytes introduced through distinct sample inputs. Light blue dashed lines indicate the fluid flow pathways through and between the layers.

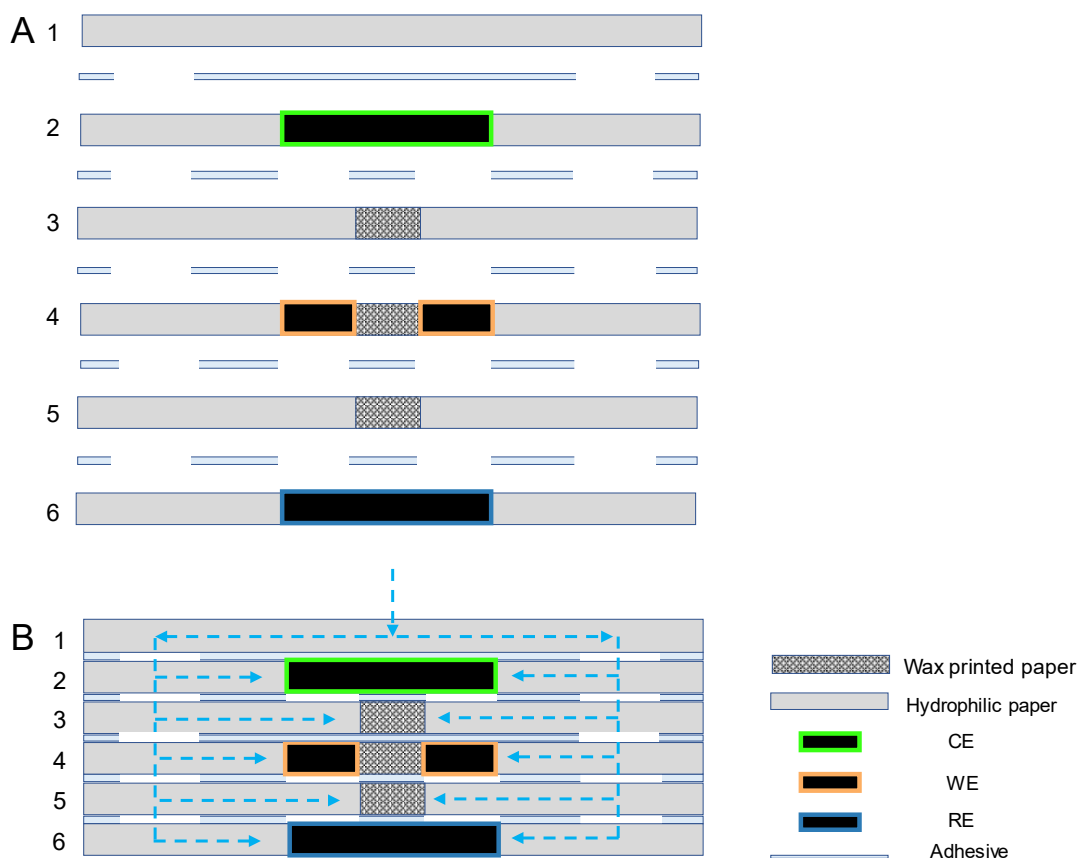


Figure 5.5. Cross-sectional illustration of the single-input compartmentalized iteration of the multiplexed 3D ePAD. (A) Detailed and (B) compact illustrations show the layer configuration and fluidic architecture. Light blue dashed lines in (B) indicate fluid flow through and between layers. After its introduction to layer 1, the sample is distributed to respective compartments within lower layers. Upon sample introduction, fluid flows in a comb-like pattern to create an electrolytic connection among the three-electrode paper-based electrochemical cell while preventing interference across assays conducted in parallel on each working electrode. Discontinuation in adhesive layers refers to laser cut holes created to direct sample flow within the device and allow electrolytic connection between electrodes.

5.1.2.4 Signal time dependance for multiplexed measurements

Since we utilized a single-channel potentiostat for running multiplexed assays, we performed sequential measurements by manually switching the working electrode clamp from WE 1 (glucose side) to WE 2 (lactate side). This approach introduces a time delay, as WE 2 inherently receives 100 seconds more than WE 1 due to the order of measurement. To determine if this additional time affects signal intensity, we designed an experiment to evaluate the correlation between signal output and elapsed time across multiple measurement cycles.

In this experiment, we employed the single-input compartmentalized 3D ePAD for multiplexed detection of glucose and lactate. Upon complete device filling, we conducted the chronoamperometric measurement by first connecting the working electrode clamp to WE 1 (glucose side) for 100 seconds. Immediately after this, we switched the clamp to WE 2 (lactate side) to perform the second chronoamperometric measurement under identical conditions. Together, these two sequential runs constituted one measurement cycle, consuming a total of 200 seconds. We repeated this process over 5 consecutive cycles, with a total duration of 1000 seconds, and monitored the current output for each working electrode.

5.1.3 Results and discussion

5.1.3.1 Initial multiplexed 3D ePAD design: Electrode position evaluation

We analyzed and compared the signals obtained from both working electrodes. In the initial trials, the correlation between current intensity and glucose concentration was weak for both electrodes. The R^2 values were 0.652 for WE 1 and 0.402 for WE 2, indicating poor linearity. A similar trend was observed when we tested the devices using lactate assays on both TFN-based and A601-based iterations. We believe the lack of strong correlation between current signal and analyte concentration could be due to two main factors.

First, interference in signals generated by each electrode which could result from having two working electrodes in close proximity. This interference could be related to the competition over the H_2O_2 molecules produced as byproducts in the detection reaction of both glucose and lactate using glucose oxidase and lactate oxidase, respectively. While H_2O_2 molecules are oxidized at the working electrode surface to generate the signal, the close proximity of the two working electrodes could lead to having both electrodes competing for the same H_2O_2 molecules. This competition could result in uneven current among the working electrodes.²⁵

Second, mass transport limitations that could arise from the close inter-working-electrode distance, ~ 1 mm. This distance could lead to diffusion layer overlap, where the analyte transport to each electrode becomes inefficient. Theoretically, under steady-state reaction conditions, the typical thickness of a diffusion layer is on the order of 100 to 500 μm .²⁶ While the two working electrodes are, by design, 1 mm apart, this distance can shift due

to manufacturing limitations that are related to printing, handling, and dimensional change after ink drying. This distance shifting could result in diffusion layer overlap which could culminate in mass transport limitations. Eventually, this overlap could probably result in competition for the available H_2O_2 , as both working electrodes are reacting with the same limited pool of reaction products.²⁶

In addition, the current output obtained from either working electrodes is a result of the same glucose oxidation reaction in the presence of the redox mediator, potassium ferricyanide. This means that if we have 2 assays that work with the same chemistry, the current device design will not have the ability to accurately tell which signal is a result from which assay. This compromises the device reliability as it leads to a higher chance of signal interference.

5.1.3.2 Compartmentalized multiplexed 3D ePAD: Dual input design

We utilized the dual-input iteration of the compartmentalized 3D ePAD for multiplexed detection of glucose and lactate. For glucose detection, we treated the sample flow layer (Layer 2) with 250 U/mL glucose oxidase in 600 mM potassium ferricyanide dissolved in 1 M KCl. The glucose assay was performed on the right side of the device, where the working electrode (WE 1) was dedicated for this detection. Consequently, treatment of the flow layers with glucose oxidase was only limited to the right-side of Layer 2. Also, glucose samples of varying concentrations were introduced on the right side of the device.

The lactate detection assay was conducted on the left side of the device, utilizing the second working electrode (WE 2). For this assay, the left side of the sample flow layer, Layer 2, was treated with 50 U/mL lactate oxidase in 200 mM potassium ferricyanide in 1

M KCl. Instantly after introducing glucose samples to the right side, lactate samples of varying concentrations were introduced to the left side. Following complete device filling with sample, we first connected the potentiostat's working electrode clamp to WE 1 to run the chronoamperometric measurement for glucose detection, over a duration of 100 seconds. Immediately after the glucose measurement, we switched the working electrode clamp to WE 2 while counter and reference electrode clamps stayed untouched. This setup allowed to perform the lactate detection assay under identical experimental conditions such as interelectrode distance and electrode connections.

Both the glucose and lactate assays demonstrated a linear relationship between the analyte concentration and the resulting chronoamperometric current (**Figure 5.6**). The linear calibration plots obtained for each assay indicate a consistent and proportional response from the respective working electrodes, validating the effectiveness of the compartmentalized design in supporting simultaneous multi-analyte detection without signal interference. This result underlines the versatility and robustness of the device, as

the compartmentalization mitigated crosstalk, allowing each assay to be performed independently even with the same redox mediator chemistry employed on both sides.

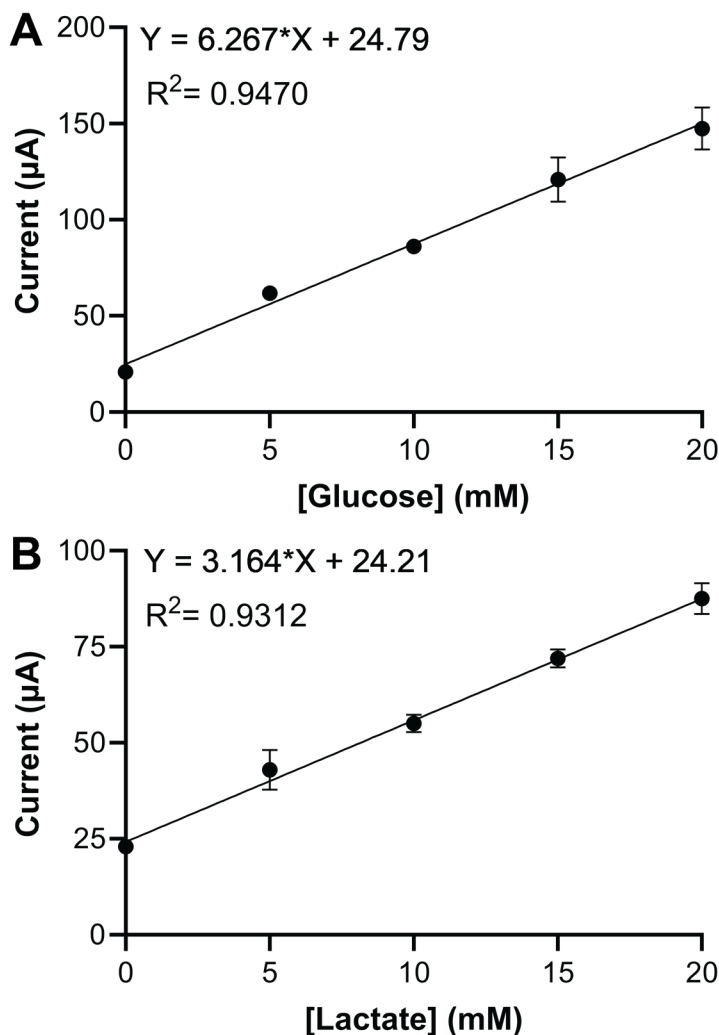


Figure 5.6. Chronoamperometric detection of both (A) glucose and (B) lactate using the dual input iteration of the multiplexed 3D ePAD with CWR electrode configuration. All paper layers are made of A601 grade. The calibration curves show a linear relationship between both analyte concentrations (X) and the average resulting current (Y) over the 0 – 20 mM range in the duration between 80 s and 100 s. Chronoamperometric measurements for glucose, WE 1, were conducted first, followed immediately by lactate measurements, WE 2. Dual samples of 75 μL each were used, containing varying concentrations of glucose and lactate in DI water. The applied step potential is 500 mV. Error bars represent the standard error of the mean (N = 3).

