



รายงานวิจัยฉบับสมบูรณ์

โครงการ: การพัฒนาระบบรับรู้ทางเคมีไฟฟ้าแบบใหม่สำหรับ
การหาปริมาณวิตามินที่ละลายในไขมันและน้ำ:

ตัวบ่งชี้ชีวภาพที่ปรากฏเพื่อใช้ควบคุมคุณภาพและแสดงความ
เป็นพิษทางคลินิก

รองศาสตราจารย์ ดร. วิณา เสียงเพราะ

ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ

พฤษภาคม 2563



Final Report

**Project: Development of novel electrochemical sensors for
investigation of fat-soluble and water-soluble vitamins:
Emerging biomarkers in clinical toxicity**

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May 2020

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
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การพัฒนากระบวนการรับรู้ทางเคมีไฟฟ้าแบบใหม่สำหรับการหาปริมาณวิตามินที่ละลายในไขมันและน้ำ: ตัวบ่งชี้ชีวภาพที่ปรากฏเพื่อใช้ควบคุมคุณภาพและแสดงความเป็นพิษทางคลินิก

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ระยะเวลาโครงการ

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สารบ่งชี้ชีวภาพหลากหลายชนิดสามารถนำมาใช้ประเมินร่วมทางคลินิกและสามารถบ่งชี้ชนิดของโรคได้ วิตามินเป็นสารบ่งชี้ชีวภาพกลุ่มแรกที่น่าสนใจเนื่องจากภาวะการพร่องวิตามินหรือการได้รับปริมาณวิตามินที่มากเกินไปเป็นปัจจัยหนึ่งที่สามารถบอกถึงโรคที่เกิดจากวิตามินแต่ละชนิดได้ ดังนั้นการติดตามปริมาณวิตามินในร่างการหรือการควบคุมปริมาณวิตามินในอาหารจึงมีความน่าสนใจ เพราะมีผลกระทบต่อสุขภาพของประชาชน โดยโครงการวิจัยนี้ได้มุ่งเป้าการศึกษาไปที่วิตามินทั้ง 2 กลุ่มคือ วิตามินที่ละลายไขมัน (วิตามินเอ ดี อี และเค) และวิตามินที่ละลายในน้ำ (กลุ่มวิตามินบี และวิตามินซี) นอกจากนี้แล้ว ได้ทำการวิจัยเพิ่มเติมเกี่ยวกับสารบ่งชี้ชีวภาพอื่น ที่มีความสำคัญ ได้แก่ โดพามีน และ แคลเซียม ถึงแม้ว่าในช่วงเวลาที่ผ่านมาพบการพัฒนาวิธีการตรวจวัดปริมาณสารบ่งชี้ชีวภาพเหล่านี้อย่างต่อเนื่อง แต่อย่างไรก็ตาม วิธีการตรวจวัดที่ง่าย รวดเร็ว ราคาถูก สามารถตรวจวัดสารปริมาณความเข้มข้นต่ำ ๆ และสามารถเคลื่อนย้ายไปใช้นอกห้องปฏิบัติการได้ ยังคงได้รับความความนิยมอย่างต่อเนื่อง เพื่อลดข้อจำกัดจากวิธีการตรวจวัดแบบดั้งเดิมที่อาศัยเครื่องมือขนาดใหญ่ ราคาแพง ใช้เวลานาน และไม่สามารถนำไปใช้ ณ จุดปฏิบัติการได้ ดังนั้นจุดมุ่งหมายของโครงการวิจัยนี้คือ การพัฒนาวิธีการตรวจวัดแบบใหม่ที่มีประสิทธิภาพเพื่อการตรวจวัดสารบ่งชี้ชีวภาพดังกล่าว โดยงานวิจัยแบ่งเป็นสองส่วน ส่วนแรกคือการพัฒนาวิธีการทางเคมีไฟฟ้าโดยการประดิษฐ์ตัวรับรู้แบบใหม่ที่มีความไวและความจำเพาะเจาะจงต่อการตรวจวัดปริมาณวิตามินและโดพามีน ส่วนที่สองเพื่อพัฒนาระบบรับรู้เชิงสีโดยใช้อนุภาคทองขนาดนาโนเมตรเป็นรีเอเจนต์เพื่อให้มีสภาพไวและความจำเพาะเจาะจงต่อการตรวจวัดปริมาณวิตามินบี 1 และปริมาณแคลเซียม ผลการทดลองชี้ให้เห็นว่าตัวรับรู้แบบใหม่ทางเคมีไฟฟ้าแสดงศักยภาพของการเป็นตัวตรวจวัดทางเลือกใหม่ที่มีประสิทธิภาพสูงและราคาย่อมเยาสำหรับวิธีการตรวจวัดเชิงสี ให้ผลที่มีความน่าเชื่อถือเปรียบเทียบเท่าวิธีมาตรฐาน แต่เป็นวิธีที่ง่าย รวดเร็ว และราคาต่อการวิเคราะห์ถูกกว่า ผลการทดลองที่ได้จากโครงการวิจัยนี้ เป็นการแสดงถึงโอกาสการสร้างตัวรับรู้แบบใหม่ทางเคมีไฟฟ้าและเชิงสีที่สามารถนำไปใช้งานได้จริงสำหรับการตรวจวัดสารบ่งชี้ชีวภาพ

คำสำคัญ ตัวรับรู้แบบใหม่ทางเคมีไฟฟ้า วิตามินละลายในไขมัน วิตามินละลายในน้ำ ภาวะพร่องวิตามิน สารบ่งชี้ชีวภาพ โดพามีน แคลเซียม อนุภาคทองขนาดนาโนเมตร

Abstract

Project Code: RSA6080060

Project title: Development of novel electrochemical sensors for investigation of fat-soluble and water-soluble vitamins: Emerging biomarkers in clinical toxicity

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There are several biomarkers that are involved in forecasting the clinical outcome of various diseases. Among these, the vitamin deficiency or over dose is one of the most important biomarkers used for investigation of many symptoms that related to their diseases. Therefore, the estimation of nutrient intake and the determination of nutritional status are important to maintain one's health. Two case studies related to fat-soluble (e.g. vitamin D and K) and water-soluble vitamins (e.g. vitamins group of B) are investigated and determined as potential nutritional biomarkers to assess their intakes and their disease status to screen and to determine for vitamin deficiency or effective treatment options as well as the quality control in food and supplements. For another important biomarker considered are dopamine and calcium. Over a period of time, various types of analytical methods have been developed for sensitive detection of numerous vitamins, dopamine and calcium. However, a simple, rapid, low cost and portable measurement at low concentrations is still challenging to solve the drawbacks from the previous works in term of using the complicated and high cost instrument as well as the long analysis time. Herein, the development of novel and sensitive method for determination of biomarkers are divided into 2 parts. First is the development of a sensitive and selective electrochemical sensors for the determination of vitamins and dopamine. Second is the development of gold nanoparticles as a sensitive and selective reagent for the determination of vitamin B1 and calcium. Results obtained demonstrated that the new electrochemical sensors provide a high potential as an alternative analytical tool for a simple and low-cost detection of target analytes in various samples. In addition, the developed colorimetric method using gold nanoparticles for the determination of vitamin B1 and calcium were in good agreement with standard method results and demonstrated that the proposed method could reliably measure vitamin B1 and calcium. Overall, this new alternative approach presents a simple, rapid and inexpensive means for the detection of vitamin B1 and calcium in artificial urine sample.

Keywords: electrochemical sensors, fat-soluble vitamin, water-soluble vitamin, vitamin deficiency, biomarker, dopamine, calcium, gold nanoparticles

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

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1. Duenchay P.; Chailapakul O.; **Siangproh W.**, A Transparency Sheet-Based Colorimetric Device for Simple Determination of Calcium Ions Using Induced Aggregation of Modified Gold Nanoparticles. International Journal of Molecular Sciences, 2019, 20, 2954. (Impact factor 4.183, Quartile 1)
2. Jesadabundit W.; Chaiyo S.; **Siangproh W.**; Chailapakul O., Simple and Cost-Effective Electrochemical Approach for Monitoring of Vitamin K in Green Vegetables. ChemElectroChem, 2019, 7(1), 155. (Impact factor 3.975, Quartile 1)

รายชื่อผลงานที่ตีพิมพ์ (Accepted)

3. Duenchay P.; Kaewjua, K.; Chailapakul O.; **Siangproh W.**, Application of Modifier-free Gold Nanoparticle Colorimetric Sensing for Rapid Screening and Detecting Vitamin B1. New Journal of Chemistry, *accepted*. (Impact factor 3.069, Quartile 1)

รายชื่อผลงานที่ตีพิมพ์ (Submitted)

4. Thangphatthanarunguang J.; Yakoh A.; Laocharoensuk R.; Chotsuwan C.; Chailapakul O.; Siangproh W., High-Efficient of Graphene Nanocomposite: Application to Rapidly Simultaneous Identification and Quantitation of Fat-Soluble Vitamins in Different Matrix Samples. Journal of electroanalytical chemistry, *submitted (under review)* (Impact factor 3.218, Quartile 1)
5. Yomthiangthae P.; Kondo, T.; Chailapakul O.; **Siangproh W.**, The effect of supporting electrolyte for the simultaneous determination of vitamin B₂, vitamin B₆, and vitamin C using a modification-free screen-printed carbon electrode. New Journal of Chemistry, *submitted (under review)*. (Impact factor 3.069, Quartile 1)
6. Khamcharoen W.; Chailapakul O.; **Siangproh W.**, A New Multilayer Microfluidic Paper-based Analytical Platform for Successive Sample Preparation and Detection of Dopamine, Analytical Chimica Acta, *submitted (under review)* (Impact factor 5.256, Quartile 1)

2. การนำผลงานวิจัยไปใช้ประโยชน์

-เชิงพาณิชย์ (มีการนำไปผลิต/ขาย/ก่อให้เกิดรายได้ หรือมีการนำไปประยุกต์ใช้โดย
ภาคธุรกิจ/บุคคลทั่วไป)

ไม่มี

-เชิงนโยบาย (มีการกำหนดนโยบายอิงงานวิจัย/เกิดมาตรการใหม่/เปลี่ยนแปลงระเบียบ
ข้อบังคับหรือวิธีทำงาน)

ไม่มี

-เชิงสาธารณะ (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง)

ไม่มี

-เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)

ผลงานได้รับการตีพิมพ์ในวารสารระดับนานาชาติใน Quartile 1 ถูกนำไปใช้เป็น application
สำหรับการสอนในระดับปริญญาโท และเอก และทางคณะวิทยาศาสตร์ มหาวิทยาลัยศรีนครินทร
วิโรฒ ได้นำส่วนผลงานจากการวิจัยเรื่อง Application of Modifier-free Gold Nanoparticle
Colorimetric Sensing for Rapid Screening and Detecting Vitamin B1 ไปใช้ในการบริการ
วิชาการแก่ชุมชน “โครงการเปิดโลกวิทยาศาสตร์สู่การพัฒนาชุมชนจังหวัดสระแก้วอย่างยั่งยืน
ครั้งที่ 4” ณ โรงเรียนวัฒนานคร อำเภอวัฒนานคร จังหวัด สระแก้ว เพื่อให้เห็นถึงความก้าวหน้า
ทางวิทยาศาสตร์นาโนและการใช้สมาร์ทโฟน เป็นเครื่องมือตรวจวัด ระหว่างวันที่ 16-18
กุมภาพันธ์ 2561

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หนังสือ การจดสิทธิบัตร)

อนุสิทธิบัตร (ยื่นจดไปแล้ว)

ชื่อสิ่งประดิษฐ์ ขั้วไฟฟ้าพิมพ์สกรีนสำหรับการตรวจวิเคราะห์วิตามินชนิดละลายในไขมัน

ผู้ประดิษฐ์

1. ดร.อับดุลฮาดี ยะโกะ
2. ศ.ดร.อรรณณ ชัยลภากุล
3. รศ.ดร.วีณา เสียงเพระ

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A Novel Graphene-Nafion Nanocomposite for Anti-Fouling of Sensors in Highly

Sensitive Determination of Vitamin D

Novel and sensitive detection of fat-soluble vitamin D is particularly important for clinical and pharmaceutical monitoring. In this contribution, an anti-fouling electrode based on graphene/nafion nanocomposite was first introduced for an electroanalysis of these compounds and explored the possibility of multiple usages of the electrode without any usual electrode adsorption restriction. The direct analysis of vitamin D could be successively analyzed with satisfying results even up to 10 repeated usages. Interestingly, a greatly enhanced in a faradaic signal current with respect to the background current (S/B ratio) was clearly achieved. Compared to conventional chromatographic methods, the electrochemical sensor outlined here demonstrated a quicker, easier, and much cheaper approach which enables a continuous monitoring of the total vitamin D in the real sample with good reliability and accuracy. This novel electrode is also promising for a fully determine of all the fat-soluble vitamins (A, D, E, and K) concurrently with no chromatographic separation needed.

Introduction

Vitamin D is one of the lipid-soluble nutrients primarily existed in two forms, vitamin D₂ (ergocalciferol) and vitamin D₃ (cholecalciferol). Vitamin D originates from two sources: endogenous, which is from the dermal synthesis from cholesterol; and exogenous, which is ingested from the diets and supplements.¹ The vital functions of vitamin D are regulating the blood level of calcium and phosphorous and also facilitating normal immune system function.^{2, 3} Insufficient amount of vitamin D can cause abnormal growth and development of bones and teeth (rickets, osteomalacia, osteoporosis), as well as increasing the risk of certain diseases.^{4, 5}

The association of official analytic chemist's international (AOACI) has established official chemical methods for vitamin D analysis based on liquid chromatography: Method 992.26—vitamin D₃ in ready-to-feed milk-based infant formula (AOAC, 2007b), Method 995.05—vitamin D in infant formulas and enteral products (AOAC, 2007a), and Method 2002.05—vitamin D₃ in selected foods (milk and cheese) (AOAC, 2007d). Likewise, several approaches involving liquid chromatography/mass spectrometry has been extensively reported and developed by many researchers.⁶⁻¹⁵ Unfortunately, these

previously reported methods suffered from the drawbacks of the tedious procedure, complex instrument, long analysis time, and the requirement for a skilled analyst. Alternatively, electrochemical detection is a promising method to circumvent those inherent shortcomings owing to their remarkable sensitivity and accuracy, experimental simplicity, fast response time, and compact setup. So far, electrochemical detection of the vitamin D has been rarely explored on account of the lipid-soluble nature of the vitamin which needs to perform in non-aqueous media. Besides, one common issue with vitamin D is that it undergoes adsorption during the oxidation steps, which blocks the surface and yields in an electrode fouling.¹⁶⁻¹⁹ This phenomenon can limit the repeated usage of the electrode, especially in continuous monitoring applications. Therefore, for a long-running electrode, electrode modification is a crucial step to eliminate or minimize this effect.

Several means have been reported to prevent the adsorption on the electrode surface for electrochemical sensing application. Electrode modification with some additives as an antifouling agent is also gaining attraction thanks to their excellent character. Poly(3,4-ethylenedioxythiophene) (PEDOT), for instance, is widely applied to the electrode through the electropolymerization methods.²⁰⁻²³ However, this material demands tight process control during polymerization procedure. Changes in the parameters can lead to widely different final results.²⁴⁻²⁶ Furthermore, nafion, a hydrophobic Teflon-like backbone with hydrophilic perfluoroether side chains bearing terminal sulfonic acid groups, is a long-standing goal in community for the development of various electrochemical sensors. Nafion can exhibit not only excellent properties of chemical inertness, catalytic activity, and high permeability to cation but also the antifouling capability.²⁷⁻²⁹ Modification with nafion offers experimental simplicity and eliminates prolonged additional steps, making it still a very attractive material in the electrochemical view.

Lately, integration of nanomaterials and nafion for preparing hybrid nanocomposites have gained popularity as they can synergistic enhance the performance of the electrochemical sensor. Graphene, the well-publicized and prominent allotrope of carbon, is notably one of the most powerful nanomaterials and widely used in the development of hybrids materials. Many graphene/nafion based electrochemical sensors reported that nafion could prevent electrode fouling and served as an effective dispersing agent to exfoliate the graphene, avoiding them from aggregation.³⁰⁻³² Due to its extraordinary electron transfer ability of graphene incorporated with the excellent

properties of nafion as mentioned earlier, graphene/nafion hybrid nanocomposite can hold a great promise for electrochemical applications.

Herein, we describe an antifouling strategy for highly sensitive determination of fat-soluble vitamin D based on graphene/nafion nanocomposite. In this paper, graphene based screen-printable ink was used to fabricate graphene/nafion nanocomposite with good reproducibility. Besides the sensitivity improvement, a graphene/nafion nanocomposite exhibited a lower background current in comparison with carbon and graphene screen-printed electrode (SPE), which resulted in a larger signal-to-background (S/B) ratio. Eventually, under the synergistic effect of graphene and nafion, a sensitive, reusable, and simple electrochemical sensor was developed for evaluating the total level of vitamin D. Diverse pharmaceutical drugs and dietary supplements were analyzed, which results obtained from the developed sensor were in good consistency with the LC-MS/MS method.

Experimental

Materials

Fat-soluble vitamins (cholecalciferol (vitamin D₃), ergocalciferol (vitamin D₂), retinyl acetate (vitamin A), DL- α -tocopherol acetate (vitamin E) and phylloquinone (vitamin K₁)), thiamine (vitamin B₁), riboflavin (vitamin B₂), nafion 117 (5% in lower alcohol), potassium ferricyanide (K₃Fe(CN)₆), sodium chloride, and potassium dihydrogen phosphate were acquired from Sigma-Aldrich (St. Louis, MO). Potassium chloride, disodium hydrogen phosphate, and ethanol were acquired from Merck (Billerica, MA). Sodium perchlorate monohydrate and tetrabutylammonium hexafluorophosphate were acquired from Fluka Chemika (Buchs, Switzerland). Potassium sulfate, calcium sulfate, and zinc nitrate were acquired from Ajax finechem (New South Wales, Australia). All chemicals were analytical grade and used as received, and all solutions were prepared by using purified water (18.2 M Ω cm) from a Milli-Q Millipore water purification system.

Transmission electron microscopy (TEM) images were recorded by an H-7650 transmission electron microscope (Hitachi model, Japan). Surface morphologies were studied using a JSM-7610F field emission scanning electron microscope (FESEM) (JEOL Ltd., Japan).

Electrode fabrication

A wax printing technique was used to define a hydrophobic barrier. Adobe illustrator CC was used to create the device design. The wax pattern was printed onto

inkjet printable PVC sheet using a wax printer (Xerox Color Qube 8570, Japan). For an electrochemical measurement, three electrodes were fabricated onto the wax-patterned sheet using a silk screen-printing method. To prepare the graphene/naion nanocomposite, 0.5 g of the graphene ink (Gwent group, United Kingdom) was finely homogenized with 100 μL of 0.1% w/v naion solution. This composite ink was then printed on a patterned substrate, serving as a working electrode (WE) and counter electrode (CE). A silver/silver chloride ink (Gwent group, United Kingdom) was subsequently printed as a reference electrode (RE) and conductive pads. After each screen-printing step, the printed electrode was allowed to dry in an oven at 65°C for 1 hour. Lastly, to complete the device, an adhesive tape was attached below to the WE to confine the surface area of the working electrode.

Electrochemical measurement

Cyclic voltammetry and square wave voltammetry were performed with a potentiostat model 1232A (CH instrument, Austin, TX) at room temperature. For vitamin D detection, standard solutions of vitamin D₃ were prepared in an ethanol:NaClO₄ (60:40), and a 30 μL aliquot was used throughout the experiments. For square wave voltammetry (SWV), amplitude of 25 mV, potential increment of 4 mV, and a frequency of 10 Hz were selected.

The electrochemical impedance spectroscopy (EIS) was carried out using an Autolab electrochemical system with a potentiostat PGSTAT 204 (Eco-Chemie, Utrecht, Netherlands) in a solution of 5.0 mM K₃Fe(CN)₆ with a sinusoidal potential modulation of 0.1 V amplitude in the 0.1 Hz to 100 kHz frequency range, and the logarithmic scale of 10 points per decade.

Results and discussion

Electrochemical characterization of screen-printed electrodes

Electrochemical properties of graphene/naion nanocomposites were initially investigated using cyclic voltammetry. Cyclic voltammograms of 5 mM K₃Fe(CN)₆ in 0.1 M KCl using various screen-printed electrode (SPE) are presented in the inset of Figure 1. As observed with graphene and graphene/naion nanocomposite (green dash line and blue line, respectively), the magnitude of current response is far greater than carbon SPE (red dash line) which can be attributed to the excellent rate of electron transfer of the graphene material. Nevertheless, the background current recorded in 0.1M KCl (Figure 1) with the graphene/naion nanocomposite was the smallest in comparison with other

materials. This behavior is considered to be due to the background reduction capability from nafion. In particular, nafion consists of three regions which are the hydrophobic fluorocarbon backbone, the hydrophilic cluster of sulfonic acid group, and the interfacial region. These structure provides a strong charge exclusion, which preventing much of redox-active interferences from affecting sensor responses.^{28, 33} Based on a large faradaic current with respect to small capacitive current, the signal-to-background (S/B) ratio can effectively enhance. The S/B ratios of the CV current for $\text{Fe}(\text{CN})_6^{3-}$ reduction in 0.1 M KCl were in the order of graphene/nafion SPE (S/B = 125.88) > graphene SPE (S/B = 90.09) > carbon SPE (S/B = 40.53).

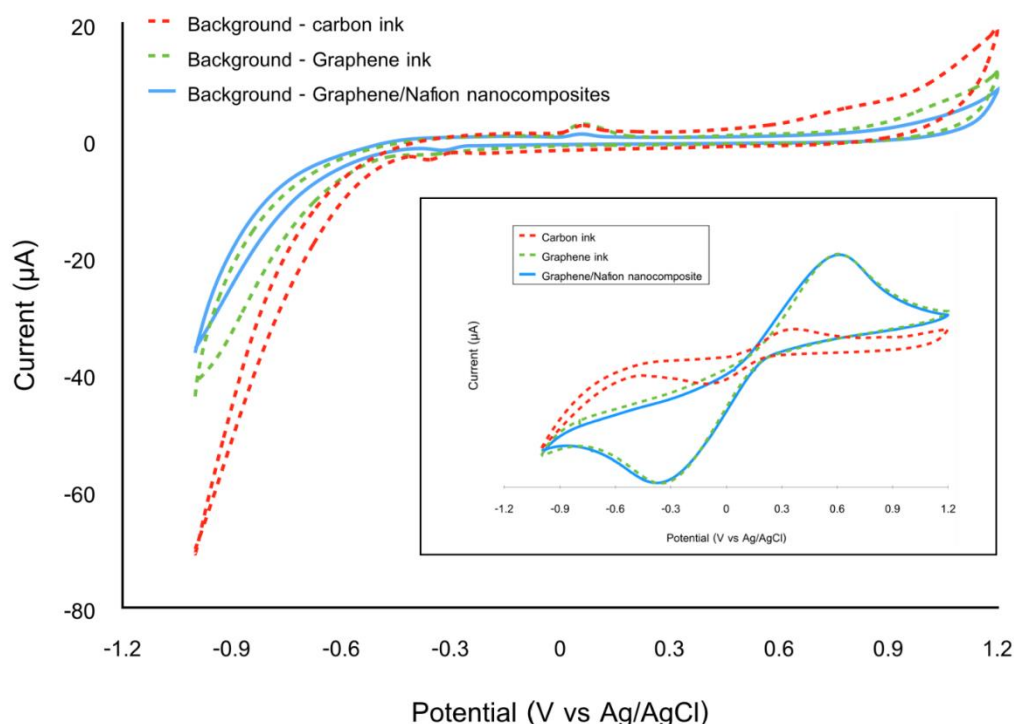


Figure 1. Cyclic voltammograms for 0.1 M KCl and 5 mM $\text{K}_3\text{Fe}(\text{CN})_6$ (inset) using the screen-printed carbon (---), graphene (---), and graphene/nafion (—) electrode. The potential sweep rate was 100 mV s^{-1} .

For a better understanding, a background electrolyte (0.1 M KCl) was measured using CV at different scan rates to assess any change in the double-layer capacitance (C_{dl}) of different SPE. The background current originates from charging to double-layer at the electrolyte/electrode interface and should be directly proportional to the total active surface exposed to the electrolyte.³⁴ As listed in the table S1, the C_{dl} value for bare carbon electrode was $23.32 \mu\text{F cm}^{-2}$ in 0.1 M KCl. For graphene SPE, the C_{dl} value substantially decreased to the $3.88 \mu\text{F cm}^{-2}$ in 0.1 M KCl system. Even though the capacitance for

carbon materials remains unclear, we believed that the oxygen functional group impurities presented on graphite material as well as the charge density in a stacked layer of graphene in carbon electrode might lead to a pseudocapacitive contribution, resulting in larger charge storage. Comparably, graphene/nafion nanocomposite SPE also decreased the C_{dl} value to 3.14 μF in 0.1 M KCl system. These results suggest that the C_{dl} value decrement of graphene/nafion nanocomposite relative to graphene SPE was related to a decrease in the electroactive surface area. This reduction in C_{dl} value could be possibly due to the partial coverage of the active surface of graphene by nafion. A similar behavior was also reported by Gayen et al, which coverage of the BDD electrode by nafion could further decrease the C_{dl} value compared to the bare BDD.³⁵ To address this issue, the further inspection of the electrode morphology and effective surface area will be investigated (section 3.2).

The electrochemical impedance characterizations of various SPE were further investigated using EIS. Figure 2 shows Nyquist plots for 5 mM $K_3Fe(CN)_6$ in 0.1 M KCl. For carbon SPE (blue dots), the plot showed a semicircle and a straight line with an angle of 45° , respectively, which indicates typical diffusion-controlled redox behavior at the planar electrode.³⁴ The decrease of semicircle radius was observed in graphene SPE (orange dots), indicating a depletion of charge transfer resistance (R_{ct}). This is due to the superior electron transfer ability of graphene, which causes the apparent reduction in R_{ct} . However, when the nanocomposite SPE was performed (green dots), the R_{ct} slightly increased which indicate the successful integration of nafion into graphene.³⁶ This phenomenon could be attributed to the nafion itself which may present an electrostatic repulsion blocking layer toward electron exchange of $Fe(CN)_6^{3-/4-}$ at the electrode-solution interface.³⁶ Despite the inconsiderably increasing in R_{ct} , the selection of electrode material may have been biased toward graphene/nafion nanocomposite regarding the S/B ratio enhancement and the anti-fouling character of the electrode.

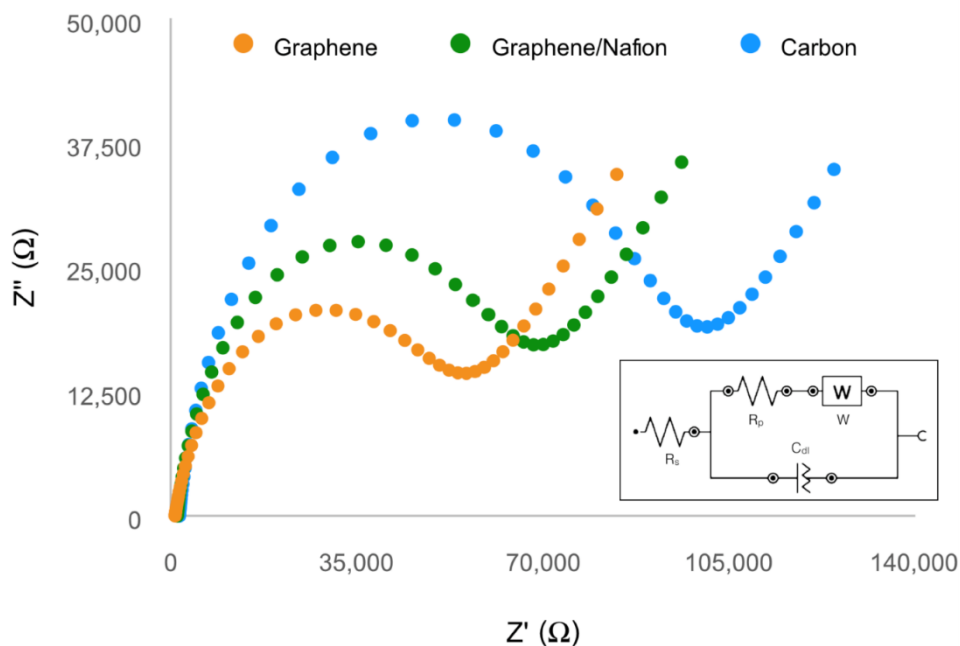


Figure 2. Nyquist plots for 5 mM $K_3Fe(CN)_6$ in 0.1 M KCl using screen-printed (●) carbon, (●) graphene, and (●) graphene/nafion nanocomposite electrode.

Morphological characterization and effective surface area estimation

The morphologies of three different electrodes; carbon, graphene and graphene/nafion nanocomposite SPE, were characterized by SEM. Figure 3, panel a and d show SEM images of carbon electrode at 5,000x and 10,000x amplification, respectively. A smooth textured region with the irregular shape of carbon was clearly seen over the surface of the SPE. Nevertheless, SEM images of graphene as displayed in panel b and e (5,000x and 10,000x amplification, respectively) illustrated a roughed and crumpled surface of graphene sheets majorly covered the surface, leading to a considerable sensitivity improvement over carbon materials. With the addition of nafion (panel c and f, 5,000x and 10,000x amplification, respectively), the prepared composite exhibited more film-like and smooth texture compared with entire graphene surface. SEM images are thus confirmed the aforementioned hypothesis, where nanocomposite possessed less surface area than graphene electrode.

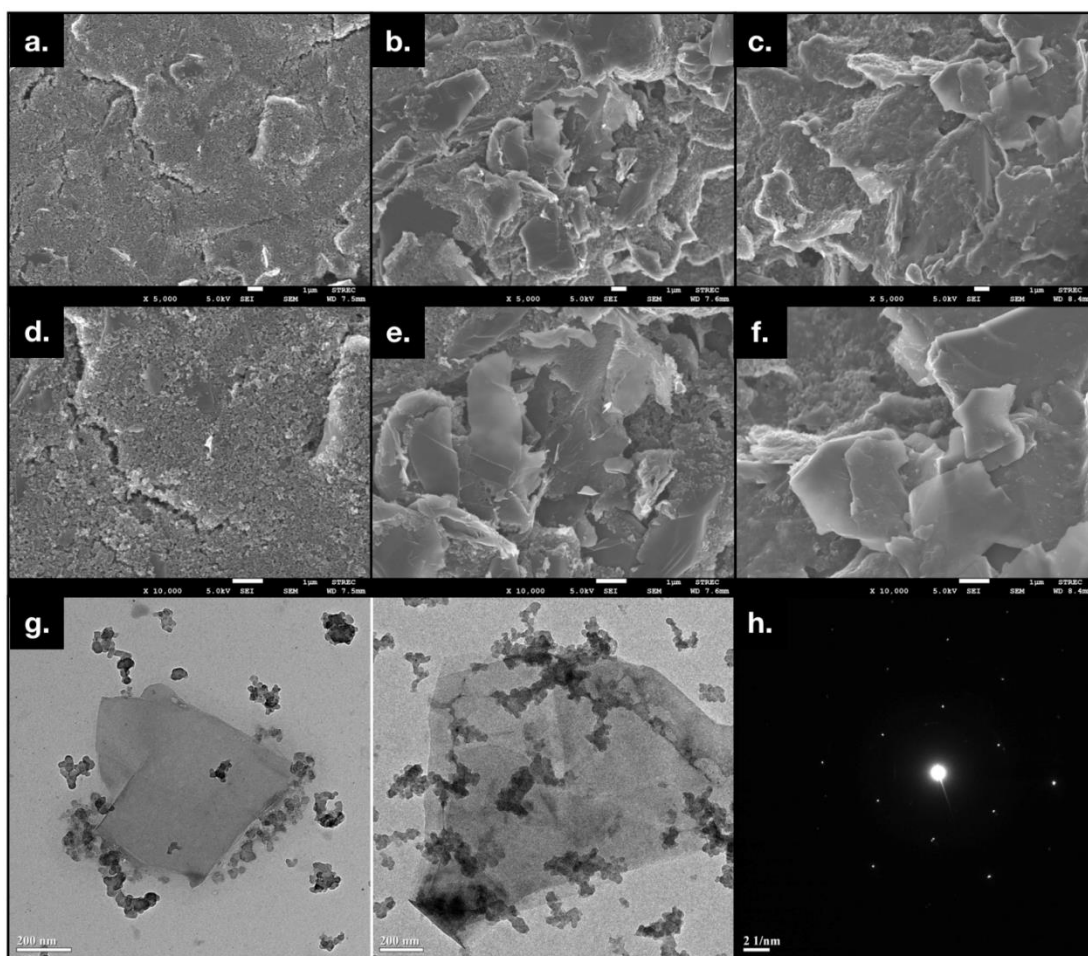


Figure 3. SEM images of the screen-printed carbon (a, d), graphene (b, e), and graphene/naion nanocomposite (c, f) electrode at 5,000X (a, b, c) and 10,000X (d, e, f) magnification. TEM images (g) and corresponding diffraction pattern (h) of the graphene sheet in the ink composition.

Figure 3, panel g and h show TEM images and corresponding diffraction pattern of the graphene sheet, respectively. These image results further confirmed the existence of the graphene in the ink composition.

Furthermore, to illustrate the effective background current reduction of nanocomposite electrode, the electroactive surface areas of studied SPE were determined employing CV in a 1.0 mM $K_3Fe(CN)_6$ solution at various scan rates (V). According to the Randles-Sevcik equation ($i_{pa} = (2.69 \times 10^5) n^{2/3} A D_0^{1/2} v^{1/2} C_0^*$, where i_{pa} is the peak current in A, A is the area of the electrode in cm^2 , C_0^* is the concentration of electroactive species in mM, and D is the diffusion coefficient in $cm^2 s^{-1}$), an approximate electrochemically active surface area of carbon, graphene, and graphene/naion composite SPE were estimated (Figure S1). The electroactive surface areas were

calculated to be 0.191, 0.342, and 0.315 cm² for the carbon, graphene, and graphene/nafion nanocomposite electrodes, respectively. Undoubtedly, nearly two-fold enlargement in active surface area promoted by graphene compared to carbon electrode is the key aspect for a sensitivity enhancement of graphene electrode. With the synergistic effect of nafion, further background current alleviation arising from a slighter active area is achieved. Some parts of the graphene electrode surface are expected to be covered with the nafion; therefore, the total surface area of the electrode should be less than that at the bare graphene electrode. Based on the experimental observations, these effective surface area results are consistent with C_{dl} evaluation, SEM images, and background current measurement obtained from cyclic voltammetry; whereby the graphene/nafion nanocomposite could synergistically enhance sensitivity, meanwhile, reduce charging current. Consequently, graphene/nafion nanocomposite SPE could clearly ameliorate the sensitivity for vitamin D determination.

Electrochemical characterization of vitamin D using different screen-printed carbon electrodes

The electrochemical measurement of vitamin D on various electrode materials was carried out using cyclic voltammetry within a potential range of 0.5 - 0.9 V and scan rate of 20 mV s⁻¹. Illustrated in Figure 4a-c are voltammograms of different concentration of vitamin D on carbon, graphene, and graphene/nafion SPE, respectively. A similar trend can be clearly observed as in the case of K₃Fe(CN)₆ in 0.1 M KCl. It should be noted that the carbon SPE presents a small peak at the only high concentration of vitamin D (10 µg mL⁻¹) while no peak was observed at lower concentrations (Figure 4a). For graphene and graphene/nafion SPE (Figure 4b and 4c, respectively), CV curves of vitamin D show a single anodic peak where the peak current is comparable. However, the background current response recorded in 60% v/v EtOH with the graphene/nafion SPE was inferior to that of graphene and carbon SPE (Figure 4d); therefore, the S/B ratio was almost 2 and 4.5 times larger at the graphene/nafion than that of graphene and carbon electrode at vitamin D₃ concentration of 10 µg mL⁻¹ (Figure 4e), respectively.

In parallel with capacitance measurement performed in 0.1 M KCl, a background solution of ethanol (EtOH) and 0.1 M sodium perchlorate (NaClO₄) (60:40) was also conducted. The electrical double layer capacitance was in the order of carbon > graphene > graphene/nafion nanocomposite SPE, which is consistent with C_{dl} value in 0.1 M KCl system (Table S1). The results indicated that the nanocomposite electrode of graphene

and nafion could be useful for high sensitivity determination of vitamin D, attributable to the larger faradaic current with respect to a lower charging current than that obtained from carbon and graphene SPE.

To further elucidate the electrode reaction of vitamin D at graphene/nafion SPE, the influence of potential scan rate (V) on peak current (I_{pa}) of $10 \mu\text{g mL}^{-1}$ vitamin D_3 was studied by cyclic voltammetry at various sweep rates ($40\text{--}200 \text{ mV s}^{-1}$), see Figure S2. The oxidation peak current of vitamin D increases with increasing of scan rate. Furthermore, a good linear relationship between I_{pa} and $V^{1/2}$ was established, indicating the redox process of vitamin D is diffusion-controlled.

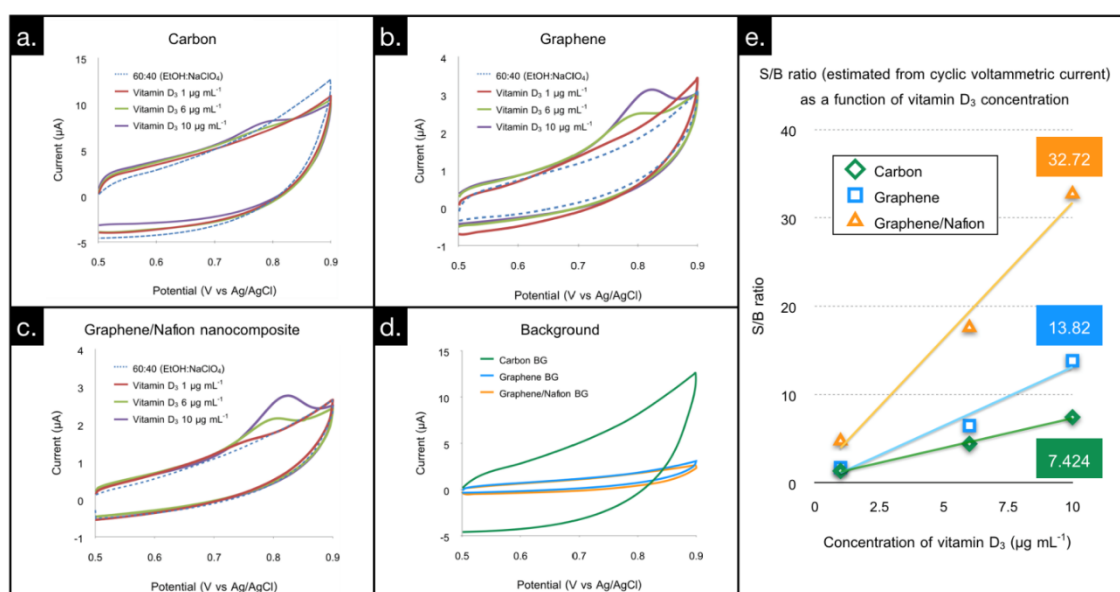


Figure 4. Cyclic voltammograms for various concentrations of vitamin D_3 using the screen-printed (a) carbon, (b) graphene, and (c) graphene/nafion nanocomposite electrode. (d) Cyclic voltammograms for ethanol:NaClO₄ (60:40) background electrolyte using the screen-printed carbon (—), graphene (—), and graphene/nafion (—) electrode. The potential sweep rate was 20 mV s^{-1} . (e) S/B ratio (estimated from cyclic voltammetric current) for vitamin D detection as a function of the vitamin D_3 concentration.

Conditions optimization

EFFECT OF EtOH/NaClO₄ ratio (% v/v)

To obtain further understanding into the electrochemical behavior of vitamin D, in this work, experiments were conducted in solution mixtures of EtOH and water in the presence of 0.1 M Sodium perchlorate electrolyte (20, 40, 60 and 80% v/v EtOH) and in

a non-aqueous system (100% EtOH). As can be seen in the cyclic voltammograms illustrated in Figure 5a, the ratio of EtOH/water influentially impact the oxidative peak current (I_{pa}) and oxidative peak potential (E_{pa}) of vitamin D. Evidently, 60% EtOH - 40% water yields the highest peak current at potential around 0.8 V. Even though less positive peak potential was observed at lower percentage of EtOH, the peak current is significantly lower than that of 60% EtOH. This could be associated with the low solubility of vitamin D in aqueous-rich solution. Meanwhile, at percentages of EtOH higher than 60% v/v, less peak current with slightly shifted toward positive potential has occurred. This is probably because of the high solution resistance in the non-aqueous system which may hinder electron transfer process. It was reported that diffusion of the ethanol-solvated molecules to the electrode surface was less efficient when the electrode surface is not preferential hydrated.³⁷ Therefore, a percentage of EtOH-water at 60% v/v was chosen as an optimal condition for the determination of vitamin D.

EFFECT OF NaClO_4 CONCENTRATION

The voltammetric responses of vitamin D in different concentrations (0.01 - 1 M of NaClO_4) of supporting electrolytes were also recorded by square-wave voltammetry (SWV) (Figure 5b). The maximum peak current was observed at a concentration of 0.1M, then remain steady above that level. Usually, the concentration of supporting electrolyte should at least be 1000 times higher than the concentration of the analyte to eliminate migration.

EFFECT OF NAFION CONCENTRATIONS (% w/v)

To explore the best sensitivity of the fabricated electrode for detection of vitamin D, varying concentrations of nafion for the composite electrode were investigated by SWV. Figure 5c shows the current response for different nafion percentage. Within the studied range (0.01% - 0.5% w/v nafion), the maximum I_{pa} occurred at 0.1% w/v. However, less current responses are observed when higher percentages of nafion were used. The lowering sensitivity is likely due to the hindered active area of graphene, which was extensively covered by nafion. Also, I_{pa} at 0.01% nafion was less than that of 0.1% probably because of the larger background current, which is almost identical to graphene electrode. Therefore, the optimal level of nafion was selected at 0.1% w/v for preparing electrode ink.

EFFECT OF EQUILIBRATION TIME

Time is another of important parameters affected an electrochemical determination of fat-soluble vitamin D. It was found that as the equilibration time before

analysis increases, the peak current increases up to 5 mins, and then slightly decreases (Figure 5d). As discussed earlier in section 3.3.3, the diffusion of the ethanol-solvated molecules to the electrode surface were much less efficient compared to the hydrated molecules.³⁷ Providing enough time thus will facilitate the diffusion of the vitamin D molecules. However, a longer time is undesirable. To avoid long analytical period, 5 mins was chosen as an optimal time for electrochemical analysis of vitamin D.

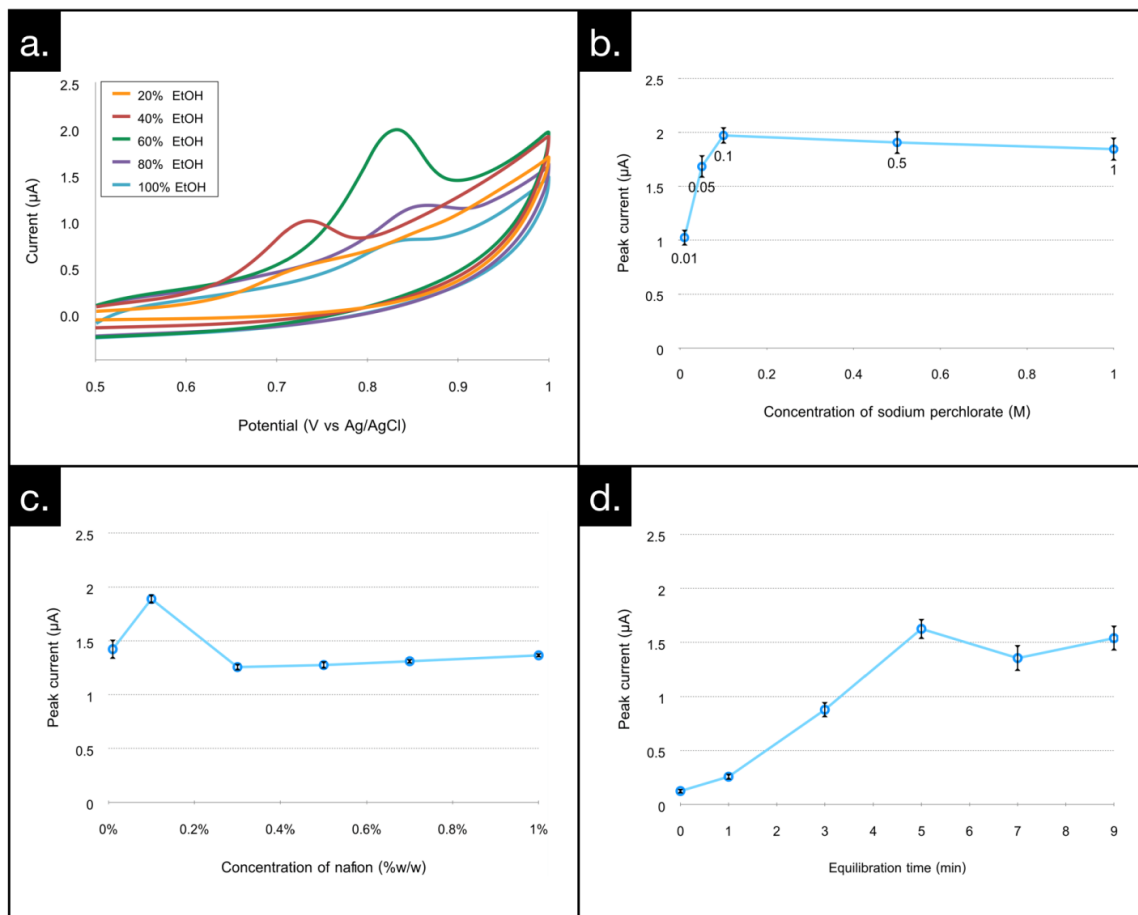


Figure 5. Effects of (a) EtOH/water ratio (% v/v), (b) concentration of supporting electrolyte, (c) concentration of nafion (% w/v), and (d) time on the electrochemical detection of 10 µg mL⁻¹ vitamin D₃. Error bar: n = 3.

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Preparation of high performance disposable electrochemical sensor based on screen-printed graphene and applied for vitamin K sensing

In this section, we have demonstrated a simple step for fabrication of large surface area and high conductivity disposable screen-printed graphene electrode (SPGE) by homemade screen printing. The prepared sensor was fully characterized and studied with scanning electron microscopy and electrochemical method. Due to the outstanding specific area and low capacitance of SPGE, this sensor could be effectively used as an alternative voltammetric sensor for total vitamin K detection using square-wave anodic stripping voltammetry (SWASV). The measurement is based on preconcentration in the first step for seconds and then the potential was scanned to a less negative direction for oxidation analysis. Under optimal conditions, the calibration graph was in the range from 1 to 15 $\mu\text{g mL}^{-1}$. The limit of detection and quantitation were observed at 0.099 and 1.000 $\mu\text{g mL}^{-1}$, respectively. The reproducibility was also evaluated at three concentrations of 5, 10 and 15 $\mu\text{g mL}^{-1}$ and the %RSDs were 3.29, 3.18 and 3.16 (n=10), respectively. Furthermore, the prepared sensor also showed good selectivity for vitamin K detection and satisfactory results in real samples detection. Therefore, the proposed sensor offers high potential to use as an alternative sensing for quality control of total vitamin K in dietary supplement samples.

Introduction

A screen-printed electrode is a kind of economical electrochemical substrate based on a screen-printing method which has many advantages, such as its small size, convenience to fabricate, disposability and rapid response due to the independence of convection and a low ohmic drop [1, 2]. These sensors can be printed by various types of ink, such as carbon, silver/silver chloride and graphene on the different substrates such as paper, ceramics and plastics. Both the selectivity and sensitivity for the analytical measurement depend on types of inks that are used as working electrodes [3]. Among various inks, graphene is a well-known conductive material which has the excellent properties. For example, it has fast electron transportation, high thermal conductivity, excellent mechanical flexibility and good biocompatibility [4].

Nowadays, healthcare is one of the most important topics with much growing concern. Dietary consumption has become an optional way for individuals to get a more

complete variety of nutrients besides the nutrients obtained directly from food. There are various kinds of commercially available supplements, such as vitamins, minerals, animal extracts or plant extracts. Therefore, the amount of active ingredients must be significantly controlled to an advisable level for an effective intake. Hence, the method for the determination of active ingredients in supplements is essential for product quality control in the pharmaceutical industry.

Among various kinds of supplements, vitamin K (VK), known as naphthoquinone compound, is one of the ingredients found in commercial products. There are mainly two forms of VK in natural sources and the human body: firstly, vitamin K₁ (Phylloquinone, PK) is produced by leaf vegetables and algae, and secondly, vitamin K₂ (Menaquinone, MK) is derived from meat, eggs, curd cheese and fermented food. VK is an essential nutrient, which plays a crucial role in the human body mechanism for blood coagulation [5, 6] and calcification of bones [7, 8]. It is a cofactor for glutamyl carboxylase, which is a catalyzed enzyme in posttranslational conversion of the protein biosynthesis mechanism. It helps to produce a protein called prothrombin which plays a crucial role in the clotting of blood [9-11]. Moreover, VK is needed to carry out the process of osteocalcin (OC) carboxylation [12]; it seems to promote the osteoblast to osteocyte transition and also prevents osteoclastogenesis [13].

The reference range of VK in healthy people is generally found to be between 0.2 and 3.2 ng mL⁻¹ as total VK in blood. Normally, a deficiency of VK occurs in infants because there are no intestinal bacteria for VK synthesis. The deficiency of VK can affect fat malabsorption syndromes, liver disease and antibiotic therapy in adults. The taking of an appropriate amount of VK is acted upon as an alternative way to maintain its normal function or for treatment. Therefore, due to such mentioned importance, it is necessary to develop a sensor for the determination of VK in supplements.

Currently, there are many methods used for the determination of VK in dietary supplements, including chemiluminescence technique [14] and HPLC cooperated with different detectors, such as ultraviolet (UV) [15-17], fluorescence [18-20], electrochemical [21, 22], chemiluminescence [23] and mass spectrometric detection [24, 25]. These methods have high sensitivity and selectivity, however; there are such disadvantages i.e. time consuming, complicated preparation and large sample volume. In order to overcome these problems, a high sensitivity, short time analysis and simple preparation method for determination of VK is still required.

Electrochemical analysis is an alternative technique for the determination of electroactive species which have a simple procedure, short analysis time, high sensitivity and selectivity [26]. There are several researches which developed the electrochemical method for the determination of VK, these works related on the large scale electrodes including hanging mercury drop electrode (HMDE) [27-29], static mercury drop electrode (SMDE) [30], carbon paste electrode (CPE) [31] and glassy carbon electrode (GCE) [32-34]. Although, these electrodes can provide a low detection limit, but they still have some drawbacks of time consuming for electrode preparation and measurement, expensive and also large sample volume requirement. Especially, mercury-based electrodes are now rarely used because of its high toxicity. Therefore, a novel electrochemical sensor that is simple to use, cheap and environmentally friendly should be continuously developed and applied for the determination of VK in dietary supplements.

Guided by above results and considerations, in this study, a simple and highly sensitive sensor for the determination of the VK as K1 and K2 in dietary supplements using screen-printed electrode was first developed. The square-wave anodic stripping voltammetry (SWASV) technique for trace analysis and screen-printed graphene electrode were used for enhancing current signal. In this work, the characterization of electrochemical behavior was studied using the cyclic voltammetry technique (CV), and the measurement of VK was carried out using SWASV. Under optimal conditions, the proposed method was applied in dietary supplements of VK successfully. The results of the real samples from this proposed method were compared with the results obtained from the HPLC-UV technique, and satisfactory results were obtained. Therefore, the developed sensor can act as quantitative control method for the determination of VK in the pharmaceutical industry, which up until now has had few studies in this area.

Experimental

Apparatus

Voltammetric experiments were performed using an Autolab electrochemical system with a potentiostat PGSTAT 30 (EcoChemie, Utrecht, Netherlands) controlled by GPES version 4.9.007 software. The three-electrode system consisted of a screen-printed graphene electrode as the working electrode and counter electrode, a screen-printed Ag/AgCl electrode as the reference electrode, and all electrodes were screened on the same substrate.

Chemical and reagents

Vitamin K1 and vitamin K2 were purchased from Sigma-Aldrich (Buchs, Switzerland). Sodium dihydrogen (NaH_2PO_4) orthophosphate was obtained from BDH Analar (Poole, UK). Ethanol, di-sodium hydrogen phosphate (Na_2HPO_4), phosphoric acid (H_3PO_4 , 85%), sodium hydroxide (NaOH) and potassium nitrate (KNO_3) were obtained from Merck (Darmstadt, Germany). Potassium ferricyanide (III) ($\text{K}_3[\text{Fe}(\text{CN})_6]$) was obtained from Sigma-Aldrich (Buchs, Switzerland). Carbon graphene ink and silver/silver chloride (Ag/AgCl) were obtained from Gwent group (Torfaen, United Kingdom). PVC laser printing sheet (thickness, 0.15 mm) was purchased from Ideecraft (Bangkok, Thailand). The stock solution of vitamin K including of K1 and K2 (2×10^{-3} M) was prepared in ethanol and kept in dark place under 20 °C. Supporting electrolyte was prepared by mixing ethanol and 0.1 M phosphate buffer pH 3.0 in the ratio of 60:40 (v/v). All chemicals were analytical grade and were used without further purification. All solutions were prepared using Milli-Q water ($R \geq 18 \text{ M}\Omega \text{ cm}^{-1}$) from Millipore (United Kingdom).

Fabrication of the screen-printed electrode

Screen-printed method was used for the fabrication of electrode according to the previous describe [35]. This platform was designed using Adobe Illustrator program and the screen blocks were fabricated by Chaiyaboon Co. (Bangkok, Thailand). For the fabrication steps, Ag/AgCl ink was firstly manual screened on PVC sheet as first layer for the reference electrode and conductive pads. Then, the screened electrode was baked at 55°C for 30 min to dry out all solvent from the electrode surface which could interfere during measurement. After that, the next step is the second layer screening of graphene as working and counter electrodes on the same substrate respectively. Finally, it was heated again at 55°C for 30 min for further solvent drying. In order to fabricate the screen-printed carbon electrode, the preparation was done in the same way using carbon ink as material for working and counter electrodes. Before measurement, the prepared screen-printed electrode was attached with small piece of tape on the graphene strip under the sample zone for limiting the contact surface area between electrode and sample constantly.

Electrochemical measurement

For the Electrochemical measurement, the screen-printed electrode was connected to the potentiostat PGSTAT30 instrument. There are two techniques used in this work; one is CV for electrochemical behavior study and the other one is SWASV for the optimization and measurement. In the determination process, a 50 μL of

standard/sample solution was dropped on three electrodes area. The CV experiments were performed in the potential range of -1.0 to +0.2 V at scan rate of 100 mV s⁻¹. For SWASV, preconcentration step was firstly carried out by applying the potential at -0.5 V for 240 s. After that, the measurement step was performed by scanning the potential to less negative direction over a potential range of -1.0 to +0.2 V at frequency of 14 Hz, step potential of 20 mV and pulse amplitude of 60 mV.

Sample preparation

The real samples for three brands of dietary supplements of VK were purchased from drugstore in Thailand. First, a tablet was weighted and grinded into powder. Then, this powder was dissolved in 30 mL of ethanol, stirred for 10 min and mixed with 20 mL of 0.1 M of PBS at pH 3.0 containing NaOH at optimized concentration of 0.01 M as precipitating agent. So, the final solution has the ratio of 60:40 % of ethanol:PBS buffer. Next, the final volume of 50 mL was centrifuged at 5000 rpm for 30 min. After that, the clear supernatant was filtered through syringe nylon filter 0.45 µm. Finally, the solution was adjusted pH using 0.1 M phosphoric acid until pH altered to 3.0 and then adjusted the final volume to 100 mL with the mixture of 60:40 % of ethanol:PBS buffer at pH 3.0, respectively.

Results and discussion

Characterization of the prepared sensors

Graphene is an attractive material which is widely used in many applications because of its extremely promising electrical characteristics. This work is an application of graphene acting as a conductive material on a screen-printed electrode for electron transfer measurement. In order to investigate the features of this ink, the scanning electron microscopy (SEM) was used for morphology characterization to illustrate the surface of the electrode material. The SEM image is shown in Fig. 1A. The screen-printed graphene electrode provides a rough and porous surface with lots of graphene sheets. These remarkable features can provide an efficient interaction between the electrode and substance.

Moreover, the double layer capacitance (C_{dl}) is an important matter which can affect the sensitivity of determination. A higher capacitance material can cause lower sensitivity and lower limit of detection for measurements owing to the accumulation of other substances. Therefore, C_{dl} is an essential criterion for choosing an electrode material. In this work, the capacitance of a screen-printed graphene electrode was

investigated and compared to a screen-printed carbon electrode with the same fabrication. For C_{dl} measurement, the CV was performed in 0.1 M KNO_3 at the potential range of -0.2 to +0.2 V with a scan rate of 100 mV s^{-1} on the different kind of electrodes. As shown in Fig. 1B, the cyclic voltammograms from two different screen-printed electrodes were exhibited as plain background currents. From the results, it can be noticed that the current signal of the graphene electrode provided a lower background current than the carbon. In addition, the capacitance of electrodes was calculated from current at 0 V against screen-printed Ag/AgCl by calculation using the equation below:

$$C_{dl} = \frac{I_{avg}}{v(A_{geometric})}$$

where I_{avg} is the current average from the forward and reverse sweep in amperes, v is the scan rate in volts per second and $A_{geometric}$ is the geometric electrode area in square centimeters. The capacitances of screen-printed graphene and carbon electrodes were evaluated at 3.45 ± 0.02 and $5.61 \pm 0.05 \mu\text{F cm}^{-2}$, respectively ($n=3$). The capacitance of the graphene electrode is lower than the carbon electrode due to its hydrophobicity of graphene [36]. The H-terminated of graphene has been predicted to localize at the edge [37]. As a result, the other ions in the supporting electrolyte do not accumulate on the electrode surface. In the case of carbon, its surface contains both hydrophobic and hydrophilic sites [38] which can cause a high charging layer on the electrode surface. Due to advantage of having low interfering of the capacitive layer of graphene, the graphene ink was selected as an electrode material for current signal enhancing.

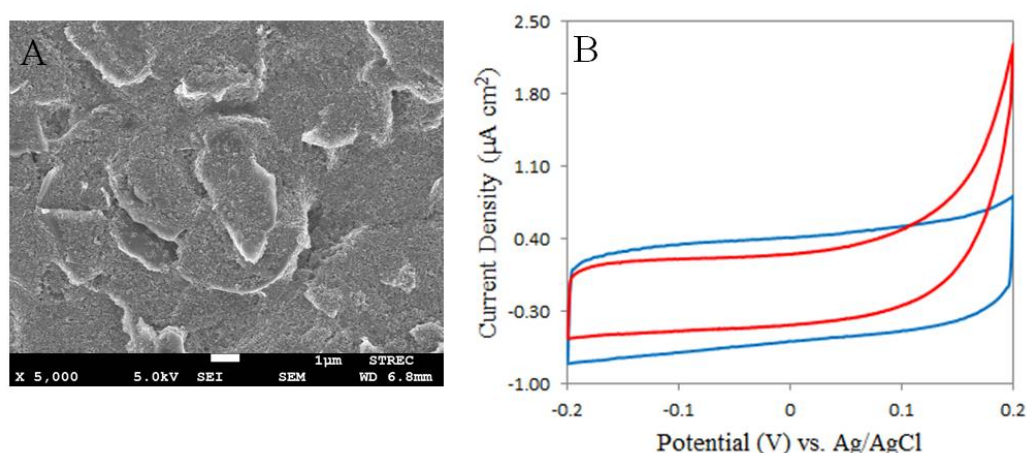


Fig. 1. (A) SEM images of surface morphology of graphene screen-printed electrodes. **(B)** Cyclic voltammograms of 0.1 M KNO_3 on carbon (blue) and graphene (red) screen-printed electrode at scan rate of 100 mV s^{-1} .

Electrochemical behavior of VK at SPGE and influence of the scan rate

To study the efficiency of the proposed electrode for VK determination, the electrochemical behavior of VK including of K1 and K2 in ethanol and phosphate buffer pH 3.0 (60:40) was individually investigated using screen-printed carbon and graphene electrodes by CV technique as shown in Fig. 2A. The potential was operated in the range of -1.0 V to +0.2 V at a scan rate of 100 mV s⁻¹. Moreover, the measurement of the background current from these electrodes was also performed at the same condition that displayed in inset. From these results, the background current of the graphene electrode was found to be lower than the current from the carbon electrode due to its low capacitance property, as mentioned previously. In the presence of VK, cyclic voltammograms were also compared between screen-printed carbon and screen-printed graphene electrodes. The CV of the screen-printed carbon electrode showed a broad and small anodic peak at -0.123 V, whereas, the screen-printed graphene electrode obviously provided the defined peak and higher current at -0.130 V. This phenomenon is due to the effect of the less charging double layer of the screen-printed graphene electrode. Thus, the high sensitivity of VK determination will be obtained from this sensor. Therefore, the screen-printed graphene electrode was chosen for this work. Furthermore, the anodic peak current was a bit higher than the cathodic one. As a result, all experiments for the determination of VK were carried out under oxidation reaction.

In the supplements, we generally found VK in two principal forms as K1 and K2, as mentioned. Hence, the investigation of the electrochemical behavior of each form is necessary. In the experiment, 1 mM solution of vitamin K1 and K2 were measured separately using CV to study the electrochemical behavior and to investigate an exact location of redox potential. The results are shown in Fig. 2B. From the results, the anodic peak current of K1 was found to be around $2.12 \pm 0.03 \mu\text{A}$ at the potential of -0.102 V, and K2 was $2.33 \pm 0.02 \mu\text{A}$ at -0.088 V. The currents and oxidation potential of both forms were not significantly different because of the similar structure with the same functional group. Hence, the developed method can be used for the determination of the VK including of K1 and K2 in dietary supplements without any discrimination of the two forms. Furthermore, these results also indicate that VK displayed a quasi-reversible property. This reaction is related to quinone-hydroquinone couple with two electrons and two proton transfer corresponding to a previous study [39], as shown in Fig. 3.

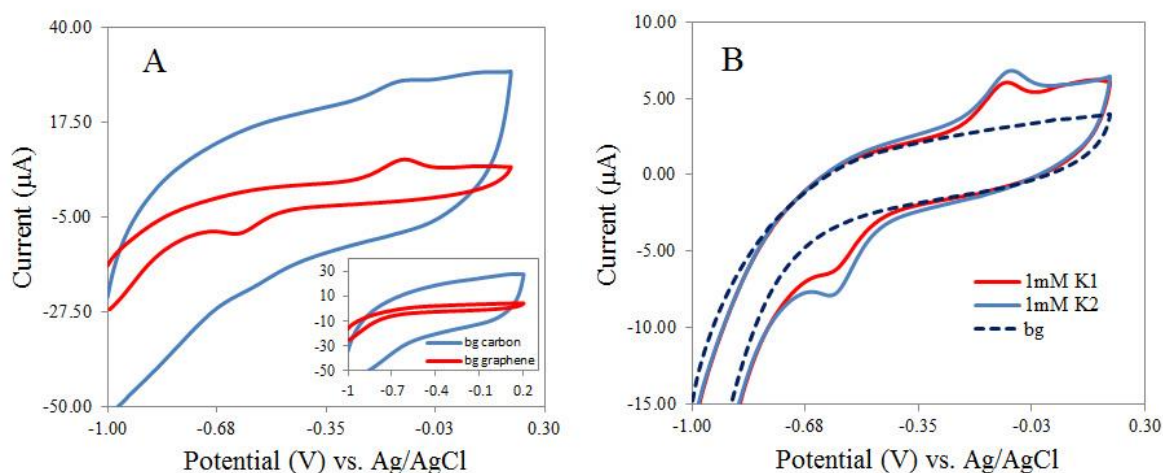


Fig. 2. Cyclic voltammograms of (A) 1 mM of the VK including of K1 and K2 at carbon (blue) and graphene (red) screen-printed electrode, comparison of background current between carbon and graphene is shown in the inset. (B) 1 mM of K1 (red) and K2 (blue) in 60:40 ratio of ethanol:PBS buffer pH 3.0 at scan rate of 100 mVs⁻¹.

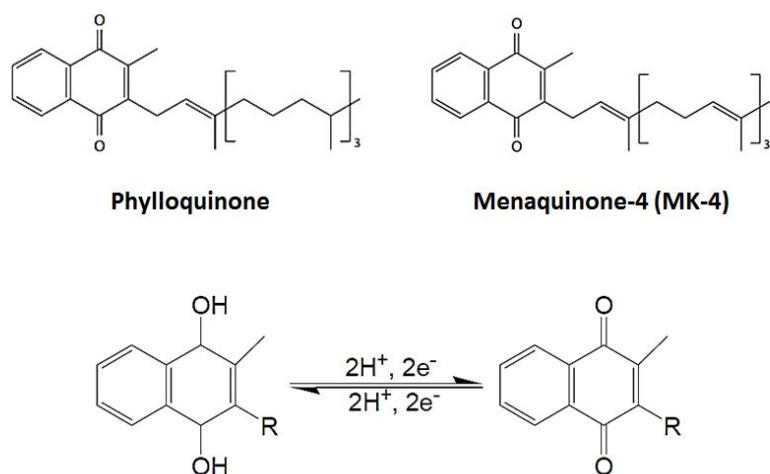


Fig. 3. The structure of K1 (Phylloquinone) and K2 (Menaquinone-4) with electrochemical oxidation mechanism of VK at screen-printed graphene electrode.

Besides electrode characterization and electrochemical behavior study, the mass transport of analyte towards the electrode surface was also investigated. The mass transfer of VK was proved by the relationship between the scan rate and anodic current signal. In the experiment, the solution of VK was measured at different scan rates in the range from 20 to 100 mV s⁻¹ at SPGE. As shown in Fig. 4, the redox currents increased with an increasing of the scan rate. When the currents in μA were plotted against the square root of scan rate in (mV s⁻¹)^{1/2}, the linear relationship was observed. Therefore, these results can indicate that the mass transport of VK at screen-printed graphene

electrode is controlled by a diffusion process [40]. Moreover, the plot between the scan rate and currents of oxidation and reduction reaction also observed in supporting information at Fig. S1. These plots showed the nonlinear relationship, so it can confirm the non-adsorption properties, but it has the diffusion process instead.

The selection of the electrochemical technique for the determination of VK is another essential topic for method development. Therefore, the efficiency of the used technique for the determination of VK was performed and compared. Square-wave voltammetry (SWV) is a popular technique due to its high sensitivity and short time analysis. Moreover, it is commonly cooperated with the anodic stripping voltammetry called square-wave anodic stripping voltammetry (SWASV) for trace determination. As a result, the efficiency of the two techniques including SWV and SWASV was investigated for VK determination at microgram level. As shown in Fig. 5, the small and quiet broad peak at -0.121 V was obtained from the SWV technique, while the higher current with a well-defined peak at -0.081 V was obtained from SWASV. When the currents of both techniques were compared together, SWASV obviously was showed to have a current signal 3.6 times higher than SWV due to the accumulation step at -0.5 V for 240 sec. This step can provide the formation change from quinone to a hydroquinone compound, which is a reducing form. The hydroquinone is a stronger and more stable adsorption property than the oxidizing one; therefore, it provides high sensitivity, as described in previous work [30]. The greater sensitivity can be improved by using more negative potential of accumulation steps before scanning towards a positive direction. From the results obtained, the SWASV was therefore used for the determination of VK in all experiments of this work.

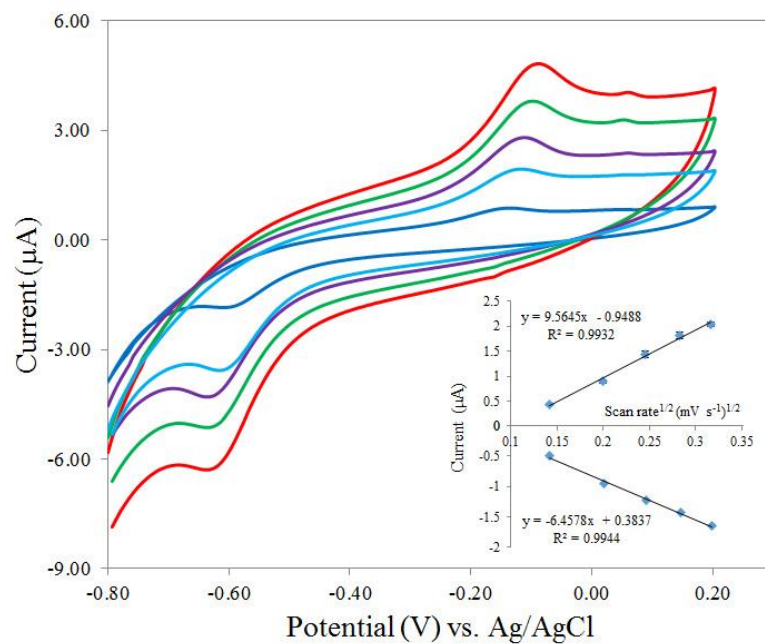


Fig. 4. Cyclic voltammograms of 1 mM of VK including of K1 and K2 in 60:40 ratio of ethanol:PBS buffer pH 3.0 at various scan rate from 20 to 100 mV s⁻¹. The relationship between current and square root of scan rate is shown in the inset.

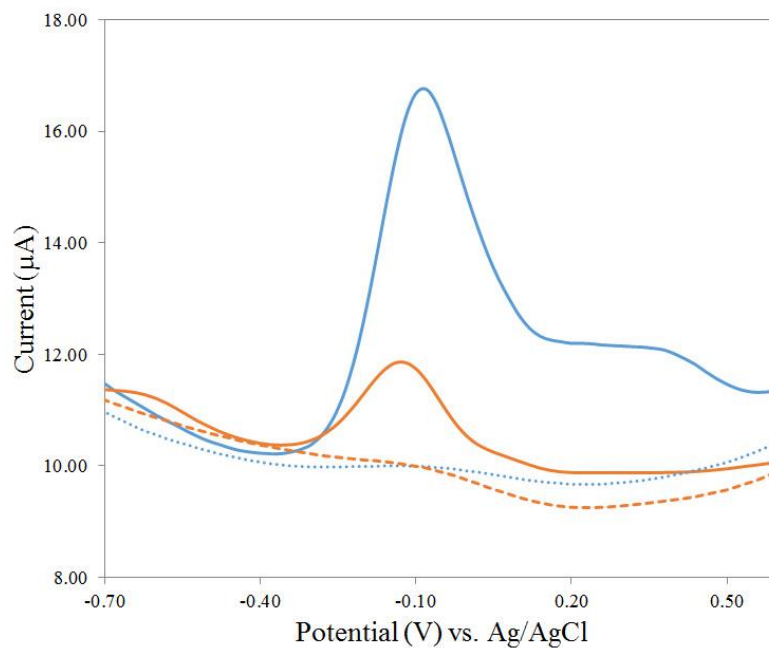


Fig. 5. Voltammograms of 1mM of VK including of K1 and K2 using SWV (orange) and SWASV (blue) technique.

Optimization of experimental and electrochemical conditions

Effect of accumulation potential, accumulation time and pH of supporting electrolyte

In order to obtain high sensitivity for the proposed method of electrochemical measurement of the VK, various experimental parameters were investigated, such as accumulation potential, accumulation time and pH of supporting electrolyte. SWASV was used to study the effect of these parameters using $10\text{ }\mu\text{g mL}^{-1}$ of VK standard solution.

Accumulation potential is one important parameter which can affect the structure of a substance. The different accumulation potentials in the range of -0.2 V to -0.7 V were studied using $10\text{ }\mu\text{g mL}^{-1}$ of VK in 60:40 ratio of ethanol:PBS buffer at pH 7.0. From the results as shown in Fig. 6A, the anodic peak currents were initially decreased at accumulation potentials of -0.3 V because the VK was partially oxidized before stripping due to the standard potential of VK at pH7.0 is -0.3 V as shown in supplementary results (Fig. S2). So, the oxidation reaction of VK during stripping step was less occurred. Then, when the system was firstly applied the accumulation potential at less negative value than standard potential at -0.2 V , the VK was changed to oxidizing form which has no adsorption property on the electrode surface, hence, it initially got a moderate current. After that, when the accumulation potential was raised towards more negative value, the anodic currents dramatically increased. From this behavior, it can suggest that the use of a more negative accumulation potential has high potential in the change from quinone to hydroquinone form, which was strongly adsorbed onto the electrode surface leading to the high sensitivity. As seen in results, at -0.5 V , the currents remained constant because all of the quinone form was absolutely converted to hydroquinone structure. Therefore, the accumulation potential at -0.5 V was chosen for the next optimization.

Then, the accumulation time for the preconcentration step was optimized in a range of 60 to 300 sec. The background current was subtracted from the VK anodic peak current before being plotted against various times as a S/B ratio. As expected, in Fig. 6B, the currents were increased with the extension of time and the highest current was obtained at 240 sec. This is because this time is enough to completely change from oxidizing to reducing form. As a result, the accumulation time at 240 sec was selected as an optimal condition.

The pH of the supporting electrolyte is another important parameter. The different pHs of 0.1 M phosphate buffer in the range of 2 to 8 mixed with ethanol at 40:60 (v/v) were investigated. The results show the higher current obtained when the pH is lower and then reaches the highest current at pH 3.0. This may be concerned that at

low pH contained a high concentration of protons, which provide a more reducing form via protonation. However, the current was then decreased at pH 2.0 (Fig. 6C) supposing that the overabundance of protons from the buffer can accumulate and interfere with the electron transfers of analytes at the electrode surface. Moreover, Furthermore, it can be noticed that its oxidation peak potentials shifted to negative values as the solution pH increased. The increasing of pH would promote the deprotonation of VK oxidation process. It is the reason that the oxidation peak potentials were shifted in less positive [41]. The voltammogram results of VK at different pH and the plot of pH versus peak potential were shown in supporting information in Fig. S2. Therefore, phosphate buffer pH 3.0 was chosen as an optimal supporting electrolyte for all experiments.

For further optimization, the effect of electrochemical parameters such as frequency, amplitude and step potential were studied. These parameters can affect the peak shape and peak current of the VK response. The frequency in the range of 8 to 16 Hz, amplitude of 20 to 100 mV and step potential of 5 to 25 mV were measured, respectively. The highest currents were observed at frequency of 14 Hz, step potential of 20 mV and amplitude of 60 mV.

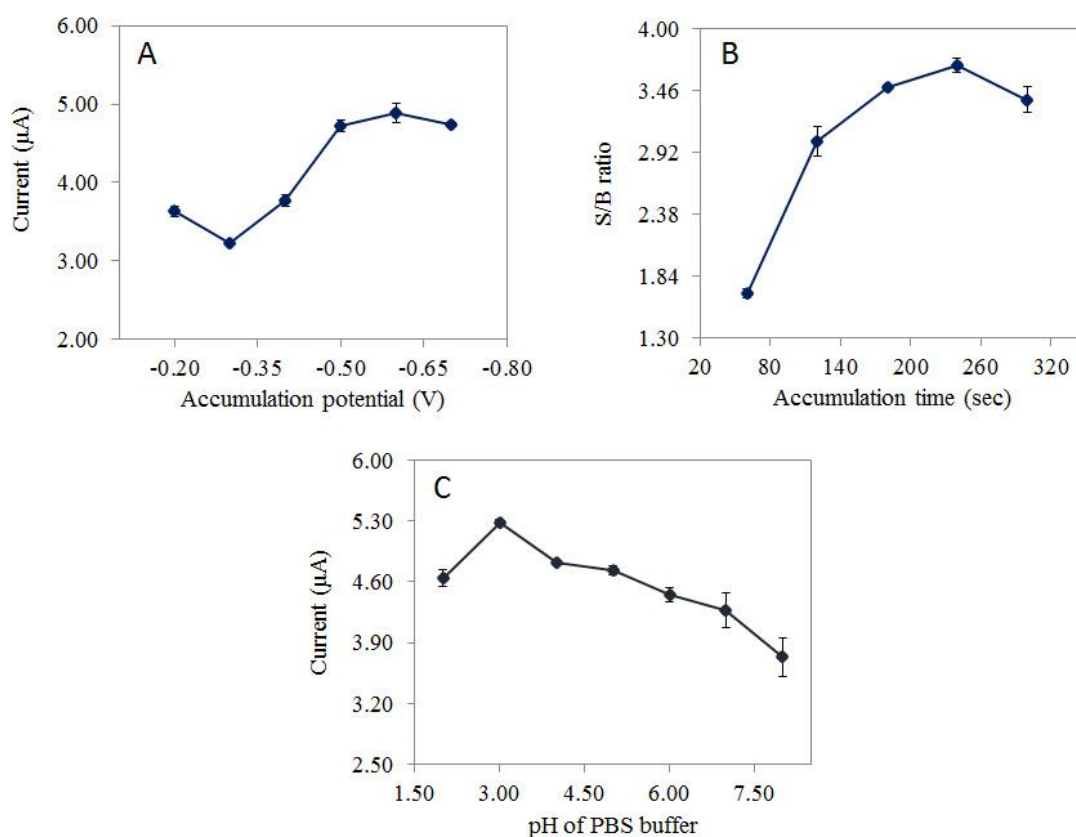


Fig. 6. Effect of (A) accumulation potential, and (B) accumulation time on the stripping peak current of $10 \mu\text{g mL}^{-1}$ of VK including of K1 and K2 in 60:40 ratio of ethanol:PBS

buffer at pH 7.0 and the effect of (C) pH of supporting electrolyte on the stripping peak current of $10 \mu\text{g mL}^{-1}$ of VK. Error bar: $n=3$

Analytical performance

To verify the analytical performance of this proposed method, the determination of the VK was evaluated using SWASV with a screen-printed graphene electrode under optimal experimental conditions. For the calibration curve, a various concentration of VK standard solution in the range of 1 to $15 \mu\text{g mL}^{-1}$ in 60:40 ratio of ethanol and 0.1 M phosphate buffer pH 3.0 was measured. The anodic peak current was increased with the increasing of the VK concentration, as shown in Fig. 7A. After that, the anodic peak current of VK was plotted against the concentration of analyte and the least square were evaluated using the linear regression method (Fig. 7B). Good linearity for VK determination was obtained in the concentration range of 1 to $15 \mu\text{g mL}^{-1}$. The regression equation was $y = 0.3055x + 1.5438$; $R^2 = 0.9904$. Then, the detection limit of the VK determination was calculated using $3 S_{\text{blank}}/\text{slope}$ and it was found to be $0.099 \mu\text{g mL}^{-1}$. This was even though this proposed method had a detection limit higher than some of previous work as shown in Table 1. However, it has some merit in terms of using screen-printed electrode with the determination of VK as K1 and K2. So, it can provide a simple operation, nontoxic, effective cost, small sample volume and short time analysis. Therefore, this developed method can be an alternative usage for the determination of the VK and applications in dietary supplement. For further performance confirmation, the reproducibility of device was investigated by measurement of VK at three concentrations of 5, 10 and $15 \mu\text{g mL}^{-1}$. Each concentration was examined using ten individual electrodes under the same optimal conditions. The %RSD was obtained at 3.29, 3.18 and 3.16, respectively, which are acceptable by AOAC guidelines.

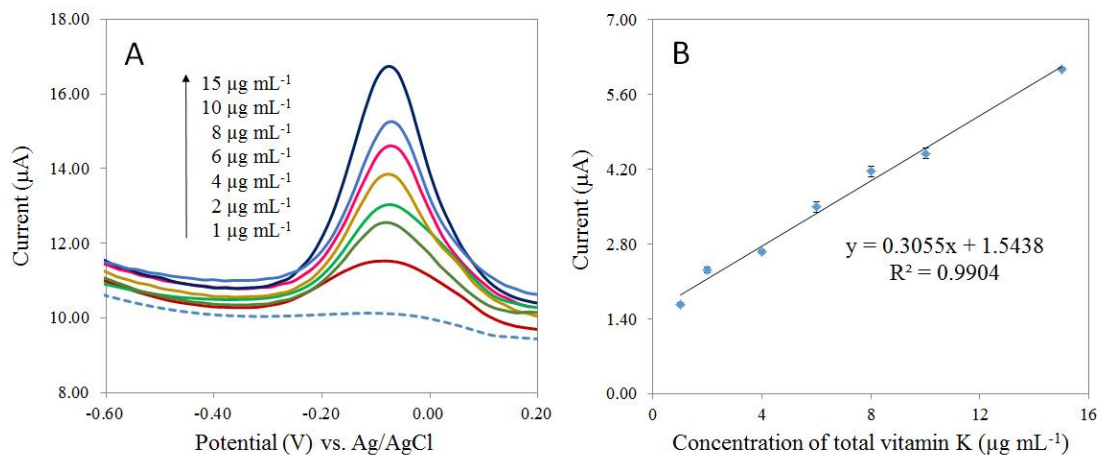


Fig. 7. (A) Square-wave voltammograms of VK standard solution at different concentrations: 1, 2, 4, 6, 8, 10 and 15 $\mu\text{g mL}^{-1}$ in 60:40 ratio of ethanol and 0.1 M phosphate buffer pH 3.0 using screen-printed graphene electrode. (B) The calibration plot of current (μA) and VK concentrations ($\mu\text{g mL}^{-1}$).

Table 1 Comparison of analytical performances of different methods for the determination of VK.

Electrode			Type of	Method	Linear range	LOD	Ref
			VK		($\mu\text{g mL}^{-1}$)	($\mu\text{g mL}^{-1}$)	
Hanging	mercury	drop	K1	DPV ^[a]	0.01 - 0.10	-	[23]
electrode							
Hanging	mercury	drop	K1	SWASV	4.5×10^{-4} - 0.45	-	[25]
electrode							
Static mercury drop electrode			K3	SWASV	3×10^{-5} - 0.09	2×10^{-5}	[26]
Hanging	mercury	drop	K3	DPV	-	0.12	[24]
electrode							
Carbon paste electrode			K1	AdSV ^[b]	0.30 – 2.00	0.18	[27]

Glassy carbon electrode		PMND ^[c] ,	SWV ^[d]	-	-	[28]
			DPV			
PEDOT:PSS-CMC-rGO@Pd composite on GCE		K3	LSV ^[e]	0.07 - 20	0.002	[29]
Glassy carbon electrode		K1	SWASV	2.3 - 45.1	0.023	[30]
				0.0045-0.45	0.004	
Screen-printed electrode	graphene	K1, K2	SWASV	1 - 15	0.099	This work

^[a] Differential pulse voltammetry

^[b] Adsorptive stripping voltammetry

^[c] (E)-2-((prop-1-enyloxy)methyl)naphthalene-1,4-dione

^[d] Square-wave voltammetry

^[e] Linear sweep voltammetry

Interference study

For the selectivity of the method for the determination of the VK, other common ingredients in dietary supplements such as Ca²⁺, Mg²⁺, vitamin C, D and E were examined separately using VK at a concentration of 5 µg mL⁻¹. The tolerance limit was estimated to be within 5% of the anodic current signal. The results show that 5-fold mass ratio of Ca²⁺, Mg²⁺ and vitamin E; 2-fold mass ratio of vitamin C and 0.12-fold mass ratio of vitamin D3 do not interfere with the determination of VK, as reported in Table 2. Nevertheless, the exact amount of these other ingredients in real samples is more available than these mass ratios. As a result, in practice, we suggest that this proposed method is not suitable for the determination of VK in multivitamin supplements. However, the developed sensor is more suitable for the determination of the VK in supplements containing VK as the main composition. In this kind of supplement, it usually has two interferences, which are Ca²⁺ and Mg²⁺. Therefore, the sample preparation is required. In order to overcome this problem, simple precipitation using NaOH was used for the elimination of Ca²⁺ and Mg²⁺ before analysis. The concentration of NaOH being used as

a precipitant was also investigated and it was found that a concentration of 0.01 M can provide an acceptable % recovery. Therefore, this condition was used as a sample preparation procedure.

