# Enzyme-based Tablet Sensor:
# A Tool for Point-of-Care Detection of Glucose in Urine

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This is to certify that the thesis is prepared,

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## Abstract

Enzyme-based Tablet Sensor: A Tool for Point-of-Care Detection of Glucose in Urine

Hasti Haji Miri

Monitoring glucose levels in biological samples is crucial to prevent complications arising from high blood glucose, such as heart-related issues, kidney problems, and vision loss. Traditional colorimetric glucose detection in urine relies on enzymatic reactions, offering a low-cost and non-invasive method for diabetes management. However, enzymes used in these assays are highly susceptible to environmental conditions, particularly elevated temperatures, which limit their transportation and storage.

In response to this challenge, this thesis presents a novel solution: the development of stable and portable enzyme-based tablet sensors. In this technique, glucose oxidase and horseradish peroxidase enzymes are encapsulated within a water-soluble dextran matrix, transforming them into solid tablets. This encapsulation enhances enzyme stability and practicality, making these sensors user-friendly. The enzymatic tablet-based sensor demonstrates remarkable capabilities, detecting glucose in urine samples within a rapid 5-minute timeframe. Utilizing 3,3',5,5'-tetramethylbenzidine (TMB) as the indicator, the tablet sensor exhibits impressive performance within the clinically relevant glucose concentration range of 0-6 mM, boasting a remarkable limit of detection at 0.013 mM. Furthermore, these enzyme tablets exhibit enhanced thermal stability, retaining their activity even at an elevated temperature of up to 60 ℃, surpassing the performance of solution-phase enzymes. This resilience under harsh conditions significantly expands the

accessibility and application of glucose sensors, especially in resource-limited settings. The development of these tablet sensors represents a significant advancement in glucose monitoring technology, offering an effective and user-friendly solution for diabetes management.

## List of publications and conference contributions

- **Hasti Hajimiri**, Seyed Hamid Safiabadi Tali, Muna Al-Kassawneh, Zubi Sadiq, Sana Jahanshahi-Anbuhi. Tablet-Based Sensor: A Stable and User-Friendly Tool for Point-Of-Care Detection of Glucose in Urine. *Biosensors,* 13, 893 (**2023**). https://doi.org/10.3390/bios13090893
- Seyed Hamid Safiabadi Tali; **Hasti Hajimiri**; Zubi Sadiq; Sana Jahanshahi-Anbuhi. Engineered detection zone to enhance color uniformity on paper microfluidics fabricated via Parafilm®-heating-laser-cutting. *Sensors and Actuators B: Chemical*, 380, 133324 (**2023**). https://doi.org/10.1016/j.snb.2023.133324
- Zubi Sadiq, Seyed Hamid Safiabadi Tali, **Hasti Hamijri**, Muna Al-Kassawneh, Sana Jahanshahi-Anbuhi. Gold nanoparticles-Based Colorimetric Assay for Environmental Monitoring and Food Safety Evaluation. *Critical Reviews in Analytical Chemistry*, 1-36 (**2022**). https://doi.org/10.1080/10408347.2022.2162331
- **Hasti Hajimiri**; Seyed Hamid Safiabadi Tali; Sana Jahanshahi-Anbuhi. Polysaccharide-based Tablets for Rapid measurement of Glucose Level in Urine. Poster Presentation, *PEOPLE 2023 International Conference*, Montreal, Canada. (**August 2023**)
- **Hasti Hajimiri**; Seyed Hamid Safiabadi Tali; Sana Jahanshahi-Anbuhi. Polysaccharide-based Tablets for Rapid measurement of Glucose Level in Urine. Poster Presentation, *Chemical Engineering Research Day*, Montreal, Canada. (**March 2023**)
- **Hasti Hajimiri**; Seyed Hamid Safiabadi Tali; Sana Jahanshahi-Anbuhi. Development of Passive Mixers for Microfluidic Paper-based Analytical Devices. Poster Presentation, *Canadian Chemical Engineering Conference*, Montreal, Canada. (**October 2021**)

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## Abbreviations

| | |
|---|---|
| POC | Point-of-care |
| $H_2O_2$ | Hydrogen peroxide |
| HRP | Horseradish peroxidase |
| GOx | Glucose oxidase |
| TMB | 3,3',5,5'-tetramethylbenzidine |
| μPADs | Microfluidic paper-based analytical devices |
| AChE | Acetylcholinesterase |
| IDA | Indoxyl acetate |
| PNR | Poly(neutral red) |
| ATP | Adenosine-5′-triphosphate |
| PDMS | Polydimethylsiloxane |
| AuNPs | Gold nanoparticles |
| AuNPs-pTab | Pullulan-stabilized gold nanoparticles |
| AuNPs-pSol | Pullulan-stabilized gold nanoparticles solution |
| UV-vis | Ultraviolet light visible |
| %R | Recovery percentage |
| SD | Standard deviation |
| %RSD | Relative standard deviation percentages |
| DMSO | Dimethyl sulfoxide |
| $v_{max}$ | Maximum velocity |
| $K_m$ | Michaelis Menten's constant |
| λ | Wavelength |

| | |
|---|---|
| sec | Second |
| min | Minutes |
| h | Hour |
| g | Gram |
| mL | Milliliter |
| mM | Millimolar |
| mg | Milligram |
| µg | Microgram |
| µM | Micromolar |
| mol | Mole |
| w/v | Weight per volume |

# Chapter 1: Introduction and objective

In this chapter a brief introduction of Point-of-Care devices and their significance in diabetes management is presented. This section continuous with the objectives of this research project and ends up with the thesis outline.

## 1.1 Background

The domain of medical diagnostics has undergone a transformative revolution, driven by the emergence of Point-of-Care (POC) analytical devices [1]. These devices extend their impact beyond medical contexts, finding application in areas like environmental monitoring—enabling real-time assessment of air, water, and soil quality—and food safety—by rapidly screening for contaminants and allergens [2]. POC devices have a history that dates back to the early 20th century when the introduction of urine testing strips laid the foundation for decentralized diagnostics [3]. Over time, technological advancements have refined these devices, rendering them invaluable tools for rapid, on-the-spot assessments. Their hallmark characteristics of portability, simplicity, and practicality have led to widespread adoption across various sectors within healthcare and beyond.

POC devices have played an essential role in diabetes management, exemplified by their application in glucose monitoring [4]. For instance, handheld glucometers have become synonymous with at-home glucose testing, empowering individuals to monitor their blood sugar levels conveniently [5]. Nevertheless, the history of glucose monitoring methods reveals a reliance on invasive procedures, such as finger pricks to obtain blood samples [6]. While undeniably accurate, these methods pose practical challenges, particularly for those coping with childhood-

onset diabetes [7]. The frequent blood draws, though necessary, disrupt daily routines, resulting in not only physical discomfort but also emotional distress. This highlights the unmet need for less intrusive alternatives that offer accurate and reliable glucose monitoring.

The exploration of non-invasive methodologies has led to the investigation of glucose detection in other body fluids, notably urine. The concept of evaluating glucose levels in urine capitalizes on the body's innate excretory processes, potentially offering a less intrusive approach. However, the success of this approach hinges on the ability to achieve accuracy and reliability that align with stringent medical diagnostic standards. This necessitates the development of robust techniques capable of consistently and precisely measuring glucose levels in urine.

### 1.2 Objective

Within the landscape of glucose detection, various methods have demonstrated success. However, this thesis addresses the need for improved non-invasive detection techniques, acknowledging the existing limitations in both invasive procedures (such as finger pricks and blood draws, which can be discomforting and emotionally taxing) and urine-based approaches (which, while accurate, often require laboratory settings and trained professionals, making them less conducive to immediate and on-the-spot applications).

The primary objective of this research is to develop and validate a novel enzyme tablet bioassay for non-invasive point-of-care glucose detection in urine. This approach aims to offer a reliable and user-friendly method for glucose monitoring, ultimately contributing to improved diabetes management.

This research explores the utilization of enzyme tablets as a solution to the challenges posed by traditional glucose detection methods. These tablets hold the promise of combining the stability of

enzymes in solution with the convenience of solid-phase platforms. The encapsulation of bioreagents within dextran, a biocompatible and biodegradable polysaccharide, offers the potential for enhanced enzyme stability under varying conditions. Moreover, these tablets can be easily deployed within complex matrices, such as urine, providing a straightforward and efficient detection platform.

### 1.3 Thesis outline

This thesis is structured into five chapters, each playing a significant role in explaining the research conducted and its outcomes.

The opening chapter of this thesis provides an essential background for the research undertaken. It offers a comprehensive introduction to the subject matter, including the background, objectives, and the overall framework of the study.

Chapter 2 undertakes a thorough examination of relevant literature related to enzyme immobilization techniques within enzyme-based biosensors. It explores the various substances employed as platforms for glucose detection within these biosensors. Moreover, this chapter introduces the concept of tablet-based sensors and explores the detection of glucose in diverse bodily fluids, setting the stage for the subsequent chapters.

Chapter 3 presents a published manuscript that encapsulates the core research findings. It begins with an abstract to provide a concise overview of the research, followed by an extensive introduction detailing the materials and methods employed in the study. This chapter comprehensively covers the preparation and characterization of enzyme-based tablets, as well as the detection of glucose in both artificial and real human urine. Furthermore, it discusses the

analytical performance, interference studies, and stability tests conducted, thereby presenting a comprehensive insight into the research outcomes.

The concluding chapter, Chapter 4, synthesizes the research journey undertaken in this thesis. It offers a concise summary of the research conclusions derived from the findings in Chapter 3. Additionally, this chapter provides valuable recommendations for guiding future research investigations in the field, serving as a logical endpoint to this research work.

Chapter 5 includes a comprehensive list of references used in the thesis, providing the sources and literature that supported and informed the research.

This structured approach ensures that the reader gains a comprehensive understanding of the research, starting from the fundamental context (Chapter 1) to the critical review of existing knowledge (Chapter 2), and culminating in the presentation of original research and its implications (Chapter 3), all ultimately leading to a thoughtful conclusion (Chapter 4) followed by Chapter 5, which provides an extensive list of references used throughout the thesis. Together, these chapters create a well-rounded and informative thesis that contributes to the body of knowledge in this field.

# Chapter 2: Literature review

In this chapter, we explore enzyme-based biosensors, examining common techniques for immobilizing enzymes and various substances employed as platforms in these biosensors for the detection of glucose. Additionally, the concept of tablet-based bioassays is introduced, and diverse bodily fluids used for glucose detection are investigated.

## 2.1 Introduction

Enzyme-based sensors have significantly advanced biosensing capabilities by offering precise detection and quantification of a wide range of analytes. These sensors utilize specific enzymes' catalytic properties to convert target analytes into measurable signals through enzymatic reactions [8]. In the 1960s, Clark and Lyons [9] pioneered the first enzyme-based glucose sensor, which marked the beginning of using enzymes for biosensing applications. This pivotal discovery paved the way for subsequent developments in enzymatic sensing, reshaping glucose monitoring, particularly for diabetes management. Consequently, enzyme-based sensors have undergone extensive research, resulting in the creation of accurate, selective, and user-friendly sensing platforms across various fields, including medical, environmental, and food monitoring [10].

An interesting aspect of enzymes is their compatibility with complex biological samples due to their ability to function effectively under physiological pH and temperature conditions. Additionally, enzyme-based sensors are highly sensitive, allowing for the detection of analytes even at low concentrations [11].

Commonly used enzymes in glucose biosensors include horseradish peroxidase (HRP) and glucose oxidase (GOx). HRP employs hydrogen peroxide ($H_2O_2$) to oxidize a range of substrates,

generating oxidized products and water. Meanwhile, GOx catalyzes the conversion of glucose into gluconolactone while simultaneously reducing molecular oxygen to hydrogen peroxide. These enzymes find integration within biosensors, where their catalytic activities generate signals directly proportional to glucose concentrations. Through the strategic integration of HRP and GOx with suitable transduction methods, glucose biosensors attain impressive levels of sensitivity, selectivity, and precision, effectively establishing them as indispensable tools for glucose monitoring across various sample types. The incorporation of these enzymes into biosensing platforms has notably propelled the field of glucose monitoring, particularly within the context of diabetes management. Consequently, this chapter will be centered around four main themes: enzyme immobilization techniques in biosensors, distinctive substances used as platforms in enzyme-based biosensors for glucose detection, introduction on tablet-based assays, and various body fluids used for the detection of glucose.

## 2.2 Enzyme immobilization techniques in enzyme-based biosensors

Enzyme immobilization techniques play a crucial role in enhancing the effectiveness and functionality of enzyme-based biosensors. By utilizing the catalytic capabilities of enzymes and attaching them to surfaces, biosensors gain the ability to convert complex biochemical interactions into measurable signals. These techniques improve biosensors' stability, selectivity, and reusability, making them valuable tools in fields like medical diagnostics and environmental monitoring. In this section, four techniques that are widely employed for enzyme immobilization in enzyme-based biosensors are discussed: encapsulation, adsorption, cross-linking, and covalent.

### 2.2.1 Encapsulation

Enzyme encapsulation, facilitated through techniques such as sol-gel, electropolymerization, and the utilization of natural polymers, offers a versatile approach for immobilizing enzymes within three-dimensional matrices. This method involves incorporating enzymes into these matrices, imparting distinct advantages for the development of biosensors. A primary strength of encapsulation lies in its straightforward fabrication process, enabling the simultaneous integration of enzymes, mediators, and additives within a single sensing layer. An important feature of encapsulation is that it requires no modifications to the enzymes themselves, thus maintaining their native activity and functionality. By entrapping enzymes within a matrix, their stability is heightened, ensuring their enduring efficacy in detecting target analytes.