5.1.3.3 Compartmentalized multiplexed 3D ePAD: Single input design

We applied the single-input iteration of the compartmentalized 3D ePAD for multiplexed detection of glucose and lactate using a sample that contained both analytes. Similar to the dual-input iteration, the sample flow layers were treated with enzyme solutions specific to each analyte: glucose oxidase on the side corresponding to the glucose assay (WE 1, right) and lactate oxidase on the side for the lactate assay (WE 2, left). This compartmentalized approach maintained the distinct chemical environment for each assay despite the single-sample input, ensuring selective interactions with the respective recognition elements.

Except for sample introduction, we conducted the multiplexed detection experiments in this device iteration exactly like we did for the dual-input iteration. Upon introducing the glucose-lactate mixture solution, the sample filled both compartments simultaneously. While filling the device, the sample rehydrated the enzyme reagents pre-stored and dried on each side. Following complete device filling, we first connected the potentiostat's working electrode clamp to WE 1 to perform the chronoamperometric measurement for glucose detection over a period of 100 seconds. Immediately after completing the glucose assay, we switched the working electrode clamp to WE 2, while keeping the counter and reference electrode connections unchanged, to run the chronoamperometric experiment for lactate detection under identical conditions.

The device exhibited a linear relationship between the concentrations of glucose and lactate and the corresponding chronoamperometric currents generated by each working electrode (**Figure 5.7**). The LOD of the glucose assay is 3.3 mM while that of the lactate assay is 2.9 mM. This outcome demonstrates that the single-input design successfully

mitigates cross-reactivity, even when the sample contains both analytes, while keeping a sensitive performance. By isolating the interactions of each analyte with its recognition enzyme in separate compartments, the device provided specific and reproducible signals for both assays. While highlighting the robustness of the 3D ePAD design, this iteration also offers a practical advantage for scenarios requiring simultaneous analysis of multiple biomarkers from a single, limited sample volume.

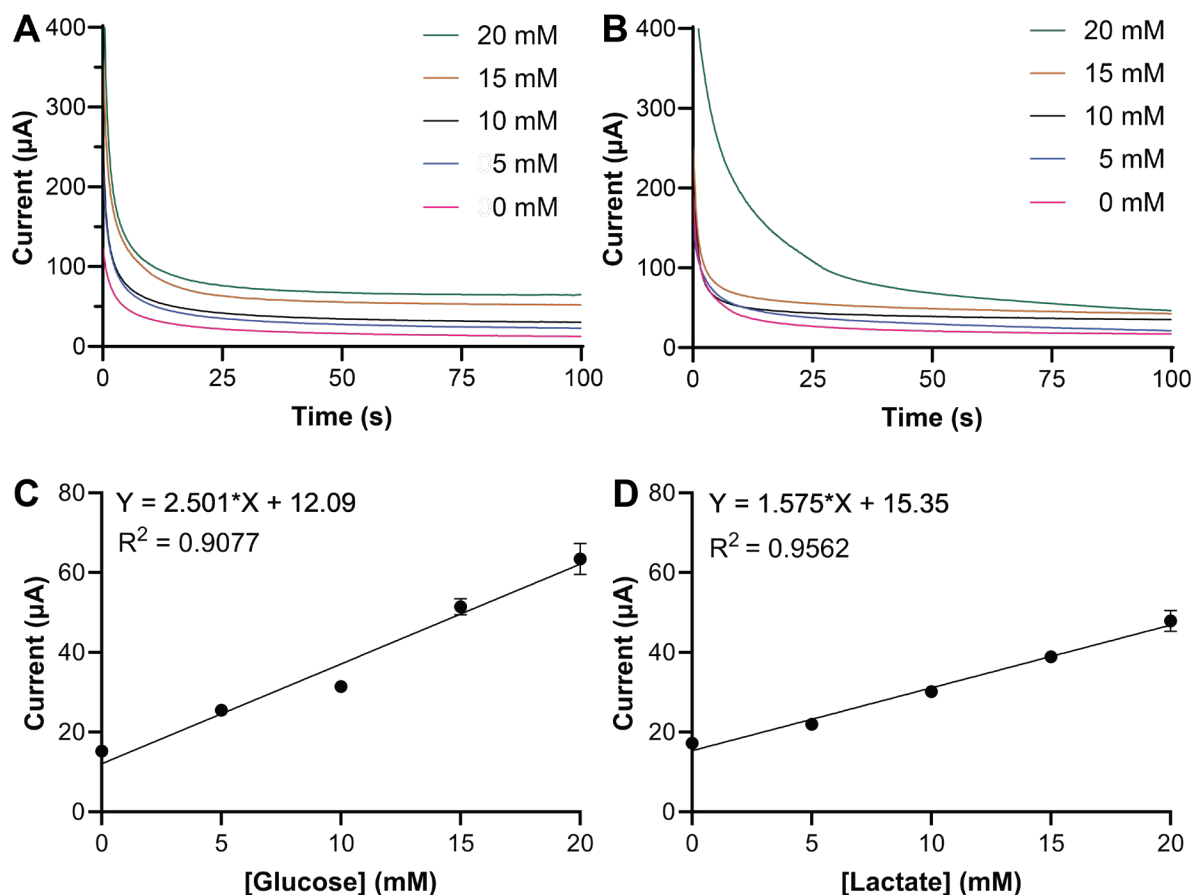


Figure 5.7. Chronoamperometric detection of both glucose and lactate using the single-input iteration of the multiplexed 3D ePAD with a CWR electrode configuration. All paper layers are made from A601 grade. The chronoamperometric response plots for (A) glucose and (B) lactate show current profiles across the 0–20 mM concentration range. The calibration curves for (C) glucose and (D) lactate indicate a linear relationship between analyte concentration (X) and the average resulting current (Y) in the duration between 80 and 100 seconds. Chronoamperometric measurements for glucose, at WE 1, were conducted first, followed immediately by lactate measurements, at WE 2. Samples are 150 μL of mixtures containing varying concentrations of glucose and lactate in DI water. In each sample mixture, both glucose and lactate were present at equimolar concentrations. The applied step potential is 500 mV. Error bars represent the standard error of the mean (N = 3).

5.1.3.4 Signal time dependance for multiplexed measurements

The results indicated that both device sides maintained consistent current intensities over the first 3 cycles – 600 seconds (**Figure 5.8**). During the subsequent two cycles, however, we observed a noticeable current decile for both glucose and lactate assays. This signal reduction could potentially be attributed to several factors: (i) enzyme deactivation or denaturation; (ii) depletion of active sites on the working electrodes, possibly caused by product buildup over time; (iii) sample evaporation, especially given the prolonged measurement duration, which might concentrate the sample and alter reagent rehydration dynamics; and/or (iv) electrode fouling, where reaction byproducts or intermediates may adsorb onto the electrode surface, inhibiting effective electron transfer and reducing the overall current response.

This experiment highlights the importance of considering time-dependent effects when using a single-channel potentiostat for multiplexed assays. Future improvements may involve employing a multi-channel potentiostat or a multiplexer unit, optimizing the reagent formulation, and/or implementing strategies to mitigate evaporation and fouling, thereby enhancing signal stability over extended measurement periods.

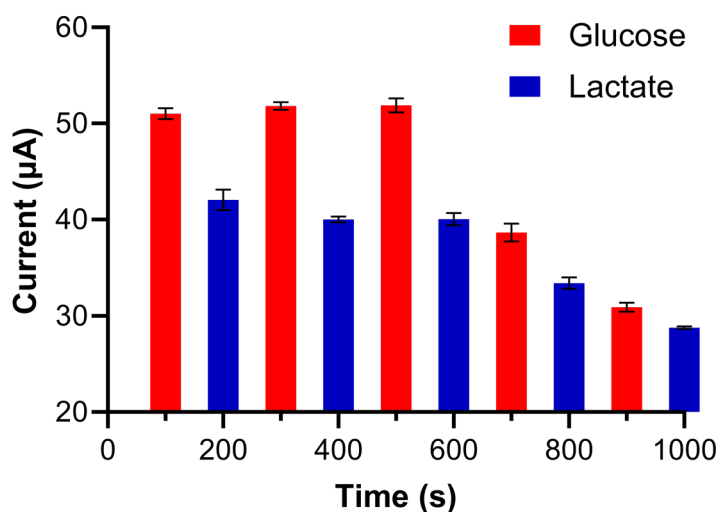


Figure 5.8. Stability assessment of the chronoamperometric current response for both glucose (red bars) and lactate (blue bars). Signals are measured using the single-input iteration of the multiplexed 3D ePAD, CWR configuration with A601 papers. Measurements were conducted over five consecutive cycles, with each cycle consisting of a 100-second glucose detection (WE 1) followed by a 100-second lactate detection (WE 2), totaling 200 seconds per cycle. The current output remains stable across the first three cycles for both analytes. Sample is 150 μL of 15 mM glucose and lactate in DI water. In each sample mixture, both glucose and lactate were present at equimolar concentrations. The applied step potential is 500 mV. Error bars represent the standard error of the mean (N = 3).

To further investigate the stability of the signal output over time, we repeated the experiment for the first 3 measurement cycles using a range of analyte concentrations for both glucose and lactate assays. This extended analysis aimed to determine if the observed consistency in current held true across different concentrations. The tested concentrations ranged from 5 mM to 20 mM for both analytes (**Figure 5.9**).

The results confirmed that the maintained signal intensity over the initial 3 cycles was consistent across nearly all tested concentrations. However, the highest concentration of 20 mM did not maintain the same signal consistency. At this elevated concentration, we observed a gradual decline in current intensity from one cycle to the next. Despite this diminishing trend, the signal remained proportional to the expected analyte response. Thus, although the current output for the 20 mM sample decreased over the cycles, it maintained a higher intensity than the 15 mM sample, indicating that the relative signal intensity was preserved.

These findings suggest that high analyte concentration might lead to additional measurement challenges. These challenges could include enzyme saturation, increased product buildup, or higher fouling rate at the electrode surface, contributing to the observed signal decline. Further optimization of the assay conditions, such as adjusting enzyme loading or altering the mediator concentration, may be necessary to mitigate these effects and ensure stable performance even at higher analyte levels. Future studies should also consider exploring strategies to enhance the longevity of the signal response, particularly when detecting high concentrations of target analytes.

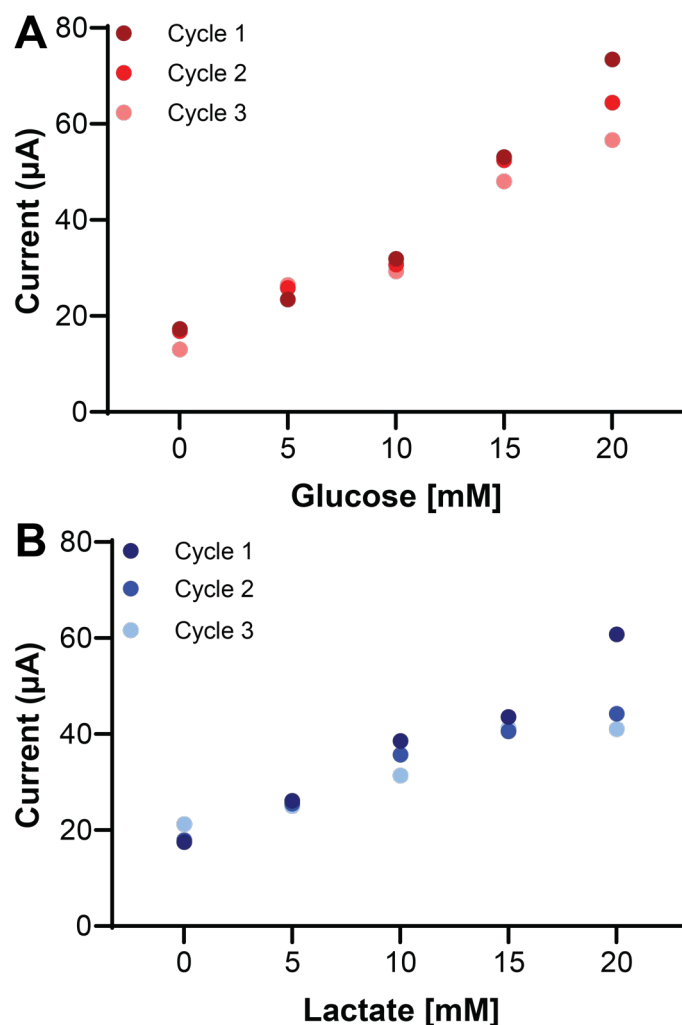


Figure 5.9. Extended analysis of chronoamperometric current stability across three measurement cycles for (A) glucose and (B) lactate. Signals are measured using the single-input iteration of the multiplexed 3D ePAD, CWR configuration with A601 papers. Samples are 150 μL of glucose and lactate mixture solutions with 0 – 20 mM concentration range. In each sample mixture, both glucose and lactate were present at equimolar concentrations. Data points represent the mean current measurements for each cycle (Cycle 1: dark, Cycle 2: medium, Cycle 3: light). Error bars represent the standard error of the mean ($N = 3$).

