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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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DEVELOPMENT OF PAPER-BASED MICROFLUIDIC DEVICES FOR THE DETERMINATION OF ALBUMIN, CREATININE, CHOLESTEROL, AND GLUCOSE.

Miss Tipawan Rungsawang

A Dissertation Submitted in Partial Fulfillment of the Requirements

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Thesis Title	DEVELOPMENT OF PAPER-BASED MICROFLUIDIC DEVICES FOR THE DETERMINATION OF ALBUMIN, CREATININE, CHOLESTEROL, AND GLUCOSE
By	Miss Tipawan Rungsawang
Field of Study	Biotechnology
Thesis Advisor	Associate Professor Orawon Chailapakul, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

……Dean of the Faculty of Science
(Professor Supot Hannongbua, Dr.rer.nat.)

THESIS COMMITTEE

…….Chairman

(Assistant Professor Warinthorn Chavasiri, Ph.D.)

…….Thesis Advisor

(Associate Professor Orawon Chailapakul, Ph.D.)

……..Examiner

(Associate Professor Nattaya Ngamrojanavanich, Ph.D.)

…….Examiner

(Associate Professor Polkit Sangvanich, Ph.D.)

…….External Examiner

(Chakorn Chinvong-amorn, Ph.D.)

ทิพย์วรรณ รุ่งสว่าง: การพัฒนาอุปกรณ์ตรวจวัดของไหลระดับไมโครบนกระดาษสำหรับการตรวจวัดแอลบูมิน ครีแอทินิน คอเลสเตอรอล และกลูโคส. (DEVELOPMENT OF PAPER-BASED MICROFLUIDIC DEVICES FOR THE DETERMINATION OF ALBUMIN, CREATININE, CHOLESTEROL, AND GLUCOSE) อ. ที่ปรึกษาวิทยานิพนธ์: รศ. ดร. อรวรรณ ชัยลภากุล, 113 หน้า.

งานวิจัยนี้เป็นการพัฒนาอุปกรณ์สำหรับการตรวจวินิจฉัยโรคหัวใจ โรคไต โรคเบาหวาน และโรคความดันโลหิตสูง ซึ่งโรคดังกล่าวเป็นผลมาจากปริมาณของสารกลูโคส คอเลสเตอรอล แอลบูมิน และ ครีแอทินิน โดยอุปกรณ์ตรวจวัดของไหลระดับไมโครบนกระดาษได้ถูกใช้ในการตรวจวัดปริมาณสารที่สนใจเหล่านี้ด้วยวิธีตรวจวัดด้วยการดูสี (colorimetric method) ในส่วนแรกของงานวิจัยได้ใช้เทคนิคโฟโตลิโทกราฟีในการสร้างอุปกรณ์ แต่เทคนิคดังกล่าวพอลิเมอร์ไวแสง (SU-8 photoresist) ที่ใช้สร้างอุปกรณ์มีราคาแพง จึงได้นำวิธีการจุ่มแว็กซ์ และการพิมพ์ด้วยแว็กซ์มาใช้ในการสร้างอุปกรณ์ ซึ่งอุปกรณ์ที่สร้างขึ้นมานั้นสามารถใช้ตรวจวัดคอเลสเตอรอล และกลูโคส ภายใต้สภาวะที่เหมาะสม ได้กราฟมาตรฐานที่มีช่วงความเป็นเส้นตรงของคอเลสเตอรอล 50-400 มิลลิกรัมต่อเดซิลิตร และกลูโคส 0-400 มิลลิกรัมต่อเดซิลิตร ในงานวิจัยนี้ได้มีการพัฒนาวิธีการใหม่สำหรับตรวจวัดกลูโคสโดยใช้ปฏิกิริยาของมาลาไคท์ กรีน ได้ช่วงความเป็นเส้นตรง 40-120 มิลลิกรัมต่อเดซิลิตร ค่าสัมประสิทธิ์สหสัมพันธ์ (R^2) 0.9924 ค่าเบี่ยงเบนมาตรฐานสัมพัทธ์ (%RSD) 1.5-2.0% นอกจากนี้อุปกรณ์นี้ได้ถูกใช้ตรวจวัดแอลบูมิน และครีแอทินิน ด้วยอนุภาคทองคำระดับนาโน ได้ภาวะที่เหมาะสมของครีแอทินิน คือ ทองคำระดับนาโนขนาด 40 นาโนเมตร และแอลบูมินขนาด 20 นาโนเมตร ความเข้มข้นของทองคำระดับนาโนที่ใช้สำหรับวิเคราะห์ทั้งสองสาร คือ 1000 มิลลิกรัมต่อลิตร

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต……..

ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์……..……

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KEYWORDS: CHOLESTEROL / GLUCOSE / ALBUMIN / CREATININE / COLORIMETRIC / PAPER-BASED MICROFLUIDIC DEVICE / SILVER NANOPARTICLES / GOLD NANOPARTICLES / SPECTROPHOTOMETRY

TIPAWAN RUNGSAWANG: DEVELOPMENT OF PAPER-BASED MICROFLUIDIC DEVICES FOR THE DETERMINATION OF ALBUMIN, CREATININE, CHOLESTEROL, AND GLUCOSE. THESIS ADVISOR: ASSOC.PROF. ORAWON CHAILAPAKUL, Ph.D., 113 pp.

This work concentrates on the development of diagnotic device for heart disease, kidney failure, diabetes, and hypertension that affected by the levels of glucose, cholesterol, albumin, and creatinine. Paper-based microfluidic devices (μPADs) with colorimetric method were used to determine these analytes. First, the Photolithography was used to fabricate the devices, but the cost of photoresist polymer is very expensive, then wax-dipping and wax-printing method were also used. μPADs were used to detect cholesterol, and glucose. Under the optimum conditions, the calibration curves between the color intensity and concentration of analytes were obtained. The plot of these relationship indicated that the cholesterol linearity was 50-400 mg dL^{-1} and the glucose linearity was 0-400 $mg.dL^{-1}$. In this work, the novel method for the determination of glucose was also proposed by malachite green reaction, the linearity of this method was in the range of 40-120 $mg.dL^{-1}$ with R^2 of 0.9924. The % RSD was found to be 1.5-2.0. In addition, μPADs were used to detect albumin and creatinine by using AuNPs. The size of AuNPs were obtained as 40 nm for determination of creatinine, and 20 nm for determination of albumin. The concentration of AuNPs used was 1000 $mg.L^{-1}$ for both albumin and creatinine detection.

Field of study Biotechnology Student's signature……

Academic year 2011 Advisor's signature……

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LIST OF ABBREVIATIONS

ppm	part per million
ppb	part per billion
mm	millimeter
cm	centrimeter
nm	nanometer
µm	micrometer
mL	milliter
µL	microliter
g	gram
mg	milligram
µg	microgram
r^2	correlation coefficient
RSD	Relative standard deviation
µPADs	Paper-based microfluidic devices
POCT	Point of care testing
i.d.	internal diameter
HPLC	High performance liquid chromatography
GC	Gas chromatography
UV/Vis	Ultraviolet/Visible
EC	Electrochemical detection
GOD	Glucose oxidase
POD	Peroxidase
CO	Cholesterol oxidase
CE	Cholesterol esterase
mM	millimolar
M	Molar
dL	deciliter
PBS	phosphate buffer solution
BSA	Bovine serum albumin
rpm	rounds per minute

UmL^{-1}	Units per milliliter
BCG	Bromocresol green
AgNP	Silver nanoparticles
AuNP	Gold nanoparticles
AP	4- aminophenol
°C	degree Celsius
Min	minute
s	second
S/B	signal to background ratio
S/N	signal to noise ration
v/v	volume by volume
w/v	weight by volume
Phe	phenol
LOD	limits of detection
LOQ	limits of quantitation
DHBS	3,5-dichloro-2-hydroxy-benzenesulfonic acid
KI	Potassium Iodide

CHAPTER I

INTRODUCTION

1.1 Introduction

High mortality rates are a serious problem for global public health. Monitoring the health of a population is very important, especially the analysis of biological fluids, which is the most necessary procedure. In the past, conventional laboratory instruments have been used to determine the marker of a disease, but they are unsuitable for real-time analyses because they are large, expensive and require trained personnel and considerable volumes of biological samples. Rapid and quantitative methods for detecting the markers of diseases are necessary for prompt and effective diagnosis and treatment.

In recent years, low-cost materials-based point of care testing (POCT) has been used to provide diagnostic devices for screening tests and self-monitoring by patients. A primary challenge for low-cost materials-based POCT is making the diagnostic devices inexpensive, simple to use, and easy to fabricate in order to preferentially benefit the developing world. Currently, paper tests or strip tests are widely used in clinical laboratories for diagnosing various diseases. Recently, Whiteside's group has developed paper-based microfluidic devices (μPADs), which are also known as a lab-on-paper technology. These μPADs are worth studying because they have many advantages; they are easy to use and inexpensive, small volumes of sample can be used, they are easy to adapt, and they are capable of rapid on-site detection. These devices are made from paper, which is a naturally abundant material, and they are biodegradable. In this work, these devices are used to detect biomarkers for serious diseases, including diabetes, renal failure, heart diseases, and hypertension.

Diabetes, renal failure, heart diseases, and hypertension are well-known diseases. Diabetes is a disorder of the body's pathway that converts food into energy. Normally, ingested sugar is digested and broken down into glucose. Then, the glucose circulates in the blood where it remains until it enters the cells to be used as a fuel. Insulin is a hormone that is produced by the pancreas. This hormone helps the glucose move into the cells. A healthy pancreas can produce insulin based on the level of glucose in the blood, but if a person has diabetes, this process breaks down, and the sugar level in the blood is higher than normal (80-110 $mg.dL^{-1}$).

Renal or kidney failure refers to the failure of the kidneys. This disease is divided into acute and chronic forms. The analysis of renal markers, such as albumin, creatinine, creatine, uric acid, urea, and p-aminohippuric acid, in biological fluids evaluates the functions of the renal and muscular systems. Creatinine is a product that is produced by the metabolism of creatine in mammals. Creatinine is an important marker for monitoring the function of the kidneys, and the amount of creatine indicates the extent of muscle damage. The determination of creatinine in the blood is also important in clinical analyses. The normal concentration range for creatinine and creatine is less than 0.14 $mmol.L^{-1}$ in serum, but their concentrations may increase to a value greater than 1 $mmol.L^{-1}$.

In addition, albuminuria is a marker of renal disease, which indicates a future risk of death by renal failure. Albuminuria is a pathological condition where albumin, which is a type of proteinuria, is present in the urine. At low levels, albumin is called micro-albuminuria. According to the American Diabetes Association (ADA), the gold standard for measuring the amount of albumin excreted in urine is through a 24-h urine collection, but this method is difficult. To eliminate any uncertainties, a more convenient method for detecting micro-albuminuria is measuring the albumin/creatinine ratio (ACR) in a random urine specimen. The ADA and the National Kidney Foundation define micro-albuminuria as an ACR ratio between 30 and 300 $\mu g.mg^{-1}$ in both men and women.

The cholesterol level is correlated with heart disease and hypertension. When the level of cholesterol is too high, there is a buildup in the walls of the arteries, which

causes a condition called atherosclerosis, a form of heart disease. During atherosclerosis, the arteries become narrow and blood flow to the heart muscles is slowed or restricted. Oxygen is carried in a blood cycle to the heart, and if sufficient amounts of blood and oxygen cannot reach the heart, chest pains may occur. If the blood supply to a portion of the heart is completely cut off by a blockage, a heart attack occurs.

Colorimetric assays have been proposed as a measurement that can be performed on μPADs. This type of measurement is widely used to quantify the color intensity of the test zone because it is easy to perform and only requires simple equipment, such as a digital camera, cell phone or scanner.

1.2 Research Objective

The aim of this thesis is to develop diagnostic devices that can be used to determine the concentrations of glucose, creatinine, albumin, and cholesterol in biological samples. The developed diagnostic devices need to be relatively simple and reliable, have a short analysis time and be inexpensive. To achieve these objectives, three sub-projects were performed, as follows;

1. To develop the paper-based microfluidic devices, SU-8 photoresist and wax were used.

2. To characterize and study the performance of the paper-based microfluidic devices.

1.3 Scope of Research

This research is focused on the fabricaiton of the paper-based microfluidic devices. The colorimetric measurement was used to determine the analytes on μPADs for the quantitative analysis. These devices were applied to use for determination of glucose, cholesterol, creatinine, and albumin in biological samples. The analysis of the same samples was simultaneously carried out with other techniques, for example, UV-Visible spectrophotometry, and gold standard method for the assessment of accuracy of the results given by the proposed method.

CHAPTER II

THEORY AND LITERATURE SURVEY

The fundamental of paper-based microfluidic devices, colorimetric measurement techniques used in this work are described in the initial section of this chapter. Consideration is then given to UV-Vis spectrophotometry and nanoparticles respectively. Finally the detail of the interested diseases and disease's marker are described.

2.1 Paper-based microfluidic devices (μPADs) [1, 2]

The paper strip tests have been commercially available for POC (point-of-care) diagnostics. These strip tests are advantageous because of their simplicity and low cost, but often suffer from the fact that they are not quantitative, not sufficiently sensitive to certain biomarkers, and lack the ability for multiplex analysis. Rapid and quantitative methods for detecting markers of disease are necessary for prompt and effective diagnosis and treatment. These issues can be potentially addressed by the recently developed paper-based microfluidic devices. These devices have many advantages: (1) they are easy-to-use (particularly suited to nontechnical personnel), inexpensive, low volume, easily adaptable, and are capable of rapid on-site detection. (2) Paper is made of naturally abundant materials, and is biodegradable.

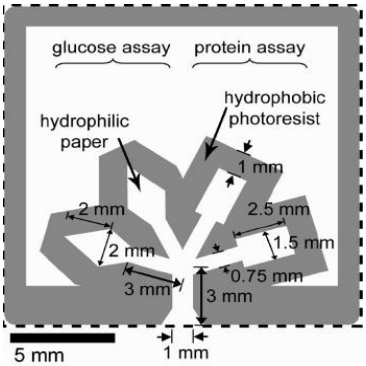


Figure 2.1 An Example of the design for a prototype paper-based microfluidic device. [2]

Figure 2.2 General strategy for performing inexpensive bioassays in remote locations and for exchanging the results of the tests with off-site technicians. [2]

2.1.1 The techniques for paper-based microfluidic device preparation.

2.1.1.1 Photolithography technique

A photoresist reagent was used in this technique such as SU-8 photoresist. This method consists of eight steps to create the paper-based microfluidic device. Firstly, soak the paper with a photoresist reagent. Secondly, bake the paper at 95°C for 5 minutes. Thirdly, align the paper under a mask and expose to UV light for 10 seconds. Then, bake the paper at 95°C for 5 minutes and develop the paper by soaking it in developer solution. After that cut out the pattern and spot the reagents on the pattern. The photoreisist method is a popular method but it has some disadvantages such as there are many steps and the photoresist reagent is very expensive.

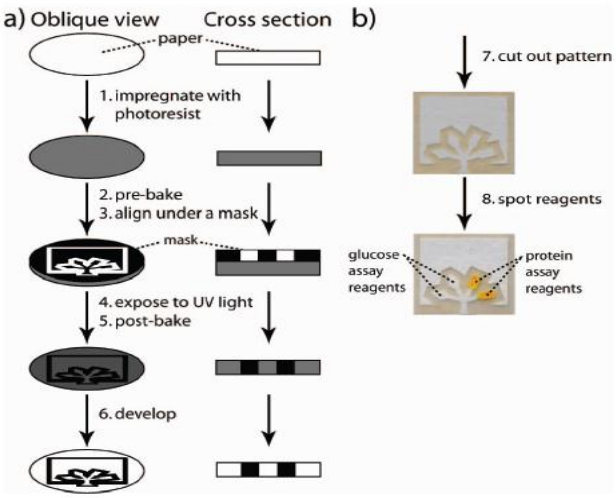


Figure 2.3 Schematic of the method for fabricating paper-based microfluidic devices. (a) Procedure for patterning paper with hydrophobic photoresist. (b) Derivatization of the device for assays. [2]

2.1.1.2 Wax dipping technique [3, 4]

The previously used technique for fabrication has some disadvantages such as time to prepare and cost of reagent (SU-8 photoresis is expensive). The new method was developed to solve these problems. To create a mould for wax dipping, cut an iron bar by laser cutting into the desired shape and size. For the wax dipping method, white Beeswax pellets were melted by heating with hotplate. Whatman No.1 paper was cut and placed onto a glass slide. Then, the iron mould was put onto the paper, and it was attached by magnetic force using a permanent magnet placed on the backside of the glass slide. Next, the assembly was dipped into a chamber of melted wax for one second. When the paper was cooled at room temperature, it was peeled off from the glass slide, and the iron mould was removed from the paper.

2.2 Colorimetric analysis [5]

Colorimetric analysis is a method determining the concentration of a chemical element of a chemical compound in a solution with the aid of a color reagent. It is applicable to both organic compounds and inorganic compounds and may be used with or without an enzymatic stage. The method is widely used in medical laboratories and for industrial purposes, for example, the analysis of water samples in connection with industrial water treatment. The equipment required is a colorimeter, some cuvettes and a suitable color reagent. The process may be automated such as an AutoAnalyzer of Flow injection analysis. This method can be divided into two types: non-enzymatic and enzymatic method.

2.2.1 Non-enzymatic method

Non-enzymatic method is the method that formed an analyte colour complex with the reagent (without enzyme). This method was used to determine several analytes such as calcium, copper, creatinine, iron and phosphate.

2.2.2 Enzymatic methods

In enzymatic analysis which is widely used in medical laboratories, the color reaction is preceded by a reaction catalyzed by an enzyme. The enzyme is specific to a substrate, and more accurate results can be obtained. Enzymatic analysis is always carried out in a buffer solution at a specified temperature (usually 37°C) to provide the optimum conditions for the enzymes to active. For example, cholesterol, glucose, triglycerides, and urea.

2.3 Ultraviolet-visible spectroscopy [6]

Ultraviolet-visible spectroscopy or ultraviolet-visible spectrophotometry (UV-Vis or UV/Vis) refers to absorption spectroscopy or reflectance spectroscopy in the ultraviolet-visible spectral region. This means it uses light in the visible and adjacent (near-UV and near-infrared (NIR)) ranges. The absorption or reflectance in the visible range directly affects the perceived color of the chemicals involved. In this region of the electromagnetic spectrum, molecules undergo electronic transitions. This technique is complementary to flurescence spectroscopy, in that fluorescence deals with transitions from the excited state to the ground state, while absorption measures transitions from the ground state to the excited state.

2.3.1 Principle of Ultraviolet-Visible Absorption

Molecules containing ¶-electrons or non-bonding electrons (n-electrons) can absorb the energy in the form of ultraviolet or visible light to excite these electrons to higher anti-bonding molecular orbitals. The more easily excited the electrons (lower energy gap between the HOMO and the LUMO) the longer the wavelength of light it can absorb.

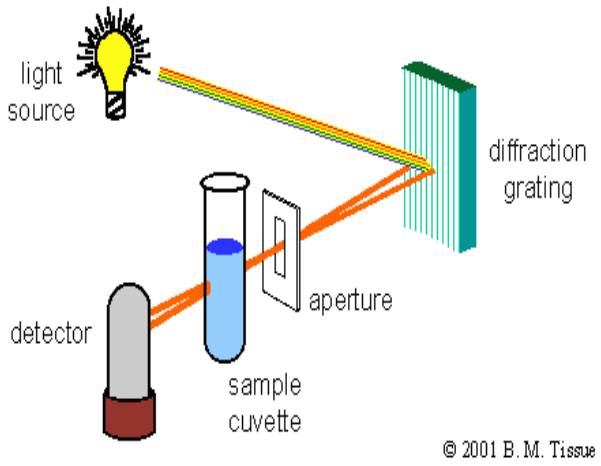


Fig. 2.4 Principle of Spectrophotometry

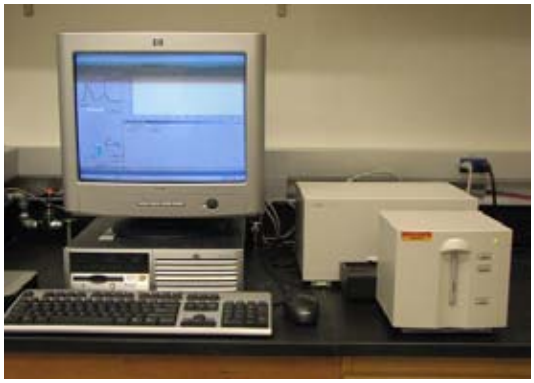


Fig. 2.5 Instrumentals of UV/Vis spectroscopy

2.3.2 Applications

UV/Vis spectroscopy is routinely used in analytical chemistry for the quantitative determination of different analytes, such as transition metal ions, highly conjugated organic compounds, and biological macromolecules. Spectroscopic analysis is commonly carried out in solutions but solids and gases may also be studied.

Solutions of transition metal ions can be colored because d electrons within the metal atoms can be excited from one electronic state to another. The colour of metal ion solutions is strongly affected by the presence of other species, such as certain anions or ligands. For instance, the colour of a dilute solution of copper sulfate is a very light blue; adding ammonia intensifies the colour and changes the wavelength of maximum absorption (λmax).

Organic compounds, especially those with a high degree of conjugation, also absorb light in the UV or visible regions of the electromagnetic spectrum. The solvents for these determinations are often water for water-soluble compounds, or ethanol for organic-soluble compounds. (Organic solvents may have significant UV absorption; not all solvents are suitable for use in UV spectroscopy. Ethanol absorbs very weakly at most wavelengths.) Solvent polarity and pH can affect the absorption spectrum of an organic compound. Tyrosine, for example, increases in absorption maxima and molar extinction coefficient when pH increases from 6 to 13 or when solvent polarity decreases.

While charge transfer complexes also give rise to colours, the colours are often too intense to be used for quantitative measurement.

The Beer-Lambert law states that the absorbance of a solution is directly proportional to the concentration of the absorbing species in the solution and the path length. Thus, for a fixed path length, UV/Vis spectroscopy can be used to determine the concentration of the absorber in a solution. It is necessary to know how quickly the absorbance changes with concentration. This can be taken from references (tables of molar extinction coefficients), or more accurately, determined from a calibration curve.

A UV/Vis spectrophotometer may be used as a detector for HPLC. The presence of an analyte gives a response assumed to be proportional to the concentration. For accurate results, the instrument's response to the analyte in the unknown should be compared with the response to a standard; this is very similar to

the use of calibration curves. The response (peak height) for a particular concentration is known as the response factor.

The wavelengths of absorption peaks can be correlated with the types of bonds in a given molecule and are valuable in determining the functional groups within a molecule. The Woodwark-Fieser rules, for instance, are a set of empirical observations used to predict λmax, the wavelength of the most intense UV/Vis absorption, for conjugated organic compounds such as dienes and ketones. The spectrum alone is not, however, a specific test for any given sample. The nature of the solvent, the pH of the solution, temperature, high electrolyte concentrations, and the presence of interfering substances can influence the absorption spectrum. Experimental variations such as the slit width (effective bandwidth) of the spectrophotometer will also alter the spectrum. To apply UV/Vis spectroscopy to analysis, these variables must be controlled or accounted for in order to identify the substances present.

2.3.3 Beer-Lambert law

The method is most often used in a quantitative way to determine concentrations of an absorbing species in solution, using the Beer-Lambert law:

$$A=\log_{10} (I_0/I)=\varepsilon cL,$$

Where A is the measured absorbance, I_0 is the intensity of the incident light at a given wavelength, I is the transmitted intensity, L the pathlength through the sample, and c the concentration of the absorbing species. For each species and wavelength, ε is a constant known as the molar absorptivity or extinction coefficient. This constant is a fundamental molecular property in a given solvent, at a particular temperature and pressure, and has units of 1/M* cm or often AU/M* cm.

