การพัฒนาชุดตรวจวัดอัลบูมินในปัสสาวะ

นางสาวลลิตพรรณ หงษ์ทะนี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเคมี ภาควิชาเคมี
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2559

DEVELOPMENT OF URINARY ALBUMIN DETECTION KIT

Miss Lalitphan Hongtanee

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Chemistry
Department of Chemistry
Faculty of Science
Chulalongkorn University
Academic Year 2016

Thesis Title	DEVELOPMENT OF URINARY ALBUMIN DETECTION KIT
By	Miss Lalitphan Hongtanee
Field of Study	Chemistry
Thesis Advisor	Luxsana Dubas, Ph.D.
Thesis Co-Advisor	Assistant Professor Fuangfa Unob, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Science
(Associate Professor Polkit Sangvanich, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Associate Professor Vudhichai Parasuk, Ph.D.)

.....Thesis Advisor
(Luxsana Dubas, Ph.D.)

.....Thesis Co-Advisor
(Assistant Professor Fuangfa Unob, Ph.D.)

.....Examiner
(Assistant Professor Pakorn Varanusupakul, Ph.D.)

.....External Examiner
(Tinnakorn Tiensing, Ph.D.)

ลลิตพรรณ หงษ์ทะนี : การพัฒนาชุดตรวจวัดอัลบูมินในปัสสาวะ (DEVELOPMENT OF URINARY ALBUMIN DETECTION KIT) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ดร.ลักษณา ดูบาส, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร.เฟื่องฟ้า อุ่นอบ, 75 หน้า.

งานวิจัยนี้เป็นการพัฒนาวิธีการตรวจวัดอัลบูมินและครีแอทินินในตัวอย่างปัสสาวะของมนุษย์ ด้วยวิธีเปรียบเทียบสี ช่วงการศึกษาคือ 0 – 30 มิลลิกรัมต่อลิตรสำหรับอัลบูมิน และ 0 – 3000 มิลลิกรัมต่อลิตรสำหรับครีแอทินิน เทคนิคเลเยอร์บายเลเยอร์ถูกนำมาใช้ดัดแปรพื้นผิวของเมมเบรนเซลลูโลสอะซีเตต เพื่อใช้ในการสกัดอัลบูมินและครีแอทินินโดยอาศัยแรงดึงดูดระหว่างประจุ คู่พอลิอิเล็กโทรไลต์ที่เหมาะสมในงานนี้ คือ พอลิไดแอลิลไดเมทิลแอมโนเนียมคลอไรด์ (PDADMAC) กับพอลิสไตรีนซัลโฟเนต (PSS) สำหรับการตรวจวัดอัลบูมิน อัลบูมินที่เตรียมในปัสสาวะเทียมถูกเจือจาง 4 เท่าด้วย 5 มิลลิโมลาร์ ฟอสเฟตบัฟเฟอร์ พีเอช 7 และกรองผ่านเมมเบรนที่ดัดแปรด้วย PDADMAC เป็นชั้นนอกสุด สารอัลบูมินที่ดูดซับอยู่บนกระดาษกรองจะถูกชะออกและตรวจวัดด้วยสีย้อมเตรตระโบรโมฟีนอลบูลในไตรตัน เอกซ์ 100 ที่พีเอช 3 ทำให้เกิดการเปลี่ยนแปลงสีของสารละลายเตรตระโบรโมฟีนอลบูลกับอัลบูมินจากสีเหลืองเป็นสีเขียว ค่าการดูดกลืนแสงตรวจวัดได้จากเครื่องยูวี-วิสิเบิลสเปกโทรโฟโตมิเตอร์ที่ความยาวคลื่น 625 นาโนเมตร พบว่ามีความสัมพันธ์ที่เป็นเส้นตรงระหว่างความเข้มข้นของอัลบูมินกับค่าการดูดกลืนแสงอยู่ในช่วง 2.5 – 7.5 มิลลิกรัมต่อลิตร ค่าความเข้มข้นต่ำสุดของการตรวจวัดอัลบูมินคือ 0.47 มิลลิกรัมต่อลิตร ร้อยละการได้กลับคืนของอัลบูมินจากตัวอย่างพบอยู่ในช่วง 81.2 – 106.9% และค่าความเที่ยงในการวิเคราะห์อยู่ในช่วง 0.8 – 3.2% สำหรับการตรวจวัดครีแอทินินนั้น ทำการสกัดครีแอทินินด้วยเมมเบรนที่ดัดแปรด้วย PSS เป็นชั้นนอกสุด ครีแอทินินที่ดูดซับบนเมมเบรนจะถูกตรวจวัดด้วยปฏิกิริยาจาฟเฟ่ สารประกอบที่เกิดขึ้นมีสีเหลืองส้ม ความเข้มสีบนเมมเบรนถูกตรวจวัดด้วยโปรแกรมอิมเมจเจ ความเป็นเส้นตรงของวิธีนี้อยู่ในช่วง 0 – 100 มิลลิกรัมต่อลิตร และค่าความเข้มข้นต่ำสุดของการตรวจวัดของครีแอทินินอยู่ที่ 3.53 มิลลิกรัมต่อลิตร ร้อยละการได้กลับคืนของครีแอทินินจากตัวอย่างพบอยู่ในช่วง 102.2 – 103.3% และค่าความเที่ยงในการวิเคราะห์ต่ำกว่า 7.6% นอกจากนั้นสีที่เปลี่ยนจากการตรวจวัดอัลบูมินและครีแอทินินสามารถสังเกตเห็นได้อย่างชัดเจน ดังนั้นวิธีที่นำเสนอสามารถประยุกต์ใช้ในการตรวจวัดอัลบูมินและครีแอทินินในตัวอย่างปัสสาวะด้วยวิธีเปรียบเทียบสีและตรวจวัดด้วยตาเปล่าได้เพื่อใช้ในการวินิจฉัยความผิดปกติของไตในระยะเริ่มต้น

ภาควิชา เคมี	ลายมือชื่อนิสิต .....
สาขาวิชา เคมี	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
ปีการศึกษา 2559	ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5772126323 : MAJOR CHEMISTRY
KEYWORDS: ALBUMIN / CREATININE / TETRABROMOPHENOL BLUE / JAFFE REACTION / POLYELECTROLYTE MULTILAYER

LALITPHAN HONGTANEE: DEVELOPMENT OF URINARY ALBUMIN DETECTION KIT. ADVISOR: LUXSANA DUBAS, Ph.D., CO-ADVISOR: ASST. PROF. FUANGFA UNOB, Ph.D., 75 pp.

This work developed the colorimetric determination of bovine serum albumin (BSA) and creatinine in urine sample. The studied ranges are 0 – 30 mg/L for BSA and 0 – 3000 mg/L for creatinine. The layer-by-layer deposition technique (LbL) was used to modify the surface of cellulose acetate membrane (CA) for albumin and creatinine extraction via electrostatic interactions. The suitable polyelectrolyte pair used in this work was poly(diallyl dimethyl ammonium chloride (PDADMAC) and poly(sodium 4-styrenesulfonate) (PSS). For the albumin determination, BSA prepared in synthetic urine was diluted 4-fold with 5 mM pH 7 phosphate buffer and filtered through the modified membrane of which PDADMAC was the outermost layer. The adsorbed BSA on the membrane was eluted and detected with tetrabromophenol blue in Triton X-100 at pH 3, resulting in a change in the color of TBPB-BSA solution from yellow to green. The absorbance intensity was measured with a UV-Vis spectrophotometer at 625 nm. The relationship between the BSA concentration and the absorbance intensity was observed in the range of 2.5 – 7.5 mg/L with the detection limit of 0.47 mg/L. The recovery of albumin from samples was 81.2 – 106.9% and the relative standard deviation was 0.8 – 3.2%. For the creatinine determination, the modified membrane of which PSS was the outermost layer was used to extract creatinine. The adsorbed creatinine on membrane was detected with Jaffé reaction, forming a yellow-orange complex. The color intensity was monitored by Image J software. The linearity of this method was in the range of 0 – 100 mg/L and the limit of detection was found to be 3.53 mg/L. The recovery of creatinine from samples was found in the range of 102.2 – 103.3% and the relative standard deviation was less than 7.6%. Moreover, the color change from albumin and creatinine determination could be observed visually. Therefore, the proposed method could be applied to determine albumin and creatinine in urine samples by both colorimetric and naked eyes detection for the early diagnosis of kidney failure.

Department:	Chemistry	Student's Signature	.....
Field of Study:	Chemistry	Advisor's Signature	.....
Academic Year:	2016	Co-Advisor's Signature	.....

ACKNOWLEDGEMENTS

First of all, I would like to express my sincere gratitude to my thesis advisor, Dr. Luxsana Dubas, and my co-advisor, Assistant Professor Dr. Fuangfa Unob for their invaluable suggestion, assistance, motivation, constructive and the continued support in my thesis. They educated me, not only in research but also everything in real life. Beside my advisor and co-advisor, I would like to acknowledge Dr. Stephan T. Dubas for his suggestion and help on the technique for fabrication in my thesis. I also would like to thank my thesis committee, Associate Professor Dr. Vudhichai Parasuk, Assistant Professor Dr. Pakorn Varanusupakul and Dr. Tinnakorn Tiensing, who give helpful comments and advice in this thesis.

Moreover, I would like to thank Miss Apisaranut Sungkaong, Miss Jarinya Sittiwong, Miss Anna Prukjareonchook and all member of Chromatography and Separation Research Unit (ChSRU), especially all members of 1253 Laboratory for their kindness, friendship, helpfulness, comment, and encouragement.

I would like to acknowledge the financial supported from Science Achievement Scholarship of Thailand (SAST) and the Ratchadaphiseksomphot Endowment Fund 2013 of Chulalongkorn University (CU-56-543-AM).

Finally, I would like to send a heartfelt acknowledgment to my family for their love, support, and especially for the encouragement they provided me throughout my thesis. Also, I would like to thank my special friend, Mr. Keerati Punaha, who always stands by me in times of need.

CONTENTS

Page

Page

Page

Page

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

PDADMAC	Poly(diallyldimethyl ammonium chloride)
PSS	Poly(sodium 4-styrenesulfonate)
PSSMA	Poly(4-styrene sulfonic acid-co-maleic acid, sodium salt)
BSA	Bovine serum albumin
PEM	Polyelectrolyte multilayer thin films
LBL	Layer-by-Layer technique
TBPB	Tetrabromophenol blue
pI	Isoelectric point
pKa	Acid dissociation constant
min	Minute
mL	Milliliter
L	Liter
mg/L	Milligram per liter
µg	Microgram
mM	Milli Molar
M	Molar
%RSD	Relative standard deviation percentage
SD	Standard deviation
LOD	Limit of detection
LOQ	Limit of quantitation
AOAC	Association of Official Analytical Chemis

CHAPTER I
INTRODUCTION

The measurements of albumin and creatinine in the urine are necessary in clinical diagnosis for kidney disease. The albumin test is typically used in conjunction with a creatinine test to provide a urinary albumin-to-creatinine ratio (UACR) due to the constant excretion of creatinine [1]. Moreover, albumin to creatinine ratio has slightly better diagnostic accuracy than urine albumin concentration alone [2]. The normal range of UACR is less than 30 mg/g, while the abnormal range is higher than 300 mg/g indicated a clinical albuminuria [3]. Microalbuminuria (defined as urinary albumin excretion of 30-300 mg/g) is used as a marker of renal damage and diabetic nephropathy. Furthermore, low levels of UACR are associated with increased risk of cardiovascular disease [4]. Therefore, it is essential for determining a urinary albumin-to-creatinine ratio below 30 mg/g to diagnose cardiovascular disease and to prevent renal failure in diabetes patient.