5.2 Standalone 3D ePADs

5.2.1 introduction

In developing innovative solutions for electrochemical POC diagnostics, we showed in the previous chapter the development process of a 3D ePAD. In this 3D ePAD, the sample fluid navigates through predefined hydrophilic pathways formed via wax-patterned hydrophobic barriers. This structural configuration allows controlled sample flow, ensuring efficient transport and interaction with the reagent-loaded electrodes. However, wax printing—a critical step in this fabrication—might have some drawbacks such as inconsistency in wax deposition that can result in sample leakage and compromised signal reproducibility. Insufficient wax printing may cause breaches in the hydrophobic barriers, altering the sample flow path and the uniformity of reagent rehydration. Consequently, variations in rehydrated reagent concentration at the electrode surface can occur, potentially leading to fluctuating electrochemical signals and inter-device inconsistencies.^{27–29}

To address the wax-printing recent challenges, multiple other fabrication approaches have been more utilized such as inkjet printing,³⁰ photolithography,³¹ and plasma treatment.³² While efficient in creating hydrophobic barriers, employing these techniques can increase the complexity and cost of fabrication. Also, it can raise a biocompatibility issue since some reagents, like surfactants used for inkjet printing, can cause protein denaturization, limiting the assay chemistries the ePAD supports.³³ To this end, we designed an advanced standalone version of the 3D ePAD. This iteration replaces the wax-printed barriers with hydrophilic channels supported by laser-cut mylar layers, eliminating the reliance on wax printing, which many manufacturers no longer support.

This modification not only improves fabrication reproducibility but also mitigates potential leakage issues, preserving the integrity of sample flow and reagent distribution. Additionally, the standalone design allows for selective replacement of the hydrophilic channels while retaining the mylar-based framework and copper electrode pads—another modification we introduce in this device iteration—thereby enabling device reusability. This modular approach promotes resource efficiency and contributes to waste reduction, aligning with the growing emphasis on sustainable practices in analytical device development.

5.2.2 Device design considerations

In this iteration of our device, we developed a standalone version of the 3D ePAD wherein the paper-based electrodes and fluid flow channels are integrated and supported by Mylar layers. This Mylar-supported 3D ePAD retains the core architecture of the fully paper-based version, featuring three electrodes—working, counter, and reference—stencil-printed on separate paper layers. In the original fully paper-based 3D ePAD, silver/silver chloride (Ag/AgCl) pads were used to connect each electrode to the corresponding metal clamps from the electrochemical workstation. However, in the Mylar-supported 3D ePAD, we replaced these connections with 3 mm-wide copper tape, which serves as the link between the stencil-printed electrodes and their respective potentiostat metal clamps. For each electrode, the copper tape is partially adhered to the surface of the electrode, while the remaining portion is affixed to the Mylar layer that supports the electrode-hosting paper layer. This dual function of the copper tape not only ensures a reliable connection between the electrode and the potentiostat clamp but also secures the electrode-hosting paper layer to the patterned Mylar layer, enhancing the structural integrity of the device.

This design modification also streamlines the manufacturing process by reducing reliance on stencil printing. In the fully paper-based version, the fabrication process involved two stages of stencil printing. The first stage involved printing carbon ink for the working and counter electrodes and Ag/AgCl ink for the reference electrode, followed by an ink drying step. The second stage involved stencil printing Ag/AgCl pads for all electrodes, with subsequent drying under the same conditions of time and temperature. Conversely, the use of copper tape in the Mylar-supported 3D ePAD eliminates the need for the second stencil printing stage. As a result, fabricating this iteration saves drying time and minimizes the potential for manufactural defects related to misalignment, ink costs, energy consumption, and material waste.

Specifically, avoiding the second stage of stencil printing significantly reduces the risk of defects that could arise from misalignment of the stencil-printed pads with their corresponding electrodes. In cases where misalignment occurs in the fully paper-based device, the entire sheet is rendered unusable, necessitating a complete reprint of both stages. The use of copper tape, on the other hand, allows for more precise manual placement, effectively mitigating this risk and reducing material waste during device fabrication associated with stencil printing. This adaptation not only enhances the efficiency and reliability of the device production process, but also underscores the potential for scaling up the manufacturing of 3D ePADs for broader applications.

Since this device iteration only modifies the hydrophilic layer support and not the interelectrode distancing, each two electrodes are still separated from one another by a distance equal to the combined thickness of the paper and adhesive used – similar to fully paper-based 3D ePAD. The overall fluidic pathway in the device maintains the

serpentine fashion: the sample wets the device inlet, travels down the top layer to the outlet, then vertically to the layer below where it changes directions. Patterned holes in the adhesive film separating channels on subsequent layers permit two electrodes to be wetted by the sample simultaneously. This process repeats until the sample reaches the bottom layer of the device. Adhesive films in between layers of paper ensure that leaks or contamination do not occur. In addition, the standalone 3D ePAD iteration still supports electrode interchangeability, since each electrode is still defined on a unique layer. Hence, this device version allows for further studying the influence of electrode configuration on different electrochemical measurements.

5.2.2.1 Standalone 3D ePADs: TFN paper channel

To accommodate the varying ink penetration extents, thoroughly described in chapter 4, we attempted testing the Mylar-supported 3D ePAD using both TFN and A601 paper grades. For TFN devices, each electrode is positioned in the center of a hydrophilic channel that also begins and ends with circular zones. In this way, every layer of the device is both a duplicate yet still uniquely addressable, maintaining the simple manufacturing and flexible device design offered in the fully paper-based 3D ePAD. While layers are affixed to each other by sheets of patterned, double-sided adhesives (**Figure 5.10**), the Mylar-supported 3D ePAD also includes two laminate sheets on top of and underneath the compacted layers. The laminate sheets serve to further affix the upper and lower paper conduits, including their electrodes, in position, since only one face of both layers is affixed with the double-sided adhesive sheet. The top laminate layer is patterned to provide access to the sample introduction port in the device. The lower laminate layer, however, is not patterned, since it serves as a preventive layer to avoid

dripping of the sample and washing the reagents away. This could be particularly helpful in case the assay run by the device needs any layer pretreatment, such as the chronoamperometric glucose assay described in chapter 4.

Layer		Material
1		laminate
2		WE/CE carbon ink
3		Mylar - copper sticker
4		adhesive
5		CE/WE carbon ink
6		Mylar - copper sticker
7		adhesive
8		RE Ag/AgCl ink
9		Mylar - copper sticker
10		laminate

Figure 5.10. Exploded schematic of the TFN-based standalone version of the 3D ePAD. The device is assembled in sequential order from bottom to top, illustrating the individual paper and adhesive layers. The TFN paper serves as the substrate for electrode stencil printing. Due to their thickness, the same TFN serve as both electrode-hosting and flow layer, allowing sample to flow underneath the deposited electrode. While RE remains the lowest electrode, the top and middle electrodes can be reconfigured as working or counter electrodes, based on potentiostat clamp attached. Copper stickers are used as electrode pads and are affixed to Mylar layers, which form the structural skeleton of the device. Top and bottom laminates aim to ensure structural integrity of device layers. Laser-cut adhesive holes guide the sample flow path to facilitate controlled wicking of all layers and electrolytic connection among electrodes.

5.2.2.2 Standalone 3D ePADs: A601 paper channel

In the A601 version of the Mylar-supported device, the hydrophilic area is confined to the two flow layers, Layers 7 and 9. The electrodes, however, are printed as rectangles on a separate sheet then cut and manually placed to be in the middle position over these hydrophilic flow layers, Layers 2, 6, and 10 (**Figure 5.11**). The placement of electrodes is a critical aspect, with each electrode being precisely positioned at the center over these flow layers. This precise alignment is facilitated by placeholders on the Mylar frame, Layers 3, 6, and 9, ensuring accurate and reproducible electrode positioning. These placeholders are etched into the Mylar frame using laser cutting at a specific power level that marks the surface without penetrating the layer entirely. This controlled engraving process is essential to prevent sample leakage, as penetration through the Mylar could compromise the device's integrity and result in signal variability.

The minimized hydrophilic area within these layers is engineered to maintain an efficient electrolytic connection between the centrally aligned electrodes while reducing the required sample volume. The reduced sample volume necessary for device filling, 50 μL as compared to 100 μL in fully paper-based device, is a substantial feature of this standalone iteration, potentially enabling the device to be used for analyte measurements where sample volume is limited.

Layer		Material
1		laminate
2		WE/CE carbon ink
3		flow layer
4		Mylar - copper sticker
5		adhesive
6		CE/WE carbon ink
7		flow layer
8		Mylar - copper sticker
9		adhesive
10		RE Ag/AgCl ink
11		Mylar - copper sticker
12		laminate

Figure 5.11. Exploded schematic of the A601-based standalone version of the 3D ePAD. The device is assembled in sequential order from bottom to top, illustrating the individual paper and adhesive layers. The A601 paper serves as the (i) substrate for electrode stencil printing and (ii) sample flow layers. Due to the deep ink penetration in A601 papers, the device features dedicated layers for sample flow. While RE remains the lowest electrode, the top and middle electrodes can be reconfigured as working or counter electrodes, based on potentiostat clamp attached. Copper stickers are used as electrode pads and are affixed to Mylar layers, which form the structural skeleton of the device. Top and bottom laminates aim to ensure structural integrity of device layers. Laser-cut adhesive holes guide the sample flow path to facilitate controlled wicking of all layers and electrolytic connection among electrodes.

As described in Chapter 4, the fully paper-based 3D ePAD iteration utilizes Ag/AgCl electrode pads which take three different directions. The different directions of electrode pads aimed to facilitate easier manual connection to external measurement systems and reduce the risk of accidental connections when attached to metal clamps. However, the Mylar-supported iteration employs copper tape-based electrode pads which are aligned next to each other such that they resemble the electrode pads in commercial SPEs. From this perspective, the aligned copper tape-pads represent a natural design evolution from a prototype-focused design to one optimized for standardized instrumentation. This Mylar-supported version is more suitable for use with commercial potentiostats and glucometers, where metal clamps are not always supported (**Figure 5.12**).

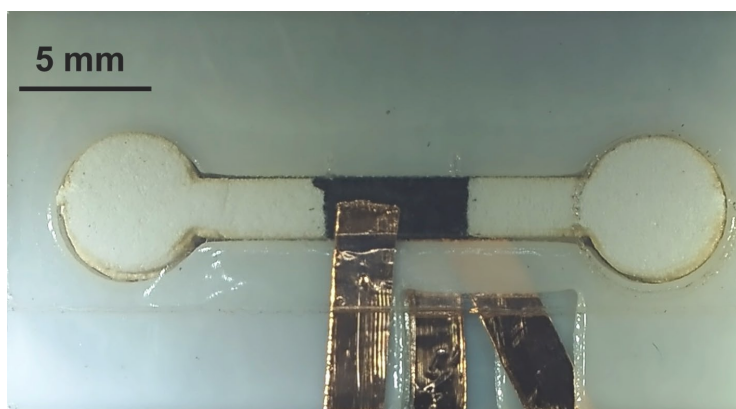


Figure 5.12. Actual image of the Mylar-supported 3D ePAD version. Paper conduits are TFN-based.