#### 2.2.1.1 Enzyme encapsulation with sol–gel

Sol-gel encapsulation involves a controlled process of entrapping enzymes within inorganic sol-gel matrices, often using sol-gel silica. As shown in Fig. 1, the method commences with the hydrolysis of metal alkoxide precursors, like tetramethoxysilane (TMOS) or tetraethoxysilane (TEOS), in the presence of water, an acid catalyst, and a mutual solvent like ethanol. This hydrolysis generates silanol groups (Si–OH), which further condense to form siloxane (Si–O–Si) polymers through a condensation reaction. The resulting colloidal suspension, termed a 'sol,' transitions into a porous gel structure. Gradual solvent removal yields the 'dry gel,' wherein the enzyme is firmly nestled within the porous network. Sol-gel encapsulation brings benefits such as heightened enzyme stability, protection from harsh conditions, and controlled enzyme release for targeted applications [12].

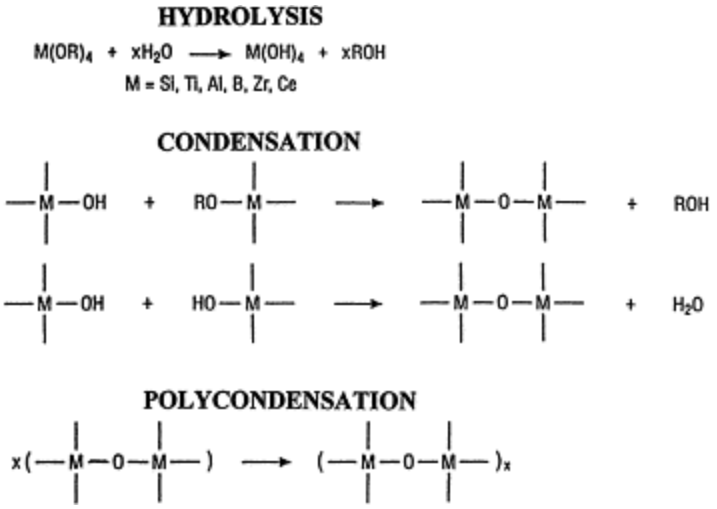


*Fig. 1 Steps involved in the sol-gel process (adopted from Ref.* [12]*).*

Raghu et al. [13] developed a sol-gel immobilized biosensor focusing on the detection of organophosphorous pesticides, specifically methyl parathion and acephate. Acetylcholinesterase (AChE) was immobilized on a carbon paste electrode using sol-gel encapsulation. This technique involves incorporating the enzyme within an inorganic matrix, enhancing its stability and activity. The inhibition of AChE by the target pesticides was measured using voltammetry, providing sensitive detection. Sol-gel immobilization allows precise enzyme immobilization, enhancing the biosensor's performance for organophosphorous compound determination.

### 2.2.1.2 Enzyme encapsulation with natural polymers

Enzyme entrapment within natural polysaccharide matrices like alginate, chitosan, or agarose constitutes an alternative strategy. The biocompatibility and non-toxicity of these polysaccharide-based gels, derived from natural sources, preserve enzymatic activity and enhance enzyme stability. Unlike sol-gel encapsulation, which utilizes inorganic materials, these gels recreate the enzyme's natural microenvironment and employ natural polymers, reinforcing its functionality.

The porous architecture of these polysaccharide-based gels further facilitates efficient electron accessibility, fostering effective electron transfer between the immobilized enzyme and the biosensor's electrode [14].

Kueng et al. [15] developed a biosensor wherein enzyme immobilization was achieved through enzyme encapsulation using the Canguard polymer. The primary objective of this biosensor was to detect adenosine-5′-triphosphate (ATP) at physiologically relevant pH levels. In this approach, GOx and hexokinase (HEX) enzymes were co-immobilized within Canguard polymer films on platinum disk electrodes. The encapsulation process employed pH-shift induced deposition, leading to the selective immobilization of enzymes while preserving stability and enzyme bioactivity. The biosensor's functionality relied on competitive enzymatic reactions between GOD and HEX for the glucose substrate, with ATP acting as a co-substrate. This enzyme encapsulation method, utilizing the Canguard polymer, significantly improved sensitivity, response time, reproducibility, and ease of fabrication for ATP detection.

#### 2.2.1.3 Electropolymerization

Electropolymerization presents another immobilization approach, where enzymes are affixed onto electrode surfaces. This process, as shown in Fig. 2, unfolds as the transducer is immersed in an enzyme-monomer solution, followed by the application of a suitable potential or current. Oxidation of monomers generates radical cations, facilitating the formation of dimers through reactions with other radicals or neutral monomers. These dimers subsequently oxidize at the electrode surface, culminating in the polymerization process. Enzymes situated nearby become incorporated into the expanding polymer network, ensuring their controlled immobilization.

Electropolymerization stands as an efficient strategy for tethering enzymes to electrodes, thereby enabling diverse biotechnological applications [14].

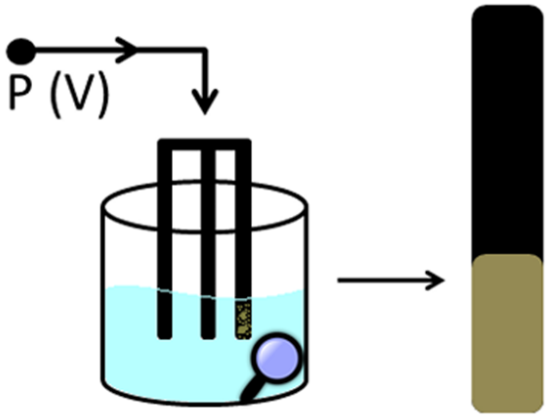


*Fig. 2 Electrodes are submerged in electrolyte containing biomolecule and monomeric molecule is showed. While potential is affecting on the electrodes, polymer entrapping biomolecule is depositing on working electrode surface (adopted from Ref.* [16]).

Pauliukaite et al. [17] constructed a glucose biosensor using electropolymerization. GOx enzymes were immobilized within a conductive polymer film created through electropolymerization of poly(neutral red) (PNR) on a carbon film electrode. The PNR film acted as a redox mediator, aiding electron transfer between GOx and the electrode surface. The enzymatic oxidation of glucose and the resulting generation of $H_2O_2$ allowed sensitive and selective glucose detection. Electropolymerization provided a stable environment for GOx enzymes, enhancing their stability and catalytic activity, promising glucose sensing applications.

### 2.2.2 Adsorption

Adsorption represents a straightforward technique for immobilizing enzymes in biosensors, involving the physical attachment of enzymes to solid supports. This method encompasses the

dissolution of enzymes in a solution, followed by their contact with a solid material for a predefined duration. Enzymes adhere to the solid support through mechanisms like electrostatic attraction, hydrophobic interaction, or van der Waal's forces, as depicted in Fig. 3. This process yields several benefits, including minimal enzyme inactivation, the preservation of enzyme activity, and heightened sensitivity of biosensors [14].

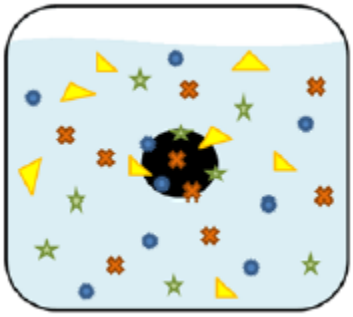

*Fig. 3 Molecules spread in solution are bounded onto surface of adsorbent/transducer by van der Waals forces(adopted from Ref.* [16]).

Soylemez et al. [18] utilized physical adsorption to immobilize cholesterol oxidase (ChOx) onto a polymer-modified graphite electrode, constructing a cholesterol biosensor. The conducting polymer, poly((Z)-4-(4-(9H-carbazol-9-yl) benzylidene)-2-(4-nitrophenyl) oxazol-5(4H)-one) (poly(CBNP)), provided an effective platform for enzyme immobilization, forming hydrogen bonds with the enzyme.

### 2.2.3 Cross-linking

Cross-linking is a well-established method for enzyme immobilization in biosensors, involving the creation of covalent bonds between different regions on the enzyme surface, as shown in Fig. 4. This technique utilizes reagents containing two or more reactive functional groups to form cross-

links within the enzyme (intramolecular), between multiple enzyme molecules (intermolecular), or among subunits of the enzyme (intersubunit). Glutaraldehyde, a widely employed cross-linking agent, is particularly favored due to its cost-effectiveness and easy accessibility in large quantities [19].

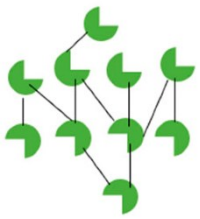

*Fig. 4 Schematic of cross-linking method for enzyme immobilization (adopted from Ref.* [20]).

Chen et al. [21] proposed a biosensor, depicted in Fig. 5, to detect $H_2O_2$-bioprecursors, with glucose as a representative $H_2O_2$-bioprecursor. They enhanced enzyme stability and reusability by immobilizing GOx onto magnetic particles through cross-linking and high-sensitivity CdTe quantum dots (QDs) as fluorescent probes. The GOx-MCLEAs (magnetic cross-linked enzyme aggregates) rapidly released $H_2O_2$ when exposed to glucose, leading to CdTe QDs' fluorescence quenching. This intensity change enabled precise glucose quantification. CdTe QDs were synthesized hydrothermally, while amino-functionalized Fe3O4 nanospheres supported efficient magnetic separation.

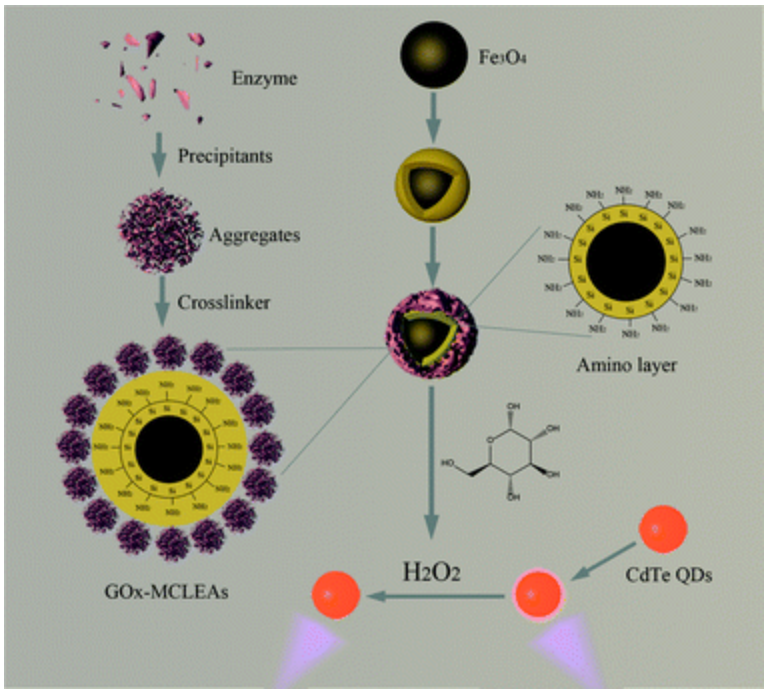


*Fig. 5 The core principle and procedure in the work presented by Chen et al.*[21]

### 2.2.4 Covalent

Covalent bonding is a robust method for creating stable enzyme-support complexes in which enzymes establish covalent bonds with support materials, as shown in Fig. 6, through the utilization of functional groups present in their side chains, such as carboxyl and amino groups, primarily found in amino acids like lysine, arginine, aspartic acid, and histidine. Crucially, these functional groups do not compromise the catalytic activity of enzymes, ensuring that their essential functions remain intact. Covalent enzyme linkage not only fortifies enzyme activity but also imparts it with heightened stability, thermostability, and an extended half-life [20].

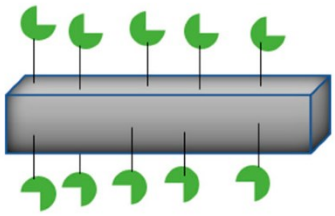

*Fig. 6 Schematic of covalent method for enzyme immobilization (adopted from Ref.* [20]).

Cortina et al. [22] developed a biosensor which utilized interdigitated electrodes and impedance spectroscopy to detect urea levels. Urease was immobilized on an enteric polymer coating, Eudragit S-100, through covalent bonding using carbodiimide coupling. Covalent immobilization enhanced enzyme-polymer interaction, resulting in improved sensor performance. Urea interaction triggered polymer coating degradation, leading to increased capacitance monitored through impedance measurements.

### 2.3 Distinctive substances as platforms for enzyme-based glucose sensors

In the realm of biosensors, enzymatic-based systems have played a pivotal role in reshaping diagnostic methodologies, particularly in glucose detection. This chapter examines substances that serve as platforms for enzyme-based glucose biosensors, each possessing unique characteristics that influence biosensor performance. With a focus on paper, thread, polydimethylsiloxane (PDMS), and gold nanoparticles (AuNPs), we discuss their individual contributions to advancing biosensing technologies.