There are several methods for albumin detection in urine such as radioimmunoassay [5], enzyme-linked immunosorbent assay (ELISA) [6], immunoturbidimetric assay [7], and dipstick tests [8]. However, there are significant drawbacks of these existing methods. ELISA system is time consuming and tedious washing steps, leading to its disfavor for routine operation. The immunoassay-based methods consume large amounts of expensive antibody reagent. A urine test strip is regularly used for semi-quantitative assay of protein. This test is based on dye binding using tetrabromophenol blue (TBPB) that is specific for albumin [9, 10]. The color changes from yellow to blue when TBPB binds with protein [9]. The urine test strip cannot detect microalbuminuria because the lower limit of detection is 250 mg/L [10], while the urine microalbumin test strip can detect. However, albumin-to-creatinine ratio below 30 mg/g is difficult to measure with the urine microalbumin test strip and the test is not absolutely specific for albumin [11]. The Jaffé reaction is the conventional standard method that involves a reaction between a creatinine and

alkaline picrate solution. The creatinine procedure is linear from 10 to 3000 mg/L and the product is a yellow-orange complex. Nevertheless, this method is time-consuming and often affected by matrix interference [12]. Hence, the high sensitivity and selectivity determination of albumin and creatinine are required.

At the present, the Layer-by-Layer (LbL) self-assembly deposition is an easy, efficient, reproducible, robust, flexible, and extremely versatile technique for surface modification [13]. Polyelectrolytes were deposited on the material's surface in this technique, leading to the formation of polyelectrolyte multilayers (PEMs). Conventionally, the main and most extensively studied driving force of LbL assembly is the electrostatic interaction [14]. The previous research has reported an application of protein adsorption on the LbL modified glass [15].

In this work, we modified cellulose acetate membranes with PEMs by using LbL technique to extract albumin and creatinine onto the modified membranes before determination process. The determination of albumin used a modified membrane of which polycations as the outermost layer to filter bovine serum albumin (BSA) solutions (pH 7). At the pH above the isoelectric point (pI) of BSA (pI = 4.7) [16], the net charges of the proteins are negative, resulting in the BSA adsorption. We proposed to reduce matrix interferences from albumin before testing with specific dye binding reagent, Tetrabromophenol blue (TBPB). TBPB in Triton X-100 solution was used to detect the amount of adsorbed BSA. In the same fashion, creatinine was converted to its cationic form by controlling pH to be below the pK_a values of creatinine (pK_{a1} = 4.83, pK_{a2} = 9.2) [17] . A modified membrane of which polyanions as the outermost layer was dipped into creatinine solution and the extracted creatinine was detected by performing a Jaffé reaction directly on the modified membrane.

1.1 Research objective

1.1.1 To modify a cellulose acetate membrane for albumin and creatinine extraction

1.1.2 To detect albumin and creatinine by colorimetric method and naked detection.

1.2 Scope of the research

The surfaces of cellulose acetate membranes (CA membrane) were modified with polyelectrolytes multilayers (PEMs) to extract albumin and creatinine from matrix. The strong polycation, strong polyanion and weak polyaion used were poly(diallyl dimethyl ammonium chloride (PDADMAC), poly(sodium 4-styrenesulfonate) (PSS) and poly (4-styrene sulfonic acid-co-maleic acid) copolymer (PSSMA), respectively. Quartz and CA membrane were used as fabricating substrate, the growth of PEMs was monitored by UV-Vis and ATR-FTIR spectrophotometer. For albumin determination, BSA solution was filtered through CA membrane modified with 13-layers of PDADMAC/PSS or PDADMAC/PSSMA multilayers. Next, the desorption and detection processes were performed by releasing the adsorbed albumin from PEMs layer and detecting with dye binding reagent (TBPB in triton X-100). The suitable condition of dye binding reagent was optimized. The albumin detection steps composed of the dipping method and the dropping method were studied. The dipping method performed by dipping the modified membrane into dye binding reagent, whereas the dropping method performed by dropping dye binding reagent into a solution that used to release BSA from PEMs layer. The color of TBPB-BSA complexes was observed by using a UV-Vis spectrophotometer.

For creatinine determination, the CA membrane modified with 12-layers of PDADMAC/PSS was immersed into creatinine standard solution to extract creatinine from matrix. After that, alkaline picrate solution was dropped onto modified membrane to react with adsorbed creatinine resulting the orange color of creatinine-picrate complex. To obtain a suitable condition for creatinine determination, the influence of picric to creatinine ratio and reaction time were evaluated. The color intensity of creatinine-picrate complex was obtained from Image J software. Finally, the proposed method was evaluated and validated.

1.3 Benefits of Research

This research aimed to obtain a selective measurement for albumin and creatinine in urine with the easy detection method using a simple instrument or naked eyes.

CHAPTER II
THEORY AND LITERATURE REVIEW

2.1 Albumin

Bovine serum albumin (BSA) is an example of well-known model protein used for protein assay. Proteins are polymers of multiple monomer units called amino acids, which have many different functional groups including alcohols, thiols, thioethers, carboxylic acids, carboxamides, and a variety of basic groups. There are four levels of protein structure including primary, secondary, tertiary and quaternary structure. The amino acid composition is generally unique and specific to each particular protein called primary structure. The secondary structure is the specific geometric shape (the alpha helix or the beta sheets) caused by intramolecular and intermolecular hydrogen bonding of amide groups. A three-dimensional shape of a protein chain is the tertiary structure. The quaternary structure refers to two or more polypeptide chains, each with its own primary, secondary, and tertiary structure [18]. The secondary structure of BSA consists of approximately 54% α-helix and 40% β-structure [19] and contains three binding domains, which are specific for metal ions, lipids, and nucleotides, respectively [20]. BSA is classified in the soft proteins class, which can easily change their structures and conformations [21]. Albumin is a protein made by the liver and normally found in the blood plasma, representing about 50% of the total protein content [22] as shown in Figure 2.1. Albumin proteins are large and water-soluble. Due to their size, a healthy kidney does not let albumin pass into the urine while a damaged kidney lets some albumin pass into the urine. The normal level of protein or albumin in human urine is lower than 30 mg/day, while clinical albuminuria is greater than 300 mg/day [23]. Moreover, the albumin excretion per day of 30–300 mg, microalbuminuria, is an important marker of early kidney damage from diabetes [23].

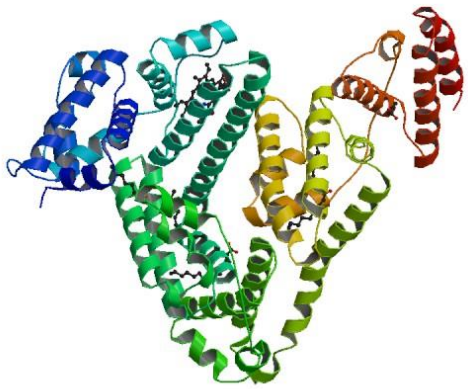

Figure 2.1 Structure of albumin [23].

2.2 Creatinine

Creatinine is a waste product produced by muscles from the breakdown of a compound called creatine [24]. The people who has the more muscle can produce the more creatinine. Almost all creatinine is filtered from the blood by the kidneys and eliminated in urine with constant throughout the day [1]. Normally, the amount of creatinine excreted between 250 – 2590 mg/L in human urine and 6 – 12 mg/L in blood [25, 26]. Therefore, creatinine in urine can be an indicator of kidney function.

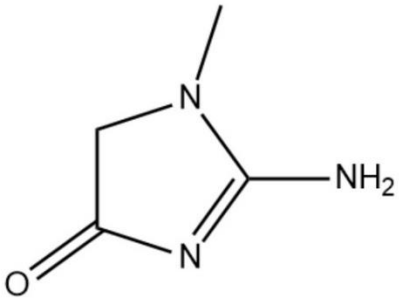

Figure 2.2 Structure of creatinine.

2.3 Urinary albumin-to-creatinine ratio

Albumin excretion is most accurately described using 24 hours urine collection, but it is cumbersome and prone to collection error [27]. To overcome this problem, albuminuria is evaluate by measuring the urinary albumin-to-creatinine ratio (UACR) in a spot urine sample [3]. This test is better than a dipstick test for albumin because it is unaffected by variation in urine concentration [28]. UACR is a ratio between two measured substances that was calculated using the following equation.

$$\frac{\text{Urine albumin (mg/L)}}{\text{Urine creatinine (g/L)}} = \text{UACR in mg/g} \approx \text{Albumin excretion in mg/day}$$

The normal level of UACR is less than 30 mg/g, while the clinical albuminuria is greater than 300 mg/g. UACR is between of 30-300 mg/g defined as microalbuminuria. Moreover, urine albumin to creatinine ratio below 30 mg/g is a predictor of kidney disease and cardiovascular disease [4].

2.4 Layer by layer (LBL) self assembly technique

The Layer-by-Layer (LbL) technique is an easy, efficient, reproducible, robust, flexible, and extremely versatile technique for surface modification [13]. This technique was reported by Decher et al in 1997 for preparation of thin films by alternate deposition of polyelectrolytes to form polyelectrolyte multilayers (PEMs) [29]. There are several reported publications incorporating polyelectrolytes since the pioneering work of Decher and co-workers [30, 31]. The LbL assembly consists of alternating sequential adsorption of polycations and polyanions on a charged surface, occurring either via electrostatic or nonelectrostatic interactions. The washing steps are needed after each adsorption to remove weakly associated polymer chains and to build as effective and reproducible film [32]. Besides, the deposition cycles are repeated in order to deposit the desired number of layers, yielding multilayer assemblies.

The LbL buildup of multilayer assemblies normally occurs through a variety of deposition methods including spin-coating, spray-coating and dip-coating. Among these ways of preparing multilayers, the dip-coating is the most widely used nowadays because it is the possibility of coating substrates with more complex geometries [14]. The advantages of the LbL method compose of their ease of processing, less equipment requirement, low cost benefit as the price of polymer and variety of materials which can be incorporated into the assembly system. In addition, there are several versatility of substrates that polyelectrolyte multilayer thin films can be grown on such as quartz, silicon wafer, glass slide, poly(dimethylsiloxane) (PDMS) and membrane. Therefore, the LbL self assembly technique was applied to various potential applications in electrical, optical, biomedical and membrane devices, such as solar cells, biosensors, drug delivery, membrane separations, etc [33-36].

2.4.1 Polyelectrolytes

Polyelectrolyte are polymers with charged groups within the monomer repeat units. They can be divided into two types by using their dissociation in polar solvents. The polyelectrolytes that fully dissociate in water or aqueous solution in total pH range between 0 and 14 are referred to as strong polyelectrolytes [37]. In contrast, the weak polyelectrolytes can be partial and strongly dependent on pH [38]. Moreover, polyelectrolytes can be classified as polycation and polyanion by using their charge. For instance, poly(diallyldimethyl ammonium chloride) (PDADMAC) and poly(sodium 4-styrenesulfonate) (PSS) are known as strong polycation and polyanion, respectively. The weak polyanion is poly (4-styrene sulfonic acid-co-maleic acid) (PSSMA). Polyelectrolytes and their chemical structures are shown in Figure 2.3.