5.2.2.3 Electrochemical characterization of standalone 3D ePADs

5.2.2.3.1 *Cyclic voltammetry*

We tested the electrochemical activity of the standalone version of the 3D ePAD by running cyclic voltammetry experiments using a range of scan rates, 50 – 500 mV/S. As a demonstration for this device design, the electrode-hosting layers in these devices were TFN-based, in which the electrode ink was only deposited on the upper side of each paper channel. We used $K_3[Fe(CN)_6]$ as a model for a redox-active compound to evaluate and compare the electrochemical activity of this Mylar-based 3D-ePADs iteration using both electrode configurations (i.e., CWR vs. WCR). For WCR configuration, we used a potential range from 0 V to 0.7 V, while for CWR configuration, we used a -0.5 V to 0.4 V potential range. For both configurations, we ran cyclic voltammetry experiments over 50 – 500 mV/s scan rate range.

5.2.2.3.2 *Electrochemical Impedance Spectroscopy*

We used electrochemical impedance spectroscopy (EIS) to evaluate the difference in resistance elements of both WCR and CWR configurations using the standalone 3D ePAD iteration. EIS is a powerful technique commonly used to investigate the electrical properties and interfacial processes at electrode surfaces. EIS measures the response of the system towards a small, alternating potential signal over a range of frequencies. These measurements provide a comprehensive analysis of both the resistive and capacitive elements in electrochemical systems. By providing insight into the resistance to charge transfer, double-layer capacitance, and diffusion processes, EIS is instrumental in characterizing various aspects of electrode performance.^{34,35} EIS measurements are relevant for analyzing our 3D ePADs as they allow us to study the complex interfacial

processes that could affect device performance and reliability. By employing EIS for both the WCR and CWR electrode configurations, we can gain a better understanding of the electrical behavior unique to each setup, especially in terms of how each configuration may impact charge transfer resistance and signal stability.

In our EIS analysis, we focus on several key electrical elements, including the solution resistance (R_s), charge transfer resistance (R_{ct}), Warburg impedance (W), and double-layer capacitance (C_{dl}), each representing distinct aspects of the electrochemical system. While the solution resistance represents the resistive component arising from the electrolyte solution, the charge transfer resistance reflects the energy barrier associated with electron transfer between the electrode and the analyte. Warburg impedance, an important element in systems involving diffusive transport, accounts for the impedance caused by mass transfer limitations, especially at lower frequencies. The double-layer capacitance represents the capacity of the electrode to store charge at the electrolyte interface.^{36,37} We employ a Warburg circuit fitting to accurately model and interpret the EIS data, as this fitting method captures the contributions of diffusive processes to the overall impedance. In addition to showing high fitting accuracy (> 85%), using the Warburg model to study our system helps quantify diffusion-related behavior and identify potential issues related to mass transport limitations in both electrode configurations. This approach not only provides a comprehensive understanding of interfacial phenomena, but also allows for comparative analysis between the WCR and CWR configurations. This output could highlight any design-specific influences on electrochemical performance of either configuration. For all EIS measurements, we employed an initial potential of 0 V, high frequency of 1×10^5 Hz, low frequency of 0.1 Hz, and amplitude of 5 mV.

5.2.3 Results and discussion

5.2.3.1 Cyclic voltammetry

The cyclic voltammograms of this Mylar-based device iteration showed an output similar to that obtained from the fully paper-based 3D ePADs in multiple ways (**Figure 5.13A**). For instance, the measured current magnitude still maintained the considerable difference with respect to the electrode configuration. In the WCR configuration, absolute values of currents were in the microampere range (approximately 20–200 μA). In opposition, absolute values of currents in the CWR configuration were in the milliampere range (approximately 0.5 – 3 mA) representing a difference of more than one order of magnitude. This result suggests that the standalone version of the device did not negatively impact the influence of interelectrode distance with respect to electrode interchangeability. As discussed in Chapter 4, the CWR configuration allows minimal WE-CE and WE-RE distance. On the other hand, WCR format allows for only minimal WE-CE distance, while that distance is doubled between WE (top layer) and RE (lowest layer). As distance between WE and RE increases, uncompensated resistance increases, which leads to lower current.^{22,38,39}

For both WCR and CWR electrode configurations, the standalone 3D ePAD iteration exhibited the anticipated linear relationship between the square root of the applied scan rate and the resulting peak current magnitude (**Figure 5.13B**). For WCR configuration, the linear relationship of the cathodic current is described as $Y = -9.298 \cdot X + 6.372$ while the anodic current equation is $Y = 9.015 \cdot X - 4.526$. On the other hand, the linear relationship of the CWR cathodic current is described as $Y = -0.1120 \cdot X + 0.3280$ while the anodic current equation is $Y = 0.1280 \cdot X - 0.6273$, where X is the square root of the

applied scan rate and Y is the corresponding peak current. The obtained linearity is characteristic of a process where the rate of electron transfer is limited by the diffusion of the analyte to the electrode surface. This relationship is described by the Randles-Sevcik⁴⁰ equation (**Equation 5.1**).

Equation 5.1. Randles-Sevcik equation.

$$i_p = (2.69 \times 10^5) n^{3/2} A D^{1/2} C \nu^{1/2}$$

where i_p is the peak current, n is the number of electrons transferred in the redox process, A is the electrode area, D is the diffusion coefficient of the analyte, C is the concentration of the analyte, and ν is the scan rate.

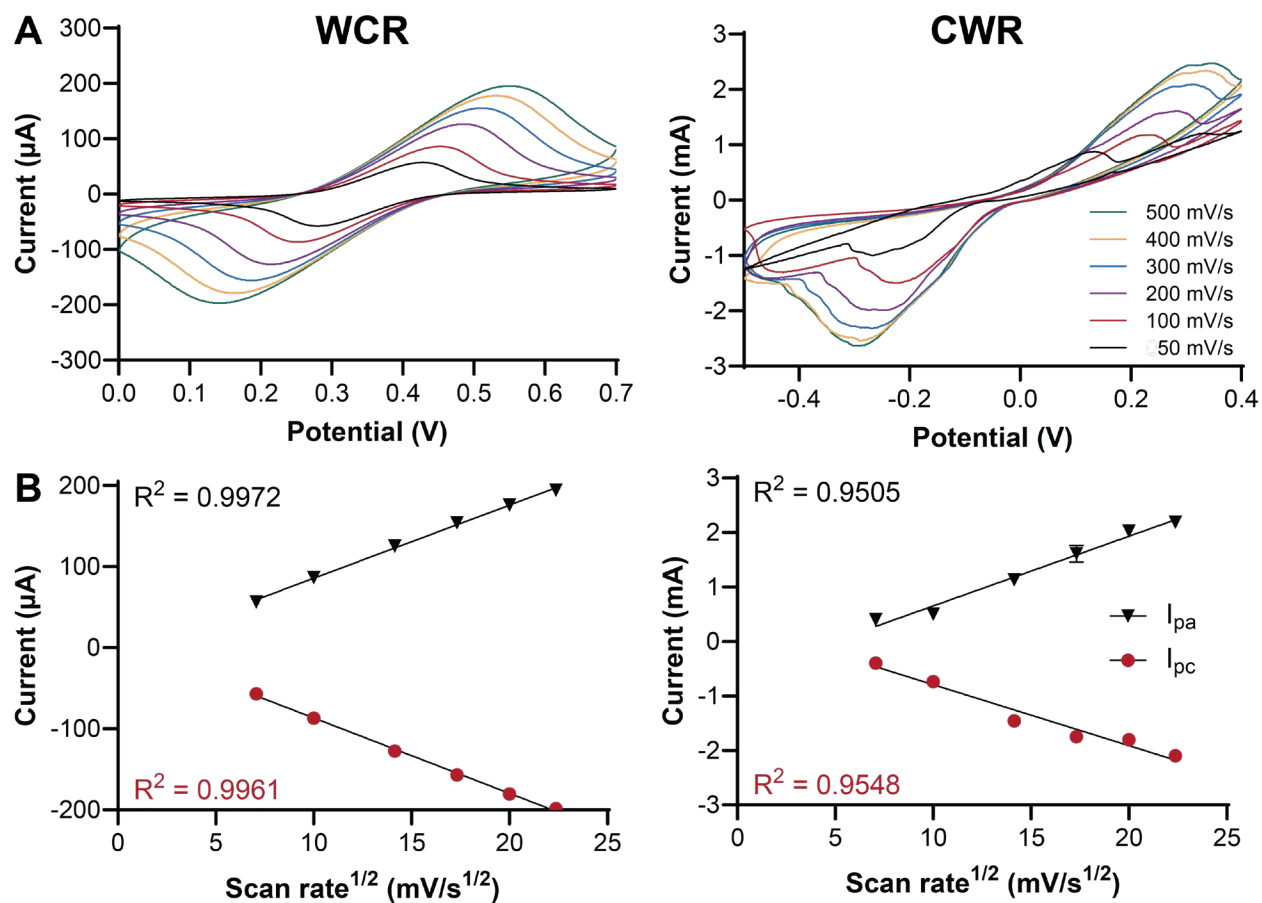


Figure 5.13. Cyclic voltammetry produced by standalone TFN-based 3D ePAD using both WCR and CWR electrode configurations. (A) Voltammograms recorded over a scan rate range of 50 – 500 mV/s as we introduced samples of 100 μ L of 10 mM $K_3[Fe(CN)_6]$ in 1 M KCl. (B) Linear relationships between square root of scan rates and their measured anodic (black triangles) and cathodic (red circles) peak currents. Error bars represent the standard error of the mean. (N = 4).

For both electrode configurations, we analyzed the peak current intensity obtained in both scan directions of cyclic voltammetry, i.e. anodic and cathodic (**Table 5.1**). For WCR configuration, all scan rates between 100 – 500 mV demonstrated ratios between the anodic and cathodic current that were close to unity ($i_{pa}/i_{pc}=1$). The produced peak shapes reveal a Nernstian electrochemical reaction whose rate is governed by electroactive species diffusion to the WE surface. Also, the WCR configuration showed an increasing peak-to-peak separation potential (ΔE_P) with increasing the CV scan rate (**Table 5.2**). As previously discussed in Chapter 4, the higher scan rates allow the electrochemical system less time to reach equilibrium at each potential point. This decreased time results in larger separation between the potential values at which oxidation and reduction of the electroactive species happen, aka polarization overpotential.

The CWR configuration, however, showed less regular cyclic voltammograms than those of WCR counterparts. For instance, CWR devices demonstrated an increase in the anodic current for both 100 and 200 mV/s scan rates (i_{pa}/i_{pc} values are 1.47 and 1.28, respectively) (**Table 5.1**). The increased anodic current might indicate a partial accumulation of the electroactive species near the working electrode. In CWR, there may be an uneven distribution of species in the diffusion layer, leading to a concentration polarization. This polarization can cause the anodic peak current to rise more than expected, as the reduced species has less chance to fully diffuse away from the electrode surface before the reverse scan.^{41,42}

In general, both electrode configurations of the Mylar-supported version showed closer peak-to-peak separations than that exhibited by fully paper-based 3D ePADs (**Table 5.2**). While this closer potential separation was only slightly shown in WCR electrode

configuration, the CWR configuration exhibited a substantial difference in peak-to-peak separation, as compared to CWR results obtained from fully paper-based cyclic voltammograms. The lower peak-to-peak separation offered by standalone CWR configuration suggests that it demonstrated higher reversibility.⁴³ This influence might be related to the fact that this device iteration does not depend on wax printing to define electrolytic path, avoiding the probability of increasing cell resistance due to wax diffusion.⁴⁴

Table 5.1. Peak current analysis of cyclic voltammetry data generated by standalone TFN-based 3D ePAD using both WCR and CWR electrode configurations.