#### 2.3.1 Paper

Paper-based platforms have gained considerable attention as enzyme-based biosensors due to their cost-effectiveness, disposability, and portability. Microfluidic concepts, notably pioneered by the Whitesides group [23], have further enhanced their utility, capitalizing on the inherent hydrophilicity of paper substrates for precise fluid manipulation and advanced biosensing capabilities. Within this framework, immobilized enzymes, like GOx, facilitate the conversion of target analytes such as glucose into detectable signals. These reactions often involve enzymatic cascades, including enzymes like HRP, which catalyze the oxidation of indicators like potassium iodide (KI) and 3,3',5,5'-tetramethylbenzidine (TMB), leading to color changes or other quantifiable outputs [24]. These advancements underscore the versatility of paper-based platforms, playing a pivotal role in rapid point-of-care diagnostics and settings with limited resources.

Zhang et al. [25] introduced an inkjet printing technique to enhance the analytical capabilities of microfluidic paper-based analytical devices (μPADs) for colorimetric measurements, and the fabrication procedure is shown in Fig. 7. In this method, enzymatic reactions take place with GOx and HRP forming a synergistic enzymatic pair for catalyzing reactions between glucose and TMB, the chromogenic substrate. This approach involved the sequential deposition of chitosan (CHI) – used for enzyme immobilization, TMB, and the GOx and HRP enzymatic mixture onto the sensing zone of chromatography paper. Notably, the incorporation of polyethylene glycol (PEG6000) serves to enhance enzyme stability, thereby enhancing the device's longevity and reliability.

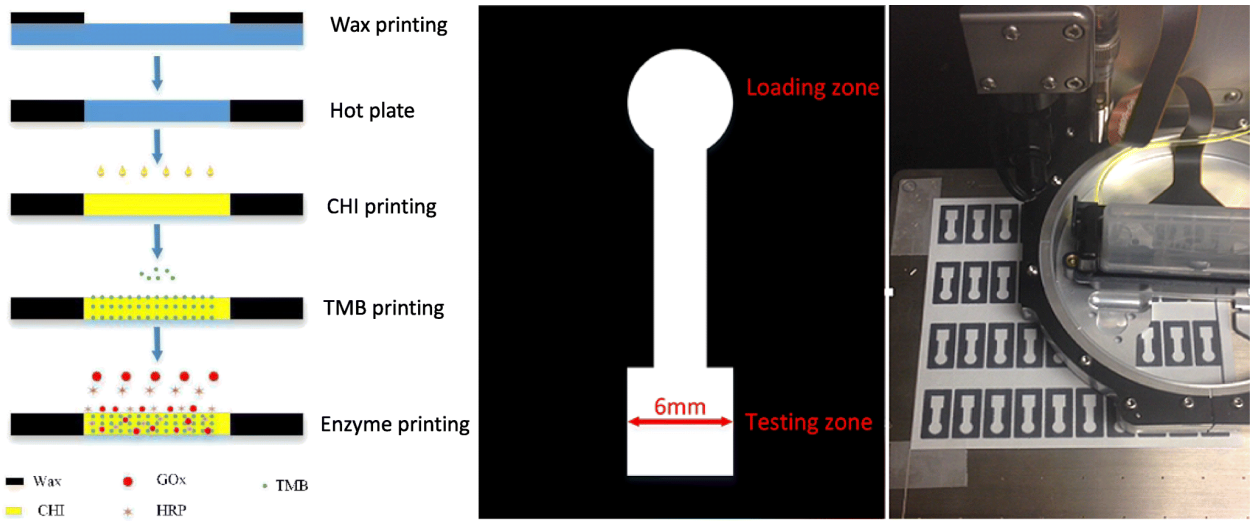


*Fig. 7 The flow chart of the μPAD fabrication procedure (left), schematic diagram of device for glucose detection (middle) and the paper device printing using DMP 2831 material printer (right) (adopted from Ref.* [25]).

### 2.3.2 Thread

Thread-based platforms are recognized for continuous glucose monitoring, leveraging thread's cost-effectiveness, wide availability, and hydrophilic nature for capillary fluid transport. Enzymatic detection is achieved through the incorporation of enzymes onto thread surfaces, enabling the conversion of analytes into detectable signals. Thread's lightweight, hydrophilic, and one-dimensional flow properties facilitate fluid transport without external power sources, and its functionalization versatility and compatibility with common processes like sewing and weaving make it suitable for mass production [26].

Thread-based biosensors emerge as a promising tool for wearable health monitoring, offering continuous and personalized healthcare insights. As Xiao et al. [27] developed a device to integrate a hydrophilic cotton thread as a microchannel to collect sweat and guide it to a paper-based colorimetric sensor for glucose detection, shown in Fig. 8. Plasma treatment was optimized to

enhance the wettability of the cotton thread. A paper-based glucose colorimetric sensor was created by sequentially depositing chitosan, GOx, HRP, and TMB onto filter paper. The hydrophilic cotton thread was then connected across the fabric to link a cotton absorbent patch and the paper-based colorimetric sensor, resulting in the wearable μTPAD, integrated with an arm guard, demonstrating its potential for real-world in situ monitoring of sweat glucose levels.

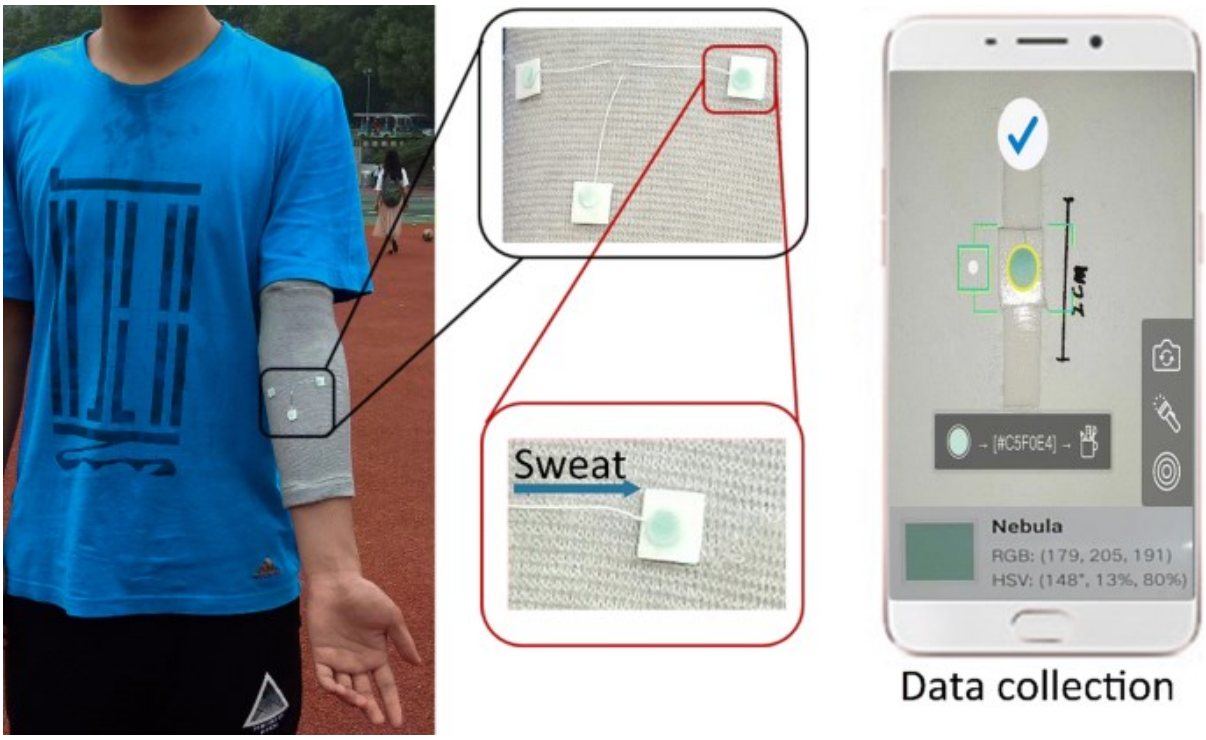


*Fig. 8 A wearable thread-based biosensor for glucose detection in sweat (adopted from Ref.* [25])

### 2.3.3 PDMS

PDMS, a widely utilized elastomeric material due to its exceptional biocompatibility and ease of fabrication, offers a versatile substrate for constructing intricate microchannels and chambers. These microstructures enable controlled fluid flow and facilitate precise interactions with analytes. PDMS-based microfluidic platforms have consistently demonstrated their proficiency in various

biological and chemical assays, making them particularly well-suited for enzyme-based biosensors designed for glucose detection. Notably, enzymes such as GOx and HRP, commonly utilized for glucose detection, can be immobilized within these microchannels, further enhancing the performance and specificity of the biosensor [28].

The work conducted by Pal et al. [29] introduces a PDMS-based biosensor designed for glucose detection. The fabrication process is shown in Fig. 9. This biosensor employs a scalable photolithographic technique, a method that employs light to selectively pattern materials onto a substrate. The conductive polymer PEDOT:PSS, a blend of poly(3,4-ethylenedioxythiophene) and polystyrene sulfonate, serves as both the conductive ink and sensing material. The substrate chosen for this purpose is PDMS, renowned for its biocompatibility and flexibility. The biosensor fabrication process involves patterning PEDOT:PSS onto thin photocurable PDMS membranes through photolithography. This technique results in the creation of high-resolution microelectrodes on the PDMS surface, enabling the accurate detection of glucose across a physiologically relevant concentration range. Within this biosensor, enzyme-loaded sensors (specifically, glucose oxidase) are covalently immobilized onto the PDMS substrate. The strength of this covalent bond ensures the biosensor's stability under mechanical flexure, allowing it to retain functionality even when bent, and when exposed to aqueous environments.

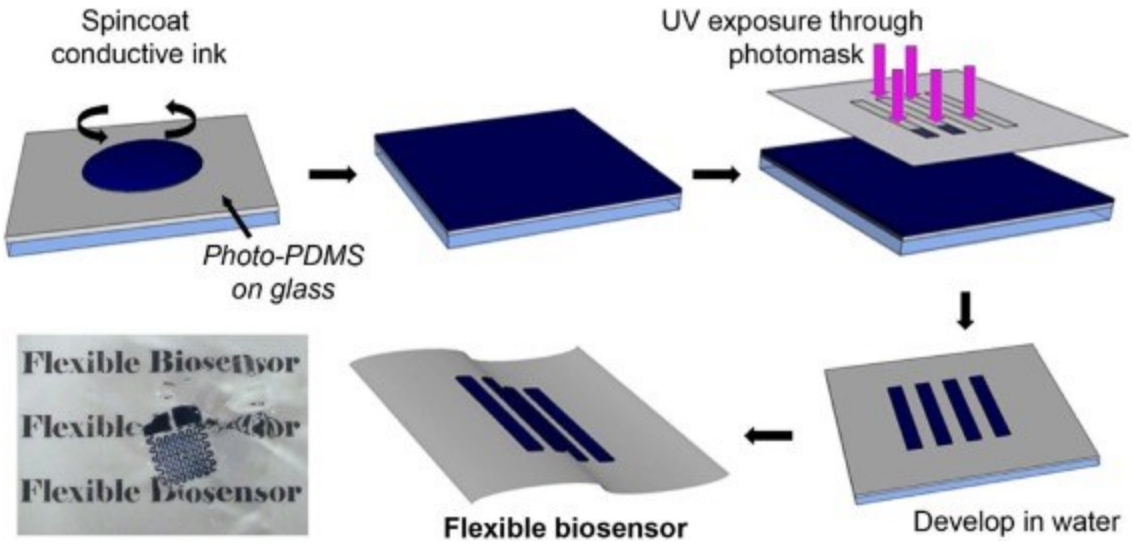


*Fig. 9 Fabrication of the flexible biosensors on PDMS substrates (adopted from Ref.* [25]).

### 2.3.4 Gold Nanoparticles

Gold nanoparticles (GNPs) have emerged as compelling platforms in enzyme-based glucose biosensors owing to their versatile characteristics. With diameters ranging from 1 to 100 nm, GNPs possess a high surface-to-volume ratio and surface energy, facilitating the stable immobilization of biomolecules while preserving their bioactivity. Beyond their biocompatibility, GNPs exhibit unique optical properties, including light-scattering capabilities and the ability to generate a locally enhanced electromagnetic field. These properties make GNPs valuable as signal amplification tags in a diverse array of biosensors. Additionally, GNPs facilitate fast and direct electron transfer, enhancing sensitivity and specificity, thus enabling their predominant use in electrochemical sensing methods [30,31].

The research presented by Şenel [32] introduces a biosensor designed for the sensitive detection of glucose. This biosensor leverages a composite film comprised of chitosan, polypyrrole, and NPs, which collectively create an effective platform for glucose detection. The biosensor operates

based on the principle of electrochemical detection, with GOx serving as the key enzyme for catalyzing the oxidation of glucose to gluconic acid and generating hydrogen peroxide in the process. The immobilization of GOx within the chitosan–polypyrrole–AuNPs matrix, as shown in Fig. 10, ensures the enzyme's stability and bioactivity under challenging conditions. The inclusion of AuNPs within the composite plays a pivotal role, as they not only enhance the conductivity of the biosensor but also offer a favorable environment for enzyme interaction.