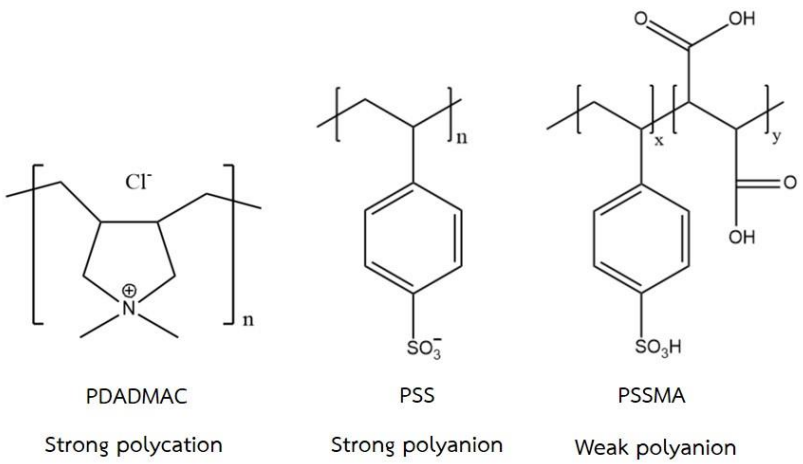

Figure 2.3 Polyelectrolytes and their chemical structures.

2.4.2 Fabrication

Polyelectrolyte multilayers (PEMs) can be prepared on the surface of a substrate by alternate deposition of a cationic polyelectrolyte and an anionic polyelectrolyte as shown in Figure 2.4. The fabrication of PEMs was performed by dipping a charged substrate into a solution of polyelectrolyte having the opposite charge as the initial substrate resulting in adsorption of first layer. Next, the substrate is rinsed by using water or buffer solution to remove excess and weakly adsorbed polyelectrolyte. After the first adsorption step, the substrate is exposed to a polyelectrolyte solution having the same charge as the initial substrate resulting in adsorption of second layer. The rinsing step is performed again and this process can be repeated several time to get a PEMs with the required thickness.

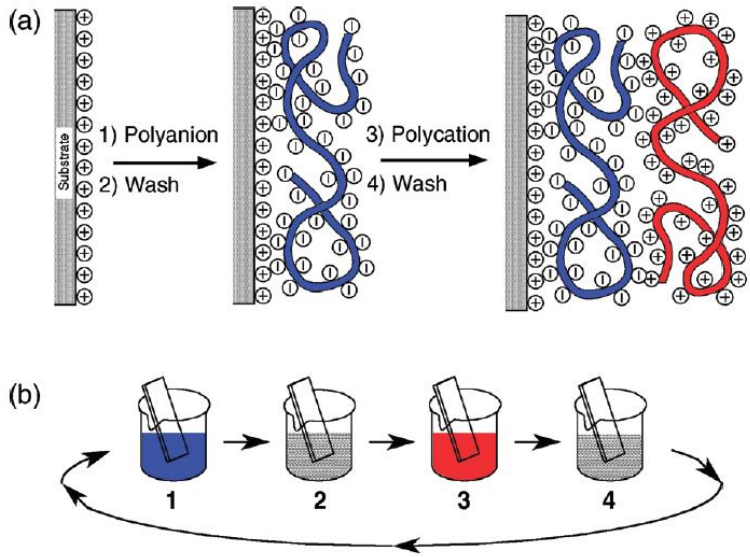

Figure 2.4 Schematic of the electrostatic self-assembly of PEMs [39].

2.4.3 Parameters affecting the thickness of polyelectrolyte multilayers

The thickness of polyelectrolyte multilayers (PEMs) depends on the number of layers and salt concentration [41]. Several previous works have reported the thickness of PEMs as a function of the number of deposited layers. For the initial coating layers on the substrate, the thickness is usually thin due to the low charge density of the surface. For higher number of layers, the deposition process is stable and the growth rate is quite fast [41, 42]. Moreover, the adding of salt into the polyelectrolyte solution increases the thickness of PEMs because salt ions can induce the chain conformation of polyelectrolyte in the solution resulting in the coil-polyelectrolyte deposition on the substrate surface [43, 44].

2.5 Albumin adsorption

The conformational change of bovine serum albumin (BSA) depending on pH values induces a change of the electrical charge. At a pH equal to the isoelectric point of BSA (pI = 4.7), BSA has zero net charge [45]. BSA carries a net positive charge at pH

below pI, whereas BSA carries a net negative charge at pH above pI. The adsorption usually occurs because of electrostatic interaction between the surface and the oppositely charged functional group along the polypeptide chain of the BSA molecules [20]. At the present, the adsorption of BSA on different surfaces has been investigated widely [46-48].

2.6 Creatinine extraction

Creatinine is monoprotonated cation at physiological pH due to its pKa of 4.8 and 9.2 [49]. Due to the positive charge on surface, creatinine can be extracted using ion exchange chromatography. The ion exchange chromatography is a technique used to separate ionizable molecules based on their total charges. This technique is classified into 2 types including cation exchange chromatography and anion exchange chromatography. A cation exchange resin is one of method chosen to extract creatinine from matrix interferences [50]. The positive charged creatinine can replace the counter ions of sulfonate acid groups on the resin (e.g. H^+, Na^+) and be adsorbed on the resin via electrostatic interactions, while negatively charged and neutral molecules are released. Afterward, creatinine are eluted with a suitable solution. From the cation exchange chromatography, the predominant driving force for creatinine extraction is electrostatic interaction.

2.7 Method for albumin and creatinine determination

2.7.1 Albumin determination

There are many different methods have been developed to quantitate proteins in a given assay for total protein content and for a single protein. The traditional methods for total protein quantitation comprise of the measurement of UV absorbance at 280 nm, Bicinchoninic acid (BCA) and Bradford assay [51]. The common method for individual protein quantitation is enzyme-linked immunosorbent (ELISA)

assay [52]. However, the immunoassay-based methods consume large amounts of expensive reagents.

The simplest way to determine albumin is dye binding. The dye binding method based on the color change of dye as it binds albumin. Among the common protein found in normal or abnormal urine, albumin gives the most color per gram protein as it binds to the dye [53]. Several dyes have been used for albumin determination such as bromocresol green [54], tetrabromophenolphthalein ethyl ester (TBPE) [55], bromophenol blue (BPB) [56], and tetrabromophenol blue (TBPB) [57]. Most of dyes are low sensitivity. Among them, TBPB as a dye reagent in urine test strip is interesting. Although, the sensitivities of TBPB was poor (32-46%), the specificities of TBPB was high (97- 100%) [58, 59].

Tetrabromophenol blue (TBPB) is a traditional reagent strip testing for protein. In acidic condition, TBPB appears yellow in the absence of protein. A positive reaction is indicated by a color change from greenish-yellow to green and then to dark-green. The reaction between TBPB and protein is showed in the following equation.

$$\underset{\text{(Yellow)}}{\text{TBPB-H}^{+}} + \text{Protein} \longrightarrow \underset{\text{(Blue-green)}}{\text{TBPB}} + \text{Protein-H}^{+}$$

2.7.2 Creatinine determination

Many methods for creatinine detection have been reported such as enzymatic method [60, 61], chromatographic method [62, 63] and spectrometric method [50, 64]. The most common method is spectrophotometry based on the Jaffé reaction which is one of the optical method [12, 65]. Jaffé reaction is the reaction between creatinine and alkaline picrate forming orange-red color product [65, 66]. The mechanism of this reaction is illustrated in Figure 2.5. The product can be measured by a UV-Visible spectrophotometer at wavelengths in the range from 490 to 520 nm [62, 65]. Jaffé method is still popular due to its simplicity and low cost [12]. However,

this method is time-consuming and lack of specificity. Some substance such as uric acid, ascorbic acid, glucose, ketones and proteins can react with alkaline picrate resulting the positive error [12, 50, 67].

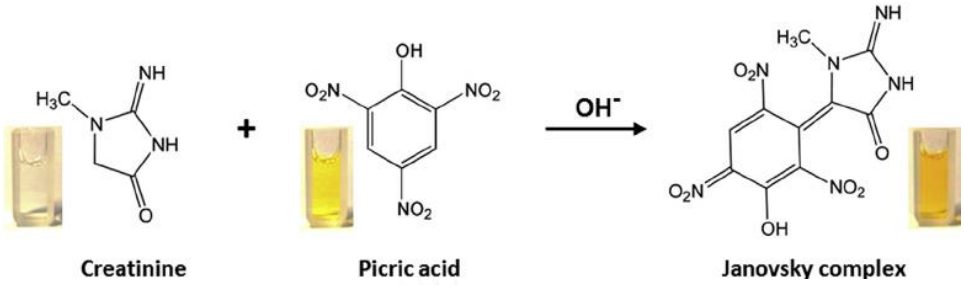

Figure 2.5 Reaction scheme of the Jaffé reaction [68].

2.8 Literature reviews

Due to a number of colorimetric methods for albumin determination, several dyes used to quantify albumin have been reported. Jung *et al.* [56] described the method for determination of microalbuminuria using bromophenol blue (BPB) as a dye reagant. The gel filtration on Sephadex G-50 was used to remove interfering components before mixing with BPB. The interfering protein, such as γ-globulin and Tamn-Horsfall protein was minimized by using short reaction time (20 s). The recovery of albumin added to sample was 98.7 ± 2.5% and the limit of detection of this method was 3 mg/L.

Wei *et al.* [69] studied the interaction between protein and bromophenol blue (BPB) in acidic solution by a spectrophotometric method. The main binding force found in this study was the electrostatic force. Under the acidic condition (pH 3.28) that pH was lower than the isoelectric point of BSA (pI=4.7), BSA in the protonated form, positively charged on molecules, could deprotonate BPB. Therefore, the color change was due to the transformation of dye species of free acidic form into bound basic form. Moreover, the researchers found that sodium chloride concentration and acidity of the solution had significant effect on the sensitivity of BPB protein assay.

Ratanawimarnwong *et al*. [57] investigated the simple automated simultaneous injection effective mixing flow analysis (SIEMA) system for the determination of urinary albumin using tetrabromophenol blue (TBPB) as the binding reagent. TBPB in the presence of Triton X-100 could be reacted with albumin at pH 3.2 resulting in a blue ion complex. The absorbance of complex was measured by a UV-Visible spectrophotometer at a wavelength of 625 nm. The range of linearity of this work was from 10 – 50 µg/mL with the detection limit of 0.53 µg/mL.

Many methods for the determination of creatinine have been reported. Mitchell developed a simple method for measuring creatinine by using cation-exchange resin to separate creatinine from interfering substances [50]. This research found that the recovery of creatinine was maximum when the adsorption buffer has a pH of 3.0. The adsorbed creatinine was eluted by using phosphate buffer solution (pH 12.2). After elution, the supernatant was detected by the Jaffé reactiom. The absorbance of mixture solution was monitored by a UV-Vis spectrophotometer at a wavelength of 505 nm after 30 minutes of reaction time.

Sittiwong and Unob [64] developed the method for detection of creatinine in human urine using gold nanoparticles (AuNPs) as colorimetric sensors. A sample preparation technique was used to eliminate the interfering effects of the urine matrix by extraction of creatinine on sulfonic acid functionalized silica gel. The decreasing of sample pH to acidic pH was performed to convert creatinine to its cation species. The positively charged amino groups on creatinine could react with the negatively charged citrate capped AuNPs. The range of linearity of this work was from 15 to 40 mg/L with a detection limit of 13.7 mg/L.

The paper-based assays for determining albumin and creatinine have been introduced in order to achieve a simple, low cost and portable method. Kaneko *et al.* developed a new visual method for albumin determination using Erythrosin B and a cellulose membrane film as the substrate [71]. Dye-protein solution containing poly(ethylene glycol) (PEG) was dropped on a membrane film, creating a red ring-shaped stain of the dye-bound protein. The addition of PEG provided a clear contrast between the reagent blank and the dye-bound human serum albumin (HSA) particle.

The studied range of this work was from 0.3 – 0.6 mg/L and the visual detection limit was 0.5 mg/L HSA.