Scan rate (mV/s)		50	100	200	300	400	500
i_{pa}/i_{pc}	WCR	0.99	0.99	0.98	0.98	0.98	0.98
	CWR	0.95	1.47	1.28	1.08	0.89	0.95

Additionally, CWR devices showed an increasing ΔE_p with most increasing CV scan rates (**Table 5.2**). However, this ΔE_p showed a drop at 300 mV/s scan rate and did not show a difference between 400 and 500 mV/s rates. In general, the irregularity of CV shapes could be related to an electrolyte pathway disturbance, in which Mylar frames could have introduced some inconsistencies in the electrolytic path. Since the hydrophilic channels are manually aligned in their Mylar frames, accidental slight misalignment could compromise the electrolyte flow path. This might affect the uniformity of electroactive species reaching the electrode surface.

Table 5.2. Peak-to-peak separation analysis of cyclic voltammetry data generated by standalone TFN-based 3D ePAD using both WCR and CWR electrode configurations.

Scan rate (mV/s)		50	100	200	300	400	500
ΔE_p (V)	WCR	0.15	0.20	0.27	0.31	0.35	0.40
	CWR	0.39	0.45	0.51	0.37	0.60	0.59

5.2.3.2 EIS analysis of standalone 3D ePADs

The Nyquist plots of the EIS measurements demonstrated major differences between the WCR and CWR electrode configurations of the 3D ePAD system (**Figure 5.14**). For the WCR configuration (**Figure 5.14A**), the data reveal a pronounced semicircle at high frequencies. The diameter of this WCR semicircle is significantly larger than in the CWR, covering two higher orders of magnitude (**Figure 5.14B**). This indicates higher charge transfer resistance, as compared to that of the CWR configuration. The higher impedance to electron transfer exhibited by the WCR format could possibly be due to increased interelectrode distances. This difference in charge transfer resistance can impact the sensitivity and response time of the WCR configuration – findings that align with the minimized current response obtained from WCR cyclic voltammograms in the previous section. These data could potentially favor the CWR configuration for applications that require rapid and efficient electron transfer.

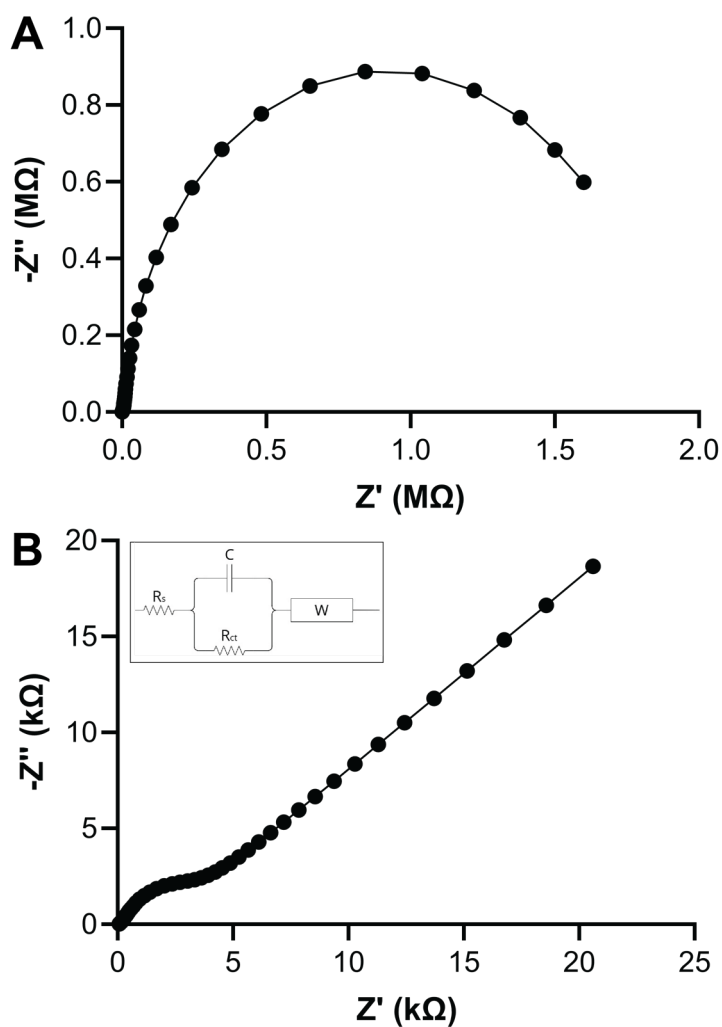


Figure 5.14. Nyquist plots representing the electrochemical impedance spectroscopy responses for both the (A) WCR and (B) CWR electrode configurations of the standalone 3D ePAD. Inset in B shows the equivalent circuit used for fitting both configurations according to the Warburg model. Circuit elements include solution resistance (R_s), double-layer capacitance (C), charge transfer resistance (R_{ct}), and Warburg impedance (W). The semicircle observed in the high-frequency region reflects the charge transfer resistance, while the linear tail at low frequencies indicates diffusion-controlled processes characterized by the Warburg impedance.

The comparison of solution resistance and double-layer capacitance values between WCR and CWR configurations reinforces the distinction in their electrochemical characteristics (**Figure 5.15A and C**). In the WCR configuration, R_s values are notably higher, indicating greater resistance through the electrolyte, which could result from a less direct electrochemical pathway across electrodes. While WCR devices showed higher R_s and R_{ct} values, they showed lower C_{dl} than CWR configuration. This observation aligns with the inverse proportionality between R_{ct} and C_{dl} .³⁷ This relationship is described by the equation:

$$C_{dl} = \frac{1}{2\pi f R_{ct}}$$

where C_{dl} is the double-layer capacitance, R_{ct} is the charge transfer resistance, and f is the frequency of the applied AC signal at which the relationship holds. This equation highlights that as R_{ct} increases, C_{dl} decreases, reflecting a reduced capacity to store charge at the interface in configurations with higher impedance to charge transfer. Accordingly, the lower capacitance in the CWR setup might lead to more stable signals. These findings suggest that there would be fewer fluctuations associated with changes in the double-layer structure during electrochemical processes performed by the CWR configuration. However, this suggestion does not perfectly align with the irregularities demonstrated by CWR cyclic voltammograms. To this end, more studies are needed to fully understand the compromise between C_{dl} and electrochemical reversibility influence of CWR devices.

Additionally, the capacitance values also vary across configurations, with the CWR configuration showing lower capacitance. This difference in capacitance could relate to differences in electrode surface area accessibility, where the CWR configuration might allow a more compact double-layer formation at the electrode interface.³⁴ Lower capacitance in the CWR format might lead to more stable signals, as there would be fewer fluctuations associated with changes in the double-layer structure during electrochemical processes.

The Warburg element, evident in the low-frequency tail of both Nyquist plots, provides additional insight into the diffusion-dominated processes in each configuration. The linear segment of the Nyquist plot for CWR extends further along the real axis than in the WCR configuration. This extension indicates that mass transport limitations could be more prominent in the CWR format. This suggests that while the CWR configuration may benefit from lower R_{ct} and R_s values, it may also encounter diffusion-related constraints, potentially impacting the device's performance under high-demand scenarios where rapid analyte diffusion to the electrode surface is required.

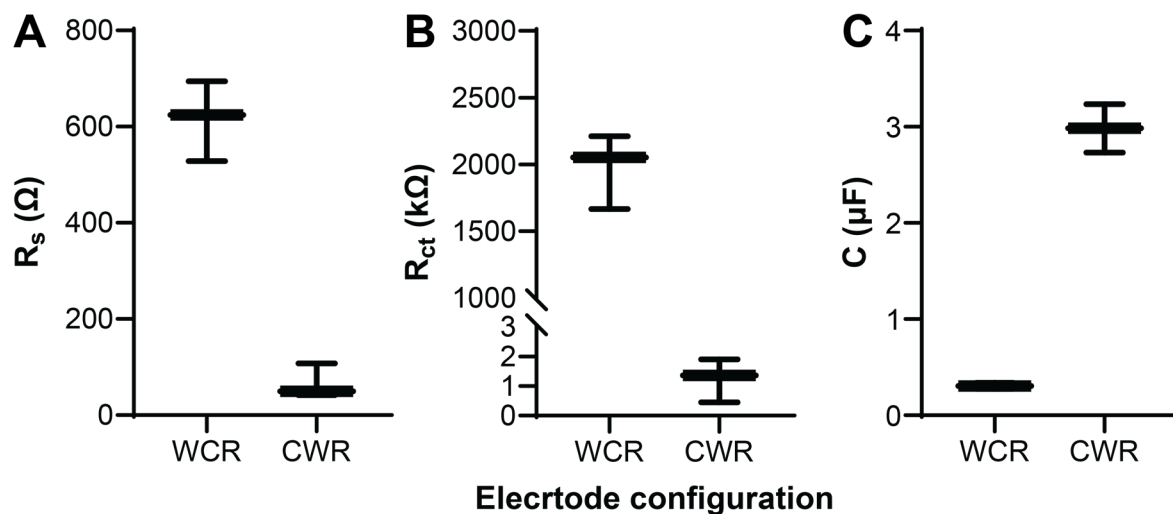


Figure 5.15. Quantitative comparison of EIS-derived parameters for WCR and CWR configurations of the standalone 3D ePAD version. Plots display (A) solution resistance, R_s , (B) charge transfer resistance, R_{ct} , and (C) double-layer capacitance, C . WCR configuration exhibits higher R_s and R_{ct} values, suggesting increased electrolyte and charge transfer resistances. CWR, however, shows a higher C value, consistent with an inverse relationship between R_{ct} and C . These differences highlight the distinct interfacial properties of each configuration. Whiskers represent the minimum and maximum values. (N = 3).

5.2.3.3 Chronoamperometric detection of glucose

We attempted using the TFN-based standalone 3D ePAD for chronoamperometric detection of glucose. In these trials, we deposited 8 μL of glucose oxidase solution in the hydrophobic midsection underneath the electrode printed on the top TFN layer. Compared to blank samples, glucose samples showed an increased chronoamperometric current. However, sample flow experienced frequent interruptions which increased flow time and manifested the sample evaporation effects. This issue likely stems from the limited hydrophilic area available beneath the stencil-printed electrode. Alternatively, we tested the A601-based standalone 3D ePAD described earlier to run the same experiment. In this device iteration, smooth sample flow was

demonstrated since the recognition enzyme was deposited on the sample flow layer while the electrode was printed on a separate layer. Despite the improved flow, we observed no correlation between glucose concentration and the measured current. Potential improvements for this experiment include using ink dilution and double-sided printing to deposit electrodes on A601 layers—techniques that were not implemented in this initial trial.

5.3 Conclusions and future work

In this chapter, we presented the development and characterization of two versions of the 3D ePAD design: the multiplexed and the standalone configurations. Each version addresses different aspects of usability and diagnostic needs for POC devices, building upon the foundational principles introduced in chapter 4.

The multiplexed version of the 3D ePAD was developed to enable simultaneous detection of multiple analytes within a single device architecture. The dual-input and single-input compartmentalized iterations showcased the versatility of this design, allowing independent assays to run in parallel without cross-interference. Despite the promising results, issues with signal reproducibility and interference from closely spaced electrodes emerged, particularly in the early iterations of the multiplexed device. The incorporation of hydrophobic barriers and compartmentalized flow paths partially addressed these challenges, but further refinement is necessary to improve device reliability.