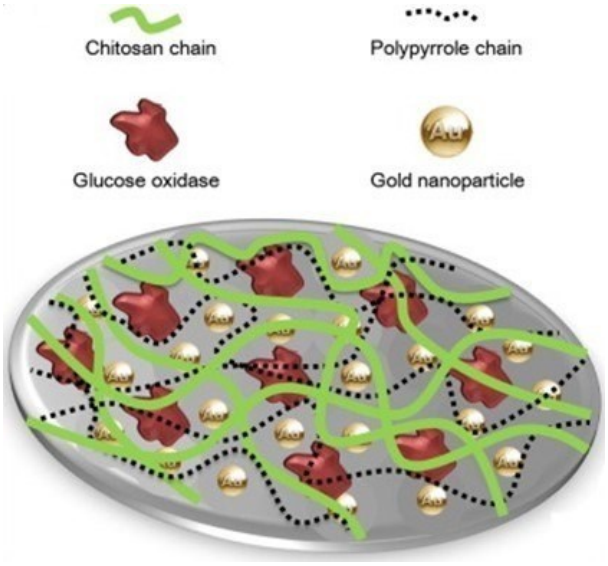


*Fig. 10 Schematization of the surface of GOx immobilized gold electrode (adopted from Ref.* [32]).

### 2.4 Tablet-based bioassays

Tablet-based bioassays were first introduced by Jahanshahi-Anbuhi et al. [33] in 2014 as a novel technique that introduced a remarkable advance in the stabilization of bioassays. This approach centered on the encapsulation of pre-measured quantities of AChE and indoxyl acetate (IDA) within separate tablets, leveraging the versatile properties of pullulan polysaccharide. Departing from conventional compression methods, the team embraced an innovative liquid solution casting approach to craft these distinct tablets. These tablets emerged as versatile tools, enabling a

convenient ready-to-use assay for the detection of malathion, a pesticide, in water, as shown in Fig. 11. A remarkable aspect of this work lay in the application of pullulan encapsulation, effectively extending the shelf life of delicate bioreagents like enzymes from weeks to months. This breakthrough not only ensured the robustness of bioassay components but also underscored the potential for transformative strides in functional bioassays.

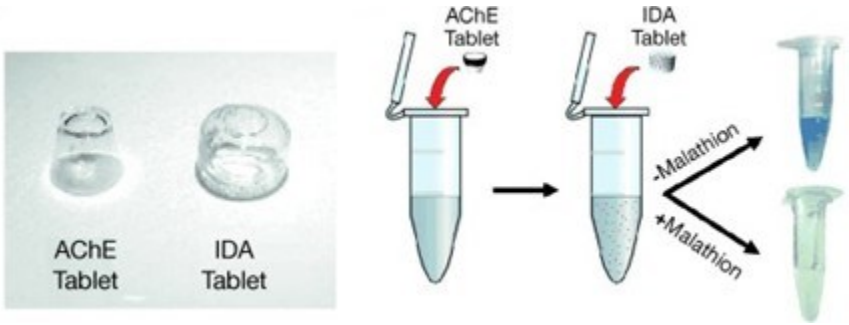


*Fig. 11 A photograph of the AChE and IDA pullulan tablets (left) and operational principal of the assay kit (adopted from Ref.* [33])

Taking inspiration from this pivotal work, Al-Kassawneh et al. [34] introduced a tablet-based sensor platform for the colorimetric detection of glucose in human saliva samples. Leveraging the peroxidase-mimic activity of pullulan-stabilized gold nanoparticles (AuNPs-pTab), the fabrication process involves the synthesis of AuNPs-pSol using pullulan as a versatile reagent, followed by the formation of AuNPs-pTab via casting and subsequent colorimetric detection of glucose using the AuNPs-pTab sensor. The method demonstrates remarkable stability, with AuNPs-pTab outperforming AuNPs-pSol in terms of stability and peroxidase-like activity. The tablet-based biosensor platform holds potential for broader applications in disease detection, making significant strides toward enhancing point-of-care diagnostics through its unique capabilities.

## 2.5 Glucose detection on body fluids

In the realm of biosensors, blood has long reigned as the primary source for glucose monitoring. However, recent research has broadened our scope, prompting exploration into alternative body fluids. Urine, saliva, sweat, and tear emerged as compelling candidates, showcasing the potential for non-invasive glucose detection. The range of glucose detected in healthy patients in various body fluids are mentioned in Table 1. These fluids, characterized by unique compositions and diverse biomarkers, offer valuable prospects for transforming diagnostic methodologies. Through their distinctive attributes and potential applications, these bodily fluids illuminate a path toward innovative healthcare management, driven by patient-centric needs and evolving diagnostic approaches.

*Table 1 Glucose concentration in body fluids of healthy patients*

| Body Fluid | Biomarker | Concentration for Healthy Patients | Ref. |
|---|---|---|---|
| Blood | Glucose | 4.9–6.9 mM | Med. Eng. Phys. 2008 [35] |
| Urine | Glucose | 2.78–5.55 mM | Diagnostics 2014 [36] |
| Saliva | Glucose | 0.23–0.38 mM | J. Diabetes Sci. Technol. 2015 [37] |
| Sweat | Glucose | 0.06–0.11 mM | Sci Adv. 2017 [38] |
| Tear | Glucose | 0.05–0.5 mM | Talanta 2005 [39] |

### 2.5.1 Blood

Blood, a crucial bodily fluid responsible for transporting nutrients, oxygen, and waste products, maintains cellular equilibrium and serves as a valuable source of information for clinical

diagnostics, with blood tests being the gold standard in medical practice for a multitude of applications. While blood-glucose monitoring remains a cornerstone for assessing metabolic health, it's worth noting that invasiveness has been a challenge in fluid extraction. Blood harbors an array of biomarkers, such as glucose, cholesterol, hemoglobin, enzymes, electrolytes, and inflammatory markers, each offering unique insights into various physiological and pathological conditions, empowering early disease detection and management [40].

The current widespread method for self-monitoring entails the commonly used "finger-pricking" technique, which relies on enzymatic principles, involves the extraction of a blood sample from a finger through pricking. The collected blood sample is subsequently subjected to in vitro analysis using test strips and a glucometer [41]. Pioneering the field, Clark and Lyons [9] introduced the first generation of glucose biosensors back in 1962. These initial biosensors adopted an electrochemical framework and utilized GOx. The choice of electrochemical sensors stemmed from their notable sensitivity within the micromolar to millimolar concentration range, coupled with their reproducibility and cost-effectiveness in fabrication. The selection of GOx as the enzymatic catalyst was driven by its distinctive specificity for glucose. In their sensor design, Clark and Lyons achieved the indirect quantification of glucose levels by immobilizing a thin GOx enzyme layer on a platinum electrode, employing a semipermeable dialysis membrane. This foundational sensor concept measured the reduction in oxygen concentration and the concurrent generation of hydrogen peroxide, the quantity of which was proportionate to the glucose concentration.

### 2.5.2 Urine

Urine, a diagnostic fluid that has been utilized for diabetes diagnosis since as early as 1841, has remained a subject of extensive study due to its unique attributes that allow for easy and non-invasive collection. Composed of essential components like glucose, proteins, nitrates, and various dissolved salts including sodium, potassium, phosphate, and sulfate, urine offers a non-invasive glimpse into the body's metabolic status, with creatinine as a notable indicator, all within a fluid matrix that is approximately 95% water. One of urine's notable advantages is its feasibility for large-scale collection with minimal invasiveness. Its relatively lower concentration of proteins, lipids, and other high molecular weight compounds simplifies the preparation process, further enhancing its utility as a valuable biofluid for diagnostic and monitoring purposes [42].

A study conducted by Zhang et al. introduces a self-powered biosensor system embedded within a diaper to detect urine composition, with a specific focus on glucose levels. The system utilizes an enzymatic biofuel cell (EBFC) that captures glucose present in urine to generate electricity. This EBFC, situated in the diaper, connects to a power management system equipped with an energy-storing capacitor. This stored energy drives a flashing light-emitting diode (LED) whose frequency corresponds to the capacitor's charging speed, linked to EBFC-generated power. As a result, the flashing LED serves as an indicator of glucose concentration in the user's urine. This technology holds promise for monitoring diabetes in individuals with urinary incontinence, offering a non-invasive means of managing glucose levels [43].

### 2.5.3 Saliva

Human saliva, this clear and viscid fluid, produced by salivary glands, has gained significant attention as a promising biofluid for non-invasive diagnostic and monitoring purposes. The fluid contains a wealth of biomarkers, such as glucose, lactate, phosphate, enzymes like alpha-amylase

(sAA), hormones such as cortisol and steroids, and antibodies like IgA and IgG, that provide valuable insights into various aspects of our health. This unique composition positions saliva as a rich source of information about our physiological and metabolic state [44].

In a study, García-Carmona et al. [45] introduce a chemical wearable sensor specifically designed for newborn monitoring. This sensor takes the form of a pacifier, utilizing the infant's natural mouth movements to pump saliva into an external electrochemical chamber. This chamber houses a GOx-based enzymatic biosensor on a Prussian Blue (PB) electrode transducer. The sensor's wireless amperometric circuitry, combined with Bluetooth communication, ensures low-power operation and real-time monitoring. The platform's pacifier design provides a baby-friendly, nonintrusive approach to chemical biomarker monitoring.

### 2.5.4 Sweat

Sweat, comprising approximately 99% water and primarily functioning for thermoregulation, presents a valuable source for diagnostic sampling due to its accessibility through eccrine glands dispersed across the body, particularly in hands, feet, and underarms. This fluid holds disease markers like sodium, potassium, calcium, phosphate, and glucose, along with small-molecule drugs and metabolites. Facilitated by close skin contact with sensors, sweat enables swift and uncontaminated processing and serves diagnostic purposes for conditions like cystic fibrosis and offers insights into electrolyte balance, diet, injury, stress, medications, and hydration. Moreover, sweat serves as a non-invasive alternative to blood sampling, containing health-relevant biomarkers including lactate, ascorbic acid, and electrolytes [42].

The wearable biosensor designed by Lee et al. [46] integrates graphene with a gold mesh which enhances electrochemical activity, enables accurate glucose monitoring through sweat analysis.

The sensor is incorporated into a flexible, skin-mounted patch that features multiple components, including sweat control layers, glucose sensors, pH sensors, and tremor sensors. Additionally, the patch includes therapeutic components such as bioresorbable microneedles for controlled transcutaneous drug delivery. These microneedles, coated with a phase-change material, release drugs into the bloodstream when a specified temperature threshold is exceeded. The patch's stretchable design ensures conformal contact with the skin, enabling stable sensing and efficient drug delivery. This wearable biosensor holds great promise for non-invasive glucose monitoring and personalized treatment of diabetes, exemplifying the integration of advanced materials and technology for improved healthcare management.

### 2.5.5 Tear

Tear, containing a variety of biomarkers including glucose, salts, proteins, enzymes, and lipids, is a biofluid that accumulates in the eyes. This unique composition provides insights into ocular conditions and systemic disorders, revealing valuable information such as elevated proline-rich protein 4 levels in dry-eye conditions, altered cancer-related biomarkers in breast cancer patients, and a close correlation between tear and blood glucose concentrations for diabetes management. Tear analysis offers a non-invasive and convenient approach for biomarker monitoring, with potential applications in biosensors that passively collect tears, although challenges remain for larger sample quantities [44].

The research conducted by Gabriel et al. [47] introduces a paper-based colorimetric biosensor designed for quantifying glucose concentrations within human tear samples, which is shown in Fig. 12. The biosensor is constructed using a wax printing technique on paper platforms, with chitosan modification using acetic acid to enhance enzyme attachment. The fundamental principle

of the device relies on a colorimetric reaction involving TMB as the chromogenic reagent. When glucose is present in the tear sample, it triggers an enzymatic reaction that leads to the production of a color change. This color change is proportional to the concentration of glucose, enabling easy visual detection. In comparison to a commercial glucometer, the paper-based colorimetric biosensor demonstrates reliable performance. This advancement could notably improve the management of diabetic patients who require continuous glucose level tracking without the need for repeated finger pricks.

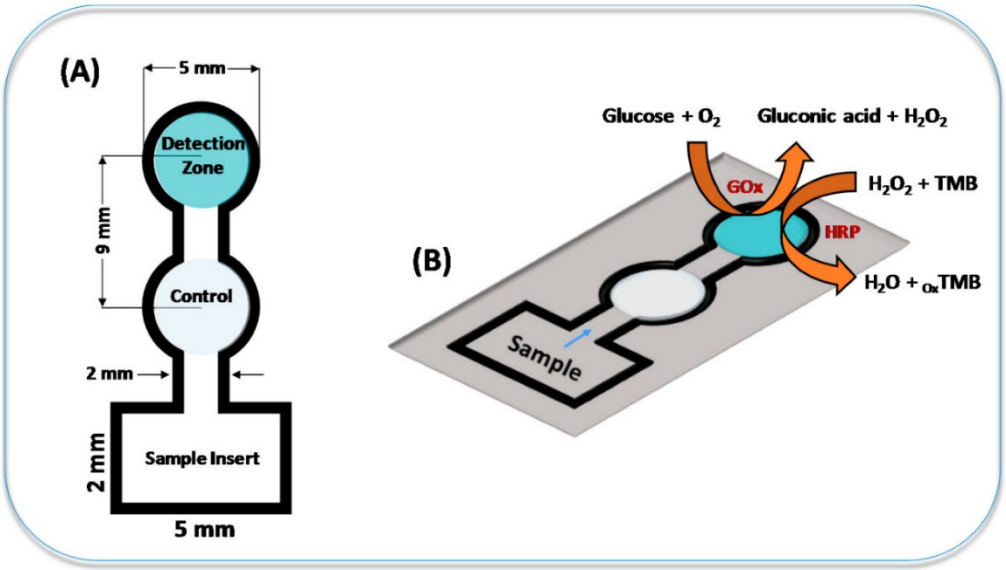


*Fig. 12 Presentation of (A) the layout of µPAD used for glucose colorimetric assays and (B) a simplified view of the enzymatic reaction involved in the presence of chromogenic agent (TMB) for glucose detection (adopted from Ref.* [47])

## 2.6 Enzyme kinetics

Enzyme kinetics plays a pivotal role in advancing biosensors and diagnostic technologies, especially in the realm of glucose detection assays. Understanding the intricacies of enzyme-substrate interactions is fundamental to the precise quantification of glucose levels.