Martinez *et al*. [72] presented the simultaneous detection of glucose and protein using patterned paper-based assay. The appropriate reagents were added to the test areas. The glucose assay was based on the enzymatic oxidation of iodide to iodine, while the protein assay was based on the color change of tetrabromophenol blue (TBPB) as it ionized and bound to proteins. This test strip was dipped in a solution having glucose and BSA, the fluid filled the entire pattern by capillary force. The studied range of this method for bovine serum albumin (BSA) was from 25.25 to 498.0 mg/L. This method could be detected protein as low as 49.8 mg/L by naked eyes.

Sittiwong and Unob [73] developed a new paper platform for the colorimetric detection of creatinine. The filter paper was coated with sulfonic acid, resulting in a negatively charged surface. The positively charged creatinine in acidic condition was extracted onto the filter paper via an electrostatic interaction. The absorbed creatinine was detected through Jaffé reaction by dropping alkaline picrate onto the filter paper, giving a yellow-orange colored complex. The color change on the paper could be observed by naked eyes and the color intensity obtained from Image J software was used for quantitative detection of creatinine. The linear range of this method was found in the range of 10 – 60 mg/L with the detection limit of 4.2 mg/L. The scheme of this method was shown in Figure 2.6.

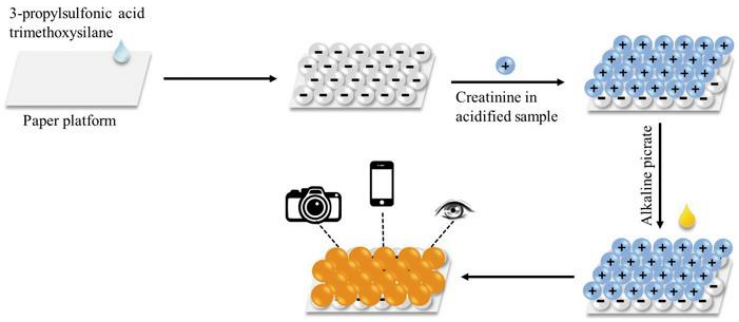

Figure 2.6 Paper-based colorimetric method for the determination of creatinine [73].

From previously several studies, the dye-binding methods were successfully used for albumin detection in many formats. Tetrabromophenol blue (TBPB) as a common dye in urine test strip was selected to use in this work due to its high specificity [58, 59]. Numbers of determining methods of creatinine using Jaffe reaction were combined sample preparation to eliminate the interfering substances. However, there is not many reports of the albumin and creatinine determination at low level using paper-based assay.

In this work, we proposed to determine albumin to creatinine ratio at low level by modifying membrane with polyelectrolyte multilayers (PEMs) for albumin and creatinine extraction. The layer-by-layer technique as an easy, efficient, and extremely versatile technique for surface modification was used to modify the cellulose acetate membranes, resulting in the specific charged surface. The detection of albumin using tetrabromophenol blue as a dye binding reagent, while creatinine using Jaffé reaction. The color change from these reactions could be observed by naked eyes.

CHAPTER III

EXPERIMENTAL

3.1 Materials and chemicals

3.1.1 Substrates

1. Cellulose acetate membrane (25 mm and 47 mm diameter and 0.2 μm pore size, Vertical Chromatography, Thailand)

2. Quartz slides (50 × 25 mm Electron Microscopy Sciences, Hatfield, PA)

3. Poly(dimethylsiloxane) (PDMS) (30 x10 mm Dow Corning, USA)

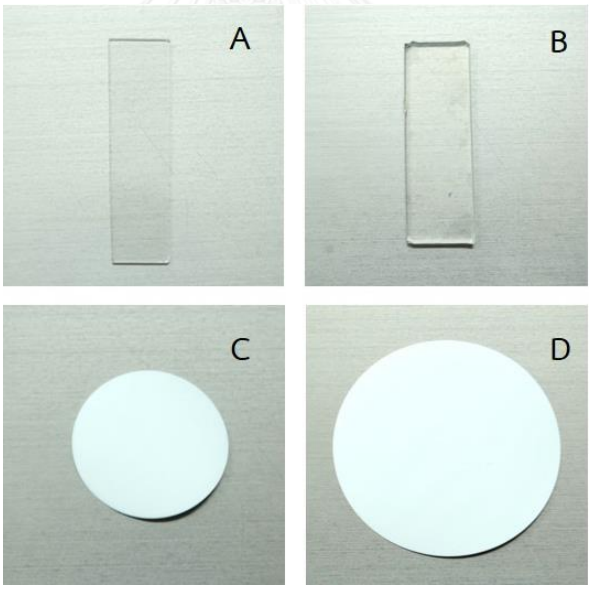

Figure 3.1 (A) Quartz slides (B) Poly(dimethylsiloxane) films (C) Cellulose acetate membrane (25 mm diameter) and (D) Cellulose acetate membrane (47 mm diameter).

3.1.2 Chemicals

Chemicals used in this research are listed in Table 3.1. All chemicals were analytical reagent grade (AR).

Table 3.1 List of chemical

Chemicals	Supplier
1. Poly(diallyldimethyl ammonium chloride) (PDADMAC) medium molecular weight, 20 wt% in water, average MW ~200,000-350,000	Sigma-Aldrich
2. Poly(sodium 4-styrenesulfonate) (PSS), average MW ~70,000	Sigma-Aldrich
3. Poly(4-styrene sulfonic acid-co-maleic acid, sodium salt) (PSSMA), average MW ~20,000	Sigma-Aldrich
4. Bovine serum albumin, M_w 66 kDa	Sigma-Aldrich
5. Globulins human blood, M_w 155-160 kDa	Sigma-Aldrich
6. Sodium chloride, A.R. Grade	Carlo Erba Reagent
7. Potassium dihydrogen phosphate, A.R. Grade	Carlo Erba Reagent
8. Di-sodium hydrogen phosphate anhydrous, A.R. Grade	Carlo Erba Reagent
9. Potassium chloride, A.R. Grade	Carlo Erba Reagent
10. Tri-sodium citrate dihydrate	Carlo Erba Reagent
11. Citric acid anhydrous	Carlo Erba Reagent
12. Hydrogen peroxide 30%, for synthesis	Merck
13. Sulfuric acid 95-99%, A.R. Grade	Merck
14. QuantiPro BCA assay kit	Sigma-Aldrich
15. Urine strip test (Microalbumin strip)	Cybow
16. Creatinine, A.R. Grade	Aldrich
17. Sodium hydroxide, A.R. Grade	Merck

Chemicals	Supplier
18. Picric acid, A.R. Grade	Sigma-Aldrich
19. Hydrochloric acid (37%w/w)	Merck
20. Urea	Sigma-Aldrich
21. Sodium bicarbonate	Carlo Erba Reagent
22. Calcium chloride	Merck
23. Magnesium sulfate	Sigma-Aldrich
24. Sodium sulfate	Sigma-Aldrich
25. Ammonium chloride	Sigma-Aldrich
26. DI and Mill-Q water	-

3.2 Instruments

The instruments used in this study are listed in Table 3.2.

Table 3.2 Apparatus list

Apparatus	Company, model
1. UV-visible spectrophotometer	Hewlett Packard 8453
2. Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR)	Nicolet 6700
3. pH meter	Precisa pH 900
4. Syringe pump	QIS, model NE1000
5. Syringe filter holder	Millipore
6. Syringe 50 mL	Nipro

3.3 Solutions preparation

3.3.1 10 mM phosphate buffer solutions at pH 5.5 and 7.0.

The 10 mM phosphate buffer solution at pH 5.5 was prepared by dissolving approximately 0.030 g of di-sodium hydrogen phosphate anhydrous (Na_2HPO_4) and 1.570 g of potassium dihydrogen (KH_2PO_4) in deionized water (DI water). The ionic strength was controlled by adding 8.00 ± 0.10 g sodium chloride and 0.20 ± 0.01 g potassium chloride into this solution. The pH 7.0 phosphate buffer solution was prepared by dissolving approximately 0.860 and 0.520 g of Na_2HPO_4 and KH_2PO_4, respectively in DI water. Before the final volume of 1.0 L was adjusted by using DI water, the pH of solution was adjusted to a desired pH value using either 0.1 M HCl or 0.1 M NaOH. The stock solution was diluted with DI water to achieve the desired final concentration of phosphate buffer solution.

3.3.2 100 mM hydrochloric-potassium chloride buffer at pH 2 and citric-citrate buffer solutions at pH 3.0 and 4.0

The 200 mM of hydrochloric and potassium chloride were prepared in DI water as stock solution. Then, 50 mL of KCl solution was mixed with 10.6 mL of HCl solution. Before the final volume of 200 mL was adjusted by DI water, the pH of solution was adjusted to pH 2.0 using either 0.1 M HCl or 0.1 M NaOH.

The 100 mM citric acid and tri-sodium citrate dihydrate were prepared in DI water as stock solutions. Then, 100 mM citric-citrate buffer solutions at pH 3.0 and 4.0 were prepared by mixing 100 mM citric acid and tri-sodium citrate dehydrate with different volume ratios (Table 3.3). The buffer solutions were adjusted to pH 3.0 and 4.0 by either 0.1 M HCl or 0.1 M NaOH.

Table 3.3 Volume of citric acid and tri-sodium citrate dihydrate for preparing buffer solutions

pH	Volume (mL)	
	100 mM Citric acid	100 mM Tri-sodium citrate dihydrate
3.0	82	18
4.0	59	41

3.3.3 Bovine serum albumin stock solution

A 1.00×10^3 mg/L bovine serum albumin (BSA) solution was prepared by dissolving approximately 0.10XX g of BSA in DI water. The final volume was adjusted to 100 mL using a volumetric flask. The working BSA solutions were obtained by diluting the stock solution with phosphate buffer at pH 7.

3.3.4 Dye binding reagent

For optimization of dye binding reagent, a 0.50 mM tetrabromophenol blue (TBPB) stock solution was prepared in 100 mL of DI water. A 2.0 % (w/v) Triton X-100 stock solution was prepared by dissolving approximately 2.0XX g of Triton X-100 in 100 mL of DI water.

3.3.5 Creatinine stock solution

A 1.00×10^4 mg/L creatinine standard stock solution was prepared by dissolving approximately 1.0XX g of creatinine power in 100 mL of DI water. The working standard solutions in the concentration range of 0 – 100 mg/L were obtained by diluting creatinine stock solution with 5 mM HCl solution.

3.3.6 Sodium hydroxide solution

Sodium hydroxide solution (NaOH) was used in the standard Jaffé method for determining creatinine. A 0.75 M sodium hydroxide solution (NaOH) was prepared by dissolving approximately 3.00 g of sodium hydroxide in 100 mL of DI water.

3.3.7 Picric acid solution

Picric acid solution was used in creatinine analysis by the standard Jaffé method. A picric acid stock solution was prepared by dissolving 1.50 g of picric acid in 100 mL of DI water. The saturated stock solution of picric acid was diluted with DI water to achieve a concentration of 0.04 M picric acid.

3.3.8 Synthetic urine

A synthetic urine solution was prepared in DI water according to the recipe provided by Brooks and Keevil [74]. The synthetic urine solution compositions are 2.0 mM citric acid, 25 mM sodium bicarbonate, 170 mM urea, 2.5 mM calcium chloride, 90 mM sodium chloride, 2.0 mM magnesium sulfate, 10 mM sodium sulfate, 7.0 mM potassium dihydrogen phosphate, 7.0 mM dipotassium hydrogen phosphate, and 25 mM ammonium chloride.

3.3.9 Polyelectrolyte solutions

100 mM of polyelectrolyte solutions (the stock solution)

Each 100 mM stock polyelectrolyte solution for multilayer thin film preparation was prepared by dissolving approximately 20.20 g of PDADMAC, 5.15 g of PSS and 8.60 g of PSSMA in DI water. The final volume of all polyelectrolyte solutions were adjusted to 250 mL using a volumetric flask.