In contrast, the standalone version was primarily optimized for singleplexed detection, with a focus on maximizing structural stability. By leveraging TFN and A601 paper substrates, we presented various strategies to improve structural integrity while maintain

controlled sample flow dynamics to ensure reliable electrochemical responses. Utilizing both WCR and CWR configurations, the standalone version demonstrated the electrode interchangeability primarily offered by fully paper-based devices while eliminating the need for wax printing. However, challenges related to sample evaporation and variability in current output persisted, highlighting the need for further improvements in device reproducibility.

To build upon the progress made with both standalone and multiplexed versions, we propose several key directions for future research. First, enhancing reproducibility for the standalone version is crucial, as variability in current output often stems from inconsistencies in electrode printing and sample flow. Implementing double-sided stencil printing of electrode inks, as highlighted in Chapter 4, is an area of improvement for signal output uniformity. Using diluted ink on A601 papers might also improve consistent ink penetration, resulting in more reproducible current responses. For the multiplexed design, refining the sample flow path is necessary to address challenges related to inconsistent fluid distribution and signal output. Manipulating flow paths can help ensure precise sample wicking and controlled interaction between sample and electrode surface. Additionally, conducting comprehensive reproducibility studies and failure analysis are essential for identifying potential weaknesses in both designs.

Future work should also focus on integrating the findings from Chapter 4 into both device iterations to tackle the observed challenges. Techniques such as double-sided printing, adjusted ink formulations, and well-defined sample flow channels could enhance electrode consistency, reduce signal fluctuations, and improve overall device performance. Exploring advanced electrode materials, including conductive

nanomaterials or composite inks, could further enhance conductivity and stability, while surface modifications may boost enzyme immobilization and decrease fouling, leading to longer-lasting signals. Moreover, addressing the limitations of the current sequential measurement approach in the multiplexed device by integrating a multi-channel potentiostat or a multiplexer unit could enable simultaneous detection, eliminate time-dependent effects, and increase assay throughput. These combined strategies are expected to significantly advance the 3D ePAD platform, making it a robust and versatile tool for point-of-care diagnostics across diverse applications.

5.4 Materials and methods

Reagents and Device Materials

We purchased Alhstrom chromatography paper grade 601 from Thomas Scientific. We purchased Munktell TFN paper from Laboratory Sales and Services (Somerville, NJ). We purchased Flexmount Select DF051521 (permanent adhesive double-faced liner) from FLEXcon. We purchased Uxcell Double-Sided Conductive Tape Copper Foil Tape Adhesive from Amazon. We purchased Banltre 7.5 mil Mylar Sheets from Amazon. We purchased 1/4" clear acrylic sheets from McMaster-Carr. We purchased Avery laminating sheets from Amazon. We purchased Elikliv 7" digital microscope with IPS screen - 1300X from Amazon. We purchased E3178 carbon ink and E2414 AG/AGCL ink from Ercon Inc. We purchased Glucose Oxidase from *Aspergillus niger* and Lactate Oxidase from *Aerococcus viridans* from Sigma. We purchased Lead(II) nitrate, 99+% from Thermo Scientific Chemicals. We purchased Samsill Economy Transparent Printer Sheets from Amazon. We purchased potassium ferricyanide from Acros Organics. We purchased

carbon screen printed electrodes (WE-D-3mm-carbon / pseudo RE-Ag/AgCl / CE-carbon) from Zensor R&D (Taiwan). We purchased potassium chloride [4 M] saturated with silver chloride from Ricca Chemical Company. We purchased glacial acetic acid and sodium acetate anhydrous from Fisher Scientific. We purchased 99.5% anhydrous ethanol from Thermo Fisher. We purchased ASTM Type I water from Ricca Chemical Company. We used CH Instrunment 650E workstation to run all electrochemical measurements.

Solution preparation

We prepared the solutions following the same conditions described in Chapter 4. For multiplexed assays, we deposited 4 μL of either 250 U/mL glucose oxidase in 600 mM potassium ferricyanide in 1 M potassium chloride solution or 50 U/mL lactate oxidase in 200 mM potassium ferricyanide in 1 M potassium chloride solution on the opposing compartments of WE/CE flow layer. We dried the treated paper layers for 20 min at 4 $^{\circ}\text{C}$ in dark over a silica gel bed. Sample is 150 μL of different concentrations of glucose and lactate mixture. The increased sample volume aims to mitigate the sample evaporation effect.

Electrode deposition on paper

Electrodes were stencil printed using both carbon and Ag/AgCl inks. For multiplexed assays, all electrodes are deposited on a single (top) side of their respective paper layers. All ink is used as received without dilutions for both A601 and TFN-based devices. We stencil printed Ag/AgCl ink to deposit all electrode pads. To dry deposited ink, we incubated their hosting device layers in the oven for 20 minutes at 65 $^{\circ}\text{C}$.

Fabrication of multiplexed paper devices

We fabricated multiplexed paper-based devices following the same protocol described in Chapter 4.

Fabrication of standalone devices

We used Adobe Illustrator to design the Mylar skeleton. We cut all Mylar, adhesive, stencil, and paper layers using a Boss laser cutter (BOSS-LS1630). Using a custom acrylic alignment jig, we belt the device layers bottom up, starting with the lower Fellowes laminating sheets. Next we introduced the RE-hosting Mylar frame. We then manually placed the laser cut hydrophilic papers in their designated spaces in the Mylar frame. We repeated the same sequence with all Mylar-paper-based layers, whether electrode-hosting or flow layers, with adhesive layers inserted between each two layers to ensure device integrity. Finally, we cut a strip of a Fellowes laminating sheet, adhered it as a top layer with a hole to access the sample introduction port. The upper and lower laminating sheet serves to further support device layer integrity and prevents evaporation or dripping of sample or product formed in the cell. After assembling the device layers, we sent the device through a laminator (Vevor) to remove any air bubbles trapped between the layers.

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Appendix

Impedimetric characterization of paper spray systems

A.1 Introduction

Paper spray mass spectrometry (PS-MS) has emerged as a powerful analytical technique for the direct analysis of complex biological samples. PS-MS aims to leverage the simplicity of paper as a substrate and the precision of mass spectrometry, eliminating the need for extensive sample preparation. PS-MS can be well suited for applications in clinical diagnostics, forensics, and environmental monitoring, especially when minimal interaction with the sample is required. The technique involves applying a high voltage to a paper substrate loaded with a small amount of sample, leading to the generation of charged analyte ions that can be analyzed directly by the mass spectrometer. This innovation bridges the gap between traditional liquid chromatography-mass spectrometry (LC-MS) and portable, rapid diagnostic solutions.¹

The choice of paper substrate is critical to the performance and reproducibility of PS-MS. While standard filter paper provides a robust and inexpensive option, its natural properties—such as porosity, surface charge, and hydrophilicity—can influence ionization efficiency and analyte recovery. The development of tailored paper substrates through surface modifications, including salinization, has proven promising to optimize performance for a wide range of analytes. By altering the paper's chemical properties, more PS-MS analytical challenges can be addressed such as ion suppression, nonspecific adsorption, and variability in signal intensity.²

Salinizing paper substrates involves introducing functional groups that modulate the paper surface chemistry to better suit specific classes of analytes. This modification can be important for analytes with low polarity or those prone to matrix effects in untreated paper. For example, salinization can improve the hydrophobicity of the paper surface,

facilitating the analysis of lipophilic compounds while maintaining the overall simplicity and cost-effectiveness of the PS-MS setup. Also, these modifications expand the applicability of PS-MS to challenge analytes in diverse sample matrices, from blood and urine to environmental samples.³

Through efforts to refine and optimize paper salinization techniques, this work contributes to advancing the versatility and quality control of PS-MS for various diagnostic and analytical applications. The following sections provide insights into the methods, challenges, and outcomes associated with these modifications, highlighting their impact on the reliability of PS-MS.

A.2 Device design

A.2.1 Testing the influence of paper spray setup parameters

To optimize the spray characteristics in PS-MS, we systematically evaluated the influence of mobile phase composition, the distance between the paper tip and the collection plate, and the applied voltage. These parameters were tested for their influence on the evenness and speed of the spray formation at the tip of Whatman Grade 4 filter paper, chosen for its high porosity and efficient liquid transport properties.

Mobile phase selection was critical due to its role in balancing solvent volatility, analyte dissolution, and ionization efficiency. We tested solvents with varying polarities, including methanol, ethanol, acetonitrile, and ethyl acetate. Additionally, we included H₂O:MeOH (5:95) with 0.1% formic acid (v/v), a commonly used solvent system in PS-MS for its ability to dissolve analytes while promoting efficient spray formation. The results provided

insights into how solvent polarity and volatility affect spray behavior, guiding the selection of conditions that enhance reproducibility and ionization efficiency.

A.2.2 Paper silanization

To modify the surface properties of paper substrates for PS-MS, we performed silanization using silane compounds with varying carbon chain lengths. Specifically, we used ethyltrimethoxysilane, n-octyltrimethoxysilane, and n-octadecyltrimethoxysilane, representing silane compounds with 2, 8, and 18 carbon chain lengths. This range was chosen to systematically study the impact of hydrophobicity changes on the paper surface and its influence on spray performance.

For the silanization process, silane compounds were mixed with hexane to prepare a 10 mL of 10% (v/v) silanization solution. Paper sheets (8 cm X 8 cm) were then placed with this mixture in a vacuum oven at 25 mmHg pressure and 140°C for 3 hours. Following the heating process, we let the oven cool overnight while the pressure was gradually released. The next day, we opened the oven and started using the silanized paper sheets for further testing.

To evaluate the effectiveness of the silanization process, we employed electrochemical impedance spectroscopy (EIS). This technique allowed us to study changes in surface properties by analyzing EIS parameters such as solution resistance (R_s) charge transfer resistance (R_{ct}) and double-layer capacitance (C_{dl}). The data were analyzed to determine correlations between these parameters and the carbon chain length of the silanization compounds, providing insights into how surface modifications influence the electrochemical and functional properties of the paper.

A.2.3 Electrochemical impedance spectroscopy cell

To evaluate EIS parameters, we designed Mylar-based cells where the paper substrate served as the connection between the electrodes. In all designs, the working electrode (WE) and counter electrode (CE) were stencil-printed using carbon ink, while the reference electrode (RE) was printed with Ag/AgCl ink. The initial design placed the WE and CE on the same Mylar plane and the RE on a separate plane. However, this configuration incorporated a large interelectrode distance (1 cm), leading to increased cell resistance and less sensitivity (**Figure A.1**).⁴

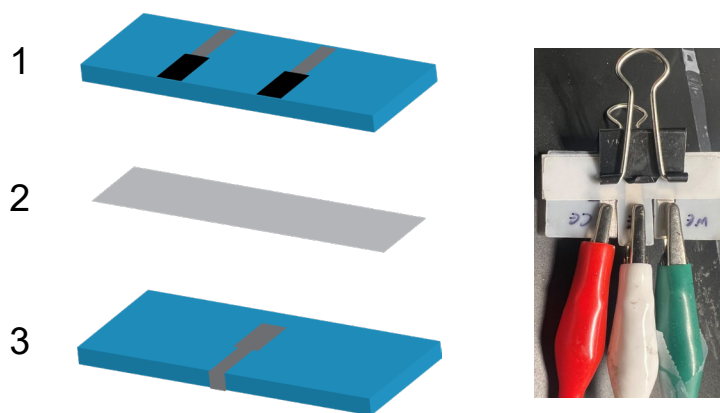


Figure A.1. Schematic and actual image of the early EIS cell to characterize paper strips used in PS-MS.