The absorbance and extinction εare sometimes defined in terms of the natural logarithm instead of the base-10 logarithm.

The Beer-Lambert Law is useful for characterizing many compounds but does not hold as a universal relationship for the concentration and absorption of all substances. A 2^{nd} order polynomial relationship between absorption and concentration and absorption of all substances. A 2^{nd} order polynomial relationship between aborption and concentration is sometimes encountered for very large, complex molecules such as organic dyes (Xylenol Orange or Neutral Red, for example).

2.4 Silver nanoparticle [7]

Silver nanoparticles are the silver particles that are small in size with particle sizes in the range of 1 nm and 100 nm. They are considered silver nanoparticles because they are composed of a large percentage of silver oxide proven in their large ratio of surface-to-bulk silver atoms.

The synthesis of AgNPs are different methods to synthesize the silver nanoparticles. They can be divided into three groups such as physical vapor deposition, ion implantation, and wet chemistry. Only the last methos was used in this dissertation.

2.4.1 Wet chemistry

This method consists of several types for creating silver nanoparticles. Normally, they involve the reduction of a silver salt, for example, silver nitrate with a reducing agent like sodium borohydrede in the presence of a colloidal stabilizer. Sodium borohydrede was used with polyvinyl alcohol, poly (vinylpyrrolidone), bovine serum albumin (BSA), citrate and cellulose as stabilizing agents, Moreover, BSA, the sulfur-, oxygen-, and nitrogen-bearing groups on the cellulose are reported to help stabilize the silver particles. Polydopamine coated on magnetic-bacterial cellulose that contains multifunctional groups, which acts as a reducing agent for preparation of in situ of reusable antibacterial Ag-nanocomposites. Citrate and

cellulose was used to create silver nanoparticles independent of a reducing agent as well. In addition, novel wet chemistry method was used to create silver nanoparticles that take advantage of β-D-glucose as a reducing sugar and a starch as the stabilizer. However, it is important to note, not all nanoparticles are created equal. The efficiency of the impact was shown in the size and shape. Crystal facet size with oxide content and other factors could also affect the antimicrobial properties.

AgNPs have many advantages then they could be applied in the types of catalysis, optics, electronics and other areas due to their unique size-dependent optical, electrical and magnetic properties. Nowadays, most of the applications of silver nanoparticles are in antibacterial/antifungal agents in biotechnology and bioengineering, textile engineering, water treatment, and silver-based consumer products.

There is also an effort to incorporate silver nanoparticles into a wide range of medical devices, including but not limited to bone cement, surgical instruments, surgical masks, and wound dressings. On the commercial side, silver nanoparticles are used on the surfaces of household appliances.

2.5 Hypertension [8]

Hypertension or high blood pressure, sometimes arterial hypertension is a chronic medical condition in which the blood pressure in the arteries is elevated. This condition causes the heart to work harder than normal. Blood pressure involves two measurements, systolic and diastolic, which depend on the heart muscle contracting (systole) or relaxing (diastole) between beats. Normal blood pressure is 120/80 mmHg. High blood pressure is said to be present if it goes above 140/90 mmHg.

Hypertension is classified as either primary (essential) hypertension or secondary hypertension. The cases of 90–95% are categorized as primary hypertension which means high blood pressure with no obvious underlying medical

cause. The remaining 5–10% of cases (secondary hypertension) are caused by the other conditions that affect the kidneys, arteries, heart or endocrine system.

Hypertension is a major risk factor for stroke, myocardial infarction (heart attacks), heart failure, aneurysms of the arteries, peripheral arterial disease and is a cause of chronic kidney disease. Even moderate elevation of arterial blood pressure is associated with a shortened life expectancy. Dietary and lifestyle changes can improve blood pressure control and decrease the risk of associated health complications, although drug treatment is often necessary in patients for whom lifestyle changes prove ineffective or insufficient.

2.5.1 Signs and symptoms

Hypertension is rarely accompanied by any symptoms, and its identification is usually through screening, or when seeking healthcare for an unrelated problem. A proportion of people with high blood pressure reports headaches (particularly at the back of the head and in the morning), as well as lightheadedness, vertigo, tinnitus (buzzing or hissing in the ears), altered vision or fainting episodes. On physical examination, hypertension may be suspected on the basis of the presence of hypertensive retinopathy detected by examination of the optic fundi using ophthalmoscopy. Classically, the severity of the hypertensive retinopathy changes is graded from grade I–IV, although the milder types may be difficult to distinguish from each other. Ophthalmoscopy findings may also indicate how long a person has been hypertensive.

2.5.1.1 Secondary hypertension

Some additional signs and symptoms may suggest secondary hypertension, for example, hypertension due to some identifiable cause such as kidney diseases or endocrine diseases. For example, truncal obesity, glucose intolerance, moon facies, a buffalo hump and purple striae suggesting Cushing's

syndrome. Thyroid disease and acromegaly can also cause hypertension and have characteristic symptoms and signs. An abdominal bruit may be an indicator of renal artery stenosis, while decreased blood pressure in the lower extremities and/or delayed or absent femoral arterial pulses may indicate aortic coarctation. Labile or paroxysmal hypertension accompanied by headache, palpitations, pallor, and perspiration should prompt suspicions of pheochromocytoma.

2.5.1.2 Hypertensive crises

Severely elevated blood pressure (systolic over 180 or diastolic over 110) is referred to as a hypertensive crisis, as blood pressures above these levels are known to confer a high risk of complications. People with blood pressures in this range may have no symptoms, but are more likely to report headaches and dizziness. Other symptoms accompanying a hypertensive crisis may include visual deterioration or breathlessness due to heart failure or a general feeling of malaise due to renal failure. Most people with a hypertensive crisis are known to have elevated blood pressure, but additional triggers may have led to a sudden rise.

A hypertensive emergency, previously malignant hypertension, is diagnosed when there is evidence of direct damage to one or more organs as a result of the severely elevated blood pressure. This may include hypertensive encephalopathy, caused by brain swelling and dysfunction, and characterised by headaches and an altered level of consciousness (confusion or drowsiness). Retinal papilloedema and/or fundal hemorrhages and exudates are another sign of target organ damage. Chest pain may indicate heart muscle damage (which may progress to myocardial infarction) or sometimes aortic dissection, the tearing of the inner wall of the aorta. Breathlessness, coughing, and the expectoration of blood-stained sputum are characteristic signs of pulmonary edema, the swelling of lung tissue due to left ventricular failure, an inability of the left ventricle of the heart to adequately pump blood from the lungs into the arterial system. Rapid deterioration of kidney function (acute kidney injury) and microangiopathic hemolytic anemia (destruction of blood

cells) may also occur. In these situations, rapid reduction of the blood pressure is mandated to stop ongoing organ damage. In contrast there is no evidence that blood pressure needs to be lowered rapidly in hypertensive urgencies where there is no evidence of target organ damage and over aggressive reduction of blood pressure is not without risks Use of oral medications to lower the blood pressure gradually over 24 to 48 h is advocated in hypertensive urgencies.

In pregnancy, hypertension occurs in approximately 8-10% of pregnancies. Most women with hypertension during pregnancy have pre-existing primary hypertension, but high blood pressure druing pregnancy may be the first sign of pre-eclampsia, a serious condition of the second half of pregnancy and puerperium. Pre-eclampsia is characterised by increased blood pressure and the presence of protein in the urine. It occurs in about 5% of pregnancies and is responsible for approximately 16% of all maternal deaths. Pre-eclampsia also doubles the risk of perinatal mortality. Usually there are no symptoms in pre-eclampsia and it is detected by routine screening. When symptoms of pre-eclampsia occur the most common are headache, visual disturbance, vomiting, epigastric pain, and edema. Pre-eclampsia can occasionally progress to a life-threatening condition called eclampsia, which is a hypertensive emergency and has several serious complications including vision loss, cerebral edema, seizures or convulsions, renal failure, pulmonary edema, and disseminated intravascular coagulation.

2.5.1.3 In neonates, infants and children

Failure to thrive, seizures, irritability, lack of energy, and difficulty breathing can be associated with hypertension in neonates and young infants. In older infants and children, hypertension can cause headache, unexplained irritability, fatigue, failure to thrive, blurred vision, nosebleeds, and facial paralysis. The bio-marker of hypertension is cholesterol.

2.5.2 Cholesterol

Cholesterol is a lipid, waxy steroid found in the cell membranes and transported in the blood plasma of all animals. It is an essential component of mammalian cell membranes, which is required to establish proper membrane permeability and fluidity. In addition, cholesterol is an important precursor molecule for the biosynthesis of bile acids, steroid hormones, and several fat-soluble vitamins. Cholesterol is the principal sterol synthesized by animals, but small quantities are synthesized in other eukaryotes, such as plants and fungi. It is almost completely absent among prokaryotes, which include bacteria.

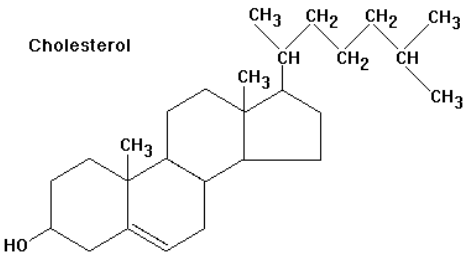


Fig. 2.6 Structure of Cholesterol.

Choresterol is only slightly soluble in water. In addition to providing a soluble means for transporting cholesterol through the blood, lipoproteins have cell-targeting signals that direct the lipids they carry to certain tissues. For this reason, there are several types of lipoproteins within the blood called, in order of increasing density, chylomicrons, very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL).

According to the lipid hypothesis, abnormally high cholesterol levels (hypercholesterolemia); that is, higher concentrations of LDL and lower concentrations of functional HDL are strongly associated with cardiovascular disease because these promote atheroma development in arteries (atherosclerosis). This

disease process leads to myocardial infarction (heart attack), stroke, and peripheral vascular disease.

2.6 Diabetes mellitus [9]

Diabetes mellitus, often simply referred to as diabetes, is a group of metabolic diseases in which a person has high blood sugar, either because the body does not produce enough insulin, or because cells do not respond to the insulin that is produced. This high blood sugar produces the classical symptoms of polyuria (frequent urination), polydipsia (increased thirst) and polyphagia (increased hunger).

There are three main types of diabetes:

Type 1 diabetes: results from the body's failure to produce insulin, and requires the person to inject insulin.

Type 2 diabetes: results from insulin resistance, a condition in which cells fail to use insulin properly, sometimes combined with an absolute insulin deficiency.

Gestational diabetes: is when pregnant women, who have never had diabetes before, have a high blood glucose level during pregnancy. It may precede development of type 2 (DM). Other forms of diabetes mellitus include congenital diabetes, which is due to genetic defects of insulin secretion, cystic fibrosis-related diabetes, steroid diabetes induced by high doses of glucocorticoids, and several forms of monogenic diabetes.

All forms of diabetes have been treatable since insulin became available in 1921, and type 2 diabetes may be controlled with medications. Both type 1 and 2 are chronic conditions that usually cannot be cured. Pancreas transplants have been tried with limited success in type 1; gastric bypass surgery has been successful in many with morbid obesity and type 2. Gestational diabetes usually resolves after delivery. Diabetes without proper treatments can cause many complications. Acute complications include hypoglycemia, diabetic ketoacidosis, or nonketotic

hyperosmolar coma. Serious long-term complications include cardiovascular disease, chronic renal failure, retinal damage. Adequate treatment of diabetes is thus important, as well as blood pressure control and lifestyle factors such as smoking cessation and maintaining a healthy body weight.

2.6.1 Signs and symptoms

The classical symptoms of diabetes are polyuria (frequent urination), polydipsia (increased thirst) and polyphagia (increased hunger). Symptoms may develop rapidly (weeks or months) in type 1 diabetes while in type 2 diabetes they usually develop much more slowly and may be subtle or absent.

Prolonged high blood glucose can cause glucose absorption in the lens of the eye, which leads to changes in its shape, resulting in vision changes. Blurred vision is a common complaint leading to a diabetes diagnosis; type 1 should always be suspected in cases of rapid vision change, whereas with type 2, change is generally more gradual, but should still be suspected. A number of skin rashes can occur in diabetes that are collectively known as diabetic dermadromes.

2.6.1.1 Diabetic emergencies

People (usually with type 1 diabetes) may also present with diabetic ketoacidosis, a state of metabolic dysregulation characterized by the smell of acetone; a rapid, deep breathing known as Kussmaul breathing; nausea; vomiting and abdominal pain; and altered states of consciousness. A rare but equally severe possibility is hyperosmolar nonketotic state, which is more common in type 2 diabetes and is mainly the result of dehydration.

2.6.1.2 Complications of diabetes mellitus

All forms of diabetes increase the risk of long-term complications. These typically develop after many years (10–20), but may be the first symptoms in those who have otherwise not received a diagnosis before that time. The major long-term complications relate to damage to blood vessels. Diabetes doubles the risk of cardiovascular disease. The main macrovascular diseases are ischemic heart disease, stroke and peripheral vascular disease.

Diabetes also causes microvascular complications that cause damage to the small blood vessels. Diabetic retinopathy, which affects blood vessel formation in the retina of the eye, can lead to visual symptoms, reduced vision, and potential blindness. Diabetic nephropathy, the impact of diabetes on the kidneys, can lead to scarring changes in the kidney tissue, loss of small or progressively larger amounts of protein in the urine, and eventually chronic kidney disease requiring dialysis. Diabetic neuropathy is the impact of diabetes on the nervous system, most commonly causing numbness, tingling and pain in the feet and also increasing the risk of skin damage due to altered sensation. Together with vascular disease in the legs, neuropathy contributes to the risk of diabetes-related foot problems that can be difficult to treat and may occasionally require amputation.

2.6.2 Glucose

Glucose ($C_6H_{12}O_6$, also known as D-glucose, dextrose, or grape sugar) is a simple sugar (monosaccharide) and an important carbohydrate in biology. Cells use it as the primary source of energy and a metabolic intermediate. Glucose is one of the main products of photosynthesis and starts cellular respiration.

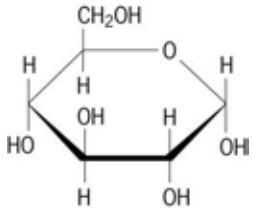


Fig. 2.7 Structure of glucose ($C_6H_{12}O_6$)

Glucose exists in several different structures, but all of these structures can be divided into two families of mirror-images (stereoisomers). Only one set of these isomers exists in nature, those derived from the "right-handed form" of glucose, denoted D-glucose. D-glucose is often referred to as dextrose. The term dextrose is derived from dextrorotatory glucose. Solutions of dextrose rotate polarized light to the right. Starch and cellulose are polymers derived from the dehydration of D-glucose. The other stereoisomer, called L-glucose, is hardly ever found in nature. Glucose is a very important carbohydrate in biology. The living cell uses it as a source of energy and metabolic intermediate. Glucose is one of the main products of photosynthesis and starts cellular respiration in both prokaryotes and eukaryotes.

Glucose is a common medical analyte measured in blood samples. Eating or fasting prior to taking a blood sample has an effect on the result. Higher than usual glucose levels may be a sign of prediabetes or diabetes mellitus. Blood sugar concentration, or glucose level, refers to the amount of glucose present in the blood of a human or animal. Normally, in mammals, the blood glucose level is maintained at a reference range between about 3.6 and 5.8 mM (mmol/L). It is tightly regulated as a part of metabolic homeostasis.

Failure to maintain blood glucose in the normal range leads to conditions of persistently high (hyperglycemia) or low (hypoglycemia) blood sugar. Diabetes mellitus, characterized by persistent hyperglycemia from any of several causes, is the most prominent disease related to failure of blood sugar regulation.

2.7 Renal failure [10]

Renal failure or kidney failure (formerly called renal insufficiency) describes a medical condition in which the kidneys fail to adequately filter toxins and waste products from the blood. The two forms are acute and chronic. A number of other diseases or health problems may cause either form of renal failure to occur.

Renal failure is described as a decrease in glomerular filtration rate. Biochemically, renal failure is typically detected by an elevated serum creatinine level. Problems frequently encountered in kidney malfunction include abnormal fluid levels in the body, deranged acid levels, abnormal levels of potassium, calcium, phosphate, and (in the longer term) anemia as well as delayed healing in broken bones. Depending on the cause, hematuria (blood loss in the urine) and proteinuria (protein loss in the urine) may occur. Long-term kidney problems have significant repercussions on other diseases, such as cardiovascular disease.

2.7.1 Classification

Renal failure can be divided into two categories: acute kidney injury or chronic kidney disease. The type of renal failure is determined by the trend in the serum creatinine. Other factors which may help differentiate acute kidney injury from chronic kidney disease include anemia and the kidney size on ultrasound. Chronic kidney disease generally leads to anemia and small kidney size.

2.7.1.1 Acute kidney injury

Acute kidney injury, previously called acute renal failure (ARF), is a rapidly progressive loss of renal function, generally characterized by oliguria (decreased urine production, quantified as less than 400 mL per day in adults, less than 0.5 mL/kg/h in children or less than 1 mL/kg/h in infants); and fluid and electrolyte imbalance. It can result from a variety of causes, generally classified as

prerenal, intrinsic, and postrenal. An underlying cause must be identified and treated to arrest the progress, and dialysis may be necessary to bridge the time gap required for treating these fundamental causes.

Acute kidney failure usually occurs when the blood supply to the kidneys is suddenly interrupted or when the kidneys become overloaded with toxins. Causes of acute failure include accidents, injuries, or complications from surgeries in which the kidneys are deprived of normal blood flow for extended periods of time. Heart-bypass surgery is an example of one such procedure.

2.7.1.2 Chronic kidney disease

Chronic kidney disease can develop slowly and, initially, show few symptoms. It can be the long term consequence of irreversible acute disease or part of a disease progression. It has numerous causes. The most common is diabetes mellitus. The second most common is long-standing, uncontrolled, hypertension, or high blood pressure. Polycystic kidney disease is another well-known cause. The majority of people afflicted with polycystic kidney disease have a family history of the disease. Other genetic illnesses affect kidney function as well. Overuse of common drugs such as aspirin, ibuprofen, and acetaminophen (paracetamol) can also cause chronic kidney damage. Some infectious diseases such as hantavirus can attack the kidneys, causing kidney failure.

2.7.1.3 Acute-on-chronic renal failure

Acute kidney injuries can be present on top of chronic kidney disease, a condition called acute-on-chronic renal failure. The acute part of it may be reversible, and the goal of treatment, as with the acute, is to return the patient to baseline renal function, typically measured by serum creatinine. Like acute, acute-on-chronic can be difficult to distinguish from chronic kidney disease if the patient has

not been monitored by a physician and no baseline blood work is available for comparison.

2.7.2 Symptoms

Symptoms can vary from person to person. Someone in early stage kidney disease may not feel sick or notice symptoms as they occur. When kidneys fail to filter properly, waste accumulates in the blood and the body, a condition called azotemia. Very low levels of azotaemia may produce few, if any, symptoms. If the disease progresses, symptoms become noticeable. Renal failure accompanied by noticeable symptoms is termed uraemia.

2.7.3 Albumin

Albumin refers generally to any protein that is water soluble, is moderately soluble in concentrated salt solutions, and experiences heat denaturation. Albumins are commonly found in blood plasma, and are unique from other blood proteins in that they are not glycosylated. Substances containing albumin, such as egg white, are called albuminoids. A number of blood transport proteins are known to be evolutionarily related, including serum albumin, alpha-fetoprotein, vitamin D-binding protein and afamin.

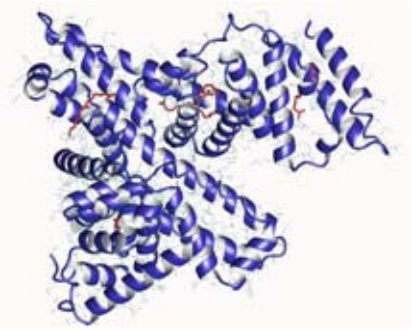


Fig. 2.8 Structure of Albumin.

Albumin is the main protein of plasma; it binds water, cations (such as Ca^{2+}, Na^{+} and K^{+}), fatty acids, hormones, bilirubin, thyroxine (T4) and drugs (including barbiturates) - its main function is to regulate the colloidal osmotic pressure of blood. Alpha-fetoprotein (alpha-fetoglobulin) is a fetal plasma protein that binds various cations, fatty acids and bilirubin. Vitamin D-binding protein binds to vitamin D and its metabolites, as well as to fatty acids. The biological role of afamin (alpha-albumin) has not yet been characterised.

Serum albumin is the most abundant blood plasma protein and is produced in the liver and forms a large proportion of all plasma protein. The human version is human serum albumin, and it normally constitutes about 60% of human plasma protein.

Serum albumins are important in regulating blood volume by maintaining the oncotic pressure (also known as colloid osmotic pressure) of the blood compartment. They also serve as carriers for molecules of low water solubility this way isolating their hydrophobic nature, including lipid soluble hormones, bile salts, unconjugated bilirubin, free fatty acids (apoprotein), calcium, ions (transferrin), and some drugs like warfarin, phenobutazone, clofibrate & phenytoin. Competition between drugs for albumin binding sites may cause drug interaction by increasing the free fraction of one of the drugs, thereby affecting potency.

Normal range of human serum albumin in adults is 3.5 to 5 $g.dL^{-1}$. For children less than three years of age, the normal range is broader, 2.9-5.5 $g.dL^{-1}$.

2.7.4 Creatinine

Creatinine is a break-down product of creatine phosphate in muscle, and is usually produced at a fairly constant rate by the body (depending on muscle mass). Creatinine starts to decompose above 295 °C.

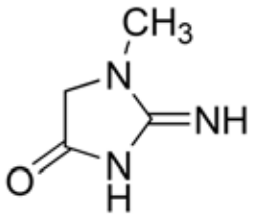


Fig. 2.9 Structure of Creatinine

In chemical terms, creatinine is a spontaneously formed cyclic derivative of creatine. Creatinine is chiefly filtered out of the blood by the kidneys (glomerular filtration and proximal tubular secretion). There is little-to-no tubular reabsorption of creatinine. If the filtering of the kidney is deficient, creatinine blood levels rise. Therefore, creatinine levels in blood and urine may be used to calculate the creatinine clearance (CrCl), which reflects the glomerular filtration rate (GFR).

The GFR is clinically important because it is a measurement of renal function. However, in cases of severe renal dysfunction, the creatinine clearance rate will be overestimated because active secretion of creatinine from the proximal tubule will account for a larger fraction of the total creatinine cleared. Ketoacids, cimetidine and trimethoprim reduce creatinine tubular secretion and therefore increase the accuracy of the GFR estimate, particularly in severe renal dysfunction. (In the absence of secretion, creatinine behaves like insulin.)

A more complete estimation of renal function can be made when interpreting the blood (plasma) concentration of creatinine along with that of urea. BUN-to-creatinine ratio (the ratio of blood urea nitrogen to creatinine) can indicate other problems besides those intrinsic to the kidney; for example, a urea level raised out of proportion to the creatinine may indicate a pre-renal problem such as volume depletion.

Men generally tend to have higher levels of creatinine because they have more skeletal muscle mass than women. Vegetarians have been shown to have lower creatinine levels.

The typical human reference ranges for serum creatinine are 0.5 to 1.0 mg/dL (about 45-90 μmol/L) for women and 0.7 to 1.2 mg/dL (60-110 μmol/L) for men. While a baseline (medicine) serum creatinine of 2.0 mg/dL (150 μmol/L) may indicate normal kidney function in a male body builder, a serum creatinine of 1.2 mg/dL (110 μmol/L) can indicate significant renal disease in an elderly female. For males, the reference range is 60-120 μmol/L and for females, it is 50-110 μmol/L.

2.8 Literature Surveys

Analytical method developed for certain analytes

The analytical methods for quantitative determination of glucose, cholesterol, albumin, and creatinine are summarized as the following.