Table 2 Tolerance ratio of interfering substances for the determination of 5 $\mu\text{g mL}^{-1}$ of VK on screen-printed graphene electrode.

Common interferences	5% Tolerance ratio
	$(W_{interferences}/W_{VK})$
Ca^{2+}	5
Mg^{2+}	5
C	2
D3	0.12
E	5

Sample analysis

In practical applications, the SWASV technique was performed with a screen-printed graphene electrode for the determination of VK in dietary supplements including three brands of VK dietary supplement. For accuracy and precision of the method, the % recovery and %RSD were examined by spiking three concentrations of VK at 4, 8 and 12 $\mu\text{g mL}^{-1}$, respectively. Each of the concentrations was measured repeatedly three times. The results are shown in Table 3. The recovery and RSD were found to be in the range of 90.1 to 108.1 % and 0.3 to 6.6 % respectively, which can indicate the capability of this method for the determination of VK in real samples. Furthermore, the results of this proposed method were compared with those obtained from a conventional HPLC technique coupled with UV detector (Table 4). The comparison of found concentrations in $\mu\text{g}/\text{tablet}$ between the proposed method and the conventional HPLC-UV shows a satisfactory agreement by paired t-test at the 95% confidence interval ($t_{\text{stat}}=0.8438$, $t_{\text{critical}}=4.3026$). Therefore, the proposed stripping method can be used as an alternative method for quality control of VK in real dietary supplements.

Table 3 Determination of VK in dietary supplement samples using the proposed method (n = 3).

Samples	Added ($\mu\text{g mL}^{-1}$)	Expected ($\mu\text{g mL}^{-1}$)	Found ($\mu\text{g mL}^{-1}$)	%Recovery	%RSD
Pharm. 1	4.0	5.0	5.1 ± 0.2	107.6	2.9
	8.0	9.0	8.4 ± 0.5	92.9	6.2
	12.0	13.0	13.8 ± 0.4	107.2	2.7
Pharm. 2	4.0	5.0	4.5 ± 0.1	92.2	0.3
	8.0	9.0	9.8 ± 0.1	108.1	0.7
	12.0	13.0	13.1 ± 0.3	102.8	2.3
Pharm. 3	4.0	5.0	4.9 ± 0.3	98.1	6.6
	8.0	9.0	8.6 ± 0.3	93.9	3.6
	12.0	13.0	11.9 ± 0.5	90.1	4.2

Note: values reported are mean of three replicates (n = 3).

Table 4 The comparison of the proposed method and standard method for the determination of VK in dietary supplement samples (n = 3).

Samples	Expected ($\mu\text{g/tablet}$)	Proposed method		HPLC-UV	
		Founded	%RSD	Founded	%RSD
		($\mu\text{g/tablet}$)		($\mu\text{g/tablet}$)	
Pharm. 1	100	93.7 ± 6.5	6.9	80.6 ± 0.1	0.1
Pharm. 2	100	80.3 ± 3.8	4.7	100.3 ± 0.4	0.4

Pharm. 3	500	507.9 ± 8.6	1.7	529.5 ± 1.27	0.2

Note: values reported are mean of three replicates (n = 3).

Conclusions

In this study, we successfully prepared a new sensor using graphene by homemade screen printing for the determination of the VK including of K1 and K2 in supplements by a square-wave anodic stripping voltammetry. This kind of sensor provides high sensitivity, simplicity, low costs, fast analysis and a low sample volume. Moreover, this proposed sensor can act as being economical and environmentally friendly, which is suited for use in the real industry. For the comparison results of the electrode, screen-printed graphene was shown to have lower conductance than the carbon type; hence, it can generate a lower charging layer accumulated on the surface of the electrode, leading to a lower background current and higher sensitivity. After the experimental parameter optimization, the best result was obtained in acidity conditions using a 60% ethanolic phosphate buffer at pH 3.0. The linearity was observed in the range from 1 to 15 $\mu\text{g mL}^{-1}$. The limit of the detection and quantitation were achieved at 0.099 $\mu\text{g mL}^{-1}$ and 1 $\mu\text{g mL}^{-1}$, respectively. Finally, this developed sensor was successfully applied for real supplement samples and results obtained were in good agreement with the results from HPLC-UV detector. The %RSD is in the range of 1.7 to 6.9 which is an acceptable level. Therefore, this proposed sensor shows high potential to be an alternative sensing for the qualitative controlling in the pharmaceutical industry in the future.

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Application of a Screen-Printed Graphene/Nafion Nanocomposite for the Sensitively Simultaneous Determination of Vitamin A, D, E and K in Dietary Supplement

The application of a sensitive and selective screen-printed graphene/Nafion nanocomposite for the simultaneous determination of vitamin A, D, E and K in dietary supplement was demonstrated. First, the scanning electron microscopy was used for morphological characterization of electrode surface followed by the electrochemical characterization by cyclic voltammetry technique using potassium ferricyanide. Then, the proposed sensor was used to study the electrochemical oxidation of vitamin A, D, E and K in a mixture of supporting electrolyte containing ethanol and sodium perchlorate monohydrate using adsorptive square-wave voltammetry. The results indicated that the oxidation peak of each vitamin appeared at different potentials leading to the possibility for the simultaneous detection. To enhance performance, the influences of experimental parameters, such as the effects of proportion of ethanol in supporting electrolyte, potential increment, amplitude, frequency, and quiet time were investigated. Under the optimal conditions, a good linear relationship was obtained between the anodic current and the concentration of vitamins within the range from $0.02 \mu\text{g mL}^{-1}$ to $1 \mu\text{g mL}^{-1}$. The limits of detection were found to be 0.0086, 0.0063, 0.0075 and $0.0071 \mu\text{g mL}^{-1}$ for vitamin A, D, E and total K, respectively. Furthermore, to evaluate the efficiency and the capability of the proposed method, this method was applied for the simultaneous determination of vitamins in real dietary supplement samples. The recoveries of vitamins in spiked sample at 0.1, 0.2 and $0.4 \mu\text{g mL}^{-1}$ were found to be in the range from 80.62% to 108.75% for vitamin A, 80% to 105.88% for vitamin D and 77.19% to 97.01% for vitamin E, which were in acceptable ranges by AOAC guidelines. In real sample, this developed assay provided results were in good agreement with labels. Therefore, the proposed sensor can be used for simple, rapid, selective and highly sensitive quantification of fat-soluble vitamins in dietary supplements.

Introduction

Vitamins are a broad group of organic nutrients which are essential for life. The human body requires these nutrients to ensure normal metabolism, growth and physical well-being. Most vitamins are not made in the body, or only in insufficient amounts to meet our needs. Therefore, they have to be obtained primarily through the food that we

eat. Vitamins are classed as micronutrients because they present in food in minute quantities compared to macronutrients as proteins, carbohydrates and fats [1].

Vitamins are classified into two groups: water-soluble vitamins (B-complex and C) and fat-soluble vitamins (A, D, E, and K). Fat-soluble vitamins are characterized by their low polarity [2] and found mainly in animal products, fatty nutrients and pharmaceuticals [3]. These vitamins are stored in the fatty tissues of the body and the liver for a long time. Therefore, the possibility for a risk of toxicity can be caused by consumption of these vitamins in excess over water-soluble vitamins. Thus, maintaining the appropriate quantity of fat-soluble vitamins in the body is very important. Currently, many people take multivitamins capsules or tablets and mineral supplements to supplement the nutrition from commercially available. Therefore, these products are required for quality control to accurate amount of active ingredients on the labeled quantities of vitamins.

Several analytical methodologies have been developed for the determination of fat-soluble vitamins in a diverse variety of biological, drug, food and dietary supplement samples such as liquid chromatography [4], capillary liquid chromatography [5], liquid chromatography with mass spectrometry [6-9], high-performance liquid chromatography [10,11], and high-performance liquid chromatography with ultraviolet [12-19], fluorescence [17-19], electrochemistry [20,21], and mass spectrometry [22,23]. Although these techniques provided high sensitivity and selectivity, however, there had several limitations such as expensiveness, time-consuming, complexity of sample preparation, need for large sample amount, sophisticated instrument and special training. In order to overcome these limitations, a simple, rapid, selective and sensitive methodology for the determination of fat-soluble vitamins is still required.

Electrochemical detection is an alternative method for the determination of electroactive species due to its simplicity, short analysis time, high sensitivity and selectivity [24]. From the literature reviews, the determination of vitamin D in a mixture of organic solvent and supporting electrolyte with differential pulse voltammetry (DPV) using glassy carbon electrode with and without modification [25-26] were proposed. The results of interference studies from others vitamins including vitamin A, E and K were also investigated. Nevertheless, these proposed methods provided a high detection limit and could not determine vitamin A, D, E and K simultaneously. Therefore, the electrochemical sensor is very attractive to be continuously developed and applied for the simultaneous determination of fat-soluble vitamins in the supplementary products.

Hence, efforts to extend electrochemical detection for the simultaneous determination of vitamin A, D, E and K have been challenging. Recently, a screen-printed electrode (SPE) is a kind of a disposable electrochemical sensor based on the screen-printing technology. It has been examined extensively in the field of electrochemical analysis. Compared to conventional electrodes, SPE offers many advantages such as easy to use, small size, convenience to fabricate, disposable device, portability, low cost and can be designed to work with micro-volume of sample [27]. Sensor can be fabricated from various types of ink on different substrates which are important for the sensitivity and selectivity obtained from the analytical procedure. Among various inks, graphene is a well-known carbon material which consists of two-dimensional structures with sp^2 bonded carbon atoms in a hexagonal configuration [28]. It has many excellent properties such as high electrical conductivity, increase electron transportation, high mechanical and chemical stability, large specific surface area and good biocompatibility [29-32]. Moreover, we also focused on the use of Nafion as the binding agent in a part of sensor. Nafion is a perfluorinated sulphonated cation exchanger which has been widely used in electrochemistry to modify electrodes. It has many unique properties such as thermal stability, chemical inertness, mechanical strength, huge cation exchange capacity, high permeability to cations and antifouling properties [33-35]. From the advantages of materials, we aim to fabricate the sensor by using nanocomposite of graphene and Nafion to increase the oxidation signals for the simultaneous determination of vitamin A, D, E and K.

Surprisingly, there is currently very little information about the determination of fat-soluble vitamins in biological or dietary supplement samples using electrochemical techniques, and to the best of our knowledge, there is no information about the simultaneous determination of vitamin A, D, E and K levels using SPE nanocomposite as the sensor in the field of electrochemical analysis. Therefore, this is the first report on the use of a screen-printed graphene/Nafion composite electrode as the sensor for the simultaneous analysis of fat-soluble vitamins in dietary supplement samples.

Experimental

Apparatus

Voltammetric experiments were performed using a model CHI660D electrochemical analyzer (CH Instruments, USA). All experiments were performed using a three-electrode system which consisted of a screen-printed silver/silver chloride

electrode as the reference electrode, a screen-printed graphene/Nafion electrode as the working electrode and counter electrode. All electrodes were screened on the same substrate by using a DEK Horizon screen printer (Weymouth, United Kingdom)

Chemicals and reagents

Vitamin A, K₁ and K₂ were purchased from Sigma-Aldrich (St. Louis, Missouri, *United States*). Vitamin D and E were obtained from Supelco (Bellefonte, Pennsylvania, *United States*). Sodium perchlorate monohydrate (NaClO₄·H₂O) was purchased from Fluka (Buchs, Switzerland). Ethanol absolute anhydrous was obtained from Carlo Erba (Val de Reuil, France). Potassium ferricyanide (K₃[Fe(CN)₆]), Potassium chloride (KCl) and Nafion were purchased from Sigma-Aldrich (St. Louis, Missouri, *United States*). Graphene, silver/silver chloride (Ag/AgCl) and polymer dielectric ink were obtained from Gwent group (Pontypool, United Kingdom). Polyvinyl chloride (PVC) substrate (thickness, 0.4 mm) was purchased from Cadillac Plastic (Swindon, United Kingdom). The stock solution of vitamin A (2 mM) and K₁ (2 mM) were prepared in ethanol and stored in dark at 2-8°C. The stock solution of vitamin D (0.1 mM), E (1 mM) and K₂ (2 mM) were provided in ethanol and kept in dark under -20°C. The working solutions of fat-soluble vitamins were prepared in a mixture of supporting electrolyte containing ethanol and 0.1 M of sodium perchlorate monohydrate in the ratio of 60:40 (v/v). All other chemical reagents were analytical grade and were used without further purification. All solutions were prepared in Milli-Q water from a Millipore water purification system ($R \geq 18.2 \text{ M}\Omega \text{ cm}$).

Fabrication of the screen-printed electrode

Screen-printed electrodes were fabricated with appropriate stencil designs by using a DEK Horizon screen printer according to the previous describe [36]. For the fabrication steps, a mixture of graphene ink and Nafion was first screened on PVC substrate as first layer to form both the working electrode and counter electrode. Next, baking this screened electrode at 60°C for 30 min to clear up all solvent from the electrode surface. Afterwards, silver/silver chloride ink was screened onto the same substrate as the second layer for the reference electrode. Then, baking it at 60°C for 30 min. After that, the last step was the third layer screening of polymer dielectric ink as the insulator to define the working area of electrode. Finally, the screen-printed electrode was baked again at 80°C for 30 min.

Morphological study

Transmission electron microscopy, TEM images, were recorded by an H-7650

transmission electron microscope)Hitachi model, <http://www.hitachi.com>). The surface morphologies were studied using a JSM-7610F field emission scanning electron microscope)FESEM) (JEOL Ltd., <https://www.jeol.co.jp>).

Electrochemical measurement

In this work, a model CHI660D electrochemical analyzer was used for cyclic voltammetry (CV) and adsorptive square-wave voltammetric (AdSWV) analysis. The screen-printed electrode was connected to this electrochemical analyzer for the measurement. In the determination process, the standard/sample solution (50 μL) was dropped onto three-electrode area. For CV analysis, characterization studies of the electrode were carried out in potential range from -0.8 V to 1.1 V with scan rate of 100 mV s^{-1} . Moreover, AdSWV experiments also were used for the optimizations and measurements in potential range from 0 V to 1.5 V. The oxidation peaks of vitamin A, D, E and K occurred within this potential window and their currents were used for the simultaneous determination of vitamins concentrations. All experiments were performed at room temperature (298 K).

Analytical performance

To obtain the validation parameters, evaluation of the anodic peak current was employed to determine the concentrations of fat-soluble vitamins. Calibration curves were constructed with linear least square regression analysis using various concentrations of standards. The limits of detection (LOD) and limits of quantification (LOQ) were determined from $3S_{bl}/S$ to $10S_{bl}/S$, respectively, where S_{bl} is the standard deviation of the blank measurement ($n = 7$) and S is the sensitivity of the method, evaluated as the slope of the linearity. To determine the repeatability and reproducibility of the analytical process, three concentrations (0.06, 0.20 and 0.80 $\mu\text{g mL}^{-1}$) of fat-soluble vitamins were measured five times on the same electrode and one time on five electrodes, respectively. In addition, the sensitivities of the proposed method were studied by varying the concentrations of only one standard solution of fat-soluble vitamin while the concentrations of other fat-soluble vitamins were assigned to a constant variable.

Sample preparation

The simultaneous determination of vitamin A, D, E and K in dietary supplement sample was carried out in a mixture of organic solvent and supporting electrolyte. Real sample was purchased from drugstore in Japan. First, a tablet was weighted and grinded into powder. Next, this powder was dissolved in a mixture of ethanol and 0.1 M of $\text{NaClO}_4 \cdot \text{H}_2\text{O}$ in the proportion of 60:40 (v/v). Then, the solution was sonicated for 10 min.

Finally, the concentrations of fat-soluble vitamins within the linear range (0.02 to $1 \mu\text{g mL}^{-1}$) were spiked into the sample solution. The labeled values were employed in comparison.

Results and discussion

Characterization of a screen-printed graphene/Nafion composite electrode (SPGNE)

Nowadays, graphene and Nafion have been widely used in many applications for surface modifications of electrode materials, especially in electrochemical research because of its excellent electrical conductivity. This work aimed to use graphene/Nafion nanocomposite as conductive material to fabricate an electrochemical sensor for electron transfer measurement. Scanning electron microscopy (SEM) was used for morphological characterization of primary studies. SEM images of the surface morphology of SPGNE (Fig. 1) shows wrinkled surface with lots of flake-like foams. Nafion can enhance the dispersion of graphene to reduce the agglomeration. This nature of graphene/Nafion is good for maintaining the nanomaterial properties and highly beneficial in increasing a surface area on the electrode surface, resulting in the increase of sensitivity.

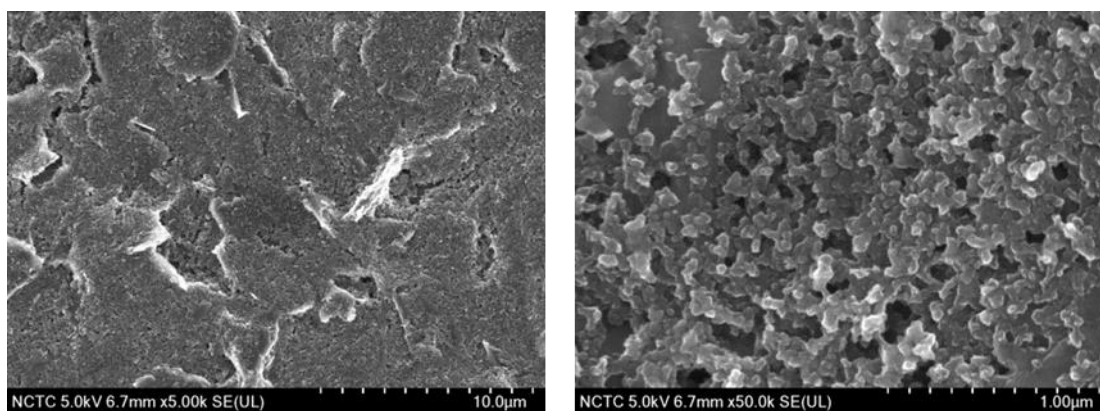


Fig. 1. SEM images 5x (left) and 50x (right) magnification of the surface morphology of SPGNE.

Electrochemical characterization of SPGNE

To study the efficiency of the proposed sensor for the simultaneous determination of vitamin A, D, E and K, the electrochemical activity of SPGNE was conducted with CV using the 5 mM of $\text{K}_3[\text{Fe}(\text{CN})_6]$ in 1 M of KCl . The potential was performed in the range from -0.8 V to $+1.1 \text{ V}$ at a scan rate of 100 mV s^{-1} . As shown in Fig. 2, we examined three different electrodes; (a) commercial electrode, (b) screen-printed graphene electrode (SPGE), (c) a screen-printed graphene/Nafion composite electrode (SPGNE).

According to the redox diagram, the oxidation peaks of each electrode appeared at the potential of +0.298 V for SPGNE, +0.309 V for SPGE, and +0.335 V for the commercial electrode. In addition, SPGNE provided the most peak current of 76.6 μA , while SPGE and the commercial electrode exhibited 51.9 μA and 36.6 μA , respectively. It was found that SPGNE can enhance the electrochemical activity over using the commercial electrode and SPGE. For electrochemical active surface area (A), we also estimated by calculating the oxidative current with adapting the Randles-Sevcik equation: $I_p = (2.69 \times 10^5) n^{3/2} A C D^{1/2} \nu^{1/2}$ [37]. From an equation, electrochemical active surface area depended on the oxidative current. The evidence indicates that SPGNE has the highest electrochemical active surface area. Therefore, SPGNE was the most superior electrode in electrochemical activity among these investigated electrodes.

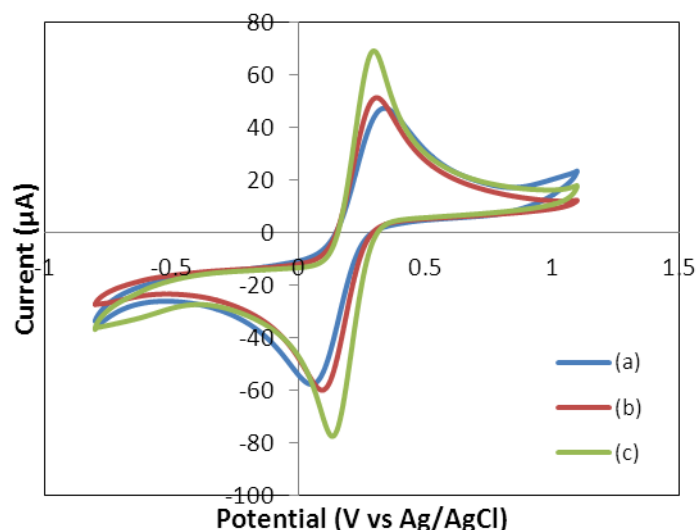


Fig. 2. Cyclic voltammograms of 5mM $\text{K}_3[\text{Fe}(\text{CN})_6]$ in 1 M KCl using (a) commercial electrode, (b) SPGE and (c) SPGNE.

Electrochemical behavior of vitamin A, D, E and K

In order to identify the general features for the electrochemical oxidation of individual fat-soluble vitamins on the proposed electrode surface, the electrochemical behavior of fat-soluble vitamins in a mixture of ethanol and 0.1 M of sodium perchlorate monohydrate (60:40 (v/v)) was investigated using SPGNE by AdSWV technique (data not shown). Fig. 3 displays the square-wave voltammograms of 10 $\mu\text{g mL}^{-1}$ vitamin A, D, E and K in the potential window from 0 V to 1.5 V. Curve (a) shows the square-wave voltammogram of a mixture of ethanol and 0.1 M of sodium perchlorate monohydrate in the proportion of 60:40 (v/v) in the absence of fat-soluble vitamins; this voltammogram not shows any peak. Moreover, the background current of SPGNE was found to be low

value because of its low capacitance property. In contrast, when fat-soluble vitamins were added into the supporting electrolyte, as can be seen in curve (b), the oxidation peak of these vitamins were appeared at 0.52 V, 0.74 V, 1.02 V and 1.20 V for vitamin A, D, E and total K, respectively, which were agreed with the peak potentials of individual vitamins. These results indicated that vitamin A, D, E and K could be detected by using this proposed sensor. Furthermore, the unique properties of graphene and Nafion nanocomposite can enhance the oxidation signals of vitamin A, D, E and K. This result illustrates that the use of the SPGNE greatly improved the electrochemical response of vitamin A, D, E and K, suggesting that this electrode will be an excellent sensor for directly simultaneous determination of vitamin A, D, E and K. To obtain the best result, all experimental conditions that influence the experimental results such as the effect of chemical and instrumental factors were next investigated. The results are reported in the following section.

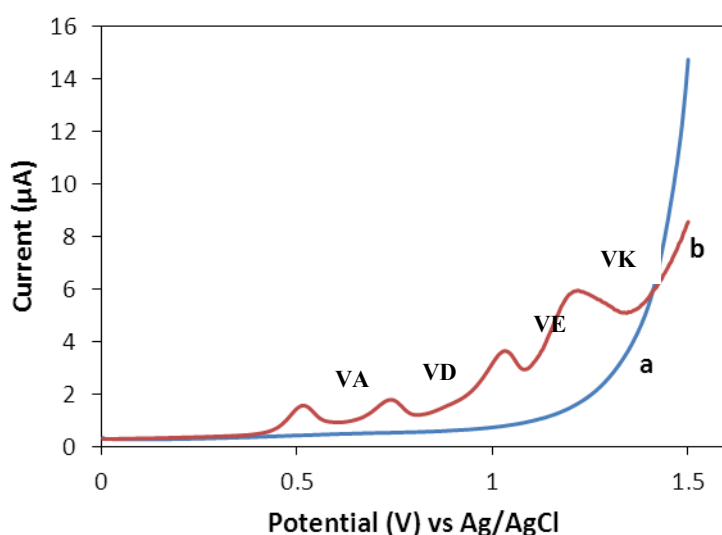


Fig. 3. Square-wave voltammograms of 10 µg mL⁻¹ vitamin A, D, E and K (b) in 60:40 (v/v) of ethanol and 0.1 M of NaClO₄.H₂O and (a) a blank of ethanol and 0.1 M of NaClO₄.H₂O 60:40 (v/v).

In addition, to evaluate whether the mass transfer of the reactant toward the electrode is diffusion or adsorption controlled, the effects of the scan rate on the electrochemical signal were investigated. Linear dependence of the anodic peak current (I_{pa}) of vitamin A, D, E and K versus the square root of the scan rate ($U^{1/2}$) was observed (data not shown). This behavior indicates that the electrochemical oxidation of vitamin A, D, E and K at the SPGNE is a diffusion-controlled process.

Optimization of electrochemical parameters

For selection of the electroanalytical methodology and optimization of parameters, the electrochemical conditions such as the effects of proportion of ethanol, potential increment, amplitude, frequency and quiet time were investigated. These parameters were a crucial factor for sensitivity of the proposed method. The electrochemical oxidations of $10\text{ }\mu\text{g mL}^{-1}$ vitamin A, D, E and K have been studied by AdSWV. For these measurements, the examined parameter was changed value while others were kept constant. The effect of potential increment was studied individually over the potential range from 0.002 V to 0.018 V. The effect of a quiet time is one important parameter which can affect the sensitivity of fat-soluble vitamins detection. For the effect of a quiet time, we examined in the range from 10 s to 500 s. The optimization of amplitude and frequency were carried out in the range from 0.005 V to 0.040 V and 5 Hz to 120 Hz, respectively. The effect of proportion of ethanol was studied in the range from 20% to 80%. The criteria for selection the optimal conditions are not only provided the highest peak current but also well-separated voltammogram of fat-soluble vitamins for the simultaneous detection. After the measurements have been already completed, the optimized conditions were achieved to use proportion of ethanol of 60%, potential increment of 0.010 V, amplitude of 0.040 V, frequency of 100 Hz, and quiet time of 300 s.

Method validation

Analytical performance

Under optimum conditions, the simultaneous determination of vitamin A, D, E and K was estimated by AdSWV with SPGNE in order to verify the analytical performance of this proposed methodology. The calibration curves relationship between the anodic peak currents and different concentrations of each fat-soluble vitamin ranged from $0.02\text{ }\mu\text{g mL}^{-1}$ to $1\text{ }\mu\text{g mL}^{-1}$. The anodic peak current increased linearly as the concentration of fat-soluble vitamins as shown in Fig. 4. Typical calibration curves provide the regression equations of $y = 3.4371x + 0.2354$ for vitamin A, $y = 3.565x + 0.1716$ for vitamin D, $y = 3.0195x + 0.1298$ for vitamin E and $y = 3.7537x + 0.1512$ for total vitamin K. The LOD and LOQ of each fat-soluble vitamin were calculated from $3S_b/S$ to $10S_b/S$, as described in the methods section, and are summarized in Table 1. Compared to previously published methods, particularly in the field of electrochemical analysis, this proposed method not only simultaneously measured fat-soluble vitamins, but also had low detection

limit. The benefits of this work were not only a simple procedure, effective cost, small sample volume, disposability and short time analysis but also offered highly sensitive and potential to apply for the simultaneous determination of fat-soluble vitamins in real samples.

The precision of the analytical process was calculated by determining the relative standard deviation (%RSD) for ten repetitive measurements of solutions that contained the complete set of standard compounds. To evaluate the repeatability and reproducibility of the analytical process, three concentrations (0.06 , 0.20 and $0.80 \mu\text{g mL}^{-1}$) were studied. These spiked concentration levels were chosen to check the results obtained from low, medium and high concentrations with respect to the probable range of interest dietary supplement samples. The RSDs of repeatability were found to vary over the ranges of 7.8 - 11.8% for vitamin A, 3.8 - 9.3% for vitamin D, 9.3 - 11.2% for vitamin E and 3.0 - 9.2% for total vitamin K, respectively, while the RSDs of reproducibility varied over the range of 3.8 - 11.7% for vitamin A, 3.4 - 19.2% for vitamin D, 11.4 - 16.3% for vitamin E and 5.5 - 15.9% for total vitamin K, respectively, which were in acceptable ranges by AOAC guidelines.

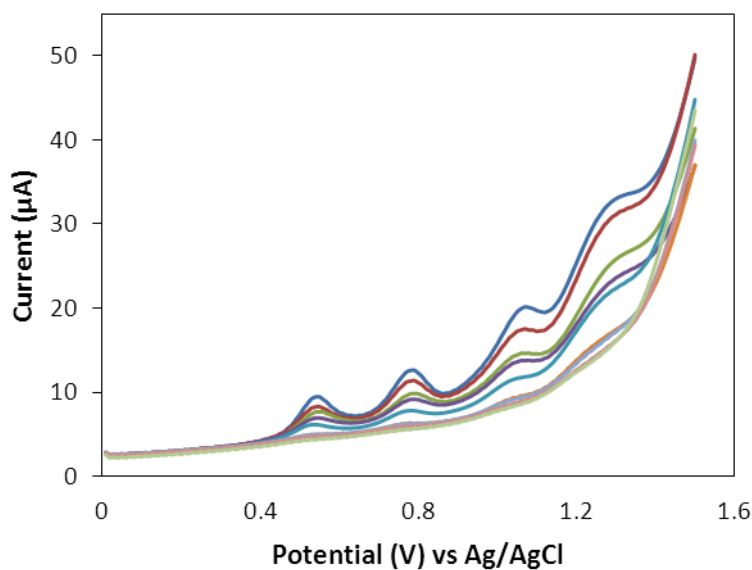


Fig. 4. Square-wave voltammograms of various concentrations of vitamin A, D, E and K: 0.02 , 0.04 , 0.06 , 0.08 , 0.20 , 0.40 , 0.60 , 0.80 and $1 \mu\text{g mL}^{-1}$.

Table 1. Linearity, limit of detection (LOD) and limit of quantification (LOQ) of the proposed method using SPGNE (n=3).

Analyte	Linear range ($\mu\text{g mL}^{-1}$)	Slope ($\mu\text{A}/\mu\text{g mL}^{-1}$)	Intercept (μA)	r^2	LOD ($\mu\text{g mL}^{-1}$)	LOQ ($\mu\text{g mL}^{-1}$)
Vitamin A	0.02-1	3.4371	0.2354	0.9959	0.0086	0.0286
Vitamin D	0.02-1	3.5650	0.1716	0.9972	0.0063	0.0211
Vitamin E	0.02-1	3.0195	0.1298	0.9940	0.0075	0.0249
Total vitamin K	0.02-1	3.7537	0.1512	0.9915	0.0071	0.0238

Sensitivity

The effects of various concentrations of vitamin A, D, E and K were studied by varying the concentrations in the range of 0.02-1 $\mu\text{g mL}^{-1}$ of only one standard solution of fat-soluble vitamins while the concentrations of other fat-soluble vitamins were kept constant at 0.10 $\mu\text{g mL}^{-1}$. The regression equations of fat-soluble vitamins are shown in Table 2. As evident in the table, the slopes of fat-soluble vitamins were consistent with linearity study of the simultaneous measurement of fat-soluble vitamins and absolute correlation coefficient values obtained were greater than 0.9500. According to the results, each fat-soluble vitamin did not affect the simultaneous detection. Therefore, these results confirmed that the proposed method provides high sensitivity for the simultaneous determination of vitamin A, D, E and K in real samples.

Table 2. Linearity of sensitivity analysis.

Analyte	Linear range ($\mu\text{g mL}^{-1}$)	Slope ($\mu\text{A}/\mu\text{g mL}^{-1}$)	Intercept (μA)	r^2
Vitamin A	0.02-1	3.5669	0.0848	0.9990
Vitamin D	0.02-1	3.4624	0.1669	0.9973
Vitamin E	0.02-1	3.1644	0.1692	0.9706
Total vitamin K	0.02-1	4.0891	0.2076	0.9615

Analytical application

In order to verify the applicability of SPGNE and the proposed method, the fabricated electrode was used for the simultaneous determination of vitamin A, D, E and K in dietary supplement using AdSWV technique. Supplement samples from local Japanese supermarket were investigated by standard addition. For accuracy and precision of this method, the percentages of recoveries and RSDs were examined by spiking three concentrations of 0.1, 0.2 and 0.4 $\mu\text{g mL}^{-1}$, respectively. The results were shown in Table 3. The recoveries and RSDs were found to be in acceptable ranges by AOAC guidelines. Additionally, the results obtained from our proposed method were compared to those obtained from labeled by the paired *t*-test at a 95% confidence interval. The paired two-tail test showed calculated *t*-values 0.66 below the critical *t*-value 2.36. These results indicate that the proposed method is acceptable and reliable for the determination of vitamin A, D, E and K in supplement samples. Therefore, this developed method provided high accuracy and precision that can be used as an alternative choice for quality control of fat-soluble vitamins in dietary supplements.

Table 3. Results obtained from the simultaneous determination of fat-soluble vitamins in dietary supplement samples.

Analyte	Added ($\mu\text{g mL}^{-1}$)	Founded ($\mu\text{g mL}^{-1}$)	Recovery (%)	RSD (%)
Vitamin A	0.1	0.156 ± 0.016	96.83	10.6
Vitamin D	0.1	0.090 ± 0.007	80.00	7.4
Vitamin E	0.1	0.087 ± 0.003	77.19	3.5
Total vitamin K	-	-	-	-

Vitamin A	0.2	0.268 ± 0.016	108.75	5.8
Vitamin D	0.2	0.212 ± 0.012	101.01	5.8
Vitamin E	0.2	0.197 ± 0.019	93.45	9.9
Total vitamin K	-	-	-	-
Vitamin A	0.4	0.372 ± 0.017	80.62	4.5
Vitamin D	0.4	0.433 ± 0.006	105.88	1.4
Vitamin E	0.4	0.398 ± 0.003	97.01	0.7
Total vitamin K	-	-	-	-

Conclusions

Application of using a novel, simple, rapid, highly sensitive and selective, and low-cost electrochemical sensor for the simultaneous determination of vitamin A, D, E and K using SPGNE was successfully demonstrated. Furthermore, this method did not require any separation steps and was directly applied for the simultaneous determination of vitamin A, D, E and K in dietary supplement samples. Composite of graphene and Nafion can significantly enhance the anodic peak currents of each fat-soluble vitamin at different potentials. Under the optimal conditions, the simultaneous determination of fat-soluble vitamins was received with a wide linear range ($0.02\text{--}1\ \mu\text{g mL}^{-1}$) and low detection limits. The methodology was sensitive, selective, accurate and precise for real dietary supplement samples, and results were in good agreement with the labeled. Therefore, this developed sensor provides high capability as an alternative choice for quality control of fat-soluble vitamins in the pharmaceutical industry.

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The effect of supporting electrolyte for the simultaneous determination of vitamin B₂, vitamin B₆, and vitamin C using a modification-free screen-printed carbon electrode

To open a new choice and to replace the complicated steps of a modified electrode, this research reported on the systematic investigation of the type of supporting electrolyte that affected to the electrochemical behavior of vitamin B₂ (VB₂), vitamin B₆ (VB₆), and vitamin C (VC) using a common screen-printed carbon electrode (SPCE) to obtain the simultaneous detection. The role of various well-known supporting electrolytes, including Britton-Robinson buffer solution (BRBS), acetate buffer solution (ABS), and phosphate buffer solution (PBS), on the electrochemical oxidation of VB₂, VB₆, and VC were carefully accomplished by square wave voltammetry (SWV). From this successive investigation based on the ionic conductivity of species containing in a supporting electrolyte, PBS at pH 3.5 was selected as a suitable supporting electrolyte for the simultaneous detection of VB₂, VB₆, and VC. Under the optimized conditions, the simultaneous detection of VB₂, VB₆, and VC provided satisfactory sensitivity, selectivity, and reproducibility despite using a common electrode. To demonstrate the practicality and reliability of the proposed analytical procedure, the simultaneous detection of VB₂, VB₆, and VC was performed in mixed vegetable and fruit juice and urine sample by SWV and the results were compared with chromatographic detection. Therefore, this finding is simple and could be a new alternative way for the simultaneous determination of VB₂, VB₆, and VC in practical applications.

Introduction

A supporting electrolyte (inert electrolyte or inactive electrolyte) is a necessary solution to preserve the conductivity and ionic strength in the electrochemical system. Chemical species containing in a supporting electrolyte are required to have a non-electroactive property within the applied potential window and their conductivities are considerably larger than those of the electroactive species or analytes.¹ The essential roles of a supporting electrolyte are to minimize the resistance of an electrochemical cell to eradicate the transportation of electrochemical species caused by migration effect in the electric field and to constrain the interfacial potential difference to the closest distance approach of solvated ions to the electrode.^{2, 3} These phenomena will diminish

uncompensated iR drop, which causes the potential-control error and the ohmic heating of a solution to acquire a factual potential measurement.⁴

Normally, the concentration of ionic species in a supporting electrolyte is at least 50-100 times of the electroactive species and is the significant source of electrically conducting ionic species.⁵ It can be inorganic or organic salt, an acid or a base, a buffer solution such as acetate, carbonate, and phosphate. Among these supporting electrolytes, buffer solutions are tremendously consumed in the electrochemical system because of the ability for preservation pH at a virtually constant value. With the advantageous property of buffer solutions, numerous reports in an electroanalytical application select the buffer solutions serving as supporting electrolytes for the detection of interesting electroactive species, especially for vitamin detection.⁶⁻⁸

Vitamins are a disparate group of compounds which are essential nutrients for human.⁹ They are classified into 2 groups by their solubility, which are water-soluble vitamins (vitamin B and vitamin C) and fat-soluble vitamins (vitamin A, vitamin D, vitamin E, and vitamin K). They play important roles in various biological functions. Vitamin B₂ (VB₂) is an important component of a flavoenzyme that catalyzes a variety of biochemical reactions of carbohydrates, protein, and fat in the human body. It promotes the growth process and reproductive system; strengthens the visibility and relieves the fatigue of eyes; and prevents inflammation of the mouth, lips, and tongue.¹⁰ Vitamin B₆ (VB₆) involves many aspects of macronutrient metabolism, histamine synthesis, hemoglobin synthesis, and gene expression. It participates in activities of many enzymes and maintenance of skin as well as nervous and immune systems.¹¹ Vitamin C (VC) is an antioxidant that is necessary for the growth, development, and repairing of all tissues. It is involved in many functions of the human body which are the formation of collagen, the absorption of iron, the immune system, the wound healing, and the maintenance of cartilage, bones, and teeth.¹² These mentioned water-soluble vitamins have to receive daily from food. Moreover, they are not stored in the human body because of the urinary excretion. Therefore, the determination of vitamins in real samples is important for various applications to monitor the quantities of vitamins in consuming products and to indicate abnormalities or diseases.

Such the importance of those mentioned vitamins, various analytical methods were developed and proposed for the determination of VB₂, VB₆, and VC in both individual and simultaneous measurements including high-performance liquid chromatography,¹³ spectrophotometry,¹⁴ chemiluminescence,¹⁵ and electrophoresis.¹⁶ Unfortunately, these

techniques still had some limitations despite their high sensitivity and selectivity for detection. For example, they were time-consuming, high cost for maintenance, complicated operation, required a large volume of solution and had several pre-concentration steps. Consequently, the other novel methodology has to explicate successively for VB₂, VB₆, and VC detection to achieve the objective of simple practicality in various field analysis.

For twenty-first-century analytical experimentation, the electrochemical method is another choice for the simultaneous determination of VB₂, VB₆, and VC due to its simplicity for operation, rapid results, direct measurement, high sensitivity and selectivity. From previous electrochemical researches,¹⁷⁻²⁷ the detection of VB₂, VB₆, and VC was determined principally by various modified electrodes which provided high precision and high sensitivity. However, they were not suitable for some applications because of some disadvantages, such as high-cost electrodes, take a long time for modification with complicated steps, and require a lot of solution volume for analysis. The most importance is that it is hard to hit the goal for field analysis and also suffered from surface modification. Therefore, the selection of a suitable, simple, and inexpensive electrode platform is currently significant for the application in real sample analysis to increase the performance of the analytical method.

Nowadays, the furtherance of screen-printing technology for the fabrication of screen-printed electrodes (SPEs) have been enormously attracted the attention due to their advantageous properties over conventional electrodes,²⁸ such as cost-effectiveness, disposability, simplicity, versatility, availability in materials and patterns, elimination of electrode maintenance, low volume of solution requirement, and appropriate for outside laboratory measurement. For these reasons, these devices were applied tremendously for electrochemical analysis in environmental monitoring,²⁹ clinical diagnoses,³⁰ and food safety assessment.³¹ Various reports demonstrated that the electrochemical devices were fabricated generally on paper as a substrate,³²⁻³⁴ so-called electrochemical paper-based analytical devices (ePAD). The main advantages of ePAD are simple, cost-effective, portable, and disposable.³⁵ However, apart from paper, other substrates are additionally consumed as platforms for the fabrication of SPEs, such as acrylic, ceramic, and PVC sheet. To ascertain the different platform for the development of an electrochemical device, commercial plastic sheets (OHP films) are recently employed to fabricate an electrode platform, named transparent film-based analytical device (TFAD).³⁶ Because of their compatibility with screen-printing technology, easily available in a local

stationery store and low cost, therefore, they are considerably noticeable for the fabrication of screen-printed electrodes.

Herein, the main objective of this research was to focus on the effect of supporting electrolyte on the simultaneous detection of VB₂, VB₆, and VC using a common screen-printed carbon electrode (SPCE) to obtain a simple way for detection and to overcome the cost of analysis as well as the complex modification from using various modern materials. Also, we proposed the manually fabricated of SPCE using a transparent-film substrate, named electrochemical transparent-film based analytical device (eTFAD), which was commercially available in a local stationery store. Under the optimal conditions, the overall electrochemical experiment was rapid, convenient, and uncomplicated in comparison to those obtained from previous works. Besides, the proposed procedure was sensitive and selective for the determination of those amounts of selected vitamin presence in real samples. For the various practical applications, the developed electrochemical electrode was successfully applied for the determination of mentioned vitamins in a mixed vegetable and fruit juice, and an artificial urine sample to demonstrate the reliability of the proposed analytical system. The results obtained show the effectiveness of method for monitoring the quantities of vitamins in consuming products or using for indicating the abnormalities and diseases. This finding could be an alternative way to open the new vision for application obtained from a basic concept and to encourage the researcher for creating their work by using their basic instruments to get rid of the limitations.

Experimental

Chemicals and reagents

All chemicals were used as received without further purification. Carbon paste (C2130307D1) and silver/silver chloride paste (C2130809D5) were purchased from Gwent Group (Torfaen, United Kingdom). Riboflavin (vitamin B₂, VB₂) and L-ascorbic acid (vitamin C, VC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pyridoxine hydrochloride (vitamin B₆, VB₆) was purchased from Hi-Media Laboratories Pvt., Ltd. (India). Sodium dihydrogen orthophosphate (NaH₂PO₄·2H₂O) and di-Sodium hydrogen orthophosphate (Na₂HPO₄·2H₂O) were acquired from Ajax Finechem Pty., Ltd. (New South Wales, Australia). Ethanol and ortho-phosphoric acid (H₃PO₄, 85% purity) were acquired from Merck (Darmstadt, Germany). Acetone (commercial grade) was purchased from CaHC (Thailand) Co., Ltd. (Bangkok, Thailand).

All solutions were prepared using Milli-Q water from a Millipore water purification system ($R = 18.2 \text{ M}\Omega \text{ cm}$ at 25°C). All of stock standard solutions (1 mM) were weekly prepared in water and kept in the dark at -20°C . As for working standard solutions preparation, the stock standard solution was diluted with supporting electrolyte and mixed using a vortex mixer. Phosphate buffer solution (PBS), which was served as a supporting electrolyte, was prepared by mixing 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4 and adjusted to the desired pH with 0.1 M H_3PO_4 . Britton-Robinson buffer solution (BRBS) was prepared by mixing equal volumes of 0.04 M CH_3COOH , 0.04 M H_3BO_3 and 0.04 M H_3PO_4 that has been adjusted to the desired pH with 0.2 M NaOH. Acetate buffer solution (ABS) was also prepared by mixing 0.1 M CH_3COOH and 0.1 M CH_3COONa and adjusted to the desired pH with 0.1 M HCl or 0.1 M NaOH.

Fabrication of electrochemical transparent-film based analytical device (eTFAD)

The electrode pattern was designed using Adobe Illustrator CC 2018. To obtain the electrode blocks for screening, the designed patterns were built by Chaiyaboon Brothers Co., Ltd. (Bangkok, Thailand). Screen-printed electrodes were fabricated by using a manual screen-printing method on the transparent sheet as a substrate which was described in the previous report.³⁷ Briefly, the clean transparent sheet was screened by carbon paste to obtain working electrodes and counter electrodes. Then, these electrodes were placed in an oven at a temperature of 55°C for 30 min to remove the solvent and to dry electrodes. Next, silver/silver chloride (Ag/AgCl) paste was screened on the same transparent sheet to obtain reference electrodes and the conductive pads. After that, they were placed in an oven at a temperature of 55°C for 30 min. Finally, the common screen-printed carbon electrodes (SPCE) were obtained and ready to use for an experiment. The picture of the obtained eTFAD was shown in Fig. 1.

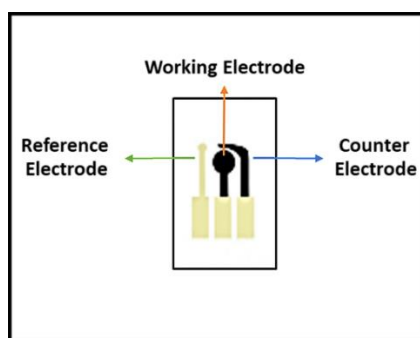


Fig. 1. A picture of electrochemical transparent film-based analytical device (eTFAD) consisting of screen-printed working, reference, and counter electrodes.

Electrochemical measurement

The electrochemical experiments were performed by potentiostat/galvanostat PGSTAT128N from Metrohm- Autolab (Switzerland) with the NOVA 1.10 software (Kanaalweg 29-G 3526 KM Utrecht, The Netherlands). A three-electrode system was fabricated on a transparent sheet which was employed throughout an experiment. The geometric area of a working electrode was 0.126 cm^2 (calculated from circular area = πr^2 ; $r = 0.2 \text{ cm}$). For the electrochemical measurement, 100 μL aliquot of a standard or a sample solution was thoroughly dropped covering all three electrodes onto the detection zone. After that, electrochemical techniques including cyclic voltammetry (CV) or square wave voltammetry (SWV) were conducted. For CV measurement, the potential was applied over the range from -1.1 V to 1.3 V with a scan rate of 100 mV/s. Similarly, SWV measurement was also performed for the simultaneously quantitative determination by applying the potential from -1.0 V to 1.3 V with a step potential of 5 mV, an amplitude of 25 mV, and a frequency of 5 Hz.

Sample preparation

To demonstrate the probability of the proposed electrode for application, two types of samples including mixed vegetable and fruit juice and artificial urine were investigated for the simultaneous determination of water-soluble vitamins.

The mixed vegetable and fruit juice were purchased from a convenience store in Thailand. 5 mL of juice sample was added into a test tube. Then, the sample was centrifuged for 15 minutes at 2500 rpm followed by filtration through a filter paper and kept in a dark container at 4°C. After that, samples were spiked at various concentrations of standard vitamins and the spiked solution was diluted 10-fold with PBS pH 3.5 (a suitable dilution order) and measured the quantity using the SWV technique as followed by the topic 2.3. Then, the average recovery percentage was calculated. Artificial urine was purchased from Carolina Biological Supply Company (Burlington, USA). For the sample preparation, the artificial urine and spiked artificial urine were merely diluted 1000-fold with PBS pH 3.5 which is a suitable dilution order for this sample. After that, the samples and spiked samples were quantitatively measured by using the same procedure to mixed vegetable and fruit juice.

Results and discussions

Electrochemical behavior for the simultaneous detection of VB₂, VB₆, and VC

Cyclic voltammetry was initially used to investigate the electrochemical properties and the possibility of simultaneous detection of VB₂, VB₆, and VC. To examine the oxidation or reduction mechanism of selected vitamins on a common SPCE surface, cyclic voltammograms of VB₂, VB₆, and VC in Britton-Robinson buffer solution (BRBS) at pH 7 were recorded at a scan rate of 100 mV/s. The obtained voltammograms from both individual and simultaneous detection were shown in Fig. 2. The cyclic voltammogram of VB₂ shown in Fig. 2A exhibited a pair of sharp and well-behaved redox peaks at -0.60 V and -0.75 V for anodic and cathodic peaks, respectively. This result revealed that VB₂ could display a reversible property because of the ratio between anodic and cathodic current is around 1.0. However, the separation of anodic and cathodic peak potential is around 0.15 V which is greater than $0.059/n$ V of the reversible process (n is a number of electron transfer). The result might be attributed to the fact that VB₂ provided a slow rate for the electron transfer reaction.³⁸ The cyclic voltammogram of VC depicted in Fig. 2B displayed a sharp and strong oxidation peak at 0.35 V with a peak current of 27.65 μ A. This result indicated that VC occurs an irreversible process at an electrode surface. Similarly, VB₆ also occurs an irreversible process due to the presence of a small and broad oxidation peak at 0.82 V as demonstrated in Fig. 2C. From these results, it could be concluded that the possibility for the simultaneous detection of VB₂, VB₆, and VC can be performed on the proposed analytical electrode because of the explicit distinction of peak potential position. Subsequently, the simultaneous detection of VB₂, VC, and VB₆ was also investigated as shown in Fig. 2D. From the voltammogram, VB₂ exhibited a pair of outstanding redox peaks at -0.48 V and -0.61 V for anodic and cathodic peaks, respectively. Meanwhile, VC and VB₆ provided a broad anodic peak at 0.41 V and 0.90 V, respectively. The obtained voltammogram confirmed that the simultaneous detection of VB₂, VC, and VB₆ in oxidative direction is possible owing to the distinction of peak potential position as same as anticipated results obtained from the individual detection. The discovered results are in good agreement with the previous critical review of all vitamins,³⁹ therefore, the simultaneous detection of VB₂, VC, and VB₆ can be performed using a common SPCE.

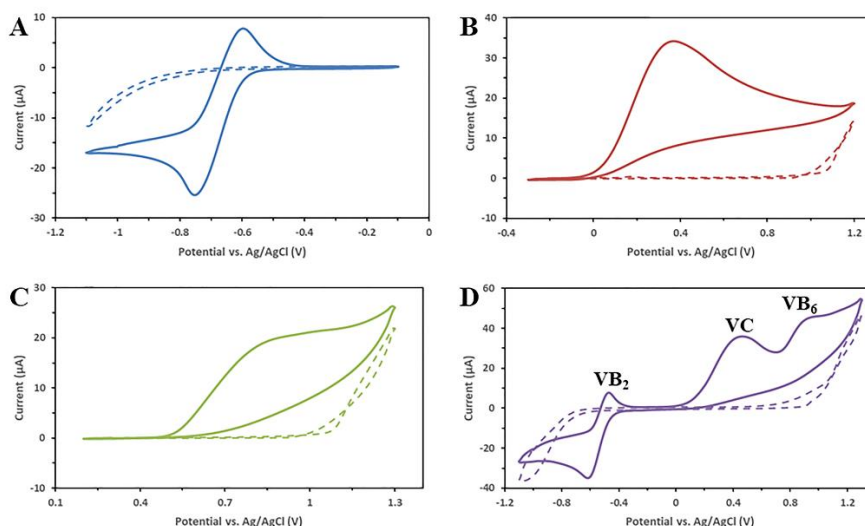


Fig. 2. Cyclic voltammograms obtained from common SPCEs in absence (dash line) and presence (solid line) of 1 mM VB₂ (A), 1 mM VC (B), 1 mM VB₆ (C), and the mixture solution of 1 mM VB₂, 1 mM VC, and 1 mM VB₆ (D). BRBS pH 7 was served as a supporting electrolyte. All cyclic voltammograms were recorded at a scan rate of 100 mV/s.

Influence of pH and supporting electrolyte on the electrooxidation of VB₂, VC, and VB₆

The effects from adjusting the pH values of supporting electrolyte on the oxidative behavior of VB₂, VC, and VB₆ disclose a significant information about the mechanisms of electrochemical reactions at a common SPCE. Therefore, the influences of pH and supporting electrolyte on the electrooxidation of VB₂, VC, and VB₆ were carefully investigated by square wave voltammetry. The suitable pH conditions were preliminarily studied using BRBS at pH 5, 7, and 9 as supporting electrolytes. The obtained voltammograms from the simultaneous detection were shown in Fig. S1. With increasing pH value, the anodic currents of VB₂, VC, and VB₆ were decreased obviously. We believed that in our case, the acidic supporting electrolyte would strongly affect to a higher current. By considering the pK_a values of each vitamin, the results might be attributed to the fact that ionic forms of target analytes affect the electrooxidation processes at the electrode surface. According to data obtained from the literature,⁴⁰ the pK_a values of each vitamins are the following: VB₂ = -0.2 and 6.0, VC = 4.2, and VB₆ = 5.6 and 9.4. These values indicated that the three compounds can exist in their ionic forms in aqueous solutions. Fig. S2 shows the charge versus pH curves (CurTiPot software)⁴¹ for VB₂, VC, and VB₆ molecules. As can be observed, VB₂ and VB₆ are amphoteric molecules (pI =

2.9 and 7.5, respectively) which can exist in cationic, anionic, and neutral forms. On the contrary, VC presents a weak acid character and is a predominantly negatively charged in solutions with a pH higher than 6.5. As the result shown in Fig. S1, vitamins in BRBS pH 5 provided the highest anodic currents. It could be reasoned that VB₂, VC, and VB₆ exist predominantly in neutral, anionic, and cationic forms, respectively. We assumed that these ionic forms easily occur electrons transfer at an electrode surface. On the other hand, anodic currents decreased when vitamins dissolved in BRBS pH 7 and 9 because VB₂ and VB₆ exist predominantly in cationic and neutral forms, respectively. VC, in the meanwhile, exists completely in anionic forms. These ionic forms in neutral and basic supporting electrolytes hardly occur electron transfer at an electrode surface. From these results, it can be concluded that acidic supporting electrolyte is a suitable electrolyte for the detection of VB₂, VC, and VB₆.

Afterward, the type of supporting electrolytes was investigated at fixed pH 5 to select the appropriate electrolyte for the detection of VB₂, VC, and VB₆. As mentioned previously, based on electrochemical researches, buffer systems were universally served as supporting electrolytes due to the ability of pH regulation. In this research, buffer systems at pH 5 consisting of Britton-Robinson buffer solution (BRBS), acetate buffer solution (ABS), and phosphate buffer solution (PBS) were studied by square wave voltammetry for the selection of appropriate supporting electrolyte. Fig. S3 shows square wave voltammograms of a mixture solution in various buffer systems at pH 5. By considering anodic currents, PBS at pH 5 provided the highest responses. We believed that in our case, the buffer properties, particularly in electrical conductivity, would get involved in the electrooxidation of these vitamins. Consequently, the electrical conductivity of species containing in the proposed buffer system was discussed as reported in references.⁴²⁻⁴³ To deeply consider, PBS at pH 5 consisted of H₃PO₄, NaH₂PO₄, and Na₂HPO₄ which each provided the specific conductance (20°C) of 5.5, 2.2, and 4.6 mS/cm, respectively. These values were higher than other species containing in other buffer systems. For example, ABS at pH 5 containing of CH₃COOH and CH₃COONa provided the specific conductance (20°C) of 0.3 and 3.9 mS/cm, respectively. Similarly, BRBS at pH 5 containing of H₃PO₄, CH₃COOH, and H₃BO₃ provided the specific conductance (25°C) of boric acid of 0.008 mS/cm. It could be ascribed that the more specific conductance was better electrical conductivity. As a result, the resistance of the electrochemical cell could decrease and eliminate the electromigration effects which were desired in controlled-potential experiments.

Accordingly, it can be concluded that PBS in acidic pH value is a suitable electrolyte for the detection of VB₂, VC, and VB₆.

To confirm the effect of acidic electrolyte, the influence of solution pH on the electrochemical behavior of VB₂, VC, and VB₆ in PBS using a common SPCE was studied in specific pH ranges from 3.5 to 6.5. Fig 3A shows the anodic peak potentials (E_{pa}) for these vitamins and their linear regression equations: $E_{pa} (V) = -0.0533pH - 0.295$ ($r^2 = 0.9929$) for VB₂; $E_{pa} (V) = 0.0556pH + 0.0334$ ($r^2 = 0.9901$) for VC; and $E_{pa} (V) = -0.054pH + 1.0377$ ($r^2 = 0.9925$) for VB₆. According to the Nernst equation (Eq. 1), which explains the relationship between E_{pa} and pH, where n and m represent the numbers of electrons and protons involved in the reaction, and a and b are coefficients of oxidant and reductant in the reaction.⁴⁴

$$E_{pa} = E^0 + \left(\frac{0.0591}{n} \right) \log \left[\frac{(OX)^a}{(R)^b} \right] - \left(0.0591 \frac{m}{n} \right) pH \quad \text{Eq. 1}$$

All of the slopes between E_{pa} and pH of each vitamin were very imminent to 59 mV/pH at 25°C, indicating that the overall electrochemical process at the proposed electrode was proton-dependent-the electron transfer which was regulated by a transfer of an equal number of protons and electrons.

Furthermore, the anodic peak currents (I_{pa}) as a function of pHs were also investigated as presented in Fig. 3B. The anodic currents of VB₂, VC, and VB₆ do not significant distinction with increasing pH value until it reaches 6.5. Consequently, the difference in anodic peak potential between VC and VB₆ was primary considered to select the appropriate pH value. The difference of anodic peak potential between these two vitamins decreases with increasing pH value leading to the overlapping of anodic peaks. PBS at pH 3.5 provided the highest difference of anodic peak potential, therefore, it was chosen as an appropriate pH value for the simultaneous detection of VB₂, VC, and VB₆. We supposed that due to these vitamins dissolved in the acidic solution (pH 3.5) in which VB₂ and VC predominantly exist in neutral forms, meanwhile, VB₆ is positively charged because of the protonation of nitrogen in the pyridine ring. These molecules and ionized form can be oxidized at differently applied potential using a common SPCE.

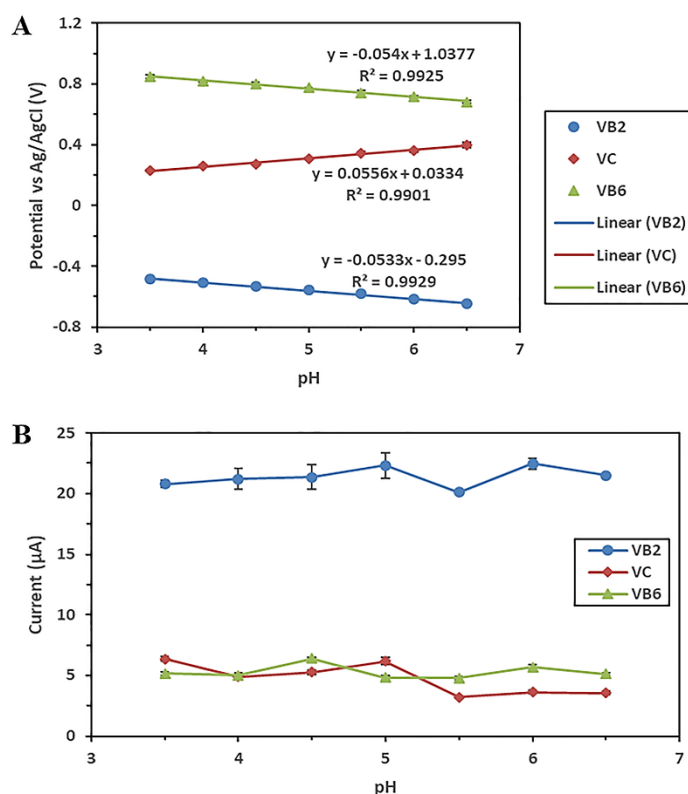


Fig 3. Influences of solution pH on anodic peak potentials (A) and peak currents (B) for the oxidation of 100 μ M VB₂, 400 μ M VC, and 400 μ M VB₆ at a common SPCE.

The effect of scan rate on the electrooxidation of VB₂, VC, and VB₆

To elucidate whether the mass transfer of the analyte toward the electrode surface is diffusion or adsorption controlled, the effect of scan rate on the electrooxidation of VB₂, VC, and VB₆ was investigated by cyclic voltammetry. Fig. 4A shows cyclic voltammograms of VB₂, VC, and VB₆ at a common SPCE using different scan rates. The results presented that the redox peak currents for VB₂ increased with increasing the scan rate, while their redox peak potentials remained the same values, suggesting the electron transfer process was reversible. On the other hand, the anodic peak currents for VC and VB₆ increased with increasing the scan rate, however, their anodic peak potentials gradually shifted to positive values, introducing the electron transfer processes were irreversible. The linear dependence of the anodic peak current of all three vitamins versus the square root of the scan rate was observed as displayed in Fig. 4B. These behaviors demonstrate that the electrochemical oxidations of all three vitamins at a common SPCE are diffusion-controlled processes.⁴⁵

Using the information received from the linear regression of the relationship between anodic peak currents (I_{pa}) and the square root of scan rates ($V^{1/2}$), the

relationship as shown in Eq. 2 was applied to estimate the diffusion coefficients (D_0) of all three vitamins.⁴⁶

$$I_{pa} = 0.496FAC_s D_0^{1/2} \left(\frac{F}{RT} \right)^{1/2} v^{1/2} \quad \text{Eq. 2}$$

When F is the Faraday constant, A is a geometric area of the working electrode (0.126 cm^2), C_s is the concentration of vitamins ($1 \times 10^{-6} \text{ mol/cm}^3$), D_0 is the diffusion coefficient of vitamins, R is a gas constant (8.31447 J/mol/K), and T is a temperature (298 K). The calculated D_0 values of VB₂, VC, and VB₆ were found to be 8.07×10^{-6} , 5.32×10^{-6} , and $1.57 \times 10^{-7} \text{ cm}^2/\text{s}$, respectively. Then, the number of electrons (n) involved in the electrooxidation of VB₂ was determined by the Randles-Sevcik relationship for a reversible process (Eq. 3).

$$I_{pa} = (2.69 \times 10^5) n^{3/2} AC_s D_0^{1/2} v^{1/2} \quad \text{Eq. 3}$$

Using Eq. 3 and the previously estimated D_0 of VB₂, the number of electrons involved in the electrooxidation of VB₂ can be calculated and was found to be $n = 1.07$ ($n \sim 1$). Next, the number of electrons involved in the electrooxidation of VC and VB₆ was determined by the Randles-Sevcik relationship for the irreversible process (Eq. 4).

$$I_{pa} = (2.99 \times 10^5) n [(1-\alpha)n]^{1/2} AC_s D_0^{1/2} v^{1/2} \quad \text{Eq. 4}$$

To calculate the n value, it is unavoidable to solve initially the factor of $(1-\alpha)n$. Using the Laviron theory for oxidative reaction (Eq. 5),⁴⁷ the calculation is based on the linear relationship between anodic peak potential (E_{pa}) and the natural logarithm of scan rates ($\ln v$), as shown in Fig. 4C. Depended on Eq. 5 and the slopes received from the linear relationship between the E_{pa} and $\ln v$, the factors of $(1-\alpha)n$ for VC and VB₆ were found to be 0.572 and 1.00, respectively.

$$E_{pa} = E^0 + \frac{RT}{(1-\alpha)nF} \ln \frac{RTk_s}{(1-\alpha)nF} + \frac{RT}{(1-\alpha)nF} \ln v \quad \text{Eq. 5}$$

After calculating the factors of $(1-\alpha)n$, using Eq. 4 and the formerly estimated D_0 of VC and VB₆, the number of electrons involved in the electrooxidation of VC and VB₆

can be calculated and were found to be $n = 1.32$ ($n \sim 2$) and $n = 0.998$ ($n \sim 1$), respectively.

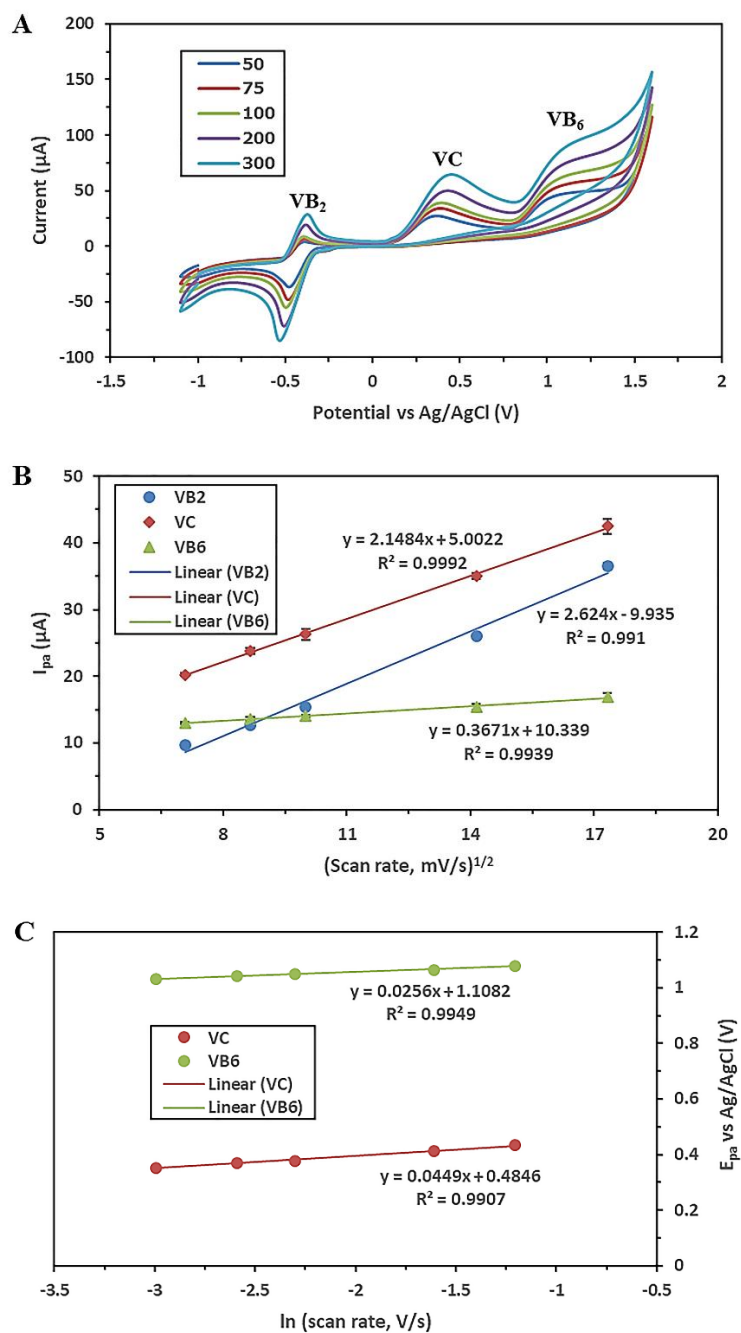


Fig. 4. (A) Cyclic voltammograms for 1 mM VB₂, 1 mM VC, and 1 mM VB₆ in PBS pH 3.5 at the common SPCE using a series of scan rate (50, 75, 100, 200, and 300 mV/s). (B) The relationship between the anodic peak currents (I_{pa}) and the square root of scan rates. (C) The variation of anodic peak potential (E_{pa}) and natural logarithm of scan rates ($\ln V$) from 50 to 300 mV/s.

Analytical performance

Square wave voltammetry was selected for the simultaneous determination of VB₂, VC, and VB₆ in mixture solutions due to its better resolution and higher sensitivity than cyclic voltammetry. Under the optimized experimental conditions, square wave voltammograms for the electrooxidation of different concentrations of VB₂, VC, and VB₆ mixture at a common SPCE were obtained, as shown in Fig. 5A. The results presented that the well-defined anodic peaks of all three vitamins are separated sufficiently to discriminate and measure the current. Besides, the increment in the peak currents of all three vitamins can be observed with increasing their concentrations at the same time. Fig. 5B displays the calibration curves of all three vitamins, indicating that the anodic peak currents for the electrooxidation of all three vitamins increased proportionally with their corresponding concentrations. From these results, the linear ranges for the simultaneous determination of VB₂, VC, and VB₆ were found to be 1-60 μ M, 10-400 μ M, and 10-400 μ M with the detection limit (calculated as the 3 σ value of the blank) of 0.37 μ M, 5.07 μ M, and 3.32 μ M, respectively. Comparison of the analytical performance to previous works and this work was shown in Table 1. Even though, detection limits and linear ranges of the proposed system are not as low as those received by other modified electrodes. However, we first proposed the simultaneous determination of VB₂, VC, and VB₆ using a common SPCE by systematically studied all experimental parameters that affected to peak separation and sensitivity. Our proposed methodology provided various advantages, such as a low-cost electrode, the modification-free, require a small sample volume for analysis. Moreover, this proposed system is easy to apply for commercialization the electrode in the future.

To investigate the practicality of a common SPCE for the simultaneous determination of VB₂, VC, and VB₆, the individual determination of each vitamin was performed by varying the concentration of one analyte while those of the others have remained constant. The results in Fig. S4A display that the anodic peak currents of VB₂ increased with concentration while the anodic peak currents of VC and VB₆ were equally unaltered at these concentrations as shown in Fig. S4B. In the same way, as can be seen in Fig. S4C and D, the anodic peak currents of VC increased linearly with increasing the concentrations. Similarly, Fig. S4E and F show that the anodic peak current signals for VB₆ also increased with increasing the concentrations. By comparison between anodic peak currents and concentrations from individual and simultaneous determinations, the slopes of each vitamin were virtually unchanged. It could be emphasized that the

electrooxidation processes of each vitamin are independent, therefore, the simultaneous determinations of these vitamins are feasible without any interference from their presence together.

The reproducibility of all three vitamins at various concentrations was calculated in the term of relative standard deviation (RSD) by using seven different electrodes ($n = 7$). The value was lower than 6.5% which can be acceptable.⁴⁸ As shown in Table S1, indicated that this electrode can be successfully employed for the simultaneous determination of water-soluble vitamins with excellent reproducibility.

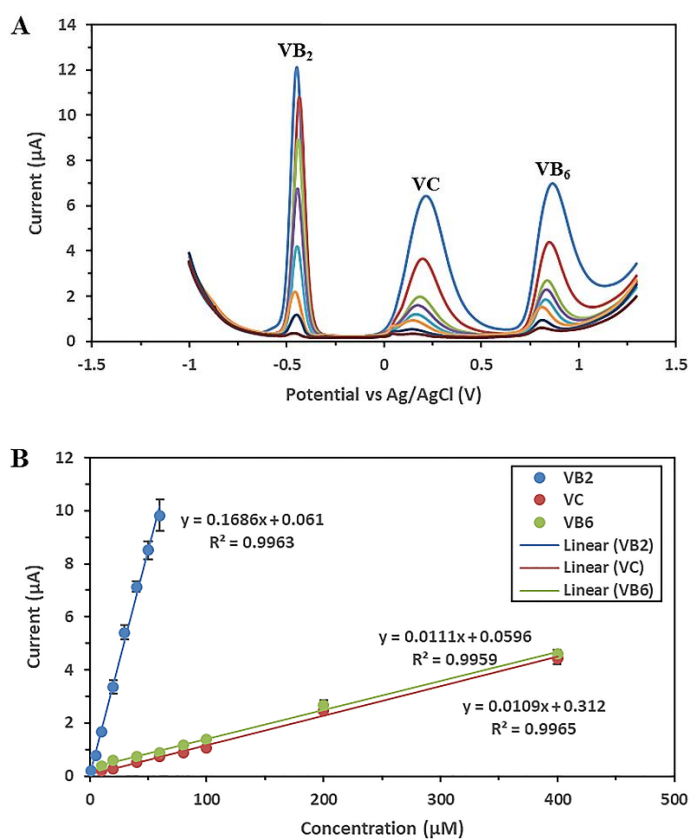


Fig. 5. (A) Square wave voltammograms of VB₂, VC, and VB₆ in concentration ranges of 1-60 μM, 10-400 μM, and 10-400 μM, respectively, in PBS pH 3.5 at common SPCEs. (B) Calibration plots between anodic peak currents of each vitamin versus concentration.

Table 1

Comparison of the analytical performance between previous works for the simultaneous determination of VB₂, VC, and VB₆.

Working electrode	Technique	Detection limit (μM)			Linear range (μM)			Ref.
		VB ₂	VC	VB ₆	VB ₂	VC	VB ₆	
PGCE	DPV	0.001	-	0.8	0.005-100	75-75000	2.5-7500	[17]
MWCNT/GCE	CV	0.7	5	10	1-100	50-2000	50-2000	[18]
PEDOT/Fc ⁻ /GCE	DPV	0.05	0.7	0.1	0.1-300	1.5-2000	0.5-1500	[19]
PEDOT/Fe(CN) ₆ ⁴⁻ /GCE		0.02	0.5	0.3	0.04-200	1-2000	0.7-1500	
PEDOT/ZrO ₂ NPs/GCE	DPV	0.012	0.45	0.2	0.05-300	1-1500	0.5-1000	[20]
Ag-PLA/GCE	LSV	0.08	3	5	0.1-23	5-4000	10-3000	[21]
<i>f</i> -MWCNT-Cu ₂ O-Ag ₂ O composite/copper substrate	DPV	0.014	0.011	0.008	0.05-1752	0.05-1628	0.02-1056	[22]
Modification-free SPCE	SWV	0.37	5.07	3.32	1-60	10-400	10-400	This work

PGCE: Pretreated glassy carbon electrode; MWCNT: Multi-wall carbon nanotubes; PEDOT: Poly(3,4-ethylenedioxythiophene); Fc⁻: Ferrocenecarboxylic acid; Fe(CN)₆⁴⁻: Ferricyanide; ZrO₂NPs: Zirconia nanoparticles; Ag-PLA: Silver doped poly(L-arginine); *f*-MWCNT-Cu₂O-Ag₂O: Functionalized multi-walled carbon nanotubes supporting Cu₂O and Ag₂O composite; DPV: Differential pulse voltammetry; LSV: Linear sweep voltammetry.

Interference study

To investigate the selectivity of the proposed electrode and developed method, the influence of various foreign species on the simultaneous determination of 20 μM VB₂,

60 μM VC, and 60 μM VB₆ in PBS pH 3.5 was evaluated. The tolerance limit was calculated to be lower than 5% of a relative error. The results revealed that 3000-fold and 1000-fold concentration ratios of Na⁺, K⁺, Mg²⁺, Ca²⁺, Al³⁺, Cl⁻, HCO₃⁻, SO₄²⁻, and PO₄³⁻, 300-fold and 100-fold concentration ratios of VB₁, VB₃, VB₅, glucose, fructose, citrate, L-lysine, L-cysteine, and L-methionine; 30-fold and 10-fold concentration ratios of Fe²⁺, Cu²⁺, VB₉, and oxalate; 15-fold and 5-fold concentration ratios of VB₁₂ and uric acid, and 5-fold concentration ratios of albumin and globulin did not interfere the measurement of VB₂, VC, and VB₆ (Table 2). No change significantly in the current signals were observed. Thus, the proposed electrodes were good selectivity towards the simultaneous determination of these three vitamins.

Table 2

Tolerance ratio of foreign species in the simultaneous determination of 20 μM VB₂, 60 μM VC, and 60 μM VB₆ on the common SPCEs.

Foreign species	Tolerance ratio ($C_{\text{species}}/C_{\text{target vitamins}}$)		
	VB ₂	VC	VB ₆
Na ⁺ , K ⁺ , Mg ²⁺ , Ca ²⁺ , Al ³⁺ , Cl ⁻ , HCO ₃ ⁻ , SO ₄ ²⁻ , PO ₄ ³⁻	3000	1000	1000
	300	100	100
VB ₁ , VB ₃ , VB ₅ , glucose, fructose, citrate, L-lysine, L-cysteine, L-methionine	30	10	10
	15	5	5
Fe ²⁺ , Cu ²⁺ , VB ₉ , oxalate	5	5	5
VB ₁₂ , uric acid			
Albumin, globulin			

Application in real samples

To examine the practicality and reliability of the proposed method, the simultaneous determinations of VB₂, VC, and VB₆ at a common SPCE were performed in mixed vegetable and fruit juice and artificial urine sample. The concentrations of these vitamins were determined by square wave voltammetry using the standard addition method to prevent any matrix influence. Recovery studies were carried out by spiking VB₂, VC, and VB₆ to the samples at various concentration levels. The experimental results revealed in Table S2, and S3 display that the recovery values were in the range

of 83.91 - 109.10%, which can be acceptable. Furthermore, to confirm the accuracy of the proposed method, the concentrations of VB₂, VC, and VB₆ in these samples were also determined by HPLC-DAD. The paired t-test between two methods disclosed that the results were in good agreement because the calculated t-value (2.15) was less than the critical value (2.31) for a two-tailed comparison. Therefore, a common SPCE can be successfully performed for the simultaneous determination of VB₂, VC, and VB₆ in real applications.

Table S1

The reproducibility calculated as relative standard deviation of VB₂, VC, and VB₆ at different concentrations obtained from common SPCEs.

Vitamins	Concentration (μM)	RSD (% , n = 7)
VB ₂	1	4.90
	20	2.78
	60	2.34
VC	10	6.43
	60	4.95
	400	3.70
VB ₆	10	6.27
	60	4.24
	400	2.44

Table S2

The determination of VB₂, VC, and VB₆ in mixed vegetable and fruit juice by SWV and HPLC.
(n=3)

Analytes	Added (μM)	Found (μM)	Recovery (%)	RSD (%)	HPLC (μM)
VB ₂	0	ND	-	-	ND
	10	9.92 ± 0.07	99.19	4.30	10.02 ± 0.09
	30	27.59 ± 0.07	91.96	1.68	29.09 ± 0.12
	50	46.81 ± 0.22	93.63	2.98	47.82 ± 0.12
VC	0	17.59 ± 0.02	-	6.37	ND
	40	50.06 ± 0.07	86.92	5.88	41.39 ± 0.07
	80	81.89 ± 0.11	83.91	6.10	82.07 ± 0.11
	200	209.06 ± 0.16	96.08	3.92	203.48 ± 0.16
VB ₆	0	ND	-	-	ND
	40	42.90 ± 0.05	107.25	4.90	42.10 ± 0.15
	80	87.23 ± 0.06	109.04	3.01	84.83 ± 0.21
	200	207.04 ± 0.19	103.52	4.86	211.04 ± 0.22

ND =Not detectable

Table S3

The determination of VB₂, VC, and VB₆ in artificial urine by SWV and HPLC. (n =3)

Analytes	Added (μM)	Found (μM)	Recovery (%)	RSD (%)	HPLC (μM)
VB ₂	0	ND	-	-	ND
	10	10.80 ± 0.13	107.96	6.92	10.53 ± 0.23
	30	32.92 ± 0.33	109.10	6.14	32.15 ± 0.33
	50	52.30 ± 0.25	104.59	2.91	51.85 ± 0.15
VC	0	ND	-	-	ND
	40	37.12 ± 0.03	92.79	7.17	38.40 ± 0.03
	80	72.89 ± 0.01	91.11	1.52	75.29 ± 0.01
	200	192.90 ± 0.9	96.45	4.51	193.10 ± 0.09
VB ₆	0	ND	-	-	ND
	40	36.73 ± 0.03	91.82	6.64	38.33 ± 0.03
	80	70.92 ± 0.04	88.65	4.05	74.12 ± 0.04
	200	198.85 ± 0.10	99.43	4.62	200.60 ± 0.10

ND =Not detectable

Conclusions

In summary, a simple and cost-effective modification-free SPCE was developed for the simultaneous determination of VB₂, VC, and VB₆. To the best of our knowledge, the measurement of these water-soluble vitamins at a common SPCE was performed for the first time. Meanwhile, continuously keeping the high efficiency, the proposed electrode still provided many advantages over the previous conventional modified-electrodes, such

as disposability, simple fabrication procedure, elimination of electrode maintenance, and low volume of solution requirement . The effect of various typical supporting electrolytes was successively accomplished to investigate the electrochemical behavior of VB₂, VC, and VB₆ .Under the optimal conditions using PBS pH 3.5 as a suitable supporting electrolyte, a common SPCE exhibited good sensitivity, selectivity, and reproducibility towards the simultaneous determination of VB₂, VC, and VB₆ in the oxidative direction without any modification at the working electrode surface .Finally, the proposed procedure was applied for the simultaneous determination of these vitamins in mixed vegetable and fruit juice and artificial urine with satisfactory results .Therefore, this proposed assay can be selected as a good and attractive choice for the simultaneous determination of water-soluble vitamins in food and clinical samples.

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A New Multilayer Microfluidic Paper-based Analytical Platform for Successive Sample Preparation and Detection of Dopamine

The development of simple and cost-effective electrochemical microfluidic paper-based analytical device (ePAD) for dopamine (DA) detection was first proposed. The device consists of two parts, the first is sample preparation, nafion was casted into sample zone and microfluidic channel to block ascorbic acid (AA) before solution flows to detection zone. The second is electrochemical detection, poly(glutamic acid) (PGA) modified screen-printed graphene (SPGE) electrode was used to enhance sensitivity for DA detection. Moreover, multilayer ePAD was fabricated by implement rounded-cotton pad onto detection zone for generating quasi-steady flow rate and packing tape was sealed on the top of device for increasing flow rate and preventing the volatilization of sample on ePAD. The DA concentrations can be determined by differential pulse voltammetry (DPV). Under optimal conditions, the linear concentration range of DA revealed over the range of 1.0-200 μM and exhibited the limit of detection of 0.41 μM (3SD/slope). This proposed platform was eventually applied for detection of DA in artificial urine sample and compared to UV-vis method. The results offered good accuracy and satisfactory method for DA detection in real sample.