10 mM of polyelectrolyte solutions in 1.0 M NaCl solution

The 10 mM polyelectrolyte in 1.0 M NaCl was prepared by pipetting 20 mL of a polyeletrolyte stock solution into a volumetric flask. NaCl (11.68 ± 0.01 g) was added in the solution. Then, the final volume of solution was adjusted to 200 mL using either DI water or an appropriated buffer solution.

3.4 Fabrication of PEMs

The substrates were coated with polyelectrolyte multilayer thin films by using the layer-by-layer electrostatic deposition technique. First, substrate was dipped into a polycationic solution (PDADMAC) for 5 minutes. After the first dipping, the substrate was rinsed 3 times with DI water or an appropriated buffer solution. After that, the substrate was dried using a hair dryer. Then, the substrate was dipped into a polyanionic solution for 5 minutes and was rinsed 3 times with DI water or an appropriated buffer solution. Hence, the substrate with the first positive layer was coated with a negative layer of the polyanions as the second layer. This process can be repeated many times to get the polyelectrolyte multilayers (PEMs) with the required thickness. For albumin determining, the number of PEMS layers on the cellulose acetate membrane (47 mm diameter) of both PDADMAC/PSS and PDADMAC/PSSMA are 13 layers, in which PDADMAC is the outermost layer. For creatinine determination, the number of PEMS layers on the cellulose acetate membrane (25 mm in diameter) of PDADMAC/PSS is 12 layers, in which PSS is the outermost layer.

3.5 Characterization of PEMs thin film

3.5.1 Ultraviolet/Visible spectrophotometer

To study the growth of PDADMAC/PSS or PDADMAC PSSMA film, quartz slide was selected as another studied material to characterize the signal from PSS or PSSMA using a UV-Vis spectrophotometer. The absorbance values at 227 nm which is

the characteristic UV absorption band of the styrene group of PSS or PSSMA was measured.

3.5.2 Attenuated Total Reflectance Fourier transform Infrared Spectrometer (ATR-FTIR)

The growth of PEMs on substrate was also monitored using ATR-FTIR. This method could be identified the functional groups of each polyelectrolyte. Both modified and non-modified surface were evaluated in triplicate and performed at three different positions per a substrate. OMNIC PROGRAM was used to measure the peak areas of each functional group deposited on substrates.

3.6 Albumin determination

The albumin determination composed of four steps including fabrication, filtration, desorption and detection step. The fabrication step using layer-by-layer technique was described in section 3.4. Next the filtration step was performed by putting the PEMs modified membrane into the filter holder and 30 mL of BSA solutions was filtered through this membrane filter using a syringe pump with flow rate at 1.0 mL/min. Experimental setup for the filtration is shown in Figure 3.2.

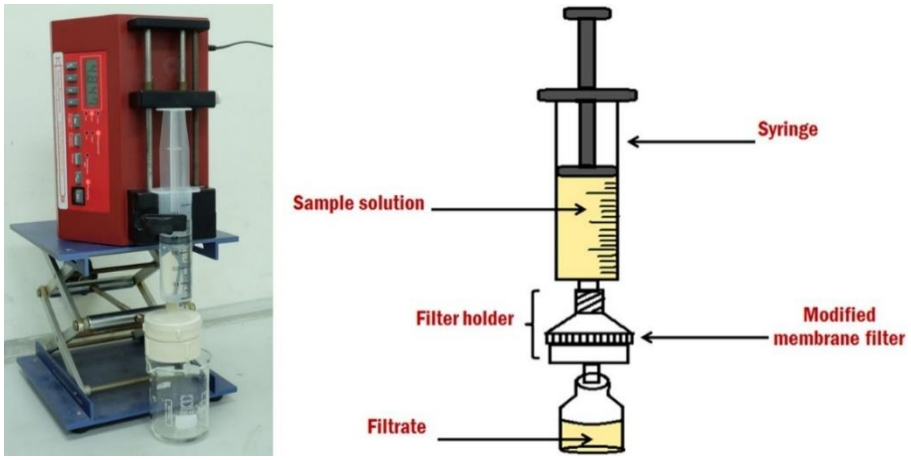

Figure 3.2 Scheme of protein adsorption on membrane filter process.

For desorption step, the adsorbed albumin on membrane was desorbed using desorption solvent. Finally, the amount of BSA was detected by using TBPB in Triton X-100 as a dye binding reagent. Two methods were developed to detect albumin including dipping method by dipping the modified membrane into the dye binding reagent and dropping method by dropping dye binding reagent into a solution having desorbed BSA. For dipping method, BSA was simultaneously desorbed and detected by using dye binding reagent. On the other hand, BSA was desorbed before testing with dye binding reagent in dropping method. The obtained product (TBPB – BSA complex) can be measured by a UV-Visible spectrophotometer at a wavelength of 625 nm.

3.6.1 Optimization of dye binding reagent

The influence of TBPB concentration was studied to obtain the optimum concentration of dye binding reagent reacted with albumin. Moreover, the concentration of Triton X-100 used to solubilize TBPB-BSA complex was also optimized.

3.6.1.1 Effect of TBPB concentration

The effect of TBPB concentration on the complex intensity was studied by varying the concentration from 0.01 to 0.10 mM with constant Triton X-100 and BSA concentration. TBPB solutions at different concentrations were prepared by diluting a 0.50 mM TBPB stock solution with 25 mM pH 3 citric-citrate buffer having 0.20 % (w/v) Triton X-100. Then, 0.120 mL of 1.00×10^3 mg/L BSA standard solutions were added to 3.00 mL of dye binding reagent to achieve the BSA concentration at 40 mg/L. The TBPB-BSA complexes were analyzed by measuring the absorbance intensity using a UV-Visible spectrophotometer at 625 nm.

3.6.1.2 Effect of Triton X-100 concentration

Triton X-100, a non-ionic surfactant, is often used to increase the solubility of the TBPB–BSA complex [57]. To study the effect of Triton X-100 concentration on the color intensity of the TBPB-BSA complexes, the amount of BSA (0 – 100 mg/L) were prepared in 3.00 mL of dye binding reagent by varying the concentration of Triton X-100 from 0 – 0.40 % (w/v) while keeping 0.03 mM TBPB constant. The linear relationship between the response and the amount of BSA (0 – 100 mg/L) were measured using a UV-Visible spectrophotometer at 625 nm.

3.6.2 Dipping method

After filtration process, the modified membrane was rinsed 3 times with DI water or an appropriated buffer solution and dipped in 3.0 mL of dye binding reagent for 10 minutes. In this study, we proposed that albumin was desorbed from modified membrane and reacted with dye binding reagent, TBPB. The absorption intensity of TBPB-BSA complexes was measured with a UV-Visible spectrophotometer at 625 nm. Due to modified membrane dipping into dye binding reagent, PEMs may be affected the colorimetric method. TBPB may be deprotonated by the charge of PEMs on membrane, resulting in the color change without albumin. Therefore, the effect of pH and concentration of buffer solution was evaluated.

The optimization of pH and buffer concentration on the color response of dye binding reagent

The pH of buffer solution used to prepare dye binding reagent was varied using the pH range of 2 – 4 because the reaction between TBPB and BSA was occurred in acidic condition. The unmodified modified and modified membranes with PDADMAC/PSS and PDADMAC/PSSMA were dipped into 3.0 mL of dye binding reagents for 10 minutes. Afterward, the color of dye bind reagent was observed and monitored by UV-Visible spectrophotometer at 625 nm.

The concentration of buffer was studied using the concentration range of 10 – 100 mM citric-citrate at optimum pH. The unmodified modified and modified membranes with PDADMAC/PSS and PDADMAC/PSSMA were dipped into 3.00 mL of optimized dye binding reagent. After dipping for 10 minutes, each solution was collected to measure absorbance intensity using a UV-Visible spectrophotometer at 625 nm.

3.6.3 Dropping method

In this method, the BSA was possessed positively charged and desorbed from PEMs layer before reacted with dye binding reagent. Desorption solvent was pH 3.0 citric-citrate buffer solution [75]. After the filtration process, the modified membrane was immersed in 2.50 mL of pH 3.0 citric-citrate buffer solution for 10 minutes to elute BSA from PEMs layer. Then, 0.10 mL of dye binding reagent obtained from the optimization of dye binding reagent in section 3.6.1 was dropped into 2.00 mL of releasing solution to form TBPB – BSA complexes.

3.6.3.1 The optimization of the concentration of citric-citrate buffer on the desorption process

To improve the efficiency of desorption process, the effect of citric-citrate buffer concentration for desorbing was evaluated at 10 mM and 25 mM. After fabrication process in section 3.4, 0.50 mL of 200 mg/L BSA was dropped onto the modified membrane to achieve 100 µg BSA and left to dry. Then, the desorption and detection as described earlier was performed. The amount of albumin was calculated from the calibration curves which BSA were prepared in either 10 mM or 25 mM citric-citrate buffer solutions.

3.6.4 The effect of urine matrix on albumin adsorption

Synthetic urine was prepared as described in section 3.3.8 to use as matrix of albumin solution. Sample dilution was used to convert BSA to negatively charge and decrease the effect of a urine matrix. Both 10 mg/L of BSA solutions were prepared in DI water and synthetic urine. After that, the BSA solutions were diluted 2, 3, 4-fold with 5 mM phosphate buffer solution at pH 7.0. Then, 30 mL of this solution was filter through the PDADMAC/PSS membrane. The adsorbed BSA was eluted from PEMs-layer and detected by dye binding reagent following the dropping method. The amount of BSA prepared in DI water was analyzed by using a UV-Vis spectrophotometer and compared to a solution of albumin in synthetic urine.

3.6.5 Quantitative measurement of albumin

The amount of BSA was quantified by using calibration curve. For dropping method, the BSA standard solutions were prepared in 2.00 mL of pH 3 10 mM citric-citrate buffer in the concentration range 0 – 100 mg/L and mixed with 0.10 mL of 0.90 mM TBPB in 6.0 % (w/v) Triton X-100. A calibration curve of BSA solutions was plotted between absorbance at 625 nm and concentrations of BSA. Then, the BSA concentration in dye binding reagent was determined from the calibration curve.

3.6.6 Method performance for albumin determination

The various concentrations of BSA were prepared in synthetic urine. Then, the solutions were diluted with appropriate dilution factor using pH 7 5 mM phosphate buffer solution. The PEMs-modified membranes were used to filter 30 mL of BSA solution. The flow rate was fixed at 1 mL/min. After filtration process, the adsorbed albumin was detected by using the optimized albumin detection method. The performance of the proposed method was evaluated under a selected condition.

3.6.6.1 The relationship between the BSA concentration and the absorbance intensity

To quantity the amount of BSA in urine sample, the relationship between BSA concentration and absorbance intensity after the extraction was investigated. BSA was prepared in synthetic urine with different concentrations and diluted with suitable dilution factor using 5 mM phosphate buffer solution at pH 7. Albumin was extracted and detected according to the protocol described previously. The absorbance of TBPB – BSA complexes at 625 nm were plotted against the concentration of albumin before filtration. The relationship between the initial BSA concentration and the absorbance intensity was investigated under the optimal condition.

3.6.6.2 Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD and LOQ were investigated by testing 10 replicates of the synthetic urine blank diluted with an appropriate dilution factor using the optimal condition found in this study. The LOD and LOQ were calculated from the signal of blank solution using UV-Vis spectrophotometer that is three and ten times the standard deviation of blank solution signal.