2.8.1 Glucose

In 2004, Garcia, Henry [11] described a new analysis strategy for microchip capillary electrophoresis with pulsed amperometric detection and its application to the determination of glucose. The addition of sodium dodecyl sulfate (SDS) to the mobile phase and detection reservoir stabilized order to improve the detection performance. Under optimum conditions, a linear relationship between the peak current and the concentration of glucose was found between 10^{-2}-10^{-5}M, with a limit of detection of 1.2 μM.

In 2004, Bernardez *et al.* [12] developed the method for detection of sugars in varieties of chestnut fruits by High Performance Liquid Chromatography (HPLC) with a universal light scattering detector. The method proposed for assessing

sucrose, glucose and fructose makes it possible to obtain a variance coefficient of 1.42 % for a content of sucrose of 22 % and a recovery coefficient of 86 %.

In 2004, Cao *et al.* [13] described a simple, reliable and reproducible method based on capillary electrophoresis with electrochemical detection, for the determination of sucrose, maltose, glucose and fructose in rice flour. Operated in a wall-jet configuration, a copper-disk electrode was used as a working electrode, which exhibits good response at +650 mV (vs. SCE) for all analytes. Under optimized conditions, four analytes were base-line separated within 15 min. The response was linear over two orders of magnitude, and the detection limit (S/N=3) is $9x10^{-7}$ g/L, $1.4x10^{-6}$ $g.L^{-1}$, $6x10^{-7}g.L^{-1}$ and $1.3x10^{-7}$ $g.L^{-1}$ for sucrose, maltose, glucose and fructose, respectively.

In 2005, Chen *et al.* [14] presented a method based on capillary electrophoresis with electrochemical detection which had been developed for the separation and determination of mannitol, sucrose, glucose, and fructose in *Ligustrum lucidum* Ait. The four analytes can be well separated within 13 min in a 40 cm length fused-silica capillary at a separation voltage of 12 kV in a 75 mM NaOH aqueous solution. The relation between peak current and analyte concentration was linear over about three orders of magnitude with detection limits (S/N=3) ranging from 1 to 2 μM for all analytes.

In 2008, Sato *et al*. [15] reported the simultaneous determination of serum mannose and glucose concentration in dog serum using High Performance Liquid Chromatography with a UV and fluorescence detection system. Under optimized conditions, detection limits were 0.09 $\mu mol.L^{-1}$ for mannose and 0.04 $mmol.L^{-1}$ for glucose. Linearity of peak areas vs. amounts of mannose and glucose in the range of 0.27-320 $\mu mol.L^{-1}$ and 0.13-64 $\mu mol.L^{-1}$ were observed, respectively.

2.8.2 Cholesterol

In 2001, Brahim *et al.* [16] presented the method for determination of cholesterol in serum using a biosensor of cholesterol oxidase contained within a polypyrrole-hydrogel membrane. The optimized cholesterol biosensor exhibited a linear response range from $5x10^{-4}$ to $1.5x\ 10^{-2}$ M and detection limit of 120 μM toward cholesterol. The response time of the biosensor was 30 s. The analytical recovery of cholesterol in the serum samples ranged from 97 to 103 % with mean coefficients of variation of 3% (within-day analyses) and 3.9% (day-to-day analyses).

In 2004, Rodriguez *et al*. [17] presented the method for the determination of creatinine and creatine in human urine by capillary zone electrophoresis with UV detection. Under optimized condition, the analysis takes less than 2.7 min. A linear response over the 3.0-120 $mg.L^{-1}$ concentration range was investigated for compounds. Detection limits of 0.7 and 1.3 $mg.L^{-1}$ for creatinine and creatine (S/N=3) were obtained.

In 2006, Salinas *et al.* [18] reported the method for determination of cholesterol by a flow injection analysis (FIA) system with multienzymatic-rotating biosensor. This method could be used to determine total cholesterol concentration in the range 1.2 μM-1mM (r=0.999). A fast response time of 2 min has been observed with this amperometric-rotating biosensor. The calculated detection limits were 11.9 nM. Reproducibility assays were made using repetitive standards solutions (n=5) and the percentage standard error was less than 4%.

In 2007, Hojo *et al*. [19] presented the method for determination of total cholesterol in serum by High Performance Liquid Chromatography with electrochemical detection. Under optimum conditions, the current peak height was linearly related to the amount of cholesterol injected from 0.5-100 μM (r>0.999). The detection limit of cholesterol was 0.36 μM (1.8 pmol). Cholesterol at 100 μM was directly detected with a relative standard deviation (RSD) of less than 1.0% (n=8). The cholesterol and free cholesterol in control human serum were determined by the

proposed method with the recovery of more than 90% and the RSD (n=6) of less than 3.0%.

2.8.3 Albumin

In 2002, Khawali *et al*. [5] presented the method for determination of albumin by a commercial kit (DCA 2000) with conventional immune turbidimetric detection. To evaluate the correlation between early-morning urinary of the albumin-to-creatinine ratio (A/C) and overnight albumin excretion rate. A/C ratios determined with the DCA 2000 kit and by the laboratory method were 13.1±20.5 and 20.4 ± 46.3 mg/g, respectively. A/C results by both methods proved to be strongly correlated ($r=0.98$, $P<0.001$). DCA 2000-determined A/C showed 50% sensitivity and 100% specificity when compared to the reference method.

In 2004, Wiberg *et al.* [20] presented the method for determination of albumin and immunoglobulin G with fluorescence spectroscopy. Under optimum conditions, linearity of albumin and IgG1 were in the range of 0.42 $\mu g.mL^{-1}$ and 0-12.7 $\mu g.mL^{-1}$, respectively.

In 2005, Zhao *et al*. [21] developed the method for determination of albumin by a different solid phase chemiluminescence ELISA. The influence of several physic-chemical parameters, such as incubation time, detergent concentration and solid phase conditions were also studied. For the two solid phases, both the linear range and the limit of detection of albumin were 0.15-15 and 0.089 $\mu g.mL^{-1}$, compared with the commercially ELISA kit, a good correlation was obtained.

In 2007, Gao *et al*. [22] proposed the method for determination of bovine serum albumin by the resonance light scattering technique with sodium dodecylbenzene sulphonate-cetyltrimethylammonium bromide probe. Under optimum conditions, the enchanced resonande light scattering (RLS) intensity is proportional to the concentration of BSA in the range from $2.5x10^{-8}$ to $2.0x10^{-6} mol.L^{-1}$. The detection limit is $9.7x10^{-9}$ $mol.L^{-1}$ for BSA.

In 2007, Giovannoli *et al.* [23] developed the method for determination of human serum albumin by capillary electrophoresis immunoassay with laser-induced fluorescence detection. Under optimum conditions two calibration curves with good run-to-run reproducibility and LOD- respectively 14.0nM for the FITC-polyclonal antibody and 9.0nM for the FITC -monoclonal antibody – were achieved.

2.8.4 Creatinine

In 2003, Hewavitharana, Brusce [24] described the method for simultaneous determination of creatinine and pseudouridine concentrations in bovine plasma by reversed-phase liquid chromatography with photodiode array detection. Under optimized conditions, the recoveries of both analytes were above 96%. Lowest detectable amounts of creatinine and pseudouridine were 0.28 nmol and 9.0 pmol, respectively.

In 2003, Stefan *et al*. [25] proposed the simultaneous determination of creatine and creatinine by an amperometric biosensor. A bienzymatic biosensor based on creatinase (CI) and sarcosine oxidase (SO) was used for the assay of creatine and a trienzymatic biosensor based on CI, SO and creatininase (CA) for the assay of creatinine. The linear concentration ranges are of pmol/L to nmol/L magnitude order, with very low limits of detection.

In 2004, Huskova *et al*. [26] presented the method for determination of creatinine in urine by tandem mass spectrometry. Under optimized conditions, the time of the analysis was 3.015 min. Linearity was obtained in the range 0.06-60 $mmol.L^{-1}$. The detection limit was 0.2 $\mu mol.L^{-1}$ and recoveries were in the range 95.1-98.3% for both the assays with and without the ion-exchange column.

In 2006, Chen *et al*. [27] described the method for the selective determination of creatinine in human urine by enzymeless electrochemical sensor. The creatinine was selectively detected in the ranges of 0.37-3.6 mM with a slope and regression coefficient deviation of 3.4%, indicating a detection limit of 8.6 μM.

In 2006, George *et al.* [28] developed the method for the simultaneous determination of allantoin, uric acid and creatinine in cattle urine by High Performance Liquid Chromatography with UV detection. The recoveries of the standard compounds added to urine samples were 94-104 %.

In 2009, Liotta *et al*. [29] presented the method for determination of creatinine in urine by capillary zone electrophoresis with UV detection. Under optimum conditions, linearity was assessed in the range 0.2-32 mM. Precision tests resulted in CVs % below 0.56 % for migration time and below 3.78 % for peak area ratios (analyte/I.S.).

In 2009, Songjaroen *et al*. [30] developed the method for the determination of urinary creatinine by a portable microfluidic system with a fiber optic spectrometer as a detector. A linear range was displayed from 0 to 40 $mg.L^{-1}$ creatinine (r^2=0.997) with a detection limit of 3.3 mg/L (S/N=3). On-chip absorbance signals are reproducible, with a relative standard deviation (RSDs) of 7.1%, when evaluated with 20 $mg.L^{-1}$ creatinine (n=10). The standard curves in which the intra-run CVs (4.7-6.8%) and inter-run CVs (7.9%) obtained were performed on three different days and exhibited good reproducibility.

2.8.5 Paper-based microfluidic devices

In 2007, Martinez *et al.* [1] reported the method for determination of glucose and protein (Bovine serum albumin, BSA) in artificial urine sample by a paper-based microfluidic device. This device is suitable for measuring multiple samples in parallel and in a relatively short period of time. It can be used for the determination of glucose and protein in clinically relevant ranges (2.5-50 mM for glucose and 0.38-7.5 μM for Bovine serum albumin,BSA).

In 2008, Dungchai *et al.* [31] developed the method for the determination of glucose, uric acid, and lactate by a paper-based microfluidic device with an electrochemical detector. Under optimized conditions, linearity was in the

range 0-100 mM, 0-50 mM ,and 0-35 mM for glucose, lactate, and uric acid, respectively. The Detection limits were 0.21 mM for glucose, 0.38 mM for lactate, and 1.38 for uric acid.

In 2008, Martinez *et al.* [2] described the method for the determination of glucose and protein by paper-based microfluidic devices and proposed information focused on the component of the system that exchanges the results of these assays with off-site experts for evaluation. This system gave accurate and quantitative results when detecting all analytes in urine, uses small volumes of the sample (≤5 μL).

CHAPTER III

EXPERIMENTAL

This chapter provides the information about instruments and equipments, chemical and reagents employed in this work.

3.1 Instruments and equipment

List of instruments and equipments used in this work is shown in Table 3.1.

Table 3.1 List of instruments and equipments and their suppliers.

No.	Instruments and Equipments	Suppliers
1	Spin coater (model WS-400A-6NPP)	Laurell technologies Corp, USA
2	UV-lithography MJB4 mask aligner	SUSS microtec, Germany
3	Oxygen plasma cleaner (PDC-32G)	Harrick scientific Corp, USA
4	Hotplate stirrer	Cole-Parmer Canada Inc., Canada
5	UV-VIS spectrophotometer	Thermo Scientific, USA
6	pH meter	Thermo Scientific, USA
7	Vortex Mixer	FINEPCR, Korea
8	Milli Q water system	Millipore, USA
9	Hot air oven	Memmert, Germany
10	Transparency film	Thailand
11	Quart cuvette	SigmaAldrich, USA
12	Auto pipette and tip	Eppendrof, Germany
13	Digital camera model ES 70	Samsung, Korea
14	Duran bottles	SCHOTT, Germany

Table 3.1 List of instruments and equipments and their suppliers. (Cont.)

No.	Instruments and Equipments	Suppliers
15	Glass slide	Thailand
16	Magnet	Thailand
17	Glassware	Pyrax, USA
18	Paper filters	Whatman, Japan
19	Metal sheet	Thailand

3.2 Chemical and reagents

All the chemicals and reagents were of analytical grade or better. Milli Q water was used for the preparation of solution. List of chemical and their suppliers is summarized in Table 3.2.

Table 3.2 List of chemicals and reagents and their suppliers

No.	Chemical/ Reagents	Suppliers
1	Photoresist (SU-8 3025)	Micro Chem, USA
2	Developer	Micro Chem, USA
3	Acetone	Merck, Germany
4	Hydrogen peroxide	Merck, Germany
5	Isopropyl alcohol	Merck, Germany
6	Picric acid	ChemAlert Guide, Fisher Scientific
7	Creatinine hydrochloride	Sigma Aldrich, USA
8	Hydrochloric acid	Merck, Germany
9	Sodium hydroxide	Merck, Germany
10	Bovine serum albumin	Sigma Aldrich, USA
11	Phosphate Buffered Saline	Sigma Aldrich, USA
12	Sodium chloride	Merck, Germany

Table 3.2 List of chemical and reagents and their suppliers (Cont.)

No.	Chemicals/ Reagents	Suppliers
13	Ascorbic acid	Sigma Aldrich, USA
14	Glucose	Sigma Aldrich, USA
15	Eosin Y disodium salt	Sigma Aldrich, USA
16	Cholesterol	Sigma Aldrich, USA
17	Glucose oxidase	Sigma Aldrich, USA
18	Cholesterol oxidase	Sigma Aldrich, USA
19	Horseradish peroxidase	Sigma Aldrich, USA
20	Potassium iodide	Sigma Aldrich, USA
21	4-Aminoantipyrine	Sigma Aldrich, USA
22	3-(N-Ethyl-3-methylanilino)-2-hydroxy-propanesulfonic acid Sodium salt	Sigma Aldrich, USA
23	Sodium 3,5-dichloro-2-hydroxybenzenesulfonate	Sigma Aldrich, USA
24	Bees wax	Thailand
25	Silver nanoparticles	Thailand
26	Potassium dihydrogen phosphate	Carlo Erba Reagenti
27	Gold nanoparticles	Thailand
28	Potassium Dihydrogen Orthophosphate	BDH, England

3.3 Preparation of solutions

3.3.1 stock solution of 100 mM PBS (100 mL)

3.5815 g of sodium dihydrogen orthophosphate was dissolved in 100 mL of Milli Q water.

3.3.2 0.1 mM PBS pH 7.1 (100 mL)

0.1 mL of the stock solution of 100 mM PBS was diluted to 100 mL using Milli Q water in a volumetric flask. Then, the 0.1 mM PBS solution was adjusted to pH 7.1 0.1 M sodium hydroxide or 0.1 M hydrochloric acid.

3.3.3 stock solution of 100 mM Phe (10 mL)

94.11 mg of phenol was dissolved in 10 mL of Milli Q water.

3.3.4 10 mM Phe (10 mL)

1mL of stock solution of 100 mM Phe was diluted to 10 mL using Milli Q water.

3.3.5 stock solution of 100 mM DHBS (10mL)

265.05 mg of DHBS was dissolved in 10 mL of Milli Q water.

3.3.6 10 mM DHBS (10 mL)

1 mL of stock solution of 100 mM DHBS was diluted to 10 mL using Milli Q water.

3.3.7 stock solution of 100 mM 4AP (10 mL)

203.25 mg of 4AP was dissolved in Milli Q water.

3.3.8 10 mM 4AP (10 mL)

1 mL of stock solution of 100 mM 4AP was diluted to 10 mL using Milli Q water.

3.3.9 Mixtures of 4AP: DHBS 1:1 v/v (2mL)

1 mL of 10 mM 4AP and 1 mL of 10 mM DHBS were mixed in a vial.

3.3.10 Mixtures of 4AP:DHBS 1:2 v/v (3mL)

1 mL of 10 mM 4AP and 2 mL of 10 mM DHBS were mixed in a vial.

3.3.11 0.03 M Potassium dihydrogenphosphate (500 mL)

2.041 g of potassium dihydrogen phosphate was dissolved in 500 mL of Milli-Q water.

3.3.12 0.12% eosin-y (50 mL)

0.0600 g of eosin-y was dissolved in 50 mL of Milli-Q water.

3.3.13 1.0 M KI (10 mL)

1.6601 g of KI was dissolved in 10 mL of Milli-Q water.

3.3.14 standard solution of 400 mg dL^{-1} of cholesterol

Cholesterol (8.0 mg) was added to 100 mg of triton-x100 and heated at 100 °C and stirred for 1 hr. Then 1,900 µL of hot water was added to the solution and stirred for 1 hr. This solution was then cooled with cold water. The final concentration of this solution was kept in the refigurature (5 °C) before use. This solution should be use within 2 days following which white particles will appear in the stock solution.

3.3.15 200 mg dL^{-1} cholesterol (1 mL)

0.05 mL of the stock standard solution of 400 mg dL^{-1} cholesterol was diluted with 0.95 mL of 0.1 mM PBS pH 7.1.

3.3.16 stock standard solution of 1,000 mg dL^{-1} of glucose

1.0 g of glucose was dissolved in 100 mL of Milli-Q water.

3.3.17 standard solution of 5 mM glucose (10 mL)

0.9 mL of stock solution of glucose was diluted using 10 mL of Milli Q water.

3.3.18 stock solution of 1,000 mgL^{-1} of creatinine (25 mL)

0.0250 g of creatinine was dissolved in 0.1 M hydrochloric acid.

3.3.19 stock solution of 1,000 mgL^{-1}albumin (25 mL)

0.0250g of BSA was dissolved in 25 mL of Milli Q water.

3.4 Preparation of paper-based microfluidic devices

3.4.1 Optimization conditions for fabrication of paper-based microfluidic devices by photolithography method

The fabrication of paper-based microfluidic devices has various factors which can effect the efficiency of these devices. These factors are the weight of the photoresist (2-5 g), a velocity rate of a spin coater (1,800-2,000 rpm), temperature of baking (90-95 °C), time of baking (5-10 min), time of plasma process (1-60 s), and time of rinsing with developer and alcohol (1-3 min).

3.4.2 Preparation of paper-based microfluidic devices by photo-lithography method.

Photolithography was used to pattern the filter paper according to previously reported methods [54-62]. Briefly, SU-8 3025 photoresist was poured onto the center of the paper and distributed using a spin-coater. The photoresist-covered paper was baked at 95°C for 5 min. The paper was then covered with a patterned transparency film generated using a laser printer and irradiated with UV lamp at 100% intensity (400 W) for 7 s. After baking at 95°C for 5 min, the unpolymerized photoresist was removed from the paper by submerging in the developer for 3 min, followed by rinsing with isopropyl alcohol. After that, the paper was dried under ambient conditions for 1 h. Prior to adding reagents, the paper microfluidic devices were exposed to an air plasma at 18 W for 30 s. Areas covered with photoresist remained hydrophobic while areas without photoresist were hydrophilic.

3.4.3 Preparation of paper-based microfluidic devices by wax-dipping method.

To create a mould for wax dipping, an iron bar was cut into the desired shape and size using a laser cutting technique. For the wax dipping method, white Beeswax pellets were put into a beaker and heated until melted using a hotplate. Whatman No.1 paper was cut into a 1.5 x 2.5 cm. piece and placed onto a glass slide. Then, the iron mould was put onto the paper, and it was temporarily attached by means of magnetic force using a permanent magnet placed on the backside of the glass slide. Next, the assembly was dipped into a chamber of melted wax for one second. After the paper was cooled to room temperature, it was peeled from the glass slide, and the iron mould was removed from the paper. The wax-dipping for fabrication of the μPAD is shown in Figure 3.2.

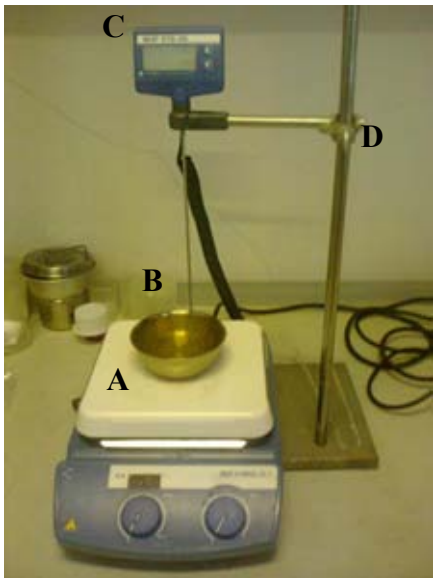


Fig. 3.2 The instrumental setting for wax-dipping fabrication that consist of (A) hotplate, (B) wax, (C) digital thermometer, and (D) stand.

3.5 Optimization of the fabrication of paper-based microfluidic devices

3.5.1 Optimization of melting temperature and dipping time

The melting temperature and dipping time influenced the penetration of the melted wax into the paper and also affected the resolution between the hydrophobic and hydrophilic areas. The melting temperature was studied in the range of 100- 130 ℃, and the dipping time was studied in the range of 1-3 s.

3.5.2 The effect of reagent and sample volume

The volumes of red food dye at 0.5, 1.0, and 1.5 µL were dropped into the detection zone for optimization of the volume of the reagent. The effect of sample volumes was studied by dropping 7.0-13.0µL of red food dye into the sample zone using a micropipette.

3.6 Optimization of cholesterol determination

3.6.1 The effect of enzyme concentration of POD and CO

The effect of concentration of POD was studied between 250 and 500 UmL^{-1} and the concentrations of CO were 30, 168, and 312 UmL^{-1}.

3.6.2 The effect of size of AgNPs

The size of AgNPs was studied at 10, 20, 40, and 80-100 nm. The concentration of AgNPs was fixed at 10 mgL^{-1}.

3.6.3 The effect of concentration of AgNPs

In this part, the concentration of AgNPs of 10 mgL^{-1} and 100 mgL^{-1} was used to study the effect of this factor. The size of AgNPs was chosen from 3.4.6.1

3.7 Optimization of conditions for glucose determination

3.7.1 The effect of concentration ratio of GOD and POD

The effect of GOD and POD ratio was studied at 1:1 and 1:2 v/v. The concentrations of all enzymes were 500 UmL^{-1}.

3.7.2 The effect of KI concentration

To study the effect of KI concentration, the concentration of KI at 0.6 M and 1.0 M.

3.7.3 The effect of %w/v of malachite green

The effect of %w/v of malachite green was studied at 0.05, 0.10, 0.15, and 0.2 %.

3.7.4 The effect of % w/v of $FeCl_3$

The effect of % $FeCl_3$ was studied by adding the Fe (III) on devices at 0.5, 1.0, 2.0, 5.0, and 10.0%, and the concentration of malachite green was fixed at 0.15 %.

3.8 Optimization of conditions for albumin determination

3.8.1 The effect of size of AuNPs

20 nm and 80 nm particle sizes of AuNPs were studied at 40 mgL^{-1}.

3.8.2 The effect of AuNPs concentration

The effect at 20, 40 and 1,000 mgL^{-1} on AuNPs was studied using the stock AuNP solution 1,000 mgL^{-1}. 20 and 40 mgL^{-1} of AuNPs were prepared by dilution with Milli Q water and protected from light. The size of AuNPs was choosen from 3.8.1.

3.8.3 The effect of pH of solutions

The effect of pH of solutions was studied in the range 2 - 5 at 0.02% of Eosin-Y.

3.8.4 The effect of % Eosin-Y

The effect of % Eosin-Y was studied in the range 0.1- 0.6% at the pH solutions from 3.4.8.3.

3.9 Optimization of conditions for creatinine determination

3.9.1 The effect of size of AuNPs

20 nm and 80 nm particle sizes of AuNPs were studied, at the concentration of 40 mgL^{-1}.

3.9.2 The effect of AuNPs concentration

The effect of AuNPs concentration was studied at 20, 40 and 1,000 mgL^{-1}. The concentrations of 20 and 40 mgL^{-1} AuNPs were prepared by dilution of Milli-Q water and protected from light. The size of AuNPs was choosen from 3.4.9.1.