Introduction

Dopamine (DA) is one of the most important catecholamine neurotransmitter that found in the central nervous system. This substance plays a major role in various functions including metabolism, cardiovascular, renal and endocrine system [1]. In addition, the presence of DA concentration in human body is mainly involved in controlling movement, attention, learning and emotional responses. As a result, the deficiency of DA level associates with various diseases such as Parkinson's disease [2], Attention-Deficit Hyperactivity Disorder [3], Huntington's disease, Alzheimer's disease [4], and Schizophrenia [5]. Hence, a simple and sensitive analytical method for the detection and monitoring of DA level in human fluid samples are significantly important for clinical applications. During the past, diverse analytical techniques have been proposed for DA detection, such as, fluorescence spectrophotometry [6, 7] high performance liquid chromatography (HPLC) [8, 9], colorimetric method [10], electrophoresis [11] and surface enhance Raman scattering [12]. Even though these techniques provided high sensitivity and selectivity, but there are some limitations because these techniques required several

sample preparation steps, time consuming, large amount of sample volume, specialist skill and expensive equipment. Therefore, the analytical method that can overcome these limitations is still in high demand.

Electrochemical technique is significantly attractive analytical method for DA detection, due to its excellent performances such as fast analysis time, high sensitivity, low cost and simple instrumentation. However, the significant problems for electrochemical DA detection is often suffered from a presence of ascorbic acid (AA) in real sample. Since the DA and AA are oxidized at nearly the same potential. Moreover, traditional electrodes also offer slow electron transfer and poor electrocatalytic properties. For these reasons, leading to the poor selectivity and sensitivity for DA detection [13]. Additionally, several types of materials have been designed for modification at electrode surface to solve these problems. The examples of modifiers are chitosan-graphene [14], layer-by-layer assembly of graphene and gold nanoparticles [15], overoxidized polyimidazole/graphene oxide copolymer [16] and graphene anchored with Pd-Pt nanoparticles [17]. Although these proposed materials provided good limit of detection and also be used to detect DA level in a presence of AA. Nevertheless, these modified electrodes require expensive materials, several steps for electrodes modification and time consuming, which make increasingly difficult for use.

The demand of simple and affordable medical diagnostics system has rapidly increased over the past decade, point-of-care (POC) is an important role for reducing time, complication and cost for detection of analytes close to the patient. POC provides more comfortable for diagnosis, monitoring and management, that could greatly help doctors to diagnose the disease at early stage [18]. Therefore, this platform offers the high beneficial impact for global health especially in developing countries. Because of the high growth rate of POC platform, World Health Organization (WHO) established the criteria ideal POC diagnostic device including ease of use, affordable, high sensitivity, specificity, equipment-free, user-friendliness, rapid, robustness and deliver [19]. Microfluidic paper-based analytical device (μ PAD) is an excellently challenging and alternative device for POC applications. μ PAD is fabricated from filter paper that generates several advantages including cost-effectiveness, ease of use, portability, easy to eliminate and using small amount of sample-reagent referring to small waste from analysis as well. Furthermore, solution is driven in μ PAD by capillary action without external pump [20, 21]. Because of these properties, μ PAD is considered to be an innovative platform for equipment-free and using as a sensing device for many kinds of

applications including environmental monitoring, food safety and clinical diagnosis. The challenge to improve efficiency of μ PAD is to achieve low limit of detection and good selectivity. Most of research proposed μ PAD coupled with colorimetric detection, however, sensitivity and selectivity are poor. Therefore, the first microfluidic paper-based analytical device integrated with electrochemical detection was designed by Dungchai et al.[22], in comparison, this platform provided lower limit of detection and generated more selective than colorimetric method. Since the first report, electrochemical microfluidic paper-based analytical device ($e\mu$ PAD) has continuously increased at an exponential growth to be one of the most popular technique for combining with μ PAD. Consequently, $e\mu$ PAD has been used for various applications including separation [23], preconcentration [24], sample preparation [25] and coupling with colorimetry for dual detection [26]. Simplifying the detection even further, μ PAD coupled with electrochemical technique has been designed for DA detection. Nafion was deposited on microfluidic paper-based channel is used to block AA as a sample preparation before solution flowed to the electrode area. For electrochemical detection, poly(glutamic acid) (PGA) was employed as modifier to alter the screen-printed graphene electrode (SPGE) surface to enhance the electrochemical response of DA. Moreover, multilayer $e\mu$ PAD was also fabricated to overcome the limiting performance from previous platforms. In this work, differential pulse voltammetry (DPV) technique was used to measure DA concentrations. To obtain the highest performance of device, various optimal conditions are investigated to provide excellent quantitative results. To the best of our knowledge, there is no report of $e\mu$ PAD for the successive sample preparation and electrochemical detection. This proposed device was finally detected DA level in artificial urine samples. The device provided simple, cost-effective, high sensitivity and selectivity for detection of DA in a presence of AA.

Experimental

Chemicals, materials and apparatus

All chemicals used in this work were of analytical grade and used without any purification. Dopamine, l-ascorbic acid, uric acid, nafion, l-glutamic acid, Bovine serum albumin (BSA), potassium ferricyanide ($K_3Fe(CN)_6$), sodium chloride (NaCl), calcium chloride ($CaCl_2$) vitamin B₂ and B₆ were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glucose was obtained from Merck (Darmstadt, Germany). Artificial urine sample was purchased from Carolina Biological Supply Company (Burlington, USA). 0.1 M

Phosphate buffer solution (PBS) as supporting electrolyte was prepared with sodium dihydrogen orthophosphate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) and di-Sodium hydrogen orthophosphate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) and were acquired from Ajax (Australia). N-propanol was purchased from Unilab (Mumbai, India). All aqueous solution was prepared in deionized (DI) water. The electrochemical techniques using cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were performed by potentiostat CHI 1200C (Austin, TX, USA). Electrochemical impedance spectroscopy (EIS) was used to ensure the successful modification of modified electrode using PalmSens4 (Houten, The Netherlands). Graphene and silver/silver chloride (Ag/AgCl) inks were purchased from Sun chemical (Slough, United Kingdom). The screen-printed blocks were generated by Chaiyaboon Co. Ltd. (Bangkok, Thailand). μPAD patterned was fabricated by Xerox Color Qube 8570 series wax printing machine (Xerox, Japan). Cotton pad was purchased from Silcot brand by Unicharm Products Co., Ltd. (Tokyo, Japan) and packing tape was purchased from 3M brand by 3M Thailand Company (Bangkok, Thailand).

μPAD design and preparation

The μPAD pattern was designed using Adobe illustrator CC. Then wax-patterned was printed onto Whatman No.1 filter paper using wax printing machine. After that, wax printed paper was melted on a hot plate at temperature of $175\text{ }^\circ\text{C}$ for 30 s for melting the wax into cellulose structure of paper to generate the difference between hydrophobic and hydrophilic area. For the electrochemical detection zone, three electrodes including working electrode, reference electrode and counter electrode were screened using in-house screen printing procedure. Firstly, graphene ink was screened on detection zone as shown in Fig. S1a to obtain working and counter electrode, and placed into oven at temperature of $55\text{ }^\circ\text{C}$ for 30 min. After that, silver/silver chloride (Ag/AgCl) ink was screened on detection zone to complete the electrochemical cell as shown in Fig. S1a and placed into oven at temperature of $55\text{ }^\circ\text{C}$ for 30 min. Fig. S1b shows the drawing of μPAD coupled with electrochemical detection consisted of sample zone (circle, diameter = 10 mm), electrochemical detection zone (circle, diameter = 12 mm) and microfluidic channel connected between sample zone and electrochemical detection zone (channel, $3 \times 5\text{ mm}$).

ePAD operation and electrochemical procedure for DA detection

For operation, ePAD was sealed with packing tape in the bottom of paper (Fig. 1a). PGA was modified on SPGE using electropolymerization of glutamic acid by preparation of 20 mM glutamic acid in 0.1 M phosphate buffer solution (PBS) pH 7.4 then 100 μ L of modifier was dropped on detection zone and applied potential using cyclic voltammetry scanned from -0.5 V to +1.6 V with scan rate of 0.1 V/s for 7 cycles (Fig. 1b). ePAD was rinsed with DI water and dried at room temperature. After that, 0.5% nafion was prepared in n-propanol and 10 μ L of solution was drop-casted on sample zone and microfluidic channel (Fig. 1c) and dried at room temperature. Last, rounded-cotton pad was placed onto electrochemical detection zone and sealed with packing tape on the top of device (Fig. 1d).

The determination of DA, 60 μ L of DA solution in 0.1 M PBS pH = 7.4 was dropped on sample zone and waited for 15 min (Fig. 1e). In this step, the solution was flowed from sample zone through microfluidic channel to electrochemical detection zone via capillary force. After that, the electrochemical detection was performed by DPV using PGA/SPGE as a working electrode, Ag/AgCl as a reference electrode and screen-printed graphene ink as a counter electrode. The parameters for DPV measurement were used follow; an increment of 0.006 V, a pulse amplitude of 0.2 V, a pulse width of 0.1 s and a pulse period of 0.6 s for scanning from -0.4 V to +0.4 V vs. Ag/AgCl. The differential pulse voltammogram of DA was shown in Fig. 1f.

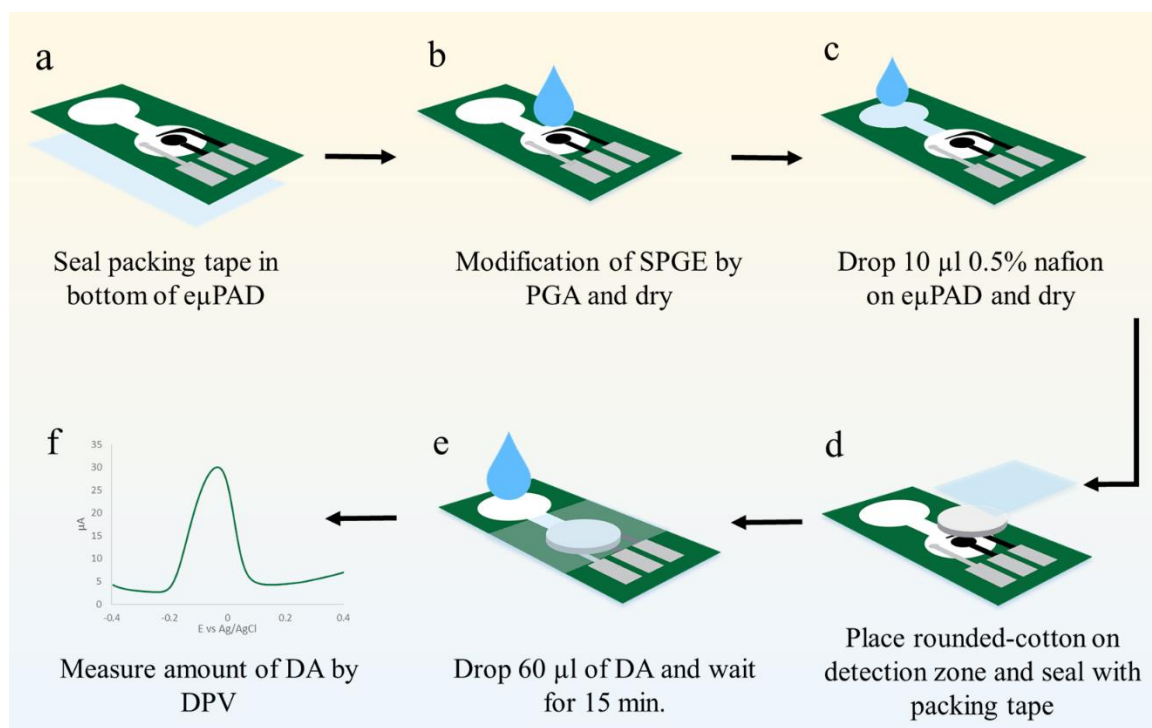


Fig. 1 The representation of μ PAD for DA detection consisting of: (a) sealed packing tape at the bottom of μ PAD, (b) PGA modified SPGE step, (c) drop casting of 0.5% nafion (d) completed multilayer μ PAD components, (e) determination of DA step and (f) differential pulse voltammogram of DA.

The components of μ PAD

The μ PAD components consist of four layers (Fig. 2); (i) packing tape was sealed on the top and at the bottom of device to increase flow rate, moreover, these layers were used to prevent the leakage and volatilization of sample solution, (ii) rounded-cotton pad (circle, diameter = 10 mm) was placed onto electrochemical detection zone to generate a quasi-steady flow rate on device and (iii) μ PAD layer, PGA was modified on SPGE and 0.5% nafion was drop-casted on sample zone and microfluidic channel.

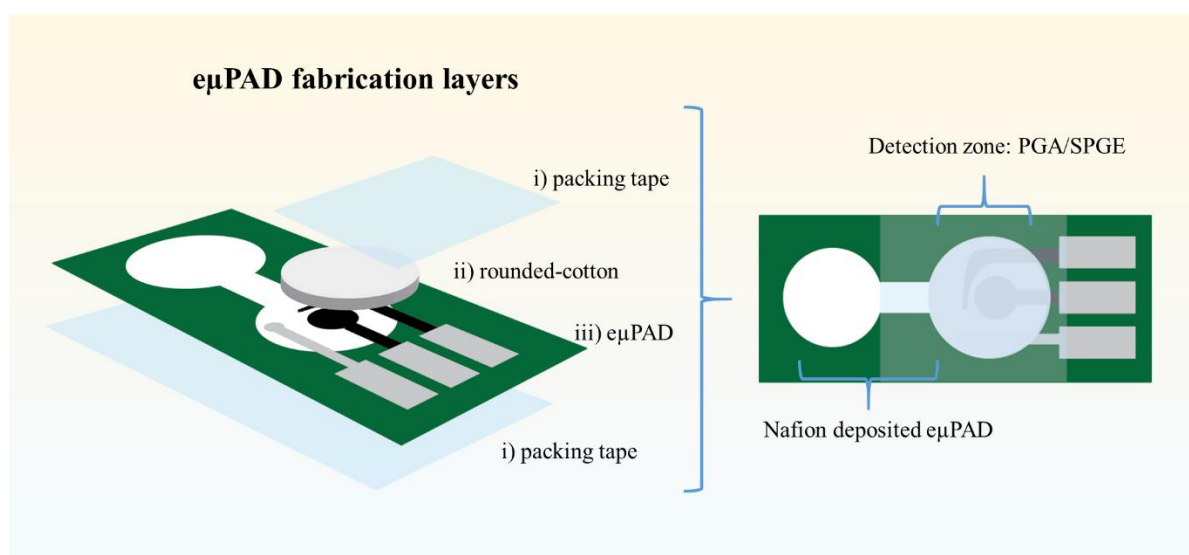


Fig. 2 Illustration of μ PAD layers, fabricated from i) packing tape, (ii) rounded-cotton pad and iii) μ PAD layer.

The determination of DA in real samples

To demonstrate the capability of this device, μ PAD was evaluated for detection of DA in artificial urine samples. The samples were diluted with PBS (pH 7.4), then DA was spiked into samples at concentrations of 10, 20, 40, 60 and 80 μ M and measured the quantity of DA using μ PAD, the recoveries of spiked standard DA were thoroughly studied. To validate the accuracy of this device, the results received from μ PAD were compared to the results those obtained from UV-vis method [27].

Results and discussion

Characterization of modified electrode

The poly(glutamic acid) modified screen-printed graphene electrode (PGA/SPGE) was prepared by electropolymerization of 20 mM glutamic acid in 0.1 M PBS pH 7.4 using CV technique scanned over the range between -0.5 and +1.6 V vs. Ag/AgCl, scan rate 100 mV/s for 7 cycles. Cyclic voltammogram during electropolymerization was showed in Fig. 3a, it can be clearly observed the well-defined peak current around 1.49 V at first three cycles, after forth cycle, the broad peak current gradually increased with number of scan, indicating that this polymer film was grown and formed at SPGE surface [28].

Electrochemical behavior of unmodified SPGE and PGA/SPGE were compared and investigated by CV and ESI techniques. Cyclic voltammograms of 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ in 0.1 M PBS (pH 7.4) were recorded in range between -0.8 and 1.0 V to exhibit the redox peak of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (Fig. 3b). The electrochemical signal of 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ obtained from PGA/SPGE increased almost 2-fold when compared with unmodified SPGE. In addition, to confirm the increased electroactive surface area in the presence of PGA at SPGE surface, the Randles-Sevcik equation (1) was applied to calculate the electroactive surface area. Where i_p is peak current, n is number of electrons, F is Faraday's constant, A is electroactive surface area, C_0^* is concentration of analyte ($[\text{Fe}(\text{CN})_6]^{3-/4-}$), ν is scan rate, D_0 is diffusion coefficient of $[\text{Fe}(\text{CN})_6]^{3-/4-}$, R is ideal gas constant and T is temperature. Based on known parameters from previous study ($n = 1$, $C_0^* = 5 \times 10^{-6}$ mol/cm³, $\nu = 100$ mV/s, $D_0 = 7.6 \times 10^{-6}$, $R = 8.13447$ J/K·mol and $T = 298$ K) [29]. The comparison of electroactive surface area shows in Fig. 3c, PGA modified SPGE provided more electroactive surface area than unmodified SPGE over 2 times. Similarly, peak separation value was decreased from 0.950 to 0.361 V in case of unmodified SPGE according to PGA/SPGE indicating that rate of electron transfer increased after the electropolymerization of PGA on SPGE [28].

$$i_p = 0.45nFAC_0^* \sqrt{\frac{nF\nu D_0}{RT}} \quad (1)$$

ESI technique is used to verify the successful modification of the proposed electrode by investigation electron transfer resistance (R_{ct}) between the redox couple of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and electrode surface. The Nyquist plot of unmodified SPGE and PGA/SPGE were obtained in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ in 0.1 M PBS pH 7.4 (Fig. 3d). Each point of Nyquist plot refers for the impedance of one frequency scanned from high to low

frequency. The semicircle diameter was equal to charge transfer resistance (R_{ct}) at electrode and solution interface [30]. The R_{ct} value of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ at unmodified SPGE and PGA/SPGE were calculated as 6.8 and 2.3 $\text{k}\Omega$, respectively. The R_{ct} value after electropolymerization of PGA on SPGE was considerably decreased. These results confirmed that PGA, which is conductive polymer, was successfully modified on SPGE.

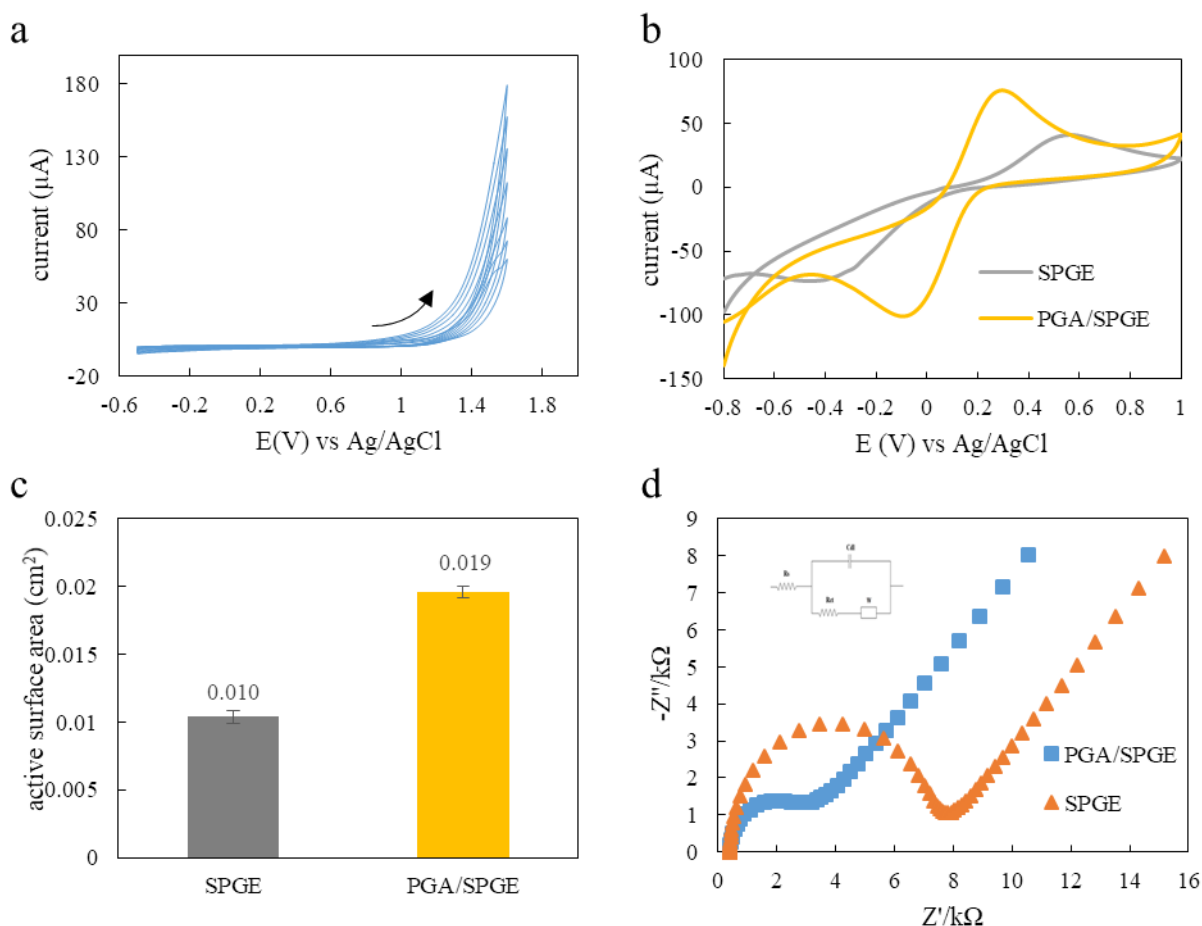


Fig. 3 (a) The electropolymerization of glutamic acid at SPGE was performed using cyclic voltammetry scanned between -0.5 and +1.6 V, scan rate 100 mV/s for 7 cycles in 20 mM glutamic acid. (b) Cyclic voltammograms of 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ in 0.1 M PBS pH 7.4 at SPGE (gray line) and PGA/SPGE (yellow line), scan rate 100 mV/s. (c) Electroactive surface area of SPGE (grey bar) and PGA/SPGE (yellow bar). (d) Nyquist plots of SPGE and PGA/SPGE obtained in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ at SPGE (orange) and PGA/SPGE (blue).

Electrochemical behavior of DA at PGA/SPGE

The electrochemical property of DA was investigated by CV and DPV using unmodified SPGE and PGA/SPGE. CV was performed to study the electrochemical

behavior of DA at working electrodes. Fig.4a shows cyclic voltammograms of DA compared between using unmodified SPGE (blue line) and PGA/SPGE (orange line). At unmodified SPGE, anodic and cathodic peak obtained at 0.3 and -0.15 V, respectively. However, PGA/SPGE provided higher current and the anodic and cathodic peak shifted to negative direction recorded at 0.089 and -0.083 V, respectively. Moreover, DPV was used to confirm better performance of PGA/SPGE over unmodified SPGE, as shown in Fig. 4b. At unmodified SPGE (blue line), the broad oxidation peak of DA was observed at 0.01 V and oxidation currents obtained was 6.096 μ A. In contrast, PGA/SPGE (orange line) provided a well-defined peak and shifted to negative direction potential at -0.07 V. Otherwise, there is significant enhancement in peak current of DA almost 2-time compared with unmodified SPGE. The results obtained from CV and DPV technique indicated that PGA/SPGE offered electrocatalytic activity toward DA oxidation. These results exhibit that PGA/SPGE is the excellent electrode for DA detection for this work.

To achieve the highest electrochemical response for DA detection, the parameters in electropolymerization of PGA steps were focused on concentration of monomer glutamic acid and numbers of scan in CV technique. These two parameters involve in controlling the thickness of polymer onto electrode surface. First, concentrations of glutamic acid were studied in range of 10 to 50 mM (Fig S2). At 20 mM of glutamic acid provided the highest oxidation current of DA. After 20 mM, the oxidation current of DA gradually decreased, this might affect from thickness film of PGA on SPGE surface is too thick that leading to low electron transfer between electrode and electrolyte interface. Therefore, 20 mM of glutamic acid was an optimal condition. Second, the numbers of scan in CV technique were investigated in range of 3 to 15 cycles (Fig S3). As can be seen that the oxidation currents of DA gradually increased by increasing of scan numbers from 5 to 7 cycles and then oxidation currents after 7 cycles almost stable. Thus, the numbers of scan at 7 cycles was selected as optimal condition.

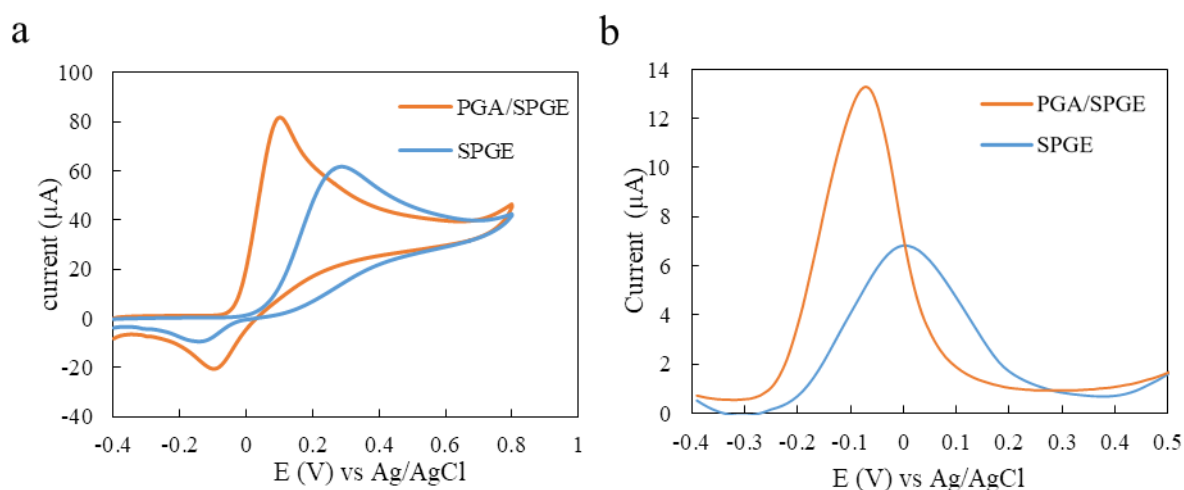


Fig. 4 Cyclic voltammograms (a) and differential pulse voltammograms (b) of DA at SPGE (blue line) and PGA/SPGE (orange line) in PBS pH 7.4.

Comparison of electrochemical configuration devices

To confirm the capability of e μ PAD for successive separation and detection, the stagnant electrochemical analysis (Fig. S4) was investigated by dropping 100 μL of DA and AA solution on PGA / SPGE and electrochemical measurement was performed by DPV. Fig. 5a shows the differential pulse voltammograms of 100 μM DA and 100 μM AA in PBS pH 7.4, the oxidation peak of two substances are almost the same potential. There is no way to distinguish between DA and AA peak. Therefore, AA is considered to be electrochemical interference for detection of DA at PGA/SPGE using stagnant electrochemical analysis.

Based on the results confirmed from stagnant electrochemical analysis, to overcome an interference problem from AA, nafion coated on e μ PAD was employed for detection of DA in the presence of AA. This material has been widely used in biosensor applications [31, 32], due to nafion structure shows a strongly permeable membrane that allows only cation passes through membrane but strongly repulses anion away from electrode surface [25]. Because of this property, nafion was used to coat into e μ PAD channel to block AA (anion) while DA in solution is allowed to flow to the electrochemical detection zone and measured by electrochemical technique. In this work, the influence of concentrations of nafion to block AA were studied. Fig. 5b shows the oxidation current of 100 μM DA (orange bar) and 100 μM DA in the presence of 100 μM AA (blue bar) obtained from different concentration of nafion coated into e μ PAD channel. As can be seen that the different oxidation current between 100 μM DA and 100 μM DA in the

presence of 100 μM AA considerably decreased when increased nafion concentration. Up to 0.5% nafion, the oxidation current value of both are almost equal. These results illustrated that using 0.5% nafion can completely block 100 μM AA and get rid of the interference from AA while DA concentration is measured. Hence, at 0.5% (v/v) nafion was selected as optimal condition for coating into ePAD channel.

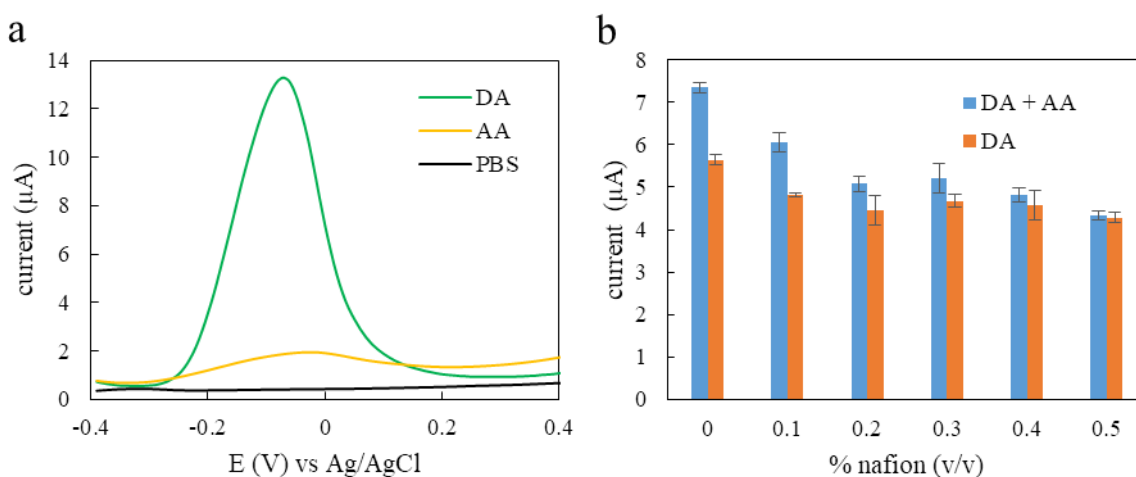


Fig. 5 (a) Differential pulse voltammogram of 100 μM DA (blue line), 100 μM AA (orange line), and PBS pH 7.4 (black line) at PGA/SPGE performed by stagnant analysis. (b) oxidation current value of 100 μM DA (blue bar) and 100 μM DA in a presence of 100 μM AA (orange bar) obtained from different concentrations of nafion coated into ePAD

Improvement of electrochemical response using ePAD

From results obtained from ePAD, the oxidation current value for DA detection was very low which caused from two main factors. The first factor is to come from nafion coated in sample zone and microfluidic channel. Based on nafion structure containing perfluoroalkyl backbones, so, it has strong hydrophobicity properties [33]. Therefore, after coating nafion into ePAD, nafion can penetrate into the pores of paper that obstruct the flow of solution on microfluidic channel following to slow flow rate and leading to low mass transport of DA from bulk solution to electrode interface. Moreover, the slow flow rate also requires more time consuming for measurement. The second factor is the flow rate on ePAD after solution migrates completely to dry region of hydrophilic area leading to the decay of flow rate, thereby flow rate is decreased within time. This phenomena on ePAD also effected to low mass transport at electrode interface [20]. Herein, multilayer of ePAD were employed to overcome those mentioned disadvantages. In recent work, the sealing method using packing tape was proposed. The packing tape was sealed on

the top of μ PAD can increase the flow rate over 2-fold compared with open μ PAD [34]. From this phenomena, packing tape was used as sealing material for increasing flow rate of μ PAD. As shown in the 2nd platform of Fig. 6a, the oxidation current of 100 μ M DA obtained from the 2nd platform (Fig. 6b) slightly increased when compared with open μ PAD (1st platform). However, it is not high enough for DA detection in biological sample. In addition, as previous reported, using absorbent pad can overcome the decay of flow rate with time. Because the absorbent pad function is used to absorb and pull the solution from sample zone through porous structure and generates continuously capillary flow (quasi-steady flow with time) on μ PAD [35]. Therefore, in this work, we applied round-cotton pad instead of absorbent pad and placed on top of detection zone (3rd platform) for making a quasi-steady flow. owing to round-cotton pad has high porosity similar to absorbent pad but offers a lower cost of material. Unfortunately, the oxidation current obtained from the 3rd platform also was slightly increased when compared with open μ PAD (1st platform) which is not high enough for detection of DA. To the best of our knowledge, multilayer μ PAD consisting of μ PAD, rounded-cotton pad and packing tape were first fabricated as shown in the 4th platform (Fig. 6a). It is clear that the multilayer μ PAD (4th platform) offered the highest electrochemical response for detection of DA. The excellent results derived from the benefits of packing tape and rounded-cotton pad which provided higher flow rate and generated a quasi-steady flow on μ PAD.

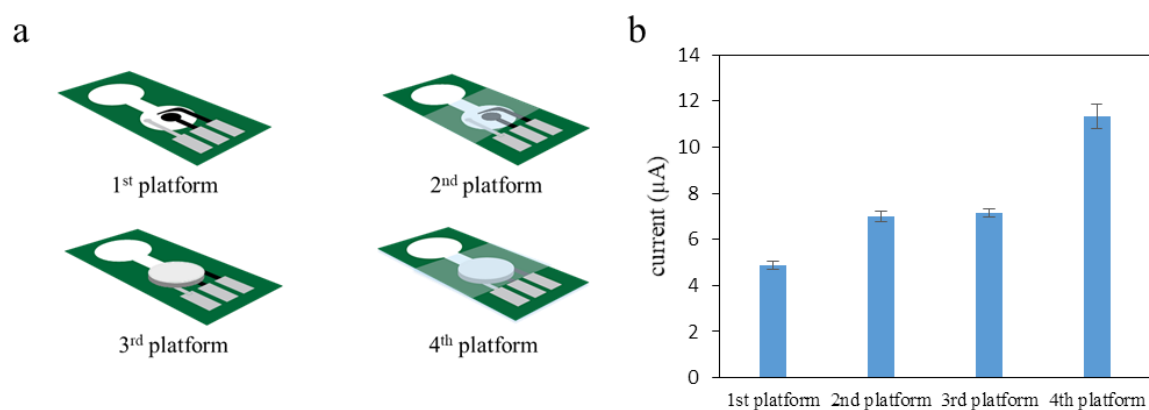


Fig. 6 (a) The schematic representation for fabrication of μ PAD in different platforms. (b) The effect of different μ PAD platforms on oxidation current for DA detection

Using this proposed device, the waiting time before electrochemical measurement also have significant effect for electrochemical response. Multilayer μ PAD was investigated in different waiting time (10, 15 and 20 min) for detection of 100 μ M DA, the oxidation current in different times was shown in Fig. S5. Increasing waiting time, the

oxidation current considerably increased as expectation. However, we observed that the oxidation current obtained between 15 and 20 min were not significant difference. Accordingly, waiting time at 15 min is selected as an optimal condition.

Analytical performance

The analytical performance of multilayer eμPAD for DA detection was evaluated, several concentrations of DA were determined using DPV technique. Fig. 7a shows differential pulse voltammogram using multilayer eμPAD, the oxidation peak currents of DA gradually increased with increasing concentrations. The linear calibration plot between oxidation current and DA concentration was found over the range from 1.0 μM to 200 μM. The linear regression equation of $i_{pa} = 0.1987C_{DA} + 1.1301$ with correlation coefficients of $R^2 = 0.9994$ (Fig. 7b) was also presented. The limit of detection (LOD = $3SD_{blank}/\text{slope}$) was found to be 0.41 μM. Besides, the analytical performance of multilayer eμPAD was compared with previous reports and summarized in Table 1. Although, the analytical performance from some previous reports offered the lower limit of detection compared with our platform, nonetheless, these methods required for several steps for modification of electrode and high cost of material. However, this developed electrochemical platform exhibited one-step sample preparation and detection integrated in a single device, that provided more simple, rapid and cost-effective device for dopamine detection.

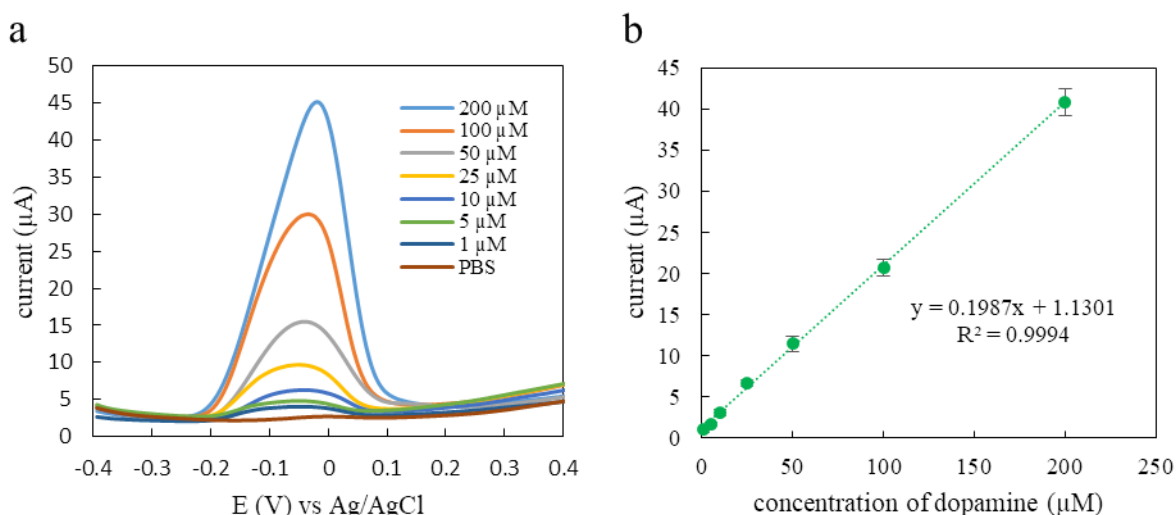


Fig. 7 (a) differential pulse voltammograms obtained from several concentrations of DA in PBS pH 7.4 using multilayer eμPAD, (b) calibration plot between concentration of DA (μM) and current (μA)

Table 1 Analytical performance of previous reports compared with proposed multilayer eμPAD based on electrochemical detection of DA

Electrode	Technique	LOD (μM)	Linear range (μM)	Ref.
Poly(SDB)/CPE	DPV	0.8	5.0–10, 20–80 and 90–1000	[36]
AgNPs/rGO/GCE	LSV	5.4	10–800	[37]
Plmox-GO/GCE	DPV	0.63	12–278	[16]
HNP-PtTi/ GCE	DPV	3.2	4.0–500	[38]
G-DHE	DPV	0.2	5.0– 2000	[39]
PtNi@MoS ₂ /GCE	DPV	0.1	0.5–150	[40]
multilayer eμPAD (PGA/SPGE)	DPV	0.41	1.0–200	This work

Abbreviaion

Poly(SDB)- poly(solochrome dark blue), CPE- carbon paste electrode, AgNPs- silver nanoparticles, rGO- reduced graphene oxide, LSV- linear sweep voltammetry, Plmox- overoxidized polyimidazole, GO- graphene oxide, HNP- hierarchical nanoporous, G-DHE- graphene–diamond hybrid electrode and PtNi@MoS₂- PtNi bimetallic naoparticles loaded MoS₂ nanosheets.

Selectivity

Aside the interference from AA, other common interferences that possibly found in urine sample including uric acid, glucose, vitamin B, BSA, Na^+ and Ca^{2+} were also investigated. As shown in Fig. 8, the oxidation current of 100 μM DA in the presence of 100 μM uric acid, 1 mM glucose, 100 μM vitamin B_2 and B_6 , 1000 $\mu\text{g/L}$ BSA, 1 mM Na^+ and 1 mM Ca^{2+} do not interfere (signal change below 5%). These results illustrated the multilayer e μ PAD offer the excellent selectivity for detection of DA.

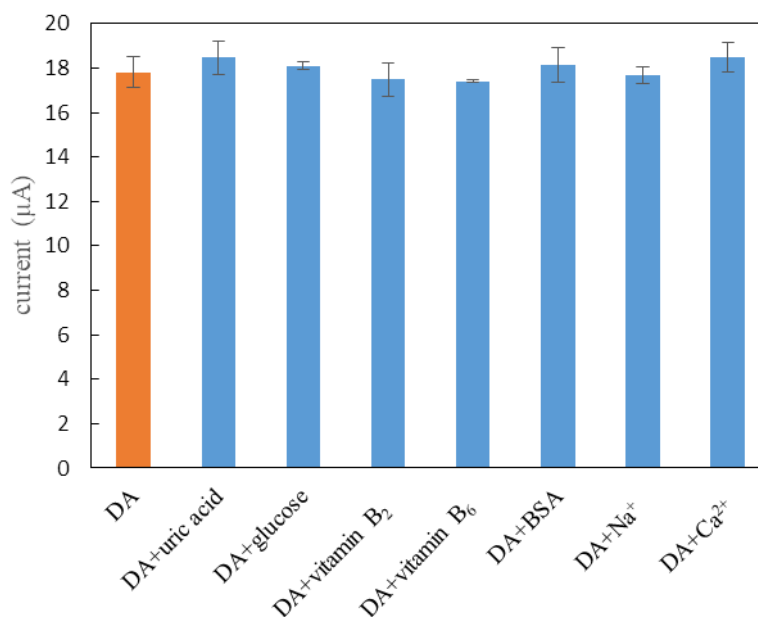


Fig. 8 The interferences effect of 100 μM uric acid, 1 mM glucose, 100 μM vitamin (B_2 and B_6), 1000 $\mu\text{g/L}$ BSA, 1 mM Na^+ and 1 mM Ca^{2+} in detection of 100 μM DA in PBS pH 7.4

Analytical application

The practicality of proposed device was demonstrated by detection of DA in artificial urine sample. The recovery test was performed by spiking specified concentration (10, 20, 40, 60 and 80 μM) of DA using standard addition method. As shown in table 2, %recovery and % relative standard deviation (RSD) with different three device were found in range from 96.6% to 108.3% and 1.4% to 5.0%, respectively. Finally, the obtained results from this device were compared with those obtained from UV-vis method [27]. The paired t-test at 95% confidence interval was evaluated, the calculated paired two tail value (0.13) of DA was below the critical t value (2.78). These results can be concluded that the proposed device is acceptable and reliable for detection of DA in urine sample.

Table 2 The analytical application using multilayer μ PAD for detection of DA in artificial urine sample (n=3).

Sample	Spiked (μ M)	Found (μ M)	Recovery (%)	RSD (%)
Artificial urine	10	10.8 $\pm$ 0.3	108.3	2.3
	20	20.2 $\pm$ 0.7	101.0	3.6
	40	38.6 $\pm$ 1.9	96.6	5.0
	60	59.2 $\pm$ 1.7	98.7	2.8
	80	81.1 $\pm$ 1.1	101.4	1.4

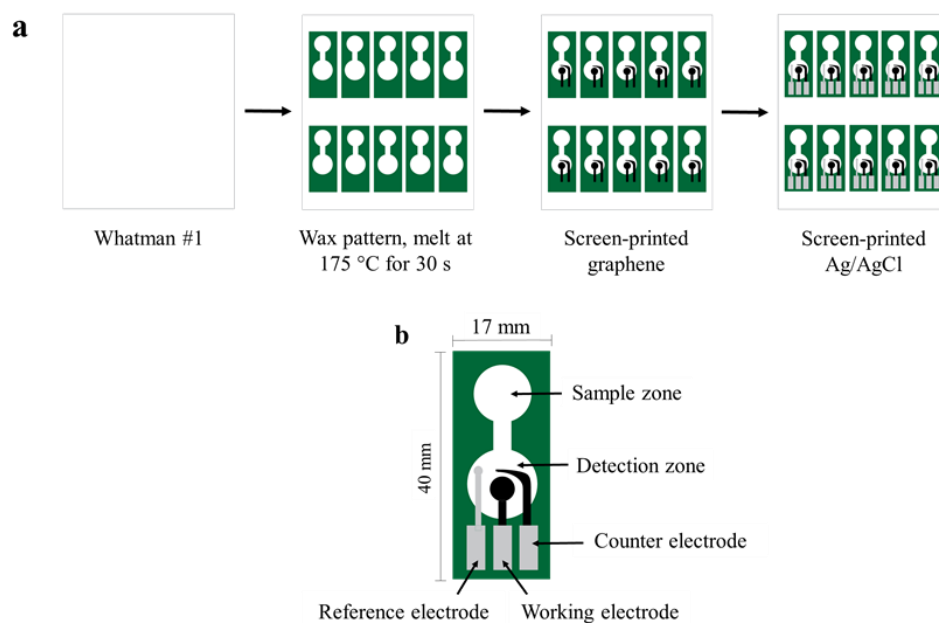


Fig. S1 (a) The preparation of wax-patterned paper (b) drawing of μ PAD coupled with electrochemical detection.

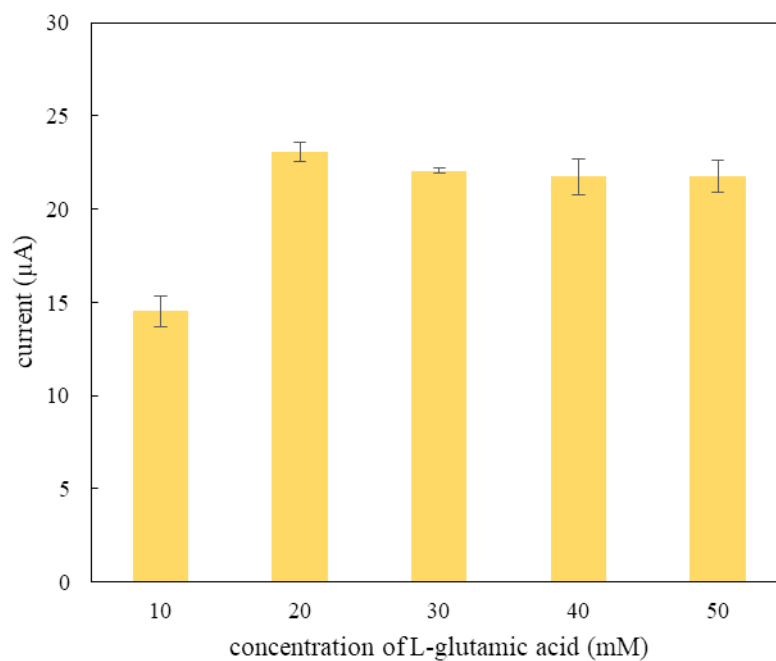


Fig. S2 the oxidation currents of 100 μM DA in PBS pH 7.4 measured by DPV using PGA/SPGE having different concentrations of L-glutamic acid.

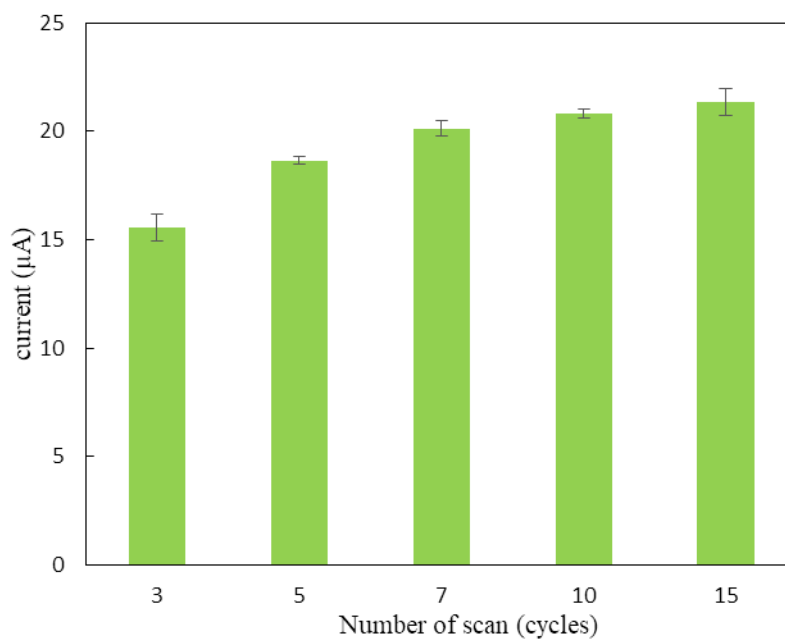


Fig. S3 the oxidation currents of 100 μM DA in PBS pH 7.4 measured by DPV using PGA/SPGE having different scan numbers.

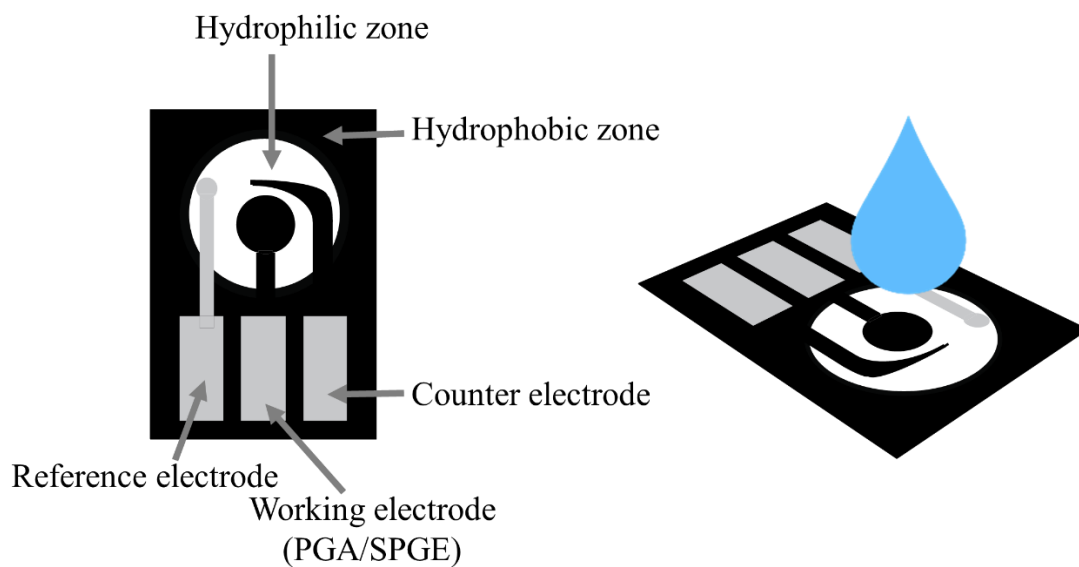


Fig. S4 The illustration of paper-based screen-printed electrode for electrochemical stagnant analysis.

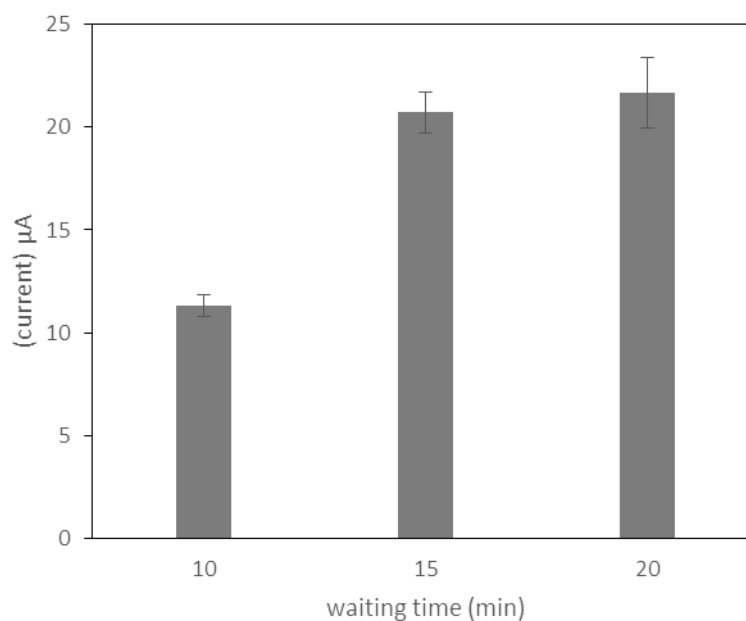


Fig. S5 the oxidation currents of 100 μM DA in PBS pH 7.4 measured by DPV using multilayer epPAD at different waiting time.

Conclusion

The successive sample preparation and detection on multilayer electrochemical microfluidic paper-based analytical device (multilayer ePAD) was successfully developed for DA detection. In this work, nafion coated on ePAD was used to block AA that can not flowed to electrochemical detection zone. The PGA / SPGE was served as working electrode offer an excellent sensitivity to quantify the amount of DA in urine sample by DPV. In addition, this new designed and fabricated multilayer of ePAD paltform leads to achievement in increasment of electrochemical response for DA detection. The developed device consists of packing tape and rounded-cotton pad for generating high and quasi-steady flow rate onto device. Finally, to demonstrate the powerfull of device, multilayer ePAD was successfully applied for detection of DA in artificial urine sample. This platform exhibits excellent advantages over previous reports including low cost, simple steps for fabrication, portability and short analysis time. Consiquently, this multilayer ePAD serves as an alternative electrochemical detection platform for point-of-care diagnostic system to detect other important biomarkers in the future.

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Sensitive and selective method for detection of vitamin B1 in urine samples using transparency sheet-based colorimetry and modifier-free gold nanoparticles

In this report, we present a transparency sheet-based colorimetric sensor as an alternative platform for the simple, sensitive and selective determination of vitamin B1 in urine samples by using unmodified gold nanoparticles (AuNPs). Samples presenting different concentrations of vitamin B1 were evaluated. The specific analysis is based on the aggregation of AuNPs by vitamin B1 due to the ability to form strong electrostatic interactions between positively charged vitamin B1 and negatively charged AuNPs. In the presence of vitamin B1, a distinctive color change in the AuNPs from red to blue was visualized by the naked eye within 10 minutes without the requirement for surface modification. For quantitative measurement using image processing, a good linear relationship ($R^2 = 0.9913$) between the vitamin B1 concentration and average red intensity was obtained in the range of 40-200 ppb. The limit of detection (LOD) and the limit of quantitation (LOQ) for vitamin B1 were found to be 3.002 ppb ($S/B=3$) and 10.006 ppb ($S/B=10$), respectively. The characteristics of the developed sensors were investigated for precision, accuracy, and sensitivity, and they were validated by classic methods. Statistical analysis proved that the developed sensors were precise, sensitive and accurate and that they can be used effectively for the analysis of vitamin B1 in urine samples.

Introduction

Vitamins are essential nutrients that must be provided to the body in small amounts on a regular basis to perform various chemical and physiological functions in the human body [1-3]. Vitamins are widely distributed in natural food sources and can be easily introduced into diets to satisfy daily needs. Vitamins are a group of organic compounds that have different structural and chemical properties, and can be conveniently categorized into two groups based on their solubility: fat-soluble vitamins and water-soluble vitamins. As the member of vitamin B group, thiamine (vitamin B1) is a sulfur-containing member of the water-soluble B-complex family, which are essential for normal development, growth, reproduction, lactation, and physical performance. Thiamine is involved in releasing energy from macronutrients that provide energy, especially from carbohydrates. Vitamin B1 is an essential vitamin that cannot be synthesized and must come from the diet. Vitamin B1 is widely distributed in small

amounts in foods, but it is easily lost during the milling, heating, canning, blanching, and storage of foods. Vitamin B1 is readily absorbed from the intestine and readily excreted through the kidneys. A deficiency in vitamin B1 can be caused by various factors and diseases, such as alcoholism, HIV, vomiting, and other gastrointestinal illnesses. Vitamin B1 deficiency may lead to chronic diseases. One of these diseases is beriberi, which is a neurological and cardiovascular disease. Brain abnormalities are also one of the common signs of vitamin B1 deficiency. These abnormalities include Wernicke-Korsakoff syndrome [2-5]. Due to the functions and stability of vitamin B1 in the body, it is necessary to develop simple, fast, low cost and sensitive analytical methods to determine vitamin B1 concentrations in clinical analysis.

Several methods have been reported for the determination of vitamin B1, including high-performance liquid chromatography (HPLC) [6, 7], chemiluminescence [8], electrochemical detection [9, 10], spectrophotometric detection [11, 12, 13], and fluorimetric analysis [14, 15]. Currently, metal nanoparticles have received significant interest in the past decade as active components for widespread application in the field of biomedicine. These nanoparticles have received considerable attention because of their distinctive properties including their optical and electronic properties, biocompatibility and long-term stability. Their excellent optical properties display brilliant color for sensing applications which can be visualized by the naked eye with no need for any additional instrumentation. Several methods for vitamin B1 determination have been described using different nanoparticles as reagents, such as gold [10, 11, 13, and 14], multiwall carbon nanotube [9], Cu nanoclusters [15], and silver nanoparticles [16]. Among these nanoparticles, gold nanoparticles (AuNPs) have been intensively studied for numerous applications as optical sensors for heavy metals [17], antioxidant [18], folic acid [19, 20], melamine [21], dopamine [22], and organophosphate pesticides [23, 24]. Although a number of methods have been proposed for the determination of vitamin B1, none of them were found to be suitable for on-site determinations. In addition, the main drawbacks of these mentioned methods are the time-consuming processes and costly instruments, which are inconvenient for routine work and on-site monitoring. Therefore, it will be very useful to have a simple tool that everyone can use for the on-site determination of vitamin B1 levels to obtain real-time determination.

Recently, portable devices have become an increasingly important goal of method development for the detection of various targets. Paper-based analytical devices (PADs) are one of the most exploited devices to be used as miniaturized analytical platforms

which are fabricated based on cellulose materials as a substrate for economical and portable sensing applications. Paper-based sensors can be advantageous for applications in diagnostics, food safety, and environmental monitoring due to their advantages of natural abundance, simplicity, disposability, low sample-reagent usage and suitability for routine analysis [17, 18]. Unfortunately, the use of paper for colorimetric detection suffers from the adsorption of the color by the paper porosities, leading to limitations in the sensitivity in comparison with that of detection methods in solution.

To solve this problem, transparency sheet was proposed to be an alternative analytical platform. The lack of porosities of polymer surface on transparency sheet leads to no adsorption of the color. All reagents can be held and reacted on the hydrophobic detection zone that seem to have the same behavior in solution. This can increase the performance to distinguish the color intensity by naked eyes and can measure of the color change at low concentration of the analyte. Therefore, we proposed for the first-time a simple, sensitive, and field-portable transparency sheet-based colorimetric devices for vitamin B1 monitoring by utilizing unmodified AuNPs in a neutral media as a remarkable colorimetric agent.

Upon the addition of a vitamin B1 solution into the AuNP solutions, a visible color change of the AuNPs from red to blue was observed within 10 minutes. To facilitate the application of this sensor for on-site monitoring, a smartphone or digital camera was also used to record the colorimetric responses in the detection platform. Furthermore, the proposed colorimetric sensor could be exploited to determine the vitamin B1 level in different urine samples, and comparisons were in good agreement with conventional spectroscopic method. The simplicity, sensitivity, selectivity, disposability, and portability of these devices provides an interesting alternative to the most frequently used conventional methods.

Experimental section

Chemicals and materials

Gold nanoparticles (AuNPs, 0.01%) with a diameter of 20 nm were purchased from Kestrel Bio Sciences (Thailand). AuNPs in an aqueous solution were synthesized by citrate reduction. The resulting nanoparticles have negatively charged citrate ions adsorbed on their surface and are thus stabilized by electrostatic repulsion. Thiamine hydrochloride (vitamin B1), potassium chloride, ammonium chloride, calcium chloride, sodium chloride, magnesium chloride, disodium hydrogen orthophosphate

($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$), sodium dihydrogen orthophosphate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) and hydrochloric acid were obtained from Ajax Finechem Pty Ltd. Riboflavin (vitamin B2), d-pantothenic acid hemicalcium (vitamin B5), folic acid (vitamin B9), vitamin B12, uric acid and ascorbic acid (vitamin C) were purchased from Sigma Aldrich. Niacin (vitamin B3) and pyridoxine hydrochloride (vitamin B6) were purchased from Himedia Laboratories Pvt Ltd. All chemicals were used as received without any purification procedures. All reagents were prepared with milli-Q water from Millipore ($R \geq 18.2 \text{ M}\Omega \text{ cm}$) throughout these experiments.

Preconcentration of received gold nanoparticles

Preconcentration of gold nanoparticles was performed by pipetting the stock solution of gold nanoparticles into 1 mL microcentrifuge tubes. After that, the microcentrifuge tubes were centrifuged at 12,000 rpm (4 °C) for 30 minutes. The 0.8 mL of supernatant was removed, and the mixture solution was continuously stirred for 10 seconds.

Wax-pattern transparency sheet-based fabrication

Circular areas with a diameter of 5 mm were designed by Adobe illustrator CS4 software (Adobe Systems, Inc.). A wax printing method was used for the fabrication of the transparency sheet-based devices. A blue wax color with an RGB value of 98/147/254 was used to complement the colorimetric reaction. The process used to fabricate the transparency-based devices was to print the wax pattern onto the surface of the transparency sheet using a commercial wax printer (Xerox Color Qube 8570, Japan). The printed wax can confine the aqueous solution onto the transparency surface to produce the detection zone.

Colorimetric measurement of vitamin B1 using the AuNP probes and image processing

For the colorimetric monitoring of vitamin B1, 15 μL of the AuNPs was first added to the detection test zone of the transparency-based devices. Next, 15 μL of either a sample solution or vitamin B1 standard solution was applied to the test zone. The color change of the AuNPs was visualized after 10 minutes by the naked eye. After, the test zones of the transparency-based devices were imaged with a mobile phone (vivo V3) under an in-house light-control box using the Camera application. The JPEG format

images were then imported into Image J software (National Institute of Health, USA) to measure the mean color intensity. The image color threshold was set to remove the blue background color by adjusting the hue and brightness window to a specific range of (100/200) and (60/200), respectively. The color signal of each zone was measured as the mean color intensity and then imported into Numbers software (v 2.0.3, Apple Inc.) for analysis.

Specificity test for interference ions

Riboflavin (vitamin B2), d-pantothenic acid hemicalcium (vitamin B5), folic acid (vitamin B9), vitamin B12, niacin (vitamin B3), pyridoxine hydrochloride (vitamin B6), uric acid, ascorbic acid (vitamin C), glucose, albumin, and ions that are likely to be found in real samples were examined as potential interferences for the determination of vitamin B1. To investigate the specificity of the proposed method, each kind of foreign substance and ion was individually tested and added into a fixed 20 ppb vitamin B1 solution.

Method validation experiments

The applicability of the proposed method was evaluated with a fluorescent method by determining vitamin B1 concentration in urine samples. Fluorescent methods are one of the most important methods used to measure the levels of vitamin B1 in samples. The fluorescent molecule, thiochrome is produced by the oxidation of vitamin B1 in an alkaline medium with potassium hexacyanoferrate(III) [25]. The following three solutions are prepared. Potassium hexacyanoferrate(III) (K_3FeCN_6) 0.001 g was dissolved in DI water, and then diluted to 100 ml to make a 10 ppm K_3FeCN_6 solution. Vitamin B1 stock solution (10,000 ppb) was made from 0.0005 g of pure vitamin B1 was dissolved in DI water and then brought to 50 mL in a volumetric flask. The standard vitamin B1 stock solution was prepared and then stored in the dark. Working standards of vitamin B1 (300 ppb, 200 ppb, 150 ppb, 100 ppb, and 50 ppb) were prepared by diluting (0.3 mL, 0.2 mL, 0.15 mL, 0.1 mL, and 0.05 mL) of the stock solution in a 10- mL volumetric flask with DI water. A pH=12.2 buffer was prepared from reagent grade NaH_2PO_4 (0.29 g) and Na_2HPO_4 (3.23 g) in 100 mL of water to make a 0.2 M buffer. Either sodium hydroxide or phosphoric acid was added to bring the pH to 12.2.

Analytical Procedure for Calibration: One milliliter of the 0.2 M, 12.2 pH buffer was added to a (fluorescence) sample cuvette. Then, 1.00 mL of the 10 ppm K_3FeCN_6

solution was added into the cuvette. After that, 1.00 mL of the standard to be analyzed was added. The cuvette was quickly capped and inverted a few times to ensure adequate mixing and then placed into the spectrofluorometer. The fluorescence intensity generated by the thiochrome produced that was immediately measured at $\lambda_{\text{ex}} = 369 \text{ nm}$ and $\lambda_{\text{em}} = 436 \text{ nm}$. The above steps were repeated for all of the working standards.

Preparation of artificial urine samples

The practical application of the proposed method was evaluated by determining vitamin B1 concentrations in urine samples. The artificial urine samples were purchased from Carolina Biological Supply Company. To determine vitamin B1 concentrations in real samples, a certain amount of vitamin B1 was spiked into the urine samples. The spiked samples were added into the AuNP solution, and after 10 min, the test zones of transparency-based devices were captured with a digital camera. Finally, the standard additions were used to examine the amount of vitamin B1. In addition, relative standard deviations and percentage recoveries were calculated.

Results and discussion

Characterization

1.1 UV-Visible spectrophotometry

To monitor the size changes of the AuNPs, UV-Vis absorption spectroscopy was first used. Fig. 1 displays the surface plasmon resonance (SPR) absorption spectra of the AuNPs. The characteristic peak of AuNPs (red line) was observed at 517 nm. Interestingly, after the addition of vitamin B1 to the AuNP solution, a slight shift towards longer wavelengths, from 517 nm to 650 nm, was observed. This change indicates an increase in the aspect size of the nanoparticles. In addition, gradual decreases in absorbance were also observed at 517 nm. Additionally, an obvious color change of the AuNP solution from red to blue could be observed by the naked eye. The color change and shift in the SPR absorption band suggests that the shape of the AuNPs was transformed by the concentration of vitamin B1 added to the solution.

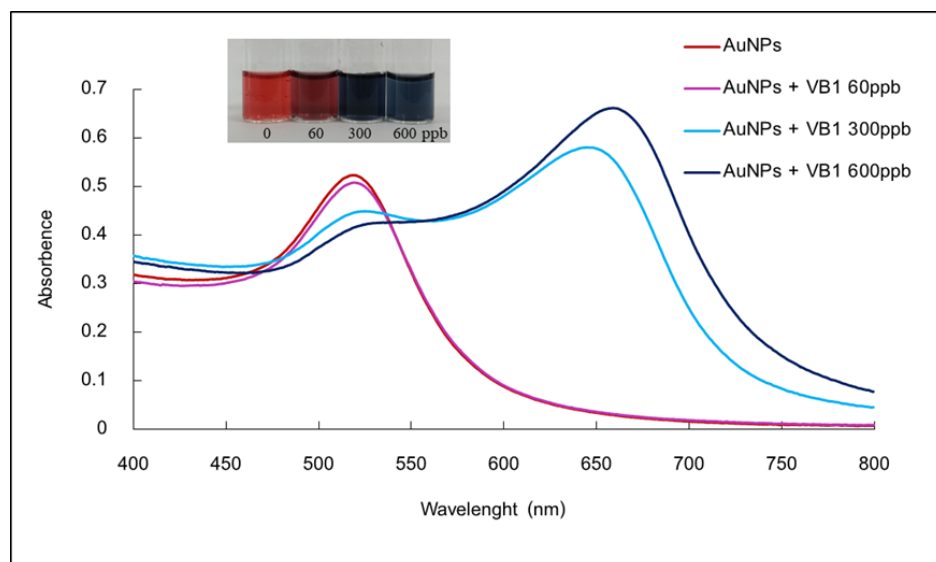


Fig. 1 UV-vis absorption spectra and photographs of the AuNPs after the addition of vitamin B1 at different concentrations, measured 10 minutes after addition.

1.2 Mechanism studies for colorimetric detection of vitamin B1 with modifier-free gold nanoparticles

In order to confirm the aggregation mechanism, the zeta potential and FITR have been used to investigate this action. From Table 1, it was found that the zeta potential clearly shows that after addition of vitamin B1 in AuNPs solution, the negative charge decreased from -53.2 mv to -44.1 mv, while the average size of AuNPs was increased from 27.8 to 61.8 nm. These results indicated that AuNPs aggregation was induced by electrostatic force between the positive charge of vitamin B1 and the negative charge of AuNPs at the optimized conditions.

Table 1. The size average and zeta potential of AuNPs, AuNPs- Buffer and AuNPs- Buffer + vitamin B1

Samples name	Scattering Angle	Z-Average (nm)	PI	Zeta Potential (mV)
AuNPs	90	26.9	0.638	-43.1
AuNPs- Buffer		27.8	0.610	-53.2

AuNPs- Buffer + vitamin B1		61.8	0.655	-44.1

Moreover, the results obtained corresponded to the FTIR spectra which showed the characteristic peak for NH_2 ($3,347\text{ cm}^{-1}$), O-H ($3,094\text{ cm}^{-1}$) and N-H ($1,623\text{ cm}^{-1}$) for vitamin B1. As shown in Fig.2, it can be seen that after addition of vitamin B1 in the solution of AuNPs, notable peak shifting and decreasing intensity was observed. This phenomenon indicated the aggregation of AuNPs by strong electrostatic from vitamin B1. The result obtained from our work was similar to the previous reports that used AuNPs and functionalized AuNPs as a color reagent of vitamin B1 detection [13, 14].

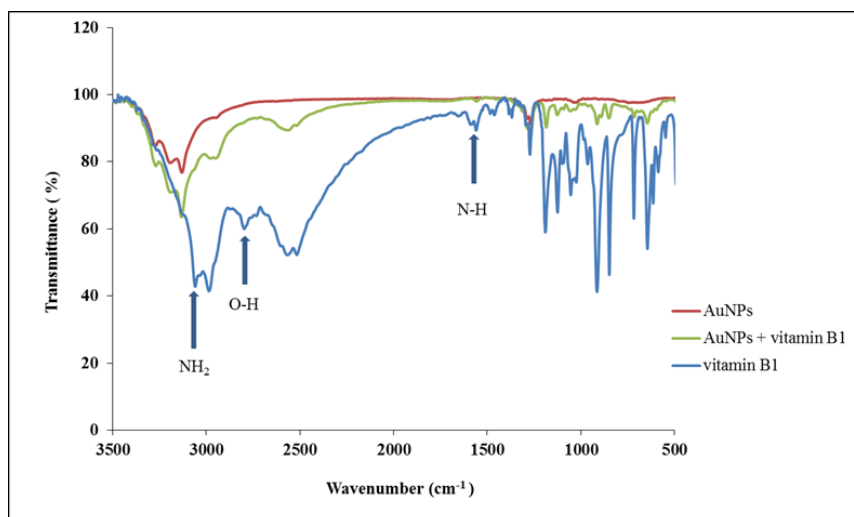


Fig. 2 FI-IR spectra of AuNPs, vitamin B1 and AuNPs after the addition of vitamin B1.

Optimization of the assay conditions

2.1 Optimization of the AuNP concentration

The concentration of AuNPs was first optimized for vitamin B1 determination. At low concentrations, the light color of AuNPs causes difficulties in monitoring the color changes, resulting in poor sensitivity of detection. Fig. 3a shows AuNP solutions at various concentrations. The preconcentration procedure and concentration of the AuNPs were performed and calculated based on a reported procedure [26] and were found to be 100, 125, 167, 250, and 500 ppm, respectively. It was clearly observed that the color density increased with increasing concentration. Subsequently, all of the AuNP solutions were tested at 100 ppb of vitamin B1, and the mean color-intensity difference $|\Delta I|$ of the AuNPs

was measured using Image J software. As shown in Fig. 3b, the use of 500 ppm AuNPs provided the highest ΔI between the absence and presence of vitamin B1. Therefore, a concentration of 500 ppm for the AuNPs was selected as the optimal value for the determination of vitamin B1.

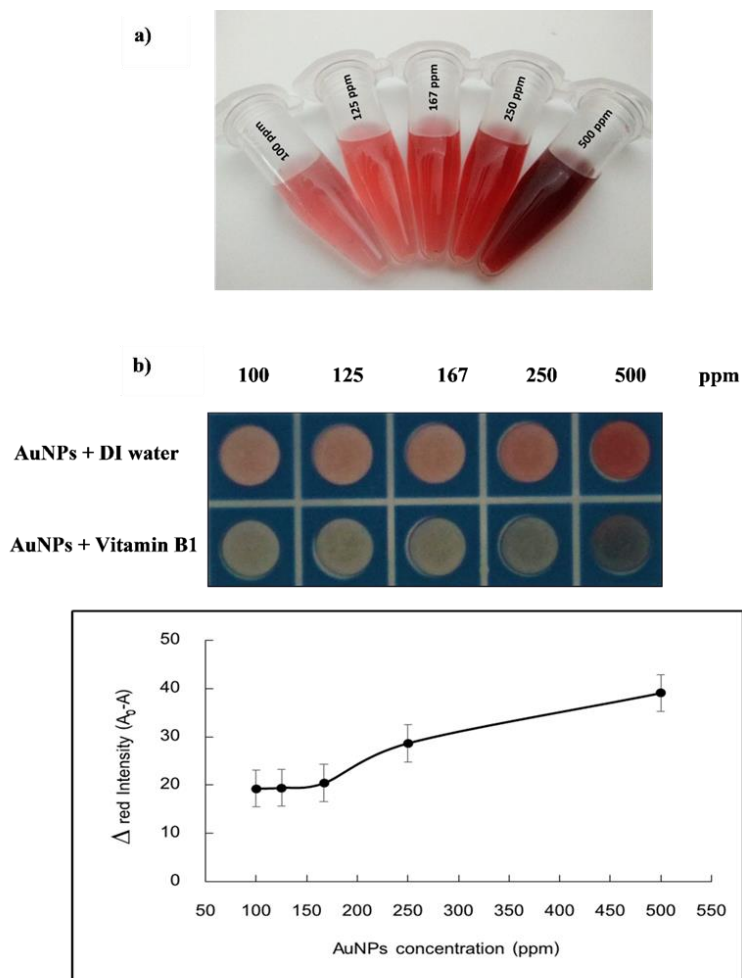


Fig. 3 (a) The color of AuNP concentrations at 100, 125, 167, 250, and 500 ppm after the preconcentration process, (b) The color intensity difference in the absence and presence of 100 ppb of vitamin B1 at 100, 125, 167, 250, and 500 ppm AuNP solutions, respectively.

2.2 Optimization of the AuNPs and vitamin B1 volume ratio

The detection zone of the transparency sheets had a maximum volume of 30 μ L. The color changes of the aggregation between the AuNPs and vitamin B1 at volume ratios of 1:5, 1:2, 1:1, 2:1, and 5:1 are shown in Fig. 4. The Δ red intensity was maximized at a 1:1 volume ratio. As such, 15 μ L of the AuNP solution and 15 μ L of the vitamin B1

solution was chosen for further experiments to provide the preferable sensitivity for vitamin B1 detection.

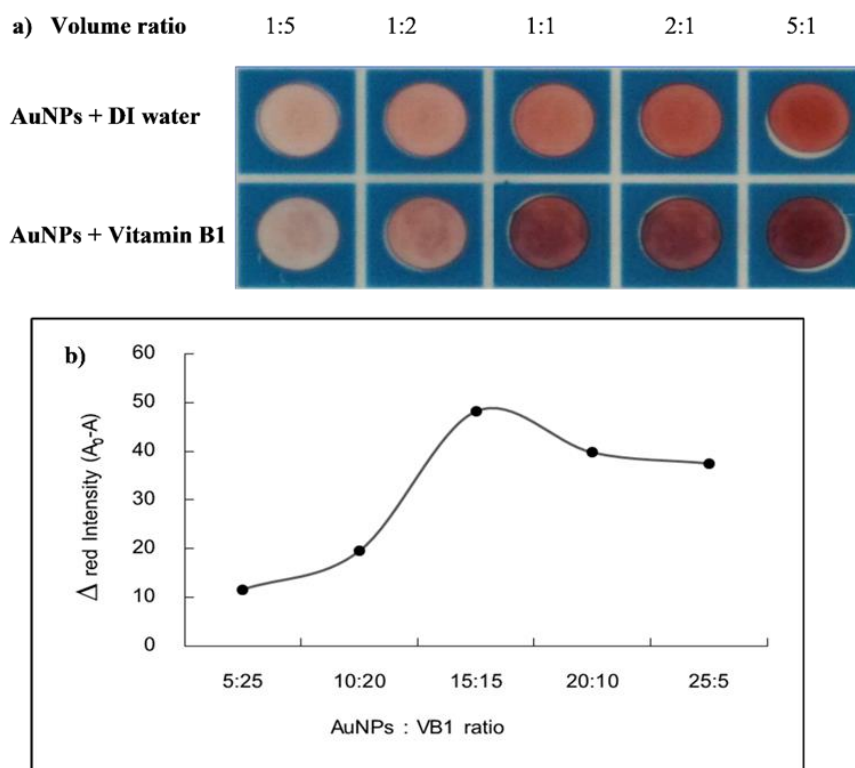


Fig. 4 (a) The color images of the aggregation between AuNPs with and without a 100 ppb solution of vitamin B1 at volume ratio 1:5, 1:2, 1:1, 2:1, and 5:1, (b) Plot of the Δ red intensity as a function of the volume ratio of AuNPs:100 ppb vitamin B1.

2.3. Optimization of pH buffer

The effects of the pH buffer on the sensitivity and selectivity after aggregation between the AuNPs and vitamin B1 were examined by varying the pH of the buffer from 2 to 10. In principle, vitamin B1 is positively charged on both the pyrimidine N1 nitrogen ($pK_{a1} \cong 4.8$) and thiazole N3 nitrogen at low pH, and vitamin B1 exists as a cation with a positive charge on the thiazole N3 nitrogen at physiological pH. As the pH further increases, vitamin B1 exists as an uncharged pseudo base intermediate, which displays a negatively charged thiol form ($pK_{a2} \cong 9.2$). As a result, the similarly charged AuNPs and vitamin B1 repelled each other when the pH was higher than 7. Therefore, pH 7.0 was chosen for the experimental buffer in the following study. In addition, it was found that when using a pH 7 buffer, the Δ red intensity of the related vitamin B samples

changed slightly, while only vitamin B1 was obviously changed, as shown in Fig. 5. From this finding, a 10 mM phosphate buffer at pH 7 was suitable for the sensitive and selective detection of vitamin B1 by this colorimetric method.

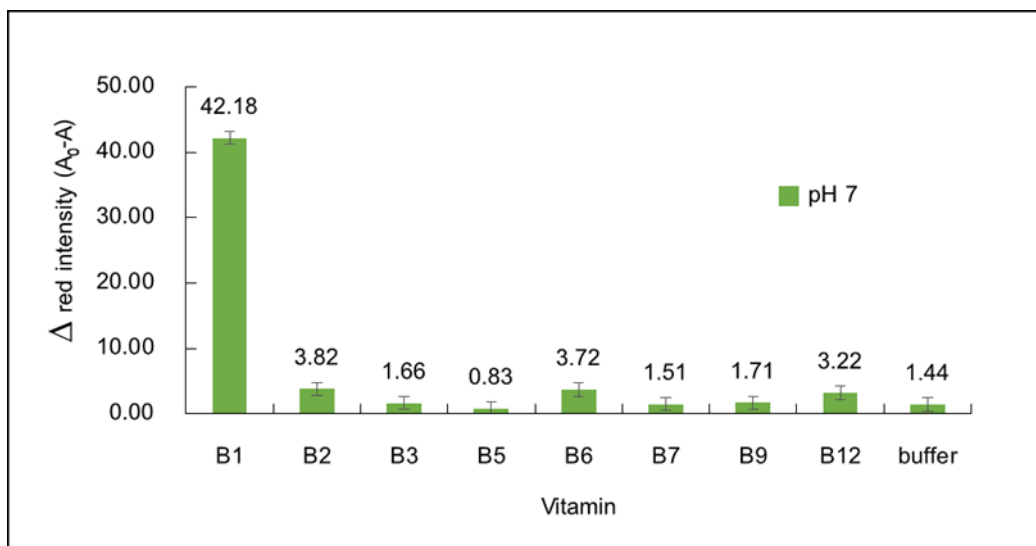


Fig. 5 Effects of a 10 mM phosphate buffer at pH 7 on the sensitivity and selectivity after aggregation between AuNPs and vitamin B1 at 100 ppb.

2.4. Optimization of the incubation time

The incubation time, within the range of 2 to 30 minutes, was optimized. The Δ red intensity was calculated from the picture that was captured and the measured intensity of the color change was monitored simultaneously, as shown in Fig. 6. The Δ red intensity increased with increasing incubation time and reached a maximum at 10 minutes. The Δ red intensity decreased after 10 minutes because evaporation of the solution may occur and background intensity also was increased. From the results, the optimal incubation time was 10 minutes. This condition provided the highest color change intensity.

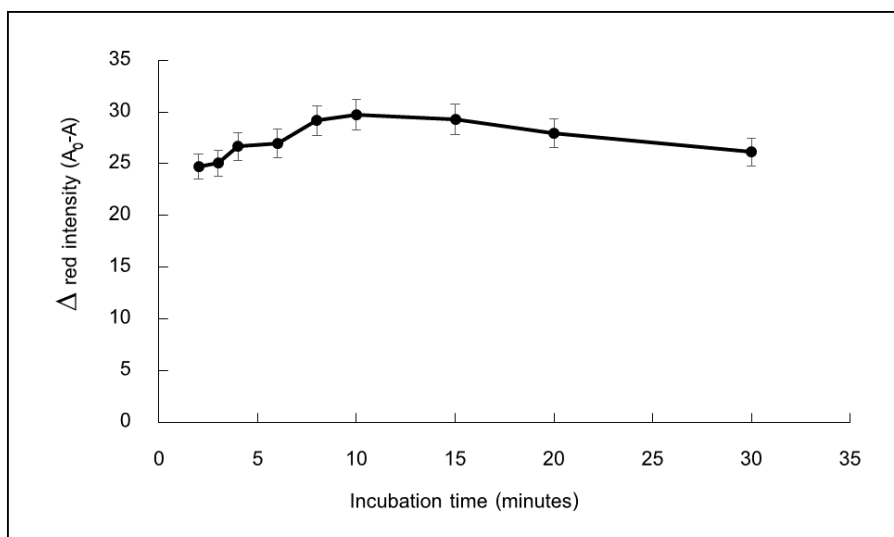


Fig. 6 The effects of incubation time on the detection of vitamin B1 at a concentration of 100 ppb.

Analytical performance of the transparency sheet-based colorimetric assay for determining vitamin B1 concentration

3.1 Calibration curve

A calibration curve was obtained from the Δ red intensity measured by mobile phone. Concentrated AuNPs (15 μ L) were dropped onto a transparency sheet, and 15 μ L of a standard vitamin B1 solution was added with a 10-minute incubation time. With an increase in the vitamin B1 concentration, the Δ red intensity of the AuNPs decreased because the AuNP color changed from red to blue, corresponding to the results from the UV-vis spectrophotometer in Fig. 1. A linear relationship between the Δ red intensity and vitamin B1 concentration was established as shown in Fig 7. The Δ red intensity of the AuNPs is directly proportional to the added vitamin B1 concentration. A good linearity was observed in the concentration range from 40 ppb to 200 ppb with a correlation coefficient of 0.9913. The limit of detection (LOD) and the limit of quantitation (LOQ) for vitamin B1 were found to be 3.002 ppb and 10.006 ppb, respectively.