The study of reaction rates, known as kinetics, reveals that in many biochemical reactions, the rate directly correlates with the concentration of the reactant, expressed as:

Velocity = $K_1 \times [A]^1$

In this equation, $K_1$ signifies the first-order rate constant, and [A] represents the concentration of substrate A. However, as reactions progress, there comes a point when the rate becomes independent of substrate concentration, denoted by $K_0$. This marks the realm of zero-order kinetics, occurring when enzymes saturate with substrate. Here, the reaction rate remains constant, unaffected by further increases in substrate concentration.

At the heart of enzyme kinetics, the Michaelis-Menten equation, introduced by Leonor Michaelis and Maude L. Menten in 1913, describes the enzymatic conversion of substrate to product. It operates under the assumption of equilibrium between the enzyme and substrate during catalysis, ensuring that the rate of product formation doesn't disrupt this balance. Mathematically, the Michaelis-Menten equation is expressed as:

$$v = \frac{V_{max} \times [A]}{K_m + [A]}$$

In this equation, $v$ represents the reaction velocity, $V_{max}$ signifies the maximal velocity, $K_m$ denotes the Michaelis constant (representing the substrate concentration at half-maximal velocity), and [A] stands for the substrate concentration. Under specific conditions, enzymes exhibit a maximum

velocity ($V_{max}$) as the substrate concentration increases. Notably, Km is a defining characteristic of every enzyme, generally hovering around physiological substrate concentrations [48].

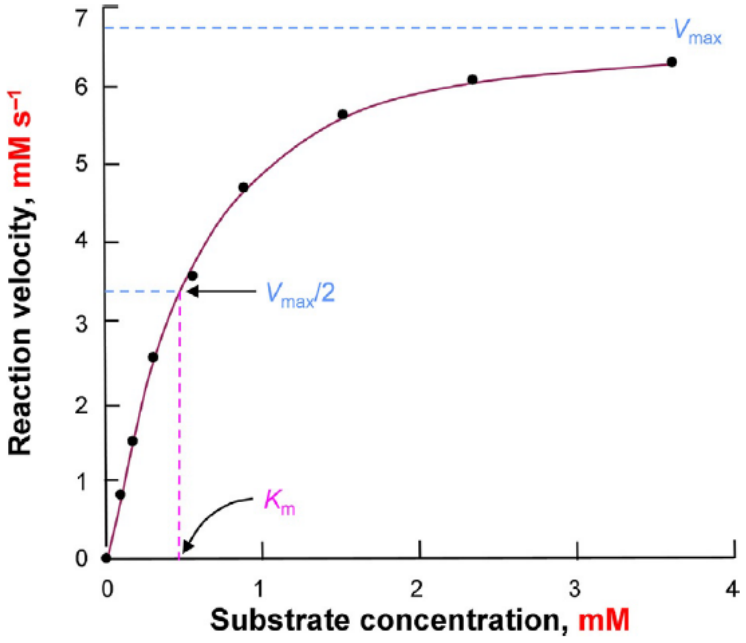


*Fig. 13 The rate, or velocity, of an enzyme-catalyzed reaction as a function of substrate concentration. The curve is a rectangular hyperbola. The $K_m$ is the substrate concentration at half-maximal velocity, which corresponds to $V_{max}/2$ (Adopted from Ref.* [48].*)*

These principles find direct application in glucose detection assays where enzymes like HRP and GOx interact with substrates. Understanding the concepts of Km and Vmax is crucial in these assays, as they provide a quantitative basis for precise glucose quantification. Enzymes operate optimally within specific substrate concentration ranges, a vital consideration in designing accurate and reliable glucose detection systems.

# Chapter 3: Enzyme-based tablet sensor for glucose detection in urine

In this chapter, I present the comprehensive research documented in a recently published manuscript. This chapter begins with a concise abstract, setting the stage for an innovative enzyme-based tablet technology. It subsequently explores tablet preparation, characterization, and practical applications in glucose detection, providing a detailed account of the research process. The chapter concludes with discussions on analytical performance, interference studies, and stability assessments, offering a comprehensive insight into the research outcomes.

## 3.1 Abstract

The colorimetric detection of glucose in urine through enzymatic reactions offers a low-cost and non-invasive method to aid in diabetes management. Nonetheless, the vulnerability of enzymes to environmental conditions, particularly elevated temperatures, and their activity loss pose significant challenges for transportation and storage. In this work, we developed a stable and portable tablet sensor as a user-friendly platform for glucose monitoring. This innovative device encapsulates glucose oxidase and horseradish peroxidase enzymes with dextran, transforming them into solid tablets and ensuring enhanced stability and practicality. The enzymatic tablet-based sensor detected glucose in urine samples within 5 min, using 3,3′,5,5′-tetramethylbenzidine (TMB) as the indicator. The tablet sensor exhibited responsive performance within the clinically relevant range of 0–6 mM glucose, with a limit of detection of 0.013 mM. Furthermore, the tablets detected glucose in spiked real human urine samples, without pre-processing, with high precision. Additionally, with regard to thermal stability, the enzyme tablets better maintained their activity at an elevated temperature as high as 60 °C compared to the solution-phase enzymes,

demonstrating the enhanced stability of the enzymes under harsh conditions. The availability of these stable and portable tablet sensors will greatly ease the transportation and application of glucose sensors, enhancing the accessibility of glucose monitoring, particularly in resource-limited settings.

### 3.2 Introduction

Diabetes is a chronic condition that affects millions of people worldwide. According to the Atlas of the International Diabetes Federation [49], there were approximately 537 million adults diagnosed with diabetes in 2022 worldwide, while the World Health Organization (WHO) [50] has estimated that in low- and middle-income countries, one in two adults has undiagnosed diabetes. While there are various types of diabetes, in all instances, there is an abnormal accumulation of glucose ($C_6H_{12}O_6$) in the bloodstream [51]. If left untreated, diabetes can lead to a variety of dangerous problems such as an increased risk of cardiovascular diseases [52], heart strokes [53], kidney failures [54], blindness [55], and amputations [56]. Therefore, addressing diabetes in the early stages of its development will greatly lower the risks involved while decreasing the future healthcare costs associated with the required treatment. Consequently, there is an increasing demand for non-invasive, user-friendly, and cost-effective glucose monitoring methods to assist in the early detection of this disease, which in turn would provide a chance for mitigating the adverse effects of diabetes [57].

One viable solution to deal with this demand is the use of point-of-care (POC) analytical devices [58]. Recent advances in POC testing have led to innovative solutions for sensitive analyte detection. For instance, Gao et al. [59] introduced a user-friendly POC tool for the on-site analysis of DNA adenine methyltransferase activity. Their approach employs DNA tetrahedra-based

hydrogel to capture glucose-producing enzymes, streamlining target recognition and signal transduction on paper. This integration of DNA hydrogel, enzyme-encapsulated substrates, and a commercial glucose test strip exemplifies the versatility of POC devices. Another recent study by Rauf et al. [60] presented a nanostructured gold-modified Laser-Scribed Graphene electrode system offering a two-fold sensitivity boost over conventional electrodes, making it ideal for POC applications. Their work also yielded a highly sensitive aptasensor for Her-2 biomarker detection, highlighting the potential for rapid and precise POC diagnostics, contributing to the field's evolution.

POC devices are compact, portable, and simple to operate, making them suitable for practical deployment in hospitals, clinics, and in-home use for consistent monitoring of glucose to regulate its level in blood. Currently, the most common POC method for glucose detection is enzymatic detection through electrochemical test strips utilized for measuring glucose level in blood [61]. Nonetheless, taking blood from patients is an invasive process, which presents a significant obstacle to frequent glucose monitoring [62]. For instance, this is a widely acknowledged difficulty in managing childhood-onset diabetes type 1, where youngsters are required to keep a check on their blood sugar levels every time they consume food [63]. In contrast, detecting glucose in urine is a non-invasive approach that can be a suitable alternative or supportive method to estimate glucose levels for diabetes management [64]. Various techniques on different POC platforms have been reported for glucose detection, including colorimetric, electrochemical, and fluorescent on paper-based, thread-based, and other types of microfluidic devices [65–72]. Still, these platforms are prone to limitations such as poor mixing of reagents and samples, non-uniform color formation and decreased precision, and the need for external equipment and cumbersome immobilization steps for the reagents.

To tackle the challenges encountered with other platforms, one possible approach involves adopting enzymatic detection in bulk solutions. Utilizing enzymes offers high sensitivity and specificity for the assays [73], while the solution phase allows for proper mixing and homogeneity and in turn better repeatability and accuracy. However, solution-based enzymatic detection still suffers from drawbacks such as the enzymes denature at higher temperatures and under altered pH conditions, as well as the necessity for specific temperature requirement during transportation that need to be addressed [74]. In this regard, tablet-based detection platform, introduced in 2014, is a viable method that puts together the advantages of solution-based detection method and long-term stability of solid-phase platforms [33]. In this technique, the reagents are encapsulated in polysaccharides such as pullulan and dextran to form solid tablets. The encapsulation of the bioreagents in polysaccharides enhances the stability of enzymes in different external conditions, while the solid tablets act as portable easy-to-use detection platform that can be inserted into the sample medium and readily dissolve to initiate the assay procedure. This approach has been successfully applied to a variety of assays, including the detection of phosphate in water [75] using AuNPs and nanozyme sensors for glucose detection in saliva with pullulan stabilized AuNPs tablets [34]. Among the various materials that have been explored for enzyme encapsulation, dextran emerges as a promising candidate. This is primarily due to its remarkable biocompatibility, biodegradability, and low toxicity [76]. Dextran is a water-soluble polysaccharide composed of glucose molecules linked by α-1,6 and α-1,3 glycosidic bonds and has been widely used in biomedical applications, such as drug delivery and tissue engineering [77].

In this work, for the first time, enzyme tablet bioassay for non-invasive point-of-care detection of glucose in urine . Our method involves encapsulating pre-measured quantities of both glucose oxidase (GOx) and horseradish peroxidase (HRP) in dextran to produce all-in-one enzyme solid

tablets. The tablets are then characterized by Fourier Transform Infrared Spectroscopy (FTIR) and Atomic Force Spectroscopy (AFM). A colorimetric assay based on enzyme tablets is then carried out using 3,3′,5,5′- tetra-methylbenzidine (TMB) as the chromogenic agent, and the analytical performance of the assay is assessed in complex matrices of artificial urine and real human urine. Lastly, the stability of the enzyme tablets was studied for storage at room temperature and through accelerated stress testing conditions at elevated temperatures.

## 3.3 Materials and methods

### 3.3.1 Chemicals and materials

Horseradish peroxidase (HRP, Cat. No. P8250), glucose oxidase from *Aspergillus niger* (GOx, Cat. No. G7141), glucose ($C_6H_{12}O_6$, Cat. No. G8270), 3,3′,5,5′- tetramethylbenzidine (TMB, Cat. No. 860336), sodium phosphate dibasic ($Na_2HPO_4$, Cat. No. S9763), sodium phosphate monobasic monohydrate ($NaH_2$-$PO_4{\cdot}H_2O$, Cat. No. S9638), citric acid ($C_6H_8O_7$, Cat. No. 251275), sodium hydroxide (NaOH, Cat. No. S5881), dextran (average Mw. ~100 kDa), fructose (Cat. No. PHR1002), maltose (Cat. No. PHR1497), trehalose (Cat. No. PHR1344), and HPLC water (Cat. No. 270733) were acquired from Sigma-Aldrich, Canada. Dimethyl sulfoxide (DMSO) (Cat. No. D128–1) was obtained from Fisher Scientific, Canada. To simulate realistic conditions, stabilized artificial urine (Cat. No. BZ104) was carefully sourced from Biochemazone, Canada. A non-stick carbon steel tray (Betty Crocker) was purchased from a local store in Canada. Real urine samples were obtained from a healthy volunteer and were employed without any further treatment. This study was conducted with the agreement of Concordia University's Institutional Review Board with the approval number SC5823 and BioPermit number B-SJA-22–01. Also, written and oral informed consent was obtained from the volunteer.

### 3.3.2 Characterizations

The tablets were characterized using Fourier Transform Infrared Spectroscopy (FTIR) to study the functional groups using Nicolet™ iS20 FTIR Spectrometer (Thermo Scientific Instrument Co., USA). Atomic Force Spectroscopy (AFM) was performed to study the tablet's surface morphology using TOSCA 400 AFM (Anton Paar, Austria).