3.9.3 The effect of concentration of NaOH

The effect of concentration of NaOH was studied in the range 1-5% at 1% picric acid.

3.9.4 The effect of concentration of picric acid

The effect of picric acid was studied in the range 0.5-5% at the suitable concentration of NaOH.

3.10 Validation method

3.10.1 Calibration curve and linear range

The standard solution of each analyte was freshly prepared and then the standard solutions were diluted to 5 concentrations. In these experiments, each concentration of analyte was measured six times. The results were used to plot the calibration curve and determine the linear range.

3.10.2 Limit of detection (LOD)

The LOD was investigated by testing blank devices ten times at the optimal condition. After that the results were obtained by calculating the signal with the following equation.

LOD = 3 SD/ slope

SD = the standard deviation of the signal from blank devices

Slope = the slope of calibration curve ($\frac{y}{x}$)

3.10.3 Limit of quantitation (LOQ)

The LOQ was investigated by testing blank devices ten times at the optimal condition. After that the results were obtained by calculating the signal using the following equation.

LOQ = 10 SD/ slope

SD = the standard deviation of the signal from blank devices

Slope = the slope of the calibration curve ($\frac{y}{x}$)

3.10.4 Precision

The precision was studied by repeating ten replicates of each analyte solution. Precision was assessed in terms of the relative standard deviation (%RSD) using the following formula.

$$\% \text{ RSD} = \frac{\text{standard deviation}}{\text{Mean}} \text{ x } 100$$

3.10.5 Accuracy

The accuracy was studied by adding the 3 concentrations of standard solution to the sample at 3 different concentrations. It was calculated by the following formula in terms of %recovery.

$$\% \text{ Recovery} = \frac{\text{Sspike} - \text{Ssample}}{\text{Sstd}} \times 100$$

S_{spike} = the signal of the sample spike with standard solution

S_{sample} = the signal of sample without the spike

S_{std} = the Ssgnal of the standard solution

CHAPTER IV

RESULTS AND DISCUSSION

PART I: Paper-based microfluidic devices

4.1 SU-8 photoresist technique

4.1.1 Designing the paper-based microfluidic device

The computer program Illustrator CS4 was used to design this device, including the shape of the test zone, the size of the test zone, the size of the sample zone, the number of test zones, and the length of the separation channel. The majority of the factors were determined with respect to the number of analytes and the reaction time of the assay that was used with the μPADs. Only the shape of the test zone was studied in this work, which is shown in Figure 4.3. After the pattern was completely designed, it was printed onto a transparency film using a laser printer. The printed pattern is called a mask, and it was used to produce the device during the fabrication step. In this work, the pattern has three test zones and one blank zone; the test zone was circular shaped (i.d. 3 mm) with a separation channel that had a length of 2 mm. The device was designed on filter paper with dimensions of 2 cm x 2 cm. Figures 4.1 and 4.2 present the design of this device used in this work.

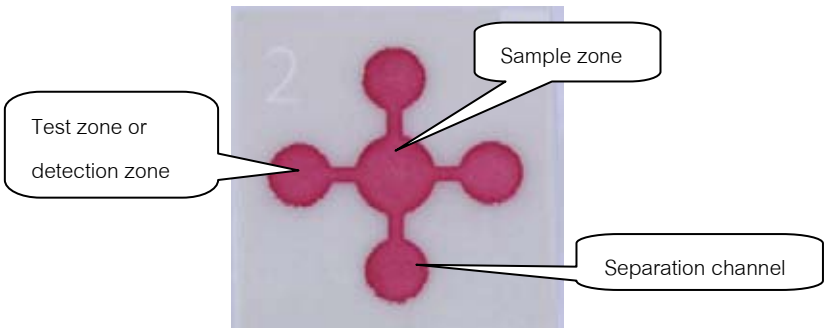


Fig. 4.1 The design of the SU-8 paper-based microfluidic device (i.d. of the detection zone was 3 mm, i.d. of the sample zone was 5 mm, the width of the separation channel was 1 mm, and the length of the separation channel was 2 mm)

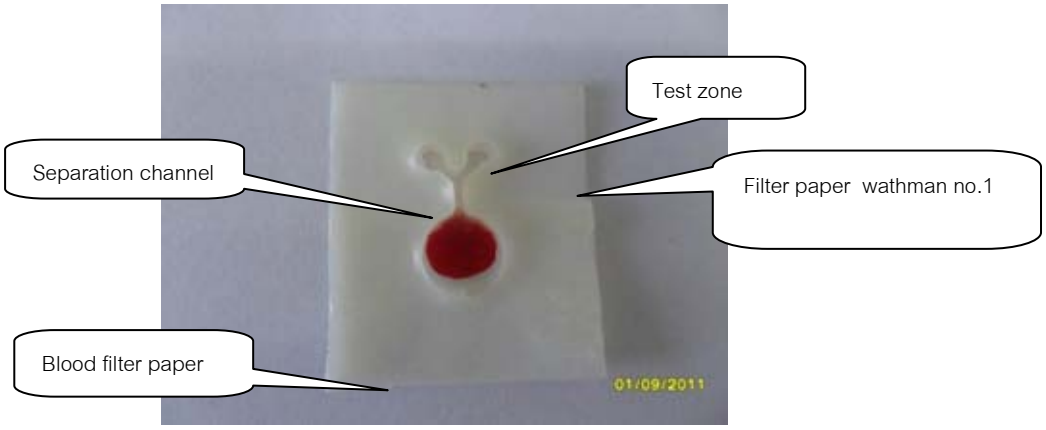


Fig. 4.2 Design of the wax paper-based microfluidic device. (i.d. of the test zone was 3 mm, i.d. of the sample zone 5 mm, and the separation channel length was 3 mm). This device has two detection test zones, and it was designed on a filter paper (Whatman no.1). Furthermore, the sample zone was designed on blood filter paper, which could separate blood serum from whole blood.

4.1.2 Fabrication of the paper-based microfluidic device

There are several factors involved in this device fabrication technique that are important for the efficiency of this device. These factors include the time in the spin coater, the rotation rate of the spin coater (rpm), the weight of the SU-8 photoresist, the time of the prebake and the postbake, the temperature of the pattern baking, and the soaking time in the developer solution and isopropanol. The factors of the time in the spin coater, the rotation rate of the spin coater, and the weight of the SU-8 photoresist were examined by spreading the SU-8 photoresist on the paper that was used to fabricate the device. Furthermore, the other factors were used to determine the suitable conditions for creating a completely clean channel without the appearance of damage to the paper. All of the results from this part are shown in table 4.1.

4.1.3 Selecting a suitable shape for the detection zone

Four shapes, including a circle, a square, a triangle, and a diamond, were designated for the detection test zones on the paper-based microfluidic device. A red food dye was used to test the suitability of the detection zone for the determination of the analytes. The result of this test is shown in Figure 4.3. The circular-shaped test zone was selected because it does not contain any angles, which makes the data collection easier.

Table 4.1 The optimal conditions for fabricating the paper-based microfluidic device using the photolithography technique.

Factor	Optimum condition
Time in the spin coater (1, 3, 5, 10 sec)	5 sec
Velocity of the spin coater (1,500-2,000 rpm)	1,900 rpm
Weight of SU-8 (2-5 g)	4 g
Temperature of baking (90-10ºC)	95 ºC
Time of baking (3-10 min)	Prebake 5 min, postbake 5 min
Time of developer soaking (1-5 min)	3 min
Time of isopropanol soaking (1-5 min)	1 min

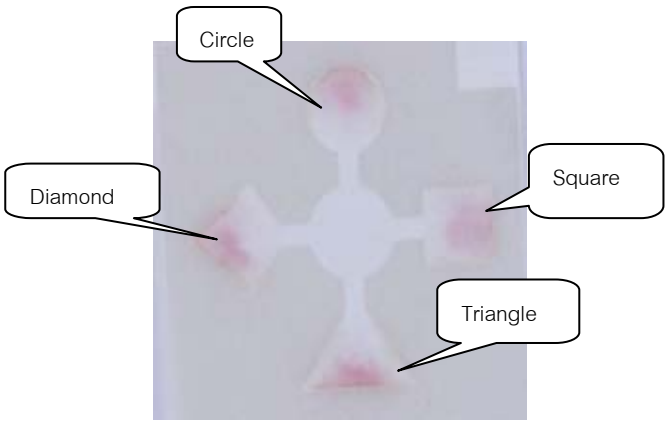
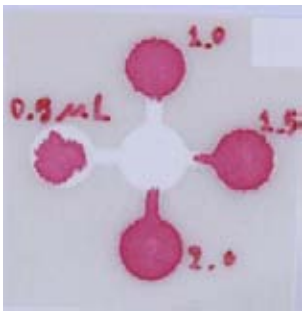


Fig. 4.3 The four shapes of the detection zones in the paper-based microfluidic device (circle, diamond, square, and triangle). A 15 μL aliquot of a red dye solution was dropped into the sample test zone, and this solution was spread throughout the device.

The result of the effect of the different shapes of the detection test zones was analyzed to select the shape that produced the best spread of the dye solution.

4.1.4 Reagent volume and sample volume

The reagent volume and sample volume are important factors to consider when using the paper-based microfluidic device with the colorimetric assay. Because this device was constructed from paper, it only absorbed a limited volume of liquid. The effects of excess liquid on the paper were destructive. When the volume of reagent or sample was not sufficient for use on this device, the color was not completely developed. Figure 4.4 presents the results from the study of the reagent volume and the sample volume, which were 1.0 µL and 13.0 µL, respectively.

a) 

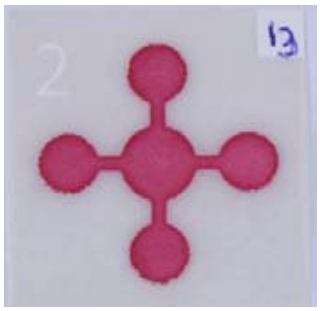
b) 

Fig. 4.4 The effects of (a) the reagent volume (0.5-2.0 µL) and (b) the sample volume. In picture a) A 1.0 µL volume of reagent was chosen because at this volume, the spread of the red dye solution completely covered the detection area. Picture b) the sample volume was studied in the range of 7-13 µL. The result shown is the suitable volume of sample (13 µL) where the red dye solution was spread throughout this device.

4.2 Wax-dipping technique

The wax-dipping technique was developed for reducing the cost of fabricating the μPAD, and it could fabricate the device within 1 min using a single step. In this work, two factors of the wax-dipping technique were examined. First, the temperature that the wax is heated at is a very important factor when using the wax-dipping technique. When an excess temperature (>120ºC) was used to fabricate the device, the area of the hydrophilic channel was decreased because more melted wax could absorb into a fiber of the cellulose paper than at the lower temperature. The melted wax could not completely absorb into cellulose fiber at the lower suitable temperature (120°C). The result of this study indicated that 120ºC was the optimal temperature for melting the wax. Second, the amount of time that the paper was dipped into the melted wax was examined under the optimal temperature for heating the wax because this factor had a direct effect on the area of the hydrophilic channel in this device. When the device was dipped into the melted wax for a long period of time, more melted wax could spread into the fiber of the paper than during a short dipping time. The result of this examination indicated that the suitable time for dipping the device into the melted wax was 1 sec. The reagent volume was 0.5 μL, and the sample volume was 11.0 μL.

CHAPTER V

RESULTS AND DISCUSSION

PART II: CHOLESTEROL

The colorimetric assay was used to determine the concentration of cholesterol. The assay was divided into 2 parts: an assay with an enzyme and a non-enzyme assay. The first part examined the color intensity with the reaction in Fig. 5.1, which included CO and POD. The last part used AgNPs, which combined with cholesterol and aggregated to form a new color complex.

5.1 Determination of cholesterol using an enzyme assay

Fig. 5.1 shows the enzyme assay reaction that was used to determine the concentration of cholesterol in this work. This reaction has two steps. In the first step, cholesterol is changed into cholestenone and hydrogen peroxide (H_2O_2) by cholesterol oxidase (CO), and in the second step, hydrogen peroxide reacts with 4-aminoantipyrine (4AP) and phenol (Phe). Then, horseradish peroxidase (POD) functions as a catalyst to form the final products of this reaction, which are a rose-colored complex and water. From this reaction, the concentration of enzymes and the concentration of the reagent complex can be the cause of a %yield of product.

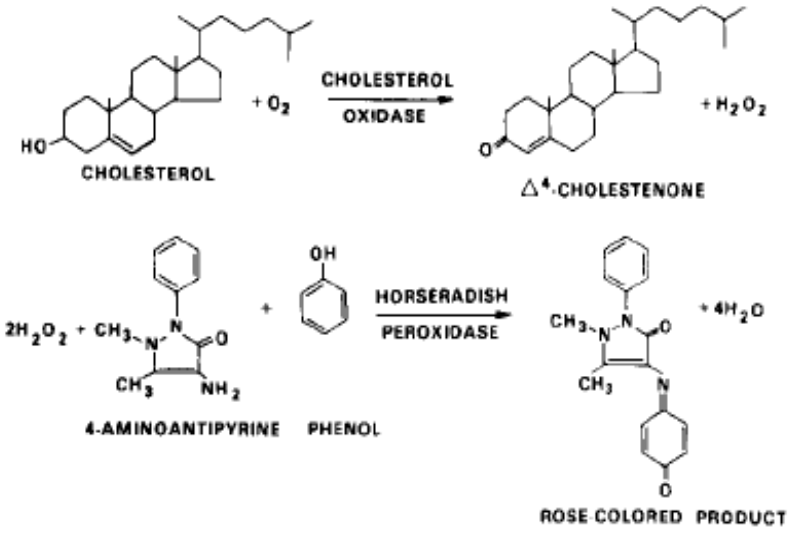


Fig. 5.1 Reaction scheme for the enzymatic measurement of cholesterol. [64]

5.1.1 The effect of the concentrations of CO and POD

The effects of the concentration of CO were examined at concentrations of 39, 169, and 312 UmL^{-1}; the results indicated that a low concentration of CO resulted in a long reaction time and a concentration of 312 $Um.L^{-1}$ could form a complex faster than the concentrations of 169 and 39 $Um.L^{-1}$. Furthermore, the results of the concentrations of 169 and 312 $Um.L^{-1}$ were not different; therefore, the concentration of 169 $Um.L^{-1}$ was selected for use in this work because it could save cost.

The intensity of the color was affected by the concentration of POD because this enzyme functioned as a catalyst during the reaction. The effects of the concentration of POD were examined at 250 and 500 $Um.L^{-1}$. The concentration of 500 $Um.L^{-1}$ of POD was selected for use in this work.

5.1.2 The effect of Phe and DHBS reagent

The results from using the same concentration (10 mM) of all reagents (Phe and DHBS) are shown in Fig. 5.2 and Fig. 5.4; the color intensity of the solution from the analyte that was complexed with DHBS was more intense than Phe. Based on this result, a structure of DHBS was forming the complex with the 4AP reagent stronger than Phe because the structure of DHBS was more rigid than Phe. The color intensity of the DHBS complex was greater than the Phe complex. When the color intensity was higher, the gray scale value was lower.

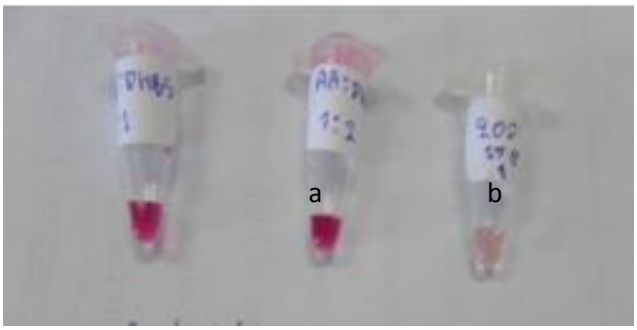


Fig. 5.2 Study of the effects of the Phe and DHBS reagents: a) 4AP: DHBS 1:1 v/v, b) 4AP: DHBS 1:2 v/v, and c) Phe. Both reagent concentrations were 10 mM, and a standard solution of cholesterol was studied at 200 $mg.dL^{-1}$. The concentrations of CO and POD were 39 $U.mL^{-1}$ and 250 $Um.L^{-1}$, respectively. Based on visual observation, the color intensity of tube b) was higher than a), and the intensity of c) was the lowest. (Gray scale value; c>b>a)

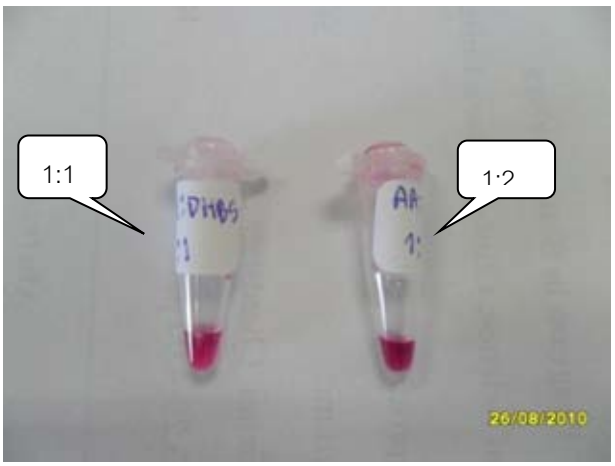


Fig. 5.3 The effect of 4AP: DHBS ratio v/v; 1:1 (left), and 1:2 (right). The concentration of all reagents was 10 mM, and a standard solution of cholesterol was studied at 200 mg.dL^{-1}. The volume of all reagents was 100 µL. The concentrations of CO and POD were 39 U.mL^{-1} and 250 U.mL^{-1}, respectively.

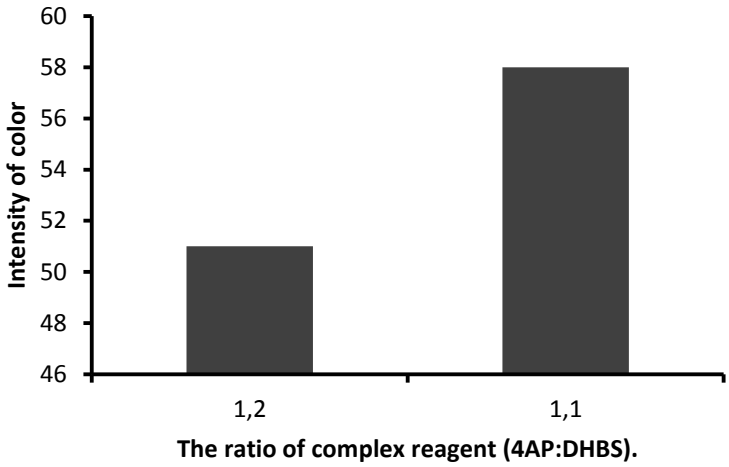


Fig 5.4 The results of the effect of type and v/v ratio of the complex reagent. The concentration of all reagents was 10 mM, and the concentration of the standard solution of cholesterol was 200 mg.dL^{-1}.

The results of the effect of the complex reagent ratio under optimum conditions are shown in Fig. 5.4. The gray scale mode was used to evaluate these data. The density of color was correlated with the inverse of the color intensity. When the color was intense, the gray scale value (intensity color) was decreased. Therefore, the 1:2 ratio of complex reagent was selected for this work.

5.1.3 The effect of 4AP: DHBS ratio v/v

The results shown in Fig.s 5.3 and 5.4 at a ratio of 1:2 v/v of 4AP:DHBS produced the red color complex with a higher intensity than at a ratio of 1:1 v/v. Therefore, a ratio of 1:2 v/v was chosen for use in this work. The suitable ratio of mixing reagent was affected by the cross-linking of complex agents and H_2O_2 that produced from cholesterol by cholesterol oxidase.

5.1.4 LOD and LOQ

The LOD and LOQ were determined by testing 10 replicates of the blank paper-based microfluidic device that only contained drops of the reagent complex without the sample. The LOD and LOQ were calculated from the standard deviation (SD); the LOD was 3 SD/slope, and the LOQ was 10 SD/slope. In this work, the LOD was 7 $mg.dL^{-1}$, and the LOQ was 21 $mg.dL^{-1}$.

5.1.5 The calibration curve and the linear range

The calibration curve and the linear range were examined over the range of 50 and 400 $mg.dL^{-1}$ of cholesterol under the optimal conditions, and the result indicated that the cholesterol linearity was 50-400 $mg.dL^{-1}$.

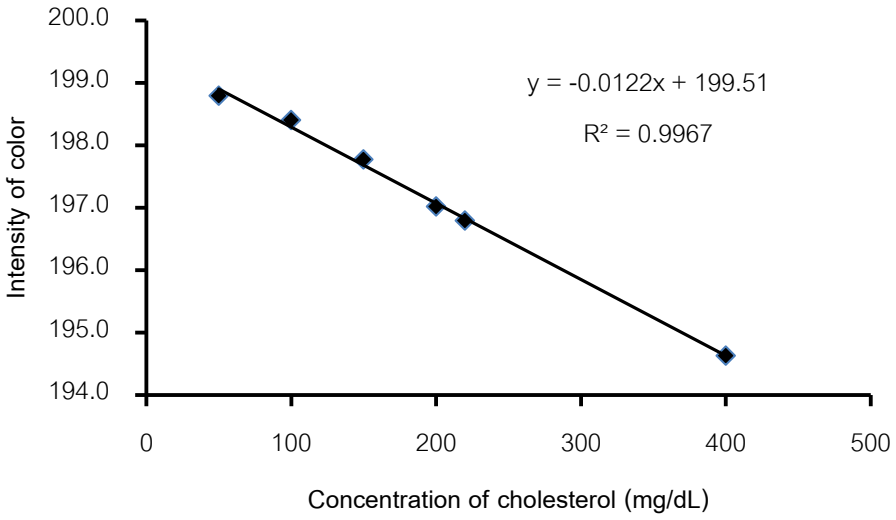


Fig. 5.5 The study of the calibration curve and the linearity in the range of 50-400 $mg.dL^{-1}$ of cholesterol under the optimal conditions; CO 169 $U.mL^{-1}$, POD 500 $U.mL^{-1}$, 10 mM of complex reagent (1:2 v/v of 4AP and DHBS)

To find the limiting of cholesterol that could dissolve in the preparation procedure, the maximum concentration of cholesterol was determined. From the results, it was found that the maximum concentration of cholesterol was 400 $mg.dL^{-1}$ and the minimum concentration of cholesterol was 50 $mg.dL^{-1}$. Therefore, to study the calibration curve and linear range, the concentration of cholesterol was investigated between 50-400 $mg.dL^{-1}$ using paper-based device.$^{-1}$The intensity of color was plotted against the concentration of cholesterol. This plot was found to be linear in the range of 50-400 $mg.dL^{-1}$ (Fig. 5.5), with a correlation coefficient (R^2) of 0.9967.

5.1.6 Precision and accuracy

The precision of this method was examined at concentrations of 50, 150, and 250 $mg.dL^{-1}$, and the results in terms of the %RSD (relative standard deviation) were 1.5, 1.4 and 2.6, respectively.

The accuracy was determined by adding 50, 100 and 150 $mg.dL^{-1}$ of a standard stock solution into the sample, and the results in terms of the % recovery were 101.5, 82.1, and 89.4, respectively.

5.1.7 Application

The concentration of cholesterol was determined in a blood serum sample. Before beginning this work, the sample was diluted ten-fold with a 0.1 M PBS solution at a pH of 7.1. The intensity of the signal was calculated using the equation derived for the calibration curve; the concentration of cholesterol in the serum sample was 63.41 $mg.dL^{-1}$ (reference value was 60 $mg.dL^{-1}$)

5.2 Determination of cholesterol with silver nanoparticles (AgNPs)

The concept of this work is illustrated in Fig. 5.6. The aggregated size of the AgNPs was reduced when they interacted with hydrogen peroxide. Different sizes of AgNPs yielded various colors of the complex; therefore, the quality of this reaction was used to study the use of AgNPs. The size and concentration of AgNPs were the primary factors concerning their use for the determination of cholesterol.

$$H_2O_2 + AgNPs\ (Ag^O) \longrightarrow complex$$

(color 1) (color 2)

Fig. 5.6 Schematic illustrating the aggregation of the AgNPs, which was affected by the various concentrations of H_2O_2.