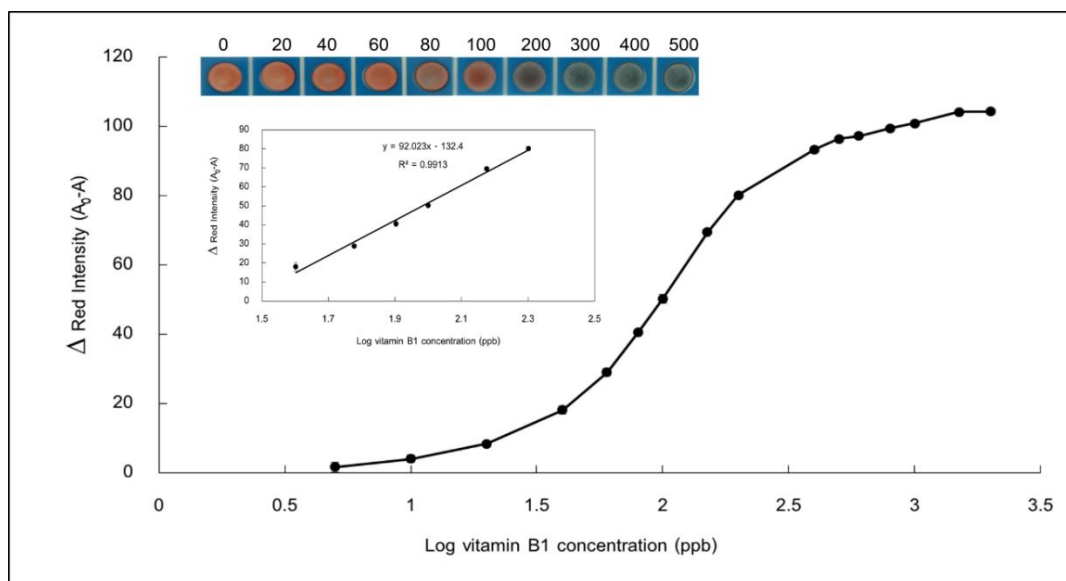


Fig. 7 Calibration curve plot of the change in the red intensity versus vitamin B1 concentration.

3.2. Selectivity of the AuNPs towards Vitamin B1 detection

To confirm the selectivity of the AuNPs towards vitamin B1 detection, related vitamin B complexes, including vitamin B2, vitamin B3, vitamin B5, vitamin B6, vitamin B7, vitamin B9, vitamin B12, and vitamin C were also tested. The color signal change in the AuNPs in the presence of other vitamin species and common interfering molecules had a less than $\pm 5\%$ change compared to that of the color signal of only vitamin B1 (20 ppb). As shown in Fig. 8, the tolerance concentration of other vitamin species and common interfering molecules have no appreciable change because at these concentrations are impossibly found in real sample. Therefore, the achieved high experimental selectivity and sensitivity is obtained.

The proposed mechanism for controlling the selectivity was discussed from results obtained. We strongly believed that it may be concerned to a positive charge on thiazole N3 nitrogen atom of vitamin B1 under the optimized conditions. It provided a high affinity for strong electrostatic interactions with the negatively charged AuNPs while other metal treated solution exhibited a negligible change. As presented in Fig. 1, after the addition of vitamin B1 to the AuNP solution, a new absorption peak appeared at higher wavelength region (650 nm) along with decrease in original SPR peak at 517 nm. While the other remained at the same position as that of AuNPs (data not shown). Quantitative measurement was performed by comparing the mean of red color-intensity difference, ΔI_{red} . This difference represents the degree of aggregation and representative of the

relative quantity of aggregated and dispersed AuNPs. In comparison, quantitative analysis of the tested solution suggested an obviously high different ΔI_{red} for vitamin B1 suggesting the maximum aggregation state, while all other metals had a mean of red color-intensity difference similar to that of AuNPs. Therefore, the proposed assay is efficient for selective detection of vitamin B1 in presence of other metal ions. However, the exact mechanism and the microscopic nature are still subject to research and discussion.

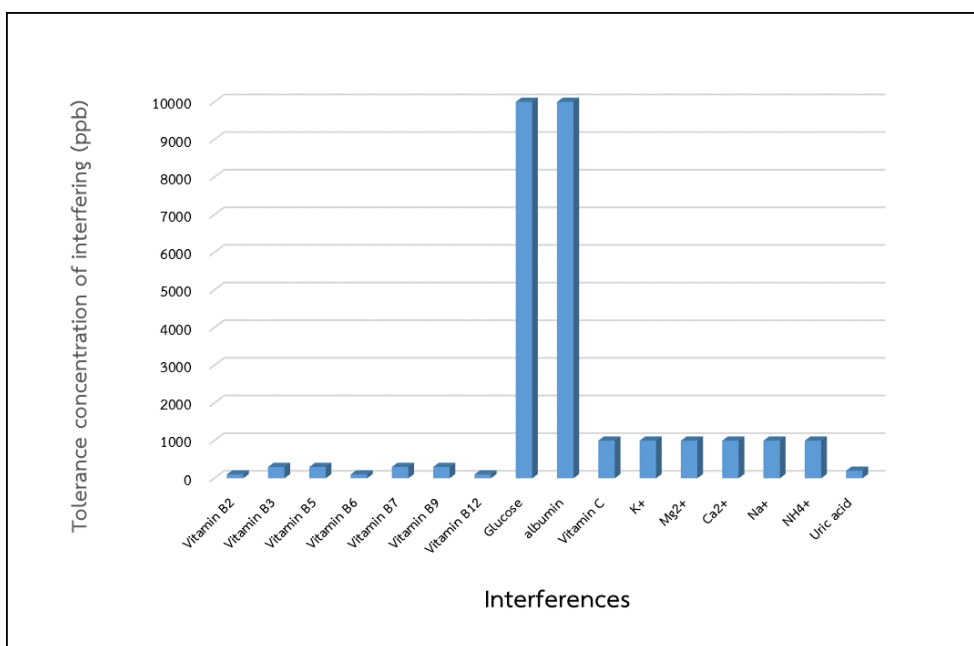


Fig. 8 The tolerance concentration of other vitamin species and common interfering molecules.

Analytical application in a real sample

To evaluate the efficiency of our method, the colorimetric sensor was used to detect vitamin B1 in urine samples. Under the optimal conditions, the determination of vitamin B1 in urine samples spiked with vitamin B1 is shown in Table 2. The recoveries and %RSDs for vitamin B1 were in the range of 84.72 - 91.08 % and 2.74 - 6.98 %, respectively. Moreover, the results for the vitamin B1 values obtained by the developed sensor were compared with the reference values obtained using a standard method. As a result, this proposed colorimetric sensor can be applied for the determination of vitamin B1 in the urine samples with satisfactory results.

Table 2. The %RSD and %Recovery for the determination of vitamin B1 in urine samples (n=10)

Vitamin B1 spiked (ppb)	Vitamin B1 (ppb)		% RSD (n=10)	% Recovery
	This Method	Fluorometric method		
40	36.43 ± 1.00	41.53 ± 0.63	2.74	91.08
60	50.83 ± 3.55	63.55 ± 0.68	6.98	84.72
80	69.06 ± 3.40	79.02 ± 0.50	4.92	86.33

Conclusion

A simple, rapid, and low-cost transparency sheet-based colorimetric assay was developed for the determination of vitamin B1 in urine samples . In addition to the direct measurement of vitamin B1, this method allows for a simple way to screen and calculate the amount of vitamin B1 in the sample. Under the optimized experimental conditions, a linear relationship was obtained between the change mean red color intensity) ΔI (and the logarithmic concentration of vitamin B1 in the concentration range of 40 to 200 ppb. A detection limit of 3.002 ppb was found for vitamin B1 detection . The usefulness of the transparency sheet-based colorimetric sensor developed in this work is its high sensitivity and selectivity, good precision, simplicity, and reproducibility. In addition, this device has been successfully applied to determine vitamin B1 in urine samples . The proposed method has major advantages, such as being equipment-free, portable for field analysis and remote sensing for oversea or sailing. This finding provides a high possibility for applications in clinical diagnostics.

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A Transparency Sheet-Based Colorimetric Device for Simple Determination of

Calcium Ions using Induced Aggregation of Modified Gold Nanoparticles

A simple and novel transparency sheet-based colorimetric detection devices using gold nanoparticles (AuNPs) modified by 4-Amino-6-hydroxy-2-mercaptopyrimidine monohydrate (AHMP) was fabricated and developed for the determination of calcium ions (Ca^{2+}). The detection was based on a colorimetric reaction as a result of the aggregation of modified AuNPs induced by Ca^{2+} due to the ability to form strong electrostatic interactions between positively charged Ca^{2+} and negatively charged modified AuNPs. Probe solution changes color from red to blue in the presence of Ca^{2+} and can be observed by the naked eyes. To verify the complete self-assembly of the AHMP onto the AuNP surface, the modified AuNPs were characterized using ultraviolet-visible spectroscopy and zeta potential measurements. Under optimal conditions, a quantitative linearity was 10 to 100 ppm ($R^2 = 0.9877$) with a detection limit of 3.05 ppm. The results obtained by the developed method were in good agreement with standard AAS results and demonstrated that this method could reliably measure Ca^{2+} . Overall, this novel alternative approach presents a low-cost, simple, sensitive, rapid, and promising device for the detection of Ca^{2+} .

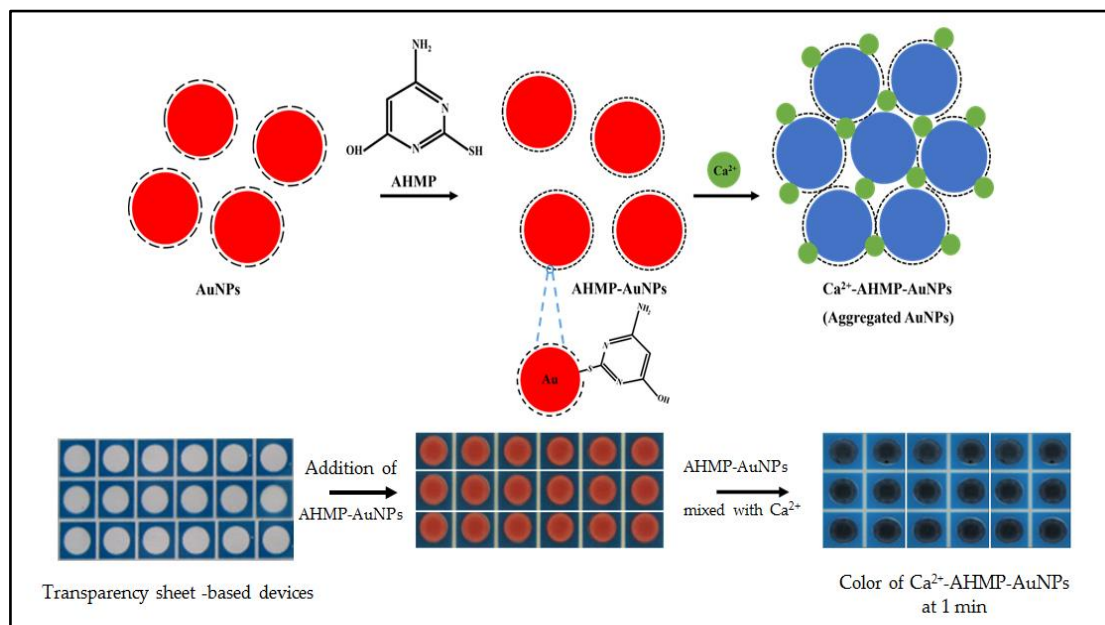
Introduction

Calcium (Ca^{2+}) is an essential nutrient and one of the most important minerals for the human body. Ca^{2+} plays a role in the growth and maintenance of bones, teeth and nails. In the ionized form, Ca^{2+} is significant in many vital processes, such as hormone secretion, nerve conduction and blood coagulation. WHO and FAO have recommended a minimum daily intake of 1,200 mg of Ca^{2+} for adults [1-2]. At the present, calcium deficiencies are taken quite seriously. Therefore, in medical diagnostics, the determination of calcium concentration is very important for the evaluation of a patient's health. A change of the Ca^{2+} concentration, which usually is relatively stable in body fluids (range in urine: 100 to 300 mg/day) [3], can indicate the presence of various pathological conditions. When calcium concentrations are lower than normal, osteoporosis, vitamin D deficiency, eclampsia or hypoparathyroidism may be present, whereas the calcium concentrations higher than normal may indicate hyperparathyroidism, vitamin D intoxication or myeloma [4]. Thus, the Ca^{2+} concentration from body fluids should be determined for the evaluation of and protection against any

disease that would be manifested by an abnormal calcium concentration. This emphasizes the need to develop highly sensitive devices for the portable, simple and rapid deficiency screening in patient care. Numerous methods have been proposed for the measurement of Ca^{2+} in biological materials. The classical method involves titration with a standardized solution of ethylenediaminetetraacetic acid (EDTA) [5-6]. However, this method is limited by sensitivity and selectivity. The preferred standard method for the determination of Ca^{2+} in body fluids is atomic absorption spectrometry (AAS) [7-10]. Other spectrophotometries, such as UV-Vis spectroscopy [11], spectrofluorimetry [12], atomic emission spectrometry (AES) [13], inductively coupled plasma [14], and fluorescence [15-17] have also been developed for Ca^{2+} determination. Currently, the determination of Ca^{2+} developed with colored complexes using metallochromic reagents, such as Eriochrome Black T (EBT), has been reported [18-19]. Even though these spectrophotometric methods provided enough sensitivity, they are difficult to design to be a portable device. Calcium ion-selective electrodes are some of the most promising methods for Ca^{2+} determination. Given their precise and selective performance, this electrochemical measurement technique has been widely developed for the detection of Ca^{2+} in biological fluids [20-22]. There have been several kinds of electrodes developed for Ca^{2+} detection, including solid-contact ion selective electrode [23] and polymer membrane ion-selective electrodes [24]. Unfortunately, most of the above-mentioned methods are still require a specialist and specific instrument. Presently, with the rapid growth of near-patient devices used at home or the hospital bedside, there is an increasing demand for portable systems utilizing small disposable sensors which is capable of the measurement of biomarkers in biological samples. Patients can collect biological samples and measure target analyt concentrations at home within a few minutes without specialized instruments. From this requirement, it is the purpose of this work to present a simple, rapid, reliable, and low-cost detection method that uses a small sample volume for the determination of Ca^{2+} in urine samples using colorimetric gold nanoparticles. Recently, paper-based analytical devices (PADs) are one of the most devices used as a miniaturized analytical platform for an economical and portable sensing applications. Paper-based platform can be benefits for applications in diagnostics, food safety, and environmental monitoring due to the advantages of natural abundance, simplicity, disposability, low sample-reagent usage and suitability for routine measurement. Unfortunately, the use of paper as substrate for colorimetric detection is usually suffer from the adsorption of color by paper lead to the low sensitivity in

comparison to solution. Therefore, the searching for new materials used as a substrate to fabricate the analytical platform is challenge. In this research, we first proposed to use transparency sheet as substrate-based for creating a new colorimetric device platform. Gold nanoparticles (AuNPs) have been widely used as colorimetric probes for metal ions, anions, small molecules, proteins, nucleic acids, and other analytes because of their unique properties, such as optical properties dependent on their spacing or surface plasmon resonances. The extinction spectrum and the wavelength at which AuNPs absorb and scatter light depends on the distance between particles [25-26]. For example, citrate-coated AuNPs were employed as colorimetric probe for organophosphorus pesticides detection [27]. Furthermore, AuNPs are easily modified by capping agents, which are specific to a given analyte. Therefore, functionalized AuNPs have been applied as colorimetric probes, receiving great attention in visual sensing applications because of the plasmonic absorbance shift. The functionalization can perform using various ligand such as AuNPs functionalized with the peptide substrates (Gly-Pro-Asp-Cys (GPDC) or Val-Pro-ethylene diamine-Asp-Cys (VP-ED-DC)) were proposed for determination of DPP-IV/CD-26 activity based on the aggregation of functionalized AuNPs [28]. 4-Amino-6-hydroxy-2-mercaptopyrimidine monohydrate (AHMP) is interesting ligand. It contains a thiol group which can self-assemble on the AuNP surface [29-30]. Additionally, the amine and hydroxyl groups available in AHMP are free from binding. The presence of the lone pair electrons of amine and hydroxyl groups lead to a negative charge around the AuNP surface. After modification, AHMP-AuNPs have different a zeta potential from unmodified indicating negative charge. From this finding, it prompts us to use AHMP-AuNPs as the probe for Ca^{2+} detection. Owing to the electrostatic force between the negatively charged AHMP-AuNPs and positively charged Ca^{2+} may induce aggregation. The color change of AHMP-AuNPs has become a useful tool in the development of colorimetric sensing, transparency sheet-based analytical devices. These devices can determine the Ca^{2+} concentration in urine samples by assessment with the naked eye. The proposed mechanism for Ca^{2+} detection, the induction of the aggregation of AHMP-AuNPs, is shown in **Scheme 1**. AHMP molecules can be absorbed onto the surface of the AuNPs via the thiol group, resulting in the enhancement of the negative charge around the surface of AuNPs after modification. Ca^{2+} can induce the aggregation of AHMP-modified AuNPs via electrostatic force. The red color of the AHMP-AuNPs changes to blue in accordance with the Ca^{2+} level added. Moreover, the proposed approach has been successfully applied to detect Ca^{2+} level in urine samples. The verified results were in good agreement

with atomic absorption spectroscopy (AAS). The simplicity, speed, suitability for real-time on-site detection and lack of required complex and expensive instruments could make this method to be a promising tool for point-of-care (POC) diagnosis.



Scheme 1. The proposed mechanism scheme and the transparency sheet-based devices for detection of Ca^{2+} using AHMP-AuNPs.

Experimental

Materials and instrumentation

Gold nanoparticles (AuNPs, 0.01% w/v (100 ppm), diameter 20 nm) were purchased from Kestrel Bio Sciences (Thailand). Calcium carbonate (CaCO_3), sodium chloride (NaCl), magnesium chloride (MgCl_2), disodium hydrogen orthophosphate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$), sodium dihydrogen orthophosphate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$), and hydrochloric acid were purchased from Ajax Finechem Pty Ltd. 4-Amino-6-hydroxy-2-mercaptopyrimidine monohydrate (AHMP), glucose, uric acid, albumin and ascorbic acid (vitamin C) were purchased from Merck (Darmstadt, Germany). Artificial urine was purchased from Carolina Biological Supply Company. Analytical grade reagents and $18 \text{ M}\Omega \text{ cm}^{-1}$ resistance deionized (DI) water (obtained from a Millipore Milli-Q purification system) were used throughout.

Phosphate buffers (pH 5–8) were prepared from 0.1 M of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and 0.1 M $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$. Phosphate buffer (pH 9–12) was prepared from 0.1 M of

$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and 0.1 M $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ and the pH was adjusted with 0.1 M sodium hydroxide (Merck) solution.

A wax printer (Xerox Color Qube 8570, Japan) was used to print wax onto transparency sheets to obtain the detection devices. Absorption spectra were measured by using a UV-2450 UV-visible spectrophotometer (SHIMADZU). The zeta potential measurements were performed on Zeta sizer Nano S90 (Malvern). An atomic absorption spectrometer (AAS) with a hollow cathode lamp and standard air/acetylene flame was also used (Analyst 300, Perkin Elmer Instruments).

Preparation of AHMP-Modified Gold Nanoparticles (AHMP-AuNPs)

First, the purchased gold nanoparticles were preconcentrated by pipetting the commercial gold nanoparticles into 1 mL micro centrifuge tubes. The tubes were centrifuged at 12,000 rpm (4°C) for 30 min. Then, 0.8 mL of supernatant was removed, and the remaining 0.2 mL of solution was stirred for 10 seconds.

AHMP-AuNPs were prepared by mixing 0.10 mL of 4 mM AHMP and 1.50 mL of the preconcentrated AuNP solution. After that, the mixture was stirred for 15 minutes to ensure the complete self-assembly of AHMP onto the surface of the AuNPs.

Fabrication of transparency sheet-based devices

Transparency sheet -based devices (Polypropylene, purchased from local stationery store) were fabricated using a wax printing method. The pattern of the device was designed as an array containing a circular area with a detection zone 5- mm in diameter using the graphic software Adobe Illustrator CS4. The fabrication process has only two steps. First, the wax pattern was printed on the transparency sheet using the wax printer (Xerox Color Qube 8570, Japan). The maximum liquid volume in the detection zone was 30 μL . The wax-covered area was hydrophobic, while the detection area without wax was hydrophilic. Second, white paper was attached the back side of the transparency sheet with tape to enhance the visibility of the color change. Using this process, the transparency sheet-based devices could be fabricated within 2 minutes.

Transparency Sheet-Based Colorimetric detection of Calcium

The working standard solution for the Ca^{2+} was made by appropriate dilution of the stock standard solution on a daily basis as required. A colorimetric analysis of Ca^{2+} was performed as follows. First, 5 μL of standard Ca^{2+} solution or samples (prepared in

pH 6.0 PBS) were added into the detection zone of a transparency sheet-based device, followed by the addition of 25 μL of AHMP-AuNPs. Then, the red/blue intensity of AHMP-AuNPs mixed with Ca^{2+} standard solution was captured by mobile phone (Vivo V3) in a light control box and measured via Image J software (<https://imagej.nih.gov>). Under optimal conditions, the relationship between the red/blue intensity of triplicate measurements and concentrations of the Ca^{2+} solution between 10 – 100 ppm were plotted to obtain the linear range.

Analysis of Ca^{2+} in artificial urine samples

Under optimal conditions, the proposed method was used for the determination of Ca^{2+} in artificial urine samples. The artificial urine was purchased from Carolina Biological Supply Company (controlled level). The proposed method was validated against atomic absorption spectrophotometry (AAS). An appropriate volume of 1000 ppm Ca^{2+} was added to 25 mL of artificial urine for preparation of the 500 ppm Ca^{2+} stock solution. Then, the stock solution was diluted with artificial urine to 320, 160 or 80 ppm Ca^{2+} concentration. The samples were adjusted to pH 6 by the addition of 3 M sodium hydroxide and additional urine was added to reach a final volume. Prior to analysis with the proposed method, the 320 and 160 ppm Ca^{2+} samples were diluted with 50 mM phosphate buffer pH 6 4-fold and 2-fold, respectively. The AAS method can detect Ca^{2+} within the range of 0.5-5 ppm. Therefore, the 320, 160 and 80 ppm Ca^{2+} samples were diluted with 50 mM phosphate buffer pH 6 150-fold, 45-fold and 80-fold, respectively.

Results and discussion

3.1. Characterization of AHMP-AuNPs

To investigate the incorporation of AHMP onto the AuNP surface, UV-vis spectroscopy and zeta potentials were determined. **Figure 1** shows the UV-vis spectra of AHMP, AuNPs and AHMP-AuNPs. The localized surface plasmon resonance (LSPR) absorption peak of AHMP has a maximum peak at 275 nm. The AuNPs alone have a maximum peak at 519 nm. After the AuNPs were modified with AHMP, the UV-vis spectra show two maximum peaks at 273 and 523 nm. We believed that the additional peak and the red shifting of the band is mainly caused by the decrease of the plasma oscillation frequency around the nanoparticles from the binding between thiol-containing compounds and the gold nanoparticles. Corresponding to the zeta potential results, the surface charge of AuNPs and AHMP-AuNPs were found at -43.1 and -49.9 mV, respectively.

The zeta potentials indicated the negative charge of AuNPs increased from -43.1 mV (intensity 32 a.u.) to -49.9 mV (intensity 38 a.u.), as shown in **Table S1**. This confirmed that the surface of the AHMP-AuNPs has more negative charge which is corresponding to the previous work. It is expected that the thiol group of AHMP is chemisorbed on the surface of AuNPs, whereas amine and hydroxyl groups are available in AHMP free from binding. The presence of lone pair electrons in amine and hydroxyl groups led to the negative charge around the AuNP surface. This result displayed that AHMP had been completely modified onto the surface of AuNPs.

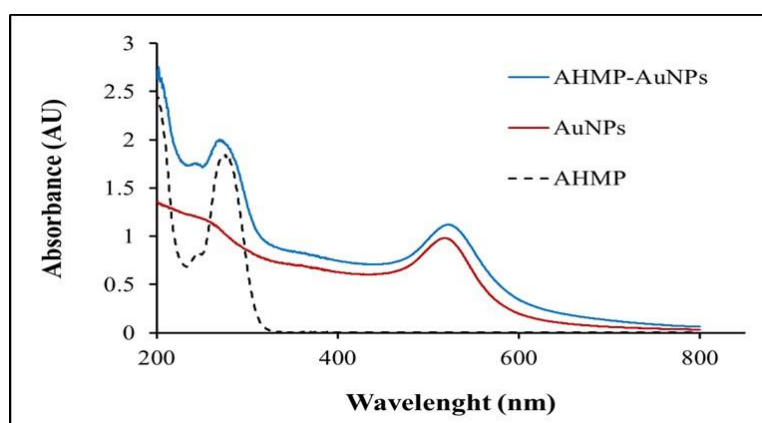


Figure 1 UV- Vis absorption spectra of AHMP, AuNPs and AHMP-AuNPs

3.2. Colorimetric assay of Ca^{2+}

Following the AuNP modification, a colorimetric analysis using AHMP-AuNPs was performed for the detection of Ca^{2+} . The color and absorption spectra of the AHMP-AuNPs with and without Ca^{2+} are shown in **Figure 2**, while their zeta potentials were displayed in **Table S1**. After addition of buffer, the negative charge of AHMP-AuNPs solution increased from -49.9 mV to -58.2 mV, while the average size of the Buffer-AHMP-AuNPs was not significant change (from 29.1 to 27.6 nm). After addition of Ca^{2+} , the zeta potential dramatically decreased from -58.2 mV, intensity 35 a.u., to -50.3 mV, intensity 26 a.u., while the average size of the AHMP-AuNPs was increased from 29.1 to 65.4 nm. These results indicated that the AHMP-AuNPs aggregation was induced by electrostatic force between the positive charge of Ca^{2+} and the negative charge of AHMP-AuNPs. Moreover, the results obtained corresponded to the decrease of the absorption bands at 273 and 523 nm along with the formation of a new absorption band at 680 nm (redshift), as illustrated in **Figure 2**. Furthermore, the color of the AHMP-AuNPs on the proposed device was clearly observed to change from red to blue immediately, which

suggested the growth of the nanoparticles' size. This indicated that Ca^{2+} could quickly bind to their functional groups ($-\text{NH}_2$ and $-\text{OH}$) onto modified AuNPs [31, 32]. These binding interactions and electrostatic force all contribute to the aggregation mechanism of the AuNPs, as schematically illustrated in **Scheme 1**. According to the considerable change in negative charge, color and LSPR absorption of AHMP-AuNPs upon the addition of Ca^{2+} , the development of a novel colorimetric AHMP-AuNPs device for Ca^{2+} determination was successfully accomplished.

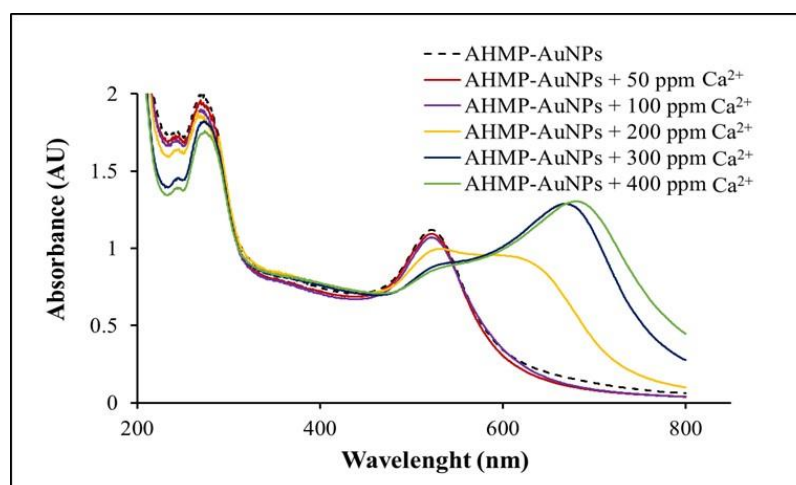


Figure 2 UV- Vis absorption spectra of AHMP-AuNPs (dot line) and AHMP-AuNPs in the presence of Ca^{2+} at 50 (red line), 100 (purple line), 200 (yellow line), 300 (navy line) and 400 ppm (green line).

3.3. Optimization for the Determination of Ca^{2+} on transparency sheet-based devices

In this section, the modifier volume, pH, incubation time and AHMP-AuNP:sample volume ratios were examined for optimization in order to accelerate the performance of the transparency sheet-based colorimetric device. The colorimetric detection of Ca^{2+} was performed at room temperature by the sequential addition of standard Ca^{2+} and AHMP-AuNPs on the detection zone of the transparency sheet. The red/blue intensity was measured and calculated by mobile phone and Image J software, respectively. The result of the aggregation capability between AHMP-AuNPs and Ca^{2+} in each condition was observed by the red/blue intensity from each transparency sheet image. First, the volume of 4 mM AHMP modifier was varied at 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.5 mL with the same volume of concentrated AuNPs (1.5 μL). Then, 15 μL of the varied AHMP-AuNPs were investigated with 15 μL of Ca^{2+} at 0, 10, 50 and 100 ppm concentration. In **Figure**

3, the red/blue intensity of the blank (AHMP-AuNPs and PBS Buffer, pH 6) decreased when the AHMP volumes were increased. It may be that the AuNPs were diluted by the AHMP solution, so that AHMP-AuNPs had reduced aggregation efficiency. The red/blue intensity of 0.1 mL AHMP was found to exhibit the most significant change from the blank, especially when tested with 100 ppm Ca^{2+} . Moreover, this change could be easily detected with the naked eye. Therefore, 0.1 mL AHMP was selected as an optimum modifier volume for further studies.

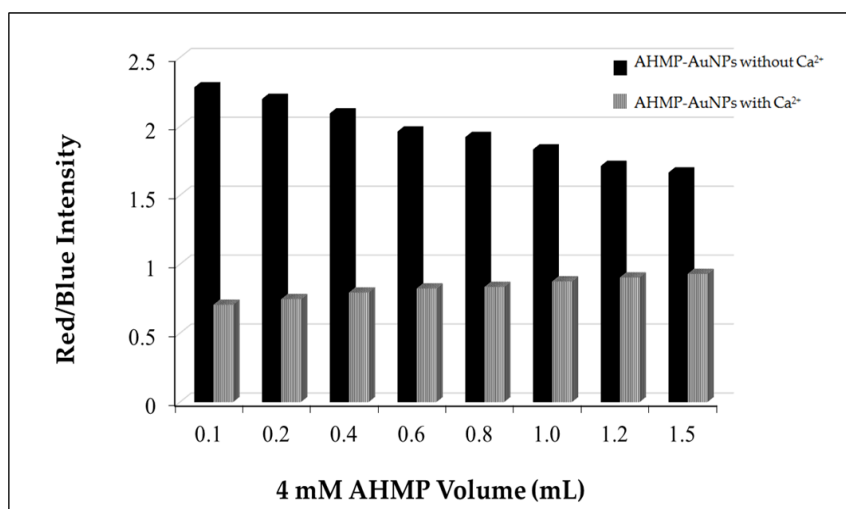


Figure 3 The red/blue intensity of the AHMP-AuNPs at different AHMP volume at 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.5 mL without (solid bar) and with (thin bar) 100 ppm Ca^{2+} , AHMP-AuNP:sample volume ratio at 1:1

Then, the effect of pH (range of 3-12) on the aggregation process was studied. As shown in **Figure 4**, the use of phosphate buffer with pH of 5.8-12 did not change the color of blank. Therefore, phosphate buffers of pH 5.8-12 were chosen to study the aggregation effect of AHMP-AuNPs and 100 ppm Ca^{2+} at a 1:1 volume ratio. The results demonstrated that pH 6 provided the maximum color change. At this pH, Ca^{2+} has a high potential to induce the aggregation of AHMP-AuNPs. Therefore, phosphate buffer pH 6 was chosen for further studies.

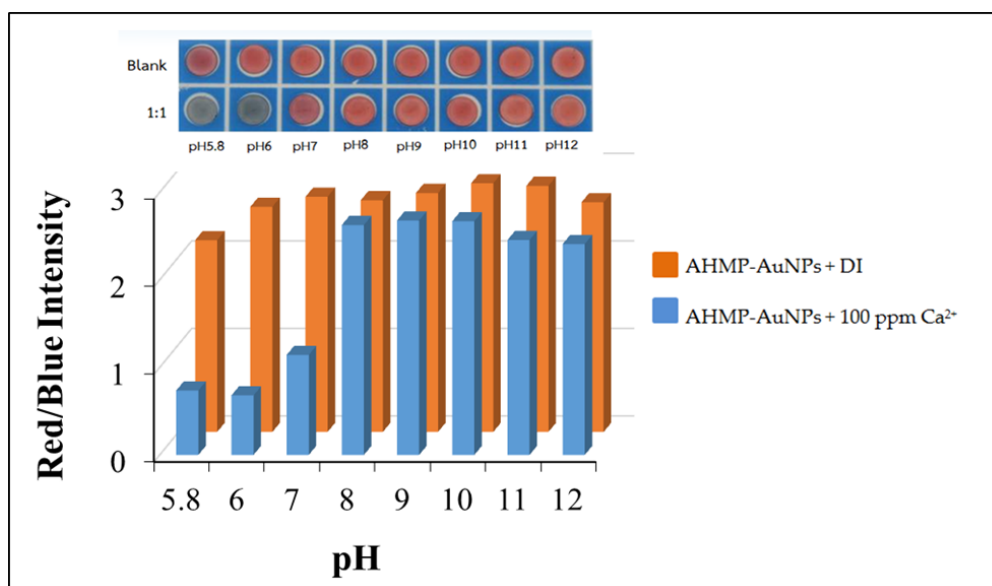


Figure 4 The influence of pH over the range 5.8-12 on the aggregation process of the AHMP-AuNPs and 100 ppm Ca²⁺, other conditions as same Figure 3

Next, the aggregation of AHMP-AuNPs induced by Ca²⁺ was investigated at various incubation times (1 – 30 min). The aggregation of the AHMP-AuNPs occurred immediately after the addition of Ca²⁺, and was completed in 1 minute. In other words, the red/blue intensity from the aggregation of AHMP-AuNPs induced by Ca²⁺ can be measured at 1 minute. When AHMP-AuNPs were left on the device for over 5 minutes, the red/blue intensity of the blank was increased from the initial (**Figure S1**) because of self-aggregation of the AHMP-AuNPs. In the case of interferences, Mg²⁺ was first selected as a representative of other positive ions, as it has an atomic radius and positive charge nearly equal to those of Ca²⁺ to investigate the possibility of using this proposed probe. From these results, it was found that the red/blue intensity of AHMP-AuNPs in the presence of Mg²⁺ were decreased more than 5%, compared to the blank, after 5 minutes. This indicated that other positive ions may also interfere with the proposed method if the incubation time was over 5 minutes. From this critical observation, we strongly believed that an incubation time of 1 minute is appropriate to specify the detection of Ca²⁺ by electrostatic force and size selective in the presence of others divalent ions. Therefore, the incubation time is critical point that has to keep in mind for selectivity study in the following part. Finally, to find a suitable quantity of the AHMP-AuNPs to sufficiently interact with Ca²⁺, the reagent volume ratio was tested between 5:25 and 25:5 (AHMP-AuNPs:Ca²⁺). The red/blue intensity of AHMP-AuNPs after mixing with Ca²⁺ was measured at various reagent volume ratios with 30 μ L maximum volume. As shown in

Figure S2, the volume ratio of AHMP-AuNPs:Ca²⁺ at 25:5 provided the highest red/blue intensity. This indicated that a 25:5 ratio of AHMP-AuNPs:Ca²⁺ was sufficient and appropriate for the determination of Ca²⁺.

3.4 Selectivity for the determination of Ca²⁺

After getting the optimal conditions, selectivity was evaluated by the $\Delta_{\text{red/blue}}$ intensity (red/blue intensity of blank – red/blue intensity of sample) of AHMP-AuNPs mixed with other substances and metal ions possibly found in urine, i.e., ascorbic acid, uric acid, albumin, glucose, Mg²⁺, K⁺, Na⁺, Cl⁻, CO₃⁻, PO₄²⁻ and SO₄²⁻, since they may also affect the aggregation of AHMP-AuNPs and might interfere with the method accuracy. An interference was defined to happen when the $\Delta_{\text{red/blue}}$ intensity in the mixture varied by more than $\pm 5\%$ of the Ca²⁺ intensity. As emphasized previously, the incubation time directly affects to the selectivity. Therefore, the change of color was measured at 1 minute after mixing to neglect the interference from Mg²⁺. As shown in **Figure 5**, the results demonstrated clearly that the $\Delta_{\text{red/blue}}$ intensity of AHMP-AuNPs mixed with foreign compounds with concentrations even 10 times greater than the concentration of Ca²⁺ did not significantly affect the signal of Ca²⁺ and the tolerance limits were shown in **Table S2**. In addition, we also applied this probe for detection of other divalent cations such as Hg²⁺, Cu²⁺, Cd²⁺ and Zn²⁺. According to the results, it can be seen that the interferences from almost all metal ions studied did not affect Ca²⁺ detection. Therefore, this probe provides high selectivity for only Ca²⁺ detection at incubation time of 1 minute.

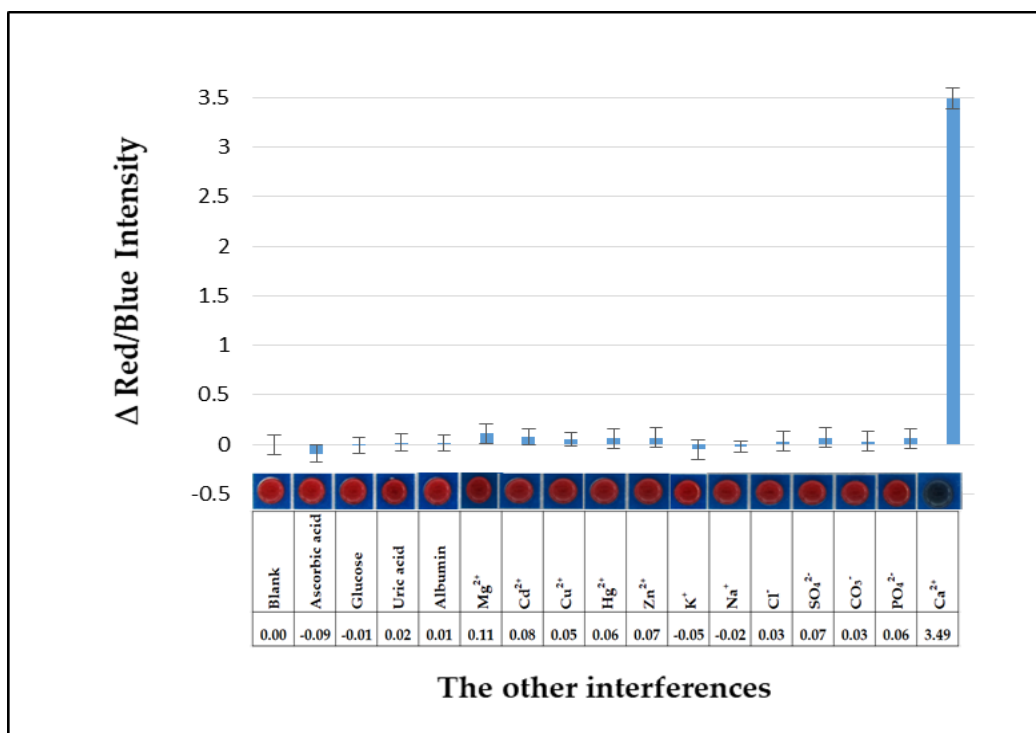


Figure 5 Responses of AHMP-AuNPs in the other interferences. The concentration of Ca²⁺ was fixed at 100 ppm while other interferences were used at 1,000 ppm. All results were repeated three times (n=3). Other conditions were used as same as optimal values

3.5 Analytical performances

Under the optimal conditions, the analytical performance for determination of Ca²⁺ was investigated by AHMP-AuNPs using transparency sheet-base devices. Ca²⁺ concentrations were studied between 0 ppm and 100 ppm. As shown in **Figure 6**, it was found that the $\Delta_{\text{red/blue}}$ intensity from the aggregation of AHMP-AuNPs by Ca²⁺ was significantly reduced when the concentration of Ca²⁺ was increased. A linear relationship between the $\Delta_{\text{red/blue}}$ intensity and log concentration of Ca²⁺ in a calibration curve was obtained. A good linearity was observed for Ca²⁺ concentrations ranged from 10 ppm to 100 ppm with a correlation coefficient of 0.9877. The detection limit from calculation by using three times the standard deviation of the blank divided by the slope of the calibration curve ($3 S_{\text{blank}}/\text{slope}$) were found to be 3.05 ppm. The proposed method provided a good recovery in the range of 91.7 - 106.7%. These recoveries were within the acceptable recovery percentages, 90–107%, for a concentration of 100 ppm (mg/kg) or at a Ca²⁺ concentration lower than 100 ppm, as recommended by the Association of Official Analytical Chemists (AOAC). The repeatability of this probe was investigated using 40

and 80 ppm standard solutions of Ca^{2+} ($n = 5$ measurements with different probes). The relative standard deviations were 2.54 and 5.16%, which indicated that there was good probe-to-probe reproducibility.

The comparisons between some previous report for the determination of Ca^{2+} and proposed devices were summarized in **Table S3**. This proposed device demonstrates the ability of transparency sheet-based colorimetric detection system to conveniently measure Ca^{2+} . In comparison, even though this proposed method had a detection limit higher than some of previous work as shown in **Table S3**. However, this limit of detection can be accepted for the determination of Ca^{2+} in urine samples. Moreover, this proposed platform offers the benefit in terms of simplicity, speed, suitability for real-time on-site detection and lack of required complex and expensive instruments. Therefore, it can provide a promising tool for Ca^{2+} diagnosis and could be an alternative choice for point-of-care analysis in the future.

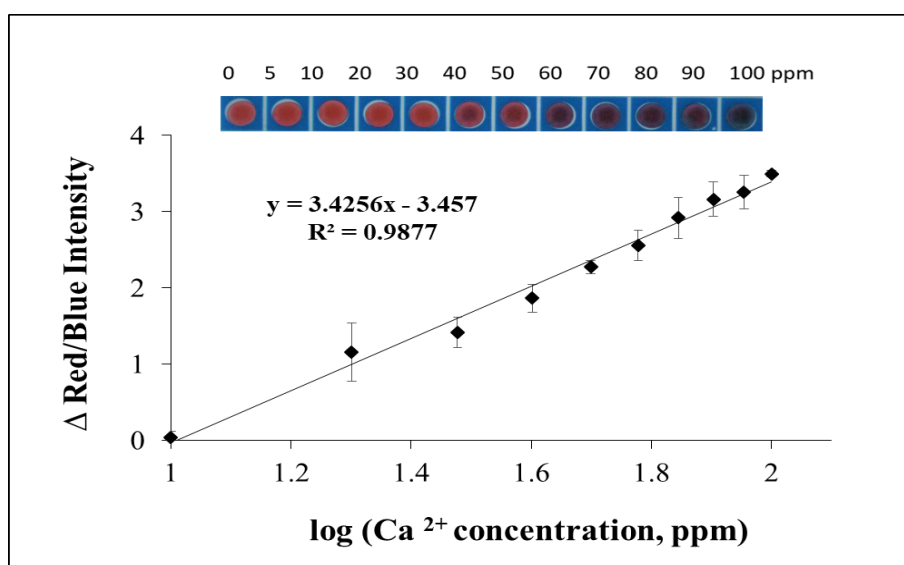


Figure 6 The transparency sheet-based devices for the quantitative analysis of Ca^{2+} and the calibration plot of $\Delta \text{red/blue}$ intensity and the log concentration of Ca^{2+} (error bar represented the standard deviation at $n = 3$). Other conditions were used as same as optimal values.

3.6. Analysis of artificial urine samples and method validation

To evaluate the efficiency of the proposed method, the transparency sheet-based devices were used to detect Ca^{2+} in artificial urine samples, and the results were

compared to the AAS method as shown in **Table S4**. The urine samples were spiked with standard Ca^{2+} at 80, 160 and 320 ppm. Then, the samples were further diluted to produce a final appropriate dilution factor before analysis. All artificial urine samples were analyzed by the AAS standard method and the proposed method. The obtained concentrations from the two methods were statistically compared using the *t*-test at the 95% confidence level. A *t*-test result was obtained with a value of 1.75, which is lower than the t_{critical} of 4.30. Thus, there was no significant difference between the concentrations obtained from these two methods. As a result, this proposed colorimetric method can be applied for the determination of Ca^{2+} in urine samples with satisfactory results, which suggests that the transparency sheet-based colorimetric device is accurate and reliable for practical applications in the clinical field.

Table S1 Zeta potential measurement of AHMP, AHMP-AuNPs, Buffer-AHMP-AuNPs and Ca^{2+} -Buffer-AHMP-AuNPs

Samples name	Z-Average (nm)	Zeta Potential (mV)	Intensity (a.u.)
AuNPs	26.9	-43.1	32
AHMP-AuNPs	29.1	-49.9	38
Buffer-AHMP-AuNPs	27.6	-58.2	35
Ca^{2+} -Buffer-AHMP-AuNPs	65.4	-50.3	26

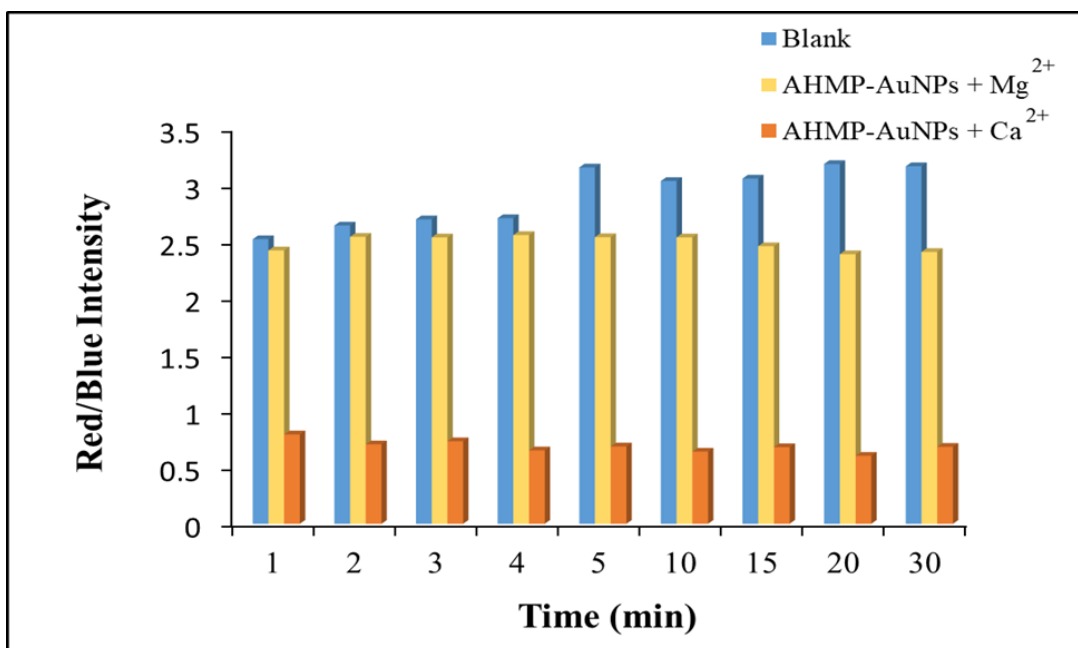


Figure S1 The Effect of the incubation time for the aggregation of AHMP-AuNPs without and with Ca²⁺ 100 ppm compare with Mg²⁺ 100 ppm

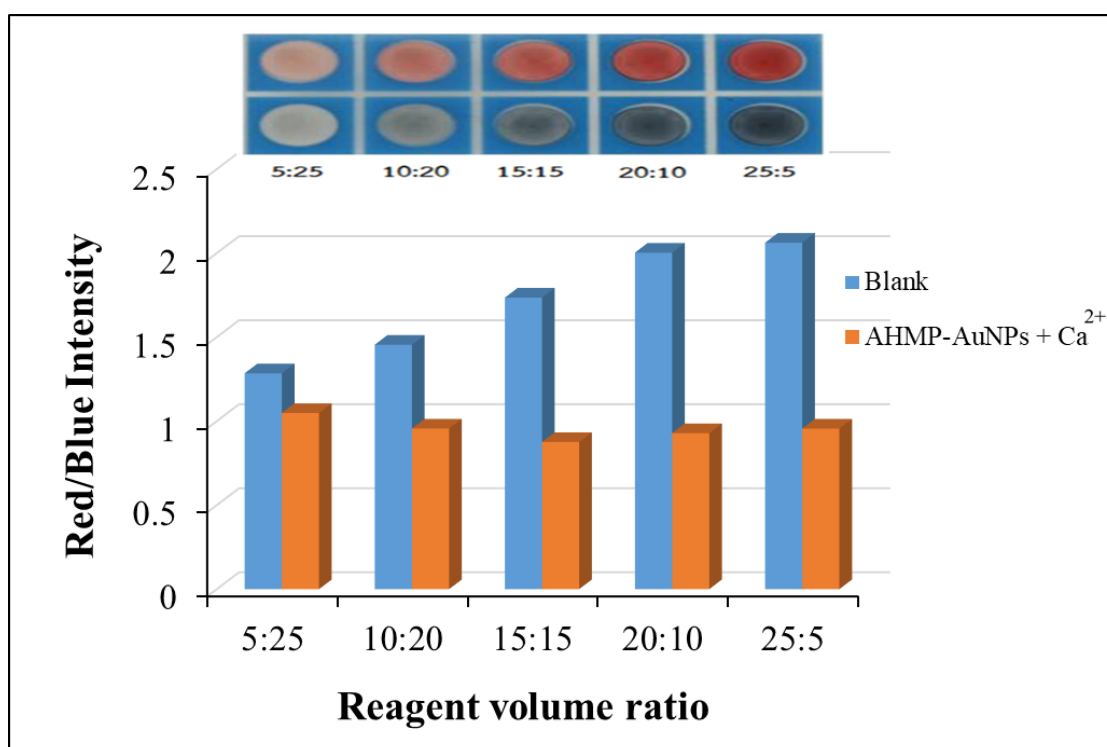


Figure S2 The Effect of the reagent volume ratio for the aggregation of AHMP-AuNPs and Ca²⁺ at 100 ppm, incubation time 1 min

Table S2 Tolerance of AHMP-AuNPs with interference as Ca^{2+} 50 ppm

Ca^{2+} : Interferences	Ratio of color change (Tolerance)
Ca^{2+} : Ascorbic acid	1:20
Ca^{2+} : Glucose	1:200
Ca^{2+} : Uric acid	1:40
Ca^{2+} : Albumin	1:200
Ca^{2+} : Mg^{2+}	1:10
Ca^{2+} : K^+	1:30
Ca^{2+} : Na^+	1:40
Ca^{2+} : Cl^-	1:40
Ca^{2+} : SO_4^{2-}	1:10
Ca^{2+} : CO_3^{2-}	1:20
Ca^{2+} : PO_4^{2-}	1:10

Table S3 Comparison of analytical performances of different methods for the determination of Ca^{2+} in biological fluid

Method	Sample	LOD (ppm)	LOQ (ppm)	Linear range (ppm)	Ref.
Atomic absorption spectrometry (AAS)	Blood	0.02	0.07	0.02-2.60	8
Sequential injection analysis/ Spectrophotometer	Pharmaceutical, Water, Urine	0.05	-	0-20	11
Atomic emission spectrometry (AES)	Gluconates oral solution, Blood	0.01–0.10	-	1–10	13
Fluorescence	Human serum	0.64	1.72	1.72-8.02	15
Fluorescence	Human serum	3.09×10^{-6}	-	4×10^{-5} - 4×10^{-4}	16
Fluorescence	Blood	10.00	-	0-80,000	17
Ion selective electrode/ electrochemistry	Blood serum	0.48	-	20.04-32.06	24

Transparency sheet-based colorimetric device	Urine	3.05	10.17	10-100	This work

Table S4 Determination of Ca^{2+} in artificial urine samples using the proposed method compared with an AAS method (n = 5)

Samples	Added Ca^{2+} (ppm)	AAS method		The proposed method	
		Found	%Recovery	Found	%Recovery
Sample 1	0	0.00±0.00	-	0.00±0.00	-
Sample 2	80	78.96±1.80	98.7	83.40±3.67	104.3
Sample 3	160	164.07±0.00	102.5	166.31±4.74	103.9
Sample 4	320	315.37±3.37	98.6	331.81±2.61	103.7

Conclusions

This paper demonstrated, for the first time, the use of AHMP-AuNPs for a transparency sheet-based colorimetric method to provide simple, rapid, inexpensive, reliable and portable devices for the determination of Ca^{2+} in urine samples. This method was based on the aggregation of AHMP-AuNPs on the device by the electrostatic force between the negatively charged of AHMP-AuNPs and the positively charge of Ca^{2+} . Under optimal conditions, the color change of the AHMP-AuNPs can be clearly observed by the naked eye. Furthermore, the proposed method was successfully applied to the quantitative analysis of Ca^{2+} in artificial urine samples. This colorimetric device provided a result within 1 minute, much faster than other mentioned methods. This simple device

is highly suitable for Ca^{2+} quantitative analysis and can act as an alternative method for the rapid screening of Ca^{2+} deficiency without the need for any special instrumentation. The advantages of the transparency sheet-based colorimetric device developed from this work include its high sensitivity and selectivity, good precision, simplicity, and reproducibility. These findings offer a high potential for applications in clinical diagnosis.

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Appendix



Article

A Transparency Sheet-Based Colorimetric Device for Simple Determination of Calcium Ions Using Induced Aggregation of Modified Gold Nanoparticles

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Abstract: A simple and novel transparency sheet-based colorimetric detection device using gold nanoparticles (AuNPs) modified by 4-Amino-6-hydroxy-2-mercaptopyrimidine monohydrate (AHMP) was fabricated and developed for the determination of calcium ions (Ca^{2+}). The detection was based on a colorimetric reaction as a result of the aggregation of modified AuNPs induced by Ca^{2+} due to the ability to form strong electrostatic interactions between positively charged Ca^{2+} and negatively charged modified AuNPs. Probe solution changes color from red to blue in the presence of Ca^{2+} and can be observed by the naked eyes. To verify the complete self-assembly of the AHMP onto the AuNP surface, the modified AuNPs were characterized using ultraviolet–visible spectroscopy and zeta potential measurements. Under optimal conditions, a quantitative linearity was 10 to 100 ppm ($R^2 = 0.9877$) with a detection limit of 3.05 ppm. The results obtained by the developed method were in good agreement with standard atomic absorption spectrometry (AAS) results and demonstrated that this method could reliably measure Ca^{2+} . Overall, this novel alternative approach presents a low-cost, simple, sensitive, rapid, and promising device for the detection of Ca^{2+} .

Keywords: gold nanoparticles; modified gold nanoparticles; colorimetric analysis; calcium; transparency sheet-based device

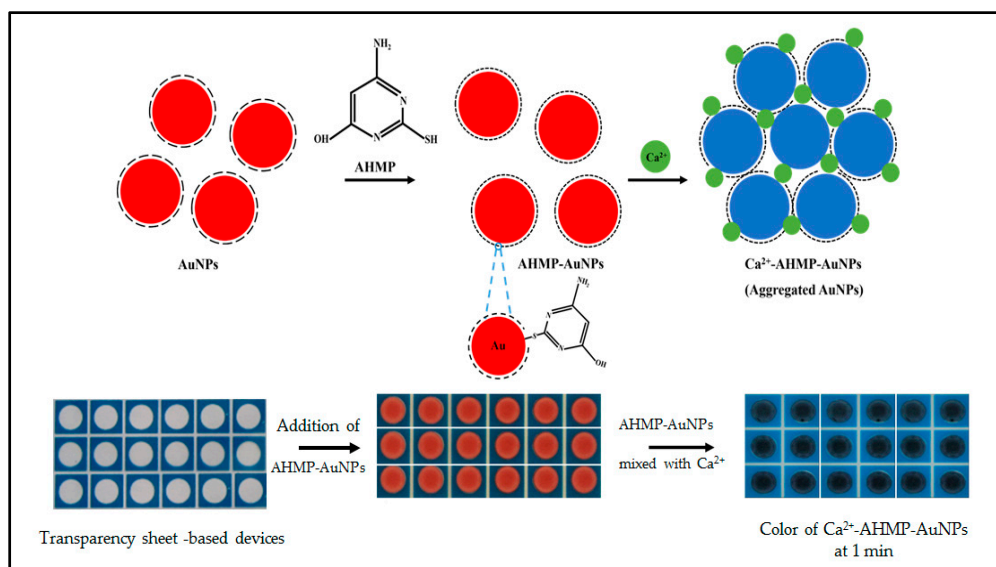
1. Introduction

Calcium (Ca^{2+}) is an essential nutrient and one of the most important minerals for the human body. Ca^{2+} plays a role in the growth and maintenance of bones, teeth, and nails. In the ionized form, Ca^{2+} is significant in many vital processes, such as hormone secretion, nerve conduction, and blood coagulation. The World Health Organization (WHO) and Food and Agriculture Organization (FAO) recommend a minimum daily intake of 1200 mg of Ca^{2+} for adults [1,2]. At the present, calcium deficiencies are taken quite seriously. Therefore, in medical diagnostics, the determination of calcium concentration is very important for the evaluation of a patient's health. A change in Ca^{2+} concentration, which usually is relatively stable in body fluids (range in urine: 100 to 300 mg/day) [3], can indicate the presence of various pathological conditions. When calcium concentrations are lower than normal, osteoporosis, vitamin D deficiency, eclampsia, or hypoparathyroidism may be present, whereas calcium concentrations higher than normal may indicate hyperparathyroidism, vitamin D intoxication, or myeloma [4]. Thus, Ca^{2+} concentration from body fluids should be determined for the evaluation of and protection against any disease that would be manifested by an abnormal calcium concentration. This emphasizes the need to develop highly sensitive devices for the portable, simple, and rapid deficiency screening in patient care. Numerous methods have been proposed for the measurement

of Ca^{2+} in biological materials. The classical method involves titration with a standardized solution of ethylenediaminetetraacetic acid (EDTA) [5,6]. However, this method is limited by sensitivity and selectivity. The preferred standard method for the determination of Ca^{2+} in body fluids is atomic absorption spectrometry (AAS) [7–10]. Other spectrophotometries, such as UV–Vis spectroscopy [11], spectrofluorimetry [12], atomic emission spectrometry (AES) [13], inductively coupled plasma [14], and fluorescence [15–17] have also been developed for Ca^{2+} determination. Currently, the determination of Ca^{2+} developed with colored complexes using metallochromic reagents, such as Eriochrome Black T (EBT), has been reported [18,19]. Even though these spectrophotometric methods provide enough sensitivity, they are difficult to design to be a portable device. Calcium ion-selective electrodes are some of the most promising methods for Ca^{2+} determination. Given their precise and selective performance, this electrochemical measurement technique has been widely developed for the detection of Ca^{2+} in biological fluids [20–22]. There have been several kinds of electrodes developed for Ca^{2+} detection, including solid-contact ion selective electrodes [23] and polymer membrane ion-selective electrodes [24]. Unfortunately, most of the above-mentioned methods still require a specialist and specific instrument. Presently, with the rapid growth of near-patient devices used at home or the hospital bedside, there is an increasing demand for portable systems utilizing small disposable sensors which are capable of measuring biomarkers in biological samples. Patients can collect biological samples and measure target analyte concentrations at home within a few minutes without specialized instruments. From this requirement, it is the purpose of this work to present a simple, rapid, reliable, and low-cost detection method that uses a small sample volume for the determination of Ca^{2+} in urine samples using colorimetric gold nanoparticles. Recently, paper-based analytical devices (PADs) are one of the most common devices used as a miniaturized analytical platform for economical and portable sensing applications. Paper-based platforms can be beneficial for applications in diagnostics, food safety, and environmental monitoring due to the advantages of natural abundance, simplicity, disposability, low sample-reagent usage, and suitability for routine measurement. Unfortunately, the use of paper as substrate for colorimetric detection is usually suffer from the adsorption of color by porosities of paper lead to difficulty in the measurement of the color change at low concentration of the analyte. Therefore, the sensitivity in detection is low in comparison to those obtained from solution. To overcome this limitation, the search for new materials to be used as a substrate to fabricate the analytical platform is a challenge. In this research, we propose to use transparency sheets as the substrate for creating a new colorimetric device platform.

Gold nanoparticles (AuNPs) have been widely used as colorimetric probes for metal ions, anions, small molecules, proteins, nucleic acids, and other analytes because of their unique properties, such as optical properties dependent on their spacing or surface plasmon resonances. The extinction spectrum and the wavelength at which AuNPs absorb and scatter light depends on the distance between particles [25,26]. For example, citrate-coated AuNPs were employed as colorimetric probes for organophosphorus pesticides detection [27]. Furthermore, AuNPs are easily modified by capping agents, which are specific to a given analyte. Therefore, functionalized AuNPs have been applied as colorimetric probes, receiving great attention in visual sensing applications because of the plasmonic absorbance shift. The functionalization can perform using various ligand; AuNPs functionalized with peptide substrates (Gly-Pro-Asp-Cys (GPDC) or Val-Pro-ethylene diamine-Asp-Cys (VP-ED-DC)) were proposed for the determination of dipeptidyl peptidase IV activity (DPP-IV/CD26 activity) based on the aggregation of functionalized AuNPs [28]. 4-Amino-6-hydroxy-2-mercaptopyrimidine monohydrate (AHMP) is an interesting ligand. It contains a thiol group which can self-assemble on the AuNP surface [29,30]. Additionally, the amine and hydroxyl groups available in AHMP are free from binding. The presence of the lone pair electrons of amine and hydroxyl groups lead to a negative charge around the AuNP surface. After modification, AHMP-AuNPs have a different zeta potential from the unmodified, indicating a negative charge. From this finding, it prompts us to use AHMP-AuNPs as the probes for Ca^{2+} detection. The electrostatic force between the negatively charged AHMP-AuNPs and positively charged Ca^{2+} may induce aggregation. The color change of AHMP-AuNPs has become

a useful tool in the development of colorimetric sensing, transparency sheet-based analytical devices. These devices can determine the Ca^{2+} concentration in urine samples by assessment with the naked eye. The proposed mechanism for Ca^{2+} detection, the induction of the aggregation of AHMP-AuNPs, is shown in Scheme 1. AHMP molecules can be absorbed onto the surface of the AuNPs via the thiol group, resulting in the enhancement of the negative charge around the surface of the AuNPs after modification. Ca^{2+} can induce the aggregation of AHMP-modified AuNPs via electrostatic force. The red color of the AHMP-AuNPs changes to blue in accordance with the Ca^{2+} level added. Moreover, the proposed approach has been successfully applied to detect Ca^{2+} levels in urine samples. The verified results were in good agreement with atomic absorption spectroscopy (AAS). The simplicity, speed, suitability for real-time on-site detection, and lack of required complex and expensive instruments could make this method a promising tool for point-of-care (POC) diagnosis.



Scheme 1. The proposed mechanism scheme and the transparency sheet-based devices for detection of Ca^{2+} using 4-Amino-6-hydroxy-2-mercaptopyrimidine monohydrate (AHMP)-gold nanoparticles (AuNPs).

2. Results and Discussion

2.1. Characterization of AHMP-AuNPs

To investigate the incorporation of AHMP onto the AuNP surface, UV-Vis spectroscopy and zeta potentials were determined. Figure 1 shows the UV-Vis spectra of AHMP, AuNPs, and AHMP-AuNPs. The localized surface plasmon resonance (LSPR) absorption peak of AHMP has a maximum peak at 275 nm. The AuNPs alone have a maximum peak at 519 nm. After the AuNPs were modified with AHMP, the UV-Vis spectra showed two maximum peaks at 273 and 523 nm. We believe that the additional peak and the red shifting of the band is mainly caused by the decrease of the plasma oscillation frequency around the nanoparticles from the binding between thiol-containing compounds and the gold nanoparticles. Corresponding to the zeta potential results, the surface charge of AuNPs and AHMP-AuNPs were found at -43.1 and -49.9 mV, respectively. The zeta potentials indicated the negative charge of AuNPs increased from -43.1 mV (intensity 32 a.u.) to -49.9 mV (intensity 38 a.u.), as shown in Table S1. This confirms that the surface of the AHMP-AuNPs has a more negative charge which corresponds to previous work. It is expected that the thiol group of AHMP is chemisorbed on the surface of AuNPs, whereas amine and hydroxyl groups are available in AHMP free from binding. The presence of lone pair electrons in amine and hydroxyl groups led to the negative charge around the AuNP surface. This result displayed that AHMP had been completely modified onto the surface of AuNPs.

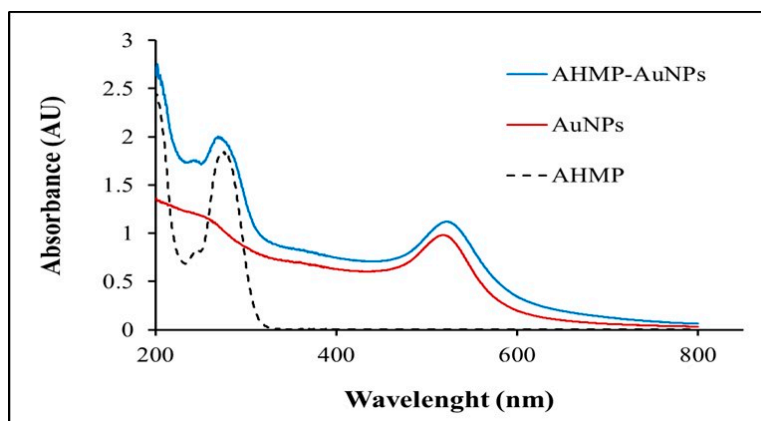


Figure 1. UV-Vis absorption spectra of AHMP, AuNPs, and AHMP-AuNPs.

2.2. Colorimetric Assay of Ca^{2+}

Following the AuNP modification, a colorimetric analysis using AHMP-AuNPs was performed for the detection of Ca^{2+} . The color and absorption spectra of the AHMP-AuNPs, with and without Ca^{2+} , are shown in Figure 2, while their zeta potentials were displayed in Table S1. After addition of buffer, the negative charge of AHMP-AuNPs solution increased from -49.9 mV to -58.2 mV, while the average size of the buffer-AHMP-AuNPs was not significantly change (from 29.1 to 27.6 nm). After addition of Ca^{2+} , the zeta potential dramatically decreased from -58.2 mV, intensity 35 a.u., to -50.3 mV, intensity 26 a.u., while the average size of the AHMP-AuNPs increased from 29.1 to 65.4 nm. These results indicate that the AHMP-AuNPs aggregation was induced by the electrostatic force between the positive charge of Ca^{2+} and the negative charge of AHMP-AuNPs. Moreover, the results obtained corresponded to the decrease of the absorption bands at 273 and 523 nm along with the formation of a new absorption band at 680 nm (redshift), as illustrated in Figure 2. Furthermore, the color of the AHMP-AuNPs on the proposed device was clearly observed to change from red to blue immediately, which suggested the growth of the nanoparticles' size. This indicates that Ca^{2+} could quickly bind to their functional groups ($-\text{NH}_2$ and $-\text{OH}$) onto modified AuNPs [31,32]. These binding interactions and electrostatic force all contribute to the aggregation mechanism of the AuNPs, as schematically illustrated in Scheme 1. According to the considerable change in negative charge, color, and LSPR absorption of AHMP-AuNPs upon the addition of Ca^{2+} , the development of a novel colorimetric AHMP-AuNPs device for Ca^{2+} determination was successfully accomplished.

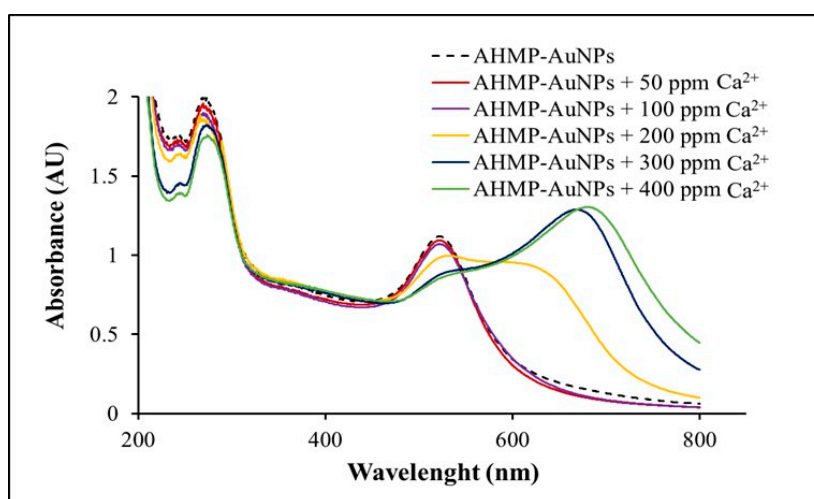


Figure 2. UV-Vis absorption spectra of AHMP-AuNPs (dot line) and AHMP-AuNPs in the presence of Ca^{2+} at 50 (red line), 100 (purple line), 200 (yellow line), 300 (navy line), and 400 ppm (green line).

2.3. Optimization for the Determination of Ca^{2+} on Transparency Sheet-Based Devices

In this section, the modifier volume, pH, incubation time, and AHMP-AuNP: sample volume ratios were examined for optimization in order to accelerate the performance of the transparency sheet-based colorimetric device. The colorimetric detection of Ca^{2+} was performed at room temperature by the sequential addition of standard Ca^{2+} and AHMP-AuNPs on the detection zone of the transparency sheet. The red/blue intensity was measured and calculated by mobile phone and Image J software, respectively. The result of the aggregation capability between AHMP-AuNPs and Ca^{2+} in each condition was observed by the red/blue intensity from each transparency sheet image. First, the volume of 4 mM AHMP modifier was varied at 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.5 mL with the same volume of concentrated AuNPs (1.5 μL). Then, 15 μL of the varied AHMP-AuNPs were investigated with 15 μL of Ca^{2+} at 0, 10, 50, and 100 ppm concentration. In Figure 3, the red/blue intensity of the blank (AHMP-AuNPs and PBS buffer, pH 6) decreased when the AHMP volumes were increased. It may be that the AuNPs were diluted by the AHMP solution, so that AHMP-AuNPs had reduced aggregation efficiency. The red/blue intensity of 0.1 mL AHMP was found to exhibit the most significant change from the blank, especially when tested with 100 ppm Ca^{2+} . Moreover, this change could be easily detected with the naked eye. Therefore, 0.1 mL AHMP was selected as an optimum modifier volume for further studies.

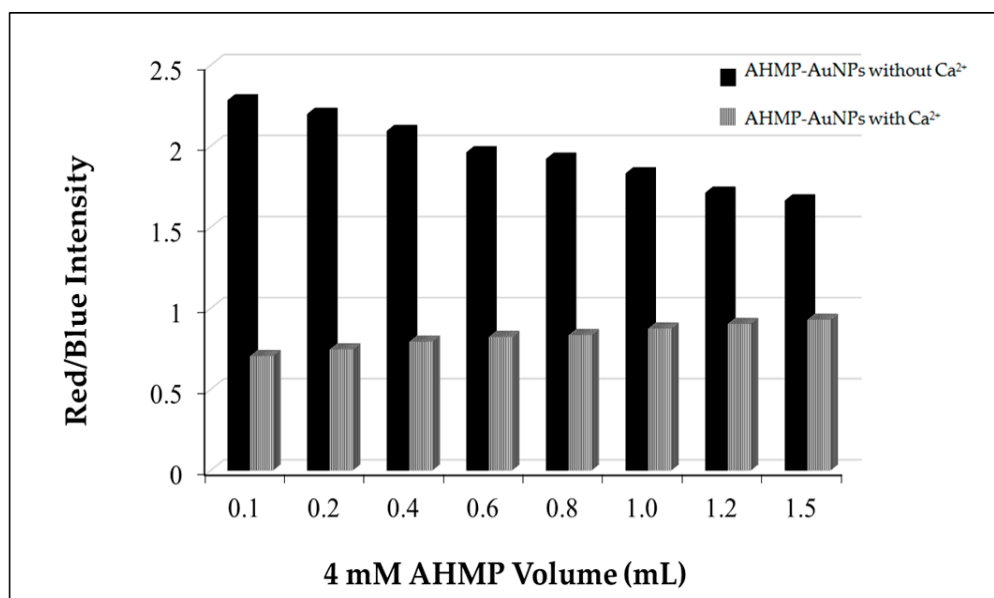


Figure 3. The red/blue intensity of the AHMP-AuNPs at different AHMP volumes at 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.5 mL without (solid bar) and with (thin bar) 100 ppm Ca^{2+} , AHMP-AuNP: sample volume ratio at 1:1.

Then, the effect of pH (range of 3–12) on the aggregation process was studied. As shown in Figure 4, the use of phosphate buffer with pH of 5.8–12 did not change the color of the blank. Therefore, phosphate buffers of pH 5.8–12 were chosen to study the aggregation effect of AHMP-AuNPs and 100 ppm Ca^{2+} at a 1:1 volume ratio. The results demonstrated that pH 6 provided the maximum color change. At this pH, Ca^{2+} has a high potential to induce the aggregation of AHMP-AuNPs. Therefore, phosphate buffer pH 6 was chosen for further studies.

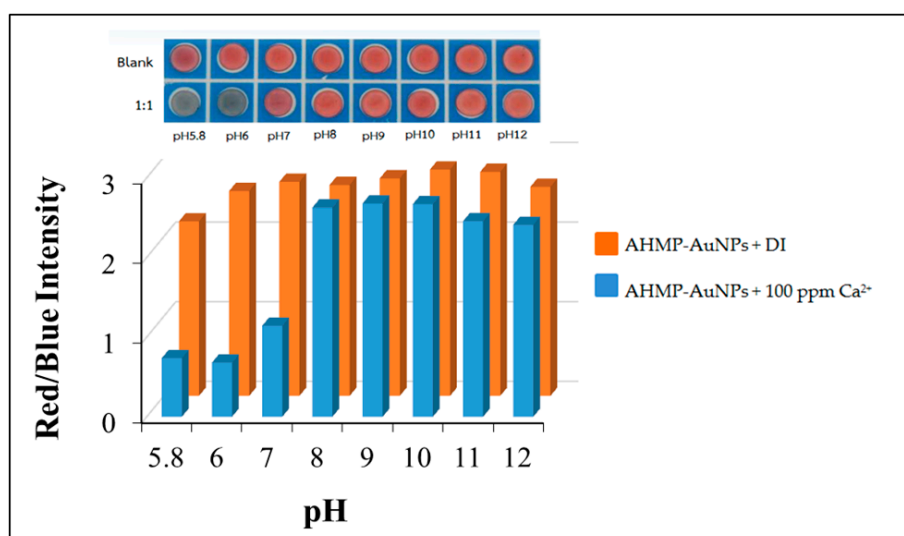


Figure 4. The influence of pH over the range 5.8–12 on the aggregation process of the AHMP-AuNPs and 100 ppm Ca^{2+} , other conditions are the same as in Figure 3.

Next, the aggregation of AHMP-AuNPs induced by Ca^{2+} was investigated at various incubation times (1–30 min). The aggregation of the AHMP-AuNPs occurred immediately after the addition of Ca^{2+} and was completed in one minute. In other words, the red/blue intensity from the aggregation of AHMP-AuNPs induced by Ca^{2+} can be measured at one minute. When AHMP-AuNPs were left on the device for over five minutes, the red/blue intensity of the blank was increased from the initial (Figure S1) because of self-aggregation of the AHMP-AuNPs. In the case of interferences, Mg^{2+} was first selected as a representative of other positive ions, as it has an atomic radius and positive charge nearly equal to those of Ca^{2+} to investigate the possibility of using this proposed probe. From these results, it was found that the red/blue intensity of AHMP-AuNPs in the presence of Mg^{2+} was decreased more than 5%, compared to the blank, after five minutes. This indicated that other positive ions may also interfere with the proposed method if the incubation time was over five minutes. From this critical observation, we strongly believe that an incubation time of one minute is appropriate to specify the detection of Ca^{2+} by electrostatic force and size selective in the presence of other divalent ions. Therefore, the incubation time is a critical point that must be kept in mind for the selectivity study in the following section. Finally, to find a suitable quantity of the AHMP-AuNPs to sufficiently interact with Ca^{2+} , the reagent volume ratio was tested between 5:25 and 25:5 (AHMP-AuNPs: Ca^{2+}). The red/blue intensity of AHMP-AuNPs after mixing with Ca^{2+} was measured at various reagent volume ratios with 30 μL maximum volume. As shown in Figure S2, the volume ratio of AHMP-AuNPs: Ca^{2+} at 25:5 provided the highest red/blue intensity. This indicated that a 25:5 ratio of AHMP-AuNPs: Ca^{2+} was sufficient and appropriate for the determination of Ca^{2+} .

2.4. Selectivity for the Determination of Ca^{2+}

After getting the optimal conditions, selectivity was evaluated by the Δ red/blue intensity (red/blue intensity of blank—red/blue intensity of sample) of AHMP-AuNPs mixed with other substances and metal ions possibly found in urine (i.e., ascorbic acid, uric acid, albumin, glucose, Mg^{2+} , K^+ , Na^+ , Cl^- , CO_3^- , PO_4^{2-} , and SO_4^{2-}), since they may also affect the aggregation of AHMP-AuNPs and might interfere with the method accuracy. An interference was defined to happen when the Δ red/blue intensity in the mixture varied by more than $\pm 5\%$ of the Ca^{2+} intensity. As emphasized previously, the incubation time directly affects the selectivity. Therefore, the change of color was measured at one minute after mixing to neglect the interference from Mg^{2+} . As shown in Figure 5, the results demonstrated clearly that the Δ red/blue intensity of AHMP-AuNPs mixed with foreign compounds with concentrations even 10 times greater than the concentration of Ca^{2+} did not significantly affect

the signal of Ca^{2+} and the tolerance limits were shown in Table S2. In addition, we also applied this probe for detection of other divalent cations such as Hg^{2+} , Cu^{2+} , Cd^{2+} , and Zn^{2+} . According to the results, it can be seen that the interferences from almost all metal ions studied did not affect Ca^{2+} detection. Therefore, this probe provides high selectivity for only Ca^{2+} detection at an incubation time of one minute.

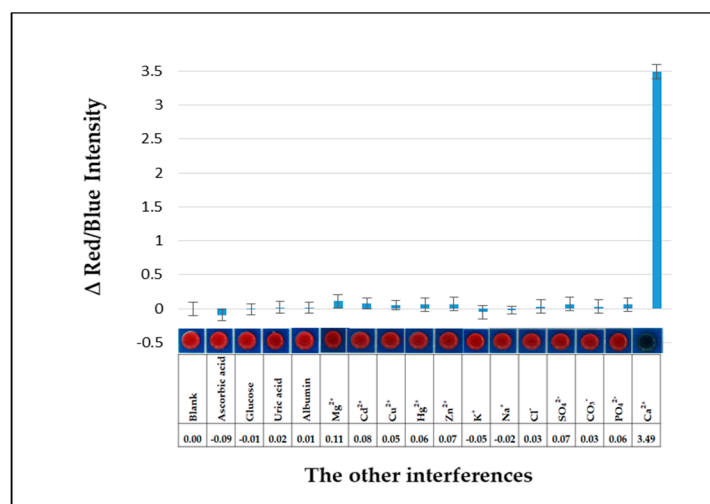


Figure 5. Responses of AHMP-AuNPs in other interferences. The concentration of Ca^{2+} was fixed at 100 ppm while other interferences were used at 1000 ppm. All results were repeated three times ($n = 3$). Other conditions were the same as the optimal values.

2.5. Analytical Performances

Under the optimal conditions, the analytical performance for determination of Ca^{2+} was investigated by AHMP-AuNPs using transparency sheet-base devices. Ca^{2+} concentrations were studied between 0 ppm and 100 ppm. As shown in Figure 6, it was found that the $\Delta\text{red}/\text{blue}$ intensity from the aggregation of AHMP-AuNPs by Ca^{2+} was significantly reduced when the concentration of Ca^{2+} was increased. A linear relationship between the $\Delta\text{red}/\text{blue}$ intensity and log concentration of Ca^{2+} in a calibration curve was obtained. A good linearity was observed for Ca^{2+} concentrations ranging from 10 ppm to 100 ppm with a correlation coefficient of 0.9877. The detection limit from calculation by using three times the standard deviation of the blank divided by the slope of the calibration curve ($3 S_{\text{blank}}/\text{slope}$) was found to be 3.05 ppm. The proposed method provided a good recovery in the range of 91.7%–106.7%. These recoveries were within the acceptable recovery percentages, 90%–107%, for a concentration of 100 ppm (mg/kg) or at a Ca^{2+} concentration lower than 100 ppm, as recommended by the Association of Official Analytical Chemists (AOAC). The repeatability of this probe was investigated using 40 and 80 ppm standard solutions of Ca^{2+} ($n = 5$ measurements with different probes). The relative standard deviations were 2.54% and 5.16%, which indicated that there was good probe-to-probe reproducibility.

The comparisons between some previous report for the determination of Ca^{2+} and proposed devices were summarized in Table S3. This proposed device demonstrates the ability of a transparency sheet-based colorimetric detection system to conveniently measure Ca^{2+} , even though this proposed method has a detection limit higher than previous works, as shown in Table S3. However, this limit of detection can be accepted for the determination of Ca^{2+} in urine samples. Moreover, this proposed platform offers the benefit in terms of simplicity, speed, suitability for real-time on-site detection, and lack of required complex and expensive instruments. Therefore, it can provide a promising tool for Ca^{2+} diagnosis and could be an alternative choice for point-of-care analysis in the future.

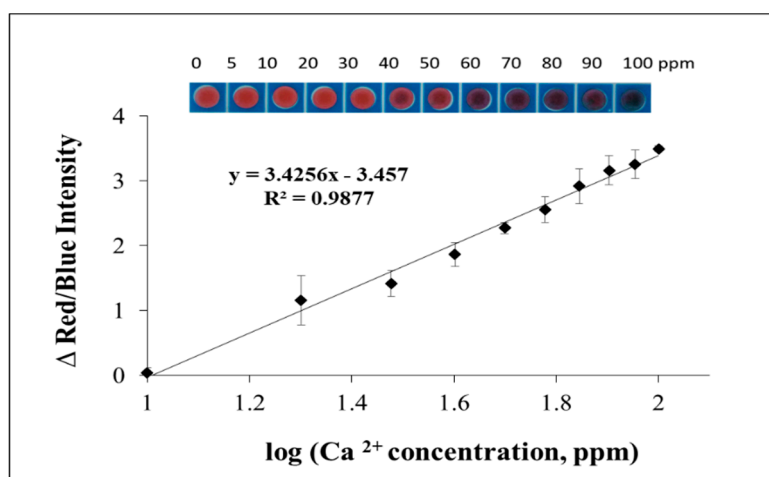


Figure 6. Transparency sheet-based devices for the quantitative analysis of Ca^{2+} and the calibration plot of $\Delta\text{red/blue}$ intensity and the log concentration of Ca^{2+} (error bar represented the standard deviation at $n = 3$). Other conditions used were the same as the optimal values.