To address this, we developed an improved design where the WE and RE were on the same plane, while the CE was positioned on a separate Mylar layer. When stacked, the CE became in contact with both the WE and RE through the silanized paper substrate, which was sandwiched between the layers (**Figure A.2**). This enhanced design reduced the interelectrode distance and enabled more sensitive detection of changes in cell resistance as a function of the silanization compounds used. This refinement facilitated gaining insights into the impact of surface modifications on paper substrates.

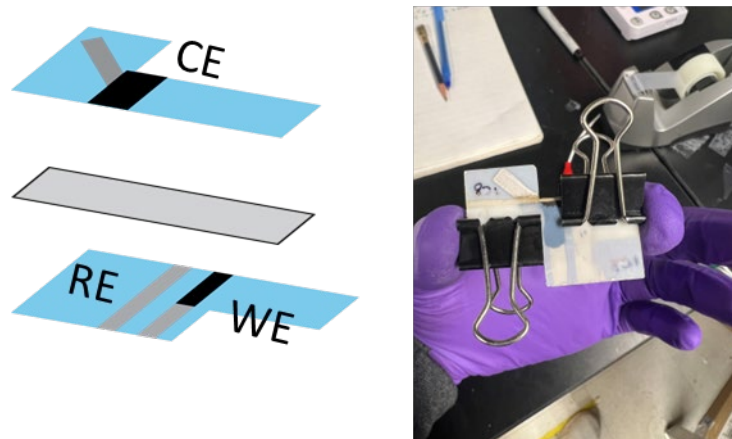


Figure A.2. Schematic and actual image of the enhanced EIS cell to characterize paper strips used in PS-MS.

For all EIS measurements, we employed an initial potential of 0 V, high frequency of 1×10^5 Hz, low frequency of 0.1 Hz, and amplitude of 5 mV. The resulting data were fitted using both Warburg and Gerischer models. The Warburg element was employed to model diffusion processes in dielectric spectroscopy, representing the diffusion of electroactive species at flat, macroscopic electrodes. Conversely, the Gerischer element was used to describe the impedance behavior of mixed ionic electronic conductors (MIECs), where oxygen reduction at the MIEC|gas|current-collector interface generates O^{2-} ions that diffuse through the MIEC to the electrolyte phase. The data fitting achieved error percentages ranging between 3% and 15%, validating the applicability of these models in interpreting the experimental results.

A.2.4 Evaluating Electrolytes for EIS Measurements

We tested multiple electrolyte combinations to identify an optimal formulation for EIS measurements on silanized paper substrates. The ideal electrolyte was to be an evaporative salt dissolved in a volatile solvent, allowing complete evaporation from the paper tip after EIS analysis. This goal was set to ensure that the paper tip could still be used for its intended purpose in the paper spray mass spectrometry (PS-MS) system. In this context, the EIS measurement served as a quality control step to verify that the silanized paper exhibited the desired hydrophilic and conductive properties.

Table A.1. Summary of electrolytes used to test EIS systems and their salt conductivities.

Salt	Solvent	Salt conductivity (S cm ² /mol)
K ₃ [Fe(CN) ₅]	EtOH	780.8
KOH	EtOH	271.6
NaOH	MeOH	248.1
KNO ₃	DMSO	145.0
CH ₃ COONH ₄	EtOH	114.5

In addition to evaporative electrolytes, we also tested non-evaporative solutions that provided higher electroactivity, despite their incompatibility with subsequent PS-MS use. The tested electrolyte combinations, which varied in conductivity and evaporation capabilities, are summarized in **Table A.1**. For each test, 100 μ L of the electrolyte was applied to the paper strip, which was then sandwiched between the two layers of the Mylar-based electrochemical cell. The electrode pads were connected to their

corresponding electrochemical workstation clamps, and EIS measurements were conducted to evaluate the performance of the paper substrate.

A.2.5 Controlling Electrolyte Evaporation During EIS Measurements

To mitigate the effects of electrolyte evaporation during EIS measurements, we employed an acrylic chamber that maintained a saturated ethanol atmosphere (**Figure A.3**). This chamber contained an uncovered glass vial filled with the tested electrolyte solvent. This design aimed to create a closed system where the ambient environment is saturated with the solvent vapor. This saturation aimed to reduce the evaporation rate of the tested electrolyte, ensuring consistent and reliable measurements throughout the duration of the experiment. The controlled environment allowed for accurate assessment of the silanized paper's electrochemical properties while avoiding the electrolyte solvent evaporation.

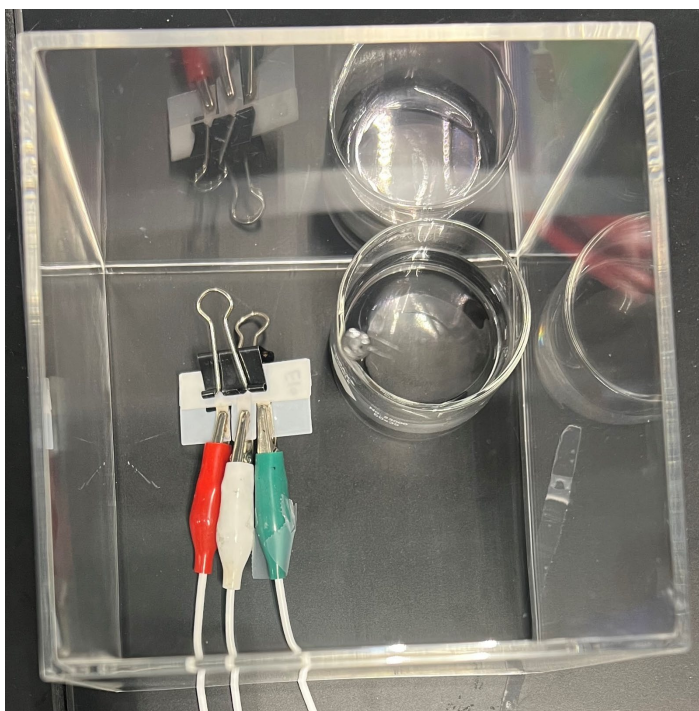


Figure A.3. Acrylic chamber used to create a solvent saturated system.

A.3 Results and discussion

A.3.1 Multivariable Analysis of Spraying Behavior

We obtained extensive results from multivariable analysis conducted to better understand the spray behavior in a PS-MS system. The analysis included several variables, such as the total distance from the electrical clamp to the collector plate, the mobile phase (MP) composition, the applied potential, and the mobile phase volume. Across the tested mobile phases, we observed that increasing the applied potential generally resulted in faster and more even spray formation compared to lower potentials. For instance, as shown in **Figure A.4**, using methanol as the mobile phase, higher voltages enhanced spray consistency.

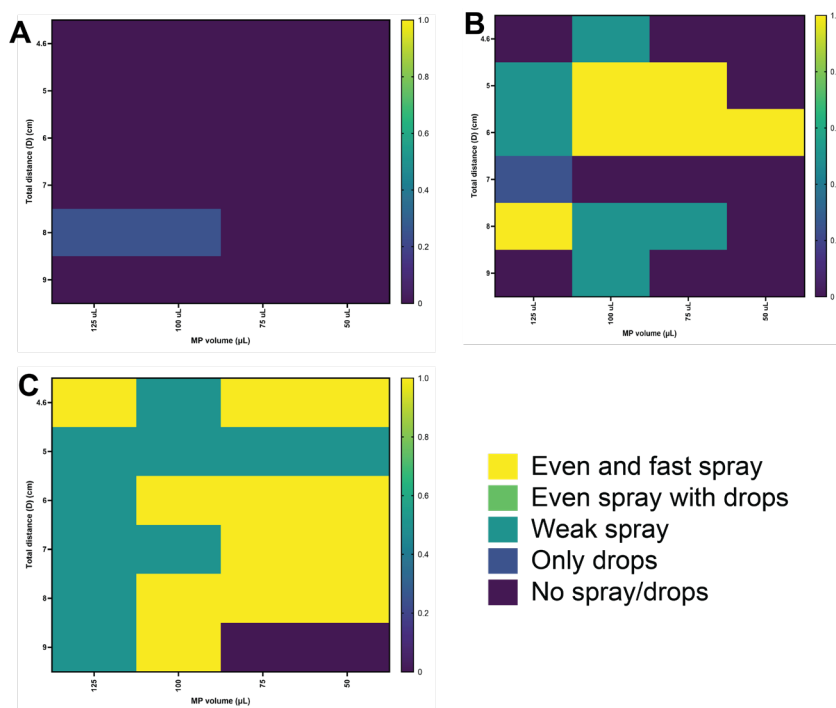


Figure A.4. Heatmap visualization of multivariate analysis using methanol as a model mobile phase to test its spraying efficiency.

Applying a potential of 7 kV yielded improved spraying behavior across most tested mobile phases, including methanol, ethanol, acetonitrile, and ethyl acetate. However, this trend was not observed with the H₂O:MeOH (5:95) + 0.1% formic acid (v/v) formulation, where the spray behavior was less consistent (**Figure A.5**). This suggests that solvent polarity influences the spray dynamics, possibly by altering the evaporation rate or surface tension of the mobile phase. Further studies are needed to confirm whether these physicochemical properties mediate the spray behavior observed under different potential settings.⁵

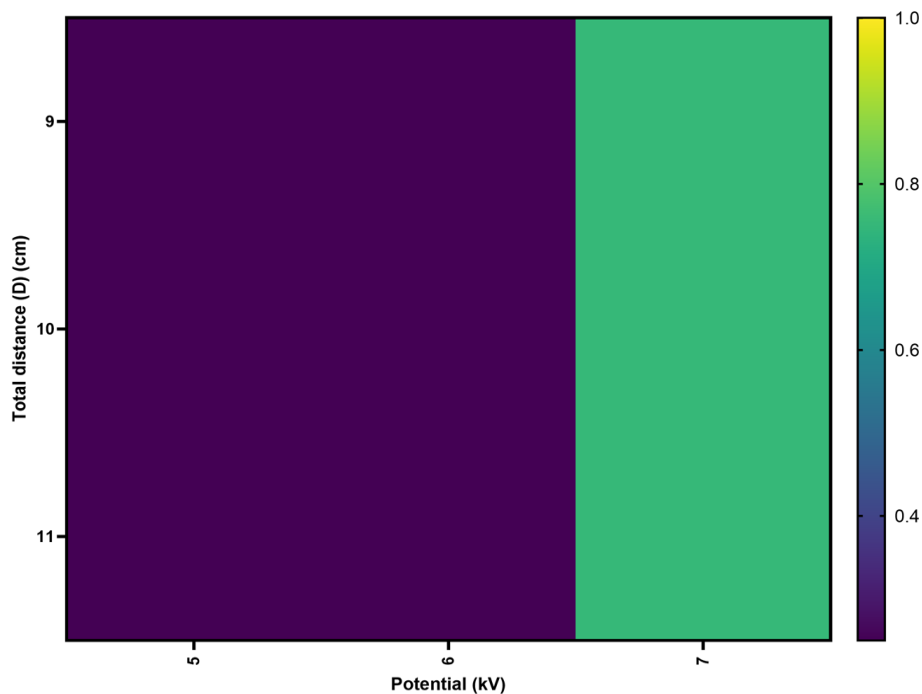


Figure A.5. Heatmap visualization of multivariate analysis using H₂O:MeOH model mobile phase to test its spraying efficiency.

To test the feasibility of collecting sprayed material, we optimized spraying conditions using ethanol as the mobile phase for 1% (w/v) Allura Red dye solution. A polystyrene 12-well plate was attached to the metal collector sheet, with holes cut in the well bottoms to provide conductive access to the collector. We engineered acrylic holders for the tested

paper strip which included reservoirs for the solution introduced to the system (**Figure A.6**). However, this configuration resulted in poor spray collection efficiency, likely due to the insulative properties of polystyrene, which reduced charge transfer at the collector interface. Future improvements could involve replacing the polystyrene components with conductive or semi-conductive materials to minimize charge buildup and enhance collection efficiency.