5.2.1 The effect of the size of the AgNPs

The result of this work, as shown in Fig. 5.7, was a) 10 mgL^{-1} of AgNP solutions and b) 10 $mg.L^{-1}$ of AgNP solutions with 2 $mg.L^{-1}$ of H_2O_2. The two sizes of AgNPs that resulted in a color change were 20 and 40 nm; therefore, these sizes were selected for study in the next step. The results of this study indicated that the color was only changed when the 40 nm AgNPs were used, and their color changed from blue to purple. Furthermore, the result of this effect that was studied on paper devices

The effect of the size of AgNPs was studied by adding H_2O_2 (2 mgL^{-1}) to AgNPs solutions. The results obtains are shown in Fig. 5.8 and Fig. 5.9. The effect of this factor was repeat to confirm that AgNPs be applied to the paper-baseddevice. The intensity of color was plot between color intensity and the concentrations of H_2O_2 with different diameter of AgNPs (10-20, 20, 40, and 80-100 nm). The results indicated that the size of 40 nm provided the extremely difference of the color intensity, then this condition was selected to use on paper-based device. The other sizes of AgNPs givesignal of intensity with no significant difference(Fig. 5.8).

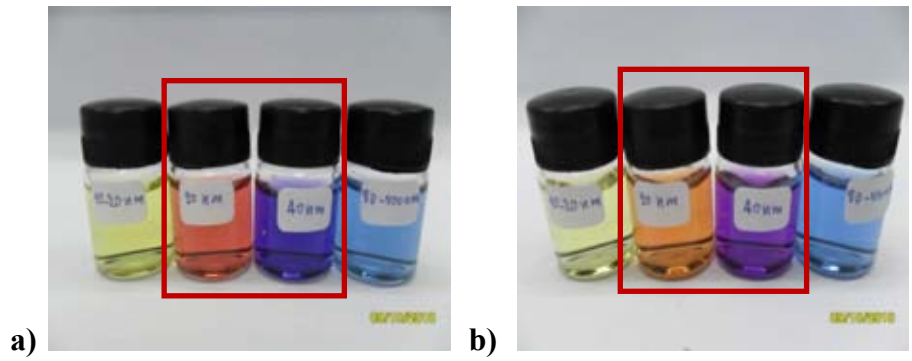


Fig. 5.7 The study of the effect of the particle size of AgNPs for 10-20, 20, 40, 80-100 nm at the same concentration 10 mg.L^{-1}. (a) AgNPs solutions without H_2O_2, and (b) AgNPs solutions with 2 mg.L^{-1} H_2O_2.

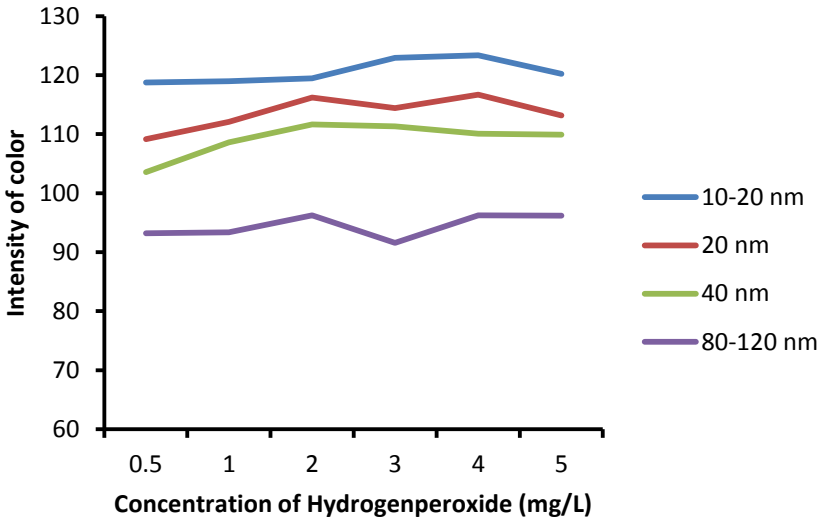


Fig. 5.8 The effect of the particle size of AgNPs that were studied on the wax-printing paper device under the optimal conditions. The concentration of all particle sizes was 10 mg.dL^{-1}, and the volume of all reagents was 1.0 µL.

5.2.2 The effect of the concentration of AgNPs

The effect of the concentration of the AgNPs was studied in vial and on paper-based device. In vial, two sizes of AgNPs (20 nm and 40 nm) were selected. The concentrations of AgNPs were studied in the range of 10-100 mgL^{-1}. Fig. 5.9 and 5.10 show the different colors of solution containing of AgNPs with and without H_2O_2. For paper-based device, although this concentration has little different tone on paper device. While testing on paper device, the concentration of 60, 100, and 400 mgL^{-1} was studied, and the results were shown in Fig. 5.11.

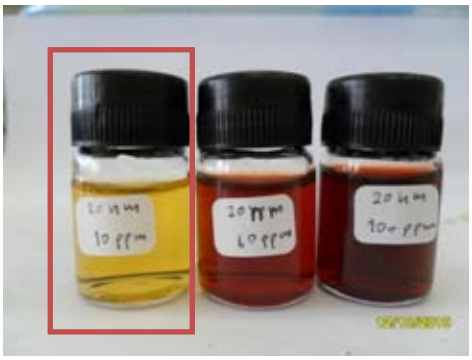
a)  b) 

Fig. 5.9 Study on the concentration of 20 nm AgNPs for 10, 60, and 100 mgL^{-1}: a) without H_2O_2, and b) with H_2O_2 (2 $mg.L^{-1}$).

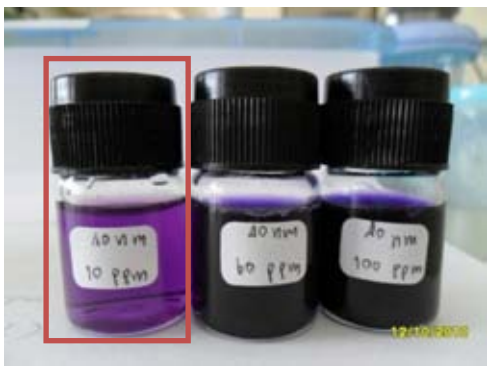
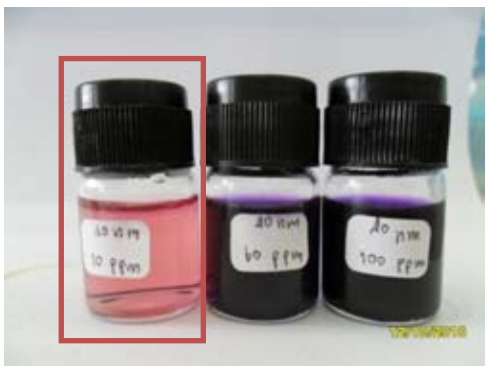
a)  b) 

Fig. 5.10 Study on the concentration of 40 nm AgNPs for 10, 60, and 100 mgL-1: a) without H_2O_2, and b) with 2 $mg.L^{-1}$ of H_2O_2.

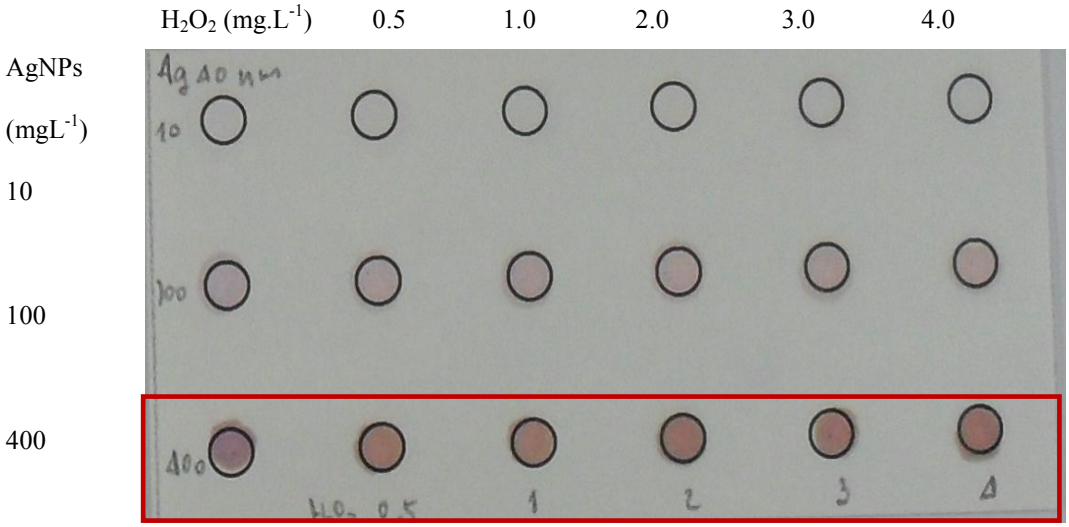


Fig. 5.11 The effect of the concentration of 40 nm AgNPs for 60, 100, and 400 mgL^{-1}. The volume of all reagents was 1.0 µL.

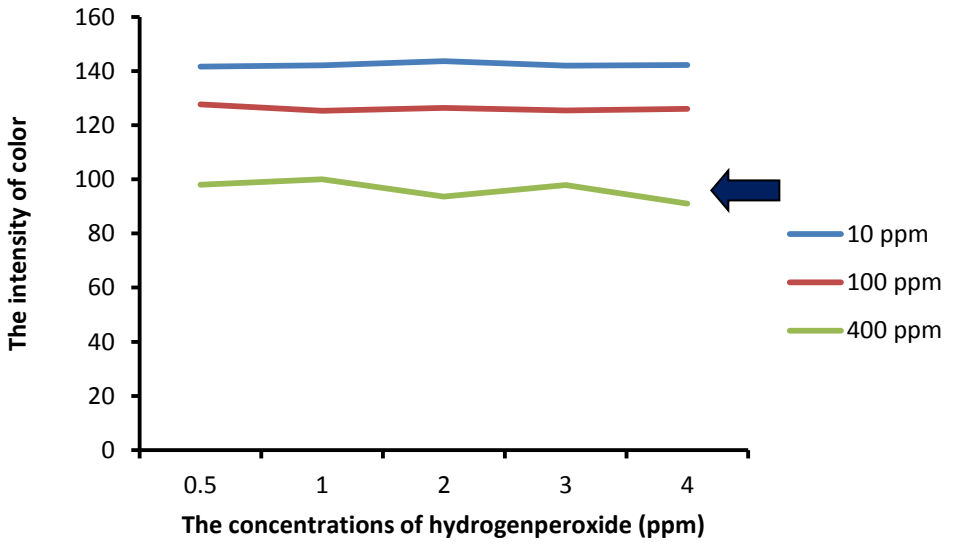
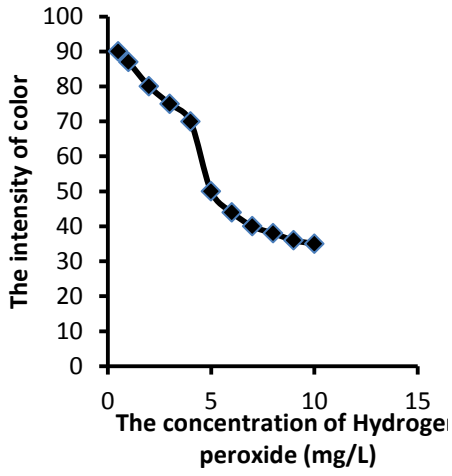


Fig. 5.12 The effect of the concentration of 40 nm AgNPs for 60, 100, and 400 mgL^{-1}. The volume of all reagents was 1.0 µL.

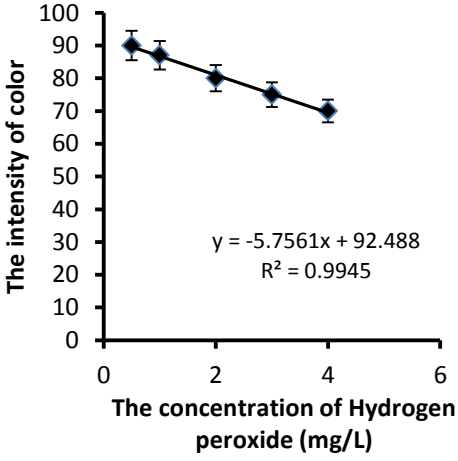
When the suitable size of AgNPs was chosen, the effect of concentration of AgNPs was studied at 40 nm diameter of AgNPs. After dropping 40 nm AgNPs at the concentration of 400 mgL^{-1} , the different concentrations of H_2O_2 (0.5-4.0 mgL^{-1}) was added on the paper-based device.It was found that the intensity of color was extremely decreased when increasing the concentration of H_2O_2 as shown in the graph in Fig. 5.12. This plot provided the optimal concentration of AgNPs that was used to determine the concentration of H_2O_2 was 400 mgL^{-1}.

5.2.3 The effect of concentration of H_2O_2

In this work, the concentration of H_2O_2 in the range of 0.5-10.0 mgL^{-1} was studied on a paper-based microfluidic device under the optimal conditions. The results are shown in Figs. 5.13 (a) and (b).



(a)



(b)

Fig. 5.13 Study on the effect of the concentration of H_2O_2 (a) 0.5-10.0 mgL^{-1} (b) the linear range of H_2O_2 in the range of 0.5-5.0 $mg.L^{-1}$.

As the previous results suggested that H_2O_2 could induce the aggregation of 40 nm diameter AgNPs, the study expectation that AgNPs could be used to quantitatively determine H_2O_2 levels, which is a product from important biomarker (cholesterol and glucose) by enzyme assay. The intensity color of AgNPs with different concentration of H_2O_2 were monitored under the optimal conditions (400 $mg.L^{-1}$ of 40 nm diameter AgNPs) and measured the levels of H_2O_2 on paper devices by using camera to capture the photo and using Adobe photoshop program after the addition of different amounts of H_2O_2 (0.5-10.0 $mg.L^{-1}$) on paper device that coated with AgNPs, the intensity value of color was plotted against the concentration of H_2O_2. This plot was found to be linear within the range of 0.5-4.0 $mg.L^{-1}$ (Fig.5.13), with a correlation coefficient (R^2) of 0.9945.

5.2.4 LOD and LOQ of H_2O_2

The LOD and LOQ were studied by adding the AgNPs onto the paper-based microfluidic device without the sample and replicating the experiment 10 times. The results were calculated from the SD value and the slope of the calibration curve (from 5. 2.3). The LOD and LOQ were 0.2 and 0.7 $mg.L^{-1}$, respectively.

5.3 Spectrophotometry

5.3.1 The effect of the concentration of H_2O_2

The effect of the concentration of H_2O_2 was studied in the range of 5-100 $mg.L^{-1}$. The result is shown in Figs. 5.14, and 5.15; the linear ranges of this study were 5-40 and 5-50 $mg.L^{-1}$. for the condition of AgNPs 20 nm and 40 nm, respectively. This part was the study the performance of paper-device when using the other technique to determine the H_2O_2 concentration, the expectation of this work was the different method would not obtained the significant different results.

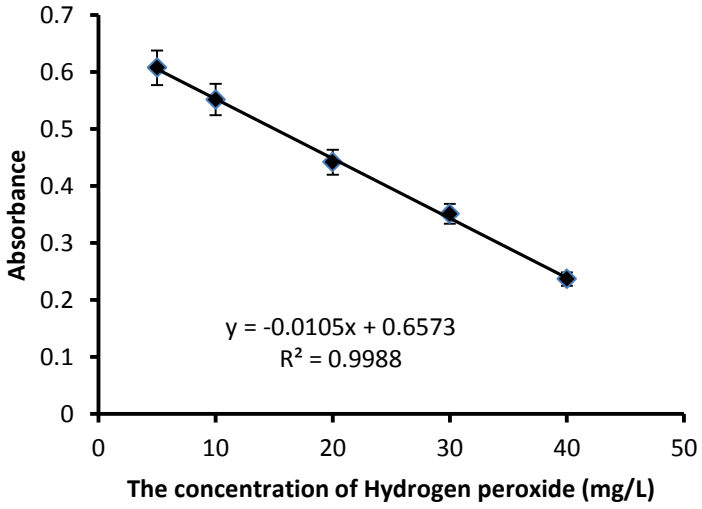


Fig 5.14 Study the effect of H_2O_2 concentration in the range of 5-40 $mg.L^{-1}$on the diameter size of 20 nm AgNPs. (wavelength 475 nm)

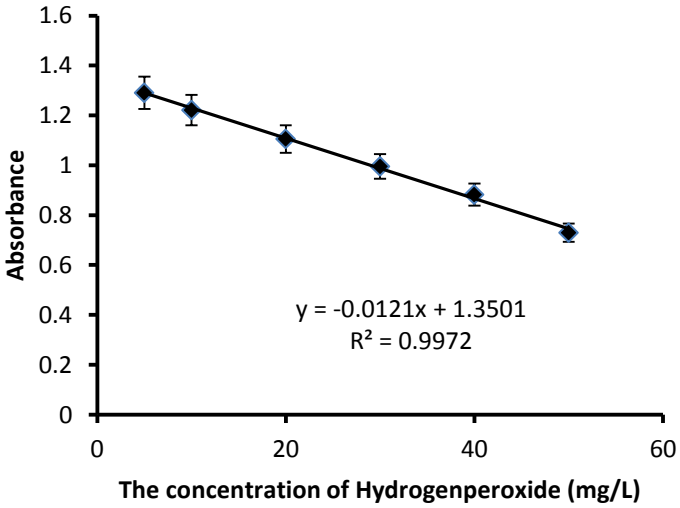


Fig 5.15 Study the effect of H_2O_2 concentration in the range of 5-50 $mg.L^{-1}$on the diameter size of 40 nm AgNPs. (wavelength 558 nm)

5.4 Determination of cholesterol in real samples

In this part, the optimum condition was used to measure the concentration of cholesterol in a real sample (blood serum). The result is shown in Table 5.1 and was compared with the result from the testing in the paper-based microfluidic device.

Table 5.1 The determination of cholesterol in blood serum (n=3)

Sample no.	Concentration of cholesterol ($mg.dL^{-1}$)	
	Standard method	μPADs
1	134.2±0.2	140.2±1.3
2	185.0±0.5	190.6±0.8
3	119.9±0.4	110.4±2.0

CHAPTER VI

RESULTS AND DISCUSSION

PART III: GLUCOSE

The study of this part was divided into two sections that consisted of an enzyme assay and a non-enzyme assay. The first part consists of two reaction steps, which are shown in Fig. 6.1. In the second part, malachite green and $FeCl_3$ were used to determine the concentration of glucose without the presence of an enzyme.

6.1 Enzyme assay for the determination of glucose

6.1.1 General standard method

The gold standard method for the determination of glucose was used as a routine screening in a hospital (Fig. 6.1a). The reaction utilizes all of the complex reagents like the reaction to determine the cholesterol assay; therefore, the final product was the same color as the cholesterol assay. Therefore, the complex reagent in this work was changed to potassium iodide (KI), which produced a brown complex. (Fig. 6.1b)

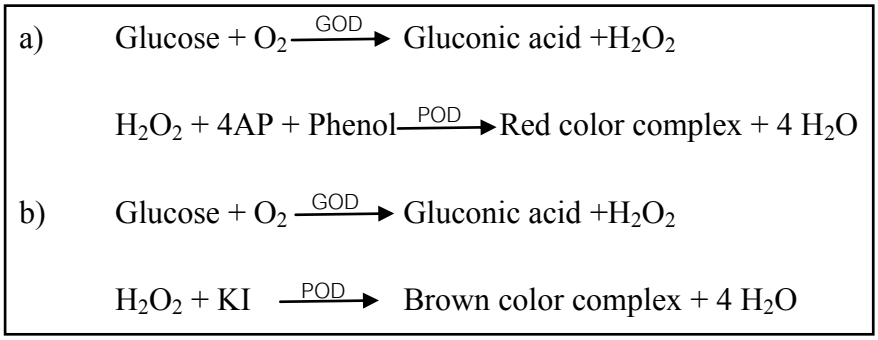
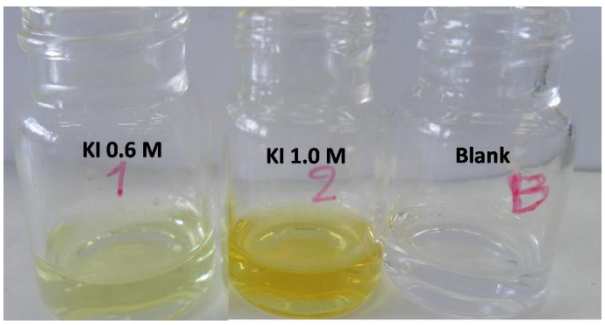


Fig. 6.1 The schematic of the colorimetric reaction with the enzyme assay for the determination of glucose; a) standard method, b) the glucose assay that was used in this work.

6.1.2 Optimization of the conditions for the determination of glucose

The effect of the concentration of KI was studied at 0.6 and 1.0 M, and the concentration of POD was studied in the range of 250 to 500 $U.mL^{-1}$. Based on the results of this work, 1.0 M KI and 500 $U.mL^{-1}$ of POD were selected. The effect of the concentration of KI (Fig. 6.2), the intense of color was increased when the concentration of KI was decreased, the the high density of color at 1.0 M of KI was selected for the determination ofe the glucose levels.

a)



b)

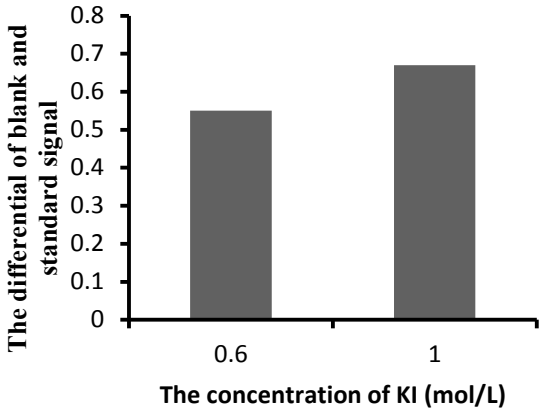


Fig. 6.2 Study the effect of the various concentration of KI (0.6 and 1.0 M) a) in standard solution of 100 mg.dL^{-1} b glucose) the plot of the signals versus the concentrations of KI, all of the these were studied under the conditions of 500 U.mL^{-1} POD, 500 U.mL^{-1} GOD and all of the volumes of reagents were 100 µL in standard solution 200 µL.

6.1.3 LOD and LOQ

The detection and quantification limit (LOD and LOQ), that is the levels producing the signal at three and ten times of the standard deviation of a blank signal, were studied by adding the complex reagent on the paper device without the sample and replicating them 10 times. Then the results were calculated from the SD value and the slope of the calibration curve. LOD and LOQ were found to be 0.8, and

2.7 mg.dL^{-1}, respectively. The results was not lower than the previous work, but it was enough for using to determine the glucose level (normal value ≥ 110 mg.dL^{-1})

6.1.4 Calibration curve and linear range

The standard addition was used to determine the linear range and calibration curve of glucose. The reason to spike the standard solution into the whole blood because matrix can be reduced. Results obtained from the standard addition method are shown in Fig.6.3. The linearity is in the range of 0-400 mg dL^{-1} with correlative coefficient (R^2) 0.9951.