2.6. Analysis of Artificial Urine Samples and Method Validation

To evaluate the efficiency of the proposed method, the transparency sheet-based devices were used to detect Ca^{2+} in artificial urine samples, and the results were compared to the AAS method as shown in Table S4. The urine samples were spiked with standard Ca^{2+} at 80, 160, and 320 ppm. Then, the samples were further diluted to produce a final appropriate dilution factor before analysis. All artificial urine samples were analyzed by the AAS standard method and the proposed method. The obtained concentrations from the two methods were statistically compared using the t -test at the 95% confidence level. A t -test result was obtained with a value of 1.75, which is lower than the t_{critical} of 4.30. Thus, there was no significant difference between the concentrations obtained from these two methods. As a result, this proposed colorimetric method can be applied for the determination of Ca^{2+} in urine samples with satisfactory results, which suggests that the transparency sheet-based colorimetric device is accurate and reliable for practical applications in the clinical field.

3. Experimental

3.1. Materials and Instrumentation

Gold nanoparticles (AuNPs, 0.01% w/v (100 ppm), diameter 20 nm) were purchased from Kestrel Bio Sciences (Bangkok, Thailand). Calcium carbonate (CaCO_3), sodium chloride (NaCl), magnesium chloride (MgCl_2), disodium hydrogen orthophosphate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$), sodium dihydrogen orthophosphate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$), and hydrochloric acid were purchased from Ajax Finechem Pty Ltd (Seven Hills, New South Wales, Australia). 4-Amino-6-hydroxy-2-mercaptopyrimidine monohydrate (AHMP), glucose, uric acid, albumin, and ascorbic acid (vitamin C) were purchased from Merck (Darmstadt, Germany). Artificial urine was purchased from Carolina Biological Supply Company (Burlington, NC, USA). Analytical grade reagents and $18 \text{ M}\Omega \text{ cm}^{-1}$ resistance deionized (DI) water (obtained from a Millipore Milli-Q purification system) were used throughout.

Phosphate buffers (pH 5–8) were prepared from 0.1 M of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and 0.1 M $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$. Phosphate buffer (pH 9–12) was prepared from 0.1 M of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and 0.1 M $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ and the pH was adjusted with 0.1 M sodium hydroxide (Merck) solution.

A wax printer (Xerox Color Qube 8570, Tokyo, Japan) was used to print wax onto transparency sheets to obtain the detection devices. Absorption spectra were measured by using a UV-2450 UV–visible spectrophotometer (SHIMADZU, Kyoto, Japan). The zeta potential measurements were performed on Zeta sizer Nano S90 (Malvern, UK). An atomic absorption spectrometer (AAS) with

a hollow cathode lamp and standard air/acetylene flame was also used (Analyst 300, Perkin Elmer Instruments, Akron, OH, USA).

3.2. Preparation of AHMP-Modified Gold Nanoparticles (AHMP-AuNPs)

First, the purchased gold nanoparticles were preconcentrated by pipetting the commercial gold nanoparticles into 1 mL micro centrifuge tubes. The tubes were centrifuged at 12,000 rpm (4 °C) for 30 min. Then, 0.8 mL of supernatant was removed, and the remaining 0.2 mL of solution was stirred for 10 s.

AHMP-AuNPs were prepared by mixing 0.10 mL of 4 mM AHMP and 1.50 mL of the preconcentrated AuNP solution. After that, the mixture was stirred for 15 min to ensure the complete self-assembly of AHMP onto the surface of the AuNPs.

3.3. Fabrication of Transparency Sheet-Based Devices

Transparency sheet-based devices (polypropylene, purchased from local stationery store) were fabricated using a wax printing method. The pattern of the device was designed as an array containing a circular area with a detection zone 5 mm in diameter using the graphic software Adobe Illustrator CS4. The fabrication process has only two steps. First, the wax pattern was printed on the transparency sheet using the wax printer (Xerox Color Qube 8570, Japan). The maximum liquid volume in the detection zone was 30 µL. The wax-covered area was hydrophobic, while the detection area without wax was hydrophilic. Second, white paper was attached to the back side of the transparency sheet with tape to enhance the visibility of the color change. Using this process, the transparency sheet-based devices could be fabricated within 2 min.

3.4. Transparency Sheet-Based Colorimetric Detection of Calcium

The working standard solution for the Ca^{2+} was made by appropriate dilution of the stock standard solution on a daily basis as required. A colorimetric analysis of Ca^{2+} was performed as follows. First, 5 µL of standard Ca^{2+} solution or samples (prepared in pH 6.0 PBS) were added into the detection zone of a transparency sheet-based device, followed by the addition of 25 µL of AHMP-AuNPs. Then, the red/blue intensity of AHMP-AuNPs mixed with Ca^{2+} standard solution was captured by mobile phone (Vivo V3, DongGuan, China) in a light control box and measured via Image J software (<https://imagej.nih.gov>). Under optimal conditions, the relationship between the red/blue intensity of triplicate measurements and concentrations of the Ca^{2+} solution between 10–100 ppm was plotted to obtain the linear range.

3.5. Analysis of Ca^{2+} in Artificial Urine Samples

Under optimal conditions, the proposed method was used for the determination of Ca^{2+} in artificial urine samples. The artificial urine was purchased from Carolina Biological Supply Company (controlled level). The proposed method was validated against atomic absorption spectrophotometry (AAS). An appropriate volume of 1000 ppm Ca^{2+} was added to 25 mL of artificial urine for preparation of the 500 ppm Ca^{2+} stock solution. Then, the stock solution was diluted with artificial urine to 320, 160, or 80 ppm Ca^{2+} concentration. The samples were adjusted to pH 6 by the addition of 3 M sodium hydroxide and additional urine was added to reach a final volume. Prior to analysis with the proposed method, the 320 and 160 ppm Ca^{2+} samples were diluted with 50 mM phosphate buffer pH 6, 4-fold and 2-fold, respectively. The AAS method can detect Ca^{2+} within the range of 0.5–5 ppm. Therefore, the 320, 160, and 80 ppm Ca^{2+} samples were diluted with 50 mM phosphate buffer pH 6, 150-fold, 45-fold, and 80-fold, respectively.

4. Conclusions

This paper demonstrated, for the first time, the use of AHMP-AuNPs for a transparency sheet-based colorimetric method to provide simple, rapid, inexpensive, reliable, and portable devices for the determination of Ca^{2+} in urine samples. This method was based on the aggregation of AHMP-AuNPs on the device by the electrostatic force between the negatively charged AHMP-AuNPs and the positively charge Ca^{2+} . Under optimal conditions, the color change of the AHMP-AuNPs can be clearly observed by the naked eye. Furthermore, the proposed method was successfully applied to the quantitative analysis of Ca^{2+} in artificial urine samples. This colorimetric device provided a result within one minute, much faster than other mentioned methods. This simple device is highly suitable for Ca^{2+} quantitative analysis and can act as an alternative method for the rapid screening of Ca^{2+} deficiency without the need for any special instrumentation. The advantages of the transparency sheet-based colorimetric device developed from this work include its high sensitivity and selectivity, good precision, simplicity, and reproducibility. These findings offer a high potential for applications in clinical diagnosis.

Supplementary Materials: Supplementary materials can be found at <http://www.mdpi.com/1422-0067/20/12/2954/s1>.

Author Contributions: P.D. performed the experiment, collected and analysis the data. O.C. contributed data and provided the evaluation comments and proof reading. W.S. designed the experiment, analysis the data, wrote and corrected the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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Simple and Cost-Effective Electrochemical Approach for Monitoring of Vitamin K in Green Vegetables

Whitchuta Jesadabundit,^[a] Sudkate Chaiyo,^[b] Weena Siangproh,^{*,[c]} and Orawon Chailapakul^{*,[a, d]}

This paper is dedicated to Dick Crooks, in celebration of his birthday.

This work presents a simple and sensitive electrochemical sensor for the determination of total vitamin K (K_1 and K_2) using a disposable screen-printed graphene electrode (SPGE). The application for the determination of vitamin K_1 in green vegetables by this sensor was developed. The proposed method is based on controlling of negative potential for seconds and scanning it to a positive for oxidation analysis. The capacitance of SPGE was studied and it showed a higher

sensitivity for vitamin K determination in comparison to carbon. The parameters, such as the pH of electrolytes, accumulation potential, accumulation time, etc. were investigated. The limit of detection was observed at $0.099 \mu\text{g mL}^{-1}$. This method was successfully applied for the determination of the vitamin K_1 in various green vegetables. Therefore, the proposed method can be used for quality control of vitamin K_1 in natural samples to create the food composition table.

1. Introduction

Vitamin K (VK) or naphthoquinone compound is one of liposoluble vitamins that found in human diet. There are mainly two forms of VK in natural sources and the human body. Vitamin K_1 (Phylloquinone, PK) is the predominant form that produced by plants and green vegetables and vitamin K_2 (Menaquinone, MK) is derived from meat, eggs, curd cheese and fermented food. VK plays a crucial role in the blood coagulation^[1–2] and calcification of bones.^[3–4] From literature reviews, a VK deficiency can cause the decrease of prothrombin and clotting factors in blood leading to hemorrhagic and excessive bleeding tendencies.^[5–6] In contrast to other vitamins, VK is irregular used as dietary supplements. Therefore, the daily intake of VK is primarily relied on the VK content in foods. Unfortunately, most of food compositions rarely presented a VK content in natural food. Thus, more detailed information on VK

in foods is particularly needed, especially for nutritional status assessment of individuals in relation to this vitamin.

In the past decades, there are many methods used for the determination of VK including chemiluminescence technique^[7] and HPLC cooperated with different detectors such as ultra-violet (UV),^[8–10] fluorescence,^[11–13] electrochemical,^[14–15] chemiluminescence^[16] and mass spectrometric detector.^[17–18] These methods have high sensitivity and selectivity, however, there are some disadvantages such as time consuming, require high cost of instrument, large sample volume requirement and difficult to design for field analysis. In order to overcome these problems, a sensitive, simple and short time analysis for determination of VK is still required.

Accordingly, electrochemical analysis becomes an alternative approach due to it is simple operation, short analysis time, high sensitivity and portability.^[19] Because of these advantages, this technique was continuously developed and applied for various applications especially in the area of food analysis.^[20–24] There are several methods developed for the determination of VK using electrochemical technique. Most of work used the square-wave adsorptive stripping voltammetry (SWASV) for the quantification. In 1981, the SWASV was first cooperated with hanging mercury drop electrode (HMDE) to determine vitamin K_1 . This method can provide a 200-fold increase of the peak current compared to differential pulse stripping voltammetry.^[25] After that, in the same year, Vire et al. developed the SWASV method for determination of vitamin K_3 (the synthetic form) using static mercury drop electrode (SMDE). They proved the important of accumulation step which helps changing of VK to more electroactive form (quinone was altered to hydroquinone which is more strongly adsorbed on the electrode surface). This process helps to promote the sensitivity.^[26] However, mercury-based electrodes are now rarely used because of its high toxicity. Then, Hart et al. proposed the method for determination of vitamin K_1 in plasma using carbon paste electrode (CPE)^[27] and the latest work, Sys et al. developed the SWASV

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An invited contribution to the Richard M. Crooks Festschrift.

method to determine vitamin K₁ in food samples using glassy carbon electrode (GCE).^[28] Although, these electrodes can provide a low detection limit, it still required time for the preparation of electrodes. Additionally, polishing and cleaning steps are required for the use of glassy carbon electrode as a substrate. Therefore, they still have some drawbacks of time consuming for electrode preparation and measurement. Therefore, the electrochemical sensor that is ready to use without pretreatment step, cheap and environmentally friendly is still greatly needed and is a great challenge for determination of VK.

Recently, a screen-printed electrode has great attention for the development of useful electrochemical sensor. It is a kind of sensor based on a screen-printing method on substrate which has advantages of small size, low sample volume, and disposability.^[29–30] These sensors can be printed by various types of ink, such as carbon, gold and so on. Among them, screen-printed graphene electrode (SPGE) is a well-known conductive sensor which has the excellent properties. For example, it has fast electron transportation, high thermal conductivity, excellent mechanical flexibility and good biocompatibility.^[31]

From the benefits of screening technology, in this study, a simple and sensitive method for the determination of the vitamin K₁, which is the only analogue of the vitamin present in plants and being predominantly found in vegetables using SWASV coupled with the disposable screen-printed electrode was developed. This work is first report on the use of SPGE for VK determination. Therefore, the characterization of electrochemical behavior was studied using cyclic voltammetric technique (CV), and the measurement of vitamin K₁ was carried out using SWASV. Under optimal conditions, the proposed method was successfully applied for the determination of vitamin K₁ in green leafy vegetables. The proposed method can be used as an alternative approach for the determination of VK in the food science.

2. Results and Discussion

2.1. Electrochemical Behavior of VK at SPGE and Influence of the Scan Rate

To study the efficiency of the proposed electrode for VK determination, the electrochemical behavior of VK mixture including of K₁ and K₂ in ethanol and phosphate solution pH 3.0 (60:40) was individually investigated using screen-printed carbon electrode (SPCE) compared with SPGE by CV technique as shown in Figure 1A. The potential was operated in the range of -1.0 V to $+0.2$ V at a scan rate of 100 mVs⁻¹. Moreover, the measurement of the background current from these electrodes was also performed at the same condition that displayed in inset. From these results, the background current of the SPGE was found to be lower than the current from the SPCE due to its low capacitance property. In the presence of VK, cyclic voltammograms were also compared between SPCE and SPGE. The CV of the SPCE showed a broad and small anodic peak at -0.123 V, whereas, the SPGE obviously provided two defined

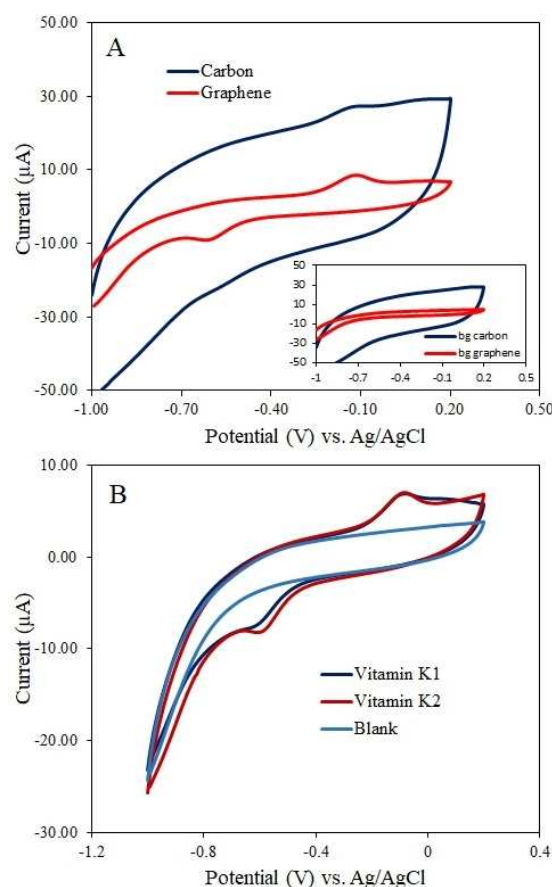


Figure 1. Cyclic voltammograms of (A) 1 mM of the VK mixture including of K₁ and K₂ at carbon (blue) and graphene (red) screen-printed electrode, comparison of background current between carbon and graphene is shown in the inset. (B) 1 mM of K₁ (red) and K₂ (blue) in 60:40 ratio of ethanol: phosphate solution pH 3.0 at scan rate of 100 mVs⁻¹.

anodic and cathodic peaks with higher peak current than that SPCE. This result also indicates that VK displayed a quasi-reversible property of VK due to the separation of cathodic and anodic peak potential is around ~ 500 mV which is greater than $59/n$ mV of reversible reaction (n is number of electron transfer).^[32] This reaction is related to quinone-hydroquinone coupled with two electrons and two protons transfer corresponding to a previous study,^[27] as shown in Figure S1. In this study, a higher peak current with lower background could be described by the effect of the less charging double layer of the SPGE. Based on graphene properties that possess a high surface area, a large number of ions or molecules can then be accumulated on its surface. Nonetheless, these non-specifically adsorbed ions can be eventually eliminated by the inherent hydrophobicity of graphene, resulting in the low capacitive current. From this reason, the VK dissolving in ethanol is capable to mainly adsorb onto the electrode surface, consequent, the high sensitivity of VK determination was obtained.^[33–34] Therefore, SPGE was chosen for this work. Furthermore, the anodic peak current was a bit higher than the cathodic one. As a result, all experiments for the determination of VK were carried out under oxidation reaction.

In some kind of real sample, VK is possible to be found in two principal forms as K_1 and K_2 , as mentioned. Hence, the investigation of electrochemical behavior for both forms is necessary. In the experiment, 1 mM solution of vitamin K_1 and K_2 were measured separately using CV to study the electrochemical behavior and to investigate an exact location of redox potential (Figure 1B). The results exhibited that the anodic peak current of K_1 was found to be around $2.14 \pm 0.03 \mu\text{A}$ at the potential of -0.102 V , and K_2 was $2.16 \pm 0.05 \mu\text{A}$ at -0.088 V . The currents and oxidation potentials of both forms were insignificantly different with %RSD of 0.66 because of the similar structure with the same functional group, as shown in Figure S1. Hence, the developed method can be used for the determination of the VK including of K_1 and K_2 without any discrimination of the two forms and there is no significant different in sensitivity from using individual vitamin K_1 , vitamin K_2 and total VK.

Besides electrode characterization and electrochemical behavior study, the mass transport of analyte towards the electrode surface was also investigated. The mass transfer of VK was proved by the relationship between the scan rate and anodic current signal. In the experiment, the solution of VK was measured at different scan rates in the range from 20 to 100 mVs^{-1} at SPGE. As shown in Figure 2, the redox currents increased with an increasing of the scan rate. When the currents in μA were plotted against the square root of scan rate in $(\text{mVs}^{-1})^{1/2}$, the linear relationship was observed. Therefore, these results can indicate that the mass transport of VK at SPGE is controlled by a diffusion process.^[35] Moreover, the plot between the scan rate and currents of oxidation and reduction reaction also observed in supporting information at Figure S2. These plots showed the nonlinear relationship, so it can confirm the non-adsorption properties, but it has the diffusion process instead.

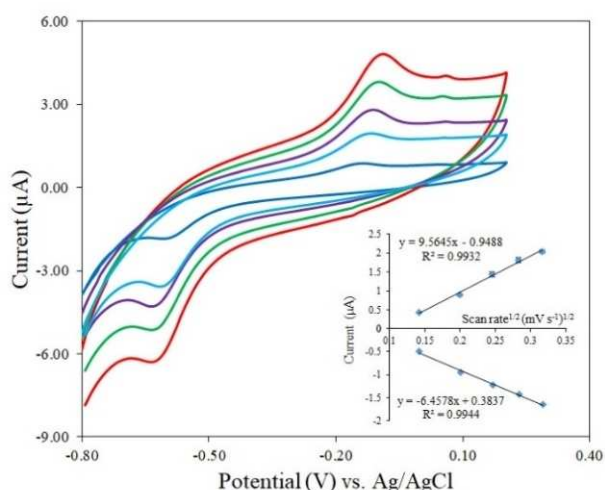


Figure 2. Cyclic voltammograms of 1 mM of VK including of K_1 and K_2 in 60:40 ratio of ethanol: phosphate solution pH 3.0 at various scan rate from 20 to 100 mVs^{-1} . The relationship between current and square root of scan rate is shown in the inset.

The selection of the electrochemical technique for the determination of VK is another essential topic for method development. Therefore, the efficiency of the used technique for the determination of VK was performed and compared. Nowadays, square-wave voltammetry (SWV) is a popular technique due to its high sensitivity and short time analysis. Moreover, it is commonly cooperated with the anodic stripping voltammetry called square-wave anodic stripping voltammetry (SWASV) for trace determination. Based on the principal, the efficiency of the two techniques including SWV and SWASV was investigated for VK determination at microgram level. As shown in Figure 3, the small and quiet broad peak at -0.121 V was obtained from the SWV technique, while the higher current with a well-defined peak at -0.081 V was obtained from SWASV. When the currents of both techniques were compared together, SWASV obviously showed a current signal 3.6 times higher than that of SWV due to the accumulation step at -0.5 V for 240 sec. This step can provide the formation change from quinone to a hydroquinone compound, which is a reducing form. The hydroquinone is a stronger and more stable adsorption property than the oxidizing one, therefore, it provides high sensitivity, as described in previous work.^[26] The greater sensitivity can be improved by using more negative potential of accumulation steps before scanning towards a positive direction. From the results obtained, the SWASV was therefore used for the determination of VK in all experiments of this work.

2.2. Characterization of the Prepared Electrodes

Graphene is an attractive material which is widely used in many applications because of its extremely promising electrical characteristics. This work is an application of graphene acting as a conductive material on a screen-printed electrode for electron transfer measurement. In order to investigate the features of this ink, the scanning electron microscopy (SEM) was used for morphology characterization to illustrate the surface of the

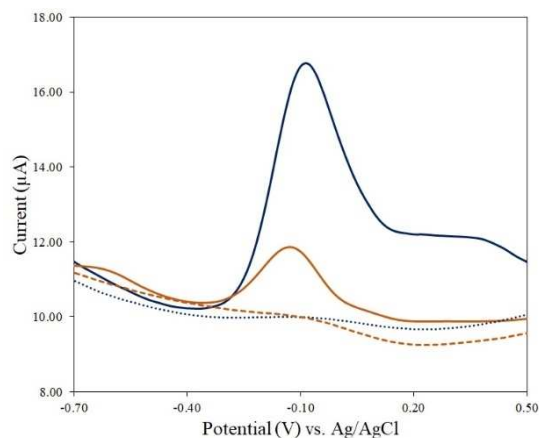


Figure 3. Voltammograms of 1 mM of VK including of K_1 and K_2 using SWV (orange) and SWASV (blue) technique, the dashed lines refer to the voltammograms of 0.1 M phosphate solution pH 3.0.

electrode material. The SEM image is shown in Figure 4A. The SPGE provides a rough and porous surface with lots of graphene sheets. These remarkable features can provide an efficient interaction between the electrode and substance. Moreover, the double layer capacitance (C_{dl}) is an important matter which can affect the sensitivity of determination. A higher capacitance material can cause lower sensitivity and higher limit of detection for measurements owing to the accumulation of other substances. Therefore, C_{dl} is an essential criterion for choosing an electrode material. In this work, the capacitance of a SPGE was investigated and compared to a SPCE with the same fabrication. For C_{dl} measurement, the CV was performed in 0.1 M KNO_3 at the potential range of -0.2 to $+0.2$ V with a scan rate of 100 mVs^{-1} on the different kind of electrodes. As shown in Figure 4B, the cyclic voltammograms from two different screen-printed electrodes were exhibited as plain background currents. From the results, it can be noticed that the current signal of the SPGE provided a lower background current than the carbon. In addition, the capacitance of electrodes was calculated from current at 0 V against screen-printed Ag/AgCl reference electrode by calculation using Equation (1):^[36]

$$C_{dl} = \frac{I_{avg}}{v(A_{geometric})} \quad (1)$$

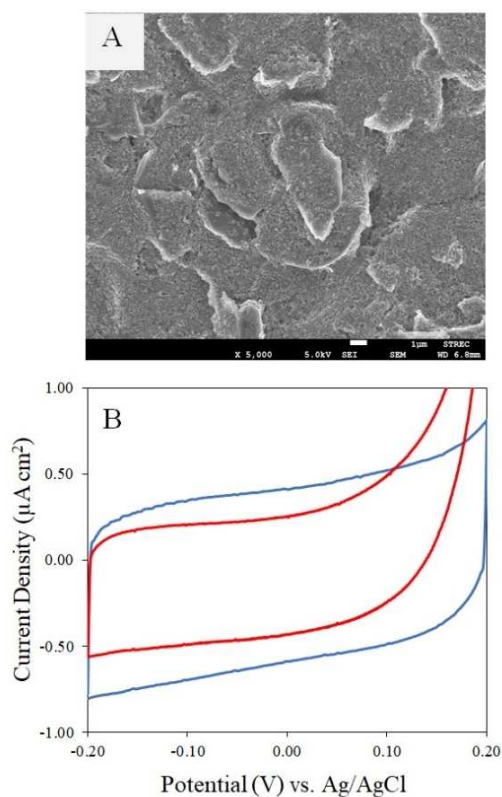


Figure 4. (A) SEM images of surface morphology of SPGE. (B) Cyclic voltammograms of 0.1 M KNO_3 on carbon (blue) and graphene (red) screen-printed electrode at scan rate of 100 mVs^{-1} .

where I_{avg} is the current average from the forward and reverse sweep in amperes, v is the scan rate in volts per second and $A_{geometric}$ is the geometric electrode area in square centimeters (0.04 cm^2). The capacitances of SPGE and SPCE were evaluated at 3.45 ± 0.02 and $5.61 \pm 0.05 \mu\text{F cm}^{-2}$, respectively ($n=3$). The capacitance of the SPGE is lower than the SPCE due to its hydrophobicity of graphene.^[33] The H-terminated of graphene has been predicted to localize at the edge^[37] As a result, the other ions in the supporting electrolyte do not accumulate on the electrode surface. In the case of carbon, its surface contains both hydrophobic and hydrophilic sites^[38] which can cause a high charging layer on the electrode surface. Due to advantage of having low interfering of the capacitive layer of graphene, the graphene ink was selected as an electrode material for current signal enhancing.

2.3. Optimization of Experimental and Electrochemical Conditions

In order to obtain high sensitivity for the proposed method of electrochemical measurement of the VK, various experimental parameters such as accumulation potential, accumulation time and pH of supporting electrolyte were investigated. SWASV was used to study the effect of these parameters using $10 \mu\text{g mL}^{-1}$ of VK standard solution.

Accumulation potential is one important parameter which can affect the form of a substance. The different accumulation potentials in the range of -0.2 V to -0.7 V were studied using $10 \mu\text{g mL}^{-1}$ of VK in 60:40 ratio of ethanol: phosphate solution at pH 7.0. From the results as shown in Figure 5A, the anodic peak currents were initially decreased at accumulation potentials of -0.3 V because the VK was partially oxidized before stripping due to the standard potential of VK at pH 7.0 is -0.3 V as shown in supplementary results (Figure S3). So, the oxidation reaction of VK during stripping step was less occurred. Then, when the system was firstly applied the accumulation potential at less negative value than standard potential at -0.2 V. It was found that the VK was changed to oxidizing form which has no adsorption property on the electrode surface. Therefore, a moderate current is observed. After that, when the accumulation potential was raised towards more negative value, the anodic currents dramatically increased. From this behavior, it can suggest that the use of a more negative accumulation potential has high potential in the change from quinone to hydroquinone form, which was strongly adsorbed onto the electrode surface leading to the high sensitivity.^[26] As seen in results, at -0.5 V, the currents remained constant because all of the quinone form was absolutely converted to hydroquinone structure. Therefore, the accumulation potential at -0.5 V was chosen for the next optimization.

Then, the accumulation time for the preconcentration step was optimized in a range from 60 to 300 sec. The background-current was subtracted from the VK anodic peak current before being plotted against various accumulation times as a S/B ratio. As expected, in Figure 5B, the currents were increased with the extension of time and the highest current was obtained at

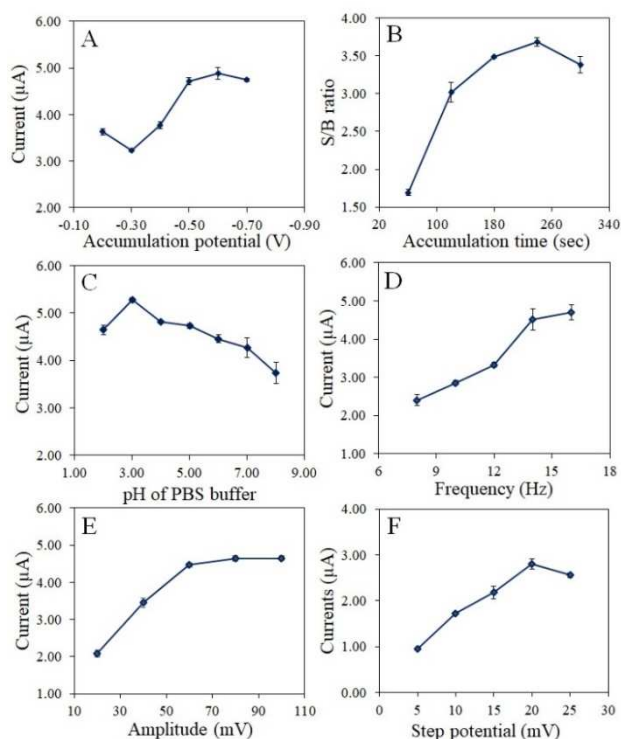


Figure 5. Effect of (A) accumulation potential, (B) accumulation time, (C) pH of supporting electrolyte, (D) Frequency, (E) Amplitude, and (F) Step potential on the stripping peak current of 10 µg mL⁻¹ of VK including of K₁ and K₂ in 60:40 ratio of ethanol: phosphate solution pH 3.0. Error bar: n = 3

240 sec. This is because this time is enough to completely covered by analyte. As results obtained, the accumulation time at 240 sec was selected as an optimal condition. The pH of the supporting electrolyte is another important parameter. The different pHs of 0.1 M phosphate solution in the range from 2 to 8 mixed with ethanol at 40:60 (v/v) were investigated. The results show the higher current obtained when the pH is lower and then reaches the highest current at pH 3.0. This may be concerned that at low pH contained a high concentration of protons which provide a more reducing form via protonation. However, the current was then decreased at pH 2.0 (Figure 5C) supposing that the overabundance of protons from the solution can accumulate and interfere the electron transfers of analytes at the electrode surface. The increasing of analytes at the electrode surface. Furthermore, it can be noticed that its oxidation peak potentials shifted to negative values as the solution pH increased. The increasing of pH would promote the deprotonation of VK oxidation process. It is the reason that the oxidation peak potentials were shifted in less positive.^[39] The voltammogram results of VK at different pH and the plot of pH versus peak potential were shown in supporting information in Figure S3. Therefore, phosphate solution pH 3.0 was chosen as an optimal supporting electrolyte for all experiments.

For further optimization, the effect of electrochemical parameters such as frequency, amplitude and step potential were studied. These parameters can affect the peak shape and peak current of the VK response. The frequency in the range

from 8 to 16 Hz, amplitude of 20 to 100 mV and step potential of 5 to 25 mV were measured, respectively. The highest currents were observed at frequency of 14 Hz, step potential of 20 mV and amplitude of 60 mV. The results are shown subsequently in the supporting information in Figure 5D–F.

2.4. Analytical Performance

To verify the analytical performance of this proposed method, the determination of the vitamin K₁ was evaluated using SWASV with a SPGE under optimal experimental conditions. For the calibration curve, a various concentration of vitamin K₁ standard solution in the range of 1 to 15 µg mL⁻¹ in 60:40 ratio of ethanol and 0.1 M phosphate solution pH 3.0 was measured. The anodic peak current was increased with the increasing of the VK concentration, as shown in Figure 6A. After that, the anodic peak current of VK was plotted against the concentration of analyte and the least square were evaluated using the linear regression method (Figure 6B). Good linearity for VK determination was obtained in the concentration range of 1 to 15 µg mL⁻¹. The regression equation was $y(\mu\text{A}) = 0.3055x(\mu\text{g mL}^{-1}) + 1.5438$, $R^2 = 0.9904$. Then, the detection limit of vitamin K₁ was calculated by using three times the standard

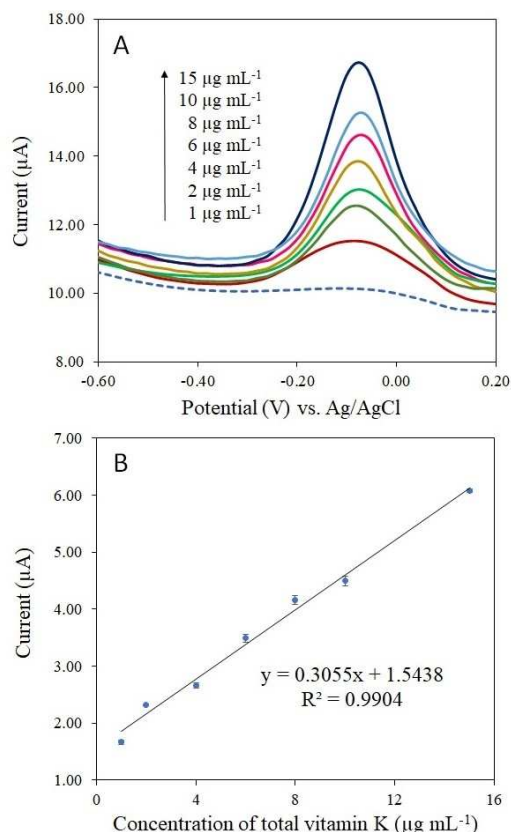


Figure 6. (A) Square-wave voltammograms of VK standard solution at different concentrations: 1, 2, 4, 6, 8, 10 and 15 µg mL⁻¹ in 60:40 ratio of ethanol and 0.1 M phosphate solution pH 3.0 using screen-printed graphene electrode. (B) The calibration plot of current (µA) and VK concentrations (µg mL⁻¹).

deviation of the blank divided by the slope of the calibration curve ($3 S_{\text{blank}}/\text{slope}$) and it was found to be $0.099 \mu\text{g mL}^{-1}$. Even though this proposed method had a detection limit higher than some of previous work as shown in Table 1, it has some merit in terms of using SPGE with the determination of vitamin K₁ such as it can provide a simple operation, nontoxic, effective cost, small sample volume and short time analysis. This developed method can be an alternative choice for the determination of the VK and applications for food analysis. For further performance confirmation, the reproducibility of device was investigated by measurement of vitamin K₁ at three concentrations of 5, 10 and $15 \mu\text{g mL}^{-1}$. Each concentration was examined using ten individual electrodes under the same optimal conditions. The %RSD was obtained at 3.29, 3.18 and 3.16, respectively, which are acceptable by AOAC guidelines, which indicated that there was good sensor-to-sensor reproducibility.

2.5. Interference Study

For the selectivity of the method, the mixture of vitamin K with other common minerals and vitamins in vegetables such as Ca^{2+} , Mg^{2+} , vitamin C, A, D and E were examined using vitamin K₁ at a concentration of $5 \mu\text{g mL}^{-1}$. The tolerance limit was estimated to be within 5% of the anodic current signal. The results show that 5-fold mass ratio of Ca^{2+} , Mg^{2+} , vitamin A and E; 2-fold mass ratio of vitamin C do not interfere with the determination of VK, as reported in Table S1. In case of vitamin D, 0.12-fold mass ratio of D₃ also do not interfere with the determination of VK. Moreover, the amount of D₃ exist in vegetables normally found at lower number than this value. So, it doesn't cause any interfering effect. In comparison to food

composition of selected vegetables, the results indicated that the assay can be used as a selective and sensitive sensor for vitamin K₁ detection in the presence of various competitive foreign compounds.

2.6. Sample Analysis

In practical applications, the SWASV technique was performed with a SPGE for the determination of vitamin K₁ in 7 green vegetables. For accuracy and precision of the method, the % recovery and %RSD were examined by spiking three concentrations of vitamin K₁ at 4, 8 and $12 \mu\text{g mL}^{-1}$, respectively. Each of the concentrations was measured repeatedly three times. The results are shown in Table S2. The recovery and RSD were found to be in the range of 95.0 to 110.0% and 0.3 to 4.6% respectively, which can indicate the capability of this method for the determination of vitamin K₁ in real samples. Furthermore, the results of this proposed method were compared with those obtained from a conventional HPLC technique coupled with DAD detector (Table 2). The comparison of found spiked concentrations in $\mu\text{g mL}^{-1}$ between the proposed method and the conventional HPLC-DAD shows a satisfactory agreement by paired t-test at the 95% confidence interval ($t_{\text{stat}}=1.1649$, $t_{\text{critical}}=2.0859$). Therefore, the proposed sensor and optimal stripping method can be used as an alternative method for determination of vitamin K₁ in real samples after using appropriate sample preparation.

Table 1. Comparison of analytical performances of different methods for the determination of VK.

Electrode	Type of VK	Method	Linear range ($\mu\text{g mL}^{-1}$)	LOD ($\mu\text{g mL}^{-1}$)	Ref
Hanging mercury drop electrode	K ₁	DPV ^[a]	0.01–0.10	–	[40]
Hanging mercury drop electrode	K ₁	SWASV	4.5×10^{-4} –0.45	–	[25]
Static mercury drop electrode	K ₃	SWASV	3×10^{-5} –0.09	2×10^{-5}	[26]
Hanging mercury drop electrode	K ₃	DPV	–	0.12	[41]
Carbon paste electrode	K ₁	AdSV ^[b]	0.30–2.00	0.18	[27]
Glassy carbon electrode	PMND ^[c]	SWV ^[d]	–	–	[42]
PEDOT:PSS-CMC-rGO@Pd composite on GCE	K ₃	DPV LSV ^[e]	0.07–20	0.002	[43]
Glassy carbon electrode	K ₁	SWASV	2.3–45.1 0.0045–0.45	0.023 0.004	[28]
Screen-printed graphene electrode	K ₁ , K ₂	SWASV	1–15	0.099	This work

^[a] Differential pulse voltammetry; ^[b] Adsorptive stripping voltammetry; ^[c] (E)-2-((prop-1-en-1-yloxy)methyl)naphthalene-1,4-dione; ^[d] Square-wave voltammetry; ^[e] Linear sweep voltammetry.

Table 2. The comparison of the proposed method and standard method for the determination of vitamin K₁ in vegetables samples.

Samples	Spiked level ($\mu\text{g mL}^{-1}$)	Amount founded ($\mu\text{g mL}^{-1}$) (x $\pm$ SD)	
		Proposed method	HPLC-DAD
Broccoli	4.0	4.1 ± 0.2	3.9 ± 0.2
	8.0	8.4 ± 0.5	8.3 ± 0.5
	12.0	11.8 ± 0.4	12.1 ± 0.1
Parsley	4.0	4.3 ± 0.1	4.4 ± 0.2
	8.0	8.5 ± 0.1	8.2 ± 0.8
	12.0	12.7 ± 0.3	12.5 ± 0.3
Chinese cabbage	4.0	4.3 ± 0.3	4.2 ± 0.3
	8.0	8.5 ± 0.3	8.3 ± 0.1
	12.0	11.9 ± 0.2	12.1 ± 0.2
Thai Basil	4.0	3.9 ± 0.3	4.1 ± 0.1
	8.0	8.6 ± 0.2	8.4 ± 0.1
	12.0	12.1 ± 0.1	12.2 ± 0.2
Iceberg lettuce	4.0	4.2 ± 0.4	4.1 ± 0.1
	8.0	8.2 ± 0.3	8.1 ± 0.1
	12.0	12.3 ± 0.2	12.2 ± 0.11
Celery	4.0	3.8 ± 0.3	3.9 ± 0.2
	8.0	8.4 ± 0.3	8.2 ± 0.3
	12.0	11.8 ± 0.5	5.02 ± 0.4
White lettuce	4.0	3.9 ± 0.5	3.8 ± 0.6
	8.0	8.3 ± 0.3	8.2 ± 0.2
	12.0	12.5 ± 0.1	12.4 ± 0.3

Note: values reported are mean of three replicates ($n=3$).

^[a] High-performance liquid chromatography with a diode-array detector.

3. Conclusions

In this study, a new sensor for the determination of the VK was proposed. The application was focused on the determination of vitamin K₁ in vegetables by a square-wave anodic stripping voltammetry technique using the SPGE. This kind of electrode provides high sensitivity, simplicity, low costs, fast analysis and a low sample volume. Moreover, this proposed method can act as being economical and environmentally friendly, which is suited for use in the food and drug industry. After the experimental parameter optimization, the best result was obtained in acidity conditions using a 60% ethanol in phosphate solution at pH 3.0. The linearity was observed in the range of 1 to 15 $\mu\text{g mL}^{-1}$. The limit of the detection was achieved at 0.099 $\mu\text{g mL}^{-1}$. The benefit of this developed sensor can apply for determination of both total VK and the individual predominant form presence in different sample. Finally, this developed method was successfully applied in natural food samples and results were in good agreement with the results from the high-performance liquid chromatography technique coupled with a DAD detector. Therefore, this proposed method shows high potential to be an alternative procedure for determination on VK in food samples to obtain the food composition table in the future.

Experimental Section

Apparatus

Voltammetric experiments were performed using an Autolab electrochemical system with a potentiostat PGSTAT 30 (EcoChemie, Utrecht, Netherlands) controlled by GPES version 4.9.007 software. The three-electrode system consisted of a SPGE as the working electrode and counter electrode, a screen-printed Ag/AgCl electrode as the reference electrode, and all electrodes were screened on the same substrate.

Chemicals and Reagents

Vitamin K₁ and vitamin K₂ were purchased from Sigma-Aldrich (Buchs, Switzerland). Sodium dihydrogen orthophosphate (NaH_2PO_4) was obtained from BDH Analar (Poole, UK). Ethanol, disodium hydrogen phosphate (Na_2HPO_4), phosphoric acid (H_3PO_4 , 85%), sodium hydroxide (NaOH) and potassium nitrate (KNO_3) were obtained from Merck (Darmstadt, Germany). Potassium ferricyanide (III) ($\text{K}_3[\text{Fe}(\text{CN})_6]$) was obtained from Sigma-Aldrich (Buchs, Switzerland). Carbon (C2130307D1), carbon graphene (C2131121D1) and silver/silver chloride (Ag/AgCl) (C2130809D5) ink were obtained from Gwent group (Torfaen, United Kingdom). PVC laser printing sheet (thickness, 0.15 mm) was purchased from Ideecraft (Bangkok, Thailand). The stock solution of vitamin K including of K₁ and K₂ (2×10^{-3} M) was prepared in ethanol and kept in dark place under 20 °C. Supporting electrolyte was prepared by mixing ethanol and 0.1 M phosphate solution pH 3.0 in the ratio of 60:40 (v/v). All chemicals were analytical grade and were used without further purification. All solutions were prepared using Milli-Q water ($R \geq 18 \text{ M}\Omega \text{ cm}^{-1}$) from Millipore (United Kingdom).

Fabrication of the Screen-Printed Electrode

Screen-printed method was used for the fabrication of electrode according to the previous describe.^[44] This platform was designed using Adobe Illustrator program as shown in Figure 7 and the screen blocks were fabricated by Chaiyaboon Co. (Bangkok, Thailand). For the fabrication steps, Ag/AgCl ink was firstly manual screened on PVC sheet as first layer for the reference electrode and conductive pads. Then, the screened electrode was baked at 55 °C for 30 min to dry out all solvent from the electrode surface which could interfere during measurement. After that, the next step is the second layer screening of graphene as working and counter electrodes on the same substrate respectively. Finally, it was heated again at 55 °C for 30 min for further solvent drying. In order to fabricate the SPCE, the preparation was done in the same way using carbon ink as material for working and counter electrodes. Before measurement, the prepared screen-printed electrode was attached with small piece of tape on the graphene strip under the sample zone for limiting the contact surface area between electrode and sample constantly.

Analytical Measurement

For the Electrochemical measurement, the screen-printed electrode was connected to the potentiostat PGSTAT30 instrument. There are two techniques used in this work; one is CV for electrochemical behaviour study and the other one is SWASV for the optimization and measurement. In the determination process, a 50 μL of standard/sample solution was dropped on three electrodes area. The CV experiments were performed in the potential range of -1.0 to $+0.2$ V at scan rate of 100 mV s^{-1} . For SWASV, preconcentration step was firstly carried out by applying the potential at -0.5 V for 240 s. After that, the measurement step was performed by scanning the potential to less negative direction over a potential range from -1.0 to $+0.2$ V at frequency of 14 Hz, step potential of 20 mV and pulse amplitude of 60 mV.

Sample Preparation

A total of seven vegetables (Broccoli, Parsley, Chinese cabbage, Thai basil, Iceberg lettuce, Celery and White lettuce) were purchased from supermarkets in Thailand. The vitamin K₁ was extracted from plant food items with 2-propanol/hexane by modification of the method of Langenberg et al.^[45] In this method, 5 g of the homogenized sample was weighed into a centrifuge tube and the standard solution of vitamin K₁ was added. Then 15 mL of 2-propanol were added. The samples were digested in a boiling water bath for 5 min. After cooling, 10 mL of 2-propanol

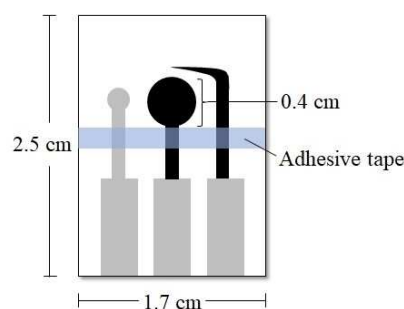


Figure 7. Screen-printed graphene electrode platform and its size including of graphene layer as working and counter electrode. A Ag/AgCl layer function as reference and conductive pads. All layers were screened on PVC substrate.

was added and the sample was homogenized with an Ultra-Thurrax T25 mixer for 2 min. Homogenization was repeated twice after adding 15 mL of n-hexane. Finally, 10 mL of water was added and after rapid shaking the extracts were centrifuged at 1500 rpm for 5 min. An appropriate amount of n-hexane phase was collected and evaporated to dryness. After that, the residue was mixed with 60:40% of ethanol: phosphate solution at pH 3.0 using 25 mL volumetric flask.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: graphene • green vegetables • screen-printed electrode • square-wave anodic stripping voltammetry • vitamin K

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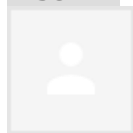
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Application of Modifier-free Gold Nanoparticle Colorimetric Sensing for Rapid Screening and Detecting Vitamin B1



[Paweenar Duenchay](#), [Kantima Kaewjua](#), [Orawon Chailapakul](#) and [Weena Siangproh](#)

Abstract

In this report, we present a transparency sheet-based colorimetric sensor as an alternative platform for the simple, sensitive and selective determination of vitamin B1 in urine samples by using unmodified gold nanoparticles (AuNPs). Samples presenting different concentrations of vitamin B1 were evaluated. The specific analysis is based on the aggregation of AuNPs by vitamin B1 due to the ability to form strong electrostatic interactions between positively charged vitamin B1 and negatively charged AuNPs. In the presence of vitamin B1, a distinctive color change in the AuNPs from red to blue was visualized by the naked eye within 10 minutes without the requirement for surface modification. For quantitative measurement using image processing, a good linear relationship ($R^2 = 0.9913$) between the vitamin B1 concentration and average red intensity was obtained in the range of 40-200 ppb. The limit of detection (LOD) and the limit of quantitation (LOQ) for vitamin B1 were found to be 3.002 ppb ($S/B=3$) and 10.006

ppb (S/B=10), respectively. The characteristics of the developed sensors were investigated for precision, accuracy, and sensitivity, and they were validated by classic methods. Statistical analysis proved that the developed sensors were precise, sensitive and accurate and that they can be used effectively for the analysis of vitamin B1 in urine samples.

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**Application of Modifier-free Gold Nanoparticle Colorimetric Sensing for Rapid
Screening and Detecting Vitamin B1**

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Abstract

In this report, we present a transparency sheet-based colorimetric sensor as an alternative platform for the simple, sensitive and selective determination of vitamin B1 in urine samples by using unmodified gold nanoparticles (AuNPs). Samples presenting different concentrations of vitamin B1 were evaluated. The specific analysis is based on the aggregation of AuNPs by vitamin B1 due to the ability to form strong electrostatic interactions between positively charged vitamin B1 and negatively charged AuNPs. In the presence of vitamin B1, a distinctive color change in the AuNPs from red to blue was visualized by the naked eye within 10 minutes without the requirement for surface modification. For quantitative measurement using image processing, a good linear relationship ($R^2 = 0.9913$) between the vitamin B1 concentration and average red intensity was obtained in the range of 40-200 ppb. The limit of detection (LOD) and the limit of quantitation (LOQ) for vitamin B1 were found to be 3.002 ppb ($S/B=3$) and 10.006 ppb ($S/B=10$), respectively. The characteristics of the developed sensors were investigated for precision, accuracy, and sensitivity, and they were validated by classic methods. Statistical analysis proved that the developed sensors were precise, sensitive and accurate and that they can be used effectively for the analysis of vitamin B1 in urine samples.

Keywords: Vitamin B1, Transparency sheet-based colorimetric sensor, Gold nanoparticles, Urine samples

1. Introduction

Vitamins are essential nutrients that must be provided to the body in small amounts on a regular basis to perform various chemical and physiological functions in the human body [1-3]. Vitamins are widely distributed in natural food sources and can be easily introduced into diets to satisfy daily needs. Vitamins are a group of organic compounds that have different structural and chemical properties, and can be conveniently categorized into two groups based on their solubility: fat-soluble vitamins and water-soluble vitamins. As the member of vitamin B group, thiamine (vitamin B1) is a sulfur-containing member of the water-soluble B-complex family, which are essential for normal development, growth, reproduction, lactation, and physical performance. Thiamine is involved in releasing energy from macronutrients that provide energy, especially from carbohydrates. Vitamin B1 is an essential vitamin that cannot be synthesized and must come from the diet. Vitamin B1 is widely distributed in small amounts in foods, but it is easily lost during the milling, heating, canning, blanching, and storage of foods. Vitamin B1 is readily absorbed from the intestine and readily excreted through the kidneys. A deficiency in vitamin B1 can be caused by various factors and diseases, such as alcoholism, HIV, vomiting, and other gastrointestinal illnesses. Vitamin B1 deficiency may lead to chronic diseases. One of these diseases is beriberi, which is a neurological and cardiovascular disease. Brain abnormalities are also one of the common signs of vitamin B1 deficiency. These abnormalities include Wernicke-Korsakoff syndrome [2-5]. Due to the functions and stability of vitamin B1 in the body, it is necessary to develop simple, fast, low cost and sensitive analytical methods to determine vitamin B1 concentrations in clinical analysis.

Several methods have been reported for the determination of vitamin B1, including high-performance liquid chromatography (HPLC) [6, 7], chemiluminescence [8], electrochemical detection [9, 10], spectrophotometric detection [11, 12, 13], and fluorimetric

analysis [14, 15]. Currently, metal nanoparticles have received significant interest in the past decade as active components for widespread application in the field of biomedicine. These nanoparticles have received considerable attention because of their distinctive properties including their optical and electronic properties, biocompatibility and long-term stability. Their excellent optical properties display brilliant color for sensing applications which can be visualized by the naked eye with no need for any additional instrumentation. Several methods for vitamin B1 determination have been described using different nanoparticles as reagents, such as gold [10, 11, 13, and 14], multiwall carbon nanotube [9], Cu nanoclusters [15], and silver nanoparticles [16]. Among these nanoparticles, gold nanoparticles (AuNPs) have been intensively studied for numerous applications as optical sensors for heavy metals [17], antioxidant [18], folic acid [19, 20], melamine [21], dopamine [22], and organophosphate pesticides [23, 24]. Although a number of methods have been proposed for the determination of vitamin B1, none of them were found to be suitable for on-site determinations. In addition, the main drawbacks of these mentioned methods are the time-consuming processes and costly instruments, which are inconvenient for routine work and on-site monitoring. Therefore, it will be very useful to have a simple tool that everyone can use for the on-site determination of vitamin B1 levels to obtain real-time determination.

Recently, portable devices have become an increasingly important goal of method development for the detection of various targets. Paper-based analytical devices (PADs) are one of the most exploited devices to be used as miniaturized analytical platforms which are fabricated based on cellulose materials as a substrate for economical and portable sensing applications. Paper-based sensors can be advantageous for applications in diagnostics, food safety, and environmental monitoring due to their advantages of natural abundance, simplicity, disposability, low sample-reagent usage and suitability for routine analysis [17, 18]. Unfortunately, the use of paper for colorimetric detection suffers from the adsorption of the

color by the paper porosities, leading to limitations in the sensitivity in comparison with that of detection methods in solution.

To solve this problem, transparency sheet was proposed to be an alternative analytical platform. The lack of porosities of polymer surface on transparency sheet leads to no adsorption of the color. All reagents can be held and reacted on the hydrophobic detection zone that seem to have the same behavior in solution. This can increase the performance to distinguish the color intensity by naked eyes and can measure of the color change at low concentration of the analyte. Therefore, we proposed for the first-time a simple, sensitive, and field-portable transparency sheet-based colorimetric devices for vitamin B1 monitoring by utilizing unmodified AuNPs in a neutral media as a remarkable colorimetric agent.

Upon the addition of a vitamin B1 solution into the AuNP solutions, a visible color change of the AuNPs from red to blue was observed within 10 minutes. To facilitate the application of this sensor for on-site monitoring, a smartphone or digital camera was also used to record the colorimetric responses in the detection platform. Furthermore, the proposed colorimetric sensor could be exploited to determine the vitamin B1 level in different urine samples, and comparisons were in good agreement with conventional spectroscopic method. The simplicity, sensitivity, selectivity, disposability, and portability of these devices provides an interesting alternative to the most frequently used conventional methods.

2. Experimental section

2.1 Chemicals and materials

Gold nanoparticles (AuNPs, 0.01%) with a diameter of 20 nm were purchased from Kestrel Bio Sciences (Thailand). AuNPs in an aqueous solution were synthesized by citrate reduction. The resulting nanoparticles have negatively charged citrate ions adsorbed on their surface and are thus stabilized by electrostatic repulsion. Thiamine hydrochloride (vitamin

B1), potassium chloride, ammonium chloride, calcium chloride, sodium chloride, magnesium chloride, disodium hydrogen orthophosphate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$), sodium dihydrogen orthophosphate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) and hydrochloric acid were obtained from Ajax Finechem Pty Ltd. Riboflavin (vitamin B2), d-pantothenic acid hemicalcium (vitamin B5), folic acid (vitamin B9), vitamin B12, uric acid and ascorbic acid (vitamin C) were purchased from Sigma Aldrich. Niacin (vitamin B3) and pyridoxine hydrochloride (vitamin B6) were purchased from Himedia Laboratories Pvt Ltd. All chemicals were used as received without any purification procedures. All reagents were prepared with milli-Q water from Millipore ($R \geq 18.2\text{ M}\Omega\text{ cm}$) throughout these experiments.

2.2 Preconcentration of received gold nanoparticles

Preconcentration of gold nanoparticles was performed by pipetting the stock solution of gold nanoparticles into 1 mL microcentrifuge tubes. After that, the microcentrifuge tubes were centrifuged at 12,000 rpm (4 °C) for 30 minutes. The 0.8 mL of supernatant was removed, and the mixture solution was continuously stirred for 10 seconds.

2.3 Wax-pattern transparency sheet-based fabrication

Circular areas with a diameter of 5 mm were designed by Adobe illustrator CS4 software (Adobe Systems, Inc.). A wax printing method was used for the fabrication of the transparency sheet-based devices. A blue wax color with an RGB value of 98/147/254 was used to complement the colorimetric reaction. The process used to fabricate the transparency-based devices was to print the wax pattern onto the surface of the transparency sheet using a commercial wax printer (Xerox Color Qube 8570, Japan). The printed wax can confine the aqueous solution onto the transparency surface to produce the detection zone.

2.4 Colorimetric measurement of vitamin B1 using the AuNP probes and image processing

For the colorimetric monitoring of vitamin B1, 15 μL of the AuNPs was first added to the detection test zone of the transparency-based devices. Next, 15 μL of either a sample solution or vitamin B1 standard solution was applied to the test zone. The color change of the AuNPs was visualized after 10 minutes by the naked eye. After, the test zones of the transparency-based devices were imaged with a mobile phone (vivo V3) under an in-house light-control box using the Camera application. The JPEG format images were then imported into Image J software (National Institute of Health, USA) to measure the mean color intensity. The image color threshold was set to remove the blue background color by adjusting the hue and brightness window to a specific range of (100/200) and (60/200), respectively. The color signal of each zone was measured as the mean color intensity and then imported into Numbers software (v 2.0.3, Apple Inc.) for analysis.

2.5 Specificity test for interference ions

Riboflavin (vitamin B2), d-pantothenic acid hemicalcium (vitamin B5), folic acid (vitamin B9), vitamin B12, niacin (vitamin B3), pyridoxine hydrochloride (vitamin B6), uric acid, ascorbic acid (vitamin C), glucose, albumin, and ions that are likely to be found in real samples were examined as potential interferences for the determination of vitamin B1. To investigate the specificity of the proposed method, each kind of foreign substance and ion was individually tested and added into a fixed 20 ppb vitamin B1 solution.

2.6 Method validation experiments

The applicability of the proposed method was evaluated with a fluorescent method by determining vitamin B1 concentration in urine samples. Fluorescent methods are one of the

most important methods used to measure the levels of vitamin B1 in samples. The fluorescent molecule, thiochrome is produced by the oxidation of vitamin B1 in an alkaline medium with potassium hexacyanoferrate(III) [25]. The following three solutions are prepared. Potassium hexacyanoferrate(III) (K_3FeCN_6) 0.001 g was dissolved in DI water, and then diluted to 100 ml to make a 10 ppm K_3FeCN_6 solution. Vitamin B1 stock solution (10,000 ppb) was made from 0.0005 g of pure vitamin B1 was dissolved in DI water and then brought to 50 mL in a volumetric flask. The standard vitamin B1 stock solution was prepared and then stored in the dark. Working standards of vitamin B1 (300 ppb, 200 ppb, 150 ppb, 100 ppb, and 50 ppb) were prepared by diluting (0.3 mL, 0.2 mL, 0.15 mL, 0.1 mL, and 0.05 mL) of the stock solution in a 10- mL volumetric flask with DI water. A pH=12.2 buffer was prepared from reagent grade NaH_2PO_4 (0.29 g) and Na_2HPO_4 (3.23 g) in 100 mL of water to make a 0.2 M buffer. Either sodium hydroxide or phosphoric acid was added to bring the pH to 12.2.

Analytical Procedure for Calibration: One milliliter of the 0.2 M, 12.2 pH buffer was added to a (fluorescence) sample cuvette. Then, 1.00 mL of the 10 ppm K_3FeCN_6 solution was added into the cuvette. After that, 1.00 mL of the standard to be analyzed was added. The cuvette was quickly capped and inverted a few times to ensure adequate mixing and then placed into the spectrofluorometer. The fluorescence intensity generated by the thiochrome produced that was immediately measured at $\lambda_{ex} = 369$ nm and $\lambda_{em} = 436$ nm. The above steps were repeated for all of the working standards.

2.7 Preparation of artificial urine samples

The practical application of the proposed method was evaluated by determining vitamin B1 concentrations in urine samples. The artificial urine samples were purchased from Carolina Biological Supply Company. To determine vitamin B1 concentrations in real samples, a

certain amount of vitamin B1 was spiked into the urine samples. The spiked samples were added into the AuNP solution, and after 10 min, the test zones of transparency-based devices were captured with a digital camera. Finally, the standard additions were used to examine the amount of vitamin B1. In addition, relative standard deviations and percentage recoveries were calculated.

3. Results and discussion

3.1 Characterization

3.1.1 UV-Visible spectrophotometry

To monitor the size changes of the AuNPs, UV-Vis absorption spectroscopy was first used. Fig. 1 displays the surface plasmon resonance (SPR) absorption spectra of the AuNPs. The characteristic peak of AuNPs (red line) was observed at 517 nm. Interestingly, after the addition of vitamin B1 to the AuNP solution, a slight shift towards longer wavelengths, from 517 nm to 650 nm, was observed. This change indicates an increase in the aspect size of the nanoparticles. In addition, gradual decreases in absorbance were also observed at 517 nm. Additionally, an obvious color change of the AuNP solution from red to blue could be observed by the naked eye. The color change and shift in the SPR absorption band suggests that the shape of the AuNPs was transformed by the concentration of vitamin B1 added to the solution.

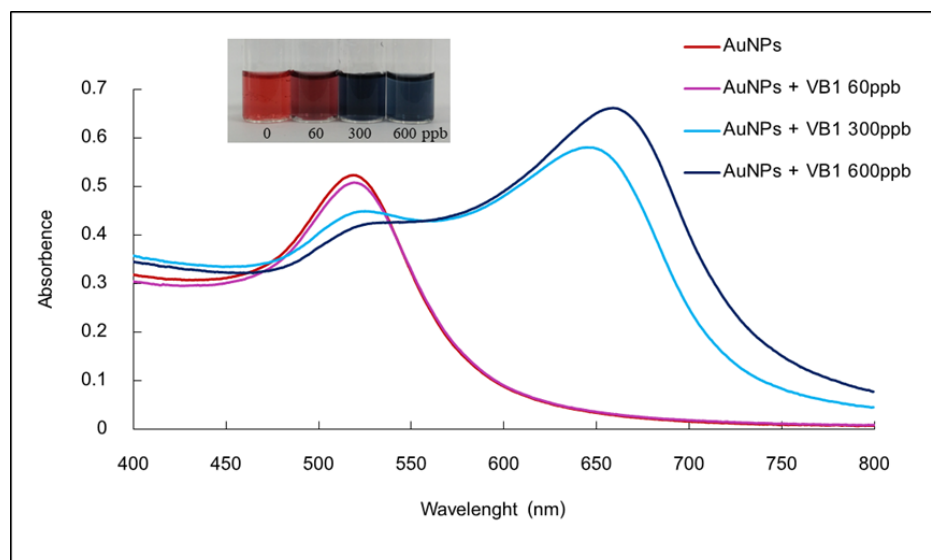


Fig. 1 UV-vis absorption spectra and photographs of the AuNPs after the addition of vitamin B1 at different concentrations, measured 10 minutes after addition.

3.1.2 Mechanism studies for colorimetric detection of vitamin B1 with modifier-free gold nanoparticles

In order to confirm the aggregation mechanism, the zeta potential and FITR have been used to investigate this action. From Table 1, it was found that the zeta potential clearly shows that after addition of vitamin B1 in AuNPs solution, the negative charge decreased from -53.2 mv to -44.1 mv, while the average size of AuNPs was increased from 27.8 to 61.8 nm. These results indicated that AuNPs aggregation was induced by electrostatic force between the positive charge of vitamin B1 and the negative charge of AuNPs at the optimized conditions.

Table 1. The size average and zeta potential of AuNPs, AuNPs- Buffer and AuNPs- Buffer + vitamin B1

Samples name	Scattering Angle	Z-Average (nm)	PI	Zeta Potential (mV)
AuNPs	90	26.9	0.638	-43.1
AuNPs- Buffer		27.8	0.610	-53.2
AuNPs- Buffer + vitamin B1		61.8	0.655	-44.1

Moreover, the results obtained corresponded to the FTIR spectra which showed the characteristic peak for NH₂ (3,347 cm⁻¹), O-H (3,094 cm⁻¹) and N-H (1,623 cm⁻¹) for vitamin B1. As shown in Fig.2, it can be seen that after addition of vitamin B1 in the solution of AuNPs, notable peak shifting and decreasing intensity was observed. This phenomenon indicated the aggregation of AuNPs by strong electrostatic from vitamin B1. The result obtained from our work was similar to the previous reports that used AuNPs and functionalized AuNPs as a color reagent of vitamin B1 detection [13, 14].

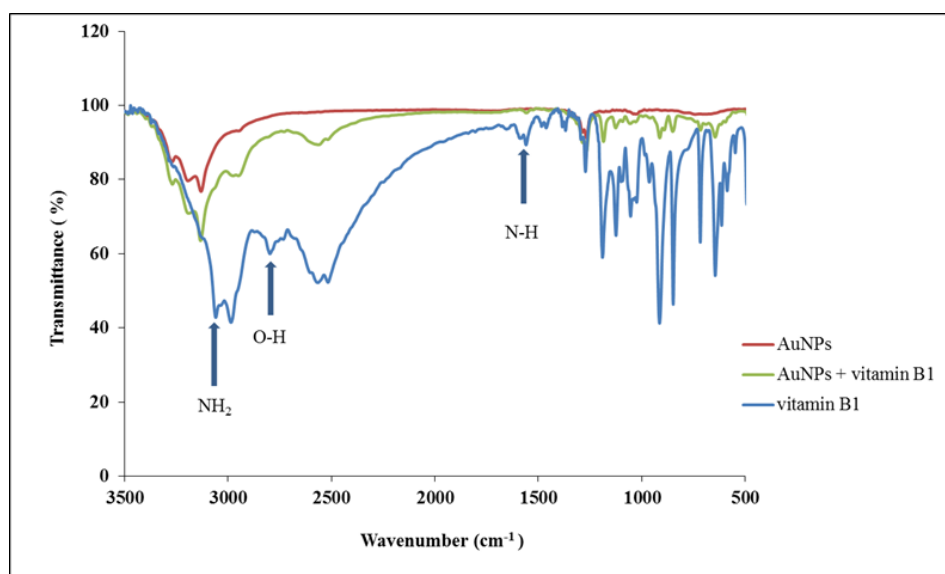


Fig. 2 FI-IR spectra of AuNPs, vitamin B1 and AuNPs after the addition of vitamin B1.

3.2 Optimization of the assay conditions

3.2.1 Optimization of the AuNP concentration

The concentration of AuNPs was first optimized for vitamin B1 determination. At low concentrations, the light color of AuNPs causes difficulties in monitoring the color changes, resulting in poor sensitivity of detection. Fig. 3a shows AuNP solutions at various concentrations. The preconcentration procedure and concentration of the AuNPs were performed and calculated based on a reported procedure [26] and were found to be 100, 125, 167, 250, and 500 ppm, respectively. It was clearly observed that the color density increased with increasing concentration. Subsequently, all of the AuNP solutions were tested at 100 ppb of vitamin B1, and the mean red color-intensity difference (ΔI_{red}) of the AuNPs was measured using Image J software. As shown in Fig. 3b, the use of 500 ppm AuNPs provided the highest ΔI_{red} between the absence and presence of vitamin B1. Therefore, a concentration of 500 ppm for the AuNPs was selected as the optimal value for the determination of vitamin B1.

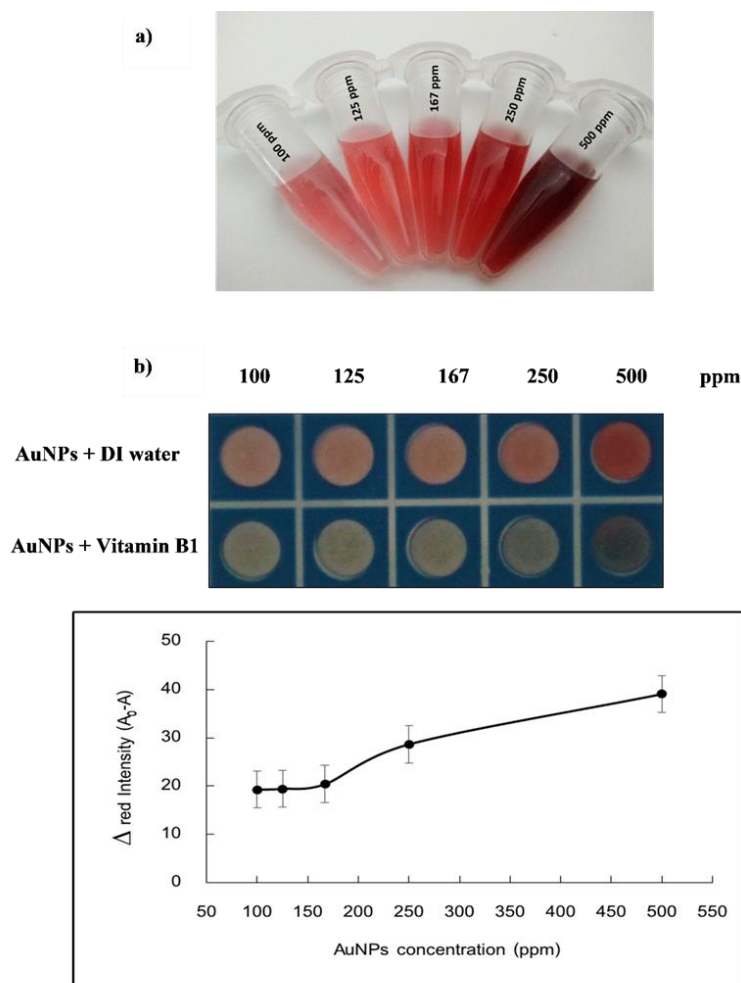


Fig. 3 (a) The color of AuNP concentrations at 100, 125, 167, 250, and 500 ppm after the preconcentration process, (b) The color intensity difference in the absence and presence of 100 ppb of vitamin B1 at 100, 125, 167, 250, and 500 ppm AuNP solutions, respectively.

3.2.2 Optimization of the AuNPs and vitamin B1 volume ratio

The detection zone of the transparency sheets had a maximum volume of 30 μ L. The color changes of the aggregation between the AuNPs and vitamin B1 at volume ratios of 1:5, 1:2, 1:1, 2:1, and 5:1 are shown in Fig. 4. The Δ red intensity was maximized at a 1:1 volume ratio. As such, 15 μ L of the AuNP solution and 15 μ L of the vitamin B1 solution was chosen for further experiments to provide the preferable sensitivity for vitamin B1 detection.

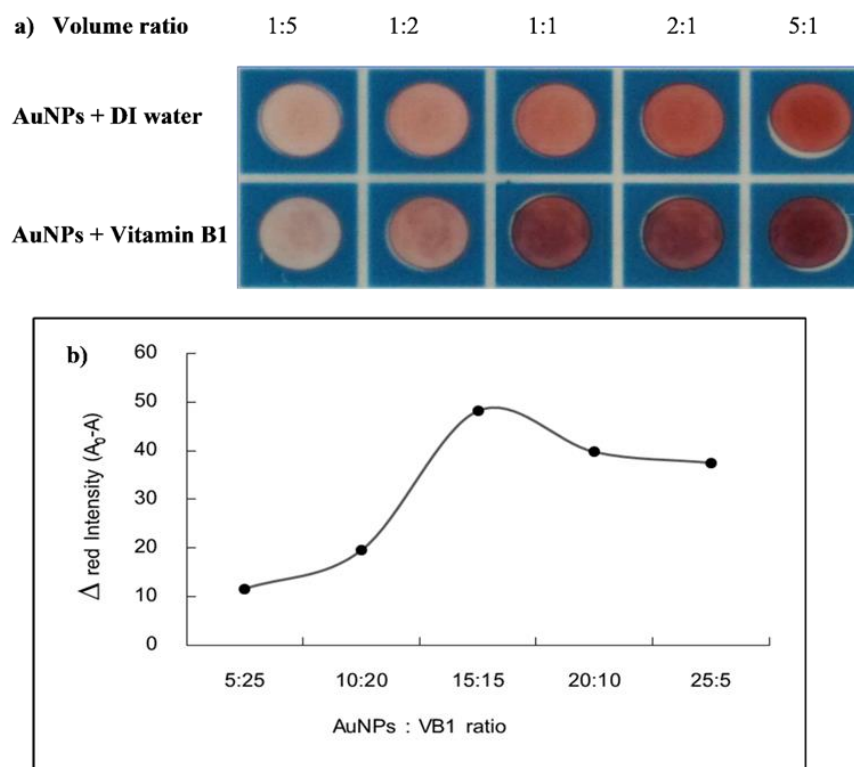


Fig. 4 (a) The color images of the aggregation between AuNPs with and without a 100 ppb solution of vitamin B1 at volume ratio 1:5, 1:2, 1:1, 2:1, and 5:1, (b) Plot of the Δ red intensity as a function of the volume ratio of AuNPs:100 ppb vitamin B1.

3.2.3. Optimization of pH buffer

The effects of the pH buffer on the sensitivity and selectivity after aggregation between the AuNPs and vitamin B1 were examined by varying the pH of the buffer from 2 to 10. In principle, vitamin B1 is positively charged on both the pyrimidine N1 nitrogen ($pK_{a1} \cong 4.8$) and thiazole N3 nitrogen at low pH, and vitamin B1 exists as a cation with a positive charge on the thiazole N3 nitrogen at physiological pH. As the pH further increases, vitamin B1 exists as an uncharged pseudo base intermediate, which displays a negatively charged thiol form ($pK_{a2} \cong 9.2$). As a result, the similarly charged AuNPs and vitamin B1 repelled each other when the pH was higher than 7. Therefore, pH 7.0 was chosen for the experimental buffer in the following study. In addition, it was found that when using a pH 7 buffer, the Δ red intensity

of the related vitamin B samples changed slightly, while only vitamin B1 was obviously changed, as shown in Fig. 5. From this finding, a 10 mM phosphate buffer at pH 7 was suitable for the sensitive and selective detection of vitamin B1 by this colorimetric method.

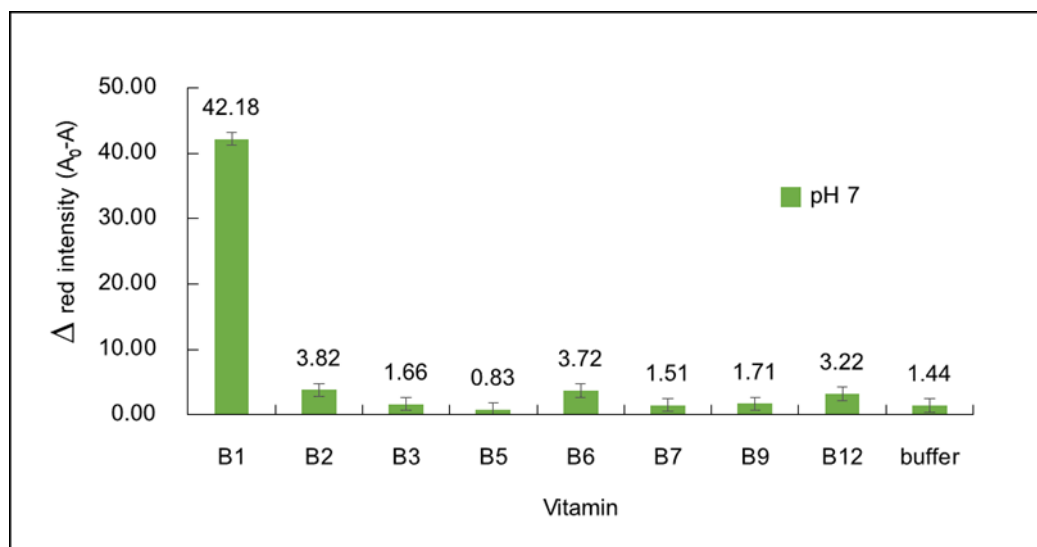


Fig. 5 Effects of a 10 mM phosphate buffer at pH 7 on the sensitivity and selectivity after aggregation between AuNPs and vitamin B1 at 100 ppb.

3.2.4. Optimization of the incubation time

The incubation time, within the range of 2 to 30 minutes, was optimized. The Δ red intensity was calculated from the picture that was captured and the measured intensity of the color change was monitored simultaneously, as shown in Fig. 6. The Δ red intensity increased with increasing incubation time and reached a maximum at 10 minutes. The Δ red intensity decreased after 10 minutes because evaporation of the solution may occur and background intensity also was increased. From the results, the optimal incubation time was 10 minutes. This condition provided the highest color change intensity.

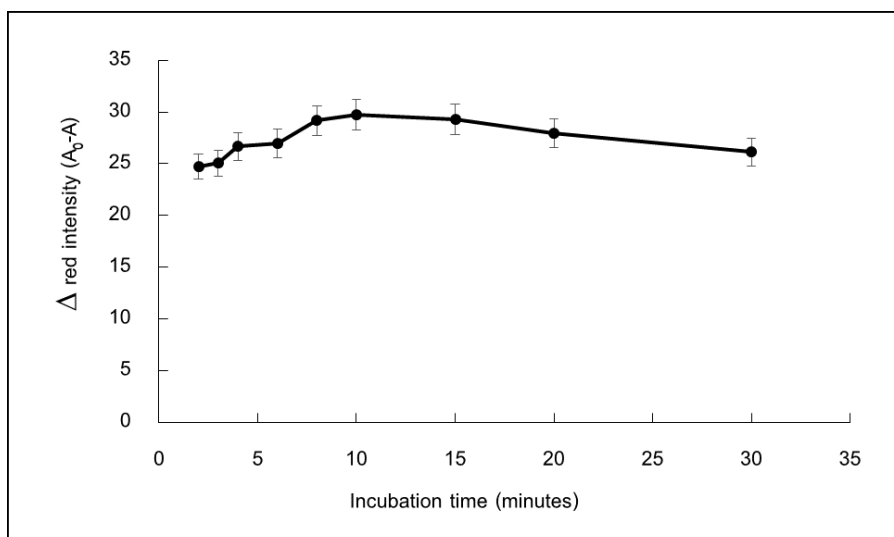


Fig. 6 The effects of incubation time on the detection of vitamin B1 at a concentration of 100 ppb.

3.3 Analytical performance of the transparency sheet-based colorimetric assay for determining vitamin B1 concentration

3.3.1 Calibration curve

A calibration curve was obtained from the Δ red intensity measured by mobile phone. Concentrated AuNPs (15 μ L) were dropped onto a transparency sheet, and 15 μ L of a standard vitamin B1 solution was added with a 10-minute incubation time. With an increase in the vitamin B1 concentration, the Δ red intensity of the AuNPs decreased because the AuNP color changed from red to blue, corresponding to the results from the UV-vis spectrophotometer in Fig. 1. A linear relationship between the Δ red intensity and vitamin B1 concentration was established as shown in Fig 7. The Δ red intensity of the AuNPs is directly proportional to the added vitamin B1 concentration. A good linearity was observed in the concentration range from 40 ppb to 200 ppb with a correlation coefficient of 0.9913. The limit of detection (LOD)

and the limit of quantitation (LOQ) for vitamin B1 were found to be 3.002 ppb and 10.006 ppb, respectively.

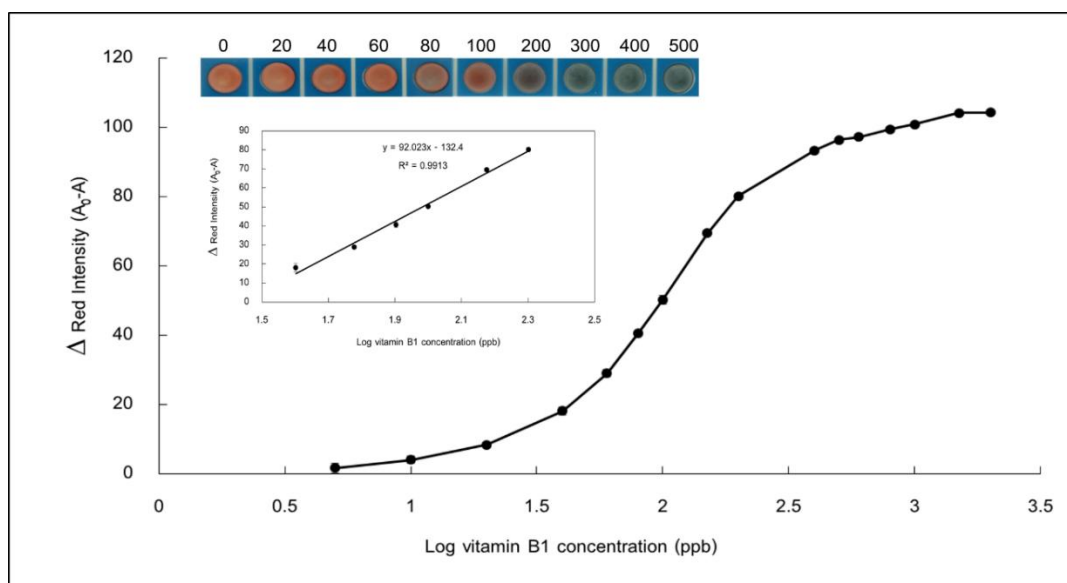


Fig. 7 Calibration curve plot of the change in the red intensity versus vitamin B1 concentration.

3.3.2. Selectivity of the AuNPs towards Vitamin B1 detection

To confirm the selectivity of the AuNPs towards vitamin B1 detection, related vitamin B complexes, including vitamin B2, vitamin B3, vitamin B5, vitamin B6, vitamin B7, vitamin B9, vitamin B12, and vitamin C were also tested. The color signal change in the AuNPs in the presence of other vitamin species and common interfering molecules had a less than $\pm 5\%$ change compared to that of the color signal of only vitamin B1 (20 ppb). As shown in Fig. 8, the tolerance concentration of other vitamin species and common interfering molecules have no appreciable change because at these concentrations are impossibly found in real sample. Therefore, the achieved high experimental selectivity and sensitivity is obtained. The proposed mechanism for controlling the selectivity was discussed from results obtained. We strongly believed that it may be concerned to a positive charge on thiazole N3 nitrogen atom of vitamin B1 under the optimized conditions. It provided a high affinity for strong electrostatic

interactions with the negatively charged AuNPs while other metal treated solution exhibited a negligible change. As presented in Fig. 1, after the addition of vitamin B1 to the AuNP solution, a new absorption peak appeared at higher wavelength region (650 nm) along with decrease in original SPR peak at 517 nm. While the other remained at the same position as that of AuNPs (data not shown). Quantitative measurement was performed by comparing the mean of red color-intensity difference (ΔI_{red}). This difference represents the degree of aggregation and representative of the relative quantity of aggregated and dispersed AuNPs. In comparison, quantitative analysis of the tested solution suggested an obviously high different ΔI_{red} for vitamin B1 suggesting the maximum aggregation state, while all other metals had a mean of red color-intensity difference similar to that of AuNPs. Therefore, the proposed assay is efficient for selective detection of vitamin B1 in presence of other metal ions. However, the exact mechanism and the microscopic nature are still subject to research and discussion.

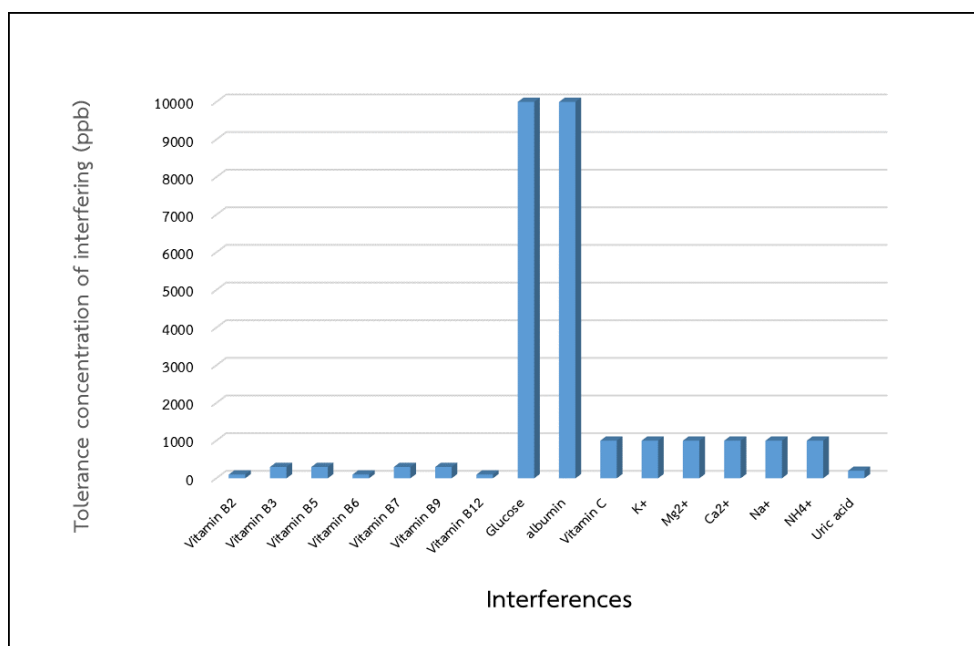


Fig. 8 The tolerance concentration of other vitamin species and common interfering molecules.