### 3.3.3 Preparation of enzyme solution and fabrication of enzymes-based tablets

First, the enzyme solution was prepared by dissolving HRP (0.15 mg $mL^{-1}$) and GOx (180 U $mL^{-1}$) in 20 ml of phosphate buffer (0.01 M) with a pH of 7.4. The buffer was prepared by dissolving 0.606 g of sodium phosphate dibasic and 0.101 g of sodium phosphate monobasic monohydrate in 24 mL of deionized water, followed by pH adjustment by the addition of sodium hydroxide and diluting to the final volume of 30 mL by deionized water. The pH was monitored using a pH meter device (AB200 pH/mV/conductivity, Fisher Scientific Accumet, Singapore). For the next step, we prepared a citrate-phosphate buffer solution (0.05 M) with a pH of 5. For this purpose, 0.5445 g of sodium phosphate dibasic (0.2 M) and 0.2882 g of citric acid (0.1 M) were added to 24 mL of deionized water, followed by the addition of sodium hydroxide for pH adjustment and dilution to 30 mL by adding deionized water. Then, a mixture solution containing 0.3 ml of the enzyme solution and 1.5 ml of citrate-phosphate buffer solution was prepared. Next, 6 % w/v of dextran was added to the mixture solution, and it was thoroughly mixed by a vortex mixer (Model# 945FIA-LUS, 50/60 Hz Fisherbrand, USA) to ensure complete dissolution of the chemical compounds. To prepare the solid polysaccharide-encapsulated enzyme tablets, the resulting solution was pipetted onto a carbon steel tray in 60 µL aliquots, per tablet. The aliquots were then allowed to dry on the tray for 24 hours at room temperature, resulting in solid tablets. The drying

process was evaluated by monitoring the weight of the aliquots, which was measured momentarily after pipetting and over 24 hours of air drying as well. The initial weights of the aliquots were determined to be 62.26±0.15 mg which reached the average weight of 5.8±0.21 mg for the formed solid tablets after full drying. The differences in weight between the tablets before and after drying correspond to the amount of water that evaporated during the drying process. This measurement allowed us to keep track of the water evaporation extents in order to ensure the consistency of tablet composition and properties. Fig. 143 represents the fabrication procedure using dextran powder as the polysaccharide encapsulating agent and demonstrates the function of dextran long-chain polymers extending over the enzymes for better preservation and stability.

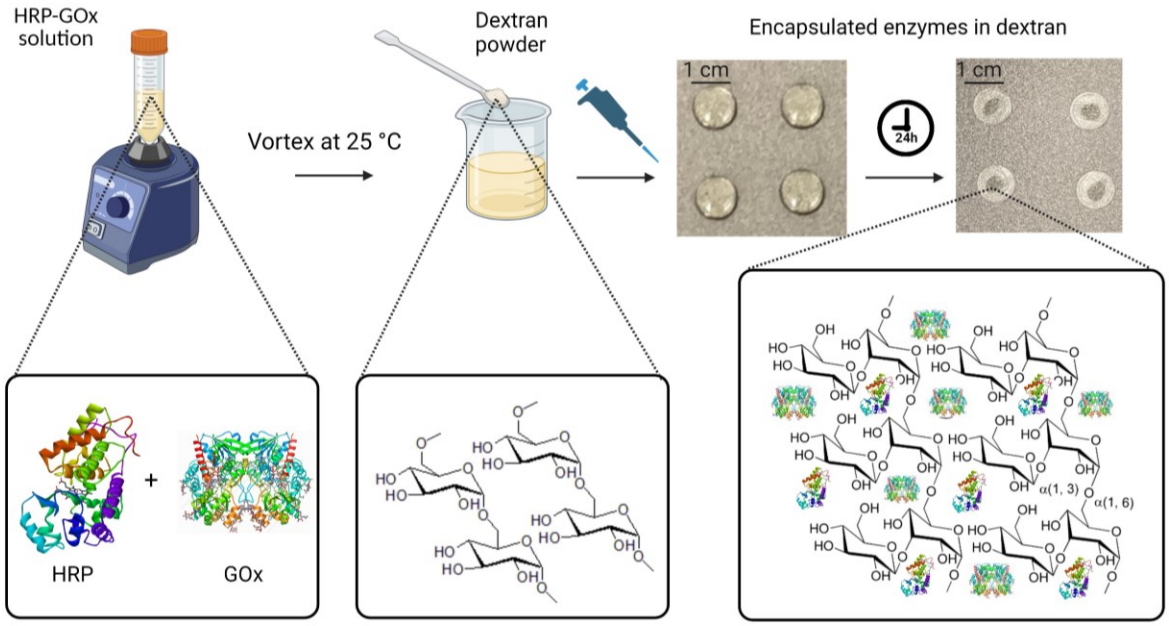


*Fig. 14 Fabrication of enzymes-based tablet sensor using dextran powder as the polysaccharide encapsulating agent. Initially, HRP and GOx were dissolved in phosphate buffer at pH 7.4. Then, 0.3 mL of this enzyme solution was mixed with 1.5 mL of the citrate-phosphate buffer of pH 5. To enable tablet formation, 6% w/v dextran was added to the mixture. In the final step, 60 µL aliquots of the resulting solution were carefully dispensed onto a non-stick tray, which was left to air dry at room temperature for 24 hours, resulting in solid tablets.*

### 3.3.4 Detection of Glucose in artificial urine using dextran-based tablets

To prepare the detection system, 8.4 mg of TMB was dissolved in 1 mL of (DMSO), re-sulting in a TMB concentration of 35 mM in DMSO. The obtained solution was kept in the fridge at 4 °C for further use. Artificial urine samples were then spiked with glucose at varying concentrations ranging from 0.1-5 mM. For running a detection test, in a 2 mL test tube pre-loaded with 5 µL of the TMB solution, 240 µL of phosphate buffer with a pH of 7.4 was added. This buffer serves a dual purpose: it dilutes the urine sample to ensure a suitable sensor response within the desired biologically relevant concentration range of glucose (0-6 mM) and adjusts the pH of the overall system for proper enzyme and TMB functioning. To initiate the colorimetric assay, a single tablet and 30 µL of the spiked arti-ficial urine sample were added to the tube, as shown in Fig. 14. Proper and consistent mixing can be achieved by using a vortex mixer for thorough mixing, allowing for the uni-form distribution of the components. Alternatively, the mixing process can be performed by gentle hand shaking. The final step was to allow the mixture to sit undisturbed for 5 minutes. During this time, the enzymatic reaction took place, leading to the development of a blueish color spectrum, corresponding to different concentrations of glucose. To develop a calibration curve and quantify the observations, the resulting color was analyzed using a UV-vis spectrometer (BioTek, Cytation 5, imaging reader).

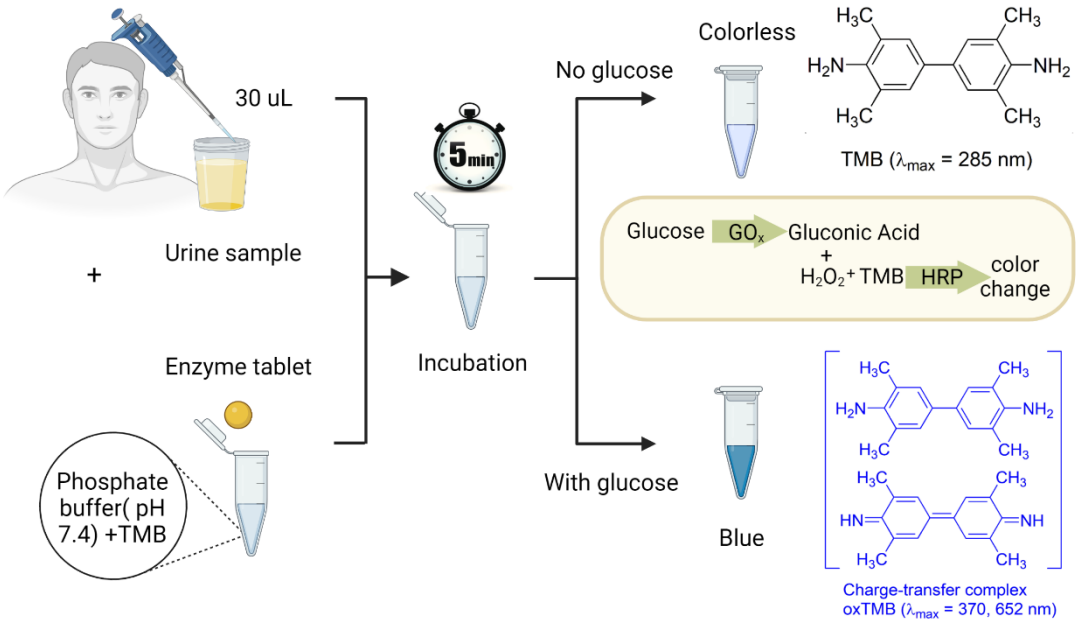


*Fig. 15 The detection procedure of glucose in urine samples. Thirty µL of the urine sample and a single enzyme-tablet are added in a test tube containing 240 µL of phosphate buffer with a pH of 7.4 and 5 µL of the TMB solution. After 5 min incubation, if the urine sample contains glucose, the GOx catalyzes glucose to gluconic acid and $H_2O_2$. Then, the HRP enzyme available in the tablet will act as a peroxidase enzyme which transfers electrons to the TMB substrate, which causes the color of the solution to turn blue due to the oxidation of the chromogenic TMB.*

### 3.3.5 Glucose detection in real human urine

To demonstrate the practical application of our tablet sensor in real-world conditions, the detection of glucose was also conducted in real human urine samples (pH 6.5-7.0). The spiking test was achieved using concentrations of 1, 2, and 3 mM of glucose, and the method was validated by calculating the percent recovery (%R) and relative standard deviation (%RSD) of the results.

### 3.3.6 Interference study for the colorimetric detection of glucose

Potential interferants such as maltose, fructose, and trehalose were studied for our proposed glucose assay following the procedure mentioned in section 2.4. All the interferants were used at 10 mM concentration in artificial urine samples. The UV-vis was used to measure the absorbance intensity of the formed blue color by taking the absorbance at 652 nm on a 96-well plate reader (Ultident Scientific, Canada).

### 3.3.7 Stability tests

To assess the stability of tablet sensors compared to the solution as well as their resistance to heat, both solid and liquid assays were put under accelerated stress testing conditions at 60°C for 24 hours in an oven without forced air convection (Thermo Scientific, Model# PR30525G, USA). Following this thermal exposure, colorimetric detection tests were performed using spiked artificial urine as explained in section 2.4. UV-vis spectroscopy was used to measure the absorbances at 652 nm over 2, 4, 6, and 24 hours, and the results were compared to those of the freshly prepared enzyme tablet and solution assays.

## 3.4 Results and discussion

### 3.4.1 Characterization of tablets

The tablet-based sensors were developed to detect glucose in urine samples through the enzymatic reaction of the tablet components with glucose and chromogenic substrate TMB. First, the enzyme solution was prepared by mixing dextran powder in a buffer containing GOx and HRP enzymes. Next, the solid tablets were created by pipetting the dextran-enzyme solution in the aliquots of 60 µL and air drying at room temperature for 24 hours. The tablets were characterized using FTIR and AFM analyses to study the presence and identify the particular functional groups of the tablets

as well as to examine the physical properties including morphology, surface texture, and roughness which have great impacts in tablet's durability.

In general, enzymes contain specific functional groups that appear in the form of weak peaks or disappear in case of losing efficiency [78,79]. Therefore, the FTIR analyses served as an appropriate method to confirm the presence of the required functional group responsible for the activity of the enzymes in our established enzymes-based tablet sensor. The FTIR analysis of pristine dextran powder indicated a peak at 3362 cm-1 due to the −OH group stretching vibrations, while it was shifted to 3301 cm−1 in the tablet. The bands at 1163, 1183, and 994 $cm^{-1}$ corresponded to the stretching vibrations of C−O bonds, the alcoholic hydroxyl (C−OH), and α-glycosidic bonds (C−O−C) in dextran [80], respectively as shown in Fig. 15A(i). The spectrum of GOx showed bands at 1643 and 1625 $cm^{-1}$ in Fig. 15A(ii) attributed to the amid I and amid II [81]. However, the band was a little shifted with less density in tablets to 1405 and 1592 $cm^{-1}$. Furthermore, similar bands were observed in the spectrum of HRP which indicated peaks between 1563 and 1644 $cm^{-1}$ assigned to the amide I (α-helix structure) [82]. Another observed spectral region of HRP was at 3293 $cm^{-1}$ attributed to O–H vibration modes as presented in Fig. 15(c). The presence of all these bands in the tablet indicated the successful encapsulation of both enzymes into the dextran matrix as shown in Fig. 15(d).

Next, the AFM analyses were performed to analyze the surface morphology of the enzymes and their dextran surroundings in a solid-state format. The results presented in Fig. 15B, C, and D show the dextran matrix containing enzymes had a smooth surface indicated by the phase trace analyses. The phase trace image in the figure revealed that the mean elevation of the tablet was between 2-8 nm, indicating that enzymes were well preserved throughout the exopolysaccharide substance which resulted in a very smooth surface. Furthermore, the surface roughness can be used to

determine how a solid material interacts with the surrounding environment [83]. In the case of our tablet sensor, the average roughness was ~1.1 μm, the average maximum height of the roughness was ~8 μm, the average maximum roughness valley depth was ~4.2 μm, the maximum peak to valley roughness was ~4 μm and the waviness average was ~0.15 μm as presented in Fig. 15. These results of FTIR and AFM analyses confirmed a strong binding connection between the dextran polymer and enzyme mixture in the tablet platform which confirms that the enzymes are well preserved in the dextran structure prolonging the shelf life, stability, effectivity, and performance of these tablet-based sensors.