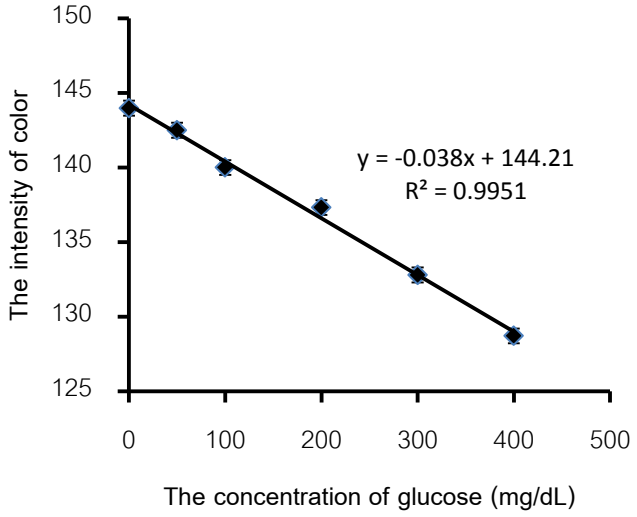


Fig. 6.3 The calibration curve of glucose was obtained under the optimum condition; 1.0 mM KI, GOD 500 U.mL^{-1}, POD 500 U.mL^{-1}, 0.5 µL of reagent volume, and 11.0 µL of sample volume.

Table 6.1 The results of the determination of glucose in the blood serum. (n=3)

Sample no.	Concentration of glucose ($mg.dL^{-1}$)	
	Spectrophotometry	μPADs
1	90.5±0.3	100.0±0.7
2	130.0±0.2	134.5±1.2
3	110.5±0.6	100.5±1.8

6.2 Determination of glucose with malachite green

Malachite green (MG) is an organic compound that is used as a dyestuff, and it has emerged as a controversial agent in aquaculture. MG is traditionally used as a dye for materials such as silk, leather, and paper. The chemical structure of MG is shown in Fig. 6.4. The aim of this work was the development of a new method for the determination of that has not been reported in previous work. Therefore, MG was a new choice for developing the new method for the determination of glucose, where MG was used to react with hydrogen peroxide and $FeCl_3$. The reaction that included hydrogen peroxide and Fe^{3+}/Fe^{2+} is called the “Fenton reaction”.

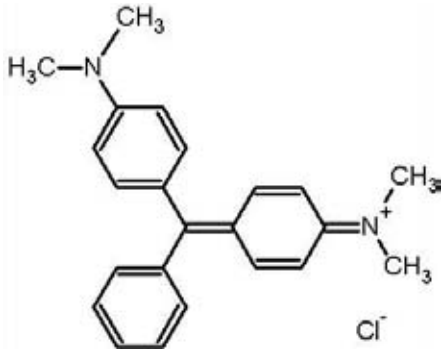


Fig. 6.4 The chemical structure of malachite green (MG)

The Fenton reaction is used to treat organic pollutants in wastewater. The mechanism and kinetics of the Fenton reaction are shown in Fig. 6.5. This reaction has two factors that were studied: the effects of the concentration of malachite green and $FeCl_3$.

(1) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\cdot + OH^-$

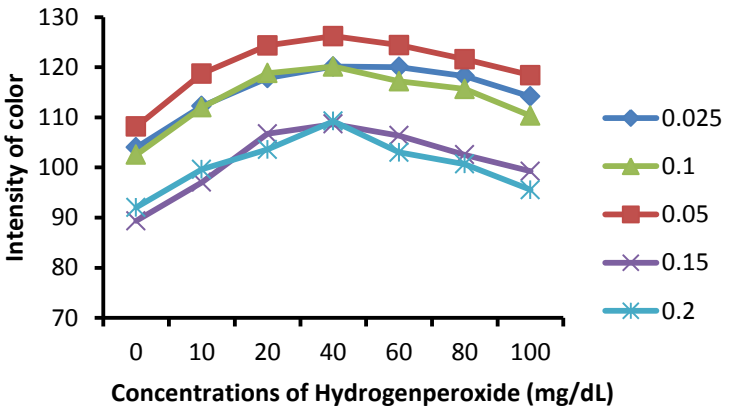
(2) $Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OOH\cdot + H^+$

Fig 6.5 The mechanism of the Fenton system involves the formation of hydroxyl and hydroproxyl radicals.

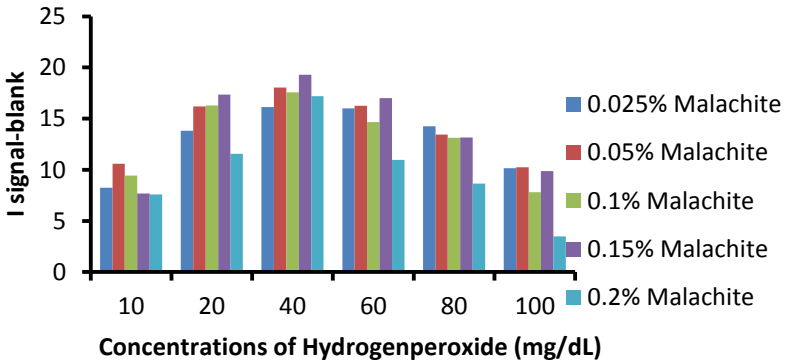
6.2.1 The effect of the concentration of malachite green

The concentration of malachite green was studied in the range of 0.025-0.2% w/v, and the malachite solutions were prepared by diluting with a 0.1 mM PBS solution at a pH of 7.1. The intensity of the color was decreased when the MG reacted with increasing concentrations of hydrogen peroxide. The results indicated that the gray scale value or the intensity of the color increased in the range of 0.5 and 40 $mg.dL^{-1}$. Fig. 6.6 a) shows only the relative between the intensity of color and %v/w of malachite green that reacted with hydrogen peroxide without the control condition (malachite green without hydrogen peroxide); therefore, the results were not

clear for determining the suitable condition. Fig. 6.6 b) and c) show the results that were calculated from the difference of the blank value and the value from the malachite green reaction. From these results, a concentration of 0.15% malachite green was chosen because this point has a maximum different value between the blank and the malachite that reacted with the hydrogen peroxide.



a)



b)

Fig. 6.6 The effect of the concentration of malachite green; a) the relative value between malachite green and hydrogen peroxide, and b) the differential value between the blank value and the value from the malachite reaction.

6.2.2 The effect of the concentration of $FeCl_3$

From the previous results, for determination of glucose by using malachite. It was found that color was decreased (intensity color was increased) when adding H_2O_2 concentration on paper device. Although the color changed ,but the intensity is not enough to distinguish the different concentration of H_2O_2. Then the study of the additive that could increase the intensity of color was very important. From the chemical structure of malachite that was a cation complex and has three aromatic rings, the reaction included aromatic ring, and H_2O_2 like a fenton reaction. Fe^{3+} was chosen to function as a catalytic agent, the aim of this work was the adding additive to catalyze the reaction to produce clear and intense color. Various concentration of Fe^{3+} (0.5-10 mM) was plotted against intensity of color (Fig. 6.7). The results show that using 1.0 mM Fe^{3+} provided the highest differential signal between blank (malachite) signal and the signal from the reaction of malachite, H_2O_2, and Fe^{3+}(Fig. 6.8).

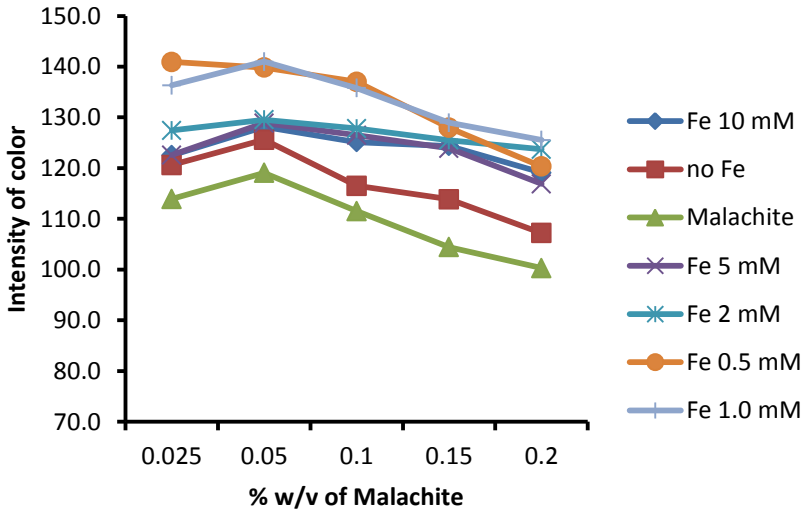


Fig. 6.7 The effect of the concentration of $FeCl_3$ that was studied under the conditions of 0.15% malachite green, 1 µL of reagent volume, pH 1.9 of $FeCl_3$ solutions, and 40 $mg.dL^{-1}$ hydrogen peroxide.

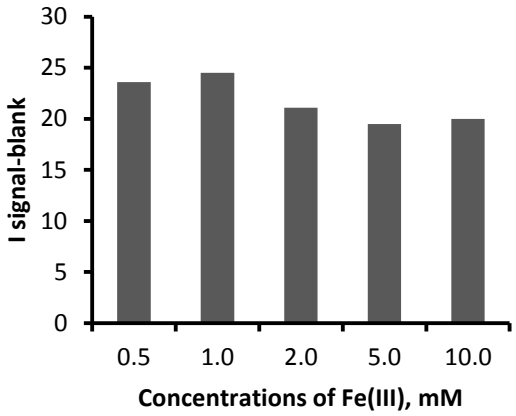


Fig. 6.8 The effect of the concentration of $FeCl_3$ that was studied under the conditions of 0.15% malachite green, 1 μL of reagent volume, pH 1.9 of $FeCl_3$ solutions, and 40 $mg.dL^{-1}$ hydrogen peroxide.

6.2.3 Linear range and calibration curve

The calibration curve and the linear range were studied in the range of 10 and 200 $mg.dL^{-1}$ under the optimal condition (see above) on the paper device, the intensity of color was plotted versus the concentration of glucose. This plot was found to be linear in the range of 40-120 mg dL^{-1}. (Fig. 6.9 and 6.10), with a correlation coefficient (R^2) of 0.9924.

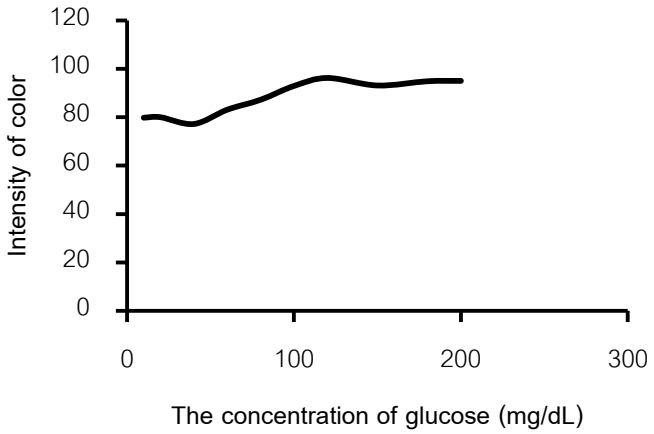


Fig. 6.9 Study the linear range of glucose in the range of 10 and 200 mg.dL^{-1}. Under the optimal conditions which included 0.15% w/v malachite green, and 1.0 mM $FeCl_3$. All of the reagents and sample volume were 1.0µL, a time to take a photo within 5 min.

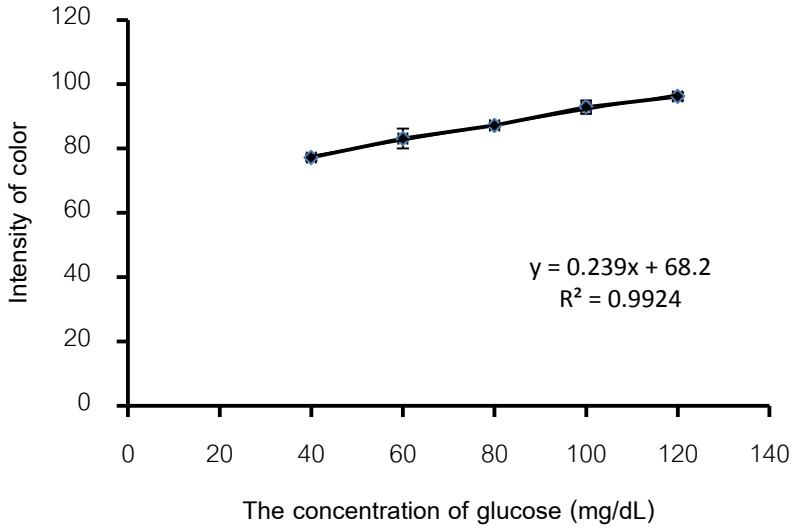


Fig. 6.10 Study the calibration curve of glucose in the range of 40 and 120 mg.dL^{-1}. Under the optimal conditions, which included 0.15% w/v malachite green, and 1.0 mM $FeCl_3$. All of the reagents and sample volume were 1.0µL, a time to take a photo within 5 min.

6.2.4 LOD and LOQ

LOD and LOQ were studied by measurement the signal of blank at 10 replicates on paper device that had only dropped of malachite without the analyte. After that the LOD and LOQ were calculate from the formula: LOD = 3SD/ slope, and LOQ = 10SD/slope. The results of this work shown that LOD was 3.1 $mg.dL^{-1}$, and LOQ was 10.2 $mg.dL^{-1}$.

6.2.5 Precision and accuracy

Precision was studied at 40, 80, and 100 $mg.dL^{-1}$ those results shown in a term of %RSD (Relative standard deviation) were 1.7, 1.5 and 2.0, respectively.

Accuracy was studied by adding a 40, 80 and 100 $mg.dL^{-1}$ stock standard solution into blood serum sample, which results shown in a term of % recovery were 81.4, 86.2 and 91.7, respectively.

6.2.6 Applications

Fig. 6.11 The wax-printing device used to determine the concentration of glucose based on colorimetric method using malachite green. The optimum conditions were 0.15% w/v of malachite green, 1.0 mM $FeCl_3$ pH 1.9, 1.0 μL of reagent volume From the results, malachite green can be used for determination of glucose levels on wax-printing paper device. The reason for chose the wax-printing device to use in this part because this device could be patterned within a short time (>50 detection zone within 1 min) and it is easy for fabrication of the device. In addition, the results of this part were in good agreement with the standard method (Spectrophotometry).

Table 6.2 The results of glucose determination by reaction with malachite green on wax-printing paper device (n=3)

Sample	Standard method ($mg.dL^{-1}$)	Wax-printing ($mg.dL^{-1}$)
1	76	72.1±0.4
2	181	188.0±1.5

CHAPTER VII

RESULTS AND DISCUSSION

PART IV: CREATININE AND ALBUMIN

The initial objective of this work was to use the Jeffe's reaction method, which involves the reaction of picric acid with creatinine under a base of the colorimetric method, for the determination of creatinine on the μPADs, and for the determination of albumin with the reaction of eosin-y to form the orange color complex on the μPADs. Although these assays could not be used to determine the analytes on the paper-based microfluidic device, they could be used with the spectrophotometry technique. Therefore, the new aim of this thesis was to solve this problem. AuNPs were used to determine the concentration of the analytes on the μPADs.

7.1 Determination of creatinine using Jeffe's reaction method

Jeffe's reaction involves the reaction of creatinine and picric acid under alkaline conditions. This work had two factors to study; the concentration of NaOH and the concentration of picric acid. The results of these examinations are shown in Figures 7.2 and 7.3.

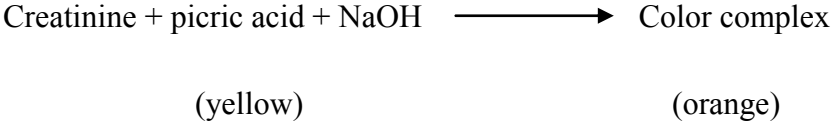


Fig. 7.1 The schematic of Jeffe's reaction.

Jeffe's reaction could produce the color complex product when it was heat at the high temperature (252ºC), then it could not used to determine the concentration of creatinine with this limiting factor because μPADs made from paper, it could be burn at the high temperature. Moreover, the product color and picric acid was the similar tone color, then it more difficult to identify the results.

7.1.1. The effect of the concentration of NaOH

Creatinine is a zwitterionic compound, there are two dissocation constants (pK_{a1} 4.8, pK_{a2} 9.2). From this reason, pH solution was very important for the Jeffe's reaction.The various concentrations of NaOH (1-5%) were mixed with 3% picric acid and standard solution of 1.0 $mg.dL^{-1}$creatinine. The absorbance of the mixing solution was measured at wavelength of the maximum absorbance from previous work. The results indicated that the 4% NaOH provided the maximum absorbance (Fig. 7.2). Therefore, the 4% NaOH was the optimum concentration for the determination of the level of creatinine.

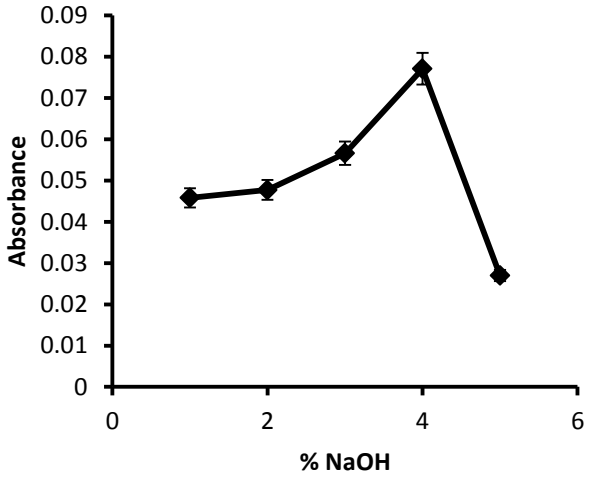


Fig. 7.2 The effect of the concentration of NaOH. The concentrations of NaOH were studied in the range of 1 to 5%. The wavelength used is 510 nm.

7.1.2 The effect of the concentration of picric acid

The effect of the concentration of picric acid was studied in the range of 1-3% by monitoring the changes in its absorbance. Fig. 7.3 presents the result of this study; 1% picric acid was selected for use in this work because this concentration resulted in the strongest signal. Then this concentration of picric acid was chosen to use in this work. The other concentrations of picric acid provided lower signal than 1% picric acid. Therefore,the sensitivity and selectivity at this concentration (1%) was better than the others.

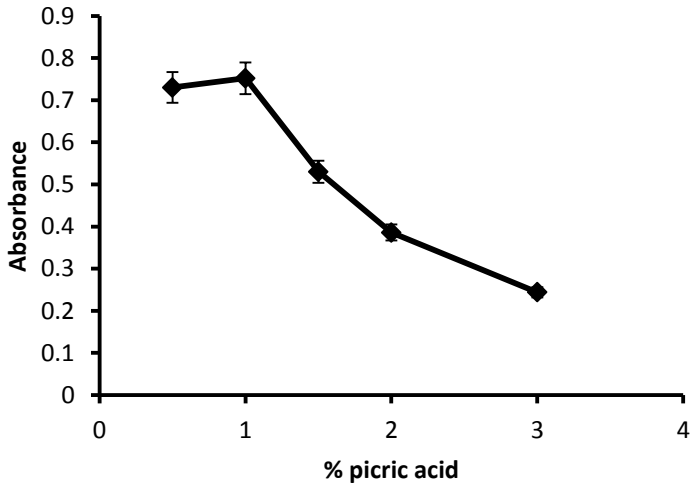
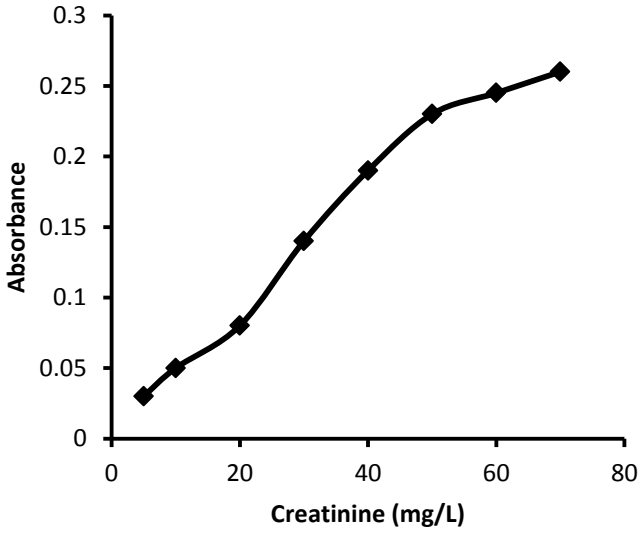


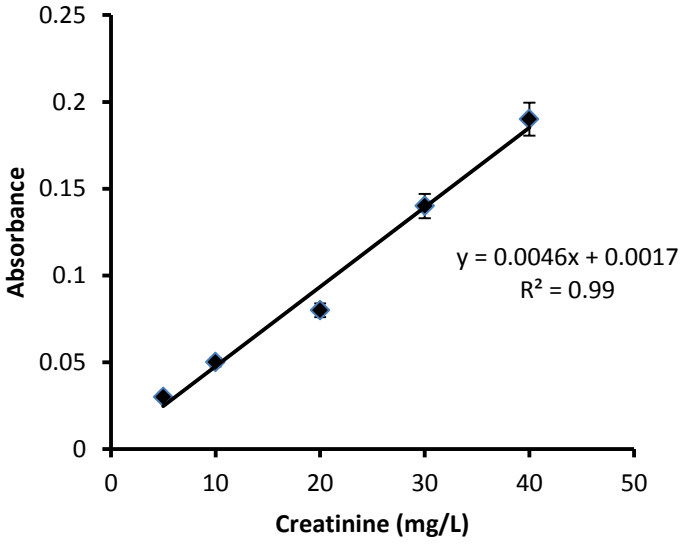
Fig. 7.3 The effect of the concentration of picric acid. The study of this effect was studied in the range of 0.5 and 3%, and 4% picric acid. The absorbance of the solution was measured at the wavelength of 510 nm.

7.1.3 Calibration curve of creatinine

The calibration curve was examined over the creatinine concentration range of 5- 70 $mg.L^{-1}$. The results were shown for the study range and the linearity range for the concentration of creatinine.



(a)



(b)

Fig. 7.4 Study of the calibration curve of creatinine (a) 5-70 $mg.L^{-1}$, and (b) 5-40 $mg.L^{-1}$. The wavelength used is 500 nm.

7.2 Determination of albumin

7.2.1 Optimization of the determination of albumin

The optimization of determination of albumin was studied the effect of pH solutions and % eosin-Y. The study of pH solution was important for dissociation of compound, and the pH has effect to the optimum condition for forming complex. From these reasons, the pH solution was studied at the first step, and follow by the effect of % eosin-Y, which effected the suitable ration to form complex. The results of this work were shown in Fig. 7.5 and 7.6.

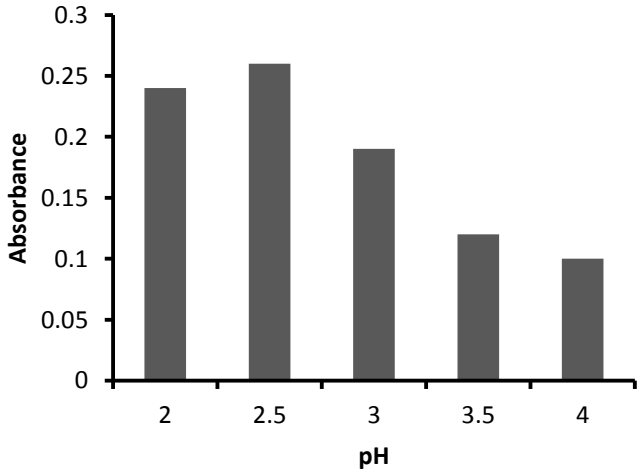


Fig. 7.5 The effect of the pH of the solutions on the determination of albumin. (pH values between 2-4) and 4 mg.dL^{-1}albumin standard solution. The absorbance of the solution was measured at the wavelength of 547 nm.

The optimum conditions of these studies were pH 2.5, and 0.03% eosin-Y. The pH solution was studied in the range of 2-4 (Fig. 7.5). The maximum signal was obtained at pH 2.5, therefore, this pH was selected for the next step of albumin determination. Moreover, the effect of concentration of eosin-Y was studied in the range of 0.01and 0.05 (Fig. 7.6), the highest absorbance was obtained at 0.03% eosin-Y.