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3.4 Analytical application in a real sample

To evaluate the efficiency of our method, the colorimetric sensor was used to detect vitamin B1 in urine samples. Under the optimal conditions, the determination of vitamin B1 in urine samples spiked with vitamin B1 is shown in Table 2. The recoveries and %RSDs for vitamin B1 were in the range of 84.72 - 91.08 % and 2.74 – 6.98 %, respectively. Moreover, the results for the vitamin B1 values obtained by the developed sensor were compared with the reference values obtained using a standard method. As a result, this proposed colorimetric sensor can be applied for the determination of vitamin B1 in the urine samples with satisfactory results.

Table 2. The %RSD and %Recovery for the determination of vitamin B1 in urine samples (n=10)

Vitamin B1 spiked (ppb)	Vitamin B1 (ppb)		% RSD (n=10)	% Recovery
	This Method	Fluorometric method		
40	36.43 ± 1.00	41.53 ± 0.63	2.74	91.08
60	50.83 ± 3.55	63.55 ± 0.68	6.98	84.72
80	69.06 ± 3.40	79.02 ± 0.50	4.92	86.33

4. Conclusions

A simple, rapid, and low-cost transparency sheet-based colorimetric assay was developed for the determination of vitamin B1 in urine samples. In addition to the direct measurement of vitamin B1, this method allows for a simple way to screen and calculate the amount of vitamin B1 in the sample. Under the optimized experimental conditions, a linear

relationship was obtained between the change mean red color intensity (ΔI_{red}) and the logarithmic concentration of vitamin B1 in the concentration range of 40 to 200 ppb. A detection limit of 3.002 ppb was found for vitamin B1 detection. The usefulness of the transparency sheet-based colorimetric sensor developed in this work is its high sensitivity and selectivity, good precision, simplicity, and reproducibility. In addition, this device has been successfully applied to determine vitamin B1 in urine samples. The proposed method has major advantages, such as being equipment-free, portable for field analysis and remote sensing for oversea or sailing. This finding provides a high possibility for applications in clinical diagnostics.

Conflict of interest

The authors declare no conflict of interest.

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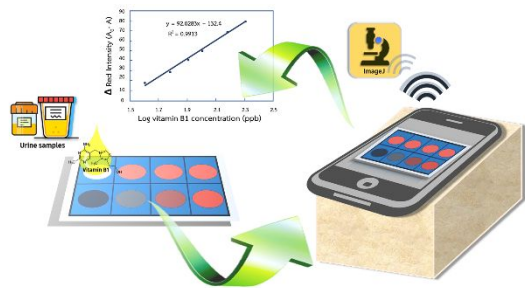
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Table of contents entry



A simple and fast alternative colorimetric sensing platform for selective determination of vitamin B1 in urine samples was proposed.

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Abstract: An alternative approach for rapid, highly sensitive and specific analytical sensor for the simultaneous determination of fat-soluble vitamins (A, D, E and K) in different matric samples was demonstrated using our developed sensor by graphene nanocomposite. The adsorptive square wave voltammetry was used to determine the amounts of vitamin A, D, E and K in infant milk, yogurt and parsley. Various analytical conditions were investigated to enhance the best performance of the proposed method. Under optimal conditions, a good linear relationship was obtained between the anodic current and the concentration of vitamins within the range from 0.02 $\mu\text{g mL}^{-1}$ to 1 $\mu\text{g mL}^{-1}$. The limits of detection were found to be 0.0086, 0.0063, 0.0075 and 0.0071 $\mu\text{g mL}^{-1}$ for vitamin A, D, E and K, respectively. The accuracy of the proposed sensor has been validated with the standard AOAC guideline. The reproducibility has also been demonstrated by repeating the experiments between sensor-to-sensor. Compared to conventional chromatographic methods, the electrochemical sensor outlined here shows a comparable performance yet faster, simpler, and cheaper approach that enables the continuous monitoring of the total quantity of vitamin A, D, E and K in various samples with good reliability, sensitivity, selectivity and accuracy.

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April 3, 2020

Dear Editor of Journal of Electroanalytical Chemistry

Please accept the submission of this manuscript entitled “**High-Efficient of Graphene Nanocomposite: Application to Rapidly Simultaneous Identification and Quantitation of Fat-Soluble Vitamins in Different Matric Samples**” for consideration for possible publication in the original research paper section of *Journal of Electroanalytical Chemistry*. In this work, we would like to demonstrate the use of our developed sensor by graphene nanocomposite for specifically simultaneous determination of fat-soluble vitamins (A, D, E and K). To the best of our knowledge, there is only short information reported by our group about the application for the simultaneous quantitation of vitamin A, D, E and K in dietary supplements. To increase the capability and open the alternative research vision obtained from our developed sensor, therefore, this is the first full report on the application of a screen-printed graphene nanocomposite electrode as a sensor for the simultaneous analysis of fat-soluble vitamins in various samples which have different matrices. Here, in this manuscript, we describe the use of graphene nanocomposite screen-printed carbon electrode as a simple and low cost electrochemical sensor for the determination of fat-soluble vitamins. We first characterize the electrodes using cyclic voltammetry to demonstrate that the graphene nanocomposite screen-printed carbon electrode exhibits lower double layer capacitance and higher signal-to-noise ratio than other carbon materials. We next demonstrate the ability of the electrode over multiple runs in the presence of fat-soluble vitamins without electrode fouling constraint. Finally, we demonstrated the use of the electrodes for possible simultaneous detection of all the fat-soluble vitamins in real samples, which is amenable for applications. We are confident that our manuscript will attract the attention of *Journal of Electroanalytical Chemistry* readership, who are interested in sensor fabrication.

We are looking forward for your valuable comments/suggestions.

Yours sincerely

Weena Siangproh, Ph.D.

Graphical abstract



Highlight

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- A sensitive electroanalysis for the simultaneous determination of fat-soluble vitamins was presented.
- Greatly enhanced in S/B ratio and low double layer capacitance were achieved from using proposed material.
- The usual fouling effect from fat-soluble vitamins analysis was not observed in the developed sensor.
- The selectivity was achieved without prior sample preparation and can be applied for various samples.

**High-Efficient of Graphene Nanocomposite: Application to Rapidly Simultaneous
Identification and Quantitation of Fat-Soluble Vitamins in Different Matric Samples**

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Abstract

An alternative approach for rapid, highly sensitive and specific analytical sensor for the simultaneous determination of fat-soluble vitamins (A, D, E and K) in different matrix samples was demonstrated using our developed sensor by graphene nanocomposite. The adsorptive square wave voltammetry was used to determine the amounts of vitamin A, D, E and K in infant milk, yogurt and parsley. Various analytical conditions were investigated to enhance the best performance of the proposed method. Under optimal conditions, a good linear relationship was obtained between the anodic current and the concentration of vitamins within the range from 0.02 $\mu\text{g mL}^{-1}$ to 1 $\mu\text{g mL}^{-1}$. The limits of detection were found to be 0.0086, 0.0063, 0.0075 and 0.0071 $\mu\text{g mL}^{-1}$ for vitamin A, D, E and K, respectively. The accuracy of the proposed sensor has been validated with the standard AOAC guideline. The reproducibility has also been demonstrated by repeating the experiments between sensor-to-sensor. Compared to conventional chromatographic methods, the electrochemical sensor outlined here shows a comparable performance yet faster, simpler, and cheaper approach that enables the continuous monitoring of the total quantity of vitamin A, D, E and K in various samples with good reliability, sensitivity, selectivity and accuracy.

Keywords: Fat-soluble vitamins; Simultaneous determination; Electrochemical sensors; Nanocomposites; Matrix samples

1. Introduction

Vitamins are a broad group of organic nutrients which are necessary for life. Sufficient level of these nutrients is a key role for a normal growth, metabolism and physical well-being. Most vitamins cannot be synthesized in the human body, or only inadequate amounts are made. Therefore, the major source of vitamins comes from a daily food.

Vitamins can be categorized into two groups: (i) water-soluble vitamins and (ii) fat-soluble vitamins (A, D, E, and K). Fat-soluble vitamins are characterized by their low polarity [1] and found mainly in animal products, fatty nutrients and pharmaceuticals [2]. These fat-soluble vitamins are stored in the fatty tissues and in the liver of the body for long periods of time. Therefore, the possibility for risk of their toxicity can be caused by consumption of these vitamins in excess over water-soluble vitamins. Thus, maintaining and controlling the appropriate quantity of fat-soluble vitamins in the body is very important. Currently, these vitamins are substances often added associated in food and pharmaceutical supplements to fulfill the nutrition. The widespread utilization of these kind of food and pharmaceutical supplements demands uncomplicated, rapid, reliable and cost-effective method for the determination of active compounds in commercial forms.

Various analytical methods involving liquid chromatographic methods coupled with mass spectrometry and spectroscopic detection have been extensively reported and developed for the determination of fat-soluble vitamins in a variety of biological, drug, food and dietary supplements samples [3-22]. Although these techniques provided high sensitivity and selectivity, unfortunately, there were suffered from the drawbacks of tedious procedures, complex instrumentation, expensiveness, long analysis time and the requirement for a skilled analyst. Alternatively, electrochemical detection is a promising method to circumvent these inherent shortcomings owing to their remarkable sensitivity and accuracy, experimental simplicity, fast response time, and compact setup. Up to date, the electrochemical detection

of fat-soluble vitamins has been rarely explored on account of the lipid-soluble nature of the vitamin and needs to be performed in non-aqueous media. In addition, one common issue with fat-soluble vitamins is that it undergoes strong adsorption during the oxidation steps, which blocks the surface and yields electrode fouling [23-25]. This phenomenon can limit the repeated usage of the electrode, especially in continuous monitoring applications. Therefore, for a long-running electrode, electrode modification is a crucial step to eliminate or minimize this effect. By means of modification, the use of some polymers as anti-fouling agents is also gaining attention because of their excellent character. For example, poly(3,4-ethylenedioxythiophene) (PEDOT) and sulfonated tetrafluoroethylene-based fluoropolymer-copolymer (Nafion) are two of the most common polymers widely applied to modify electrode surface [26-29]. Moreover, there are previously reported on the determination of vitamin D in a mixture of organic solvent and supporting electrolyte by differential pulse voltammetry (DPV) using glassy carbon electrode with and without modification step [30-31]. Nevertheless, these proposed methods provided a high detection limit and could not determine vitamin A, D, E and K simultaneously. In this regard, we particularly emphasize the use of Nafion owing to its superior anti-fouling capability and other features in term of thermal stability, chemical inertness, mechanical strength, catalytic activity, high permeability to the cation [32-34]. Furthermore, modification with Nafion offers experimental simplicity and eliminates prolonged additional steps in electrode pretreatment, making it a very attractive material from an electrochemical viewpoint for the simultaneous determination of fat-soluble vitamins in a variety of products.

Currently, the integration of nanomaterials and Nafion for the preparation of hybrid nanocomposites has gained popularity, as these materials can synergistically enhance the performance of the electrochemical sensor. Graphene, the well-publicized and prominent allotrope of carbon, is notably one of the most powerful nanomaterials and is widely used in

the development of hybrid materials. It has many outstanding advantages such as high electrical conductivity, excellent electron transportation, high mechanical and chemical stability, large specific surface area and good biocompatibility [35-38]. Many studies of graphene/Nafion-based electrochemical sensors have shown that the use of Nafion prevented electrode fouling and served as an effective dispersing agent to exfoliate graphene sheets to prevent aggregation [39-41].

Surprisingly, there is no report on the use of graphene/Nafion nanocomposite for the determination of fat-soluble vitamins in real samples using electrochemical techniques, and to the best of our knowledge, there is only short information reported by our group [42] about the application for the simultaneous quantitation of vitamin A, D, E and K in dietary supplements. To increase the capability and open the alternative research vision obtained from our developed sensor, therefore, this is the first full report on the application of a screen-printed graphene nanocomposite electrode as a sensor for the simultaneous analysis of fat-soluble vitamins in various samples which have different matrices.

2. Experimental

2.1. Apparatus

Voltammetric experiments were performed using a model CHI660D electrochemical analyzer (CH Instruments, USA). All experiments were performed using a three-electrode system which consisted of a screen-printed silver/silver chloride electrode as the reference electrode, a screen-printed graphene/Nafion nanocomposite electrode as the working electrode and counter electrode, respectively. All electrodes were screened on the same polyvinyl chloride substrate by using a DEK Horizon screen printer (Weymouth, United Kingdom)

2.2. Chemicals and reagents

Vitamin A, K₁ and K₂ were purchased from Sigma-Aldrich (Missouri, United States). Vitamin D and E were obtained from Supelco (Pennsylvania, United States). Sodium perchlorate (NaClO₄) was purchased from Fluka (Buchs, Switzerland). Ethanol was obtained from Carlo Erba (Val de Reuil, France). Potassium ferricyanide (K₃[Fe(CN)₆]), Potassium chloride (KCl) and Nafion were purchased from Sigma-Aldrich (Missouri, United States). Graphene, silver/silver chloride (Ag/AgCl) and polymer dielectric ink were obtained from Gwent group (Pontypool, United Kingdom). Polyvinyl chloride (PVC) substrate was purchased from Cadillac Plastic (Swindon, United Kingdom). The stock solutions of vitamin A (2.0 mM) and K₁ (2.0 mM) were prepared in ethanol and stored in dark at 2-8°C. The stock solutions of vitamin D (0.1 mM), E (1.0 mM) and K₂ (2.0 mM) were provided in ethanol and kept in dark under -20°C. The working solutions of fat-soluble vitamins were prepared in a mixture of supporting electrolyte containing ethanol and 0.1 M of sodium perchlorate in the ratio of 60:40% v/v. All other chemical reagents were analytical grade and were used without further purification. All solutions were prepared in Milli-Q water from a Millipore water purification system ($R \geq 18.2 \text{ M}\Omega \text{ cm}$).

2.3. Electrode fabrication

Screen-printed electrodes were fabricated with a DEK Horizon screen printer according to the previous describe [43]. For the fabrication steps, a mixture of graphene ink and Nafion was first screened on a PVC substrate as the first layer to form both the working electrode and counter electrode. Next, baking this screen-printed electrode at 60°C for 30 min to clear up all solvent from the electrode surface. Afterward, silver/silver chloride ink was screened onto the same substrate as the reference electrode and conductive pads. Then, baking it at 60°C for 30 min. After that, the last step was the layer screening of polymer dielectric ink as the insulator to define area of the working electrode. Finally, the screen-printed electrode was baked again at 80°C for 30 min.

2.4. Morphological study

Transmission electron microscopy (TEM) images were recorded by an H-7650 transmission electron microscope (Hitachi model, Japan). The surface morphologies were studied using a JSM-7610F field emission scanning electron microscope (FESEM) (JEOL Ltd., Japan).

2.5. Electrochemical measurement

In this work, a model CHI660D electrochemical analyzer was used for cyclic voltammetry (CV) and adsorptive square wave voltammetry (AdSWV). The screen-printed electrode was connected to this electrochemical analyzer for the measurement. In the measurement process, the standard/sample solution (50 μ L) was dropped onto the three-electrode area. For CV investigation, characterization studies of the electrode were carried out in the potential range from -1.0 V to 1.2 V with a scan rate of 100 mV s⁻¹. For AdSWV, the experiments were studied for the optimizations and recorded in potential range from 0.3 V to 1.5 V. The oxidation peaks of vitamin A, D, E and K occurred within this potential window and their currents were used for the simultaneous determination of vitamins concentrations. All experiments were performed at room temperature (298 K).

Electrochemical impedance spectroscopy (EIS) was carried out using an Autolab electrochemical system with a potentiostat PGSTAT 204 (Utrecht, Netherlands) in a solution of 5.0 mM K₃[Fe(CN)₆] with a sinusoidal potential modulation of 0.1 V amplitude in the 0.1 Hz to 100 kHz frequency range and a logarithmic scale of 10 points per decade.

2.6. Sample preparation

Details for a sample preparation have been presented in experimental section of supporting information.

3. Results and discussion

3.1. Electrochemical characterization of the screen-printed graphene nanocomposite electrodes

The electrochemical properties of the screen-printed graphene nanocomposite were initially investigated using CV. Cyclic voltammograms of 5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ in 0.1 M KCl at various screen-printed carbon electrodes (SPEs) are presented in the inset of Fig. 1A. As observed with graphene and the graphene nanocomposite (green dashed line and blue line, respectively), the magnitude of current response is far greater than that of the carbon SPE (red dashed line), which can be attributed to the excellent rate of electron transfer of the graphene material. In addition, in a background electrolyte (0.1 M KCl) (Fig. 1A), the background current recorded by the graphene nanocomposite was smaller than the currents measured using the other materials. This behavior is likely due to the background reduction capability of Nafion. Basically, Nafion comprises of three regions: the hydrophobic fluorocarbon backbone, the hydrophilic cluster of sulfonic acid group, and the interfacial region. These structures provide a strong charge exclusion, which prevents much of the redox-active interferents from affecting the sensor responses [44,45]. Based on the large faradaic current with respect to the small capacitive current, the signal-to-background (S/B) ratio can effectively be enhanced. The S/B ratios of the CV current for $\text{Fe}(\text{CN})_6^{3-/4-}$ reduction in 0.1 M KCl were exhibited in the following order: the graphene nanocomposite SPE (S/B = 126.20) > the graphene SPE (S/B = 90.48) > the carbon SPE (S/B = 15.05), as shown in Table 1. However, the peak separation of the carbon electrode is less than those modified electrodes. We believed that the additive substances added in the composition of the graphene ink might play a key role in a large peak separation for graphene-based electrodes.

For a better understanding, a background electrolyte was measured using CV at different scan rates to assess any change in the double-layer capacitance (C_{dl}) of different

SPEs. The background current originates from a charging of the double layer at the electrolyte/electrode interface and should be directly proportional to the total active surface exposed to the electrolyte [46]. As listed in the Table 1, the C_{dl} value for the bare carbon electrode was $23.32 \mu\text{F cm}^{-2}$ in 0.1 M KCl. For the graphene SPE, the C_{dl} value substantially decreased to $3.88 \mu\text{F cm}^{-2}$ in 0.1 M KCl system. Even though the capacitance for the carbon materials remains unclear, we believe that the oxygen functional group impurities presented on the graphite material and the charge density in a stacked layer of graphite in the carbon electrode might lead to a pseudocapacitive contribution, resulting in larger charge storage. In comparison, the graphene nanocomposite SPE also decreased the C_{dl} value to $3.14 \mu\text{F cm}^{-2}$ in 0.1 M KCl system. These results suggest that the decrease in the C_{dl} value of the graphene nanocomposite relative to the graphene SPE was related to a decrease in the electroactive surface area. This reduction in the C_{dl} value can possibly be due to the partial coverage of the active surface of graphene by Nafion. A similar behavior was also reported by Gayen et al., in which the coverage of boron-doped diamond (BDD) electrode by Nafion could further decrease the C_{dl} value compared to that of the bare BDD [47]. To confirm this issue, a further inspection of the electrode morphology and effective surface area will be investigated in the next section.

The electrochemical impedance characterizations of various SPEs were further investigated using EIS. Fig. 1B shows the Nyquist plots for 5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ in 0.1 M KCl. For the carbon SPE (blue dots), the plot shows a semicircle and a straight line with an angle of 45° , which indicates typical diffusion-controlled redox behavior at the planar electrode. A decrease in the semicircle radius was observed in the graphene SPE (orange dots), indicating a depletion in the charge transfer resistance (R_{ct}). This depletion is due to the superior electron transfer ability of graphene, which causes the apparent reduction in R_{ct} . However, when the graphene nanocomposite SPE was performed (green dots), the R_{ct} slightly

increased, which indicates the successful integration of Nafion into graphene. This phenomenon could be described by the Nafion itself, which may present an electrostatic repulsion blocking layer toward the electron exchange of $\text{Fe}(\text{CN})_6^{3-/4-}$ at the electrolyte/electrode interface [48]. Despite the considerable increase in R_{ct} , the selection of the electrode material may have been biased toward the graphene nanocomposite regarding the S/B ratio enhancement and the anti-fouling character of the electrode.

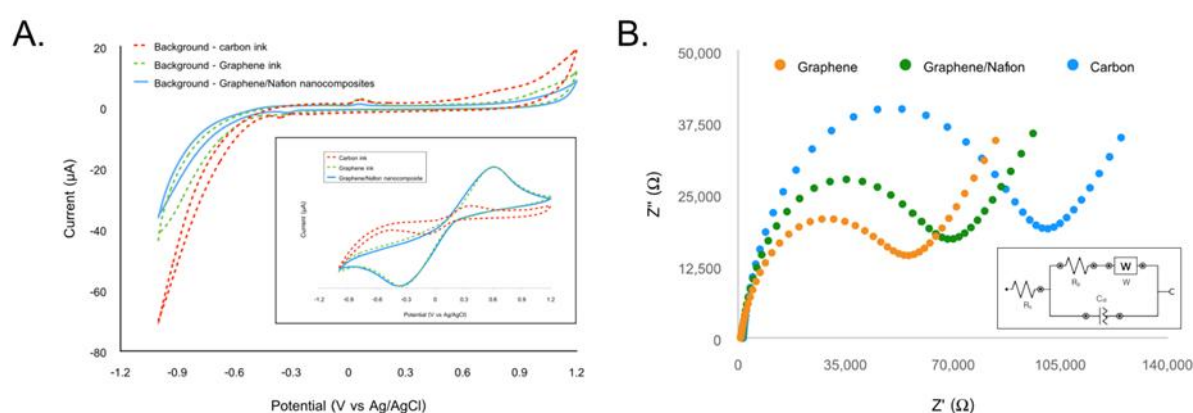


Fig. 1. (A) Cyclic voltammograms for 0.1 M KCl and 5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ (inset) using the screen-printed carbon (---), graphene (---), and graphene nanocomposite (—) electrodes. The potential sweep rate was 100 mV s⁻¹. (B) Nyquist plots for 5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ in 0.1 M KCl using the screen-printed (●) carbon, (●) graphene, and (●) graphene nanocomposite electrodes.

236

237 **Table 1.**

238 S/B ratio (estimated from the CV current for $\text{Fe}(\text{CN})_6^{3-/4-}$ reduction) and double layer
 239 capacitance in 0.1 M KCl and EtOH:NaClO₄ system at different SPEs.

Electrode materials	Potential (V vs Ag/AgCl)	Signal current (μA)	Background current (μA)	S/B ratio	Capacitance ($\mu\text{F cm}^{-2}$) (0.1 M KCl)	Capacitance ($\mu\text{F cm}^{-2}$) (60:40 EtOH: NaClO ₄)
Carbon	-0.096	-23.91	-1.589	15.05	23.32	208.30
Graphene	-0.331	-104.50	-1.155	90.48	3.88	40.89
Graphene nanocomposite	-0.353	-107.00	-0.848	126.20	3.14	24.81

240

241 3.2. Morphological characterization and effective surface area estimation

242 The morphologies of the three different electrodes, carbon, graphene and graphene
 243 nanocomposite, were characterized by SEM. In Fig. 2, panels **a** and **d** show SEM images of
 244 the carbon electrode at 5,000x and 10,000x amplification, respectively. A smooth textured
 245 region with the irregular shape of carbon was clearly seen over the surface of the SPE.
 246 Nevertheless, the SEM images of graphene, as displayed in panels **b** and **e** (5,000x and
 247 10,000x amplification, respectively), illustrated a rough and crumpled surface of the graphene
 248 sheets that majorly covered the surface, leading to a considerable sensitivity improvement
 249 over the carbon materials. With the addition of Nafion (panels **c** and **f** at 5,000x and 10,000x
 250 amplification, respectively), the prepared composite exhibited a smoother and more film-like
 251 texture than the entire graphene surface. The SEM images thus confirmed the
 252 aforementioned hypothesis, in which the nanocomposite possessed less surface area than the
 253 graphene electrode.

In Fig. 2, panels **g** and **h** show TEM images and the corresponding diffraction patterns of the graphene sheet, respectively. These image results further confirm the existence of graphene in the ink composition.

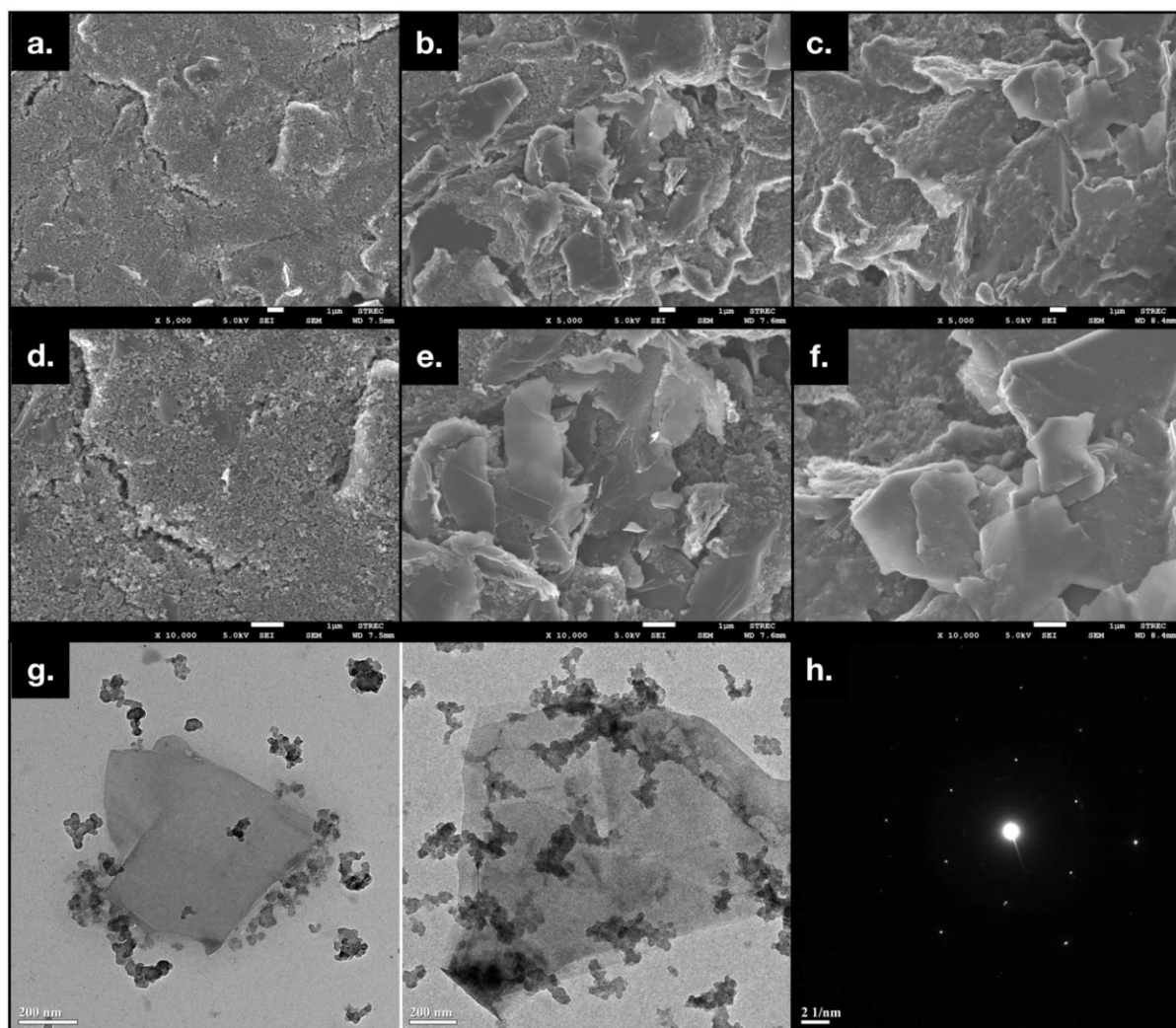


Fig. 2. SEM images of the screen-printed carbon (a, d), graphene (b, e), and graphene nanocomposite (c, f) electrodes at 5,000X (a, b, c) and 10,000X (d, e, f) magnification. TEM images (g) and corresponding diffraction pattern (h) of the graphene sheet in the ink composition.

Furthermore, to illustrate the effective background current reduction in the nanocomposite electrode, the electroactive surface areas of the studied SPEs were determined employing CV in a 1.0 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ solution at various scan rates (v). According to the

Randles-Sevcik equation ($i_{pa} = (2.69 \times 10^5)n^{2/3}AD_0^{1/2}\nu^{1/2}C_o^*$, where i_{pa} is the peak current in A, A is the area of the electrode in cm^2 , C_o^* is the concentration of the electroactive species in mM and D is the diffusion coefficient in $\text{cm}^2 \text{s}^{-1}$), the approximate electrochemically active surface areas of the carbon, graphene, and graphene composite SPEs were estimated (Fig. S1). The electroactive surface areas were calculated to be 0.191, 0.342, and 0.315 cm^2 for the carbon, graphene, and graphene nanocomposite screen-printed electrodes, respectively. Undoubtedly, a nearly two-fold enlargement in the active surface area promoted by graphene compared to the carbon electrode is important for enhancing the sensitivity of the graphene electrode. With the synergistic effect of Nafion, a further background current alleviation arising from a slighter active area was achieved. Some parts of the graphene electrode surface are expected to be covered with Nafion; therefore, the total surface area of the electrode should be less than that of the bare graphene electrode. Based on the experimental observations, these effective surface area results are consistent with the C_{dl} evaluation, SEM images, and background current measurements obtained from CV, whereby the graphene nanocomposite could synergistically enhance the sensitivity while reducing the charging current. Consequently, the graphene nanocomposite SPE could clearly enhance the sensitivity for fat-soluble vitamins determination.

3.3. Electrochemical characterization of vitamin D at different SPEs

For a better understanding, vitamin D was chosen as a fat-soluble model analyte for an electrochemical characterization of different SPEs. The electrochemical measurement of vitamin D at various electrode materials was carried out using CV within a potential range from 0.5 V to 0.9 V and a scan rate of 20 mV s^{-1} . In Fig. 3a-c, voltammograms at various concentrations of vitamin D at the carbon, graphene, and graphene nanocomposite SPEs are shown. A similar trend can be clearly observed in the case of $\text{Fe}(\text{CN})_6^{3-/4-}$ in 0.1 M KCl.

Note that the carbon SPE presents a small peak for only the high concentration of vitamin D ($10\text{ }\mu\text{g mL}^{-1}$), and no peak was observed at lower concentrations (Fig. 3a). It was suggested in earlier studies that vitamin D₂ and D₃ were likely undergoing oxidation at the common site of the triene moiety in their structure and appeared at the same oxidation potential. For graphene and the graphene/Nafion nanocomposite SPE (Fig. 3b and 3c, respectively), the CV curve of vitamin D show a single anodic peak, where the peak current is comparable. However, the background current response recorded in 60% v/v ethanol with the graphene nanocomposite SPE was inferior compared to the responses with the graphene and carbon SPEs (Fig. 3d); therefore, the S/B ratio with the graphene nanocomposite SPE was almost 2 and 4.5 times larger than the ratios with the graphene and carbon electrodes, respectively, at a vitamin D₃ concentration of $10\text{ }\mu\text{g mL}^{-1}$ (Fig. 3e).

In parallel with capacitance measurement performed in 0.1 M KCl, a background solution of ethanol and 0.1 M sodium perchlorate (60:40% v/v) was also utilized. The electrical double-layer capacitance was exhibited in the following order: the carbon SPE > the graphene SPE > the graphene nanocomposite SPE, which is consistent with C_{dl} value in the 0.1 M KCl system (Table 1). The results indicated that the nanocomposite electrode of graphene and Nafion could be useful for the high sensitivity determination of vitamin D, which is attributable to the larger faradaic current with respect to a lower charging current comparative to the values obtained from the carbon and graphene SPEs.

To further explain the electrode reaction of vitamin D at the graphene nanocomposite SPE, the influence of the potential scan rate (v) on a peak current (i_{pa}) of $10\text{ }\mu\text{g mL}^{-1}$ vitamin D₃ was studied by CV at various sweep rates ($40\text{--}200\text{ mV s}^{-1}$), as shown in Fig. S2. The oxidation peak current of vitamin D₃ increases with an increasing scan rate. Furthermore, a good linear relationship between i_{pa} and $v^{1/2}$ was established, indicating that the mass transport of vitamin D₃ is diffusion controlled.

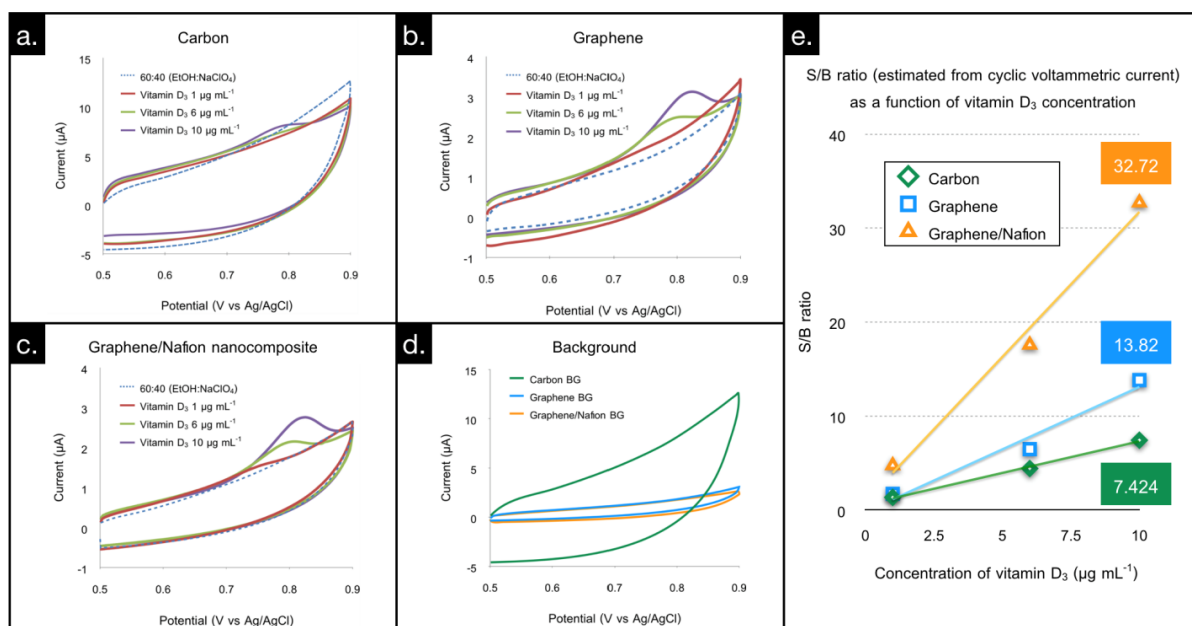


Fig. 3. Cyclic voltammograms for various concentrations of vitamin D₃ using the screen-printed (a) carbon, (b) graphene, and (c) graphene/Nafion nanocomposite electrodes. (d) Cyclic voltammograms for ethanol:NaClO₄ (60:40% v/v) background electrolyte using the screen-printed carbon (—), graphene (—), and graphene/Nafion nanocomposite (—) electrodes. The potential sweep rate was 20 mV s⁻¹. (e) S/B ratio (estimated from cyclic voltammetric current) for vitamin D detection as a function of the vitamin D₃ concentration.

3.4. Electrode fouling effect from the analysis of vitamin D

To investigate the fouling effect in the detection of fat-soluble vitamins, 10 µg mL⁻¹ vitamin D₃ in 60% v/v ethanol selected as a model analyte was consecutively analyzed using the same electrode with graphene, glassy carbon and graphene/Nafion nanocomposite electrode. For comparison, the peak height of the first run in each case was normalized to 100%. As shown in Fig. 4, the normalized current of the consecutive run was significantly elevated with the graphene electrode (orange line) and glassy carbon electrode (green line), leading to a high error in the measurement. Therefore, the repetitive usage of the electrode is

limited. Interestingly, a smaller fouling effect occurred with the graphene/Nafion nanocomposite electrode (blue line), which could be successively analyzed up to 10 times with a normalized current less than 105% (dash line). Note that a decrease in a surface area of the graphene nanocomposite plays a key role in anti-adsorption on the electrode surface by preventing macromolecules from entering inside and blocking the adsorption of debris [49]. This comparison indicates that the graphene nanocomposite offers an improvement in repeatability and robustness and is a good alternate material for the continuous monitoring of fat-soluble vitamins with no electrode fouling restrictions.

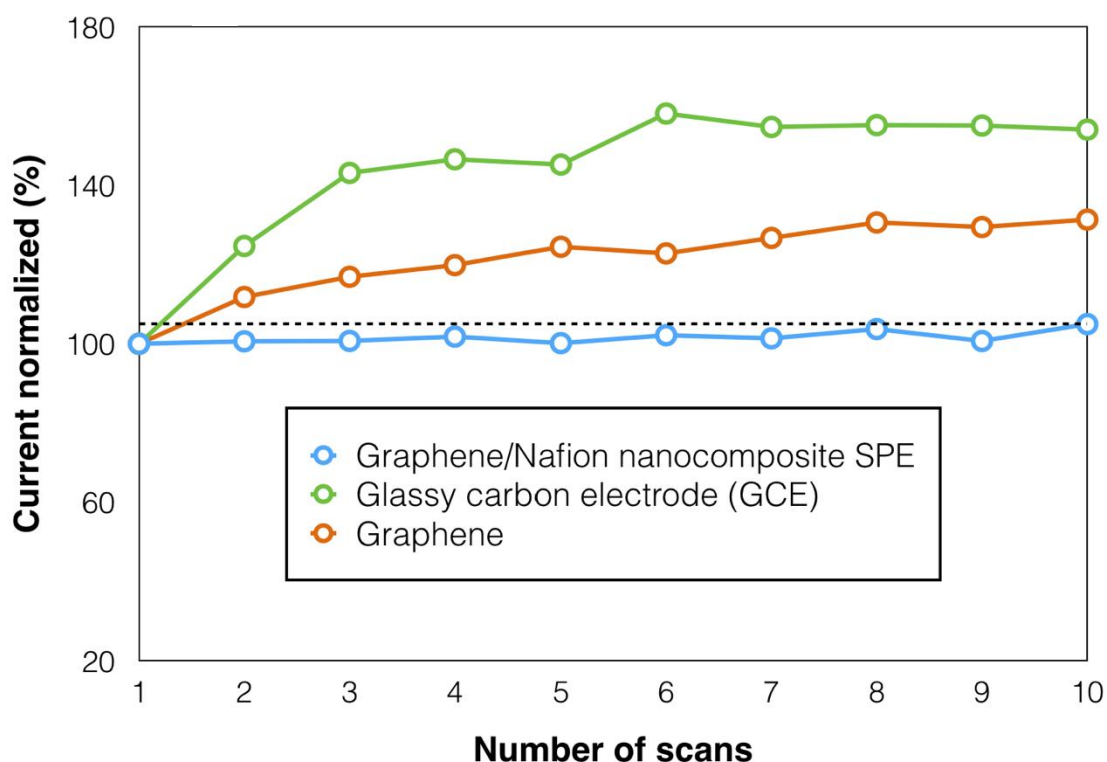


Fig.4. The plots between AdSWV voltammetric normalized current of $10 \mu\text{g mL}^{-1}$ vitamin D and number of successive usages of the (—) graphene, (—) glassy carbon and (—) graphene/Nafion nanocomposite electrodes. The first peak current in each case has been normalized to 100%.

3.5. Simultaneous electrochemical behavior of vitamin A, D, E and K

In order to accomplish the utilization of the proposed electrode to a further application of the simultaneous determination of four fat-soluble vitamins, the electrochemical behaviors of vitamin A, D, E and K in a mixture of ethanol and 0.1 M of sodium perchlorate (60:40% v/v) were investigated using the graphene nanocomposite SPE by AdSWV technique (data not shown). Fig. S3 displays the AdSWV voltammograms of 10 $\mu\text{g mL}^{-1}$ vitamin A, D, E and K in the potential window from 0.3 V to 1.5 V. The AdSWV voltammogram of a mixture of ethanol and 0.1 M of sodium perchlorate in the proportion of 60:40% v/v in the absence of fat-soluble vitamins (dashed line); this voltammogram not shows any peak. Moreover, the background current of the graphene nanocomposite SPE was found to be low value because of its low capacitance property. In contrast, when fat-soluble vitamins were added into the supporting electrolyte, as can be seen in the blue line, the oxidation peaks of these vitamins appeared at different peak potentials, which were agreed with the peak potentials of individual vitamins. These results indicated that vitamin A, D, E and K could be detected by using this proposed sensor. Furthermore, the unique properties of the graphene/Nafion nanocomposite SPE can enhance the oxidation signals of vitamin A, D, E and K. This result illustrates that the use of the graphene/Nafion nanocomposite SPE greatly improved the electrochemical response of vitamin A, D, E and K, suggesting that this electrode will be an excellent sensor for directly simultaneous determination of vitamin A, D, E and K. To obtain the best results, all experimental conditions that influence the experimental results such as the effect of chemicals and instrumental factors were next investigated. The results are reported in the following section.

In addition, to examine whether the mass transfer toward the graphene nanocomposite SPE is diffusion or adsorption controlled, the influence of the scan rate (ν) on the peak

current (i_{pa}) was investigated using CV. It was found that the anodic peak currents of vitamin A, D, E and K showed a good linear dependence ($R^2 = 0.99$) with the square root of the scan rate (data not shown). This behavior clearly demonstrates that the electrochemical oxidation of vitamin A, D, E and K at the graphene nanocomposite SPE is a diffusion-controlled process.

3.6. Optimization of electrochemical parameters

For the selection of the electroanalytical methodology and optimization of parameters, the electrochemical conditions such as the effect of the proportion of ethanol, potential increment, amplitude, frequency and quiet time were investigated. These parameters were a crucial factor for the sensitivity of the proposed method. The electrochemical oxidation of $10 \mu\text{g mL}^{-1}$ vitamin A, D, E and K has been studied by AdSWV. For these measurements, the examined parameter was changed while others were kept constant. The effect of potential increment was studied individually over the potential range from 0.002 V to 0.018 V. The effect of quiet time is one important parameter which can affect the sensitivity of fat-soluble vitamins detection. For the effect of quiet time, we examined in the range from 10 s to 500 s. The optimizations of amplitude and frequency were carried out in the range from 0.005 V to 0.040 V and 5 Hz to 120 Hz, respectively. The effect of the proportion of ethanol was studied in the range from 20% to 80%. The criteria for selecting the optimal conditions are not only provided the highest peak current but also well-separated voltammogram of fat-soluble vitamins for the simultaneous detection. After the measurements have been already completed, the optimal conditions were achieved to use the proportion of ethanol of 60%, potential increment of 0.010 V, amplitude of 0.040 V, frequency of 100 Hz, and quiet time of 300 s.

3.7. Analytical performance

Under optimum conditions, the simultaneous determination of vitamin A, D, E and K was estimated by AdSWV with the graphene nanocomposite SPE to verify the analytical performance. (Fig. 5A). The calibration curves plotted between the anodic peak currents and different concentrations of each fat-soluble vitamin are relative in the range from 0.02 $\mu\text{g mL}^{-1}$ to 1 $\mu\text{g mL}^{-1}$. The anodic peak currents increased linearly as the concentrations of fat-soluble vitamins (inset of Fig. 5A). Typical calibration curves provide the regression equations of $y = 3.4371x + 0.2354$ for vitamin A, $y = 3.565x + 0.1716$ for vitamin D, $y = 3.0195x + 0.1298$ for vitamin E and $y = 3.7537x + 0.1512$ for vitamin K. The limits of detection (LODs) and limits of quantification (LOQs) of each fat-soluble vitamin were calculated from $3S_{bl}/S$ to $10S_{bl}/S$ (where S_{bl} is the standard deviation of the blank measurement ($n = 7$) and S is the sensitivity of the method), and are summarized in Table 2. Compared to previously published methods, particularly, in the field of electrochemical analysis, this proposed method not only simultaneously measured fat-soluble vitamins, but also provided a low detection limits. The benefits of this work were not only a simple procedure, effective cost, small sample volume, disposability and short-time analysis but also offered a highly sensitive potential to apply for the simultaneous determination of fat-soluble vitamins in real samples.

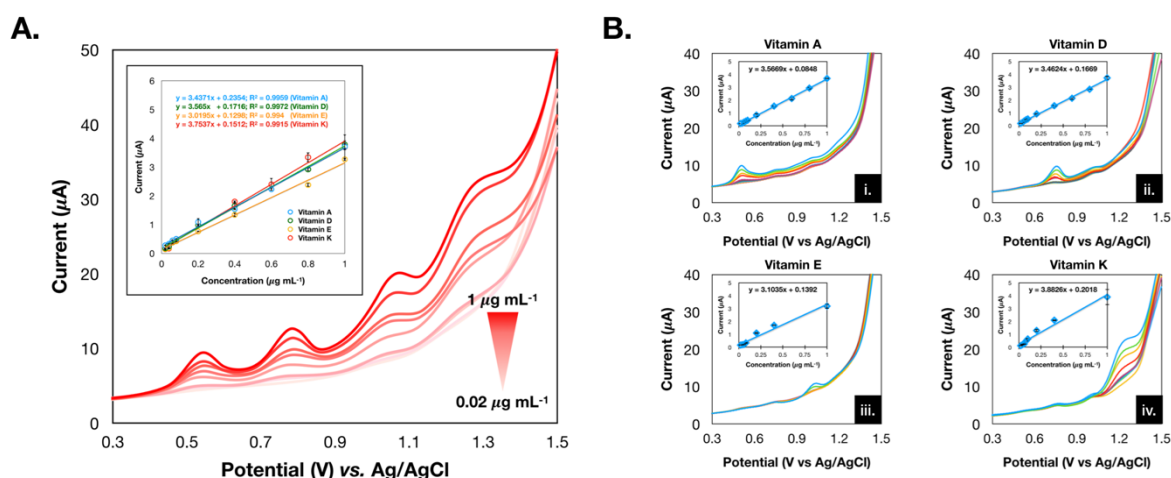


Fig. 5. (A) AdSWV voltammograms showing the electrochemical oxidation of vitamin A, D, E and K and the representative calibration graph (inset) between the anodic peak current (μA) and concentration of vitamin A, D, E and K ($0.02 - 1 \mu\text{g mL}^{-1}$). (B) AdSWV voltammograms of different concentrations of (i) vitamin A in the presence of vitamin D, E, K, each $0.10 \mu\text{g mL}^{-1}$, (ii) vitamin D in the presence of vitamin A, E, K, each $0.10 \mu\text{g mL}^{-1}$, (iii) vitamin E in the presence of vitamin A, D, K, each $0.10 \mu\text{g mL}^{-1}$, and (iv) vitamin K in the presence of vitamin A, D, E, each $0.10 \mu\text{g mL}^{-1}$. Inset: the plots between peak currents and concentrations of corresponding vitamins.

The precision of the analytical procedure was investigated by determining the relative standard deviations (RSDs) for ten replicated measurements of solutions that contained the complete set of standard compounds. To assess the repeatability and reproducibility of the proposed sensor, three concentrations (0.06 , 0.20 and $0.80 \mu\text{g mL}^{-1}$) with respect to the probable range of interest samples were studied ($n=5$). The RSDs of repeatability were found to vary over the ranges of 7.8 - 11.8% for vitamin A, 3.8 - 9.3% for vitamin D, 9.3 - 11.2% for vitamin E and 3.0 - 9.2% for vitamin K, respectively, while the RSDs of reproducibility varied over the range of 3.8 - 11.7% for vitamin A, 3.4 - 19.2% for vitamin D, 11.4 - 16.3% for vitamin E and 5.5 - 15.9% for vitamin K, respectively, which were in acceptable ranges by AOAC guideline.

Table 2.

Linearity, limits of detection (LODs) and limits of quantification (LOQs) of the proposed method using the graphene nanocomposite SPE ($n=3$).

Analytes	Linear range	Slope	Intercept	r^2	LODs	LOQs
	($\mu\text{g mL}^{-1}$)	($\mu\text{A}/\mu\text{g mL}^{-1}$)	(μA)		($\mu\text{g mL}^{-1}$)	($\mu\text{g mL}^{-1}$)

Vitamin A	0.02-1	3.4371	0.2354	0.9959	0.0086	0.0286
Vitamin D	0.02-1	3.5650	0.1716	0.9972	0.0063	0.0211
Vitamin E	0.02-1	3.0195	0.1298	0.9940	0.0075	0.0249
Vitamin K	0.02-1	3.7537	0.1512	0.9915	0.0071	0.0238

3.8. Sensitivity

The effect of various concentrations of vitamin A, D, E and K was studied by varying the concentrations in the range of 0.02-1 $\mu\text{g mL}^{-1}$ of only one standard solution of fat-soluble vitamins while the concentrations of other fat-soluble vitamins were kept constant at 0.10 $\mu\text{g mL}^{-1}$ (Fig. 5B). The peak currents present a good linearity with consecutive introduction of each compound without influencing the others. As evident in the plots, the slopes of fat-soluble vitamins were consistent with linearity study from the simultaneous measurement of fat-soluble vitamins. Therefore, these results confirmed that the proposed sensor offers excellent potential for the simultaneous determination of vitamin A, D, E and K in real samples.

3.9. Analytical application

To verify the applicability of the graphene nanocomposite SPE and the proposed method, the fabricated electrode was used for the simultaneous determination of vitamin A, D, E and K in different matrix samples using AdSWV technique. Infant milk, yogurt and parsley from local supermarket were selected to investigate standard addition. For accuracy and precision of this method, the percentages of recoveries and RSDs were examined by spiking three concentrations of 0.1, 0.2 and 0.4 $\mu\text{g mL}^{-1}$, respectively. The recoveries and RSDs were found to be in acceptable ranges by AOAC guideline. Additionally, the results

obtained from our proposed method were compared to those obtained from HPLC-UV as shown in Table 3. Using the paired *t*-test at a 95% confidence interval, the paired two-tail test showed calculated *t*-values 0.080, 0.035, 0.038 below the critical *t*-value 2.07. These results indicate that the proposed method is acceptable and reliable for the determination of vitamin A, D, E and K in different matrix samples. Therefore, this developed sensor provided high accuracy and precision that can be used as an alternative choice for quality control of fat-soluble vitamins in different matrix samples.

Table 3.

Results obtained from the simultaneous determination of fat-soluble vitamins in different matrix samples.

Samples	Analytes	Added ($\mu\text{g mL}^{-1}$)	Mean of founded ($\mu\text{g mL}^{-1}$) (n=3)		Recovery (%)		RSD (%)	
			Present method	HPLC-UV	Present method	HPLC- UV	Present method	HPLC- UV
Infant milk	VA	0.1	0.1022	0.1017	102.2	101.7	2.4	2.2
	VD	0.1	0.1032	0.1012	103.2	101.2	2.2	1.9
	VE	0.1	0.0998	0.1001	99.8	100.1	2.7	2.0
	VK	0.1	0.1001	0.0999	100.1	99.9	1.9	1.7
	VA	0.2	0.2050	0.2022	102.5	101.1	1.8	1.3
	VD	0.2	0.2100	0.2046	105.0	102.3	2.3	1.7
	VE	0.2	0.1986	0.2006	99.3	100.3	2.6	2.1
	VK	0.2	0.2150	0.2046	107.5	102.3	1.6	1.5
	VA	0.4	0.4252	0.4068	106.3	101.7	1.2	1.1
	VD	0.4	0.4232	0.4128	105.8	103.2	1.6	1.4
	VE	0.4	0.4220	0.4180	105.5	104.5	1.6	1.3
	VK	0.4	0.4252	0.4224	106.3	105.6	1.2	1.2
Yogurt	VA	0.1	0.0997	0.1001	99.7	100.1	1.5	1.3
	VD	0.1	0.0995	0.1001	99.5	100.1	1.3	1.1
	VE	0.1	0.1025	0.1013	102.5	101.3	2.2	1.9
	VK	0.1	0.1038	0.1018	103.8	101.8	3.1	2.7
	VA	0.2	0.2100	0.2076	105.0	103.8	2.9	2.6
	VD	0.2	0.2050	0.2018	102.5	100.9	2.2	2.1
	VE	0.2	0.2050	0.2016	102.5	100.8	2.9	2.8
	VK	0.2	0.2090	0.2058	104.5	102.9	3.0	2.8
	VA	0.4	0.4072	0.4028	101.8	100.7	1.9	1.7
	VD	0.4	0.4028	0.4012	100.7	100.3	2.2	2.0
	VE	0.4	0.3988	0.4004	99.7	100.1	2.3	2.2
	VK	0.4	0.4072	0.4036	101.8	100.9	2.1	2.0

Parsley	VA	0.1	0.1050	0.1023	105.0	102.3	3.1	2.9
	VD	0.1	0.1027	0.1019	102.7	101.9	3.2	2.8
	VE	0.1	0.1035	0.1026	103.5	102.6	2.8	2.6
	VK	0.1	0.0998	0.1005	99.8	100.5	3.6	3.4
	VA	0.2	0.2010	0.2004	100.5	100.2	2.6	2.4
	VD	0.2	0.2150	0.2074	107.5	103.7	3.2	3.0
	VE	0.2	0.2036	0.2018	101.8	100.9	2.5	2.7
	VK	0.2	0.2020	0.2018	101.0	100.9	2.6	2.5
	VA	0.4	0.4008	0.4004	100.2	100.1	1.6	1.5
	VD	0.4	0.3996	0.4004	99.9	100.1	2.2	2.0
	VE	0.4	0.4048	0.4020	101.2	100.5	1.9	1.7
	VK	0.4	0.4136	0.4056	103.4	101.4	1.6	1.8

460

461 4. Conclusion

462 Application of using a simple and rapid method with highly sensitive and selective
463 electrochemical sensor for the simultaneous determination of vitamin A, D, E and K using the
464 graphene nanocomposite SPE was successfully demonstrated. Furthermore, no
465 chromatographic separation technique is needed to isolate compounds before analysis and
466 can be directly applied for the simultaneous determination of vitamin A, D, E and K in
467 different matrix samples with good selectivity. The nanocomposite between graphene and
468 Nafion can synergistically enhance the anodic peak currents of each fat-soluble vitamin at
469 different potentials. Under optimum conditions, the simultaneous determination of fat-
470 soluble vitamins was received with a wide linear range ($0.02\text{-}1\text{ }\mu\text{g mL}^{-1}$) and low detection
471 limits. The methodology was sensitive, selective, accurate and precise for different matrix in
472 real samples, and results were in good agreement with the HPLC-UV. Therefore, this
473 developed sensor provides a high capability as an alternative choice for quality control and
474 quantitative analysis of fat-soluble vitamins in the pharmaceutical and food industry.

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☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



NJC

The effect of supporting electrolyte for the simultaneous determination of vitamin B₂, vitamin B₆, and vitamin C using a modification-free screen-printed carbon electrode

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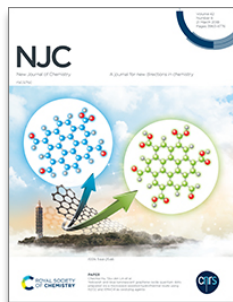
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30 April 2020

Dear Editor,

Please accept the submission of this manuscript entitled “**The effect of supporting electrolyte for the simultaneous determination of vitamin B₂, vitamin B₆, and vitamin C using a modification-free screen-printed carbon electrode**” for consideration for possible publication in the original research paper section of *New Journal of Chemistry*. In this work, we proposed a new choice by studying the effect of supporting electrolyte system and pH for increasing the performance of simultaneous detection instead of using modified electrode. Owing to a supporting electrolyte is a necessary solution to preserve the conductivity and ionic strength in the electrochemical system. We used vitamins as sample model because they are essential nutrients for human body which play important roles in various biological functions. Deficiencies in vitamins can be initiated by various factors and diseases, therefore, the development of a simple and cost-effective analytical procedure for the determination of vitamins is considerably significant and fascinating. This paper first proposed a modification-free screen-printed carbon electrode for the simultaneous determination of vitamin B₂, vitamin B₆, and vitamin C using a transparent-film based analytical device as an electrochemical platform. Also, the effect of supporting electrolyte on these vitamins was successively investigated to compensate a complex surface modification. We believe the readers of *New Journal of Chemistry* will be captivated in this manuscript because of its impact on generating simple and easy way for the analysis in various practical applications. In addition, high performance and the results obtained are in good agreement to those from a conventional method. Do not hesitate to contact me if you have any questions.

We are looking forward for your valuable comments/suggestions.

Yours sincerely

Weena Siangproh, Ph.D.

The effect of supporting electrolyte for the simultaneous determination of vitamin B₂, vitamin B₆, and vitamin C using a modification-free screen-printed carbon electrode

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Abstract

To open a new choice and to replace the complicated steps of a modified electrode, this research reported on the systematic investigation of the type of supporting electrolyte that affected to the electrochemical behavior of vitamin B₂ (VB₂), vitamin B₆ (VB₆), and vitamin C (VC) using a common screen-printed carbon electrode (SPCE) to obtain the simultaneous detection. The role of various well-known supporting electrolytes, including Britton-Robinson buffer solution (BRBS), acetate buffer solution (ABS), and phosphate buffer solution (PBS), on the electrochemical oxidation of VB₂, VB₆, and VC were carefully accomplished by square wave voltammetry (SWV). From this successive investigation based on the ionic conductivity of species containing in a supporting electrolyte, PBS at pH 3.5 was selected as a suitable supporting electrolyte for the simultaneous detection of VB₂, VB₆, and VC. Under the optimized conditions, the simultaneous detection of VB₂, VB₆, and VC provided satisfactory sensitivity, selectivity, and reproducibility despite using a common electrode. To demonstrate the practicality and reliability of the proposed analytical procedure, the simultaneous detection of VB₂, VB₆, and VC was performed in mixed vegetable and fruit juice and urine sample by SWV and the results were compared with chromatographic detection. Therefore, this finding is simple and could be a new alternative way for the simultaneous determination of VB₂, VB₆, and VC in practical applications.

Keywords: Supporting electrolyte; Simultaneous determination; Screen-printed electrode; Electrochemical transparent film-based analytical devices; Square wave voltammetry

1. Introduction

A supporting electrolyte (inert electrolyte or inactive electrolyte) is a necessary solution to preserve the conductivity and ionic strength in the electrochemical system. Chemical species containing in a supporting electrolyte are required to have a non-electroactive property within the applied potential window and their conductivities are considerably larger than those of the electroactive species or analytes.¹ The essential roles of a supporting electrolyte are to minimize the resistance of an electrochemical cell to eradicate the transportation of electrochemical species caused by migration effect in the electric field and to constrain the interfacial potential difference to the closest distance approach of solvated ions to the electrode.^{2,3} These phenomena will diminish uncompensated iR drop, which causes the potential-control error and the ohmic heating of a solution to acquire a factual potential measurement.⁴

Normally, the concentration of ionic species in a supporting electrolyte is at least 50-100 times of the electroactive species and is the significant source of electrically conducting ionic species.⁵ It can be inorganic or organic salt, an acid or a base, a buffer solution such as acetate, carbonate, and phosphate. Among these supporting electrolytes, buffer solutions are tremendously consumed in the electrochemical system because of the ability for preservation pH at a virtually constant value. With the advantageous property of buffer solutions, numerous reports in an electroanalytical application select the buffer solutions serving as supporting electrolytes for the detection of interesting electroactive species, especially for vitamin detection.⁶⁻⁸

Vitamins are a disparate group of compounds which are essential nutrients for human.⁹ They are classified into 2 groups by their solubilities, which are water-soluble vitamins (vitamin B and vitamin C) and fat-soluble vitamins (vitamin A, vitamin D, vitamin E, and vitamin K). They play important roles in various biological functions. Vitamin B₂ (VB₂) is an important component of a flavoenzyme that catalyzes a variety of biochemical reactions of carbohydrates, protein, and fat in the human body. It promotes the growth process and

reproductive system; strengthens the visibility and relieves the fatigue of eyes; and prevents inflammation of the mouth, lips, and tongue.¹⁰ Vitamin B₆ (VB₆) involves many aspects of macronutrient metabolism, histamine synthesis, hemoglobin synthesis, and gene expression. It participates in activities of many enzymes and maintenance of skin as well as nervous and immune systems.¹¹ Vitamin C (VC) is an antioxidant that is necessary for the growth, development, and repairing of all tissues. It is involved in many functions of the human body which are the formation of collagen, the absorption of iron, the immune system, the wound healing, and the maintenance of cartilage, bones, and teeth.¹² These mentioned water-soluble vitamins have to receive daily from food. Moreover, they are not stored in the human body because of the urinary excretion. Therefore, the determination of vitamins in real samples is important for various applications to monitor the quantities of vitamins in consuming products and to indicate abnormalities or diseases.

Such the importance of those mentioned vitamins, various analytical methods were developed and proposed for the determination of VB₂, VB₆, and VC in both individual and simultaneous measurements including high-performance liquid chromatography,¹³ spectrophotometry,¹⁴ chemiluminescence,¹⁵ and electrophoresis.¹⁶ Unfortunately, these techniques still had some limitations despite their high sensitivity and selectivity for detection. For example, they were time-consuming, high cost for maintenance, complicated operation, required a large volume of solution and had several pre-concentration steps. Consequently, the other novel methodology has to explicate successively for VB₂, VB₆, and VC detection to achieve the objective of simple practicality in various field analysis.

For twenty-first-century analytical experimentation, the electrochemical method is another choice for the simultaneous determination of VB₂, VB₆, and VC due to its simplicity for operation, rapid results, direct measurement, high sensitivity and selectivity. From previous electrochemical researches,¹⁷⁻²⁷ the detection of VB₂, VB₆, and VC was determined principally by various modified electrodes which provided high precision and high sensitivity. However, they were not suitable for some applications because of some disadvantages, such as high-cost electrodes, take a long time for modification with complicated steps, and require a

lot of solution volume for analysis. The most importance is that it is hard to hit the goal for field analysis and also suffered from surface modification. Therefore, the selection of a suitable, simple, and inexpensive electrode platform is currently significant for the application in real sample analysis to increase the performance of the analytical method.

Nowadays, the furtherance of screen-printing technology for the fabrication of screen-printed electrodes (SPEs) have been enormously attracted the attention due to their advantageous properties over conventional electrodes,²⁸ such as cost-effectiveness, disposability, simplicity, versatility, availability in materials and patterns, elimination of electrode maintenance, low volume of solution requirement, and appropriate for outside laboratory measurement. For these reasons, these devices were applied tremendously for electrochemical analysis in environmental monitoring,²⁹ clinical diagnoses,³⁰ and food safety assessment.³¹ Various reports demonstrated that the electrochemical devices were fabricated generally on paper as a substrate,³²⁻³⁴ so-called electrochemical paper-based analytical devices (ePAD). The main advantages of ePAD are simple, cost-effective, portable, and disposable.³⁵ However, apart from paper, other substrates are additionally consumed as platforms for the fabrication of SPEs, such as acrylic, ceramic, and PVC sheet. To ascertain the different platform for the development of an electrochemical device, commercial plastic sheets (OHP films) are recently employed to fabricate an electrode platform, named transparent film-based analytical device (TFAD).³⁶ Because of their compatibility with screen-printing technology, easily available in a local stationery store and low cost, therefore, they are considerably noticeable for the fabrication of screen-printed electrodes.

Herein, the main objective of this research was to focus on the effect of supporting electrolyte on the simultaneous detection of VB₂, VB₆, and VC using a common screen-printed carbon electrode (SPCE) to obtain a simple way for detection and to overcome the cost of analysis as well as the complex modification from using various modern materials. Also, we proposed the manually fabricated of SPCE using a transparent-film substrate, named electrochemical transparent-film based analytical device (eTFAD), which was commercially available in a local stationery store. Under the optimal conditions, the overall electrochemical

experiment was rapid, convenient, and uncomplicated in comparison to those obtained from previous works. Besides, the proposed procedure was sensitive and selective for the determination of those amounts of selected vitamin presence in real samples. For the various practical applications, the developed electrochemical electrode was successfully applied for the determination of mentioned vitamins in a mixed vegetable and fruit juice, and an artificial urine sample to demonstrate the reliability of the proposed analytical system. The results obtained show the effectiveness of method for monitoring the quantities of vitamins in consuming products or using for indicating the abnormalities and diseases. This finding could be an alternative way to open the new vision for application obtained from a basic concept and to encourage the researcher for creating their work by using their basic instruments to get rid of the limitations.

2. Experimental

2.1. Chemicals and reagents

All chemicals were used as received without further purification. Carbon paste (C2130307D1) and silver/silver chloride paste (C2130809D5) were purchased from Gwent Group (Torfaen, United Kingdom). Riboflavin (vitamin B₂, VB₂) and L-ascorbic acid (vitamin C, VC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pyridoxine hydrochloride (vitamin B₆, VB₆) was purchased from Hi-Media Laboratories Pvt., Ltd. (India). Sodium dihydrogen orthophosphate (NaH₂PO₄.2H₂O) and di-Sodium hydrogen orthophosphate (Na₂HPO₄.2H₂O) were acquired from Ajax Finechem Pty., Ltd. (New South Wales, Australia). Ethanol and ortho-phosphoric acid (H₃PO₄, 85% purity) were acquired from Merck (Darmstadt, Germany). Acetone (commercial grade) was purchased from CaHC (Thailand) Co., Ltd. (Bangkok, Thailand).

All solutions were prepared using Milli-Q water from a Millipore water purification system (R = 18.2 MΩ cm at 25 °C). All of stock standard solutions (1 mM) were weekly prepared in water and kept in the dark at -20 °C. As for working standard solutions

preparation, the stock standard solution was diluted with supporting electrolyte and mixed using a vortex mixer. Phosphate buffer solution (PBS), which was served as a supporting electrolyte, was prepared by mixing 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4 and adjusted to the desired pH with 0.1 M H_3PO_4 . Britton-Robinson buffer solution (BRBS) was prepared by mixing equal volumes of 0.04 M CH_3COOH , 0.04 M H_3BO_3 and 0.04 M H_3PO_4 that has been adjusted to the desired pH with 0.2 M NaOH. Acetate buffer solution (ABS) was also prepared by mixing 0.1 M CH_3COOH and 0.1 M CH_3COONa and adjusted to the desired pH with 0.1 M HCl or 0.1 M NaOH.

2.2. Fabrication of electrochemical transparent-film based analytical device (eTFAD)

The electrode pattern was designed using Adobe Illustrator CC 2018. To obtain the electrode blocks for screening, the designed patterns were built by Chaiyaboon Brothers Co., Ltd. (Bangkok, Thailand). Screen-printed electrodes were fabricated by using a manual screen-printing method on the transparent sheet as a substrate which was described in the previous report.³⁷ Briefly, the clean transparent sheet was screened by carbon paste to obtain working electrodes and counter electrodes. Then, these electrodes were placed in an oven at a temperature of 55 °C for 30 min to remove the solvent and to dry electrodes. Next, silver/silver chloride (Ag/AgCl) paste was screened on the same transparent sheet to obtain reference electrodes and the conductive pads. After that, they were placed in an oven at a temperature of 55 °C for 30 min. Finally, the common screen-printed carbon electrodes (SPCE) were obtained and ready to use for an experiment. The picture of the obtained eTFAD was shown in Fig. 1.

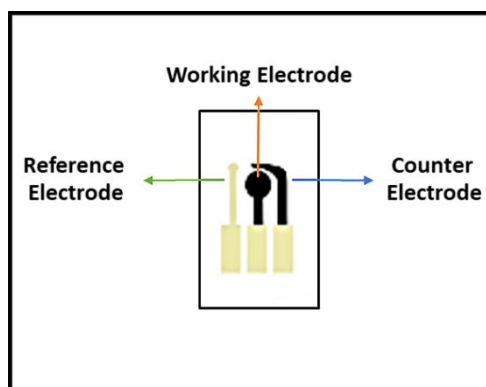


Fig. 1. A picture of electrochemical transparent film-based analytical device (eTFAD) consisting of screen-printed working, reference, and counter electrodes.

2.3. Electrochemical measurement

The electrochemical experiments were performed by potentiostat/galvanostat PGSTAT128N from Metrohm-Autolab (Switzerland) with the NOVA 1.10 software (Kanaalweg 29-G 3526 KM Utrecht, The Netherlands). A three-electrode system was fabricated on a transparent sheet which was employed throughout an experiment. The geometric area of a working electrode was 0.126 cm^2 (calculated from circular area = πr^2 ; $r = 0.2 \text{ cm}$). For the electrochemical measurement, $100 \text{ }\mu\text{L}$ aliquot of a standard or a sample solution was thoroughly dropped covering all three electrodes onto the detection zone. After that, electrochemical techniques including cyclic voltammetry (CV) or square wave voltammetry (SWV) were conducted. For CV measurement, the potential was applied over the range from -1.1 V to 1.3 V with a scan rate of 100 mV/s . Similarly, SWV measurement was also performed for the simultaneously quantitative determination by applying the potential from -1.0 V to 1.3 V with a step potential of 5 mV , an amplitude of 25 mV , and a frequency of 5 Hz .

2.4. Sample preparation

To demonstrate the probability of the proposed electrode for application, two types of samples including mixed vegetable and fruit juice and artificial urine were investigated for the simultaneous determination of water-soluble vitamins.

The mixed vegetable and fruit juice were purchased from a convenience store in Thailand. 5 mL of juice sample was added into a test tube. Then, the sample was centrifuged for 15 minutes at 2500 rpm followed by filtration through a filter paper and kept in a dark container at 4°C. After that, samples were spiked at various concentrations of standard vitamins and the spiked solution was diluted 10-fold with PBS pH 3.5 (a suitable dilution order) and measured the quantity using the SWV technique as followed by the topic 2.3. Then, the average recovery percentage was calculated.

Artificial urine was purchased from Carolina Biological Supply Company (Burlington, USA). For the sample preparation, the artificial urine and spiked artificial urine were merely diluted 1000-fold with PBS pH 3.5 which is a suitable dilution order for this sample. After that, the samples and spiked samples were quantitatively measured by using the same procedure to mixed vegetable and fruit juice.

3. Results and discussions

3.1. Electrochemical behavior for the simultaneous detection of VB₂, VB₆, and VC

Cyclic voltammetry was initially used to investigate the electrochemical properties and the possibility of simultaneous detection of VB₂, VB₆, and VC. To examine the oxidation or reduction mechanism of selected vitamins on a common SPCE surface, cyclic voltammograms of VB₂, VB₆, and VC in Britton-Robinson buffer solution (BRBS) at pH 7 were recorded at a scan rate of 100 mV/s. The obtained voltammograms from both individual and simultaneous detection were shown in Fig. 2. The cyclic voltammogram of VB₂ shown in Fig. 2A exhibited a pair of sharp and well-behaved redox peaks at -0.60 V and -0.75 V for anodic and cathodic peaks, respectively. This result revealed that VB₂ could display a reversible property because

of the ratio between anodic and cathodic current is around 1.0. However, the separation of anodic and cathodic peak potential is around 0.15 V which is greater than $0.059/n$ V of the reversible process (n is a number of electron transfer). The result might be attributed to the fact that VB₂ provided a slow rate for the electron transfer reaction.³⁸ The cyclic voltammogram of VC depicted in Fig. 2B displayed a sharp and strong oxidation peak at 0.35 V with a peak current of 27.65 μ A. This result indicated that VC occurs an irreversible process at an electrode surface. Similarly, VB₆ also occurs an irreversible process due to the presence of a small and broad oxidation peak at 0.82 V as demonstrated in Fig. 2C. From these results, it could be concluded that the possibility for the simultaneous detection of VB₂, VB₆, and VC can be performed on the proposed analytical electrode because of the explicit distinction of peak potential position.

Subsequently, the simultaneous detection of VB₂, VC, and VB₆ was also investigated as shown in Fig. 2D. From the voltammogram, VB₂ exhibited a pair of outstanding redox peaks at -0.48 V and -0.61 V for anodic and cathodic peaks, respectively. Meanwhile, VC and VB₆ provided a broad anodic peak at 0.41 V and 0.90 V, respectively. The obtained voltammogram confirmed that the simultaneous detection of VB₂, VC, and VB₆ in oxidative direction is possible owing to the distinction of peak potential position as same as anticipated results obtained from the individual detection. The discovered results are in good agreement with the previous critical review of all vitamins,³⁹ therefore, the simultaneous detection of VB₂, VC, and VB₆ can be performed using a common SPCE.

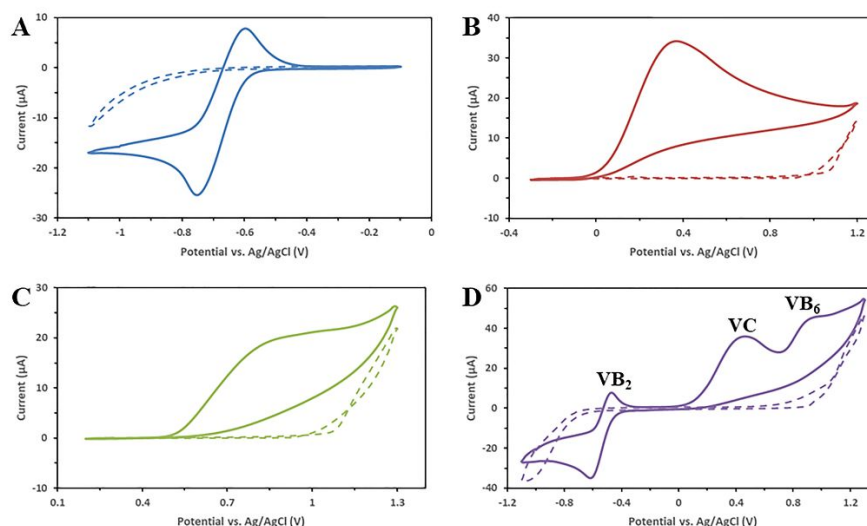


Fig. 2. Cyclic voltammograms obtained from common SPCEs in absence (dash line) and presence (solid line) of 1 mM VB₂ (A), 1 mM VC (B), 1 mM VB₆ (C), and the mixture solution of 1 mM VB₂, 1 mM VC, and 1 mM VB₆ (D). BRBS pH 7 was served as a supporting electrolyte. All cyclic voltammograms were recorded at a scan rate of 100 mV/s.

3.2. Influence of pH and supporting electrolyte on the electrooxidation of VB₂, VC, and VB₆

The effects from adjusting the pH values of supporting electrolyte on the oxidative behavior of VB₂, VC, and VB₆ disclose a significant information about the mechanisms of electrochemical reactions at a common SPCE. Therefore, the influences of pH and supporting electrolyte on the electrooxidation of VB₂, VC, and VB₆ were carefully investigated by square wave voltammetry. The suitable pH conditions were preliminarily studied using BRBS at pH 5, 7, and 9 as supporting electrolytes. The obtained voltammograms from the simultaneous detection were shown in Fig. S1. With increasing pH value, the anodic currents of VB₂, VC, and VB₆ were decreased obviously. We believed that in our case, the acidic supporting electrolyte would strongly affect to a higher current. By considering the pK_a values of each vitamin, the results might be attributed to the fact that ionic forms of target analytes affect the electrooxidation processes at the electrode surface. According to data obtained from the

literature,⁴⁰ the pK_a values of each vitamins are the following: $VB_2 = -0.2$ and 6.0 , $VC = 4.2$, and $VB_6 = 5.6$ and 9.4 . These values indicated that the three compounds can exist in their ionic forms in aqueous solutions. Fig. S2 shows the charge versus pH curves (CurTiPot software)⁴¹ for VB_2 , VC , and VB_6 molecules. As can be observed, VB_2 and VB_6 are amphoteric molecules ($pI = 2.9$ and 7.5 , respectively) which can exist in cationic, anionic, and neutral forms. On the contrary, VC presents a weak acid character and is a predominantly negatively charged in solutions with a pH higher than 6.5 . As the result shown in Fig. S1, vitamins in BRBS pH 5 provided the highest anodic currents. It could be reasoned that VB_2 , VC , and VB_6 exist predominantly in neutral, anionic, and cationic forms, respectively. We assumed that these ionic forms easily occur electrons transfer at an electrode surface. On the other hand, anodic currents decreased when vitamins dissolved in BRBS pH 7 and 9 because VB_2 and VB_6 exist predominantly in cationic and neutral forms, respectively. VC , in the meanwhile, exists completely in anionic forms. These ionic forms in neutral and basic supporting electrolytes hardly occur electron transfer at an electrode surface. From these results, it can be concluded that acidic supporting electrolyte is a suitable electrolyte for the detection of VB_2 , VC , and VB_6 .

Afterward, the type of supporting electrolytes was investigated at fixed pH 5 to select the appropriate electrolyte for the detection of VB_2 , VC , and VB_6 . As mentioned previously, based on electrochemical researches, buffer systems were universally served as supporting electrolytes due to the ability of pH regulation. In this research, buffer systems at pH 5 consisting of Britton-Robinson buffer solution (BRBS), acetate buffer solution (ABS), and phosphate buffer solution (PBS) were studied by square wave voltammetry for the selection of appropriate supporting electrolyte. Fig. S3 shows square wave voltammograms of a mixture solution in various buffer systems at pH 5. By considering anodic currents, PBS at pH 5 provided the highest responses. We believed that in our case, the buffer properties, particularly in electrical conductivity, would get involved in the electrooxidation of these vitamins. Consequently, the electrical conductivity of species containing in the proposed buffer system was discussed as reported in references.⁴²⁻⁴³ To deeply consider, PBS at pH 5

consisted of H_3PO_4 , NaH_2PO_4 , and Na_2HPO_4 which each provided the specific conductance (20°C) of 5.5, 2.2, and 4.6 mS/cm, respectively. These values were higher than other species containing in other buffer systems. For example, ABS at pH 5 containing of CH_3COOH and CH_3COONa provided the specific conductance (20°C) of 0.3 and 3.9 mS/cm, respectively. Similarly, BRBS at pH 5 containing of H_3PO_4 , CH_3COOH , and H_3BO_3 provided the specific conductance (25°C) of boric acid of 0.008 mS/cm. It could be ascribed that the more specific conductance was better electrical conductivity. As a result, the resistance of the electrochemical cell could decrease and eliminate the electromigration effects which were desired in controlled-potential experiments. Accordingly, it can be concluded that PBS in acidic pH value is a suitable electrolyte for the detection of VB_2 , VC , and VB_6 .

To confirm the effect of acidic electrolyte, the influence of solution pH on the electrochemical behavior of VB_2 , VC , and VB_6 in PBS using a common SPCE was studied in specific pH ranges from 3.5 to 6.5. Fig 3A shows the anodic peak potentials (E_{pa}) for these vitamins and their linear regression equations: $E_{\text{pa}} (\text{V}) = -0.0533\text{pH} - 0.295$ ($r^2 = 0.9929$) for VB_2 ; $E_{\text{pa}} (\text{V}) = 0.0556\text{pH} + 0.0334$ ($r^2 = 0.9901$) for VC ; and $E_{\text{pa}} (\text{V}) = -0.054\text{pH} + 1.0377$ ($r^2 = 0.9925$) for VB_6 . According to the Nernst equation (Eq. 1), which explains the relationship between E_{pa} and pH, where n and m represent the numbers of electrons and protons involved in the reaction, and a and b are coefficients of oxidant and reductant in the reaction.⁴⁴

$$E_{\text{pa}} = E^0 + \left(\frac{0.0591}{n}\right) \log \left[\frac{(\text{OX})^a}{(\text{R})^b} \right] - \left(0.0591 \frac{m}{n}\right) \text{pH} \quad \text{Eq. 1}$$

All of the slopes between E_{pa} and pH of each vitamin were very imminent to 59 mV/pH at 25°C, indicating that the overall electrochemical process at the proposed electrode was proton-dependent-the electron transfer which was regulated by a transfer of an equal number of protons and electrons.

Furthermore, the anodic peak currents (I_{pa}) as a function of pHs were also investigated as presented in Fig. 3B. The anodic currents of VB₂, VC, and VB₆ do not significant distinction with increasing pH value until it reaches 6.5. Consequently, the difference in anodic peak potential between VC and VB₆ was primary considered to select the appropriate pH value. The difference of anodic peak potential between these two vitamins decreases with increasing pH value leading to the overlapping of anodic peaks. PBS at pH 3.5 provided the highest difference of anodic peak potential, therefore, it was chosen as an appropriate pH value for the simultaneous detection of VB₂, VC, and VB₆. We supposed that due to these vitamins dissolved in the acidic solution (pH 3.5) in which VB₂ and VC predominantly exist in neutral forms, meanwhile, VB₆ is positively charged because of the protonation of nitrogen in the pyridine ring. These molecules and ionized form can be oxidized at differently applied potential using a common SPCE.

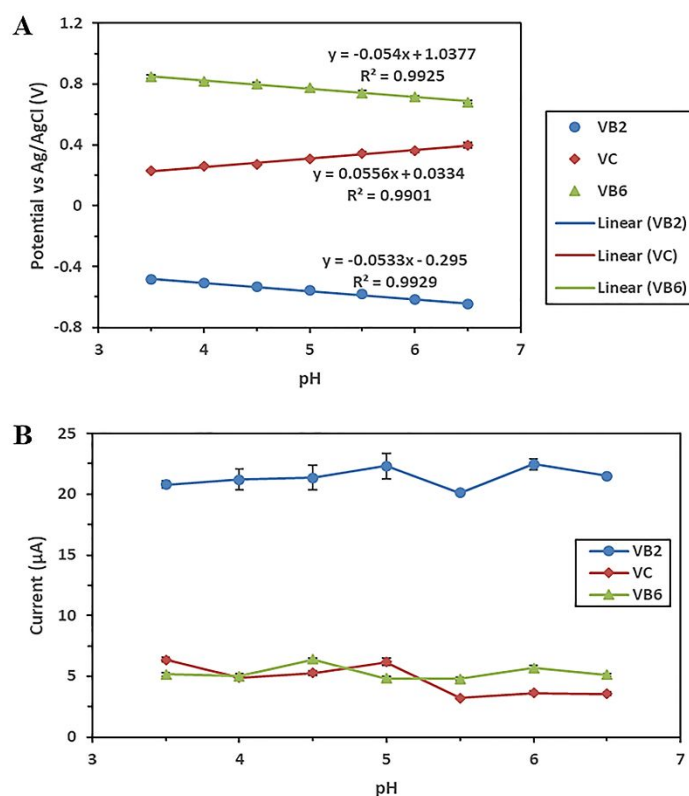


Fig 3. Influences of solution pH on anodic peak potentials (A) and peak currents (B) for the oxidation of 100 μ M VB₂, 400 μ M VC, and 400 μ M VB₆ at a common SPCE.