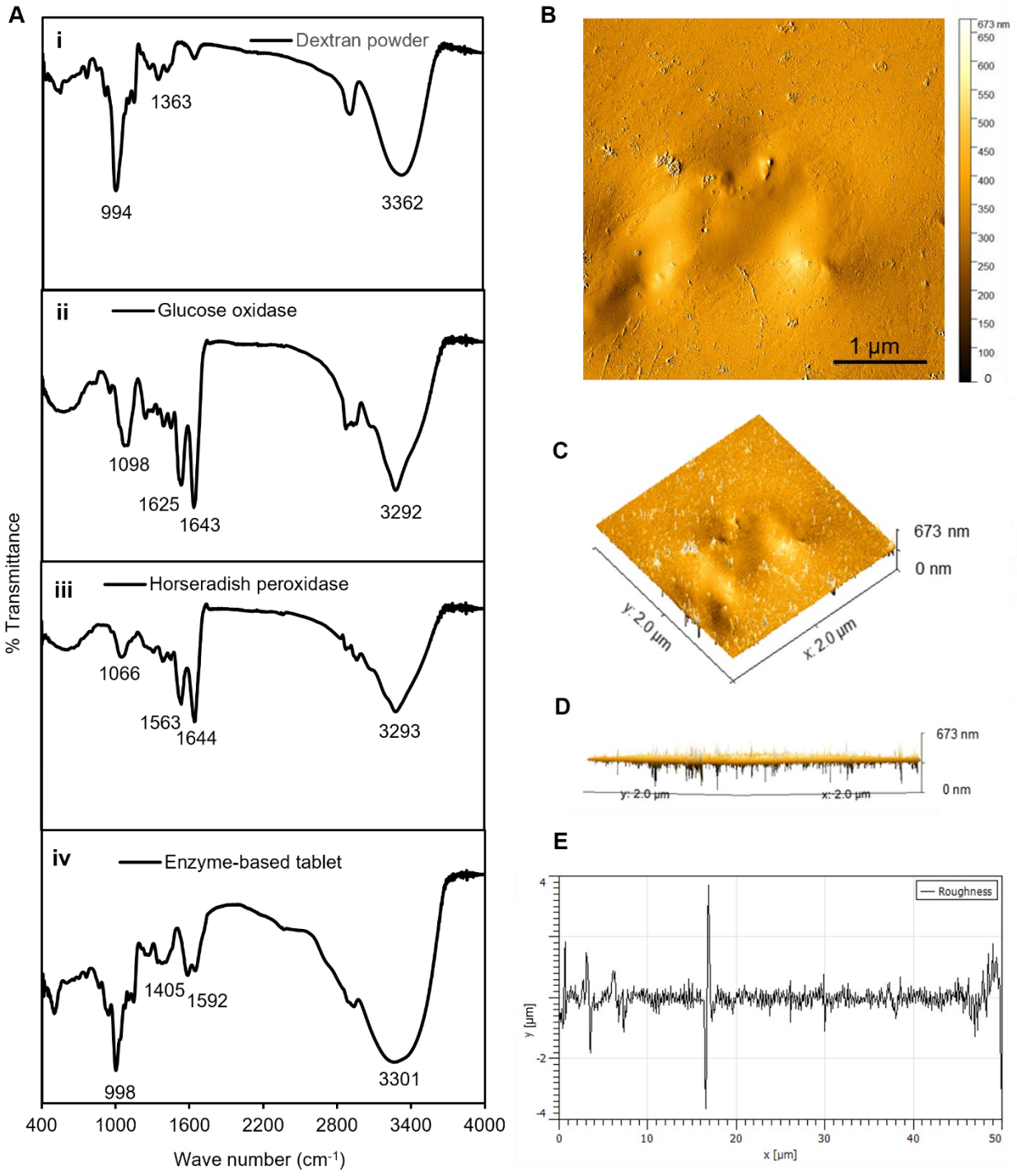


*Fig. 16 Characterization of tablets. A) The FTIR analyses of (i) dextran powder, (ii) GOx, (iii) HRP, and (iv) solid tablets. B) The AFM analyses of tablets demonstrating the 2D image of the height profile in an amplitude trace; C and D) 3D image of the height profile of B; E) Height distribution as surface*

*roughness and texture description of image B. The average roughness was ~1.1 µm, the average maximum height of the roughness was ~8 µm, the average maximum roughness valley depth was ~4.2 µm, the maximum peak to valley roughness was ~4 µm, and the waviness average was ~0.15 µm. The FTIR and AFM results confirmed a strong binding connection between the dextran polymer and enzyme mixture in the tablet platform.*

### 3.4.2 Optimization of the experimental conditions

Before proceeding with the colorimetric detection assay, it is crucial to optimize various experimental conditions to ensure the most accurate and sensitive results. In this section, we describe the optimization of five key parameters: TMB concentration, sample volume, enzyme concentration in tablets, pH of the system, and dextran concentration. Each parameter played a critical role in the assay's performance and deviations from the optimal values led to compromised accuracy or reduced sensitivity.

The TMB concentration played a crucial role in the colorimetric detection assay as it directly influenced the formation of the blue-colored product through oxidation by HRP in the presence of $H_2O_2$. Excessive TMB concentrations led to over-oxidation resulting in black-blue color [84], causing the signal to saturate and give false readings, while a low TMB concentration resulted in a weak signal and decreased assay sensitivity. To determine the ideal TMB concentration, we conducted experiments using a range of TMB concentrations from 5 to 40 mM dissolved in DMSO. The sample utilized in these experiments consisted of spiked artificial urine with a 2 mM of glucose, with a total working solution containing 60 µL of the sample, 200 µL of phosphate buffer pH 7.4, and one tablet sensor. Our investigations revealed that a TMB concentration of 35 mM provided the best blue color, as presented in Fig. 16A, indicating its suitability for use in the assay.

Next, the amount of sample volume in the assay protocol was optimized, presented in Fig. 16B. We observed that low sample volume (10 µL) resulted in low color intensity, and thus less sensitivity, while high sample volumes (60 and 90 µL) resulted in color saturation and compromised the working range of the assay. We found that a sample volume of 30 µL produced the most intense blue color, when using one tablet sensor and adding 5 mM of glucose to 200 µL of phosphate buffer pH 7.4 and 5 µL of TMB at a concentration of 35 mM, indicating its suitability for use in the assay.

Optimization of the enzyme ratios was also performed using a sample of water spiked with glucose (5 mM), totaling 60 µL, along with 200 µL of phosphate buffer pH 7.4, 5 µL of TMB at a concentration of 35 mM, and one tablet. Our investigation revealed that a ratio of 5 µL of 0.15 mg $mL^{-1}$ HRP to 5 µL of 180 U $mL^{-1}$ GOx in 50 µL of 0.05 M citrate-phosphate buffer (pH 5) produced the desired blue color, as shown in Fig. 16C and Fig. 16D, without excess TMB accumulation (black-blue color) or incomplete oxidation (faint-blue color).

Next, we optimized the pH of the reaction mixture since it showed a significant impact on the rate of TMB oxidation by HRP, and in turn assay results. In our experiments, we utilized a sample consisting of water spiked with 5 mM glucose, with a total volume of 60 µL of the sample, 200 µL of phosphate buffer, 5 µL of TMB at a concentration of 35 mM, and one tablet sensor. Our findings indicated that the optimal pH for the HRP/TMB system was 7.4, as pH values outside of this range resulted in weaker signals or undesirable color changes in the final product (Fig. 16E). Therefore, a significant decline in the effectiveness of producing blue colored products occurs when the pH of the samples reduces or exceeds 7.4 due to the comparatively elevated redox potential of the substrate system, which leads to a decreased vulnerability to oxidation [85].

The concentration of dextran, utilized as an encapsulating agent in the fabrication of tablets, is a crucial factor that influences the preservation and performance of the enzymes. In this optimization study, different concentrations of dextran (2, 4, 6, and 8 %w/v) were tested in solution and tablet formats. Our findings revealed that a dextran concentration of 6% w/v provided the optimal conditions for preserving the enzymatic activity, resulting in the desired blue color development as depicted in Fig. 16F. However, lower concentrations of dextran (2% and 4% w/v) failed to adequately preserve the enzymes, leading to reduced enzymatic activity and compromised colorimetric detection performance. On the other hand, while a dextran concentration of 8% w/v successfully preserved the enzymatic activity, it caused the solution to become excessively thick and viscous. This increased viscosity made it challenging to handle the solution during the pipetting and drying process, affecting the uniform distribution of the solution, and impeding the subsequent drying process. Similar results were observed for the solution format without drying to create tablets when the same concentrations of dextran were used.

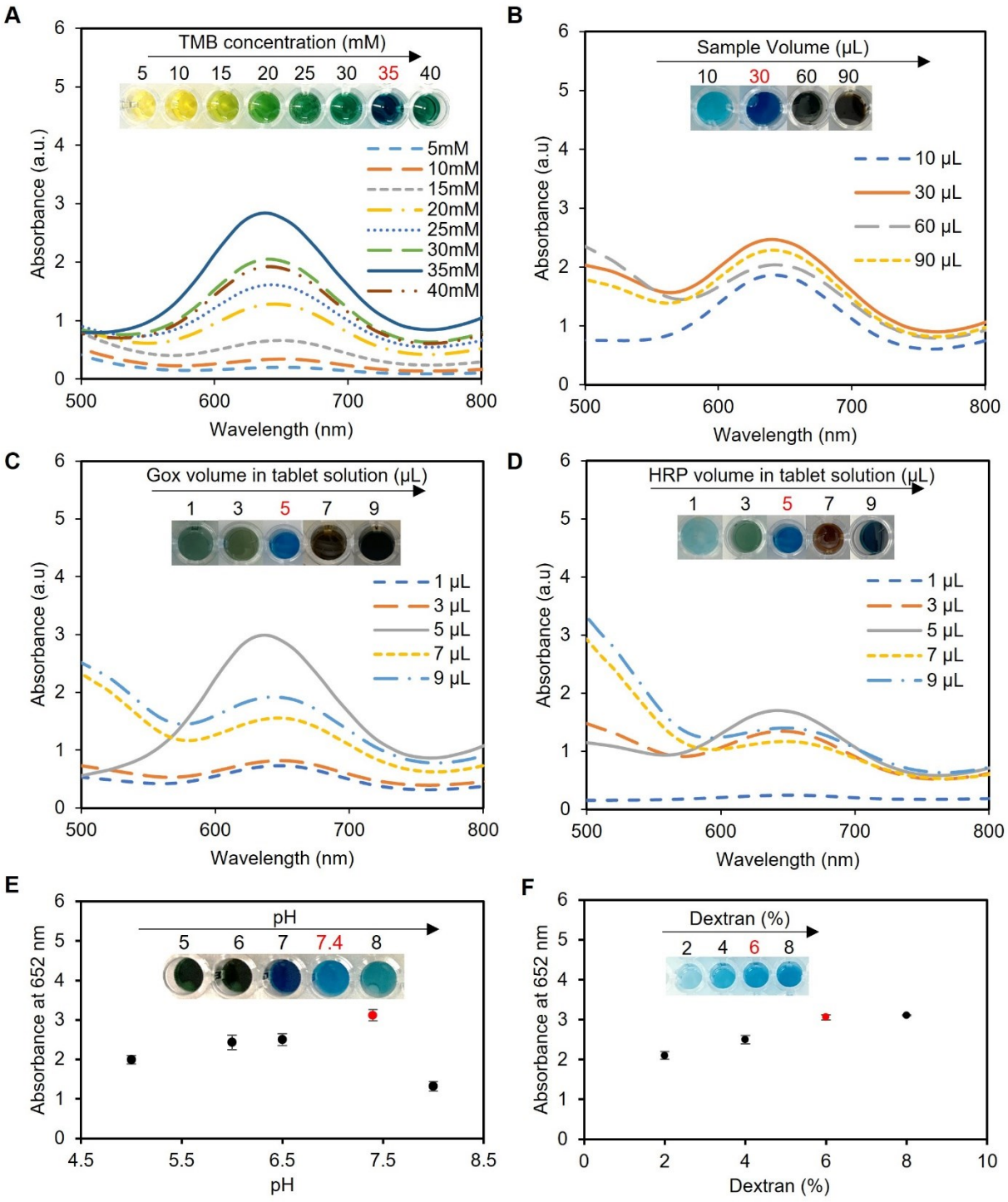


*Fig. 17 Optimization of the experimental conditions for glucose detection using enzymes-based tablets. A) optimization of the TMB concentration using various concentrations of TMB (5-40 mM). The results indicated that 35 mM of TMB was the best to give the desired blue color, while concentrations from 5-15 mM gave a yellow color, a greenish color was obtained in the concentrations between 20-30 mM, and a*

*very dark blue was observed using 40 mM of TMB. B) The effect of the sample volume in the blue color development. A sample volume of 30 µL produced the most intense blue color. C and D) Optimization of enzyme concentration in tablets. The enzyme ratio was carefully optimized using 5 µL of HRP and 5 µL of GOx in 50 µL of citrate-phosphate buffer (pH 5) to prepare the solution of one tablet. This ratio achieved the desired blue color without excess accumulation or incomplete oxidation of TMB. E) pH optimization of the system. The pH of the reaction mixture was investigated, and a pH of 7.4 was identified as the optimal condition for the system. F) Optimization of dextran concentration for better enzyme encapsulation. The results indicated that a 6% w/v concentration effectively preserved enzymatic activity and led to the desired blue color development. Lower concentrations (2% and 4% w/v) compromised enzymatic activity, while an 8% w/v concentration increased viscosity, making the solution challenging to handle during pipetting and drying processes. Therefore 6% dextran w/v solution was used to create the tablet sensor and used throughout the study.*

Next, we performed a kinetic study with different concentrations of glucose (0.5, 1, and 2 mM) to evaluate the optimum time for reading the results. The time course of light absorbance was measured for three samples spiked with glucose using a UV-vis spectrometer. The absorbance at 652 nm was measured at 1-minute intervals over a total time of 20 minutes. After 5 minutes of reagents incubation, there were no significant changes in the absorbance of the samples, indicating that the sensor response had stabilized, and reached saturation as presented in Fig. 17. Therefore, 5 minutes was chosen as the optimum time to measure the readings of the assay after adding the tablet sensor to the samples.