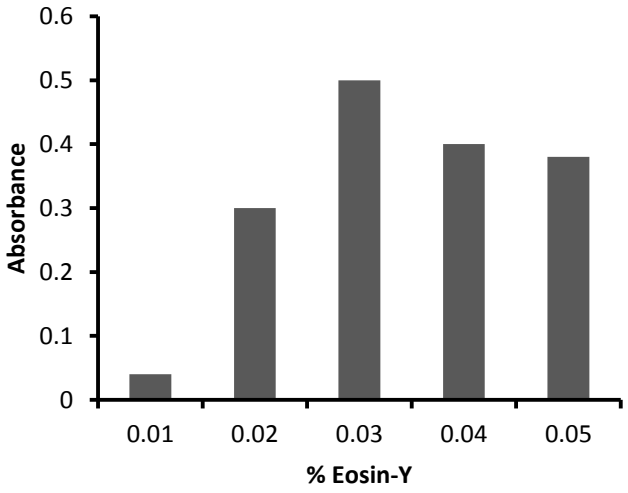


Fig. 7.6 The effect of the % eosin-Y on the determination of albumin (0.01-0.05%). The absorbance of the solution was measured at the wavelength of 547 nm.

7.3 Gold nanoparticles

From the previous work, the determination of albumin and creatinine was not successful in the realtime diagnostic device. Nowadays nanoparticle was used in the various fieldfor example, gold nanoparticles (AuNPs) was used to qualify fresh milk. In this work, the AuNPs at various size of diameter NPs (20 nm, and 80 nm at 1000 $mg.L^{-1}$) were used to determine the creatinine and albumin on paper-based microfluidic device. The concentration of the analytes were expected to affect the color due to the change of the aggregation of the analyes and AuNPs.

7.3.1 Colorimetric assay with gold nanoparticles

The following reaction shows the pathway of the aggregation of AuNPs and protein in milk. The various concentrations of protein induced the aggregation of AuNPs that produced the color complex. The color is different from the original color of AuNPs. The important factors that have effected on the performance of the aggregation were the diameter size of AuNPs, and the concentration of AuNPs.

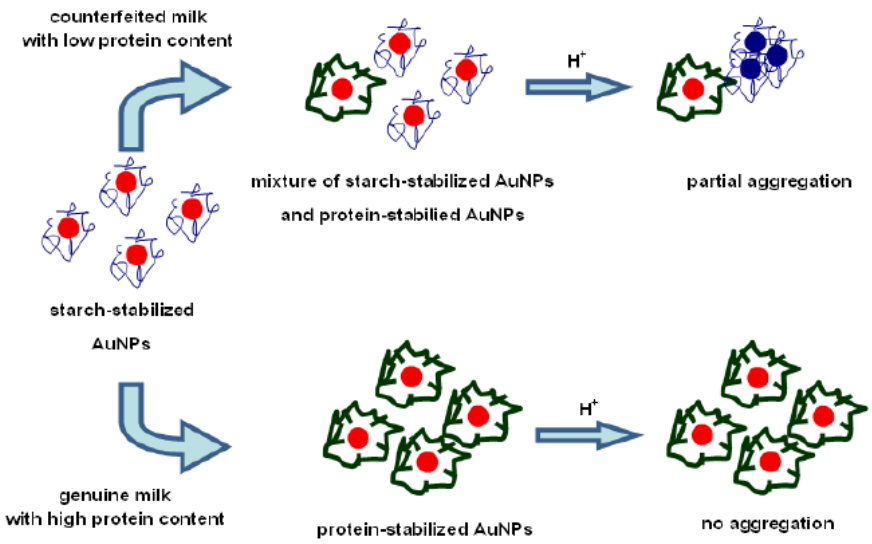


Fig. 7.7 Schematic representation of the strategy for the colorimetric assay. [65]

7.3.2 The effect of concentration and particle size of gold nanoparticles for creatinine determination

The effect of the concentration of AuNPs with sizes of 20 and 80 nm was studied for concentrations of 40 and 1000 ppm. The results of this study are shown in Figs. 7.8 and 7.9. The color of the 20 nm AuNPs changed from light pink to purple, and the color of the 80 nm AuNPs changed from light pink to dark red. Therefore, the 80 nm AuNPs at a concentration of 1000 $mg.L^{-1}$ was selected for use in this work.

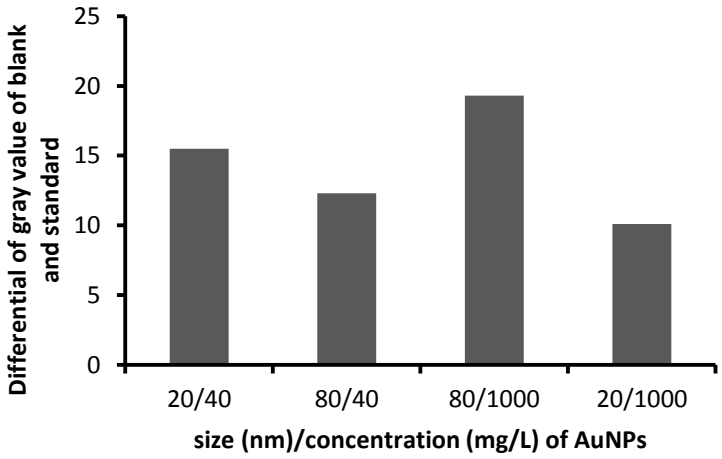


Fig. 7.8 The effects of the size and the concentration of gold nanoparticles that were examined with a 2 $mg.dL^{-1}$ concentration of creatinine. The volume of all of the reagents and samples were decreased to 5 µL for the paper device. The result was presented in terms of the difference of the gray value of the blank (AuNPs) and the standard. (AuNPs and 2 $mg.dL^{-1}$ creatinine)

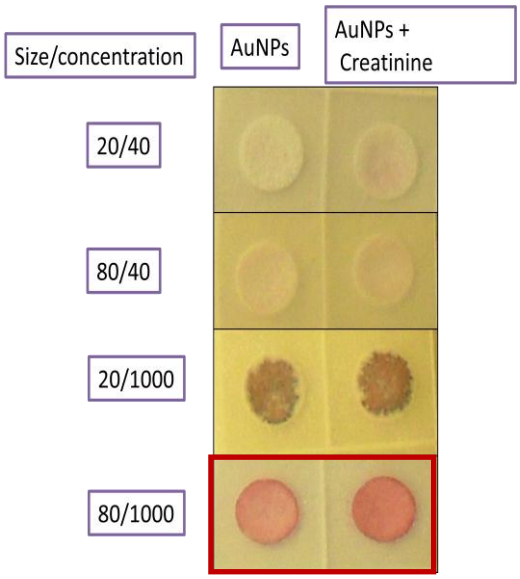


Fig. 7.9 The effects of the size and the concentration of gold nanoparticles that were examined using a concentration of 2 $mg.dL^{-1}$ of creatinine, and the volumes of all of the reagents and samples were decreased to 5 µL for the paper device. The result shown on the left hand is the color of the AuNPs without creatinine, and the color of the AuNPs with 2 $mg.dL^{-1}$ of creatinine is shown on the right-hand side.

While studied the size and concentration of AuNPs, the results show that the low concentration of both sizes provides the low color intensity, so it is difficult to identify the change of color. At high concentration (1000 $mg.L^{-1}$), color intensity was high, then the change of color was easy to visualize, and measure with the less mistake.

7.3.3 The effect of concentration and particle size of gold nanoparticles for albumin determination

The effect of the concentration of 20 and 80 nm AuNPs was studied at the concentration of 40 and 1000 $mg.L^{-1}$. The results were shown in Fig. 7.10, the color of 20 nm AuNPs changed from light pink to purple, and the color of 80 nm AuNPs changed from light pink to dark red. The highest differentintensity of color was 20 mm AuNPs at 1000 $mg.L^{-1}$ as shown in Fig. 7.7. Then the size of 80 nm AuNPs at the concentration of 1000 $mg.L^{-1}$was chosen in this work.

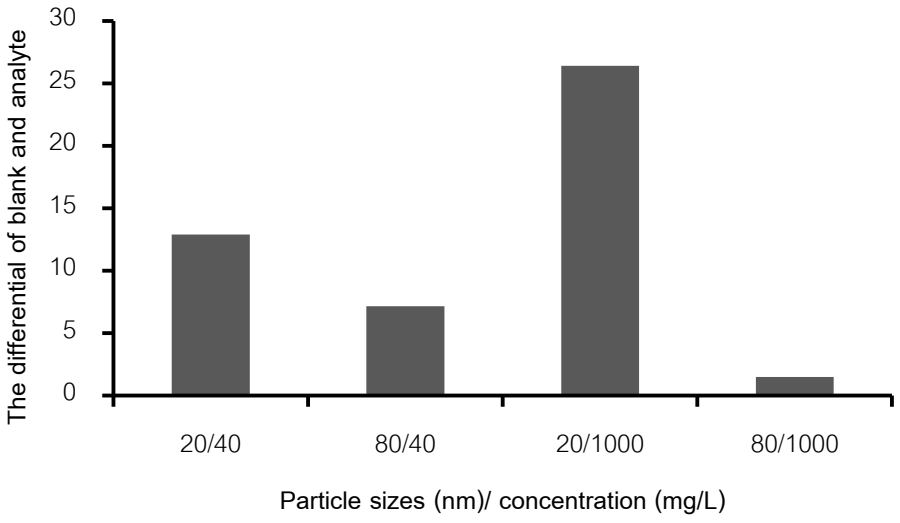


Fig. 7.10 The effect of size and the concentration of gold nanoparticles was studied for 4 $mg.dL^{-1}$ creatinine. The volume of reagent and sample dropped were 5 µL on paper device. The results was shown in a term of differential of gray value of blank (AuNPs) and standard (AuNPs and 4 $mg.dL^{-1}$albumin).

7.4 Applications

The optimal conditions for the determination of albumin and creatinine using the colorimetric assay can determine the concentration of these analytes using only the photometry technique. These assays cannot be used with the paper-based microfluidic device because of the limited color change. These analytes were determined in red blood samples obtained from a hospital, and the results of these analyses are shown in Table 7.1.

Table 7.1 The determination of the concentration of creatinine and albumin

Sample no.	Analyte	Concentration ($mg.dL^{-1}$)	
		Spectrophotometry	Standard method
1	Creatinine	10.1	9.4
	Albumin	3.8	3.6
2	Creatinine	1.1	0.9
	Albumin	3.0	2.8
3	Creatinine	0.7	0.9
	Albumin	4.5	4.2

CHAPTER VIII

CONCLUSIONS AND FUTURE PERSPECTIVE

8.1 Conclusions

The primary work in this dissertation focused on the development of a diagnostic device based on a paper-based microfluidic device with a colorimetric assay for biological sample applications.

First, a photoresist (SU-8) paper-based microfluidic device was used to determine the concentration of cholesterol and glucose based on the colorimetric method with an enzyme assay and without an enzyme assay (silver nanoparticles were used).

The photoresist method is a simple, rapid, and inexpensive method when compared with the previous techniques. However, the photoresist method has some disadvantages, such as being very expensive (60,000 BTH/ 500 mL), having an odor, requiring an organic solvent, and requiring several steps to use it. Therefore, the wax-dipping method was developed to avoid these problems.

Second, a wax paper-based microfluidic device was used to determine the concentration of cholesterol and glucose based on colorimetric method with an enzyme assay. In addition, this part studied the concentration of albumin and creatinine based on the colorimetric method without an enzyme assay (gold nanoparticles).

The wax-dipping method is a simple, rapid, and inexpensive method for the fabrication of μPADs. Other advantages of this pioneering method include no requirement for complicated and expensive instruments or organic solvents. Therefore, this technique provides an alternate and inexpensive platform for the fabrication of clinical diagnostic devices in developing countries. A single dipping

step can create microfluidic channels on paper within 1 minute. Good resolution of the hydrophilic channel of the μPAD was obtained. The crucial parameters to determine the resolution of the μPAD were the melting temperature of the wax and the dipping time. Multiple colorimetric assays can be simultaneously performed on the μPAD, and the results revealed the ability to analyze cholesterol, glucose, albumin and creatinine in real samples.

The wax-dipping procedure preserves the native paper surface; therefore, this technique does not face the problem of interference from residues that remain in the hydrophilic channel. A new method for the simple, portable, rapid, and inexpensive analysis of cholesterol, glucose, creatinine, and albumin in human serum and that could hold blood using a paper-based microfluidic device with colorimetric assay was developed.

8.2 Future perspective

Future work will focus on the development of a paper-based microfluidic device based on the colorimetric method without an enzyme assay for measuring other biological samples. Future work will also improve the diagnostic device for simultaneously diagnosing different diseases, which is a very important procedure. These future devices will save cost, reduce time-consuming procedures, and can be used for real-time analyses in field applications.

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APPENDICES

APPENDIX A

Standard method for glucose determination

GLUCOSE (GLUC-PAP)

GOD/PAP
RX SERIES

INTENDED USE

A glucose test system is a device intended for the quantitative in *vitro* determination of Glucose concentration in serum, plasma and urine. This product is suitable for use on RX series instruments which includes the RX daytona and the RX imola.

Cat. No.

GL 3815	R1. Buffer/Enzyme	9 x 51 ml

CLINICAL SIGNIFICANCE

The accurate measurement of glucose in serum or plasma is important in the diagnosis and treatment of carbohydrate metabolism disorders such as diabetes mellitus, neonatal hypoglycemia, idiopathic hypoglycemia and of pancreatic islet cell carcinoma. Glucose is often measured in conjuction with various tolerance tests after the administration of doses of leucine, insulin, glucagon or glucose.

PRINCIPLE[1]

COLORIMETRIC METHOD WITHOUT DEPROTEINISATION

Glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide produced, reacts catalysed by peroxidase, with phenol and 4-aminophenazone to form a red - violet quinoneimine dye as indicator. The intensity of the final colour is directly proportional to the glucose concentration and is measured at 505 nm.

$$\text{Glucose} + O_2 + H_2O \xrightarrow{GOD} \text{gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{POD} \text{quinoneimine} + 4H_2O$$

This assay uses an endpoint method and single point calibration.

SPECIMEN COLLECTION AND PREPARATION[2,4]

Glucose is stable for 24 hours at +2 to +8°C if the serum is prepared within 30 min after collection. By adding a glycolysis inhibitor (NaF, KF) the sample can be stored up to 24 hours at +15 to +25°C or 3 days at +2 to +8°C. For long term storage the samples should be placed in sealed containers and frozen at -20°C.

Haemolysed samples must not be used since haemolysis interferes with this test.

Plasma: use of lithium heparin as an anticoagulant is allowed.

Urine: 24 hour urine should be collected in a dark bottle and kept on ice.

Random urine – a fresh sample should be used. Sample should be stored at +2 to +8°C if not used immediately.

REAGENT COMPOSITION

Contents	Initial Concentration of Solutions
R1. Buffer/Enzyme	
Phosphate Buffer	50 mmol/l, pH 7.0
MOPS Buffer	50 mmol/l, pH 7.0
Phenol	11 mmol/l
4-aminophenazone	0.77 mmol/l
Glucose oxidase [EC 1.1.3.4, *Aspergillus niger*, @ 25°C]	≥1.5 kU/l
Peroxidase [EC 1.11.1.7, Horse radish, @ 20°C]	≥1.5 kU/l

SAFETY PRECAUTIONS AND WARNINGS

For *in vitro* diagnostic use only. Do not pipette by mouth. Exercise the normal precautions required for handling laboratory reagents.

Solution R1 contains Sodium Azide. Avoid ingestion or contact with skin or mucous membranes. In case of skin contact, flush affected area with copious amounts of water. In case of contact with eyes or if ingested, seek immediate medical attention.

Sodium Azide reacts with lead and copper plumbing, to form potentially explosive azides. When disposing of such reagents flush with large volumes of water to prevent azide build up. Exposed metal surfaces should be cleaned with 10% sodium hydroxide.

Health and Safety Data Sheets are available on request.

Please dispose of all Biological and Chemical materials according to local guidelines.

The reagents must be used only for the purpose intended by suitably qualified laboratory personnel, under appropriate laboratory conditions.

STABILITY AND PREPARATION OF REAGENT

R1. Buffer/Enzyme
Contents ready for use. Stable up to the expiry date when stored at +2 to +8°C.

MATERIALS PROVIDED

Glucose (GOD/PAP) Buffer/Enzyme

MATERIALS REQUIRED BUT NOT PROVIDED

RX series Saline (Cat. No. SA 3854)
Randox Assayed Multisera Level 2 (Cat. No. HN 1530) and Level 3 (Cat. No. HE 1532)
Randox Calibration Serum Level 3 (Cat. No. CAL 2351).

APPENDIX A

Standard method for glucose determination (cont.)

PROCEDURE NOTE

The Chemistry Parameters for Randox Dedicated RX series Assays are predefined on the hard drive of the analyser PC. The required programmes should be downloaded to the analyser software. Please note that the predefined chemistry parameters use SI units. If alternative units are required these can be edited by the user. In this case the technical range should be edited in accordance with the users selected units. All necessary instructions are encoded on the bar code. If the barcode cannot be read by the analyser, enter manually the series of numbers given beneath the barcode. If problems continue contact Randox Laboratories RX Support, Northern Ireland (028) 94451070.

NOTE

If measurement of urine samples is required, please ensure that the appropriate urine programme on the parameters disk is used.

CALIBRATION

The use of Saline and Randox Calibration Serum Level 3 is recommended for calibration. This assay uses a **linear** calculation and a **reagent blank**. Ensure that on the [Calibration] [Checks (F10)] screen the following are selected for this test:

Sampling Method for Standards
- **Duplicate**

Blank mesurement
- **Enable Reagent Blank**
- **None**

Reagent blank measurement at calibration
- **Reagent blank (system water)**

STANDARDISATION

Randox Calibration Serum Level 3 is traceable to Glucose reference materials NIST 917b and NIST 965a.

QUALITY CONTROL

Randox Assayed Multisera, Level 2 and Level 3 are recommended for daily quality control. Two levels of controls should be assayed at least once a day. Values obtained should fall within a specified range. If these values fall outside the range and repetition excludes error the following steps should be taken:

1. Check instrument settings and light source.
2. Check cleanliness of all equipment in use.
3. Check water, contaminants ie bacterial growth may contribute to inaccurate results.
4. Check reaction temperature.
5. Check expiry date of kit and contents.
6. Contact Randox Laboratories RX Support, Northern Ireland (028) 94451070.

Quality control requirements should be determined in conformance with government regulations or accreditation requirements.

INTERFERENCES

The analytes below were tested up to the following levels and were found not to interfere:

Haemoglobin	1000 mg/dl
Free Bilirubin	25 mg/dl
Conjugate Bilirubin	25 mg/dl
Triglycerides	1000 mg/dl
Intralipid	400 mg/dl

NORMAL VALUES[4,5]

Serum/Plasma	4.16-6.38 mmol/l (75-115 mg/dl)
Random urine	0.1 – 0.8 mmol/l (1 – 15 mg/dl)
24 hour urine	<2.78 mmol/d (<0.5 g/d)

It is recommended that each laboratory establish its own reference range to reflect the age, sex, diet and geographical location of the population.

SPECIFIC PERFORMANCE CHARACTERISTICS

The following performance characteristics were obtained using the RX **daytona**.

SERUM

LINEARITY

The test is linear up to a glucose concentration of 34.1 mmol/l (614 mg/dl). In the event of a rerun the upper limit of the assay range is increased to 341 mmol/l (6140 mg/dl).

RX **imola USERS**: The test is linear up to a glucose concentration of 32.1 mmol/l (578 mg/dl). In the event of a rerun the upper limit of the assay range is increased to 321 mmol/l (5780 mg/dl).

SENSITIVITY

The minimum detectable concentration of Glucose with an acceptable level of precision was determined as 0.335 mmol/l (6.04 mg/dl)

PRECISION

Intra assay

	Level 1	Level 2	Level 3
Mean (mmol/l)	1.70	5.77	16.8
SD (mmol/l)	0.076	0.113	0.375
CV(%)	4.48	1.96	2.23
n	20	20	20

Inter assay

	Level 1	Level 2	Level 3
Mean (mmol/l)	1.87	4.88	15.8
SD (mmol/l)	0.066	0.286	0.657
CV(%)	3.51	5.87	4.09
n	20	20	20

APPENDIX A

Standard method for glucose determination (cont.)

CORRELATION
This method (Y) was compared with another commercially available method (X) and the following linear regression equation obtained:

Y = 1.04 X - 0.15
and a correlation coefficient of $r = 0.99$

40 patient samples were analyzed spanning the range 3.9 mmol/l to 11.6 mmol/l.

URINE

LINEARITY
The test is linear up to a glucose concentration of 35 mmol/l (630 mg/dl). In the event of a rerun the upper limit of the assay range is increased to 350 mmol/l (6300 mg/dl).

RX **imola USERS**: The test is linear up to a glucose concentration of 29.4 mmol/l (529 mg/dl). In the event of a rerun the upper limit of the assay range is increased to 294 mmol/l (5290 mg/dl).

SENSITIVITY
The minimum detectable concentration of Glucose with an acceptable level of precision was determined as 0.54 mmol/l (9.73 mg/dl)

PRECISION

Intra Assay

	Level 1	Level 2	Level 3
Mean (mmol/l)	2.57	6.53	15.44
SD (mmol/l)	0.04	0.10	0.29
CV(%)	1.64	1.52	1.86
n	20	20	20

Inter Assay

	Level 1	Level 2	Level 3
Mean (mmol/l)	2.3	7.2	15.99
SD (mmol/l)	0.04	0.08	0.28
CV(%)	1.64	1.13	1.76
n	20	20	20

CORRELATION
This method (Y) was compared with another commercially available method (X) and the following linear regression equation obtained:

Y = 1.08 X - 0.04
and a correlation coefficient of $r = 1.00$

57 patient samples were analyzed spanning the range 0 mmol/l to 31 mmol/l.

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APPENDIX B

Standard method for creatinine determination

CREATININE (CREA)

RX SERIES

INTENDED USE

For the quantitative in vitro determination of Creatinine in serum, plasma and urine. This product is suitable for use on RX series instruments which includes the RX daytona and the RX imola.

Cat. No.		
CR 3814	R1. Sodium hydroxide	6 x 51 ml
	R2. Picric acid	3 x 28 ml

CLINICAL SIGNIFICANCE

Creatinine is derived from creatine and creatine phosphate in muscle tissue and may be defined as a nitrogenous waste product. Creatinine is not reutilised but is excreted from the body in the urine via the kidney. It is produced and excreted at a constant rate which is proportional to the body muscle mass. As a consequence of the way in which creatinine is excreted by the kidney, creatinine measurement is used almost exclusively in the assessment of kidney function. Creatinine is regarded as the most useful endogenous marker in the diagnosis and treatment of kidney disease.

Creatinine is measured primarily to assess kidney function and has certain advantages over the measurement of urea. The plasma level of creatinine is relatively independent of protein ingestion, water intake, rate of urine production and exercise. Since its rate of production is constant, elevation of plasma creatinine is indicative of under-excretion, suggesting kidney impairment. Depressed levels of plasma creatinine are rare and not clinically significant.

COLORIMETRIC METHOD

PRINCIPLE[1,2]

Creatinine in alkaline solution reacts with picrate to form a coloured complex. The rate of formation of the complex is measured.

SAMPLE

Serum or heparinised plasma.

Urine: diluted 1+8 with 0.9% NaCl solution. If the urinary creatinine program is used then this dilution is performed automatically.

REAGENT COMPOSITION

Contents	Initial Concentrations of Solutions
R1. Sodium Hydroxide	0.2 mol/l
R2. Picric acid	25 mmol/l

SAFETY PRECAUTIONS AND WARNINGS

For in vitro diagnostic use only. Do not pipette by mouth. Exercise the normal precautions required for handling laboratory reagents.