3.6.6.3 Accuracy and precision

The two real sample urines were used to evaluate the method performance. The 10 and 15 mg/L BSA standard solutions were spiked into urine samples. Before extraction, both spiked and non-spiked samples were diluted with the suitable dilution factor using 5 mM phosphate buffer pH 7. After that, BSA was filtered and detected by dye binding reagent. The amount of extracted BSA was found as described in section 3.6.5. Furthermore, %recovery and %RSD were evaluated from this data. %Recovery was calculated by the following formula.

$$\%\text{Recovery} = \frac{C_{spike} - C_{sample}}{C_{STD}} \times 100$$

C_{spike} : the concentration of the sample spike with standard solution

C_{sample} : the concentration of the sample without the spike

C_{STD} : the concentration of the standard solution

3.6.6.4 Repeatability

The repeatability of five runs of our developed method were tested using urine sample in the same-day precision.

3.7 Creatinine determination

The modified membranes with PSS as the outermost layer were dipped into creatinine standard solutions for 10 minutes. Then, the modified membrane was rinsed 3 times with de-ionized water (DI water). After creatinine extraction, 0.2 mL of alkaline picrate solution (1 mL of 0.04 M picric acid: 2 mL of 0.75 M NaOH) was dropped on the membrane to react with absorbed creatinine giving the orange color of creatinine-picrate complex. To obtain the value of color intensity, a photo of the modified membrane was taken using a digital camera under daylight and subjected to Image J program to measure the color intensity in gray scale mode. The proposed method was applied for creatinine determination in synthetic urine and human urine samples.

3.7.1 The study of the working range for creatinine determination

To determine the linear working range, the linear relationship between the creatinine concentration and the color intensity was evaluated in this work. Creatinine standard solutions of various concentrations were prepared in de-ionized water. Before extraction, the creatinine standard solutions were diluted with 1 mM HCl solution. The extraction process was performed by immersing the PDADMAC/ PSS

membrane into 2.00 mL of creatinine solution for 10 minutes. After that, the modified membrane was rinsed 3 times with de-ionize water and detected by dropping 0.2 mL of alkaline picrate solution. The change of color on membrane was observed by taking a photo and the color intensity was evaluated using Image J software.

The effect of reaction time on creatinine detection

The reaction time between adsorbed creatinine on the membrane and alkaline picrate solution on color development was studied in the time period of 2 – 10 minutes. The color intensity of creatinine complex on the modified membrane was evaluated using Image J software.

3.7.2 The determination of creatinine in synthetic urine

The solutions containing creatinine in the concentration range of 0 – 3000 mg/L were prepared in synthetic urine matrix. Before extraction, these solutions were diluted with 5 mM HCl solution to achieve concentrations within the studied range (0 – 100 mg/L) and to convert creatinine to its protonated form. The PDADMAC/PSS membranes were dipped into this creatinine solution for 10 minutes to extract creatinine. After that, Jaffé reaction was performed to analyze the extracted creatinine following previously mentioned procedure. The color intensity of creatinine complex on the modified membrane was monitored using Image J software. The parameters effecting creatinine extraction and detection including the effect of matrix and the effect of reaction time were studied.

3.7.2.1 The effect of urine matrix on creatinine extraction

To apply the proposed method in urine sample, the urine matrix on creatinine extraction was investigated. The modified membranes with PDADMAC/PSS were dipped into 2.0 mL of creatinine in synthetic urine solution for 10

minutes. After extraction, the membrane filter was washed with DI water before reacting with alkaline picrate solution. The color intensity of creatinine complexes obtained from Image J software were compared between creatinine in DI water and creatinine in synthetic urine under the same condition.

3.7.2.2 The effect of reaction time

The effect of reaction time between creatinine and alkaline picrate solution was investigated in the time period of 2 – 15 minutes. The modified membrane was immerged in 4.00 mL of creatinine in synthetic urine solution. After extraction process, 0.2 mL of alkaline picrate solution was dropped on the modified membrane having absorbed creatinine. The color intensity of creatinine complex was observed at different reaction times.

3.7.3 Method validation

The validation of the proposed method for creatinine determination in human urine sample was performed. The validation parameters including the working linear range, the limit of quantitation, accuracy, and precision of proposed method were evaluated. Moreover, the results obtained from proposed method were compared with the standard Jaffé method.

3.7.3.1 Calibration curve and linearity

The linear relationship between the color intensity and the concentration of creatinine were investigated in the concentration range of 0 and 100 mg/L under the optimal condition from section 3.7.2. Creatinine was extracted according to the protocol described in section 3.7.2. The intensity of color was plotted against the concentration of creatinine.

3.7.3.2 Limit of detection (LOD) and limit of quantitation (LOQ)

The blank synthetic urine solution without creatinine was extracted and analyzed by the proposed method in 10 replicates. The results were calculated from the signal that is three and ten times the standard deviation of blank solution signal.

3.7.3.3 Accuracy and precision in urine sample analysis

The accuracy of proposed method was evaluated by spiking creatinine standard solution into urine sample to achieve the spiked concentration of 20 and 40 mg/L in sample solution. After that, creatinine was extracted and detected according to the protocol described previously. The concentration of creatinine in non-spiked urine sample and spike urine sample were calculated by comparison with standard calibration curve. The percent recovery and percent relative standard deviation that represented the accuracy and precision of the proposed method were determined.

Moreover, the creatinine concentrations in urine sample obtained from the proposed method were compared with those from the standard Jaffé method. To analyze creatinine by standard Jaffé reaction, 1.0 mL of 0.75 M NaOH solution and 1.0 mL of 0.04 M picric acid solution were mixed into 3.0 mL of creatinine standard solution or urine sample diluted with HCl solution under stirring at room temperature for 30 minutes. After that, the absorbance intensity of creatinine complex was monitored using a UV-Vis spectrophotometer at a wavelength of 505 nm.

3.8 Application to human urine sample for albumin and creatinine determination

The proposed method was applied to determine albumin and creatinine in urine sample. For albumin determination, the urine sample was diluted with optimal dilution factor with pH 7 5 mM phosphate buffer solution before the filtration step. Then, 30 mL of BSA solution was pumped through the PEMs-modified membrane using

a syringe pump. The flow rate was fixed at 1.0 mL/min. After filtration, 2.5 mL of pH 3 citric-citrate buffer solution was used to elute BSA from PEMs layer. To detect albumin adsorbed on the modified membrane, the eluent was mixed with the optimized dye binding reagent using dropping method. The TBPB-BSA complexes was analyzed using a UV-Visible spectrophotometer at 625 nm. To calculate the concentration of albumin, the absorbance intensity obtained from urine sample was compared with the linear relationship between BSA concentration and the absorbance intensity. Moreover %recovery and %RSD were evaluated.

For creatinine determination, the freshly collected urine samples were diluted 30-fold with 5 mM HCl solution before the extraction process. Then, the modified membrane with PDADMAC/PSS was immersed into the sample solution. To detect creatinine adsorbed on the modified membrane, the Jaffé reaction was performed by dropping 0.2 mL of alkaline picrate solution onto the modified membrane. The color intensity of the modified membrane was obtained from Image J software after 10 minutes of reaction time. To calculate the concentration of creatinine, the obtained color intensity were compared with standard calibration curve from section 3.7.3.1. Moreover, percent recovery and the relative standard deviation of the results were evaluated.

CHAPTER IV
RESULTS AND DISCUSSION

In this work, both albumin and creatinine were extracted via electrostatic interactions by using modifying surface of cellulose acetate membrane (CA) with PEMs and analyzed by employing a UV-Vis spectrophotometer and visual detection. The membrane surface modified with PDADMAC as the outermost layer was used for albumin extraction, whereas the modified membrane with PSS was used for creatinine extraction. In detection process, tetrabromophenol blue (TBPB) in Triton-X was used as a dye binding reagent to detect the amount of albumin and the Jaffé method was used to detect creatinine. Finally, the analytical performance of PEMs modified membranes on the detection of albumin and creatinine was evaluated and validated.

4.1 Characterization of PEMs thin film

The fabrication of PEMs on CA membrane was characterized using ATR-FTIR. Another studied material used to investigate the PEMs fabrication was a quartz slide because it is easy to follow the growth of layer by layer film and easy to fabricate PEMs on membrane resulting in the shorten process. The multilayer build-up on quartz slide was monitored using UV-Vis spectrophotometer.

4.1.1 The fabrication of PEMs on a quartz slide

From UV-Vis spectrophotometry, the spectra are shown only for PEMs with the even layers having PSS or PSSMA as the outermost layers, while PDADMAC showed no absorption band in this region. The fabricating pH of weak polyelectrolyte, PSSMA, was fixed at 5.5 because this pH is higher than the $p\mathrm{Ka}_1$ of PSSMA ($p\mathrm{Ka}_1$= 2.7 $p\mathrm{Ka}_2$= 8.3) [76]. Therefore, the charges of PSSMA are negative. The absorbance

spectrum of the styrene group of PSS and PSSMA is approximately 227 nm [77]. The results show that with the increase in the number of deposition layers, the absorbance peaks at 227 nm of PSS and PSSMA also increase as shown in Figure 4.1 and 4.2, respectively. Furthermore, the first eight layers of PDADMAC/PSS and PDADMAC/PSSMA films shows the absorption spectra slowly increased, and then the absorbance dramatically increased. It indicates the assembly of both PEMs types on quartz slide.

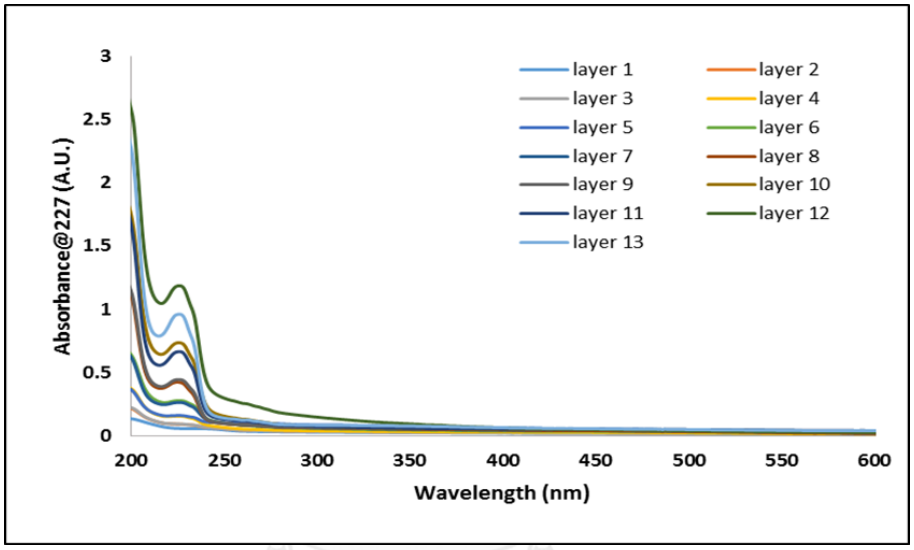

Figure 4.1 The multilayer thin film growth of PDADMAC/PSS on a quartz substrate.