Overall, the best experimental conditions to show superlative tablet sensor performance with higher blue color formations were in pH 7.4 using 35 mM TMB, 30 µL sample volume, 5 µL of 180 U $mL^{-1}$ GOx, 5 µL of 0.15 mg $mL^{-1}$ HRP, and 6% dextran (w/v) with result reading after 5 min. Therefore, these conditions were used in the following tests to detect glucose in artificial and human urine samples.

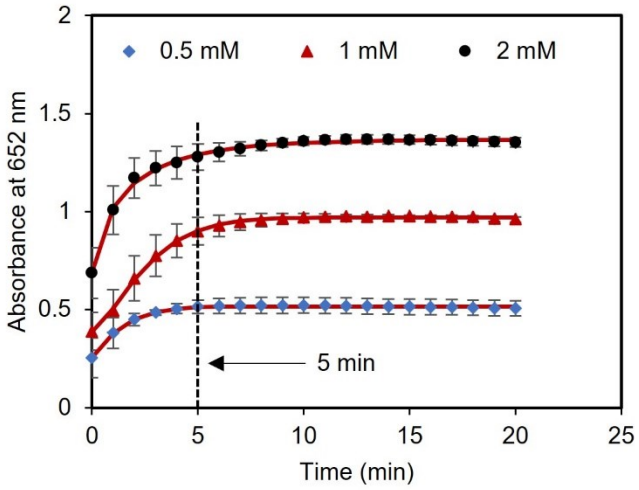


*Fig. 18 Kinetic analysis using tablets. The time duration for glucose detection using concentrations of 0.5, 1, and 2 mM was found to be 5 minutes.*

### 3.4.3 Analytical performance

The analytical performance of the enzyme-tablet colorimetric detection system was evaluated by generating a calibration curve by adding tablets to the samples of known concentrations of spiked artificial urine containing glucose. This approach allowed us to measure the correlation between glucose concentration and absorbance at 652 nm, which is proportional to the amount of glucose present in the sample. The Michaelis-Menten equation was used to plot the relationship between glucose concentration in the samples and absorbance, which resulted in a calibration curve with an $R^2$ value of 0.9899 as presented in Fig. 18A. This value indicates a strong correlation between glucose concentration and absorbance and suggests that our system's results fit well with a typical saturation model. Also, the working range of our detection system was found to be 0-6 mM in artificial human urine samples, which is a clinically relevant range of glucose concentration found

in human urine [86]. Furthermore, we determined the limit of detection for our system using the formula $3\sigma/\mu$, where $\sigma$ and $\mu$ represent the relative standard deviation and slope of the linear calibration plot, respectively. Our system was found to have a limit of detection of 0.013 mM, which is a highly sensitive detection threshold compared to other reported results in the literature [87–89]. Thus, our system can detect glucose at very low concentrations, which expands the sensor's applications in clinical settings.

To evaluate the practical suitability of our suggested tablet sensor, glucose detection was also accomplished in human urine samples. Three human samples were collected and used as is without any previous treatments. The samples were spiked with 1, 2, and 3 mM glucose, and the tests were measured at 652 nm using UV-vis spectroscopy using three replicas as presented in Fig. 18A with blue triangles. The validity of our tablet sensor was confirmed by percent recovery (%R) and percent relative standard deviation (%RSD) values for these samples. The %R provides an estimation of the proportion of the original substance that's retrieved in a solution while %RSD helps to analyze the precision of the method by calculating the dispersion of values around the mean. The %R was found to be 81±2.6, 91±1.5, and 98±2.2% while %RSD was found to be less than 3% for all replicas. These calculated %R and %RSD of spiked samples showed the applicability of our sensor for glucose detection in human urine for real-world applications.

### 3.4.4 Interference study tests

After assessing the analytical performance of our detection system, to understand whether other sugar compounds interfere with our glucose detection system, we investigated the impact of common sugars such as fructose, maltose, and trehalose, on the enzymatic assay of the spiked artificial urine samples. To assess potential interference, we mixed one tablet sensor with 20 µL

of 10 mM of fructose, maltose, and trehalose, in 240 µL of phosphate buffer pH 7.4. The results revealed that no color was produced for all three sugars, while the system turned blue in the presence of glucose (Fig. 18B). This is an important finding as the presence of interfering substances in biological samples can lead to inaccurate results. Therefore, the ability of the developed enzymatic assay to work with high specificity towards glucose is promising for its potential application in the detection of glucose in urine samples.

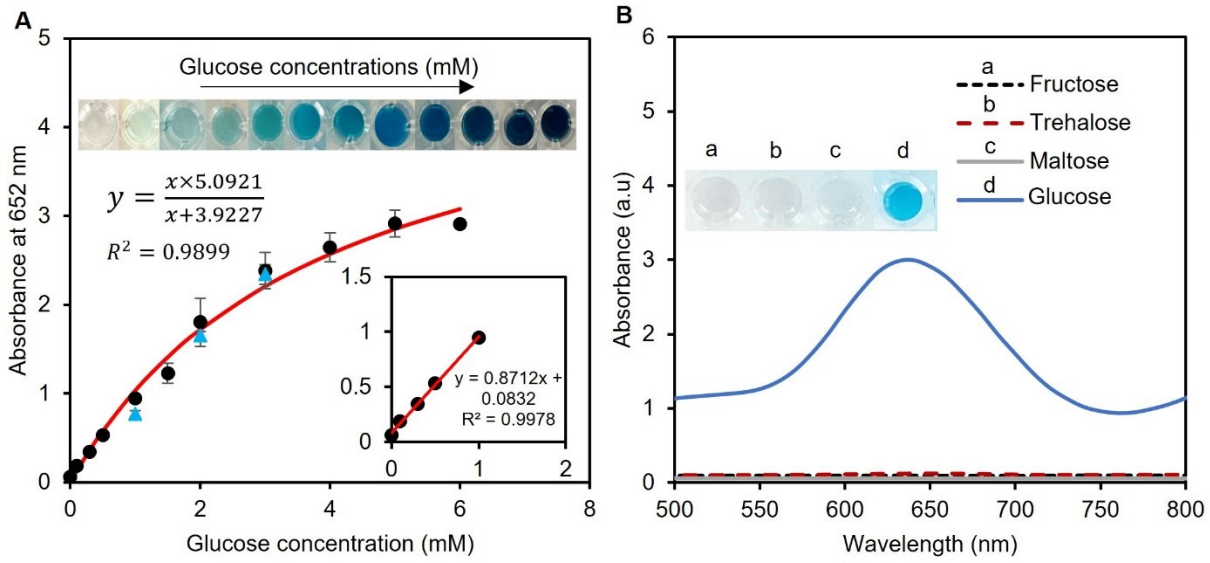


*Fig. 19 Analytical performance of the assay and interference study. A) Glucose concentrations between (0.1-6) mM were used to create the calibration curve. The curve was found to follow the Michalis-Menten equation with an $R^2 > 0.98$, showing the compatibility of the data with a standard saturation model. The inset shows the linear range of the detection system between 0 to1 mM. Real human urine samples were also tested with spiked glucose concentrations of 1, 2, and 3 mM (blue triangle points). Each data point is the mean ± standard deviation of the replications (n = 3). B) The potential interferants with glucose were tested using 10 mM of fructose, trehalose, and maltose. While the working solution remained colorless for all three sugars, a glucose sample of 0-6 mM was able to turn the color of the solution into blue indicating the sensor specificity towards glucose detection.*

### 3.4.5 Stability tests

To assess the stability of the tablets and resistance to elevated temperatures, the performance of the tablets was compared with the solution reagents put under accelerated stress testing conditions. For this mean, both the solid tablets and the solutions were subjected to a temperature of 60°C for a duration of 24 hours, followed by colorimetric detection tests using 1.5 mM glucose in spiked artificial urine. The results revealed a significant difference in enzyme activity between the solution and the tablets after the thermal exposure procedure. The solution exhibited a considerable loss of 86% enzymatic activity, whereas the tablets experienced a lower activity loss of only 41% in 24 hours as presented in Fig. 19. To further evaluate the stability of the tablets, we conducted storage tests at room temperature. The results revealed that the tablets retained 90% and 78% of their activity after two and four weeks, respectively. These findings demonstrated that the developed tablets suitably maintain their activity for up to one month at room temperature and exhibit superior thermal stability compared to the solution-based platforms. The remarkable stability of these tablets ensures the reliability of the enzymatic assay and facilitates their storage and transportation to remote locations, even by non-experts, in a cost-effective manner.

It is also worth noting that alternative techniques such as freeze-drying, which have the potential to enhance stability, would result in a powdered form. However, powdered form can be more challenging to handle and less user-friendly compared to the solidified tablets, which provide stable reagents in their pre-measured quantities.

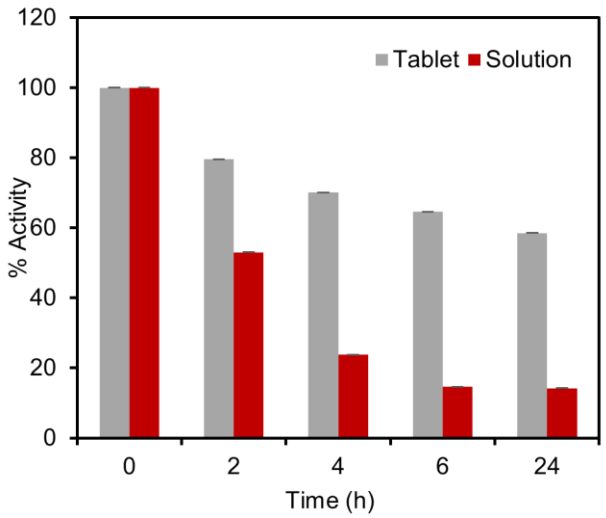


*Fig. 20 Stability tests of the enzymatic tablet-based assay under stress testing conditions at 60 ℃ compared with the similar enzymatic assay stored in the solution phase. The thermal activity test was done by comparing the activity of tablet and solution-based sensors over a duration of 24 hours. The solution exhibited a loss of 86% enzymatic activity, whereas the tablets demonstrated a lower activity loss of only 41%.*

In the upcoming chapter, the extensive research findings presented in this manuscript will be synthesized, offering conclusive insights into the innovative enzyme-based tablet technology developed. Furthermore, recommendations and prospective directions for future research within this domain will be outlined. The aim is to establish a seamless connection between the current findings and the broader landscape of advancements in biosensing technologies, emphasizing their potential implications across diverse fields.

## Chapter 4: Conclusion and future work

- **Conclusion**

This study presents a novel approach for detecting glucose in human urine samples, offering notable advantages over traditional solution-based enzymatic detection methods. The encapsulation of GOx and HRP enzymes using 6%w/v dextran was used to enhance the stability of the enzymes while providing portable solid tablets suitable for point-of-care detection systems. The detection procedure involved inserting the tablet sensor into urine samples, followed by colorimetric analysis after 5 minutes. The encapsulation of all-in-one pre-measured concentrations of enzymes in each tablet simplified the detection procedure and eliminated the need for measuring equipment. The developed tablet system exhibited an LoD of 0.013 mM and a working range of 0-6 mM, covering clinically relevant range of glucose concentrations found in human urine. The assay demonstrated excellent precision with an %RSD value of less than 3% for all samples. The recovery percentage (%R) values of 81±2.6, 91±1.5, and 98±2.2 further validated the assay's accuracy with real human urine samples, without the need for pre-processing steps. The interference study revealed that the developed enzymatic assay has a high specificity towards glucose showing no response for other potential interferent sugars such as maltose, trehalose, and fructose. Stability studies further confirmed that the developed enzymes-based tablet sensors have a higher stability under accelerated stress testing conditions at 60 ℃ compared to solution-based enzymes. This exceptional stability facilitates storage, transportation, and practical applications, particularly outside of laboratory environments.

- **Future work**

As the research has demonstrated, optimizing the detection workflow for end users is crucial for the adva ncement of diagnostic technologies. To continue building upon these findings and

further enhance the user experience, it is worth exploring innovative approaches. One promising avenue for future work is the potential inclusion of other key reagents such as TMB and DMSO into solid tablets. This approach could further simplify the detection process, reducing the reliance on liquid solutions and intricate handling steps. By encapsulating these reagents into solid tablets, we can potentially enhance the portability, stability, and user-friendliness of diagnostic assays. While challenges in formulation and compatibility may arise, the potential benefits in terms of accessibility and efficiency make this an avenue worthy of thorough investigation. Therefore, we encourage researchers in the field to consider this novel direction for future studies, as it holds promise for advancing diagnostic techniques and ultimately improving healthcare outcomes.

# Chapter 5: References


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