Figure A.6. Preliminary system for spray collection.

A.3.2 Early cell design

We employed multiple cell designs to test the conductivity of paper strips using EIS. While these early designs were not fully optimized, they provided an opportunity to practice circuit fitting of Nyquist plots using CH Instrument software. Initial results demonstrated a correlation between charge transfer resistance and the increase in the carbon chain length of the silanization compounds (**Figure A.7**). These findings were reproducible when the same cell and paper strip were used under identical conditions.

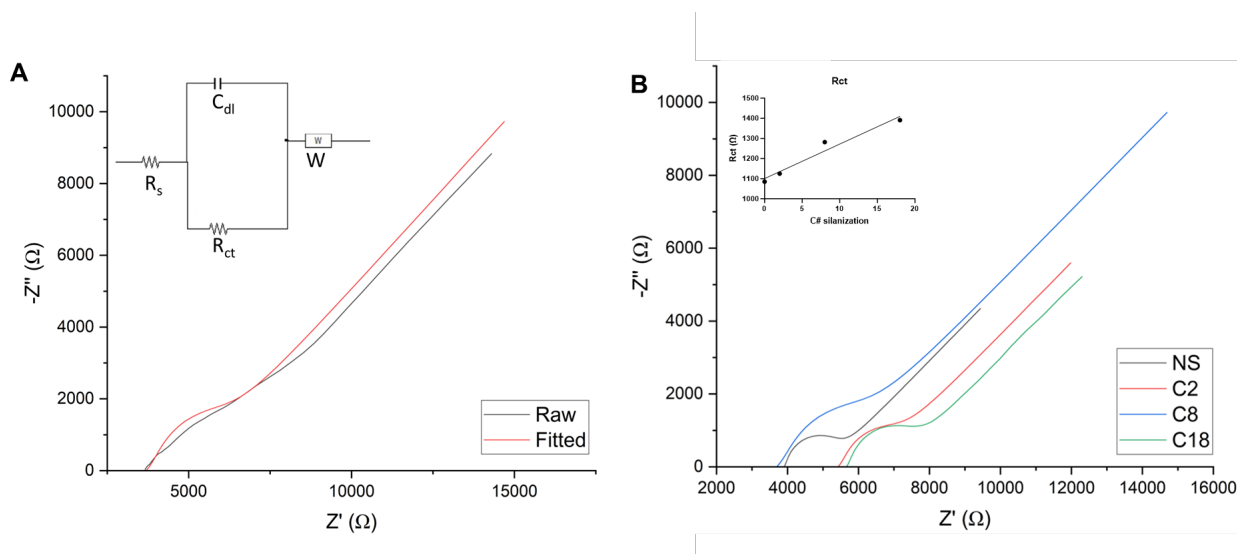


Figure A.7. Nyquist plots for early EIS cell designs. (A) Nyquist plot of raw and fitted data for early EIS cell designs, with an inset showing the Warburg-fitted circuit model. (B) Nyquist plots comparing non-silanized paper (NS) and silanized substrates (C2, C8, C18), with the inset showing the correlation between R_{ct} and silane chain length.

However, reproducibility was not achieved across different cells or paper strips. This inconsistency was likely due to the unoptimized EIS cell design, which introduced significant variability in electrochemical output. The increased interelectrode distance in these early designs may have contributed to higher cell resistance and a lack of uniform current distribution, impacting the reliability of the measurements. Additionally, irreproducibility could be attributed to uneven silanization of paper surfaces, leading to variations in the conductive properties of the substrates. To address these limitations, we developed a more optimized cell design with minimal interelectrode distance and enhanced structural precision, as described in **Figure A.2**.

A.3.3 Enhanced cell design for EIS parameter analysis

In the improved Mylar-based cell design, we analyzed key EIS elements, including solution resistance, charge transfer resistance, capacitance, Warburg impedance, and Gerischer impedance. We conducted ANOVA for each EIS element to determine whether their values showed significant differences across the carbon chain lengths used during paper silanization. **Figure A.8.** presents the p-values associated with changing different EIS elements as a function of carbon chain length in silanization compounds. The EIS elements are derived from fitting Nyquist plots using both Warburg and Gerischer models. Parameters with statistically significant differences ($p < 0.05$ at a 95% confidence level) are marked with yellow-star indicators in the heatmap plots.

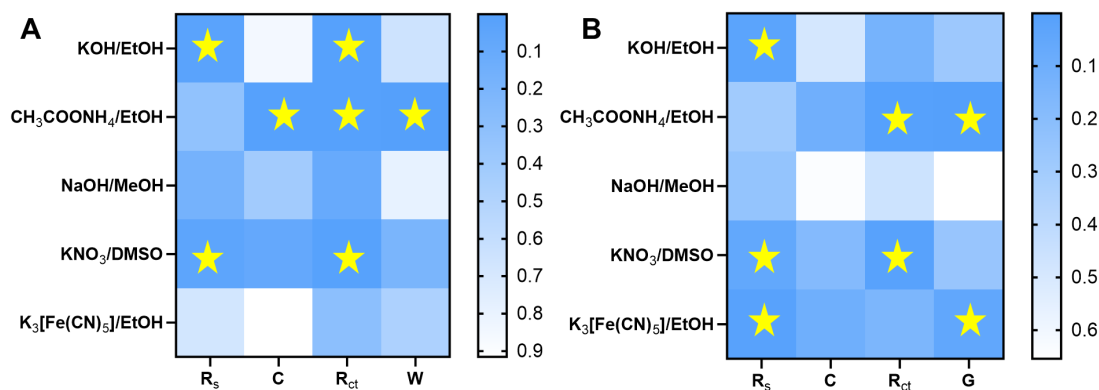


Figure A.8. Heatmap of ANOVA results for EIS parameters. Heatmaps of p-values showing statistical differences in EIS parameters across different silane chain lengths. (A) Warburg model data and (B) Gerischer model data. Yellow stars indicate parameters with significant differences ($p < 0.05$) based on ANOVA analysis. Results highlight the variability in EIS behavior influenced by silanization and electrolyte interactions.

For Warburg modeling, most EIS parameters exhibited significant variance when the ammonium acetate/ethanol electrolyte was used, with the notable exception of R_s . R_{ct} also demonstrated variance across carbon chain lengths in 3 out of the 5 electrolytes tested. Nonetheless, this variance did not consistently translate into clear correlations or regressions. For example, while R_{ct} increased with longer carbon chain lengths when ammonium acetate/ethanol was the electrolyte, this trend reversed when potassium nitrate/DMSO was used. These contradictory trends highlight the complexity of interactions between the silanized paper surface, electrolyte composition, and ion conduction pathways, suggesting a strong electrolyte dependency for observed correlations.

The change in R_s values provided a more consistent trend across the tested electrolytes, typically decreasing with longer silanization carbon chains. This observation is rather unexpected, as longer carbon chains are generally associated with increased hydrophobicity and potentially reduced ionic conduction due to surface insulation effects. One probable explanation for this trend is that longer carbon chains might improve electrolyte distribution or capillary action on the paper surface, facilitating ion movement. Alternatively, changes in surface morphology or uniformity induced by the longer silanization chains might have created more favorable conditions for ionic conduction. Future work is needed to investigate these possibilities, including studies of electrolyte-paper interaction dynamics and surface morphology analysis.

Despite these insights, the reproducibility of R_{ct} results across different Mylar-based cells was limited, as shown in the nested graphs in **Figure A.9**. Variability in electrode alignment, interelectrode distance, or uneven silanization of paper substrates likely

contributed to this inconsistency. These limitations underscore the need for improved fabrication processes to enhance the uniformity of cell assembly and reduce variability across devices.

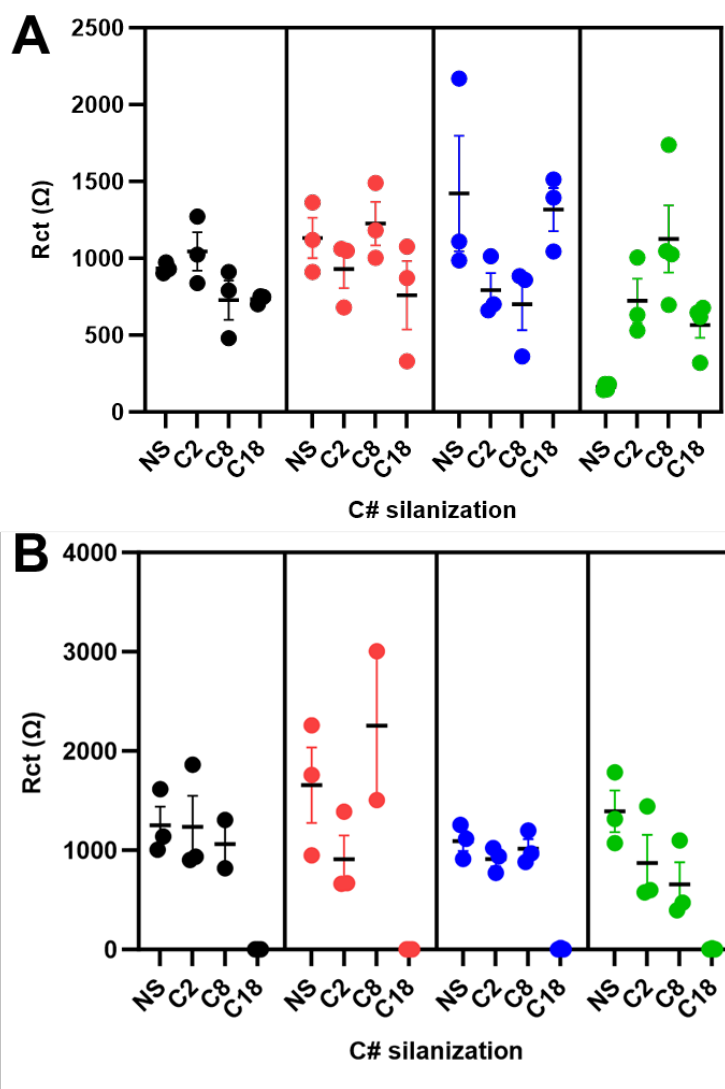


Figure A.9. Variability in Rct across Mylar-based cells. (A) Charge transfer resistance (Rct) for CH₃COONH₄ in EtOH across non-silanized (NS) and silanized paper strips (C2, C8, C18), showing variability with Mylar cell differences. (B) Rct for KNO₃ in DMSO with similar conditions. Different colors represent data from distinct Mylar cells.

Further optimization efforts could focus on refining cell design, standardizing silanization protocols, fitting equivalent circuits more accurately, and exploring alternative electrolytes with more stable conductivity properties. These improvements would enable more reliable identification of correlations between EIS parameters and silanization chemistry, paving the way for reproducible and meaningful results in future studies.

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Epilogue

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

"رَبِّ قَدْ آتَيْتَنِي مِنَ الْمُلْكِ وَعَلَّمْتَنِي مِنْ تَأْوِيلِ الْأَحَادِيثِ ۚ فَاطِرَ السَّمَاوَاتِ وَالْأَرْضِ أَنْتَ وَلِيِّي فِي الدُّنْيَا وَالْآخِرَةِ تَتَوَقَّئِي مُسْلِمًا

وَأَلْحَقْنِي بِالصَّالِحِينَ"

القرآن الكريم، سورة يوسف، آية 101

In the name of Allah, the Entirely Merciful, the Especially Merciful

"My Lord, You have given me [something] of sovereignty and taught me of the interpretation of visions and events. Creator of the heavens and earth, You are my guardian in this world and in the Hereafter. Cause me to die in a state of submission [to You] and join me with the righteous."

The Noble Qur'an, Chapter Yusuf (Joseph), Verse 101