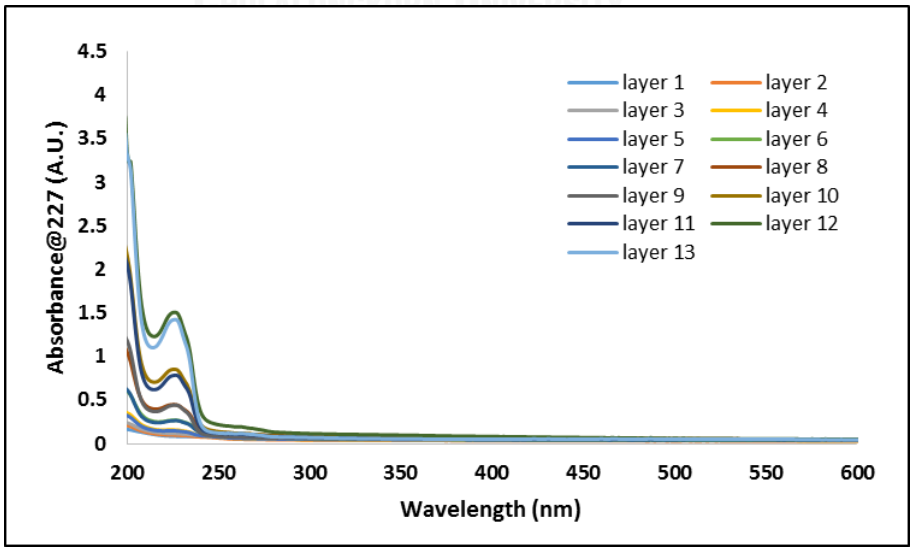

Figure 4.2 The multilayer thin film growth of PDADMAC/PSSMA on a quartz substrate.

4.1.2 The fabrication of PEMs on CA membrane

To confirm the growth of PEMs on CA membrane, the unmodified CA membrane and the modified membrane with PDADMAC/PSS or PDADMAC/PSSMA were investigated using ATR-FTIR. Figure 4.3 shows the ATR-FTIR spectrum of unmodified CA membrane. The C–H stretching vibrations for methylene groups occur between 2,800 and 3,000 cm^{-1} and symmetric and antisymmetric bending of methylene groups occur at 1,370 cm^{-1} and 1,435 cm^{-1}. A strong stretching peak at 1,746 cm^{-1} is attributed to the vibration of carbonyl in acetyl groups on the CA chain. The band at 1,234 cm^{-1} and 1,049 cm^{-1} are characteristic of asymmetric stretching of a carboxylate group and asymmetric C–O–C bond stretching from the pyranose ring [78], respectively.

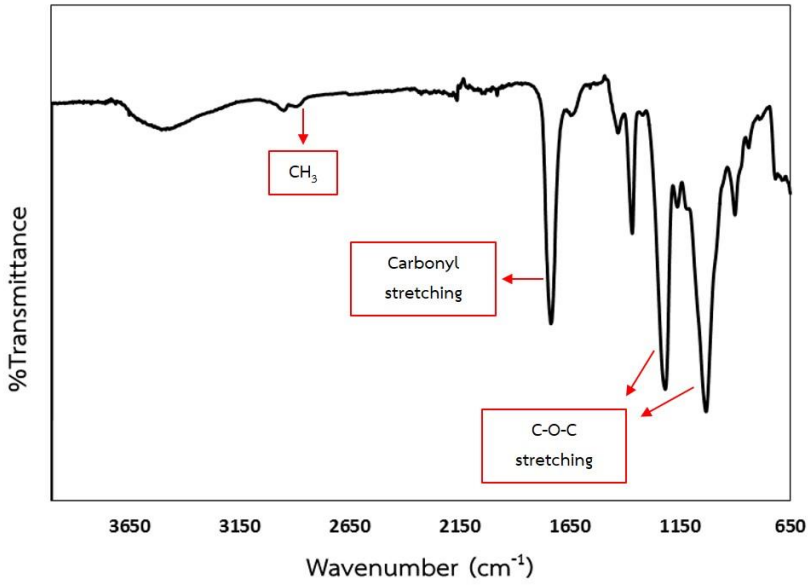

Figure 4.3 ATR-FTIR spectra of unmodified CA membranes.

The PDADMAC/PSS and PDADMAC/PSSMA multilayer thin films fabricated on CA membranes were characterized by ATR-FTIR. However, the spectrums of PSS and PSSMA cannot be investigated by ATR-FTIR due to the overlay of the

spectrum band at ~ 1000 - 1100 cm^{-1} between CA membrane and PSS [62]. Therefore, ATR-FTIR could be monitored only PDADMAC as the outermost layer. The presence of PDADMAC as the outermost layers (13 layers) on CA membrane as shown in Figure 4.4 and 4.5 is clearly demonstrated by the following characteristic peaks: the stretching of C–N group at ~1125 cm^{-1}, the stretching of NR_4 at ~1640 cm^{-1} and the broad peaks of O-H and N-H appeared at the position of ~3300 cm^{-1}. Moreover, the peak height of C-N and NR_4 increased with the number of the coating layers. From the results obtained, it can be indicated the growth of PEMs on the membrane surface.

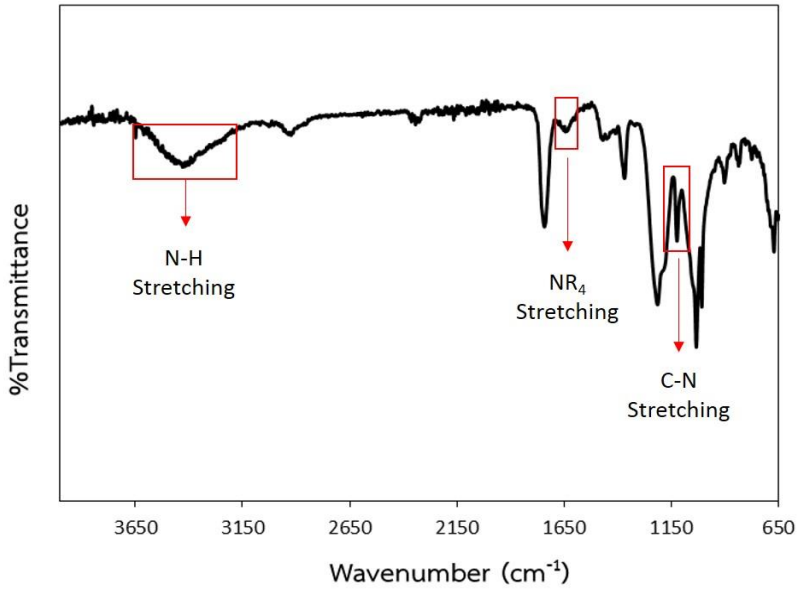

Figure 4.4 ATR-FTIR spectra of modified CA membranes with PDADMAC/PSS.

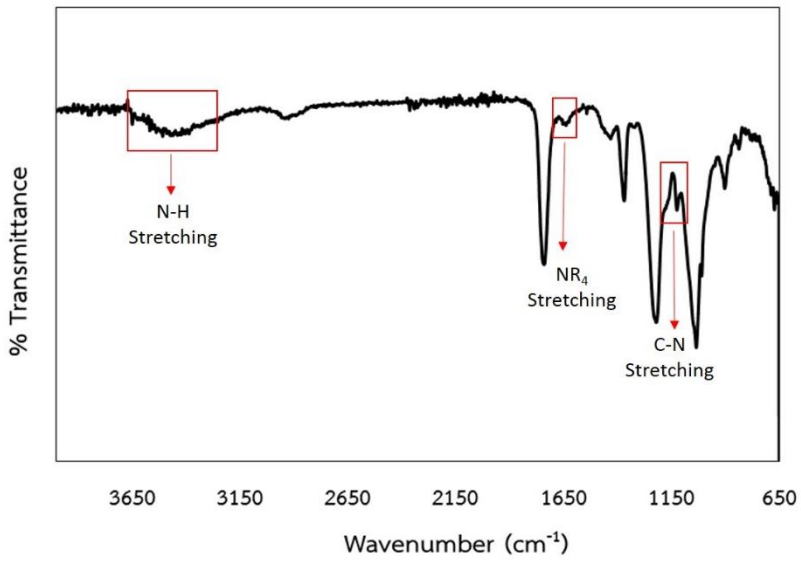

Figure 4.5 ATR-FTIR spectra of modified CA membranes with PDADMAC/PSSMA.

4.2 Albumin determination

The bovine serum albumin (BSA) solutions at pH above the isoelectric point (pI) of BSA (pI = 4.7) was filtered through the PDADMAC modified membrane possessing the positive charged surface resulting in the adsorption of BSA on the modified membrane via electrostatic interaction. After filtration process, the adsorbed BSA was released and detected by using TBPB dye binding reagent. There are two detection methods investigated in this study. First, the dipping method, the modified membranes were immersed into TBPB solution after filtration process directly. In this method the adsorbed BSA will be eluted and reacted with TBPB in one step. The second method is that the adsorbed BSA will be eluted first with acidic buffer solution and detected using TBPB afterward. The absorbance intensity of TBPB-BSA complex was measured by using a UV-Visible spectrophotometer at 625 nm. Moreover, the color of product can be observed by naked eyes.

4.2.1 Optimization of dye binding reagent on albumin detection

To obtain the suitable condition for dye binding reagent, the influence of TBPB concentration and triton X-100 concentration were investigated.

4.2.1.1 Effect of TBPB concentration

The TBPB concentration was varied from 0.01 – 0.10 mM while keeping 0.2 % (w/v) Triton X-100 and 40 mg/L BSA constant. From Figure 4.6, the result indicated that the absorbance intensity of TBPB – BSA complex increased with the TBPB concentration, but the response at a higher concentration slightly increased. It may be because the reaction between TBPB and BSA reach to equilibrium. At the 0.03 mM TBPB, the color was clearly observed by the naked eyes and it is enough to react with BSA. Therefore, the concentration at 0.03 mM TBPB was chosen in this work.

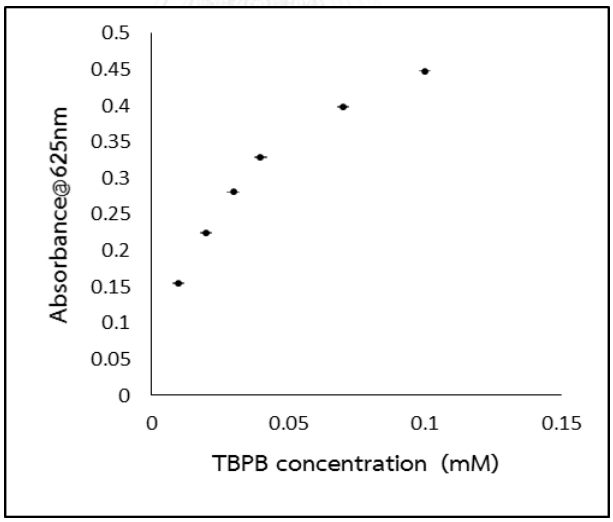

Figure 4.6 The relationship between absorbance intensity of TBPB – BSA complex at 625 nm and TBPB concentration.

4.2.1.2 Effect of Triton X-100 concentration

Because Triton X-100 is a typical nonionic surfactant that is used to dissolve the TBPB-BSA complexes, the suitable concentration had to be studied.

The Triton X-100 concentration ranging from 0 – 0.40 % (w/v) was mixed into the BSA concentration range of 0 – 100 mg/L.

The solubility of the TBPB-BSA complexes was assessed in the term of linearity of calibration curves (R^2). The results showed that the calibration curve of a system with no Triton X-100 (0 % (w/v) Triton X-100) delivered a poorest linearity because TBPB-BSA complexes were insoluble. With increasing Triton X-100 concentration from 0.10 – 0.40 % (w/v) in the system, linearity of calibration curves (R^2) also increases as shown in Table 4.1. Therefore, the increasing concentration of Triton X-100 can enhance the complex solubility. However, the sensitivity of this study obtained from slope of calibration curve was found to be smaller with increasing Triton X-100 concentrations. It may be because a higher Triton X-100 concentration can interfere with the ability of dye to bind to the albumin. To obtain linearity of calibration curves and high sensitivity, the concentration of 0.20 % (w/v) Triton X-100 was used in this work.

Table 4.1 Effects of concentration of Triton X-100 on the albumin detection.