Solution R1 contains sodium hydroxide which is caustic. Solution 2 contains picric acid which is poisonous.

Health and Safety Data Sheets are available on request.

The reagents must be used only for the purpose intended by suitably qualified laboratory personnel, under appropriate laboratory conditions.

STABILITY AND PREPARATION OF REAGENTS

R1. Sodium Hydroxide

Contents supplied ready for use. Stable up to the expiry date when stored at +15 to +25°C. Once opened the reagent should be capped and stored in the refrigerator at +2 to +8°C overnight or when not in use. If capped and stored in the refrigerator overnight the reagent is stable for 21 days.

R2. Picric Acid

Contents supplied ready for use. Stable up to the expiry date when stored at +15 to +25°C. Once opened the reagent should be capped and stored in the refrigerator at +2 to +8°C overnight or when not in use. If capped and stored in the refrigerator overnight the reagent is stable for 21 days.

MATERIALS PROVIDED

Sodium Hydroxide
Picric Acid

MATERIALS REQUIRED BUT NOT PROVIDED

Randox Assayed Multisera Level 2 (Cat. No. HN 1530) and Level 3 (Cat. No. HE 1532)
Randox Calibration Serum Level 3 (Cat. No. CAL 2351) and Level 2 (Cat. No. CAL 2350)
RX series Saline (Cat. No. SA 3854) if urine samples are used

PROCEDURE NOTES

The Chemistry Parameters for Randox Dedicated RX series Assays are predefined on the hard drive of the analyser PC. The required programmes should be downloaded to the analyser software. Please note that the predefined chemistry parameters use SI units. If alternative units are required these can be edited by the user. In this case the technical range should be edited in accordance with the users selected units. All necessary instructions are encoded on the bar code. If the barcode cannot be read by the analyser, enter manually the series of numbers given beneath the barcode. If problems continue contact Randox Laboratories RX Support, Northern Ireland (028) 94451070.

If measurement of urine samples is required, please ensure that the separate urine programme on the parameters disk is used.

Reaction rate and absorptivity of the reaction product are very sensitive to temperature.

To minimise the exposure or the reagent to air it should not be placed in reagent positions 1, 2, 19 or 20.

APPENDIX B

Standard method for creatinine determination (cont.)

CALIBRATION

The use of Randox Calibration Sera Level 2 and Level 3 is recommended for calibration. A 1+2 dilution of CAL 2351 (1part CAL 2351 + 2 parts saline) may be used as the level 2 Calibrator but it should be noted the level of performance is compromised. Recalibration is required with change of reagent lot or bottle and as indicated by quality control procedures.

This assay uses a **linear** calibration. Ensure that on the Calibration Check Screen the following are selected for this test:

Reagent Blank Measurement
- **Enable reagent blank**
- **None**

Reagent Blank Measurement at Calibration
- **Reagent Blank (System Water)**

STANDARDISATION

Randox Calibration Serum Level 3 is traceable to creatinine reference materials NIST 909b and NIST 967.

QUALITY CONTROL

Randox Assayed Multisera, Level 2 and Level 3 are recommended for daily quality control. Two levels of controls should be assayed at least once a day. Values obtained should fall within a specified range. If these values fall outside the range and repetition excludes error, the following steps should be taken:

1. Check instrument settings and light source.
2. Check cleanliness of all equipment in use.
3. Check water, contaminants i.e. bacterial growth may contribute to inaccurate results.
4. Check reaction temperature.
5. Check expiry date of kit and contents.
6. Contact Randox Laboratories Customer Rx Support, Northern Ireland (028) 94451070.

Quality control requirements should be determined in conformance with government regulations or accreditation requirements.

CALCULATION

Creatinine clearance =

$$\frac{\text{mg creatinine/dl urine} \times \text{ml urine 24 hrs}}{\text{mg creatinine/dl serum} \times 1440}$$

INTERFERENCES

The method is subject to interferences from high levels of reducing substances. The interference can be partly eliminated in assays on urine by briefly boiling the urine specimen.

The analytes below were tested up to the following levels and were found not to interfere at Creatinine concentrations of 60 µmol/l and 260 µmol/l.

Haemoglobin	750 mg/dl
Free Bilirubin	15 mg/dl
Conjugated Bilirubin	15 mg/dl
Triglycerides	1000 mg/dl
Intralipid®	800 mg/dl
Glucose	300 mg/dl
Ascorbate	3 mg/dl

NORMAL VALUES[2]

Serum:	Men	53 - 97	µmol/l
		0.6 - 1.1	mg/dl
	Women	44 - 80	µmol/l
		0.5 - 0.9	mg/dl
Urine:		8.84 - 13.3	mmol/24 hrs
		1 - 1.5	g/24 hrs

It is recommended that each laboratory establish its own reference range to reflect the age, sex, diet and geographical location of the population.

SPECIFIC PERFORMANCE CHARACTERISTICS

The following performance characteristics were obtained using a **RX daytona** analyser.

SERUM OR PLASMA

LINEARITY

This method is linear up to 2844 µmol/l (32.5 mg/dl) in serum or plasma. In the event of a rerun the linearity is extended to 7821 µmol/l (88.9 mg/dl) for serum or plasma.

SENSITIVITY

The minimum detectable concentration of Creatinine with an acceptable level of precision was determined as 26.4 µmol/l (0.29 mg/dl) in serum or plasma.

APPENDIX B

Standard method for creatinine determination (cont.)

PRECISION

Within Run Precision

	Level 1	Level 2	Level 3
Mean (µmol/l)	65.3	131	384
SD	2.64	3.38	8.35
CV(%)	4.0	2.6	2.2
n	88	87	88

Total Precision

	Level 1	Level 2	Level 3
Mean (µmol/l)	65.3	131	384
SD	3.27	4.29	14.8
CV(%)	5.0	3.3	3.8
n	88	87	88

CORRELATION VS COMPETITOR

This method (Y) was compared with another commercially available method (X) and the following linear regression equation obtained:

Y = 0.95X + 4.38
and a correlation coefficient of r = 1.00

90 patient samples were analyzed spanning the range 60.18 to 1874.3 µmol/l.

SERUM vs. PLASMA

Plasma (Y) was compared with serum (X) and the following linear regression equation obtained:

Y = 1.09X - 10.32
and a correlation coefficient of r = 0.98

53 patient samples were analyzed spanning the range 36.49 to 153.75 µmol/l.

URINE

LINEARITY

This method is linear up to 66554 µmol/l (761 mg/dl) in urine

SENSITIVITY

The minimum detectable concentration of Creatinine in urine with an acceptable level of precision was determined as 311 µmol/l (3.51 mg/dl).

PRECISION

Within Run Precision

	Level 1	Level 2	Level 3
Mean (µmol/l)	4459	8925	17753
SD	92.5	184	266
CV(%)	2.1	2.1	1.5
n	88	88	88

Total Precision

	Level 1	Level 2	Level 3
Mean (µmol/l)	4459	8925	17753
SD	133	264	499
CV(%)	3.0	3.0	2.8
n	88	88	88

CORRELATION VS COMPETITOR

This method (Y) was compared with another commercially available method (X) and the following linear regression equation obtained:

Y = 0.96X + 768.17
and a correlation coefficient of r = 0.99

100 patient samples were analyzed spanning the range 2168.52 to 55302.48 µmol/l.

REFERENCES

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APPENDIX C

Standard method for cholesterol determination

CHOLESTEROL (CHOL)

Enzymatic Endpoint Method

FX series

INTENDED USE

For the quantitative *in vitro* determination of cholesterol in serum and plasma. This product is suitable for use on the *FX series* instruments which includes the Rx Daytona and Rx Imola.

Cat. No.

CH 3810	R1. Reagent	9 x 51 ml

CLINICAL SIGNIFICANCE[1,2,3]

Cholesterol measurements are used in the diagnosis and treatments of lipid lipoprotein metabolism disorders. Lipids play an important role in the body; they serve as hormones or hormone precursors, aid in digestion, provide energy, storage and metabolic fuels, act as functional and structural components in biomembranes and form insulation to allow nerve conduction and prevent heat loss.

In clinical chemistry, over the last decade however, lipids have become associated with lipoprotein metabolism and atherosclerosis. The Abell Kendell method, reported by Abell *et al* (1952) involved extraction of cholesterol by organic solvents and subsequent alkaline hydrolysis of the cholesterol esters. This reaction is highly specific but the reagents involved are corrosive and the method cumbersome, rendering it impractical for routine laboratory use.

The use of cholesterol oxidase following specimen saponification as described by Richmond (1973) provided the first step toward a totally enzymatic procedure. In 1974 Allain *et al.* and Roeschlaw *et al.* published the first fully enzymatic procedure for cholesterol determinations replacing chemical saponification with enzymatic saponification.

ASSAY PRINCIPLE[4]

The cholesterol is determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase.

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{Fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholestane-3-one} + H_2O_2$$

$$2H_2O_2 + \text{phenol} + \text{4-Aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{quinoneimine} + 4H_2O$$

SPECIMEN COLLECTION AND PREPARATION[5]

Serum: may be used.
Plasma: EDTA (1 mg/ml) or heparin (up to 75 U/ml) may be used. Do not use citrate, oxalate or fluoride.
Plasma and Serum samples may be stored for up to 4 days at +4°C.

REAGENT COMPOSITION

Contents	Concentrations in the Test
R1. Reagent	
Pipes Buffer	80 mmol/l, pH 6.8
4-Aminoantipyrine	0.25 mmol/l
Phenol	6 mmol/l
Peroxidase (E.C.1.11.1.7, Horse Radish, +25°C)	≥ 0.5 U/ml
Cholesterol esterase (E.C.3.1.1.13, Pseudomonas, 37°C)	≥ 0.15 U/ml
Cholesterol oxidase	≥ 0.10 U/ml

SAFETY PRECAUTIONS AND WARNINGS

For *in vitro* diagnostic use only. Do not pipette by mouth. Exercise the normal precautions required for handling laboratory reagents.

Solution R1 contains Sodium Azide. Avoid ingestion or contact with skin or mucous membranes. In case of skin contact, flush affected area with copious amounts of water. In case of contact with eyes or if ingested, seek immediate medical attention.

Sodium Azide reacts with lead and copper plumbing, to form potentially explosive azides. When disposing of such reagents flush with large volumes of water to prevent azide build up. Exposed metal surfaces should be cleaned with 10% sodium hydroxide.

Health and Safety Data Sheets are available on request.

The reagents must be used only for the purpose intended by suitably qualified laboratory personnel, under appropriate laboratory conditions.

STABILITY AND PREPARATION OF REAGENT

R1. Reagent

Contents ready for use. The reagent is stable up to the expiry date when stored at +2 to +8°C in the absence of contamination, protected from light.

MATERIALS PROVIDED

Cholesterol Reagent

MATERIALS REQUIRED BUT NOT PROVIDED

Randox Assayed Multisera Level 2 (Cat. No. HN 1530) and Level 3 (Cat. No. HE 1532)
Randox Calibration Serum Level 3 (Cat. No. CAL 2351)
FX series Saline (Cat. No. SA 3854)

CALIBRATION

The use of Randox Calibration Serum Level 3 is recommended for calibration.

This assay uses a **linear** calculation and no **reagent blank**. Ensure that on the Calibration Checks screen the following are selected for this test:

Sampling Method for Standards
- **Duplicate**

Reagent Blank measurement
- **Enable reagent blank –None**

Reagent blank measurement at calibration
- **Reagent Blank (system water)**

PROCEDURE NOTES

The Chemistry parameters for Randox Dedicated *FX series* Assays are predefined on the hard drive of the analyser PC. The required programs should be downloaded to the analyser software. Please note that the predefined chemistry parameters use SI units. If alternative units are required, these can be edited by the user. In this case, the technical range should be edited in accordance with the users selected units. All necessary instructions are encoded on the bar code. If the barcode cannot be read by the analyser, enter manually the series of numbers given beneath the barcode. If problems continue contact Randox Laboratories RX Support, Northern

APPENDIX C

Standard method for cholesterol determination (cont.)

RX daytona - CHOLESTEROL - CH 3810 PAGE 2 OF 2

STANDARDISATION
Randox Calibration Serum Level 3 is traceable to Cholesterol reference material NIST 909b and NIST 1952a.

QUALITY CONTROL
Randox Assayed Multisera, Level 2 and Level 3 are recommended for daily quality control. Two levels of controls should be assayed at least once a day. Values obtained should fall within a specified range. If these values fall outside the range and repetition excludes error the following steps should be taken:

1. Check instrument settings and light source.
2. Check cleanliness of all equipment in use.
3. Check water, contaminants ie bacterial growth may contribute to inaccurate results.
4. Check reaction temperature.
5. Check expiry date of kit and contents.
6. Contact Randox Laboratories RX Support, Northern Ireland (028) 94451070.

Quality control requirements should be determined in conformance with government regulations or accreditation requirements.

SPECIFICITY/LIMITATIONS
Cholesterol oxidase from *Nocardia* is not absolutely specific for cholesterol as it will oxidise several cholesterol analogues such as dihydrocholesterol or 7-dehydrocholesterol. Since these derivatives do not exist in serum in significant concentrations, the cholesterol oxidase from *Nocardia erythropolis* is suitable for a reliable cholesterol determination.

INTERFERENCE
The analytes below were tested up to the following levels and were found not to interfere:

Haemoglobin	1000 mg/dl
Free Bilirubin	25 mg/dl
Conjugate Bilirubin	25 mg/dl
Triglycerides	600 mg/dl
Intralipid®	800 mg/dl

NORMAL VALUES IN SERUM/PLASMA[4]
Risk levels

Value	Interpretation
< 5.17 mmol/l (200 mg/dl)	Desirable blood cholesterol
5.17 - 6.18 mmol/l (200-239 mg/dl)	Borderline-high blood cholesterol
≥ 6.20 mmol/l (240 mg/dl)	High blood cholesterol

It is recommended that each laboratory establish its own reference range to reflect the age, sex, diet and geographical location of the population.

SPECIFIC PERFORMANCE CHARACTERISTICS
The following performance characteristics were obtained using the RX Daytona analyser.

LINEARITY
The test is linear up to a cholesterol concentration of 16.6 mmol/l (640 mg/dl). In the event of a rerun the linearity is extended to 166 mmol/l (6407 mg/dl).

SENSITIVITY
The minimum detectable concentration of cholesterol with an acceptable level of precision was determined as 0.865 mmol/l (33.4 mg/dl).

PRECISION
Within run precision

	Level 1	Level 2	Level 3
Mean (mmol/l)	1.71	4.73	7.70
SD	0.064	0.079	0.295
CV(%)	3.73	1.67	3.84
n	20	20	20

Between run precision

	Level 1	Level 2	Level 3
Mean (mmol/l)	1.67	3.91	7.52
SD	0.022	0.039	0.105
CV(%)	1.33	1.00	1.39
n	20	20	20

CORRELATION
This method (Y) was compared with another commercially available method (X) and the following linear regression equation obtained:

$Y = 1.00X + 0.11$
and a correlation coefficient of $r = 0.99$

40 patient samples were analyzed spanning the range 3.25 to 9.55 mmol/l.

REFERENCES

1. Abell, L.L, Levey, B.B., Brodie B.B., *et al*, J. Biol Chem **195** : 357, 1952
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4. Trinder, P., Ann clin Biochem **6** : 24, 1969.
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6. Third Report of the National Cholesterol Education Programme (NCEP) Expert Panel on Detection, Evaluation and treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). JAMA Publication, Vol 285, No. 19, P2486 - 2497; 2001.

APPENDIX C

Standard method for cholesterol determination (cont.)

PRECISION

Intra Assay

	Level 1	Level 2	Level 3
Mean (µmol/l)	629	2668	7114
SD	21.07	52.0	103.15
CV(%)	3.35	1.95	1.45
n	20	20	20

Inter Assay

	Level 1	Level 2
Mean (µmol/l)	655	2679
SD	29.5	58.7
CV(%)	4.43	2.19
n	20	20

REFERENCES

1. Barham, D., and Trinder, P., Analyst (1972) **97**, 142-145.
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3. Tietz, NW, Textbook of clinical chemistry, W.B. Saunders, Co., Philadelphia, P.A. (1994) pp 58-69 (specimen collection and storage recommendations), P2210 (reference values).
4. Thefeld, W. *et al.* Dtsch. Med. Wschr. (1973) **98**, 380.
5. Krieg, M. *et al.* J. Clin. Chem. Clin. Biochem.(1986) **24**, 863.

APPENDIX D

Standard method for albumin determination

INTENDED USE
An Albumin test system is a device intended for the quantitative *in vitro* determination of Albumin concentration in serum and plasma. This product is suitable for use on Rx series instruments which includes the Rx Daytona and the Rx Imola.

Cat. No.
AB 3800 R1. Bromocresol Green 51 ml

CLINICAL SIGNIFICANCE[1]
Albumin is the most abundant serum protein representing 55-65% of the total protein. It is synthesised in the liver and has a half-life of 2 to 3 weeks.
The main biological functions of albumin are to maintain the water balance in serum and plasma and to transport and store a wide variety of ligands e.g. fatty acids, calcium, bilirubin and hormones such as thyroxine. Albumin also provides an endogenous source of amino acids.
Albumin measurements are used in the diagnosis and treatment of numerous diseases involving primarily the liver or kidneys.

PRINCIPLE[2]
The measurement of serum albumin is based on its quantitative binding to the indicator 3,3',5,5'-tetrabromo-m cresol sulphonphthalein (bromocresol green). The albumin-BCG-complex absorbs maximally at 578 nm.

SAMPLE COLLECTION AND PREPARATION[3,4]
Serum, heparinized plasma or EDTA plasma. Normal procedures for collecting and storing serum may be used for samples to be analysed by this method. Serum is stable for 3 days at + 2 to +8°C, or 6 months at -20°C.

REAGENT COMPOSITION

Contents		Concentrations in the Test
R1.	**Bromocresol Green**	
	Succinate buffer	75 mmol/l; pH 4.2
	Bromocresol green	0.15 mmol/l
	Brij 35	
	Preservative	

SAFETY PRECAUTIONS AND WARNINGS
For *in vitro* diagnostic use only. Do not pipette by mouth. Exercise the normal precautions required for handling laboratory reagents.

Health and Safety Data Sheets are available on request.

The reagents must be used only for the purpose intended by suitably qualified laboratory personnel, under appropriate laboratory conditions.

STABILITY AND PREPARATION OF REAGENTS

R1. **Bromocresol Green**
Contents supplied ready for use. Stable up to the expiry date when stored at +15 to +25°C.

MATERIALS PROVIDED
Albumin Bromocresol Green Reagent

MATERIALS REQUIRED BUT NOT PROVIDED
Randox Assayed Multisera Level 2 (Cat. No. HN 1530) and Level 3 (Cat. No. HE 1532)
Randox Calibration Serum Level 3 (Cat. No. CAL 2351)
RX Daytona Saline (Cat. No. SA 3854)

PROCEDURE NOTES
The Chemistry parameters for Randox Dedicated Rx series Assays are predefined on the hard drive of the analyser PC. The required programs should be downloaded to the analyser software. Please note that the predefined chemistry parameters use SI units. If alternative units are required, these can be edited by the user. In this case, the technical range should be edited in accordance with the users selected units. All necessary instructions are encoded on the bar code. If the barcode cannot be read by the analyser, enter manually the series of numbers given beneath the barcode. If problems continue contact Randox Laboratories RX Support, Northern Ireland (028) 94451070.

CALIBRATION
0.9% NaCl as zero calibrator and Randox Calibration Serum Level 3 are recommended for calibration. A 2 point calibration is recommended.

This assay uses a linear calculation and a reagent blank at calibration only. Ensure that on the [Calibration] [Checks (F10)] screen the following are selected for this test:

Sampling Method for Standards
- Duplicate

Reagent Blank measurement
- Enable Reagent Blank – None

Reagent Blank measurement at calibration
- Reagent Blank (system water)

STANDARDISATION
Randox Calibration Serum Level 3 is traceable to Albumin reference material DA470 (IFCC).

QUALITY CONTROL
Randox Assayed Multisera, Level 2 and Level 3 are recommended for daily quality control. Two levels of controls should be assayed at least once a day. Values obtained should fall within a specified range. If these values fall outside the range and repetition excludes error the following steps should be taken:

1. Check instrument settings and light source.
2. Check cleanliness of all equipment in use.
3. Check water, contaminants ie bacterial growth may contribute to inaccurate results.
4. Check reaction temperature.
5. Check expiry date of kit and contents.
6. Contact Randox Laboratories Rx Support, Northern Ireland (028) 94451070.

Quality control requirements should be determined in conformance with government regulations or accreditation requirements.

APPENDIX D

Standard method for albumin determination (cont.)

INTERFERENCE
The analytes below were tested up to the following levels and were found not to interfere:

Haemoglobin	500 mg/dl
Free Bilirubin	25 mg/dl
Conjugate Bilirubin	25 mg/dl
Triglycerides	1000 mg/dl
Intralipid®	800 mg/dl

NORMAL VALUES IN SERUM[2]

Adults	38 - 44 g/l (3.8 - 4.4 g/dl)
Neonates	38 - 42 g/l (3.8 - 4.2 g/dl)

It is recommended that each laboratory establish its own reference range to reflect the age, sex, diet and geographical location of the population.

SPECIFIC PERFORMANCE CHARACTERISTICS
The following performance characteristics were obtained using an RX Daytona analyser.

LINEARITY
This method is linear to 50.6 g/l (5.06 g/dl). In the event of a rerun the upper limit of the assay range is increased to 300 g/l (30.0 g/dl).

SENSITIVITY
The minimum detectable concentration of Albumin with an acceptable level of precision was determined as 3.20 g/l.

PRECISION

Within Run Precision

	Level 1	Level 2	Level 3
Mean (g/l)	14.6	35.6	43.1
SD	0.24	0.43	0.53
CV%	1.62	1.20	1.23
N	20	19	20

Between Run Precision

	Level 1	Level 2	Level 3
Mean (g/l)	14.0	30.6	45.4
SD	0.49	0.67	0.79
CV%	3.48	2.19	1.74
N	20	20	20

CORRELATION
This method (Y) was compared with another commercially available method (X) and the following linear regression equation obtained:

$Y = 1.00 X - 0.39$
and a correlation coefficient of $r = 0.97$

40 patient samples were analyzed spanning the range 19.9 to 52.3 g/l.

REFERENCES

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3. Tietz, NW, Textbook of Clinical Chemistry, W.B. Saunders, Co., Philadelphia, P.A. 1986 pp 478-497 (specimen collection and storage recommendations).
4. Tietz, NW, Clinical Guide to Laboratory Tests (Second Edition), W.B. Saunders Co., Philadelphia, P.A. 1990 p.26

VITA

Miss Tipawan Rungsawang was born on September 14, 1976 in Mukdahan, Thailand. She graduated with high school degree from Singburi School, Singburi in 1993. She received her Bachelor's degree of Science (Chemistry) from Naresuan University in 1999. Then she has been appointed as a Chemist at Pan Asia Leather co. Ltd. until 2000, and an instructor at Walai-alongkorn Rajabhat University, Pathum thani in 2001. She received her Master's degree of Science (Chemistry) in 2005 from Burapha Universityi. She has been appointed as an instructor at Nakorn pathom Rajabhat University, Nakorn pathom until 2007.

Since 2007, she has become a graduate student at the program in Biotechnology, Faculty of Science, Chulalongkorn University and worked under the supervision of Assoc. Prof. Dr. Orawon Chailapakul. In 2008, she has received the scholarship from Strategic Scholarship Fellowships Frontier Research Network, Office of the Higher Education Commitsion, and she had an opportunity to do the research for nine months in Assoc. Prof. Charls Henry chuck at Colorado state University in United States of America. She graduated the Doctor of Science degree from Chulalongkorn University in the academic year 2011.