3.3. The effect of scan rate on the electrooxidation of VB₂, VC, and VB₆

To elucidate whether the mass transfer of the analyte toward the electrode surface is diffusion or adsorption controlled, the effect of scan rate on the electrooxidation of VB₂, VC, and VB₆ was investigated by cyclic voltammetry. Fig. 4A shows cyclic voltammograms of VB₂, VC, and VB₆ at a common SPCE using different scan rates. The results presented that the redox peak currents for VB₂ increased with increasing the scan rate, while their redox peak potentials remained the same values, suggesting the electron transfer process was reversible. On the other hand, the anodic peak currents for VC and VB₆ increased with increasing the scan rate, however, their anodic peak potentials gradually shifted to positive values, introducing the electron transfer processes were irreversible. The linear dependence of the anodic peak current of all three vitamins versus the square root of the scan rate was observed as displayed in Fig. 4B. These behaviors demonstrate that the electrochemical oxidations of all three vitamins at a common SPCE are diffusion-controlled processes.⁴⁵

Using the information received from the linear regression of the relationship between anodic peak currents (I_{pa}) and the square root of scan rates ($V^{1/2}$), the relationship as shown in Eq. 2 was applied to estimate the diffusion coefficients (D_0) of all three vitamins.⁴⁶

$$I_{pa} = 0.496FAC_sD_0^{1/2}\left(\frac{F}{RT}\right)^{1/2}v^{1/2} \quad \text{Eq. 2}$$

When F is the Faraday constant, A is a geometric area of the working electrode (0.126 cm^2), C_s is the concentration of vitamins ($1 \times 10^{-6} \text{ mol/cm}^3$), D_0 is the diffusion coefficient of vitamins, R is a gas constant (8.31447 J/mol/K), and T is a temperature (298 K). The calculated D_0 values of VB₂, VC, and VB₆ were found to be 8.07×10^{-6} , 5.32×10^{-6} , and $1.57 \times 10^{-7} \text{ cm}^2/\text{s}$, respectively. Then, the number of electrons (n) involved in the electrooxidation of VB₂ was determined by the Randles-Sevcik relationship for a reversible process (Eq. 3).

$$I_{pa} = (2.69 \times 10^5) n^{3/2} A C_s D_0^{1/2} \nu^{1/2} \quad \text{Eq. 3}$$

Using Eq. 3 and the previously estimated D_0 of VB_2 , the number of electrons involved in the electrooxidation of VB_2 can be calculated and was found to be $n = 1.07$ ($n \sim 1$). Next, the number of electrons involved in the electrooxidation of VC and VB_6 was determined by the Randles-Sevcik relationship for the irreversible process (Eq. 4).

$$I_{pa} = (2.99 \times 10^5) n [(1 - \alpha)n]^{1/2} A C_s D_0^{1/2} \nu^{1/2} \quad \text{Eq. 4}$$

To calculate the n value, it is unavoidable to solve initially the factor of $(1 - \alpha)n$. Using the Laviron theory for oxidative reaction (Eq. 5),⁴⁷ the calculation is based on the linear relationship between anodic peak potential (E_{pa}) and the natural logarithm of scan rates ($\ln \nu$), as shown in Fig. 4C. Depended on Eq. 5 and the slopes received from the linear relationship between the E_{pa} an $\ln \nu$, the factors of $(1 - \alpha)n$ for VC and VB_6 were found to be 0.572 and 1.00, respectively.

$$E_{pa} = E^0 + \frac{RT}{(1 - \alpha)nF} \ln \frac{RTk_s}{(1 - \alpha)nF} + \frac{RT}{(1 - \alpha)nF} \ln \nu \quad \text{Eq. 5}$$

After calculating the factors of $(1 - \alpha)n$, using Eq. 4 and the formerly estimated D_0 of VC and VB_6 , the number of electrons involved in the electrooxidation of VC and VB_6 can be calculated and were found to be $n = 1.32$ ($n \sim 2$) and $n = 0.998$ ($n \sim 1$), respectively.

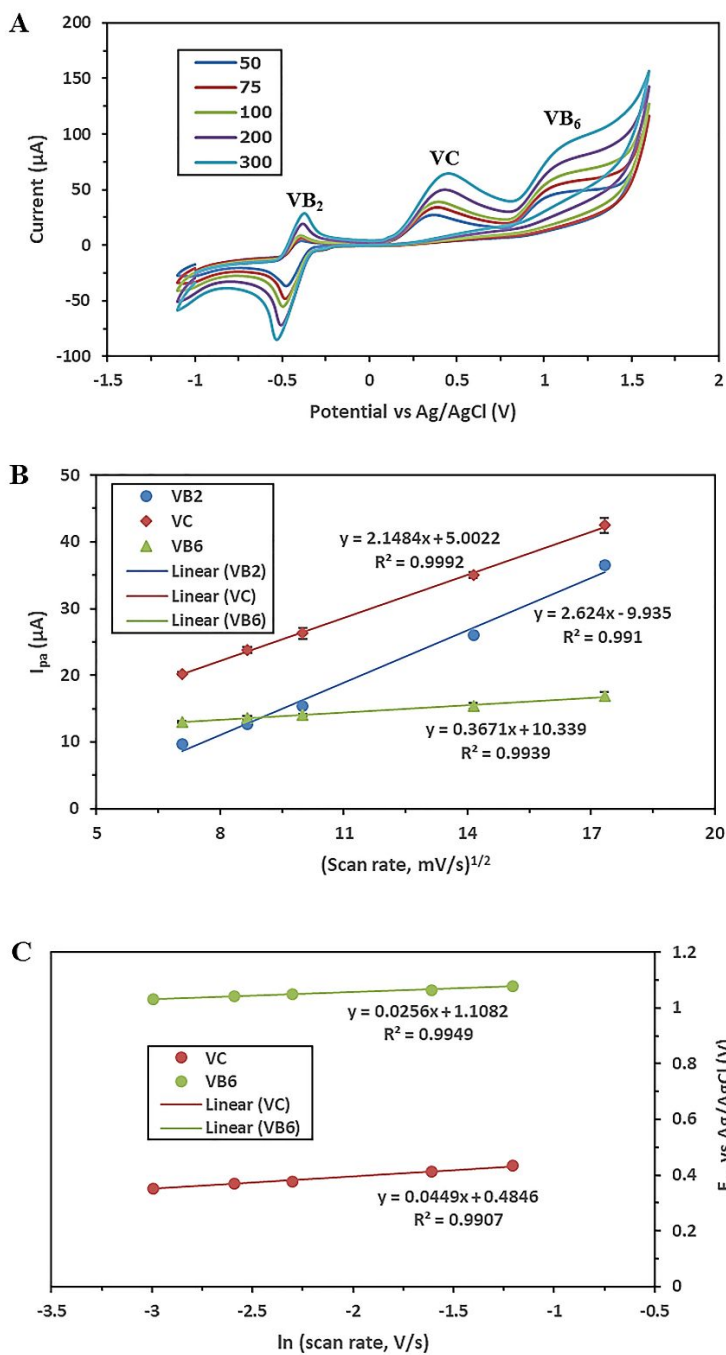


Fig. 4. (A) Cyclic voltammograms for 1 mM VB₂, 1 mM VC, and 1 mM VB₆ in PBS pH 3.5 at the common SPCE using a series of scan rate (50, 75, 100, 200, and 300 mV/s). (B) The relationship between the anodic peak currents (I_{pa}) and the square root of scan rates. (C) The variation of anodic peak potential (E_{pa}) and natural logarithm of scan rates ($\ln V$) from 50 to 300 mV/s.

3.4. Analytical performance

Square wave voltammetry was selected for the simultaneous determination of VB₂, VC, and VB₆ in mixture solutions due to its better resolution and higher sensitivity than cyclic voltammetry. Under the optimized experimental conditions, square wave voltammograms for the electrooxidation of different concentrations of VB₂, VC, and VB₆ mixture at a common SPCE were obtained, as shown in Fig. 5A. The results presented that the well-defined anodic peaks of all three vitamins are separated sufficiently to discriminate and measure the current. Besides, the increment in the peak currents of all three vitamins can be observed with increasing their concentrations at the same time. Fig. 5B displays the calibration curves of all three vitamins, indicating that the anodic peak currents for the electrooxidation of all three vitamins increased proportionally with their corresponding concentrations. From these results, the linear ranges for the simultaneous determination of VB₂, VC, and VB₆ were found to be 1-60 μ M, 10-400 μ M, and 10-400 μ M with the detection limit (calculated as the 3 σ value of the blank) of 0.37 μ M, 5.07 μ M, and 3.32 μ M, respectively. Comparison of the analytical performance to previous works and this work was shown in Table 1. Even though, detection limits and linear ranges of the proposed system are not as low as those received by other modified electrodes. However, we first proposed the simultaneous determination of VB₂, VC, and VB₆ using a common SPCE by systematically studied all experimental parameters that affected to peak separation and sensitivity. Our proposed methodology provided various advantages, such as a low-cost electrode, the modification-free, require a small sample volume for analysis. Moreover, this proposed system is easy to apply for commercialization the electrode in the future.

To investigate the practicality of a common SPCE for the simultaneous determination of VB₂, VC, and VB₆, the individual determination of each vitamin was performed by varying the concentration of one analyte while those of the others have remained constant. The results in Fig. S4A display that the anodic peak currents of VB₂ increased with concentration while the anodic peak currents of VC and VB₆ were equally unaltered at these concentrations as shown in Fig. S4B. In the same way, as can be seen in Fig. S4C and D, the anodic peak

currents of VC increased linearly with increasing the concentrations. Similarly, Fig. S4E and F show that the anodic peak current signals for VB₆ also increased with increasing the concentrations. By comparison between anodic peak currents and concentrations from individual and simultaneous determinations, the slopes of each vitamin were virtually unchanged. It could be emphasized that the electrooxidation processes of each vitamin are independent, therefore, the simultaneous determinations of these vitamins are feasible without any interference from their presence together.

The reproducibility of all three vitamins at various concentrations was calculated in the term of relative standard deviation (RSD) by using seven different electrodes ($n = 7$). The value was lower than 6.5% which can be acceptable.⁴⁸ As shown in Table S1, indicated that this electrode can be successfully employed for the simultaneous determination of water-soluble vitamins with excellent reproducibility.

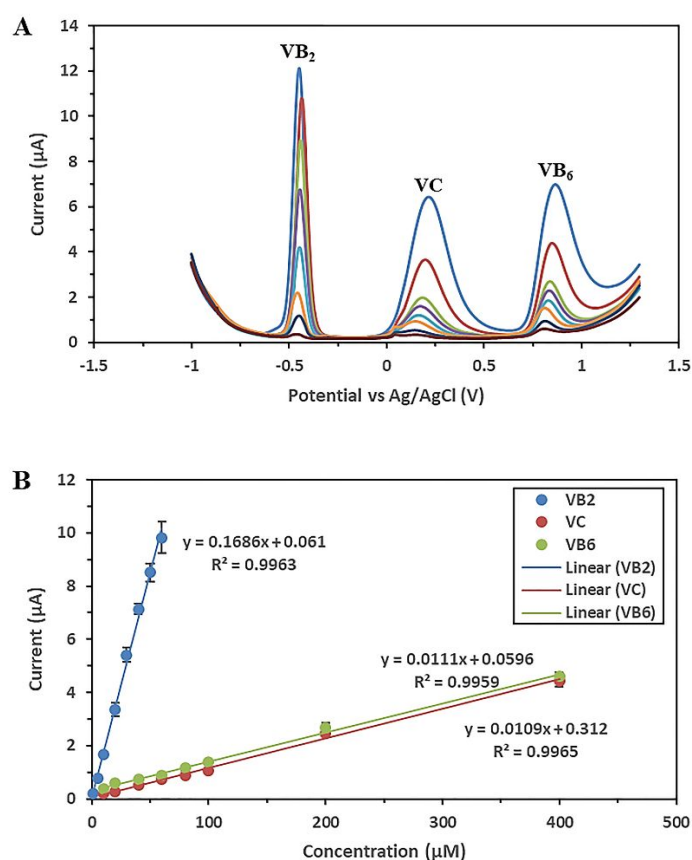


Fig. 5. (A) Square wave voltammograms of VB₂, VC, and VB₆ in concentration ranges of 1-60 μ M, 10-400 μ M, and 10-400 μ M, respectively, in PBS pH 3.5 at common SPCEs. (B) Calibration plots between anodic peak currents of each vitamin versus concentration.

Table 1

Comparison of the analytical performance between previous works for the simultaneous determination of VB₂, VC, and VB₆.

Working electrode	Technique	Detection limit (μ M)			Linear range (μ M)			Ref.
		VB ₂	VC	VB ₆	VB ₂	VC	VB ₆	
PGCE	DPV	0.001	-	0.8	0.005-100	75-75000	2.5-7500	[17]
MWCNT/GCE	CV	0.7	5	10	1-100	50-2000	50-2000	[18]
PEDOT/Fc ⁻ /GCE	DPV	0.05	0.7	0.1	0.1-300	1.5-2000	0.5-1500	[19]
PEDOT/Fe(CN) ₆ ⁴⁻ /GCE		0.02	0.5	0.3	0.04-200	1-2000	0.7-1500	
PEDOT/ZrO ₂ NPs/GCE	DPV	0.012	0.45	0.2	0.05-300	1-1500	0.5-1000	[20]
Ag-PLA/GCE	LSV	0.08	3	5	0.1-23	5-4000	10-3000	[21]
<i>f</i> -MWCNT-Cu ₂ O-Ag ₂ O composite/copper substrate	DPV	0.014	0.011	0.008	0.05-1752	0.05-1628	0.02-1056	[22]
Modification-free SPCE	SWV	0.37	5.07	3.32	1-60	10-400	10-400	This work

PGCE: Pretreated glassy carbon electrode; MWCNT: Multi-wall carbon nanotubes; PEDOT: Poly(3,4-ethylenedioxythiophene); Fc⁻: Ferrocenecarboxylic acid; Fe(CN)₆⁴⁻: Ferricyanide; ZrO₂NPs: Zirconia nanoparticles; Ag-PLA: Silver doped poly(L-arginine); *f*-MWCNT-Cu₂O-Ag₂O: Functionalized multi-walled carbon nanotubes supporting Cu₂O and Ag₂O composite; DPV: Differential pulse voltammetry; LSV: Linear sweep voltammetry.

3.5. Interference study

To investigate the selectivity of the proposed electrode and developed method, the influence of various foreign species on the simultaneous determination of 20 μM VB₂, 60 μM VC, and 60 μM VB₆ in PBS pH 3.5 was evaluated. The tolerance limit was calculated to be lower than 5% of a relative error. The results revealed that 3000-fold and 1000-fold concentration ratios of Na⁺, K⁺, Mg²⁺, Ca²⁺, Al³⁺, Cl⁻, HCO₃⁻, SO₄²⁻, and PO₄³⁻, 300-fold and 100-fold concentration ratios of VB₁, VB₃, VB₅, glucose, fructose, citrate, L-lysine, L-cysteine, and L-methionine; 30-fold and 10-fold concentration ratios of Fe²⁺, Cu²⁺, VB₉, and oxalate; 15-fold and 5-fold concentration ratios of VB₁₂ and uric acid, and 5-fold concentration ratios of albumin and globulin did not interfere the measurement of VB₂, VC, and VB₆ (Table 2). No change significantly in the current signals were observed. Thus, the proposed electrodes were good selectivity towards the simultaneous determination of these three vitamins.

Table 2

Tolerance ratio of foreign species in the simultaneous determination of 20 μM VB₂, 60 μM VC, and 60 μM VB₆ on the common SPCEs.

Foreign species	Tolerance ratio (C _{species} /C _{target} vitamins)		
	VB ₂	VC	VB ₆
Na ⁺ , K ⁺ , Mg ²⁺ , Ca ²⁺ , Al ³⁺ , Cl ⁻ , HCO ₃ ⁻ , SO ₄ ²⁻ , PO ₄ ³⁻	3000	1000	1000
VB ₁ , VB ₃ , VB ₅ , glucose, fructose, citrate, L-lysine, L-cysteine, L-methionine	300	100	100
Fe ²⁺ , Cu ²⁺ , VB ₉ , oxalate	30	10	10
VB ₁₂ , uric acid	15	5	5
Albumin, globulin	5	5	5

3.6. Application in real samples

To examine the practicality and reliability of the proposed method, the simultaneous determinations of VB₂, VC, and VB₆ at a common SPCE were performed in mixed vegetable and fruit juice and artificial urine sample. The concentrations of these vitamins were determined by square wave voltammetry using the standard addition method to prevent any matrix influence. Recovery studies were carried out by spiking VB₂, VC, and VB₆ to the samples at various concentration levels. The experimental results revealed in Table S2, and S3 display that the recovery values were in the range of 83.91 -109.10%, which can be acceptable. Furthermore, to confirm the accuracy of the proposed method, the concentrations of VB₂, VC, and VB₆ in these samples were also determined by HPLC-DAD. The paired t-test between two methods disclosed that the results were in good agreement because the calculated t-value (2.15) was less than the critical value (2.31) for a two-tailed comparison. Therefore, a common SPCE can be successfully performed for the simultaneous determination of VB₂, VC, and VB₆ in real applications.

4. Conclusions

In summary, a simple and cost-effective modification-free SPCE was developed for the simultaneous determination of VB₂, VC, and VB₆. To the best of our knowledge, the measurement of these water-soluble vitamins at a common SPCE was performed for the first time. Meanwhile, continuously keeping the high efficiency, the proposed electrode still provided many advantages over the previous conventional modified-electrodes, such as disposability, simple fabrication procedure, elimination of electrode maintenance, and low volume of solution requirement. The effect of various typical supporting electrolytes was successively accomplished to investigate the electrochemical behavior of VB₂, VC, and VB₆. Under the optimal conditions using PBS pH 3.5 as a suitable supporting electrolyte, a common SPCE exhibited good sensitivity, selectivity, and reproducibility towards the simultaneous determination of VB₂, VC, and VB₆ in the oxidative direction without any modification at the working electrode surface. Finally, the proposed procedure was applied for the simultaneous

determination of these vitamins in mixed vegetable and fruit juice and artificial urine with satisfactory results. Therefore, this proposed assay can be selected as a good and attractive choice for the simultaneous determination of water-soluble vitamins in food and clinical samples.

Conflicts of interest

There are no conflict to declare.

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Notes and References

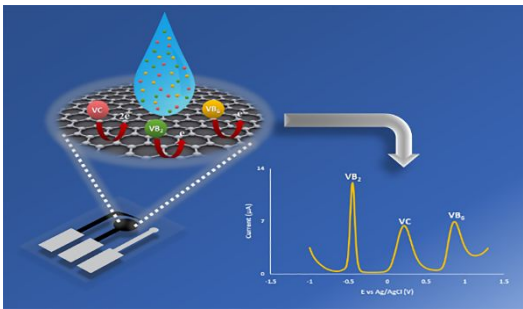
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Table of contents entry



A simple and modification-free SPCE performed the high efficiency for the simultaneous detection of VB₂, VB₆, and VC in various practical applications.

Electronic Supplementary Information

The effect of supporting electrolyte for the simultaneous determination of vitamin B₂, vitamin B₆, and vitamin C using a modification-free screen-printed carbon electrode

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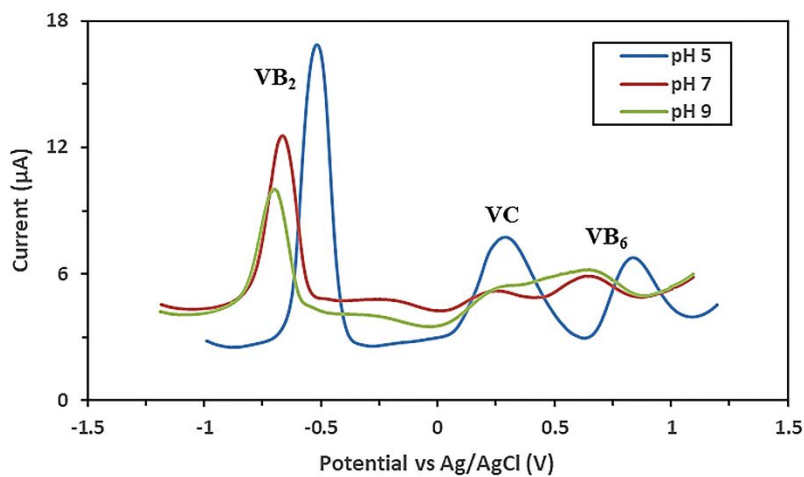


Fig. S1. Square wave voltammograms of mixture vitamins solution (0.1 mM VB₂, 0.4 mM VC, and 0.4 mM VB₆) in BRBS at pH 5 (blue line), 7 (red line), and 9 (green line) obtained from common SPCEs. All square wave voltammograms were recorded at a step potential of 5 mV, an amplitude of 25 mV, and a frequency of 5 Hz.

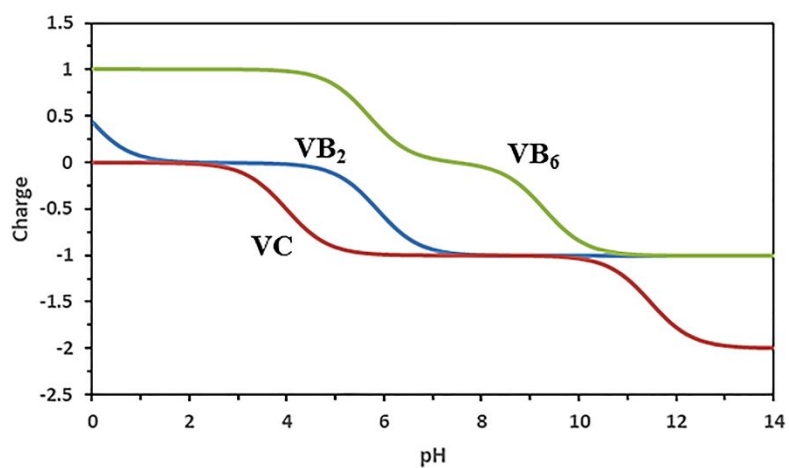


Fig. S2. Charge versus pH curves (CurTiPot software) for VB₂ (blue line), VC (red line), and VB₆ (green line).

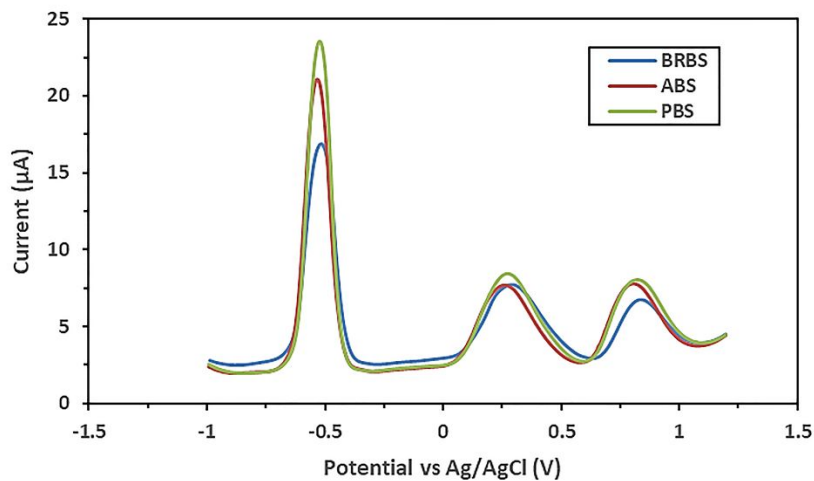


Fig. S3. Square wave voltammograms of mixture vitamins solution (0.1 mM VB₂, 0.4 mM VC, and 0.4 mM VB₆) in BRBS (blue line), ABS (red line), and PBS (green line) at pH 5 obtained from common SPCEs. All square wave voltammograms were recorded at a step potential of 5 mV, an amplitude of 25 mV, and a frequency of 5 Hz.

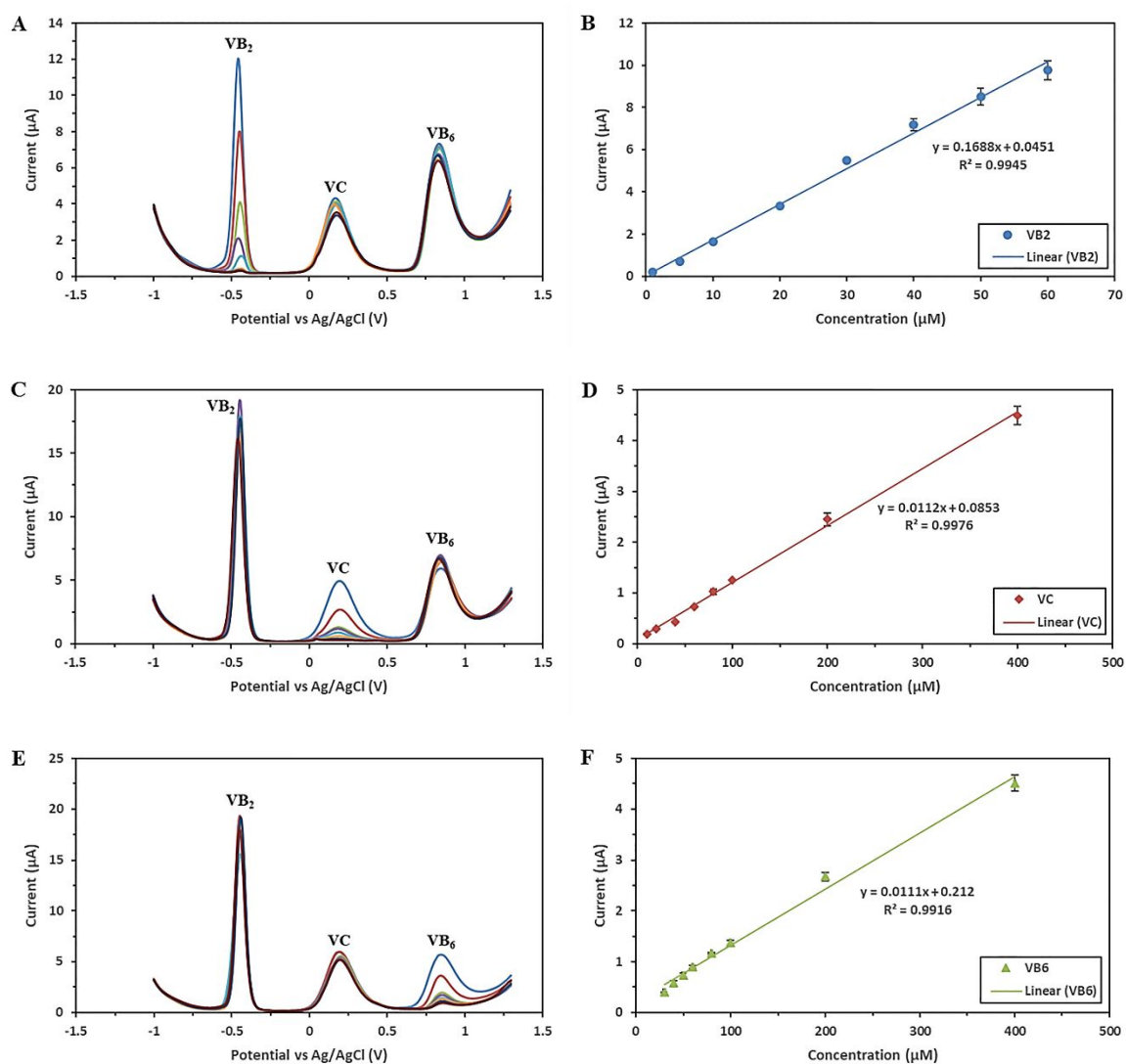


Fig. S4. Square wave voltammograms obtained from common SPCEs of (A) VB₂, (C) VC, and (E) VB₆ in PBS pH 3.5 at different concentrations in the range of 1-60 μM, 10-400 μM, and 10-400 μM, respectively. Calibration plots of their anodic peak currents versus concentrations for (B) VB₂, (D) VC, and (F) VB₆.

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Table S1

The reproducibility (calculated as relative standard deviation) of VB₂, VC, and VB₆ at different concentrations obtained from common SPCEs.

Vitamins	Concentration (μM)	RSD (% , n = 7)
VB ₂	1	4.90
	20	2.78
	60	2.34
VC	10	6.43
	60	4.95
	400	3.70
VB ₆	10	6.27
	60	4.24
	400	2.44

Table S2

The determination of VB₂, VC, and VB₆ in mixed vegetable and fruit juice by SWV and HPLC (n = 3).

Analytes	Added (μM)	Found (μM)	Recovery (%)	RSD (%)	HPLC (μM)
VB ₂	0	ND	-	-	ND
	10	9.92 ± 0.07	99.19	4.30	10.02 ± 0.09
	30	27.59 ± 0.07	91.96	1.68	29.09 ± 0.12
	50	46.81 ± 0.22	93.63	2.98	47.82 ± 0.12
VC	0	17.59 ± 0.02	-	6.37	ND
	40	50.06 ± 0.07	86.92	5.88	41.39 ± 0.07
	80	81.89 ± 0.11	83.91	6.10	82.07 ± 0.11
	200	209.06 ± 0.16	96.08	3.92	203.48 ± 0.16
VB ₆	0	ND	-	-	ND
	40	42.90 ± 0.05	107.25	4.90	42.10 ± 0.15
	80	87.23 ± 0.06	109.04	3.01	84.83 ± 0.21
	200	207.04 ± 0.19	103.52	4.86	211.04 ± 0.22

ND = Not detectable

Table S3

The determination of VB₂, VC, and VB₆ in artificial urine by SWV and HPLC (n = 3).

Analytes	Added (μM)	Found (μM)	Recovery (%)	RSD (%)	HPLC (μM)
VB ₂	0	ND	-	-	ND
	10	10.80 ± 0.13	107.96	6.92	10.53 ± 0.23
	30	32.92 ± 0.33	109.10	6.14	32.15 ± 0.33
	50	52.30 ± 0.25	104.59	2.91	51.85 ± 0.15
VC	0	ND	-	-	ND
	40	37.12 ± 0.03	92.79	7.17	38.40 ± 0.03
	80	72.89 ± 0.01	91.11	1.52	75.29 ± 0.01
	200	192.90 ± 0.9	96.45	4.51	193.10 ± 0.09
VB ₆	0	ND	-	-	ND
	40	36.73 ± 0.03	91.82	6.64	38.33 ± 0.03
	80	70.92 ± 0.04	88.65	4.05	74.12 ± 0.04
	200	198.85 ± 0.10	99.43	4.62	200.60 ± 0.10

ND = Not detectable

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Research Data Related to this Submission

There are no linked research data sets for this submission. The following
reason is given:
Data will be made available on request

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Dear Editor of *Analytica Chimica Acta*

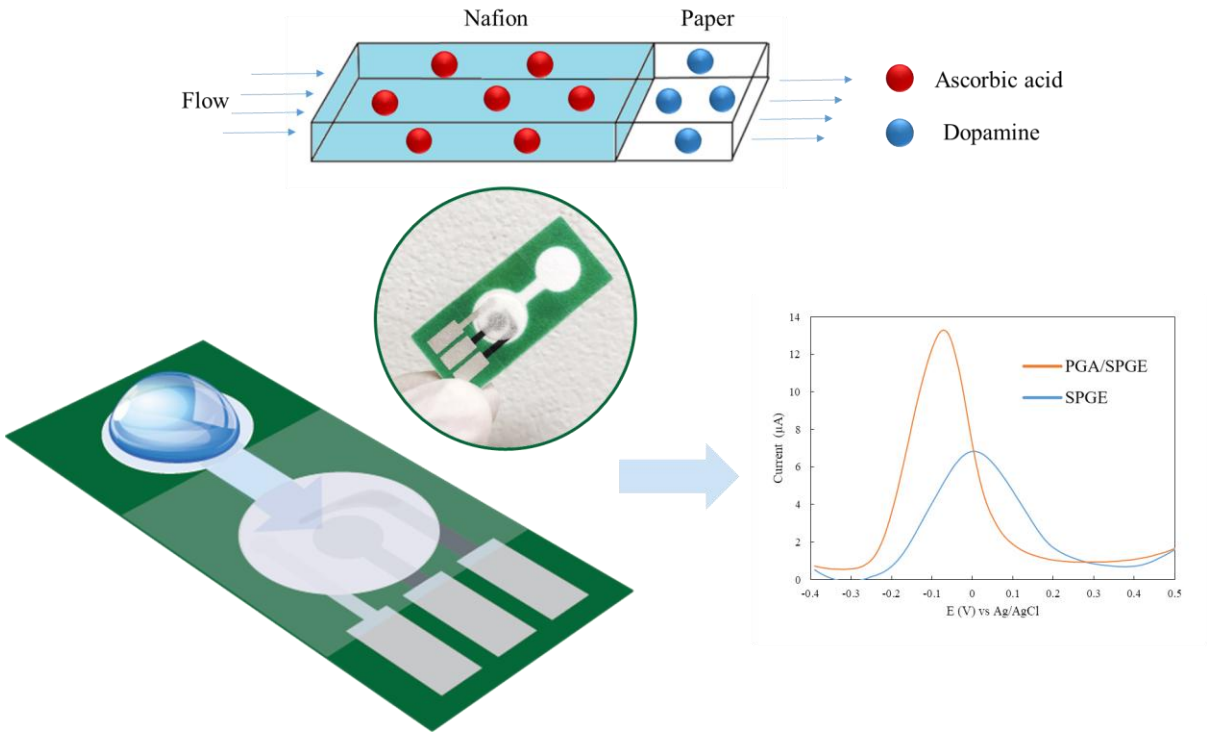
Please accept the submission of this manuscript entitled “**A New Multilayer Microfluidic Paper-based Analytical Platform for Successive Sample Preparation and Detection of Dopamine**” for consideration for possible publication in the original research paper section of *Analytica Chimica Acta*. In this work, we demonstrated a new microfluidic platform coupled with electrochemical technique for detection of dopamine. To the best of our knowledge, we designed and fabricated the electrochemical microfluidic paper-based platform to overcome inconvenience procedure and steps of electrode modification that reported from previous works for quantitation of dopamine level in biological sample. To show a powerful of this platform for a highly sensitive and selective detection, therefore, a simple and cost-effective device was proposed. In this manuscript, we described a fabrication method for making device to provide high selectivity by taking the advantages of nafion and microfluidic paper-based platform. Moreover, we proposed poly(glutamic acid) modified screen-printed graphene electrode to offer excellent sensitivity for dopamine detection. We next demonstrated the use of different microfluidic paper-based platforms to improve the electrochemical responses. The analytical performance of this device was also performed, this platform provides a good linearity, precision and limit of detection compared to previous reports. Finally, we demonstrated the use of our device for detection of dopamine in real sample, which offered reliable results. We are confident that our manuscript will attract the attention of *Analytica Chimica Acta* readership, who are interested in field of electroanalysis and new platform devices for variety of applications.

We are looking forward for your valuable comments/suggestions.

Yours sincerely

Weena Siangproh, Ph.D.

Graphical abstract



A New Multilayer Microfluidic Paper-based Analytical Platform for Successive Sample Preparation and Detection of Dopamine

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Highlights

- Nafion deposited microfluidic paper-based analytical device was used for blocking ascorbic acid to detection zone
- Poly(glutamic acid) modified screen-printed graphene electrode was used for highly sensitive dopamine detection
- Multilayer electrochemical microfluidic paper-based analytical device offered high flow rate and electrochemical response
- A simple and inexpensive device exhibited high accuracy and precision

**A New Multilayer Microfluidic Paper-based Analytical Platform for Successive Sample
Preparation and Detection of Dopamine**

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Abstract

The development of simple and cost-effective electrochemical microfluidic paper-based analytical device (μ PAD) for dopamine (DA) detection was first proposed. The device consists of two parts, the first is sample preparation, nafion was casted into sample zone and microfluidic channel to block ascorbic acid (AA) before solution flows to detection zone. The second is electrochemical detection, poly(glutamic acid) (PGA) modified screen-printed graphene (SPGE) electrode was used to enhance sensitivity for DA detection. Moreover, multilayer μ PAD was fabricated by implement rounded-cotton pad onto detection zone for generating quasi-steady flow rate and packing tape was sealed on the top of device for increasing flow rate and preventing the volatilization of sample on μ PAD. The DA concentrations can be determined by differential pulse voltammetry (DPV). Under optimal conditions, the linear concentration range of DA revealed over the range of 1.0-200 μ M and exhibited the limit of detection of 0.41 μ M (3SD/slope). This proposed platform was eventually applied for detection of DA in artificial urine sample and compared to UV-vis method. The results offered good accuracy and satisfactory method for DA detection in real sample.

Keywords: Microfluidic paper-based analytical device; Screen-printed graphene electrode; Poly(glutamic acid); Dopamine

1. Introduction

Dopamine (DA) is one of the most important catecholamine neurotransmitter that found in the central nervous system. This substance plays a major role in various functions including metabolism, cardiovascular, renal and endocrine system [1]. In addition, the presence of DA concentration in human body is mainly involved in controlling movement, attention, learning and emotional responses. As a result, the deficiency of DA level associates with various diseases such as Parkinson's disease [2], Attention-Deficit Hyperactivity Disorder [3], Huntington's disease, Alzheimer's disease [4], and Schizophrenia [5]. Hence, a simple and sensitive analytical method for the detection and monitoring of DA level in human fluid samples are significantly important for clinical applications. During the past, diverse analytical techniques have been proposed for DA detection, such as, fluorescence spectrophotometry [6, 7] high performance liquid chromatography (HPLC) [8, 9], colorimetric method [10], electrophoresis [11] and surface enhance Raman scattering [12]. Even though these techniques provided high sensitivity and selectivity, but there are some limitations because these techniques required several sample preparation steps, time consuming, large amount of sample volume, specialist skill and expensive equipment. Therefore, the analytical method that can overcome these limitations is still in high demand.

Electrochemical technique is significantly attractive analytical method for DA detection, due to its excellent performances such as fast analysis time, high sensitivity, low cost and simple instrumentation. However, the significant problems for electrochemical DA detection is often suffered from a presence of ascorbic acid (AA) in real sample. Since the DA and AA are oxidized at nearly the same potential. Moreover, traditional electrodes also offer slow electron transfer and poor electrocatalytic properties. For these reasons, leading to the poor selectivity

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4 and sensitivity for DA detection [13]. Additionally, several types of materials have been
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6 designed for modification at electrode surface to solve these problems. The examples of
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8 modifiers are chitosan-graphene [14], layer-by-layer assembly of graphene and gold
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10 nanoparticles [15], overoxidized polyimidazole/graphene oxide copolymer [16] and graphene
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12 anchored with Pd-Pt nanoparticles [17]. Although these proposed materials provided good limit
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14 of detection and also be used to detect DA level in a presence of AA. Nevertheless, these
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16 modified electrodes require expensive materials, several steps for electrodes modification and
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18 time consuming, which make increasingly difficult for use.
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24 The demand of simple and affordable medical diagnostics system has rapidly increased over
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26 the past decade, point-of-care (POC) is an important role for reducing time, complication and
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28 cost for detection of analytes close to the patient. POC provides more comfortable for diagnosis,
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30 monitoring and management, that could greatly help doctors to diagnose the disease at early
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32 stage [18]. Therefore, this platform offers the high beneficial impact for global health especially
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34 in developing countries. Because of the high growth rate of POC platform, World Health
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36 Organization (WHO) established the criteria ideal POC diagnostic device including ease of use,
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38 affordable, high sensitivity, specificity, equipment-free, user-friendliness, rapid, robustness and
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40 deliver [19]. Microfluidic paper-based analytical device (μ PAD) is an excellently challenging
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42 and alternative device for POC applications. μ PAD is fabricated from filter paper that generates
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44 several advantages including cost-effectiveness, ease of use, portability, easy to eliminate and
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46 using small amount of sample-reagent referring to small waste from analysis as well.
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48 Furthermore, solution is driven in μ PAD by capillary action without external pump [20, 21].
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50 Because of these properties, μ PAD is considered to be an innovative platform for equipment-free
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52 and using as a sensing device for many kinds of applications including environmental
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4 monitoring, food safety and clinical diagnosis. The challenge to improve efficiency of μ PAD is
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6 to achieve low limit of detection and good selectivity. Most of research proposed μ PAD coupled
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8 with colorimetric detection, however, sensitivity and selectivity are poor. Therefore, the first
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10 microfluidic paper-based analytical device integrated with electrochemical detection was
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12 designed by Dungchai et al.[22], in comparison, this platform provided lower limit of detection
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14 and generated more selective than colorimetric method. Since the first report, electrochemical
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16 microfluidic paper-based analytical device (μ PAD) has continuously increased at an
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18 exponential growth to be one of the most popular technique for combining with μ PAD.
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20 Consequently, μ PAD has been used for various applications including separation [23],
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22 preconcentration [24], sample preparation [25] and coupling with colorimetry for dual detection
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24 [26].
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31 Simplifying the detection even further, μ PAD coupled with electrochemical technique has
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33 been designed for DA detection. Nafion was deposited on microfluidic paper-based channel is
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35 used to block AA as a sample preparation before solution flowed to the electrode area. For
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37 electrochemical detection, poly(glutamic acid) (PGA) was employed as modifier to alter the
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39 screen-printed graphene electrode (SPGE) surface to enhance the electrochemical response of
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41 DA. Moreover, multilayer μ PAD was also fabricated to overcome the limiting performance
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43 from previous platforms. In this work, differential pulse voltammetry (DPV) technique was used
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45 to measure DA concentrations. To obtain the highest performance of device, various optimal
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47 conditions are investigated to provide excellent quantitative results. To the best of our
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49 knowledge, there is no report of μ PAD for the successive sample preparation and
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51 electrochemical detection. This proposed device was finally detected DA level in artificial urine
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4 samples. The device provided simple, cost-effective, high sensitivity and selectivity for
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6 detection of DA in a presence of AA.
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8 9 **2. Experimental**

10 11 2.1. Chemicals, materials and apparatus

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13 All chemicals used in this work were of analytical grade and used without any
14 purification. Dopamine, l-ascorbic acid, uric acid, nafion, l-glutamic acid, Bovine serum albumin
15 (BSA), potassium ferricyanide ($K_3Fe(CN)_6$), sodium chloride (NaCl), calcium chloride ($CaCl_2$)
16 vitamin B₂ and B₆ were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glucose was
17 obtained from Merck (Darmstadt, Germany). Artificial urine sample was purchased from
18 Carolina Biological Supply Company (Burlington, USA). 0.1 M Phosphate buffer solution
19 (PBS) as supporting electrolyte was prepared with sodium dihydrogen orthophosphate
20 ($NaH_2PO_4 \cdot 2H_2O$) and di-Sodium hydrogen orthophosphate ($Na_2HPO_4 \cdot 2H_2O$) and were acquired
21 from Ajax (Australia). N-propanol was purchased from Unilab (Mumbai, India). All aqueous
22 solution was prepared in deionized (DI) water.
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38 The electrochemical techniques using cyclic voltammetry (CV) and differential pulse
39 voltammetry (DPV) were performed by potentiostat CHI 1200C (Austin, TX, USA).
40 Electrochemical impedance spectroscopy (EIS) was used to ensure the successful modification
41 of modified electrode using PalmSens4 (Houten, The Netherlands). Graphene and silver/silver
42 chloride (Ag/AgCl) inks were purchased from Sun chemical (Slough, United Kingdom). The
43 screen-printed blocks were generated by Chaiyaboon Co. Ltd. (Bangkok, Thailand). epPAD
44 patterned was fabricated by Xerox Color Qube 8570 series wax printing machine (Xerox, Japan).
45 Cotton pad was purchased from Silcot brand by Unicharm Products Co., Ltd. (Tokyo, Japan) and
46 packing tape was purchased from 3M brand by 3M Thailand Company (Bangkok, Thailand).
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2.2. epPAD design and preparation

The epPAD pattern was designed using Adobe illustrator CC. Then wax-patterned was printed onto Whatman No.1 filter paper using wax printing machine. After that, wax printed paper was melted on a hot plate at temperature of 175 °C for 30 s for melting the wax into cellulose structure of paper to generate the difference between hydrophobic and hydrophilic area. For the electrochemical detection zone, three electrodes including working electrode, reference electrode and counter electrode were screened using in-house screen printing procedure. Firstly, graphene ink was screened on detection zone as shown in Fig. S1a to obtain working and counter electrode, and placed into oven at temperature of 55 °C for 30 min. After that, silver/silver chloride (Ag/AgCl) ink was screened on detection zone to complete the electrochemical cell as shown in Fig. S1a and placed into oven at temperature of 55 °C for 30 min. Fig. S1b shows the drawing of μ PAD coupled with electrochemical detection consisted of sample zone (circle, diameter = 10 mm), electrochemical detection zone (circle, diameter = 12 mm) and microfluidic channel connected between sample zone and electrochemical detection zone (channel, 3 x 5 mm).

2.3. epPAD operation and electrochemical procedure for DA detection

For operation, epPAD was sealed with packing tape in the bottom of paper (Fig. 1a). PGA was modified on SPGE using electropolymerization of glutamic acid by preparation of 20 mM glutamic acid in 0.1 M phosphate buffer solution (PBS) pH 7.4 then 100 μ L of modifier was dropped on detection zone and applied potential using cyclic voltammetry scanned from -0.5 V to +1.6 V with scan rate of 0.1 V/s for 7 cycles (Fig. 1b). epPAD was rinsed with DI water and

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4 dried at room temperature. After that, 0.5% nafion was prepared in n-propanol and 10 μL of
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6 solution was drop-casted on sample zone and microfluidic channel (Fig. 1c) and dried at room
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8 temperature. Last, rounded-cotton pad was placed onto electrochemical detection zone and
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10 sealed with packing tape on the top of device (Fig. 1d).

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14 The determination of DA, 60 μL of DA solution in 0.1 M PBS pH = 7.4 was dropped on
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16 sample zone and waited for 15 min (Fig. 1e). In this step, the solution was flowed from sample
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18 zone through microfluidic channel to electrochemical detection zone via capillary force. After
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20 that, the electrochemical detection was performed by DPV using PGA/SPGE as a working
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22 electrode, Ag/AgCl as a reference electrode and screen-printed graphene ink as a counter
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24 electrode. The parameters for DPV measurement were used follow; an increment of 0.006 V, a
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26 pulse amplitude of 0.2 V, a pulse width of 0.1 s and a pulse period of 0.6 s for scanning from -0.4
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28 V to +0.4 V vs. Ag/AgCl. The differential pulse voltammogram of DA was shown in Fig. 1f.

29 30 31 32 33 2.4. The components of μPAD

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36 The μPAD components consist of four layers (Fig. 2); (i) packing tape was sealed on the
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38 top and at the bottom of device to increase flow rate, moreover, these layers were used to prevent
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40 the leakage and volatilization of sample solution, (ii) rounded-cotton pad (circle, diameter = 10
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42 mm) was placed onto electrochemical detection zone to generate a quasi-steady flow rate on
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44 device and (iii) μPAD layer, PGA was modified on SPGE and 0.5% nafion was drop-casted on
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46 sample zone and microfluidic channel.

47 48 49 50 2.5. The determination of DA in real samples

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53 To demonstrate the capability of this device, μPAD was evaluated for detection of DA
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55 in artificial urine samples. The samples were diluted with PBS (pH 7.4), then DA was spiked
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57 into samples at concentrations of 10, 20, 40, 60 and 80 μM and measured the quantity of DA
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using epPAD, the recoveries of spiked standard DA were thoroughly studied. To validate the accuracy of this device, the results received from epPAD were compared to the results those obtained from UV-vis method [27].

3. Results and discussion

3.1. Characterization of modified electrode

The poly(glutamic acid) modified screen-printed graphene electrode (PGA/SPGE) was prepared by electropolymerization of 20 mM glutamic acid in 0.1 M PBS pH 7.4 using CV technique scanned over the range between -0.5 and +1.6 V vs. Ag/AgCl, scan rate 100 mV/s for 7 cycles. Cyclic voltammogram during electropolymerization was showed in Fig. 3a, it can be clearly observed the well-defined peak current around 1.49 V at first three cycles, after forth cycle, the broad peak current gradually increased with number of scan, indicating that this polymer film was grown and formed at SPGE surface [28].

Electrochemical behavior of unmodified SPGE and PGA/SPGE were compared and investigated by CV and ESI techniques. Cyclic voltammograms of 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ in 0.1 M PBS (pH 7.4) were recorded in range between -0.8 and 1.0 V to exhibit the redox peak of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (Fig. 3b). The electrochemical signal of 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ obtained from PGA/SPGE increased almost 2-fold when compared with unmodified SPGE. In addition, to confirm the increased electroactive surface area in the presence of PGA at SPGE surface, the Randles-Sevcik equation (1) was applied to calculate the electroactive surface area. Where i_p is peak current, n is number of electrons, F is Faraday's constant, A is electroactive surface area, C_0^* is concentration of analyte ($[\text{Fe}(\text{CN})_6]^{3-/4-}$), v is scan rate, D_0 is diffusion coefficient of $[\text{Fe}(\text{CN})_6]^{3-/4-}$, R is ideal gas constant and T is temperature. Based on known parameters from

previous study ($n = 1$, $C_0^* = 5 \times 10^{-6}$ mol/cm³, $v = 100$ mV/s, $D_0 = 7.6 \times 10^{-6}$, $R = 8.13447$ J/K·mol and $T = 298$ K) [29]. The comparison of electroactive surface area shows in Fig. 3c, PGA modified SPGE provided more electroactive surface area than unmodified SPGE over 2 times. Similarly, peak separation value was decreased from 0.950 to 0.361 V in case of unmodified SPGE according to PGA/SPGE indicating that rate of electron transfer increased after the electropolymerization of PGA on SPGE [28].

$$i_p = 0.45nFAC_0^* \sqrt{\frac{nFvD_0}{RT}} \quad (1)$$

ESI technique is used to verify the successful modification of the proposed electrode by investigation electron transfer resistance (R_{ct}) between the redox couple of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and electrode surface. The Nyquist plot of unmodified SPGE and PGA/SPGE were obtained in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ in 0.1 M PBS pH 7.4 (Fig. 3d). Each point of Nyquist plot refers for the impedance of one frequency scanned from high to low frequency. The semicircle diameter was equal to charge transfer resistance (R_{ct}) at electrode and solution interface [30]. The R_{ct} value of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ at unmodified SPGE and PGA/SPGE were calculated as 6.8 and 2.3 k Ω , respectively. The R_{ct} value after electropolymerization of PGA on SPGE was considerably decreased. These results confirmed that PGA, which is conductive polymer, was successfully modified on SPGE.

3.2. Electrochemical behavior of DA at PGA/SPGE

The electrochemical property of DA was investigated by CV and DPV using unmodified SPGE and PGA/SPGE. CV was performed to study the electrochemical behavior of DA at working electrodes. Fig.4a shows cyclic voltammograms of DA compared between using unmodified SPGE (blue line) and PGA/SPGE (orange line). At unmodified SPGE, anodic and

cathodic peak obtained at 0.3 and -0.15 V, respectively. However, PGA/SPGE provided higher current and the anodic and cathodic peak shifted to negative direction recorded at 0.089 and -0.083 V, respectively. Moreover, DPV was used to confirm better performance of PGA/SPGE over unmodified SPGE, as shown in Fig. 4b. At unmodified SPGE (blue line), the broad oxidation peak of DA was observed at 0.01 V and oxidation currents obtained was 6.096 μ A. In contrast, PGA/SPGE (orange line) provided a well-defined peak and shifted to negative direction potential at -0.07 V. Otherwise, there is significant enhancement in peak current of DA almost 2-time compared with unmodified SPGE. The results obtained from CV and DPV technique indicated that PGA/SPGE offered electrocatalytic activity toward DA oxidation. These results exhibit that PGA/SPGE is the excellent electrode for DA detection for this work.

To achieve the highest electrochemical response for DA detection, the parameters in electropolymerization of PGA steps were focused on concentration of monomer glutamic acid and numbers of scan in CV technique. These two parameters involve in controlling the thickness of polymer onto electrode surface. First, concentrations of glutamic acid were studied in range of 10 to 50 mM (Fig S2). At 20 mM of glutamic acid provided the highest oxidation current of DA. After 20 mM, the oxidation current of DA gradually decreased, this might affect from thickness film of PGA on SPGE surface is too thick that leading to low electron transfer between electrode and electrolyte interface. Therefore, 20 mM of glutamic acid was an optimal condition. Second, the numbers of scan in CV technique were investigated in range of 3 to 15 cycles (Fig S3). As can be seen that the oxidation currents of DA gradually increased by increasing of scan numbers from 5 to 7 cycles and then oxidation currents after 7 cycles almost stable. Thus, the numbers of scan at 7 cycles was selected as optimal condition.

3.3. Comparison of electrochemical configuration devices

To confirm the capability of μ PAD for successive separation and detection, the stagnant electrochemical analysis (Fig. S4) was investigated by dropping 100 μ L of DA and AA solution on PGA / SPGE and electrochemical measurement was performed by DPV. Fig. 5a shows the differential pulse voltammograms of 100 μ M DA and 100 μ M AA in PBS pH 7.4, the oxidation peak of two substances are almost the same potential. There is no way to distinguish between DA and AA peak. Therefore, AA is considered to be electrochemical interference for detection of DA at PGA/SPGE using stagnant electrochemical analysis.

Based on the results confirmed from stagnant electrochemical analysis, to overcome an interference problem from AA, nafion coated on μ PAD was employed for detection of DA in the presence of AA. This material has been widely used in biosensor applications [31, 32], due to nafion structure shows a strongly permeable membrane that allows only cation passes through membrane but strongly repulses anion away from electrode surface [25]. Because of this property, nafion was used to coat into μ PAD channel to block AA (anion) while DA in solution is allowed to flow to the electrochemical detection zone and measured by electrochemical technique. In this work, the influence of concentrations of nafion to block AA were studied. Fig. 5b shows the oxidation current of 100 μ M DA (orange bar) and 100 μ M DA in the presence of 100 μ M AA (blue bar) obtained from different concentration of nafion coated into μ PAD channel. As can be seen that the different oxidation current between 100 μ M DA and 100 μ M DA in the presence of 100 μ M AA considerably decreased when increased nafion concentration. Up to 0.5% nafion, the oxidation current value of both are almost equal. These results illustrated that using 0.5% nafion can completely block 100 μ M AA and get rid of the interference from

AA while DA concentration is measured. Hence, at 0.5% (v/v) nafion was selected as optimal condition for coating into epPAD channel.

3.4. Improvement of electrochemical response using epPAD

From results obtained from epPAD, the oxidation current value for DA detection was very low which caused from two main factors. The first factor is to come from nafion coated in sample zone and microfluidic channel. Based on nafion structure containing perfluoroalkyl backbones, so, it has strong hydrophobicity properties [33]. Therefore, after coating nafion into epPAD, nafion can penetrate into the pores of paper that obstruct the flow of solution on microfluidic channel following to slow flow rate and leading to low mass transport of DA from bulk solution to electrode interface. Moreover, the slow flow rate also requires more time consuming for measurement. The second factor is the flow rate on epPAD after solution migrates completely to dry region of hydrophilic area leading to the decay of flow rate, thereby flow rate is decreased within time. This phenomena on epPAD also effected to low mass transport at electrode interface [20]. Herein, multilayer of epPAD were employed to overcome those mentioned disadvantages. In recent work, the sealing method using packing tape was proposed. The packing tape was sealed on the top of μ PAD can increase the flow rate over 2-fold compared with open μ PAD [34]. From this phenomena, packing tape was used as sealing material for increasing flow rate of epPAD. As shown in the 2nd platform of Fig. 6a, the oxidation current of 100 μ M DA obtained from the 2nd platform (Fig. 6b) slightly increased when compared with open epPAD (1st platform). However, it is not high enough for DA detection in biological sample. In addition, as previous reported, using absorbent pad can overcome the decay of flow rate with time. Because the absorbent pad function is used to absorb and pull the solution from sample zone through porous structure and generates continuously

capillary flow (quasi-steady flow with time) on epPAD [35]. Therefore, in this work, we applied round-cotton pad instead of absorbent pad and placed on top of detection zone (3rd platform) for making a quasi-steady flow. owing to round-cotton pad has high porosity similar to absorbent pad but offers a lower cost of material. Unfortunately, the oxidation current obtained from the 3rd platform also was slightly increased when compared with open epPAD (1st platform) which is not high enough for detection of DA. To the best of our knowledge, multilayer epPAD consisting of epPAD, rounded-cotton pad and packing tape were first fabricated as shown in the 4th platform (Fig. 6a). It is clear that the multilayer epPAD (4th platform) offered the highest electrochemical response for detection of DA. The excellent results derived from the benefits of packing tape and rounded-cotton pad which provided higher flow rate and generated a quasi-steady flow on epPAD.

Using this proposed device, the waiting time before electrochemical measurement also have significant effect for electrochemical response. Multilayer epPAD was investigated in different waiting time (10, 15 and 20 min) for detection of 100 μ M DA, the oxidation current in different times was shown in Fig. S5. Increasing waiting time, the oxidation current considerably increased as expectation. However, we observed that the oxidation current obtained between 15 and 20 min were not significant difference. Accordingly, waiting time at 15 min is selected as an optimal condition.

3.5. Analytical performance

The analytical performance of multilayer epPAD for DA detection was evaluated, several concentrations of DA were determined using DPV technique. Fig. 7a shows differential pulse voltammogram using multilayer epPAD, the oxidation peak currents of DA gradually increased with increasing concentrations. The linear calibration plot between oxidation current and DA

concentration was found over the range from 1.0 μM to 200 μM . The linear regression equation of $i_{pa} = 0.1987C_{DA} + 1.1301$ with correlation coefficients of $R^2 = 0.9994$ (Fig. 7b) was also presented. The limit of detection ($\text{LOD} = 3SD_{\text{blank}}/\text{slope}$) was found to be 0.41 μM . Besides, the analytical performance of multilayer epPAD was compared with previous reports and summarized in Table 1. Although, the analytical performance from some previous reports offered the lower limit of detection compared with our platform, nonetheless, these methods required for several steps for modification of electrode and high cost of material. However, this developed electrochemical platform exhibited one-step sample preparation and detection integrated in a single device, that provided more simple, rapid and cost-effective device for dopamine detection.

3.6. Selectivity

Aside the interference from AA, other common interferences that possibly found in urine sample including uric acid, glucose, vitamin B, BSA, Na^+ and Ca^{2+} were also investigated. As shown in Fig. 8, the oxidation current of 100 μM DA in the presence of 100 μM uric acid, 1 mM glucose, 100 μM vitamin B₂ and B₆, 1000 $\mu\text{g/L}$ BSA, 1 mM Na^+ and 1 mM Ca^{2+} do not interfere (signal change below 5%). These results illustrated the multilayer epPAD offer the excellent selectivity for detection of DA.

3.7. Analytical application

The practicality of proposed device was demonstrated by detection of DA in artificial urine sample. The recovery test was performed by spiking specified concentration (10, 20, 40, 60 and 80 μM) of DA using standard addition method. As shown in table 2, %recovery and % relative standard deviation (RSD) with different three device were found in range from 96.6% to 108.3% and 1.4% to 5.0%, respectively. Finally, the obtained results from this device were

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4 compared with those obtained from UV-vis method [27]. The paired t-test at 95% confidence
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6 interval was evaluated, the calculated paired two tail value (0.13) of DA was below the critical t
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8 value (2.78). These results can be concluded that the proposed device is acceptable and reliable
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10 for detection of DA in urine sample.
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13 14 **4. Conclusion**

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16 The successive sample preparation and detection on multilayer electrochemical microfluidic
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18 paper-based analytical device (multilayer epPAD) was successfully developed for DA detection.
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20 In this work, nafion coated on epPAD was used to block AA that can not flowed to
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22 electrochemical detection zone. The PGA / SPGE was served as working electrode offer an
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24 excellent sensitivity to quantify the amount of DA in urine sample by DPV. In addition, this new
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26 designed and fabricated multilayer of epPAD paltform leads to achievement in increasment of
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28 electrochemical response for DA detection. The developed device consists of packing tape and
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30 rounded-cotton pad for generating high and quasi-steady flow rate onto device. Finally, to
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32 demonstrate the powerfull of device, multilayer epPAD was successfully applied for detection of
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34 DA in artificial urine sample. This platform exhibits excellent advantages over previous reports
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36 including low cost, simple steps for fabrication, portability and short analysis time.
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38 Consiquently, this multilayer epPAD serves as an alternative electrochemical detection platform
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40 for point-of-care diagnostic system to detect other important biomarkers in the future.
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Figure captions

Fig. 1 The representation of eμPAD for DA detection consisting of: (a) sealed packing tape at the bottom of eμPAD, (b) PGA modified SPGE step, (c) drop casting of 0.5% nafion (d) completed multilayer eμPAD components, (e) determination of DA step and (f) differential pulse voltammogram of DA.

Fig. 2 Illustration of eμPAD layers, fabricated from i) packing tape, (ii) rounded-cotton pad and iii) eμPAD layer.

Fig. 3 (a) The electropolymerization of glutamic acid at SPGE was perform using cyclic voltammetry scanned between -0.5 and +1.6 V, scan rate 100 mV/s for 7 cycles in 20 mM glutamic acid. (b) Cyclic voltammograms of 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ in 0.1 M PBS pH 7.4 at SPGE (gray line) and PGA/SPGE (yellow line), scan rate 100 mV/s. (c) Electroactive surface area of SPGE (grey bar) and PGA/SPGE (yellow bar). (d) Nyquist plots of SPGE and PGA/SPGE obtained in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ at SPGE (orange) and PGA/SPGE (blue).

Fig. 4 Cyclic voltammograms (a) and differential pulse voltammograms (b) of DA at SPGE (blue line) and PGA/SPGE (orange line) in PBS pH 7.4.

Fig. 5 (a) Differential pulse voltammogram of 100 μM DA (blue line), 100 μM AA (orange line), and PBS pH 7.4 (black line) at PGA/SPGE performed by stagnant analysis. (b) oxidation

current value of 100 μM DA (blue bar) and 100 μM DA in a presence of 100 μM AA (orange bar) obtained from different concentrations of nafion coated into e μ PAD.

Fig. 6 (a) The schematic representation for fabrication of e μ PAD in different platforms. (b) The effect of different e μ PAD platforms on oxidation current for DA detection.

Fig. 7 (a) diferential pulse voltamograms obtained from several concentrations of DA in PBS pH 7.4 using multilayer e μ PAD, (b) calibration plot between concentration of DA (μM) and current (μA)

Fig. 8 The interferences effect of 100 μM uric acid, 1 mM glucose, 100 μM vitamin (B_2 and B_6), 1000 $\mu\text{g/L}$ BSA, 1 mM Na^+ and 1 mM Ca^{2+} in detection of 100 μM DA in PBS pH 7.4

Figure 1

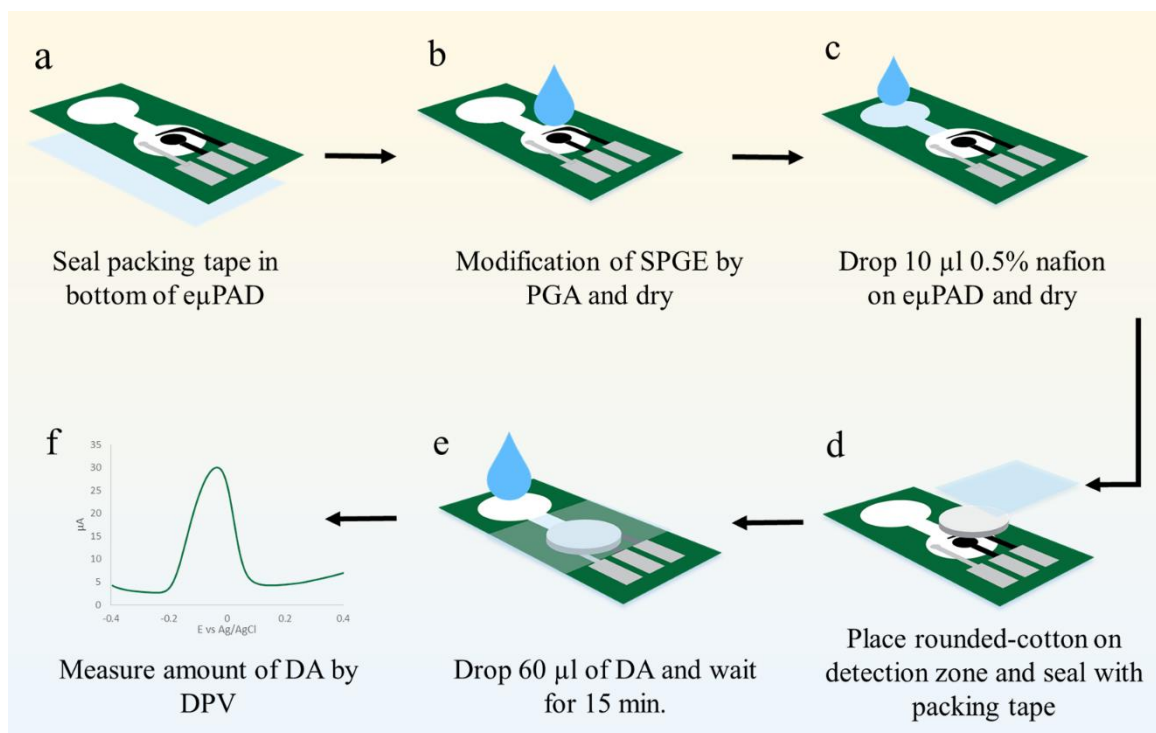


Figure 2

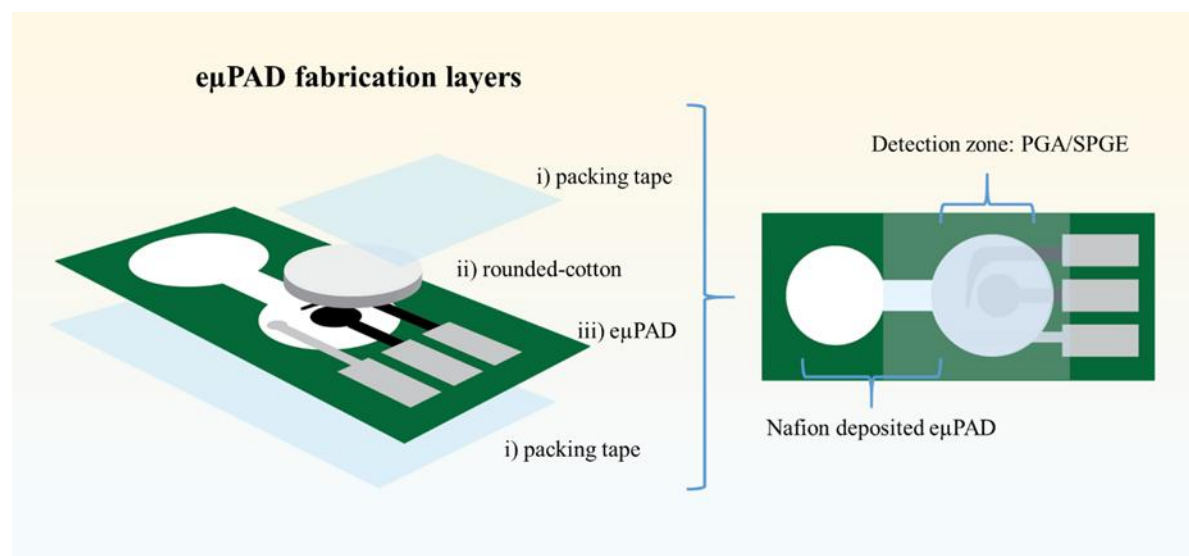


Figure 3

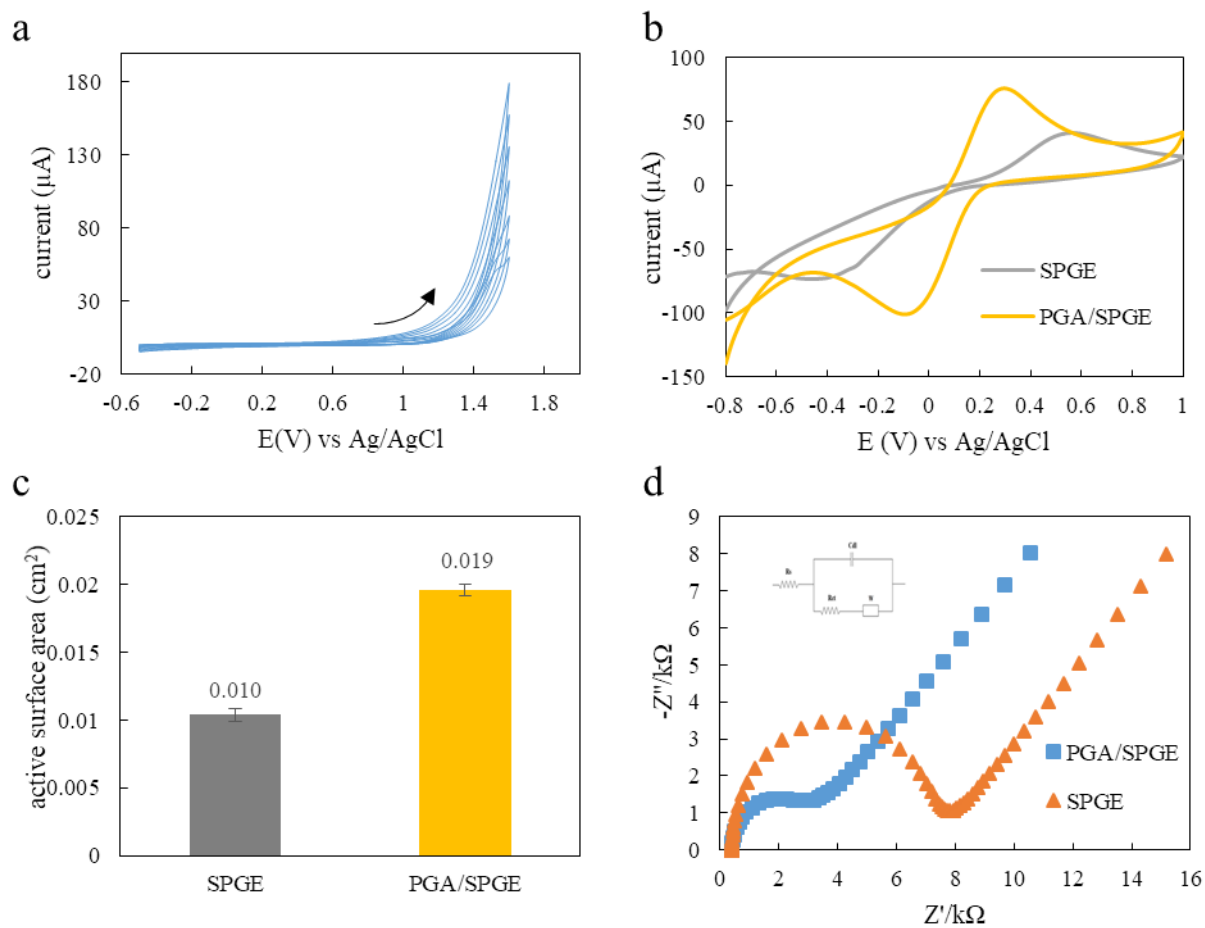


Figure 4

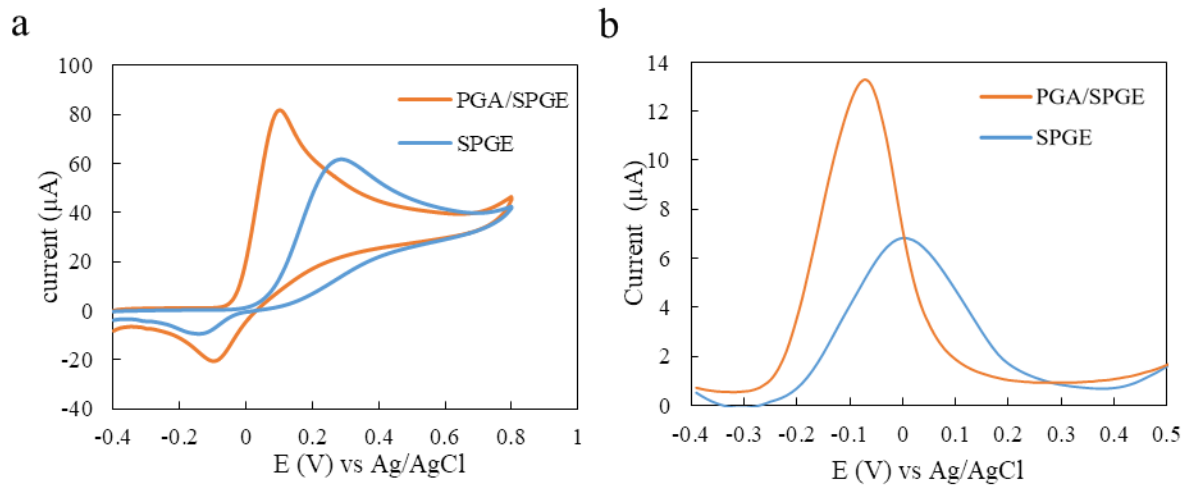


Figure 5

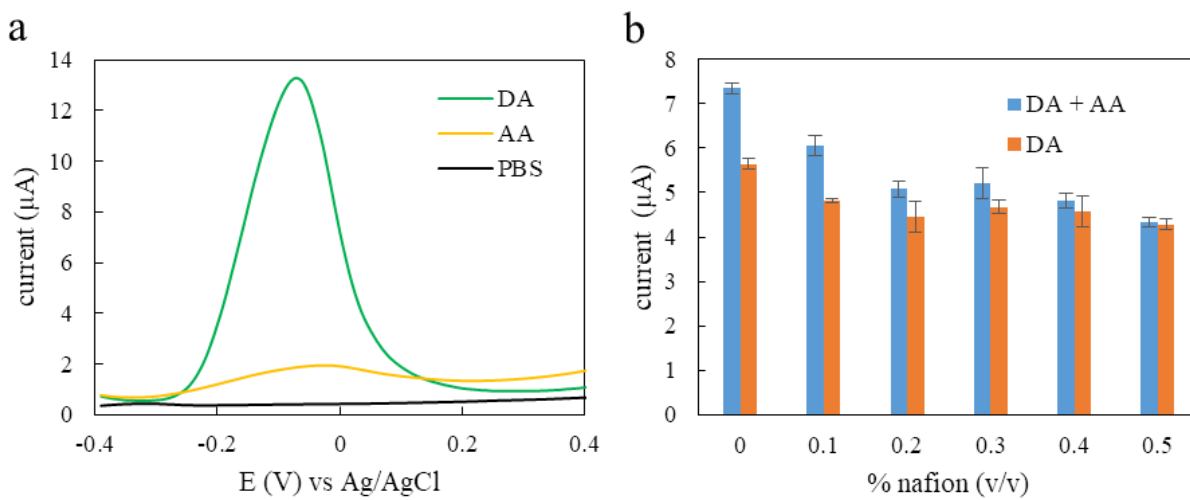
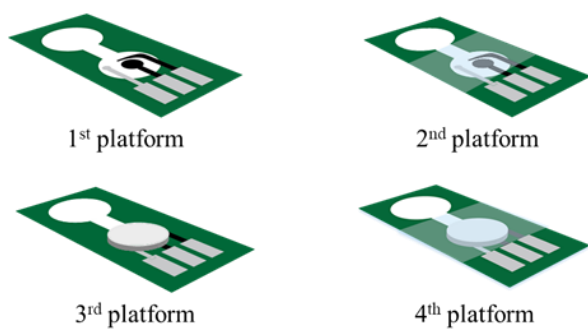


Figure 6

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b

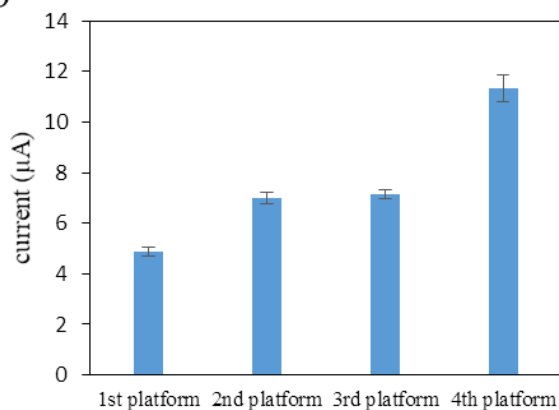
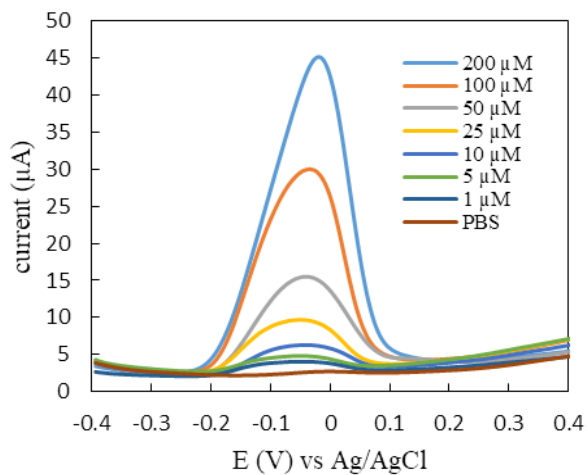


Figure 7

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b

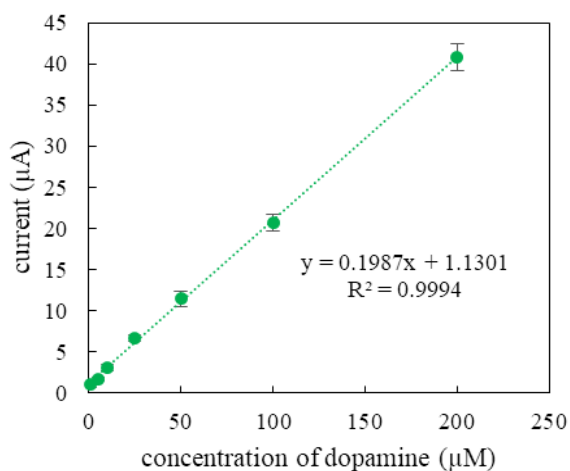


Figure 8

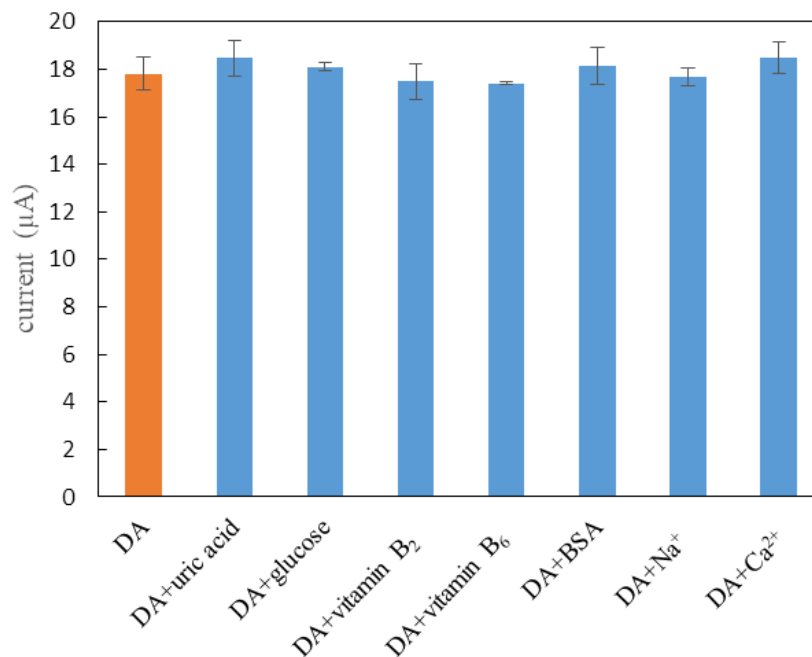


Table 1 Analytical performance of previous reports compared with proposed multilayer epPAD based on electrochemical detection of DA

Electrode	Technique	LOD (μM)	Linear range (μM)	Ref.
Poly(SDB)/CPE	DPV	0.8	5.0–10, 20–80 and 90–1000	[36]
AgNPs/rGO/GCE	LSV	5.4	10–800	[37]
PI _{mox} -GO/GCE	DPV	0.63	12–278	[16]
HNP-PtTi/ GCE	DPV	3.2	4.0–500	[38]

G-DHE	DPV	0.2	5.0– 2000	[39]
PtNi@MoS ₂ /GCE	DPV	0.1	0.5–150	[40]
multilayer epPAD (PGA/SPGE)	DPV	0.41	1.0–200	This work

Abbreviaion

Poly(SDB)- poly(solochrome dark blue), CPE- carbon paste electrode, AgNPs- silver nanoparticles, rGO- reduced graphene oxide, LSV- linear sweep voltammetry, Plmox- overoxidized polyimidazole, GO- graphene oxide, HNP- hierarchical nanoporous, G-DHE- graphene–diamond hybrid electrode and PtNi@MoS₂- PtNi bimetallic naoparticles loaded MoS₂ nanosheets.

Table 2 The analytical application using multilayer epPAD for detection of DA in artificial urine sample (n=3).

Sample	Spiked (μM)	Found (μM)	Recovery (%)	RSD (%)
Artificial urine	10	10.8±0.3	108.3	2.3
	20	20.2±0.7	101.0	3.6
	40	38.6±1.9	96.6	5.0
	60	59.2±1.7	98.7	2.8
	80	81.1±1.1	101.4	1.4

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Electronic Supplementary Material (online publication only)

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แบบฟอร์มเปิดเผยการประดิษฐ์และแสดงความจำนงขอให้จดทะเบียนสิทธิบัตร/อนุสิทธิบัตร (Invention Disclosure Form)

NO:.....

Receive date:.....

1. ชื่อที่แสดงถึงการประดิษฐ์ (ไทยและอังกฤษ)

ขั้วไฟฟ้าพิมพ์สกรีนสำหรับการตรวจวิเคราะห์วิตามินที่ละลายในไขมัน

A screen-printed electrode for fat-soluble vitamins determination.....

ลักษณะโดยย่อของการประดิษฐ์ (แบบเอกสารประกอบได้)

รูปแบบของขั้วไฟฟ้าที่ประดิษฐ์ขึ้นนี้ประกอบด้วยขั้วไฟฟ้าพิมพ์สกรีนบนฐานพีวีซี (PVC: polyvinyl chloride) จำนวน 3 ขั้ว คือ 1) ขั้วไฟฟ้าทำงาน 2) ขั้วไฟฟ้าเผชิญหน้า และ 3) ขั้วไฟฟ้าอ้างอิง โดยขั้วไฟฟ้าทำงานและขั้วไฟฟ้าเผชิญหน้าประดิษฐ์ขึ้นจากการพิมพ์สกรีนหมึกของวัสดุคอมโพสิตระดับนาโนเมตร (nanocomposite) ของกราฟีนและนาฟิออน ส่วนขั้วไฟฟ้าอ้างอิงนั้นประดิษฐ์ขึ้นจากการพิมพ์สกรีนหมึกของซิลเวอร์/ซิลเวอร์คลอไรด์ ลักษณะของขั้วไฟฟ้าที่ประดิษฐ์ขึ้นนี้มีลักษณะเป็นขั้วไฟฟ้าทำงานรูปแบบวงกลมที่มีเส้นผ่านศูนย์กลาง 5 มิลลิเมตร ซึ่งถูกล้อมรอบด้วยขั้วไฟฟ้าเผชิญหน้าและขั้วไฟฟ้าอ้างอิง นอกจากนี้ยังมีการพิมพ์ขั้วไฟฟ้าเส้นผ่านศูนย์กลาง 12 มิลลิเมตรล้อมรอบขั้วไฟฟ้าทั้งสามเพื่อกำหนดขอบเขตการไหลของสารละลาย

2. ชื่อผู้ประดิษฐ์ / ผู้ประดิษฐ์ร่วม (ระบุตำแหน่งหน้าที่ หรือ ตำแหน่งทางวิชาการ)	สัดส่วนการประดิษฐ์/การ แบ่งปันผลประโยชน์ (%)	สังกัด / ที่อยู่ (ระบุให้ครบถ้วน)	โทรศัพท์ / โทรสาร / อีเมล	เลขประจำตัว ประชาชน 13 หลัก
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3. การประดิษฐ์นี้ได้รับการสนับสนุนทางการเงินจากแหล่งเงินทุนหรือร่วมวิจัยกับ องค์กร บริษัท หรือหน่วยงานใดหรือไม่



ได้รับ โปรดระบุชื่อหน่วยงานที่เกี่ยวข้อง สำนักงานกองทุนสนับสนุนการวิจัย



ไม่ได้รับ

4. ผลงานนี้เคยมีการตีพิมพ์ หรือเผยแพร่ผลงานมาก่อนหรือไม่



มี โปรดระบุลักษณะการเผยแพร่.....เมื่อ.....



ไม่มี

5. ศักยภาพของเทคโนโลยีในเชิงพาณิชย์ (โปรดอธิบาย แบบเอกสารประกอบได้)

ผลิตภัณฑ์หรือเทคโนโลยีที่ใกล้เคียงที่มีอยู่แล้วในตลาด

ในปัจจุบัน ขั้วไฟฟ้าที่สามารถลดการดูดซับที่ผิวหน้าขั้วจากการตรวจวิเคราะห์นิยมนำใช้ขั้วไฟฟ้าแผ่นเพชรเจือโบรอน (boron-doped diamond: BDD). โดยขั้วไฟฟ้าชนิดนี้มักถูกนำมาใช้ในการตรวจวัดสารทางชีวภาพเนื่องจากสภาพผิวหน้าขั้วไฟฟ้าที่มีความไม่ขรุขระ จึงป้องกันการเกาะติดหรือดูดซับของสารได้ แต่ขั้วไฟฟ้าประเภทนี้มักมีราคาที่สูงมาก เนื่องจากกระบวนการประดิษฐ์ขั้วไฟฟ้าที่ยังยาก ขั้นตอนต้องใช้เทคโนโลยีขั้นสูงในการผลิต เช่น การใช้เครื่อง Chemical Vapor Deposition (CVD) เป็นต้น

ลักษณะการใช้ประโยชน์จากผลงาน(Application)

ขั้วไฟฟ้าพิมพ์สกรีนที่ประดิษฐ์ขึ้นนี้สามารถใช้ในการตรวจวัดวิตามินดีได้อย่างมีประสิทธิภาพ ทั้งยังสามารถนำไปประยุกต์ใช้กับการตรวจวัดวิตามินที่ละลายในไขมันชนิดอื่น ๆ ได้ในคราวเดียวกัน โดยขั้วไฟฟ้าชนิดนี้สามารถตรวจวัดหรือใช้งานซ้ำได้มากถึง 10 ครั้ง โดยไม่ประสบปัญหาการดูดซับของสารที่ผิวหน้าขั้วไฟฟ้าเมื่อเทียบกับขั้วไฟฟ้าชนิดอื่น ๆ จึงทำให้เหมาะสมกับการใช้งานที่ต้องมีการตรวจวัดซ้ำ ๆ เช่น ในอุตสาหกรรมอาหารและยาที่ต้องมีการควบคุมคุณภาพตลอดเวลา

จุดเด่นของผลิตภัณฑ์หรือเทคโนโลยีเมื่อเปรียบเทียบกับผลิตภัณฑ์หรือเทคโนโลยีปัจจุบัน

จุดเด่นของขั้วไฟฟ้าที่ประดิษฐ์ขึ้นนี้เมื่อเปรียบเทียบกับขั้วไฟฟ้าที่นิยมใช้สำหรับการตรวจวัดสารที่มักมีการดูดซับที่ผิวหน้าขั้วไฟฟ้า เช่น ขั้วไฟฟ้าเพชรเจือโบรอน คือ ราคาต้นทุนในการประดิษฐ์ที่ถูกกว่า เนื่องจากการประดิษฐ์ขั้วไฟฟ้าสามารถทำได้ง่ายกว่าโดยอาศัยเพียงการพิมพ์สกรีนเท่านั้นโดยไม่ต้องใช้เครื่องมือที่มีราคาแพง นอกจากนี้ผู้ประดิษฐ์ยังได้ทำการทดสอบการตรวจวัดวิตามินดีด้วยขั้วไฟฟ้าเพชรเจือโบรอน และพบว่ายังคงประสบปัญหาการดูดซับของสารที่ผิวหน้าขั้วไฟฟ้าเช่นเดียวกับขั้วไฟฟ้าอื่น ๆ ดังนั้นขั้วไฟฟ้าที่ประดิษฐ์จากการพิมพ์สกรีนด้วยวัสดุคอมโพสิตระดับนาโนเมตรของกราฟีนและนาฟิออน จึงสามารถตอบโจทย์ในการใช้งานสำหรับการตรวจวัดวิตามินดีและวิตามินที่ละลายในไขมันอื่น ๆ เนื่องจากสามารถใช้งานซ้ำได้มากถึง 10 ครั้งโดยที่ยังมีประสิทธิภาพคงเดิม

โปรดระบุชื่อบริษัทที่ท่านคิดว่าน่าจะมีความสนใจในเทคโนโลยีหรือสิ่งประดิษฐ์ของท่าน

บริษัท.....โทรศัพท์ (ถ้ามี).....
บริษัท.....โทรศัพท์ (ถ้ามี).....

6. การสืบค้นข้อมูลสิทธิบัตร

6.1 Keyword ที่ใช้ในการสืบค้น...graphene*nafion.. graphene*nafion*electrode.. anti-fouling. electrode

6.2 ผลการสืบค้นพบว่า ☐ เหมือนหรือคล้ายกับงานที่ปรากฏอยู่แล้ว ☒ ไม่เหมือนหรือคล้ายกับงานที่ปรากฏอยู่แล้ว

6.3 Website ที่ใช้ในการสืบค้น ☒ ประเทศไทย: www.ipthailand.go.th ☒ สหรัฐอเมริกา : www.uspto.gov

☒ ญี่ปุ่น : www.jpo.go.jp ☒ ยุโรป : <http://ep.espacenet.com> ☒ อื่นๆ ระบุ WIPO / DPMA / KIPO

(ในกรณีที่พบว่าเหมือนหรือคล้ายกับงานที่ปรากฏอยู่แล้ว กรุณาแนบเอกสารที่สืบค้นได้มาให้สถาบันฯ ด้วย)

7. ข้าพเจ้าขอยืนยันว่าข้อมูลดังกล่าวข้างต้นครบถ้วนและถูกต้อง

ลงนาม.....หัวหน้าโครงการ

(.....)

...../...../.....

กรุณาส่งแบบฟอร์มนี้กลับมายัง

สถาบันทรัพย์สินทางปัญญาแห่งจุฬาลงกรณ์มหาวิทยาลัย

254 อาคารเฉลิมราชกุมารี 60 พรรษา (จามจุรี 10) ชั้น 2 ถนนพญาไท แขวงวังใหม่ เขตปทุมวัน กรุงเทพฯ 10330

เลขหมายติดต่อ 0-2218-4195-7

เลขหมายโทรสาร 0-2218-4198

บันทึกข้อตกลงการรับคำขอ

ส่วนราชการ กองสิทธิบัตร

เลขที่บันทึกข้อตกลง A256301918

วันที่ 12 เดือน พฤษภาคม พ.ศ. 2563

ส่วนที่ 1: สำหรับผู้ยื่นคำขอ

ข้าพเจ้า (ชื่อ-สกุล) นายมงคล วรรณพพิทักษ์ ที่อยู่ สถาบันทรัพย์สินทางปัญญาแห่งจุฬาลงกรณ์มหาวิทยาลัย 254

หมู่ที่ ตระก/ซอย ถนน พญาไท ตำบล/แขวง วังใหม่
อำเภอ/เขต ปทุมวัน จังหวัด กรุงเทพมหานคร รหัสไปรษณีย์ 10330 เบอร์โทรศัพท์ 02-2184180

อีเมล หมายเลขบัตรประจำตัวประชาชน 3101600911378

ได้มายื่นคำขอ คำขอรับสิทธิบัตร/อนุสิทธิบัตร เลขที่ 2003000999

ส่วนที่ 2: สำหรับพนักงานเจ้าหน้าที่

ข้าพเจ้า (ชื่อ-สกุล) นางสาววนิดา ชุ่มวิจิตร ตำแหน่ง นักวิชาการฝ่ายส่งเสริมการค้า
ได้ตรวจสอบเอกสารประกอบการยื่นคำขอ คำขอรับสิทธิบัตร/อนุสิทธิบัตร เลขที่ 2003000999

แล้วเห็นว่าผู้ยื่นคำขอ ได้ยื่นเอกสาร แบบรายการเอกสาร (Checklist) ตามที่แนบมาแล้วปรากฏว่า



เอกสารไม่ครบถ้วนโดยมีรายละเอียดตามแบบรายการเอกสาร (Checklist) ตามแบบ

จึงได้แจ้งให้ผู้ยื่นคำขอและผู้รับมอบอำนาจแก้ไขให้ถูกต้องครบถ้วนแล้ว และผู้ยื่นคำขอตกลงว่าจะดำเนินการ

การแก้ไขหรือยื่นเอกสารเพิ่มเติมภายในวันที่ 10 เดือน สิงหาคม พ.ศ. 2563

(ระยะเวลาที่แก้ไขเพิ่มเติมหรือส่งเอกสารให้ครบถ้วน จะต้องไม่เกิน 90 วันนับแต่วันที่ผู้ยื่นคำขอฯ)

"บันทึกข้อตกลงการรับคำขอ" นี้ทำขึ้นสองฉบับ โดยมีข้อความถูกต้องตรงกัน ทั้งสองฝ่ายได้อ่าน และเข้าใจโดยตลอด จึงได้ลงลายมือชื่อไว้เป็นหลักฐานสำคัญ และพนักงานเจ้าหน้าที่ได้มอบสำเนาให้ผู้ยื่นคำขอไว้เป็นหลักฐานหนึ่งฉบับด้วยแล้ว

ลงชื่อ (นางสาววนิดา ชุ่มวิจิตร) พนักงานเจ้าหน้าที่ ลงชื่อ (นายมงคล วรรณพพิทักษ์) ผู้ยื่นคำขอ

ลงชื่อ (นางสาววนิดา ชุ่มวิจิตร) พนักงานเจ้าหน้าที่

หมายเหตุ 1. ในกรณีที่ผู้ยื่นคำขอไม่แก้ไขเพิ่มเติมคำขอหรือไม่ส่งเอกสารหรือหลักฐานเพิ่มเติมตามที่พนักงานเจ้าหน้าที่แจ้งหรือตามที่ปรากฏ
ในบันทึกข้อตกลงการรับคำขอ พนักงานเจ้าหน้าที่จะคืนคำขอให้แก่ผู้ยื่นคำขอ พร้อมทั้งแจ้งเป็นหนังสือถึงเหตุแห่งการคืนคำขอและสิทธิในการ
อุทธรณ์ให้ผู้ยื่นคำขอทราบ ทั้งนี้ เมื่อผู้ยื่นคำขอได้ชำระค่าธรรมเนียมใดๆ ให้แก่ กรมทรัพย์สินทางปัญญาไปแล้ว จะขอคืนค่าธรรมเนียม
ดังกล่าวไม่ได้ไม่ว่าในกรณีใดๆ เว้นแต่ (1) มีกฎหมายบัญญัติไว้ให้ คืนค่าธรรมเนียม หรือ (2) ชำระค่าธรรมเนียมเข้าช้อนหรือชำระเกินซึ่งการ
ชำระดังกล่าว เกินเนื่องจากความผิดพลาดของเจ้าหน้าที่รัฐ โดยมีใช้ความผิดของผู้ชำระซึ่งกรมทรัพย์สินทางปัญญาจะได้พิจารณาเป็นกรณีไป

2. ในกรณีที่ผู้ยื่นคำขอหรือตัวแทนผู้รับมอบอำนาจไม่ได้เป็นผู้ยื่นคำขอด้วยตนเองโดยมอบหมายให้บุคคลอื่นเป็นผู้ยื่นคำขอแทน
ควรมีหนังสือมอบอำนาจช่วงหรือหนังสือมอบอำนาจเฉพาะการให้บุคคลนั้นมีอำนาจยื่นคำขอและลงนามในบันทึกข้อตกลงการรับคำขอแทน
ผู้ยื่นคำขอหรือตัวแทนได้เพราะหากคำขอไม่ถูกต้องหรือเอกสารหลักฐานไม่ครบถ้วนและบุคคลผู้ยื่นคำขอไม่มีอำนาจลงนามในบันทึกดังกล่าว
เจ้าหน้าที่ไม่อาจรับคำขอของท่านไว้ได้

รายการเอกสารหลักฐาน (Checklist)

ประกอบการยื่นคำขอ

วันที่รับคำขอ

12 พฤษภาคม 2563

เลขที่คำขอ

2003000999

ประเภทคำขอ	☒ ผู้ขอเป็นนิติบุคคล	☐ ผู้ขอเป็นบุคคลธรรมดา
☐ สิทธิบัตรการประดิษฐ์ ☒ อนุสิทธิบัตร	☒ ไทย ☐ ต่างประเทศ	ครบกำหนดระยะเวลายื่นแก้ไขพร้อมส่งเอกสารเพิ่มเติม วันที่ 10 สิงหาคม 2563
☒ ยกเว้นค่าธรรมเนียม ☒ หนังสือมอบอำนาจช่วงหรือหนังสือมอบอำนาจเฉพาะกาล** (กรณีผู้ได้รับการมอบอำนาจจากผู้ยื่นคำขอได้มอบอำนาจช่วงให้บุคคลอื่นเป็นผู้ยื่นคำขอแทน) (ถ้ามี)	รายการเอกสาร ☒ แบบพิมพ์คำขอรับสิทธิบัตร/อนุสิทธิบัตร** ☒ (รายละเอียดการประดิษฐ์ ☒ ข้อถ้อยสิทธิ ☒ รูปเขียน ☒ บทสรุปการประดิษฐ์) ☐ รายละเอียดภาษาต่างประเทศ (☐ มี ☐ ไม่มี) ☐ คำแปล (☐ มี ☐ ไม่มี) ☐ คำขอถ้อยสิทธิวันยื่นคำขอในต่างประเทศเป็นครั้งแรก เอกสารประกอบคำขอ ☒ ดัชนีฉบับหรือสำเนาของหนังสือมอบอำนาจ ☒ ตามกฎหมายไทย (☐ มี ☒ ไม่มี) ☐ ตามกฎหมายต่างประเทศ/รับรองโดยโนตารีปับลิก (☐ มี ☐ ไม่มี) ☐ คำแปล (☐ มี ☐ ไม่มี) ☒ หนังสือ/สัญญาโอนสิทธิ ☒ ตามกฎหมายไทย (☐ มี ☒ ไม่มี) ☐ ตามกฎหมายต่างประเทศ/รับรองโดยโนตารีปับลิก (☐ มี ☐ ไม่มี) ☐ คำแปล (☐ มี ☐ ไม่มี) ☒ คำรับรองเกี่ยวกับสิทธิขอรับสิทธิบัตร/อนุสิทธิบัตร (ใช้ในกรณีที่ผู้ประดิษฐ์เป็นผู้ขอเองหรือขอร่วมกับบุคคลอื่นๆ) ☐ ดัชนีฉบับหรือสำเนาหนังสือรับรองนิติบุคคล ซึ่งออกให้ไม่เกิน 6 เดือน หรือเอกสารตามกฎหมายว่าด้วยการจัดตั้งนิติบุคคล (☐ มี ☐ ไม่มี) ☒ ส่วนบัตรประจักษ์ตัวประชาชนพร้อมรับรองสำเนาถูกต้อง ☒ ผู้ประดิษฐ์ (☐ มี ☒ ไม่มี) ☒ ผู้ขอรับสิทธิบัตร (☐ มี ☒ ไม่มี) (เช่น กรรมการบริษัท หัวหน้าส่วนราชการ ผู้ได้รับมอบอำนาจให้ลงนามแทน อธิการบดี ประธานมูลนิธิ เป็นต้น) ☒ เอกสารอื่นใดที่ผู้ยื่นคำขอได้อ้างถึงในคำขอและเป็นประโยชน์ในการพิจารณาคำขอ (ถ้ามี) : ขาดคำสั่งแต่งตั้งฯ, ขาดราชกิจจานุเบกษา.....	

หมายเหตุ : 1. กรณีที่คำขอหรือเอกสารหลักฐานไม่ถูกต้องครบถ้วน เจ้าหน้าที่จะจัดทำบันทึกความบกพร่องของรายการเอกสารหรือเอกสารหลักฐานที่ต้องยื่นเพิ่มเติม โดยผู้ยื่นคำขอจะต้องดำเนินการแก้ไขและยื่นเอกสารเพิ่มเติมภายในกำหนดระยะเวลา 90 วันนับแต่วันที่ได้นำคำขอรับสิทธิบัตรหรืออนุสิทธิบัตร และในกรณีที่ผู้ยื่นคำขอไม่ดำเนินการส่งเอกสารเพิ่มเติมให้ครบถ้วนภายในกำหนดระยะเวลาดังกล่าวให้ถือว่าผู้ยื่นคำขอละทิ้งคำขอ โดยเจ้าหน้าที่จะดำเนินการคืนคำขอให้แก่ผู้ยื่นคำขอพร้อมทั้งแจ้งเหตุแห่งการคืนคำขอและสิทธิในการอุทธรณ์ให้ทราบด้วย

2. ในกรณีที่ผู้ยื่นคำขอต้องนำเอกสารหรือหลักฐานหลายรายการมายื่นเพิ่มเติมให้ครบถ้วน ให้ผู้ยื่นคำขอนำเอกสารหรือหลักฐานมายื่นพร้อมกันทั้งหมดในคราวเดียวกัน

3. ในกรณีที่จะต้องส่งสำเนาเอกสารหลักฐาน ให้ผู้ยื่นคำขอรับรองความถูกต้องของสำเนาเอกสารหลักฐานนั้นด้วย

4. ในกรณีที่จะต้องส่งเอกสารเป็นภาษาต่างประเทศ ให้ผู้ยื่นคำขอส่งเอกสารนั้นพร้อมด้วยคำแปลเป็นภาษาไทยโดยมีคำรับรองของผู้แปลว่าเป็นคำแปลที่ถูกต้อง

5. ในกรณีที่ผู้ยื่นคำขอหรือตัวแทนผู้รับมอบอำนาจไม่ได้เป็นผู้ยื่นคำขอด้วยตนเองโดยมอบหมายให้บุคคลอื่นเป็นผู้ยื่นคำขอแทน ควรมีหนังสือมอบอำนาจช่วงหรือหนังสือมอบอำนาจเฉพาะการให้บุคคลนั้นมีอำนาจยื่นคำขอและลงนามในบันทึกข้อตกลงการรับคำขอแทนผู้ยื่นคำขอหรือตัวแทนได้ เพราะหากคำขอไม่ถูกต้องหรือเอกสารหลักฐานไม่ครบถ้วนและบุคคลผู้ยื่นคำขอไม่มีอำนาจลงนามในบันทึกดังกล่าว เจ้าหน้าที่ไม่อาจรับคำขอของท่านไว้ได้



สถาบันทรัพย์สินทางปัญญาแห่งจุฬาลงกรณ์มหาวิทยาลัย
254 อาคารเฉลิมราชกุมารี 60 พรรษา (อาคารจามจุรี 10) ชั้น 2 ถนนพญาไท แขวงวังใหม่ เขตปทุมวัน กรุงเทพฯ 10330
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พฤษภาคม 2563

สำเนา

เรื่อง ขอฟ่อนผันการส่งเอกสารประกอบคำขอรับอนุสิทธิบัตร

12 พ.ค. 2563

อนท.

เรียน ผู้อำนวยการกองสิทธิบัตร

ด้วยสถาบันทรัพย์สินทางปัญญาแห่งจุฬาลงกรณ์มหาวิทยาลัยได้รับมอบหมายจาก จุฬาลงกรณ์มหาวิทยาลัยให้ดำเนินการยื่นคำขอรับอนุสิทธิบัตร เรื่อง “ข้าวไฟฟ้าพิมพ์สกรีนสำหรับการตรวจวิเคราะห์วิตามินชนิดละลายในไขมัน และกระบวนการผลิต” โดยมีข้าพเจ้า นายวี วงษ์เทียนกุล ตัวแทนสิทธิบัตรเลขที่ 2520 เป็นผู้ดำเนินการ ซึ่งขณะนี้อยู่ระหว่างดำเนินการให้ผู้ที่เกี่ยวข้องลงนามในสัญญาโอนสิทธิในการขอรับอนุสิทธิบัตร และหนังสือมอบอำนาจซึ่งจำเป็นต้องใช้ระยะเวลาหนึ่งในการดำเนินการ แต่เนื่องจากการผลงานนี้ทางผู้ประดิษฐ์มีความประสงค์จะเผยแพร่ผลงานวิจัย ซึ่งหากมีการนำไปเผยแพร่ผลงานก่อนที่จะได้มีการยื่นคำขอรับอนุสิทธิบัตร ข้าพเจ้าเกรงว่าจะมีผลต่อการพิจารณารับจดทะเบียนของคำขอรับอนุสิทธิบัตรฉบับนี้ได้

ด้วยเหตุผลดังกล่าวข้างต้น ข้าพเจ้าจึงเรียนมาเพื่อโปรดพิจารณาอนุมัติขอฟ่อนผันการยื่นเอกสารประกอบคำขอรับอนุสิทธิบัตรพร้อมกับคำขอรับสิทธิบัตรด้วย จักขอบพระคุณยิ่ง

ขอแสดงความนับถือ

(นายวี วงษ์เทียนกุล)

ผู้รับมอบอำนาจ

ตัวแทนสิทธิบัตร เลขที่ 2520



คำขอรับสิทธิบัตร/อนุสิทธิบัตร

สำเนา

- ☐ การประดิษฐ์
☐ การออกแบบผลิตภัณฑ์
☒ อนุสิทธิบัตร

ข้าพเจ้าผู้ลงลายมือชื่อในคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้
ขอรับสิทธิบัตร/อนุสิทธิบัตร ตามพระราชบัญญัติสิทธิบัตร พ.ศ. 2522
แก้ไขเพิ่มเติมโดยพระราชบัญญัติสิทธิบัตร (ฉบับที่ 2) พ.ศ. 2535
และ พระราชบัญญัติสิทธิบัตร (ฉบับที่ 3) พ.ศ. 2542

สำหรับเจ้าหน้าที่

วันรับคำขอ 12 พ.ค. 2563

เลขที่คำขอ

วันยื่นคำขอ 12 พ.ค. 2563

2003000999

สัญลักษณ์จำแนกการประดิษฐ์ระหว่างประเทศ

ใช้กับแบบผลิตภัณฑ์

ประเภทผลิตภัณฑ์

วันประกาศโฆษณา

เลขที่ประกาศโฆษณา

วันออกสิทธิบัตร/อนุสิทธิบัตร

เลขที่สิทธิบัตร/อนุสิทธิบัตร

ลายมือชื่อเจ้าหน้าที่

1. ชื่อที่แสดงถึงการประดิษฐ์/การออกแบบผลิตภัณฑ์ ชื่อไฟฟ้าพืชมัสกรีนสำหรับการตรวจวิเคราะห์ความชื้นดินและปริมาณไนโตรเจน และกระบวนการผลิต

2. คำขอรับสิทธิบัตรการออกแบบผลิตภัณฑ์นี้เป็นคำขอสำหรับแบบผลิตภัณฑ์อย่างเดียวกันและเป็นคำขอลำดับที่
ในจำนวน คำขอ ที่ยื่นในคราวเดียวกัน

3. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ☐ บุคคลธรรมดา ☐ นิติบุคคล ☒ หน่วยงานรัฐ ☐ มูลนิธิ ☐ อื่นๆ

ชื่อ จุฬาลงกรณ์มหาวิทยาลัย

ที่อยู่ 254 ถนนพญาไท

ตำบล/แขวง วังใหม่

อำเภอ/เขต ปทุมวัน

จังหวัด กรุงเทพฯ

รหัสไปรษณีย์ 10330 ประเทศ ไทย

อีเมล

☐ เลขประจำตัวประชาชน ☐ เลขทะเบียนนิติบุคคล ☒ เลขประจำตัวผู้เสียภาษีอากร

9 1 0 0 0 1 5 9 0 7 2

☒ เพิ่มเติม (ตั้งแนบ)

ในกรณีที่มีการทำ สือสารกับท่าน ท่านสะดวกใช้หนังสือ ☐ อีเมล ☒ อื่นๆ

4. สิทธิในการขอรับสิทธิบัตร/อนุสิทธิบัตร

☐ ผู้ประดิษฐ์/ผู้ออกแบบ ☒ ผู้รับโอน ☐ ผู้ขอรับสิทธิโดยเหตุอื่น

5. ตัวแทน (ถ้ามี)

ชื่อ นายวี วัชย์เย็นกุล

ที่อยู่ สถาบันทรัพยากรชีวภาพแห่งจุฬาลงกรณ์มหาวิทยาลัย 254 อาคารเฉลิมราชกุมารี 60 พรรษา (อาคารจามจุรี 10) ชั้น 2 ถนนพญาไท

ตำบล/แขวง วังใหม่

อำเภอ/เขต ปทุมวัน

จังหวัด กรุงเทพฯ

รหัสไปรษณีย์ 10330 ประเทศ ไทย

อีเมล Veepayupong.W@chula.ac.th

เลขประจำตัวประชาชน 3 1 9 1 1 0 0 0 7 1 7 1 1

☒ เพิ่มเติม (ตั้งแนบ)

6. ผู้ประดิษฐ์/ผู้ออกแบบผลิตภัณฑ์ ☐ ชื่อและที่อยู่เดียวกันกับผู้ขอ

ชื่อ ดร.อับดุลฮาดี้ ยะโก๊ะ

ที่อยู่ ภาควิชาเคมี คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย 254 ถนนพญาไท

ตำบล/แขวง วังใหม่

อำเภอ/เขต ปทุมวัน

จังหวัด กรุงเทพฯ

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อีเมล y.abdulhadee@outlook.com

เลขประจำตัวประชาชน 1 9 6 9 9 0 0 1 3 3 6 0 9

☒ เพิ่มเติม (ตั้งแนบ)

7. คำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้แยกจากหรือเกี่ยวข้องกับคำขอเดิม

ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอให้ถือว่าได้ยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ ในวันเดียวกับคำขอรับสิทธิบัตร

เลขที่

วันยื่น

เพราะคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้แยกจากหรือเกี่ยวข้องกับคำขอเดิมเพราะ

☐ คำขอเดิมมีการประดิษฐ์หลายอย่าง ☐ ถูกคัดค้านเนื่องจากผู้ขอไม่มีสิทธิ ☐ ขอเปลี่ยนแปลงประเภทของสิทธิ

หมายเหตุ ในกรณีที่ไม่มีอาชญากรรมละเมิดได้ครบถ้วน ให้จัดทำเป็นเอกสารแนบท้ายแบบพิมพ์นี้โดยระบุหมายเลขกำกับข้อและหัวข้อที่แสดงรายละเอียดเพิ่มเติมดังกล่าวด้วย

สำหรับเจ้าหน้าที่

จำแนกประเภทสิทธิบัตร/อนุสิทธิบัตร

☐ กลุ่มวิศวกรรม

สิทธิบัตรการประดิษฐ์ (วิศวกรรม)

สิทธิบัตรการประดิษฐ์ (ไฟฟ้า)

สิทธิบัตรการประดิษฐ์ (ฟิสิกส์)

☐ กลุ่มเคมี

สิทธิบัตรการประดิษฐ์ (เคมีเทคนิค)

สิทธิบัตรการประดิษฐ์ (ปิโตรเคมี)

สิทธิบัตรการประดิษฐ์ (เทคโนโลยีชีวภาพ)

สิทธิบัตรการประดิษฐ์ (เภสัชภัณฑ์)

สิทธิบัตรการออกแบบ

☐ สิทธิบัตรการออกแบบ (ออกแบบผลิตภัณฑ์ 1)

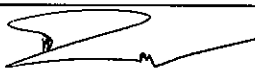
☐ สิทธิบัตรการออกแบบ (ออกแบบผลิตภัณฑ์ 2)

☐ สิทธิบัตรการออกแบบ (ออกแบบผลิตภัณฑ์ 3)

อนุสิทธิบัตร

☐ อนุสิทธิบัตร (วิศวกรรม)

☐ อนุสิทธิบัตร (เคมี)

8. การยื่นคำขออนุญาตนำเข้า ☐ PCT ☐ เพิ่มเติม (ดังแนบ)				
วันยื่นคำขอ	เลขที่คำขอ	ประเทศ	สัญลักษณ์จำแนกการประดิษฐ์ระหว่างประเทศ	สถานะคำขอ
8.1				
8.2				
8.3				
8.4 ☐ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรขอสิทธิให้ถือว่าได้ยื่นคำขอนี้ในวันที่ได้ยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรในต่างประเทศเป็นครั้งแรกโดย ☐ ได้ยื่นเอกสารหลักฐานพร้อมคำขอนี้ ☐ ขอยื่นเอกสารหลักฐานหลังจากวันยื่นคำขอนี้				
9. การแสดงการประดิษฐ์หรือการออกแบบผลิตภัณฑ์ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรได้แสดงการประดิษฐ์ที่หน่วยงานของรัฐเป็นผู้จัด วันแสดง วันเปิดงานแสดง ผู้จัด				
10. การประดิษฐ์เกี่ยวกับจุลชีพ				
10.1 เลขทะเบียนฝากเก็บ		10.2 วันที่ฝากเก็บ		10.3 สถาบันฝากเก็บ/ประเทศ
11. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอยื่นเอกสารภาษาต่างประเทศก่อนในวันยื่นคำขอนี้ และจะจัดยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ที่จัดทำเป็นภาษาไทยภายใน 90 วัน นับจากวันยื่นคำขอนี้ โดยขอเป็นภาษา ☐ อังกฤษ ☐ ฝรั่งเศส ☐ เยอรมัน ☐ ญี่ปุ่น ☐ อื่นๆ				
12. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอให้อธิบดีประกาศโฆษณาคำขอรับสิทธิบัตร หรือรับจดทะเบียน และประกาศโฆษณาอนุสิทธิบัตรนี้ หลังจากวันที่ ☐ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรขอให้ใช้รูปเขียนหมายเลข ในการประกาศโฆษณา				
13. คำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ประกอบด้วย			14. เอกสารประกอบคำขอ	
ก. แบบพิมพ์คำขอ		3	หน้า	☐ เอกสารแสดงสิทธิในการขอรับสิทธิบัตร/อนุสิทธิบัตร
ข. รายละเอียดการประดิษฐ์ หรือคำพรรณนาแบบผลิตภัณฑ์		4	หน้า	☐ หนังสือรับรองการแสดงการประดิษฐ์/การออกแบบผลิตภัณฑ์
ค. ข้อถ้อยสิทธิ		1	หน้า	☐ หนังสือมอบอำนาจ
ง. รูปเขียน	5	รูป	3	หน้า
จ. ภาพแสดงแบบผลิตภัณฑ์ ☐ รูปเขียน ☐ ภาพถ่าย		รูป	หน้า	☐ เอกสารรายละเอียดเกี่ยวกับจุลชีพ
ฉ. บทสรุปการประดิษฐ์		1	หน้า	☐ เอกสารการขอนับวันยื่นคำขอในต่างประเทศเป็นวันยื่นคำขอในประเทศไทย
				☐ เอกสารขอเปลี่ยนแปลงประเภทของสิทธิ
				☐ เอกสารอื่นๆ
15. ข้าพเจ้าขอรับรองว่า ☒ การประดิษฐ์นี้ไม่เคยยื่นขอรับสิทธิบัตร/อนุสิทธิบัตรมาก่อน ☐ การประดิษฐ์นี้ได้พัฒนาปรับปรุงมาจาก				
16. ลายมือชื่อ ☐ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ☒ ตัวแทน  (นายวี วงษ์เทียนกุล)				

หมายเหตุ บุคคลใดยื่นขอรับสิทธิบัตรการประดิษฐ์หรือการออกแบบผลิตภัณฑ์ หรืออนุสิทธิบัตร โดยการแสดงข้อความอันเป็นเท็จแก่พนักงานเจ้าหน้าที่ เพื่อให้ได้ไปซึ่งสิทธิบัตรหรืออนุสิทธิบัตร ต้องระวางโทษจำคุกไม่เกินหกเดือน หรือปรับไม่เกินห้าพันบาท หรือทั้งจำทั้งปรับ

ใบต่อแนบท้าย แบบ สป/สพ/อสป/001-ก

3. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร

ชื่อ สำนักงานคณะกรรมการส่งเสริมวิทยาศาสตร์ วิจัยและนวัตกรรม (สกสว.)
อยู่ที่ 9797/17-21 ชั้น 14 อาคาร เอส เอ็ม ทาวเวอร์ ถนนพหลโยธิน
ตำบล/แขวง สามเสนใน อำเภอ/เขต พญาไท จังหวัด กรุงเทพฯ รหัสไปรษณีย์ 10400 ประเทศไทย
อีเมล -
เลขประจำตัวผู้เสียภาษีอากร 009-4-00250119-3
3.1 สัญชาติ ไทย 3.2 โทรศัพท์ 02-278-8200 5.3 โทรสาร 02-298-0476

5. ตัวแทน(ถ้ามี)

ชื่อ นายมงคล แก้วมหา
อยู่ที่ สถาบันทรัพยากรสิ่งมีชีวิตแห่งจุฬาลงกรณ์มหาวิทยาลัย 254 อาคารเฉลิมราชกุมารี 60 พรรษา (จามจุรี 10) ชั้น 2 ถนนพญาไท
ตำบล/แขวง วังใหม่ อำเภอ/เขต ปทุมวัน จังหวัด กรุงเทพฯ รหัสไปรษณีย์ 10330 ประเทศไทย
อีเมล Mongkol.K@chula.ac.th
เลขประจำตัวประชาชน 3840100084785
5.1 ตัวแทนเลขที่ 1453 5.2 โทรศัพท์ 02-218-4195-7 5.3 โทรสาร 02-218-4198

6. ผู้ประดิษฐ์/ผู้ออกแบบผลิตภัณฑ์

ชื่อ ศ.ดร.อรรณพ ชัยลาภกุล
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ตำบล/แขวง วังใหม่ อำเภอ/เขต ปทุมวัน จังหวัด กรุงเทพฯ รหัสไปรษณีย์ 10330 ประเทศไทย
อีเมล corawon@chula.ac.th
เลขประจำตัวประชาชน 3101200984114

ชื่อ รศ.ดร.วีณา เสียงเพระ
อยู่ที่ ภาควิชาเคมี คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย 254 ถนนพญาไท
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อีเมล weenasi@hotmail.com
เลขประจำตัวประชาชน 3760500408244

รายละเอียดการประดิษฐ์

ชื่อที่แสดงถึงการประดิษฐ์

ขั้วไฟฟ้าพิมพ์สกรีนสำหรับการตรวจวิเคราะห์วิตามินชนิดละลายในไขมัน และกระบวนการผลิต
สาขาวิทยาการที่เกี่ยวข้องกับการประดิษฐ์

- 5 เคมี ในส่วนที่เกี่ยวข้องกับขั้วไฟฟ้าพิมพ์สกรีนสำหรับการตรวจวิเคราะห์วิตามินชนิดละลายในไขมัน และกระบวนการผลิต

ภูมิหลังของศิลปะหรือวิทยาการที่เกี่ยวข้อง

- วิตามินถูกแบ่งประเภทออกเป็น 2 ประเภทคือ วิตามินที่ละลายในน้ำ และวิตามินที่ละลายในไขมัน ขึ้นอยู่กับความสามารถในการดูดซึม และการนำไปใช้ของร่างกาย วิตามินละลายในน้ำต้องใช้น้ำในการดูดซึม
10 วิตามินเข้าร่างกาย ในขณะที่วิตามินละลายไขมัน ต้องใช้ไขมันที่มาจากอาหารช่วยในการดูดซึมวิตามินเข้าสู่ร่างกาย โดยทั่วไปปริมาณของวิตามินชนิดละลายในน้ำที่มากเกินไปจะถูกร่างกายขับปริมาณส่วนเกินออกมาทางปัสสาวะและเหงื่อได้ ในขณะที่วิตามินชนิดละลายในไขมัน (วิตามินเอ ดี อี และเค) บางชนิดจะถูกสะสมในร่างกายหากได้รับในปริมาณที่มากเกินไปจนก่อให้เกิดผลข้างเคียงต่าง ๆ หรือบางชนิดอาจก่อให้เกิดโรคหากได้รับในปริมาณที่ไม่เพียงพอ เช่น วิตามินดี โดยจากงานวิจัยพบว่า ระดับของวิตามินดีในร่างกายที่ไม่เพียงพอ
15 อาจจะทำให้เกิดปัญหาในเรื่องกระดูกและฟัน เช่น โรคกระดูกอ่อน กระดูกพรุน รวมถึงเพิ่มความเสี่ยงต่อบางโรค

- วิธีมาตรฐานทางเคมีวิเคราะห์สำหรับการตรวจวัดวิตามินดีได้ถูกกำหนดโดยสถาบัน AOACI (The association of official analytic chemists international) โดยอาศัยหลักการของโครมาโตกราฟีของเหลว (liquid chromatography) เช่นวิธี 992.26 สำหรับการตรวจวัดวิตามินดี 3 ในนมพร้อมดื่มของเด็กทารก (AOAC, 2007b) วิธี 995.05 สำหรับการตรวจวัดวิตามินดีในนมและอาหารเหลวสำหรับเด็กทารก (AOAC, 2007a) และวิธี 2002.05 สำหรับการตรวจวัดวิตามินดี 3 ในอาหาร (นมและชีส) (AOAC, 2007d) ด้วยเหตุนี้จึงทำให้หลายงานวิจัยมุ่งเน้นที่จะพัฒนาวิธีสำหรับการตรวจวัดวิตามินดีโดยอาศัยเครื่องโครมาโตกราฟีร่วมกับเทคนิคต่าง ๆ เช่น เทคนิคแมสสเปกโตรเมทรี (mass spectrometry) เป็นต้น แต่เนื่องจากวิธีเหล่านี้ล้วนแล้วแต่ต้องอาศัยเครื่องมือที่มีราคาแพง ยุ่งยากซับซ้อน และใช้เวลาในการวิเคราะห์นาน ด้วยเหตุนี้
25 ทำให้นักวิจัยมุ่งเน้นที่จะพัฒนาวิธีวิเคราะห์อื่น ๆ ที่จะสามารถทดแทนวิธีมาตรฐานเหล่านั้น ทั้งนี้การตรวจวัดด้วยวิธีทางเคมีไฟฟ้า เป็นอีกหนึ่งวิธีที่ได้รับความนิยมเนื่องจากข้อดีของวิธีในเรื่องของความไวและความถูกต้องในการตรวจวิเคราะห์ การทดสอบที่มารทำได้ง่าย การตอบสนองที่รวดเร็ว และเครื่องมือขนาดเล็กพกพาได้ ในปัจจุบันการตรวจวิเคราะห์วิตามินดีด้วยเทคนิคทางเคมีไฟฟ้า ยังไม่ค่อยแพร่หลายนัก เนื่องจากข้อจำกัดในเรื่องของความสามารถในการละลายของวิตามินดีที่จะต้องละลายในไขมัน จึงต้องใช้สารละลาย
30 อินทรีย์ซึ่งนำไฟฟ้าได้น้อยมากเป็นตัวทำละลาย นอกจากนั้น จากรายงานการวิจัยที่ผ่านมา พบว่า วิตามินดีมีแนวโน้มที่จะเกิดการดูดซับที่บริเวณขั้วไฟฟ้าในระหว่างกระบวนการถ่ายโอนอิเล็กตรอน ทำให้เกิดการกีดขวางขั้วไฟฟ้า (electrode fouling) จากปรากฏการณ์ที่เกิดขึ้นส่งผลให้ไม่สามารถใช้ขั้วไฟฟ้าเดิมสำหรับการตรวจวิเคราะห์ซ้ำได้อีก ซึ่งไม่เป็นผลดีสำหรับการวิเคราะห์ที่ต้องมีการตรวจวัดซ้ำๆ ในปริมาณมาก ดังนั้น การดัดแปรพื้นผิวของขั้วไฟฟ้าเพื่อให้สามารถตรวจวัดซ้ำได้จึงเป็นทางออกที่ดีในการแก้ปัญหาที่เกิดขึ้น

- 35 ผู้ประดิษฐ์ได้ทำการค้นคว้าหาข้อมูลในการดัดแปรพื้นผิวขั้วไฟฟ้าเพื่อป้องกันการกีดขวางขั้วไฟฟ้าที่อาจเกิดขึ้น จากการค้นคว้าพบว่าการเติมสารเคมีบางชนิดสามารถลดการเกิดการกีดขวางขั้วไฟฟ้าได้ เช่น การดัดแปรพื้นผิวด้วย Poly(3,4-ethylenedioxythiophene) หรือ PEDOT โดยอาศัยการโพลีเมอไรซ์ด้วยเทคนิคทางเคมีไฟฟ้า แม้ว่าวิธีนี้จะได้รับความนิยมสำหรับการดัดแปรขั้วไฟฟ้า แต่วิธีนี้ต้องมีการควบคุมการโพลีเมอ

ไรซ์ที่แน่นอน เนื่องจากมีหลายปัจจัยที่ส่งผลต่อการเกิดโพลีเมอร์ ส่งผลให้เกิดความไม่แน่นอนของผลการวิเคราะห์ นอกเหนือจาก PEDOT แล้ว นาฟิออนก็จัดเป็นอีกหนึ่งสารดัดแปรที่ได้รับความนิยมในเชิงเคมีไฟฟ้า โดยนาฟิออนนั้นจะมีโครงสร้าง 2 ส่วน คือส่วนที่มีความไม่ชอบน้ำเป็นแกนหลัก และมีกิ่งก้านในโครงสร้างเป็นหมู่ซัลโฟนิก (sulfonic) ซึ่งเป็นหมู่ที่ความชอบน้ำ นาฟิออนมีคุณสมบัติที่โดดเด่นด้วยกันหลายประการ เช่น

5 มีความเฉื่อยต่อสารเคมีอื่น สามารถเร่งปฏิกิริยาและสามารถป้องกันการเกิดการกัดกร่อนของขั้วไฟฟ้าได้ นอกเหนือจากนี้ การดัดแปรพื้นผิวด้วยนาฟิออนยังสามารถทำได้ง่าย จึงลดข้อจำกัดที่ยุ่งยากในการเตรียมขั้วไฟฟ้า

จากคุณสมบัติที่ดีของนาฟิออนทำให้หลายๆงานวิจัยมุ่งเน้นที่จะพัฒนาวัสดุผสมระหว่างนาฟิออนและวัสดุระดับนาโนเพื่อสร้างให้เกิดเป็นวัสดุคอมโพสิตระดับนาโนเมตร ที่มีคุณสมบัติที่ดีของทั้งสองวัสดุในวัสดุชิ้นเดียว เช่น การนำเอากราฟีนซึ่งเป็นหนึ่งในอีกรูปของคาร์บอนซึ่งมีคุณสมบัติที่โดดเด่นด้วยกันหลายประการในเชิงเคมีไฟฟ้า จากรายงานที่ผ่านมาพบว่ากราฟีนนาฟิออนเข้าไปในกราฟีนจะช่วยเพิ่มการกระจายตัวของกราฟีนให้ดีขึ้นซึ่งจะช่วยเพิ่มประสิทธิภาพและเสถียรภาพให้กับกราฟีน ด้วยข้อดีดังที่กล่าวมาไม่ว่าจะเป็นคุณสมบัติในการรับส่งอิเล็กตรอนที่ดีของกราฟีนร่วมกับคุณสมบัติของนาฟิออนทำให้วัสดุผสมนาโนชนิดนี้เป็นที่น่าสนใจ

15 ลักษณะและความมุ่งหมายของการประดิษฐ์

การประดิษฐ์นี้เกี่ยวกับขั้วไฟฟ้าพิมพ์สกรีนสำหรับการตรวจวิเคราะห์วิตามินชนิดละลายในไขมัน ซึ่งประกอบด้วย ขั้วไฟฟ้าทำงาน (1) มีลักษณะเป็นวงกลมที่มีเส้นผ่านศูนย์กลาง 5 มิลลิเมตร ที่พิมพ์สกรีนด้วยวัสดุคอมโพสิตระดับนาโนเมตรของกราฟีนและนาฟิออน และเชื่อมต่ออยู่กับแผ่นนำไฟฟ้า (6) ที่พิมพ์สกรีนด้วยหมึกของ ซิลเวอร์/ซิลเวอร์คลอไรด์สำหรับการต่อเข้ากับเครื่องโพเทนชิโอสแตท (potentiostat),

20 ขั้วไฟฟ้าเผชิญหน้า (2) มีลักษณะเป็นเส้นโค้งความหนา 1 มิลลิเมตร ที่พิมพ์สกรีนด้วยวัสดุคอมโพสิตระดับนาโนเมตรของกราฟีนและนาฟิออน ซึ่งล้อมรอบขั้วไฟฟ้าทำงาน (1) อยู่ 3 ใน 4 ส่วน และเชื่อมต่ออยู่กับแผ่นนำไฟฟ้า (6) สำหรับการต่อเข้ากับเครื่องโพเทนชิโอสแตท (potentiostat), ขั้วไฟฟ้าอ้างอิง (3) มีลักษณะเป็นเส้นโค้งความหนา 1 มิลลิเมตร พิมพ์สกรีนด้วยหมึกของ ซิลเวอร์/ซิลเวอร์คลอไรด์ที่ล้อมรอบขั้วไฟฟ้าทำงาน (1) อยู่ 1 ใน 4 ส่วน และเชื่อมต่ออยู่กับแผ่นนำไฟฟ้า (6) สำหรับการต่อเข้ากับเครื่องโพเทนชิโอสแตท

25 (potentiostat) โดยขั้วไฟฟ้าทั้งสาม (1,2,3) พิมพ์สกรีนอยู่บนฐานพีวีซี (5) ที่มีความไม่ชอบน้ำ ทำหน้าที่เพิ่มความแข็งแรงของขั้วไฟฟ้า และทำหน้าที่รองรับวิตามินชนิดละลายในไขมันที่ต้องการทดสอบ และมีการกันขอบเขตการไหลด้วยการพิมพ์ ไซลิ่ง (4) ที่ทำหน้าที่กันขอบเขตของวิตามินชนิดที่ละลายในไขมันให้ทดสอบให้อยู่เฉพาะในบริเวณที่มีการพิมพ์ไซลิ่งเท่านั้น ซึ่งผลการวิเคราะห์พบว่าขั้วไฟฟ้าที่ประดิษฐ์ขึ้นสามารถเพิ่มประสิทธิภาพการตรวจวัดวิตามินชนิดละลายในไขมันได้ และเมื่อทดสอบประสิทธิภาพในการใช้ขั้วไฟฟ้าพบว่าสามารถใช้งานซ้ำได้มากถึง 10 ครั้งโดยไม่พบปัญหาจากการดูดซับที่ผิวหน้าขั้วของสาร เหมาะสำหรับงานที่ต้องมีการตรวจวัดซ้ำ ๆ เช่นในอุตสาหกรรมอาหารและยาที่ต้องการควบคุมคุณภาพตลอดเวลา

ความมุ่งหมายของการประดิษฐ์นี้ คือ การพัฒนาขั้วไฟฟ้าที่สามารถลดการเกิดการดูดซับของสารที่ผิวหน้าของขั้วไฟฟ้าที่เกิดจากการตรวจวัดวิตามินที่ละลายในไขมัน โดยอาศัยขั้วไฟฟ้าที่ดัดแปรพื้นผิวด้วยวัสดุคอมโพสิตระหว่างกราฟีนและนาฟิออน

35 การเปิดเผยการประดิษฐ์โดยสมบูรณ์

การประดิษฐ์นี้เกี่ยวกับ ขั้วไฟฟ้าพิมพ์สกรีนสำหรับการตรวจวิเคราะห์วิตามินชนิดละลายในไขมันซึ่งประกอบด้วย

- ขั้วไฟฟ้าทำงาน (1) มีลักษณะเป็นวงกลมที่มีเส้นผ่านศูนย์กลาง 5 มิลลิเมตร ที่พิมพ์สกรีนด้วยวัสดุคอมโพสิตระดับนาโนเมตรของกราฟีนและนาฟิออน และเชื่อมต่ออยู่กับแผ่นนำไฟฟ้า (6) ที่พิมพ์สกรีนด้วยหมึกของ ซิลเวอร์/ซิลเวอร์คัลโรไต์สำหรับการต่อเข้ากับเครื่องโพเทนชิโอสแตท (potentiostat)

5 - ขั้วไฟฟ้าเผชิญหน้า (2) มีลักษณะเป็นเส้นโค้งความหนา 1 มิลลิเมตร ที่พิมพ์สกรีนด้วยวัสดุคอมโพสิตระดับนาโนเมตรของกราฟีนและนาฟิออน ซึ่งล้อมรอบขั้วไฟฟ้าทำงาน (1) อยู่ 3 ใน 4 ส่วน และเชื่อมต่ออยู่กับแผ่นนำไฟฟ้า (6) สำหรับการต่อเข้ากับเครื่องโพเทนชิโอสแตท (potentiostat)

- ขั้วไฟฟ้าอ้างอิง (3) มีลักษณะเป็นเส้นโค้งความหนา 1 มิลลิเมตร พิมพ์สกรีนด้วยหมึกของซิลเวอร์/ซิลเวอร์คัลโรไต์ที่ล้อมรอบขั้วไฟฟ้าทำงาน (1) อยู่ 1 ใน 4 ส่วน และเชื่อมต่ออยู่กับแผ่นนำไฟฟ้า (6) สำหรับการต่อเข้ากับเครื่องโพเทนชิโอสแตท (potentiostat)

10 โดยขั้วไฟฟ้าทั้งสาม (1,2,3) พิมพ์สกรีนอยู่บนฐานพีวีซี (5) ที่มีความไม่ชอบน้ำ ทำหน้าที่เพิ่มความแข็งแรงของขั้วไฟฟ้า และทำหน้าที่รองรับวิตามินชนิดละลายในไขมันที่ต้องการทดสอบ และมีการกันขอบเขตการไหลด้วยการพิมพ์ ไซมิง (4) ที่ทำหน้าที่กันขอบเขตของวิตามินชนิดที่ละลายในไขมันให้ทดสอบให้อยู่เฉพาะในบริเวณที่มีการพิมพ์ไซมิงเท่านั้น (ดังแสดงในรูปที่ 1)

15 ขั้วไฟฟ้าพิมพ์สกรีนสำหรับการตรวจวิเคราะห์วิตามินชนิดละลายในไขมัน ตามการประดิษฐ์นี้ ที่ซึ่งวิตามินชนิดละลายในไขมัน ได้แก่ วิตามินเอ วิตามินดี วิตามินอี และวิตามินเค ซึ่งถูกทำละลายด้วยตัวทำละลายผสมระหว่าง 55-65% เอทานอล และ 35-45% โซเดียมเปอร์คลอเรตความเข้มข้น 0.1-1 โมลาร์

กระบวนการผลิตขั้วไฟฟ้าพิมพ์สกรีนสำหรับการตรวจวิเคราะห์วิตามินชนิดละลายในไขมันตามการประดิษฐ์นี้ ซึ่งมีขั้นตอนประกอบรวมด้วยดังนี้

20 ก. ทำการออกแบบขั้วไฟฟ้าด้วยโปรแกรมอโดบีอิลลัสเตรเตอร์ (Adobe illustrator) โดยลักษณะของขั้วไฟฟ้ามีลักษณะเป็นขั้วไฟฟ้าทำงาน (1) รูปแบบวงกลมที่มีเส้นผ่านศูนย์กลาง 5 มิลลิเมตร ซึ่งถูกล้อมรอบด้วยขั้วไฟฟ้าเผชิญหน้า (2) และขั้วไฟฟ้าอ้างอิง (3) และทำการพิมพ์ไซมิง (4) เส้นผ่านศูนย์กลาง 12 มิลลิเมตรล้อมรอบขั้วไฟฟ้าทั้งสาม (1,2,3) เพื่อกำหนดขอบเขตการไหลของสารละลาย

ข. ทำการพิมพ์ไซมิง (4) เพื่อกันขอบเขตการไหลบนฐานพีวีซี (5) ด้วยเครื่องพิมพ์ไซมิง

25 ค. ทำการผสมหมึกของวัสดุคาร์บอน-กราฟีน และ นาฟิออน 0.05-0.15 % โดยปริมาตรต่อปริมาตร ในอัตราส่วน 0.45-0.55 กรัมต่อ 80-120 ไมโครลิตร แล้วจึงคนจนหมึกของวัสดุผสมระหว่างกราฟีนและฟิออนผสมเป็นเนื้อเดียวกัน

ง. ทำการพิมพ์สกรีนขั้วไฟฟ้าทำงาน (1) และขั้วไฟฟ้าเผชิญหน้า (2) ที่พิมพ์สกรีนจากหมึกของวัสดุคอมโพสิตระดับนาโนเมตรของกราฟีนและนาฟิออนลงบนฐานพีวีซี (5) ที่ผ่านการพิมพ์ไซมิง (4) ลงไป จากนั้นนำไปอบที่อุณหภูมิ 45-60 องศาเซลเซียส เป็นเวลา 45-60 นาที

30 จ. ทำพิมพ์สกรีนขั้วไฟฟ้าอ้างอิงด้วยหมึกซิลเวอร์/ซิลเวอร์คัลโรไต์ ลงบนฐานพีวีซี (5) ที่ผ่านการพิมพ์สกรีนขั้วไฟฟ้าทำงาน (1) และขั้วไฟฟ้าเผชิญหน้า (2) จากนั้นจึงนำไปอบที่อุณหภูมิ 45-60 องศาเซลเซียส เป็นเวลา 45-60 นาที ได้เป็นขั้วไฟฟ้าพิมพ์สกรีนสำหรับการตรวจวิเคราะห์วิตามินชนิดละลายในไขมัน

ฉ. ทำการตัดขั้วไฟฟ้าที่ผ่านการอบเรียบร้อยแล้วเพื่อให้พร้อมต่อการใช้งาน

35 ช. ทำการเชื่อมต่อขั้วไฟฟ้าทั้ง 3 ขั้วเข้ากับโพเทนชิโอสแตท (potentiostat) และหยดสารละลายตัวอย่างที่ต้องการตรวจวัดซึ่งละลายอยู่ในตัวทำละลายผสมระหว่าง 55-65% เอทานอล และ 35-45% โซเดียมเปอร์คลอเรตความเข้มข้น 0.1-1 โมลาร์

การตรวจวัดและวิเคราะห์ผลทางเคมีไฟฟ้า

- ทำการตรวจวัดด้วยเทคนิคสแควร์เวฟโวลแทมเมทรี (square-wave voltammetry) โดยการให้ศักย์ไฟฟ้าจาก 0.5 โวลต์ถึง 1 โวลต์ และมีการตั้งค่าแอมพลิจูด ค่าการเพิ่มขึ้นของศักย์ไฟฟ้า และค่าความถี่ที่ 25 มิลลิโวลต์ 4 มิลลิโวลต์ และ 10 เฮิรตซ์ ตามลำดับ โดยในการตรวจวัดจะต้องรอให้สารละลายเข้าสู่สถานะที่สมดุลเป็นเวลา 250-350 วินาทีก่อนทำการตรวจวัดเพื่อประสิทธิภาพที่สูงสุด

- ผลการตรวจวิเคราะห์ด้วยเทคนิคสแควร์เวฟโวลแทมเมทรี (square-wave voltammetry) จะแสดงผลดังกราฟแสดงความสัมพันธ์ระหว่างกระแส (แกน y) และศักย์ไฟฟ้า (แกน x) (รูปที่ 2.1) จากกราฟจะพบว่าค่ากระแสที่ศักย์ไฟฟ้า 0.7 – 0.8 โวลต์แสดงความสัมพันธ์เป็นเส้นตรงกับความเข้มข้นของวิตามินดีในช่วง 0.5 ถึง 30 ไมโครกรัมต่อมิลลิลิตร ดังแสดงในรูปที่ 2.2 โดยมีค่าขีดจำกัดการตรวจวัดที่ 68.8 นาโนกรัมต่อมิลลิลิตร นอกจากนี้ยังพบว่าขั้วไฟฟ้าที่ประดิษฐ์ขึ้นนี้สามารถเพิ่มประสิทธิภาพในการตรวจวัดทางเคมีไฟฟ้า (กระแสที่ได้จากการตรวจวัดวิตามินดีต่อกระแสที่ได้จากการตรวจวัดสารละลายพื้นหลัง) เมื่อเทียบกับขั้วไฟฟ้าที่ไม่ได้รับการดัดแปรและขั้วไฟฟ้าที่ดัดแปรด้วยกราฟีนเท่านั้น (รูปที่ 3) ยิ่งไปกว่านั้นขั้วไฟฟ้าที่ประดิษฐ์ขึ้นยังสามารถใช้งานซ้ำได้มากถึง 10 ครั้งโดยไม่พบปัญหาการดูดซับที่ผิวหน้าขั้วของสารเมื่อเปรียบเทียบกับขั้วไฟฟ้าที่ได้รับการดัดแปรด้วยกราฟีนเท่านั้นและขั้วไฟฟ้ามาตรฐานกลาสซีคาร์บอน (glassy carbon) ซึ่งนิยมใช้สำหรับการตรวจวัดวิตามินดี (รูปที่ 4) เพื่อแสดงถึงประสิทธิภาพในการตรวจวัดในตัวอย่างจริง ผู้ประดิษฐ์ได้ทำการทดสอบขั้วไฟฟ้าที่ประดิษฐ์ขึ้นกับการตรวจวัดในตัวอย่างยาและผลิตภัณฑ์อาหารเสริมที่มีส่วนผสมของวิตามินดี ผลการวิเคราะห์พบว่า วิธีการตรวจวัดทางเคมีไฟฟ้าด้วยขั้วไฟฟ้าที่ประดิษฐ์ขึ้นไม่มีความแตกต่างทางสถิติอย่างมีนัยสำคัญที่ระดับความเชื่อมั่น 95 % เมื่อเทียบกับการตรวจวัดด้วยวิธีทางโครมาโตกราฟีแบบวิเคราะห์มวล (LC-MS/MS) นอกจากนี้ผู้ประดิษฐ์ยังทำการทดสอบขั้วไฟฟ้าที่ประดิษฐ์ขึ้นกับการตรวจวิเคราะห์วิตามินที่ละลายในไขมันอื่น ๆ พบว่าขั้วไฟฟ้าที่ประดิษฐ์ขึ้นนี้ยังสามารถตรวจวัดวิตามินที่ละลายในไขมันทั้ง 4 ชนิด (วิตามินเอ ดี อี และ เค) ได้ในคราวเดียวกันโดยไม่ต้องอาศัยวิธีทางโครมาโตกราฟีเพื่อแยกสารทั้ง 4 ชนิดออกจากกันก่อนการวิเคราะห์ (รูปที่ 5)

คำอธิบายรูปเขียนโดยย่อ

รูปที่ 1 แสดงลักษณะของขั้วไฟฟ้าพิมพ์สกรีนสำหรับการตรวจวิเคราะห์วิตามินชนิดละลายในไขมัน
รูปที่ 2 แสดงผลการวิเคราะห์วิตามินดีด้วยขั้วไฟฟ้าพิมพ์สกรีนจากวัสดุคอมโพสิตระดับนาโนเมตรของกราฟีนและนาฟิออน

รูปที่ 3 แสดงประสิทธิภาพการตรวจวิเคราะห์วิตามินดีด้วยขั้วไฟฟ้าพิมพ์สกรีนจากวัสดุคอมโพสิตระดับนาโนเมตรของกราฟีนและนาฟิออนเทียบกับขั้วไฟฟ้าที่ไม่ได้รับการดัดแปร (คาร์บอน) และขั้วไฟฟ้าที่ดัดแปรด้วยกราฟีนเท่านั้น

รูปที่ 4 แสดงผลการใช้งานขั้วไฟฟ้าซ้ำสำหรับการตรวจวัดวิตามินดีจากการวิเคราะห์ด้วยขั้วไฟฟ้าพิมพ์สกรีนจากวัสดุคอมโพสิตระดับนาโนเมตรของกราฟีนและนาฟิออน เทียบกับที่ดัดแปรด้วยกราฟีนเท่านั้นและขั้วไฟฟ้ามาตรฐานกลาสซีคาร์บอน (glassy carbon)

รูปที่ 5 แสดงผลการวิเคราะห์วิตามินที่ละลายในไขมันทั้ง 4 ชนิด (วิตามินเอ ดี อี และ เค) ด้วยขั้วไฟฟ้าพิมพ์สกรีนจากวัสดุคอมโพสิตระดับนาโนเมตรของกราฟีนและนาฟิออน

วิธีการในการประดิษฐ์ที่ดีที่สุด

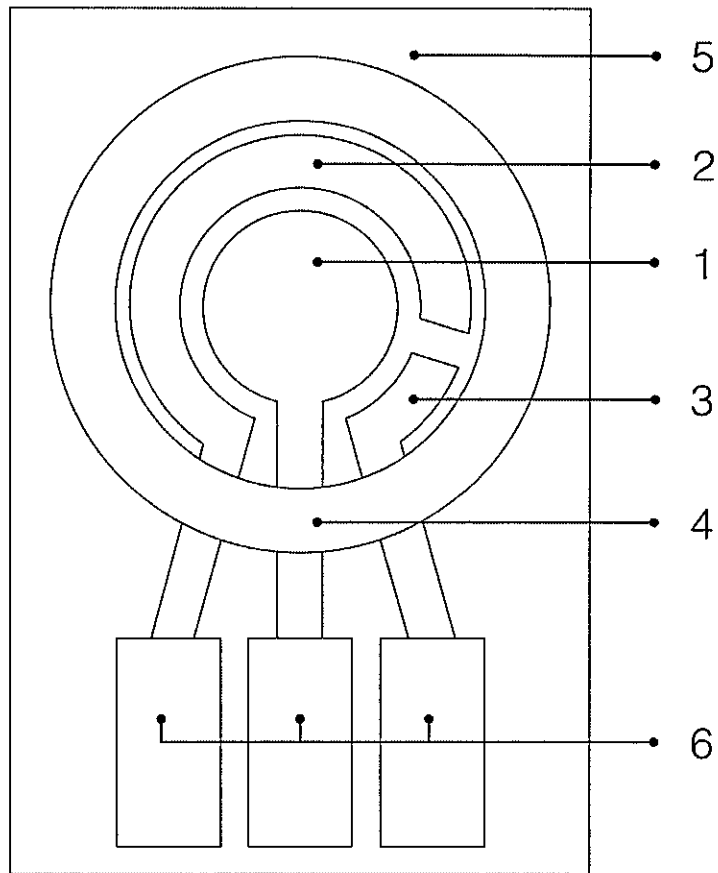
เหมือนกับที่ได้กล่าวไว้แล้วในหัวข้อการเปิดเผยการประดิษฐ์โดยสมบูรณ์

ข้อถ้อยสัญญา

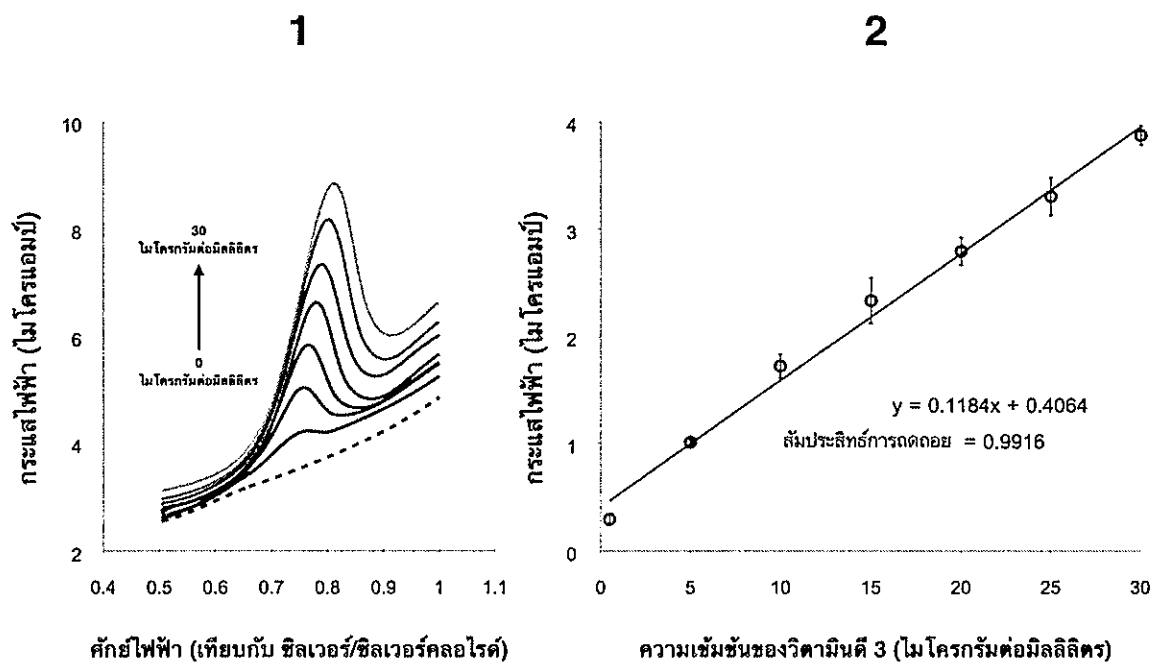
1. ขั้วไฟฟ้าพิมพ์สกรีนสำหรับการตรวจวิเคราะห์วิตามินชนิดละลายในไขมัน ซึ่งประกอบรวมด้วย
 - ขั้วไฟฟ้าทำงาน (1) มีลักษณะเป็นวงกลมที่มีเส้นผ่านศูนย์กลาง 5 มิลลิเมตร ที่พิมพ์สกรีนด้วยวัสดุคอมโพสิตระดับนาโนเมตรของกราฟีนและนาฟิออน และเชื่อมต่ออยู่กับแผ่นนำไฟฟ้า (6) ที่พิมพ์สกรีนด้วยหมึกของ ซิลเวอร์/ซิลเวอร์คลอไรด์สำหรับการต่อเข้ากับเครื่องโพเทนชิโอสแตท (potentiostat)
 - ขั้วไฟฟ้าเผชิญหน้า (2) มีลักษณะเป็นเส้นโค้งความหนา 1 มิลลิเมตร ที่พิมพ์สกรีนด้วยวัสดุคอมโพสิตระดับนาโนเมตรของกราฟีนและนาฟิออน ซึ่งล้อมรอบขั้วไฟฟ้าทำงาน (1) อยู่ 3 ใน 4 ส่วน และเชื่อมต่ออยู่กับแผ่นนำไฟฟ้า (6) สำหรับการต่อเข้ากับเครื่องโพเทนชิโอสแตท (potentiostat)
 - ขั้วไฟฟ้าอ้างอิง (3) มีลักษณะเป็นเส้นโค้งความหนา 1 มิลลิเมตร พิมพ์สกรีนด้วยหมึกของซิลเวอร์/ซิลเวอร์คลอไรด์ที่ล้อมรอบขั้วไฟฟ้าทำงาน (1) อยู่ 1 ใน 4 ส่วน และเชื่อมต่ออยู่กับแผ่นนำไฟฟ้า (6) สำหรับการต่อเข้ากับเครื่องโพเทนชิโอสแตท (potentiostat)
- 5 10 โดยขั้วไฟฟ้าทั้งสาม (1,2,3) พิมพ์สกรีนอยู่บนฐานพีวีซี (5) ที่มีความไม่ชอบน้ำ ทำหน้าที่เพิ่มความแข็งแรงของขั้วไฟฟ้า และทำหน้าที่รองรับวิตามินชนิดละลายในไขมันที่ต้องการทดสอบ และมีการกันขอบเขตการไหลด้วยการพิมพ์ ไซมิง (4) ที่ทำหน้าที่กันขอบเขตของวิตามินชนิดที่ละลายในไขมันให้ทดสอบให้อยู่เฉพาะในบริเวณที่มีการพิมพ์ไซมิงเท่านั้น
- 15 2. ขั้วไฟฟ้าพิมพ์สกรีนสำหรับการตรวจวิเคราะห์วิตามินชนิดละลายในไขมัน ตามข้อถ้อยสัญญา 1 ที่ซึ่งวิตามินชนิดละลายในไขมัน ได้แก่ วิตามินเอ วิตามินดี วิตามินอี และวิตามินเค ซึ่งถูกทำละลายด้วยตัวทำละลายผสมระหว่าง 55-65% เอทานอล และ 35-45% โซเดียมเปอร์คลอเรตความเข้มข้น 0.1-1 โมลาร์
3. กระบวนการผลิตขั้วไฟฟ้าพิมพ์สกรีนสำหรับการตรวจวิเคราะห์วิตามินชนิดละลายในไขมัน ตามข้อถ้อยสัญญา 1-2 ข้อใดข้อหนึ่ง ซึ่งมีขั้นตอนประกอบรวมด้วยดังนี้
- 20 ก. ทำการออกแบบขั้วไฟฟ้าด้วยโปรแกรมออดิเอสส์เตอร์เตอร์ (Adobe illustrator) โดยลักษณะของขั้วไฟฟ้ามีลักษณะเป็นขั้วไฟฟ้าทำงาน (1) รูปแบบวงกลมที่มีเส้นผ่านศูนย์กลาง 5 มิลลิเมตร ซึ่งถูกล้อมรอบด้วยขั้วไฟฟ้าเผชิญหน้า (2) และขั้วไฟฟ้าอ้างอิง (3) และทำการพิมพ์ไซมิง (4) เส้นผ่านศูนย์กลาง 12 มิลลิเมตรล้อมรอบขั้วไฟฟ้าทั้งสาม (1,2,3) เพื่อกำหนดขอบเขตการไหลของสารละลาย
- 25 ข. ทำการพิมพ์ไซมิง (4) เพื่อกันขอบเขตการไหลบนฐานพีวีซี (5) ด้วยเครื่องพิมพ์ไซมิง
- ค. ทำการผสมหมึกของวัสดุคาร์บอน-กราฟีน และ นาฟิออน 0.05-0.15 % โดยปริมาตรต่อปริมาตรในอัตราส่วน 0.45-0.55 กรัมต่อ 80-120 ไมโครลิตร แล้วจึงคนจนหมึกของวัสดุผสมระหว่างกราฟีนและฟิออนผสมเป็นเนื้อเดียวกัน
- ง. ทำการพิมพ์สกรีนขั้วไฟฟ้าทำงาน (1) และขั้วไฟฟ้าเผชิญหน้า (2) ที่พิมพ์สกรีนจากหมึกของวัสดุคอมโพสิตระดับนาโนเมตรของกราฟีนและนาฟิออนลงบนฐานพีวีซี (5) ที่ผ่านการพิมพ์ไซมิง (4) ลงไปจากนั้นนำไปอบที่อุณหภูมิ 45-60 องศาเซลเซียส เป็นเวลา 45-60 นาที
- 30 จ. ทำพิมพ์สกรีนขั้วไฟฟ้าอ้างอิงด้วยหมึกซิลเวอร์/ซิลเวอร์คลอไรด์ ลงบนฐานพีวีซี (5) ที่ผ่านการพิมพ์สกรีนขั้วไฟฟ้าทำงาน (1) และขั้วไฟฟ้าเผชิญหน้า (2) จากนั้นจึงนำไปอบที่อุณหภูมิ 45-60 องศาเซลเซียส เป็นเวลา 45-60 นาที ได้เป็นขั้วไฟฟ้าพิมพ์สกรีนสำหรับการตรวจวิเคราะห์วิตามินชนิดละลายในไขมัน
- 35 ฉ. ทำการตัดขั้วไฟฟ้าที่ผ่านการอบเรียบร้อยแล้วเพื่อให้พร้อมต่อการใช้งาน

บทสรุปการประดิษฐ์

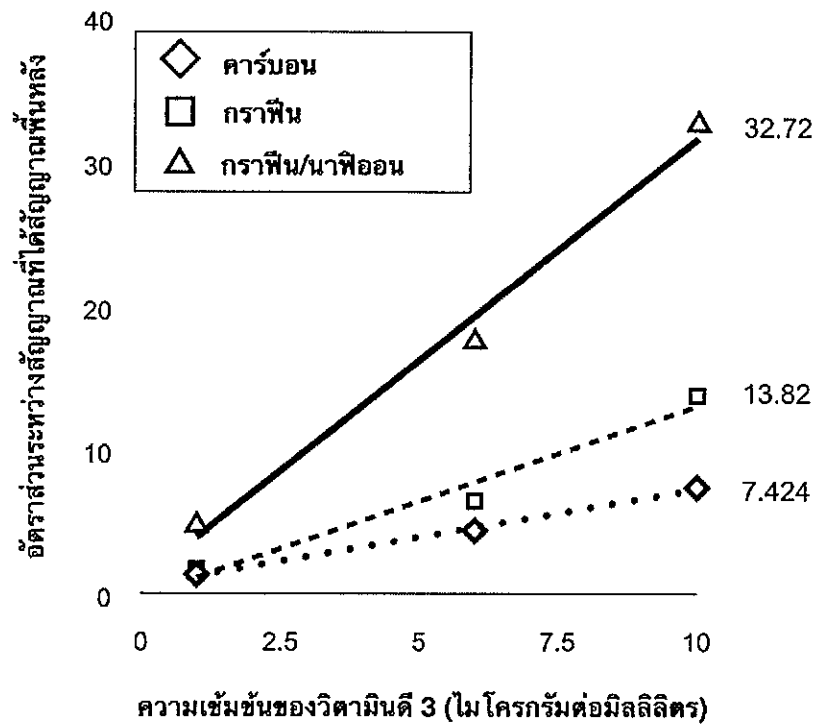
- ข้อ 5 ขั้วไฟฟ้าสำหรับการตรวจวิเคราะห์วิตามินชนิดละลายในไขมัน ตามการประดิษฐ์นี้ ซึ่งประกอบด้วย ขั้วไฟฟ้าทำงาน (1) มีลักษณะเป็นวงกลมที่มีเส้นผ่านศูนย์กลาง 5 มิลลิเมตร ที่พิมพ์สกรีนด้วยวัสดุคอมโพสิตระดับนาโนเมตรของกราฟีนและนาฟิออน และเชื่อมต่ออยู่กับแผ่นนำไฟฟ้า (6) ที่พิมพ์สกรีนด้วยหมึกของ ซิลเวอร์/ซิลเวอร์คลอไรด์สำหรับการต่อเข้ากับเครื่องโพเทนชิโอสแตท (potentiostat), ขั้วไฟฟ้าเผชิญหน้า (2) มีลักษณะเป็นเส้นโค้งความหนา 1 มิลลิเมตร ที่พิมพ์สกรีนด้วยวัสดุคอมโพสิตระดับนาโนเมตรของกราฟีนและนาฟิออน ซึ่งล้อมรอบขั้วไฟฟ้าทำงาน (1) อยู่ 3 ใน 4 ส่วน และเชื่อมต่ออยู่กับแผ่นนำไฟฟ้า (6) สำหรับการต่อเข้ากับเครื่องโพเทนชิโอสแตท (potentiostat), ขั้วไฟฟ้าอ้างอิง (3) มีลักษณะเป็นเส้นโค้งความหนา 1 มิลลิเมตร พิมพ์สกรีนด้วยหมึกของ ซิลเวอร์/ซิลเวอร์คลอไรด์ที่ล้อมรอบขั้วไฟฟ้าทำงาน (1) อยู่ 1 ใน 4 ส่วน
- ข้อ 10 และเชื่อมต่ออยู่กับแผ่นนำไฟฟ้า (6) สำหรับการต่อเข้ากับเครื่องโพเทนชิโอสแตท (potentiostat) โดย ขั้วไฟฟ้าทั้งสาม (1,2,3) พิมพ์สกรีนอยู่บนฐานพีวีซี (5) ที่มีความไม่ชอบน้ำ ทำหน้าที่เพิ่มความแข็งแรงของ ขั้วไฟฟ้า และทำหน้าที่รองรับวิตามินชนิดละลายในไขมันที่ต้องการทดสอบ และมีการกันขอบเขตการไหลด้วยการพิมพ์ ไซมิ่ง (4) ที่ทำหน้าที่กันขอบเขตของวิตามินชนิดที่ละลายในไขมันให้ทดสอบให้อยู่เฉพาะในบริเวณที่มีการพิมพ์ไซมิ่งเท่านั้น ซึ่งผลการวิเคราะห์พบว่าขั้วไฟฟ้าที่ประดิษฐ์ขึ้นสามารถเพิ่มประสิทธิภาพการตรวจวัด
- ข้อ 15 วิตามินชนิดละลายในไขมันได้ และเมื่อทดสอบประสิทธิภาพในการใช้ขั้วไฟฟ้าซ้ำพบว่าสามารถใช้งานซ้ำได้มากถึง 10 ครั้งโดยไม่พบปัญหาจากการดูดซับที่ผิวหน้าขั้วของสาร เหมาะสำหรับงานที่ต้องมีการตรวจวัดซ้ำ ๆ เช่นในอุตสาหกรรมอาหารและยาที่ต้องมีการควบคุมคุณภาพตลอดเวลา



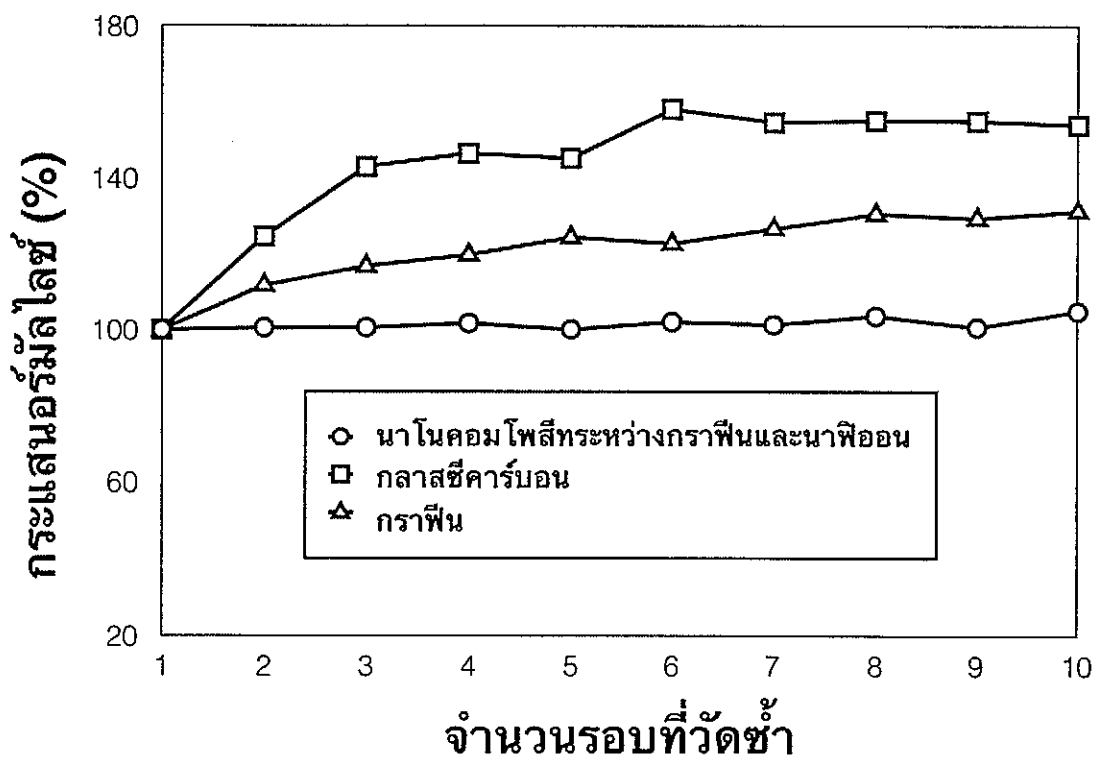
รูปที่ 1



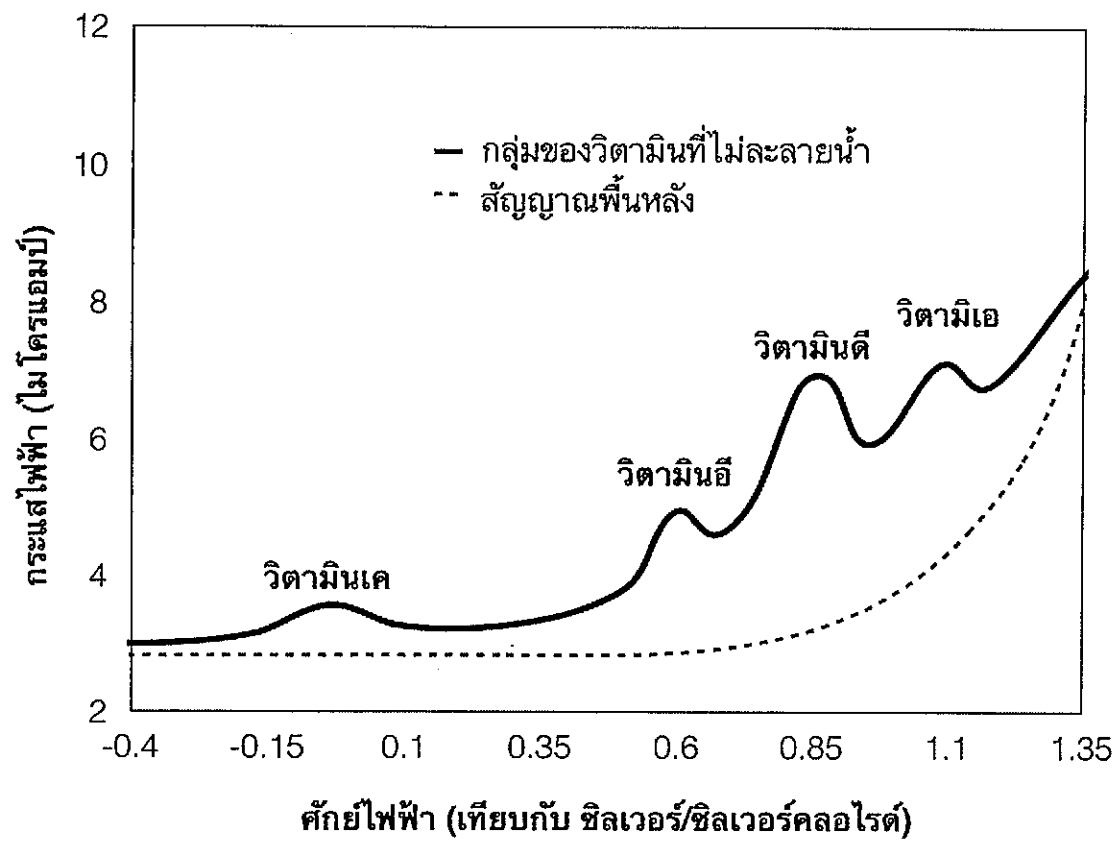
รูปที่ 2



รูปที่ 3



รูปที่ 4



รูปที่ 5