Concentration of Triton X-100 (% w/v)	Absorbance at 625 nm[a] (AU)	Sensitivity[b]	R^2
0.00	0.4436	0.0065	0.9659
0.10	0.3587	0.0056	0.9790
0.20	0.2987	0.0050	0.9865
0.30	0.2605	0.0043	0.9898
0.40	0.2345	0.0039	0.9925

a. The absorbance intensity measured at 40 mg/L BSA.

b. The slope of the calibration curve in the range of 0-100 mg/L BSA.

4.2.2 Dipping method

The adsorbed BSA from the filtration process was simultaneously desorbed and detected by dipping the modified membrane into 3 mL of dye binding reagent. Normally, the color of TBPB in acidic environment without protein appears to be yellow but in the presence of albumin, the TBPB will turn to blue-green. The obtained color of TBPB-BSA complexes was monitored by using UV-Vis spectrophotometer. According to the dipping of modified membrane into dye binding reagent, the polyelectrolyte may affect the color of dye binding reagent. The dye binding reagent may be deprotonated by the charge of polyelectrolyte. The effect of pH and concentration of buffer solution was investigated in this study.

4.2.2.1 The optimization of pH and concentration of buffer solution on the color response of dye binding reagent

The reaction between TBPB and BSA occurred in acidic environment, which the color of TBPB without BSA appears yellow. Therefore, the pH of buffer solution of dye binding reagent is crucial. The pH of buffer solution was optimized by dipping the unmodified membrane (bare membrane) and modified membranes with PDADMAC/PSS and PDADMAC/PSSMA into 3.00 mL of dye binding reagents prepared in 25 mM buffer having pH varying from 2 to 4.

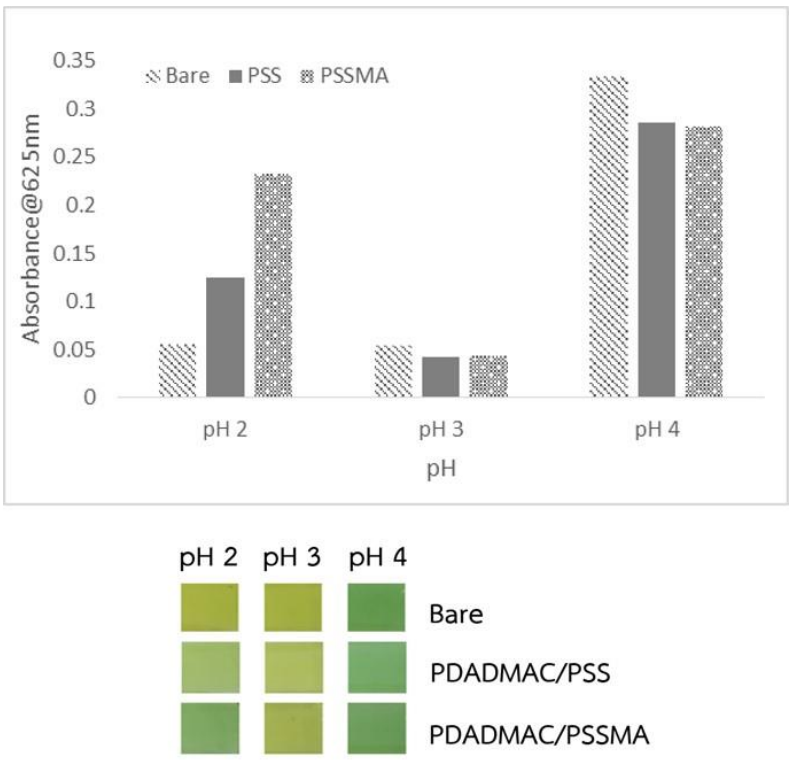

Figure 4.7 The absorbance intensity and color observed of dye binding reagent having different pH after membrane dipping.

This study proposed to obtain the optimum pH that gave the lowest absorbance signal at 625 nm and the yellow color of dye solution without the presence of BSA. The results in Figure 4.7 showed that at pH 2 HCl-KCl buffer, the absorbance signals of the modified membranes with PDADMAC/PSS and PDADMAC/PSSMA are higher than the unmodified membrane due to the low capacity of hydrochloric acid-potassium chloride buffer. At pH 3 citric-citrate buffer, the colors of dye solution of the modified membranes with PDADMAC/PSS and PDADMAC/PSSMA appear yellow as same as the bare membrane. At pH 4 citric-citrate buffer for all cases, the absorbance signals are higher than pH 2 and 3 and the color of dye solutions appears green without the presence of albumin due to the proton dissociation of TBPB. Therefore, pH 3 was selected as the suitable pH for TBPB-BSA reaction.

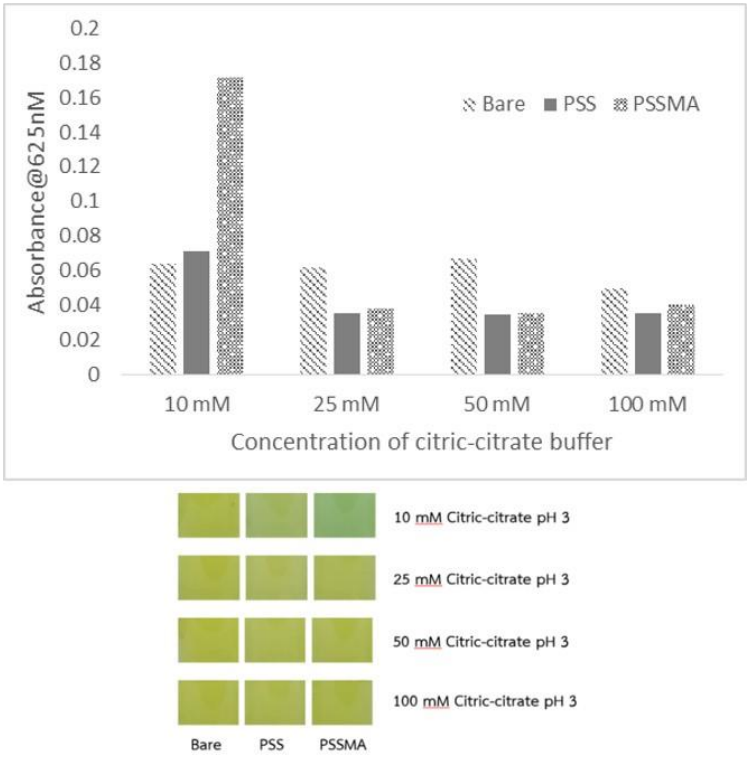

Figure 4.8 The absorbance intensity and observed color of dye binding reagent having different buffer concentrations after membrane dipping.

Based on the previous result, the concentration of pH 3 citric-citrate buffer was optimized. The results showed that at 10 mM citric-citrate buffer, the absorbance signal of the unmodified and modified membrane were higher than other concentrations, especially PDADMAC/PSSMA. It may be because TBPB is deprotonated by PDADMAC/PSSMA. The increasing of citric-citrate buffer concentration from 25 – 100 mM gave the lower absorbance signals and yellow color of dye solutions. Therefore, a concentration of 25 mM citric-citrate buffer was selected to control the lowest absorbance of modified membrane with PDADMAC/PSSMA.

However, the dipping method using the optimum composition of dye binding reagent, 0.03 mM TBPB, 0.20 % (w/v) Triton X-100 and 25 mM citric-citrate pH 3, has delivered the poor reproducibility with %RSD of 14.8, which is not accepted for AOAC (%RSD < 11). This might be the result of the reabsorbing the BSA-complex back on the surface.

4.2.3 Dropping method

As described earlier that the second detection method was composed of two steps: eluting BSA from PEMs and testing with dye binding reagent. The pH 3 citric-citrate buffer solution was selected as a desorption agent. At this pH which is lower than the *p*I of the BSA (*p*I = 4.7), the BSA possessed positively charged. Thus, BSA will be desorbed from PEMs layer by electrostatic repulsion between positively charged BSA and PDADMAC film. The dye binding reagent, 0.90 mM TBPB in 6.0 % (w/v) Triton X-100 calculated from the optimized composition of dye binding reagent in section 4.2.1 was prepared in pH 3 citric-citrate buffer solution. To achieve the higher capability for BSA desorption, the concentration of pH 3 citric-citrate buffer solution was optimized.

4.2.3.1 The optimization of the concentration of citric-citrate buffer on the desorption process

The effect of 10 and 25 mM pH 3 citric-citrate buffer solution on desorption process was studied. The results show that the amount of BSA released from PEMs layer by using 10 and 25 mM pH 3 citric-citrate buffer were 62.98 ± 3.01 and 40.54 ± 1.44 µg, respectively. The concentration of citric-citrate buffer at 10 mM showed higher capability for BSA elution comparing to 25 mM. This might be due to the lower competition between ions. Therefore, the 10 mM citric-citrate buffer solution was chosen as a suitable desorption solvent.

4.2.4 The effect of urine matrix on albumin adsorption

The urine matrix may interfere the albumin adsorption in the filtration process. Synthetic urine was used as matrix of albumin solution because its composition closes to human urine. The 10 mg/L of BSA solution was prepared in either DI water or synthetic urine. Then, the solutions were diluted with different

dilution factor (2 to 4-fold) using pH 7 5 mM phosphate buffer to covert BSA into negative charge. The amount of BSA was evaluated and compared between BSA in DI water and BSA in synthetic urine.

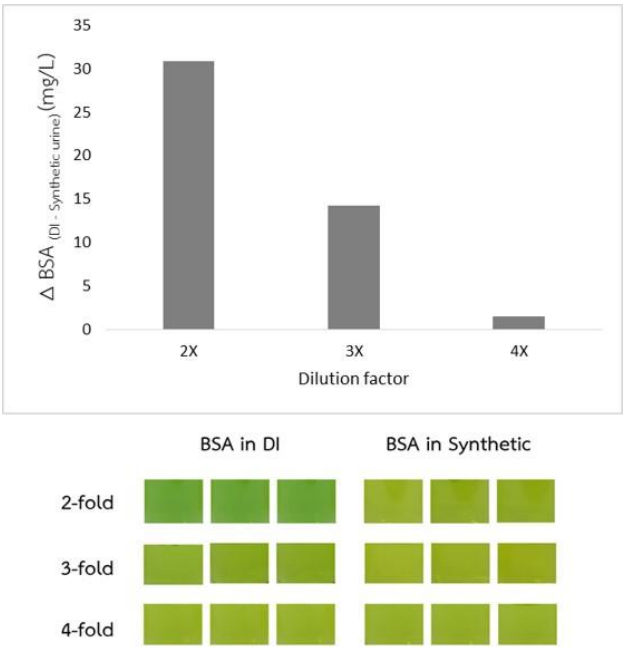

Figure 4.9 The different absorbance intensity between BSA in DI water and BSA in synthetic urine and observed color with different dilution factor.

The different between the amount of BSA in DI water and in synthetic urine after extraction was found to be larger for the BSA solutions with 2 and 3-fold dilution (Figure 4.9) comparing to the solutions with 4 times dilution. Moreover, from based on the color chart of solution, the amount of BSA in DI water was greater than BSA in synthetic urine. This might be because the higher interfering substances such as NaCl and urea in urine can interfere the adsorption of BSA on modified membrane. Therefore, a 4-fold dilution of synthetic urine was used in this work.

4.2.5 Quantitative measurement of albumin

The quantitation of albumin in the dipping method was not studied herein due to the poor reproducibility. For the dropping method, the calibration curve for BSA quantitation was construction by preparing the BSA standard solutions in 10 mM pH 3 citric-citrate buffer (desorption solvent). As shown in Figure 4.10, the absorbance of TBPB – BSA complexes at 625 nm (A_{625}) were potted versus the concentration of BSA. The graph is illustrated a good linear relationship in the range of 0 – 100 mg/L with the linear regression equation of y = 0.0061X + 0.0624 and correlation (R^2 value) of 0.9928. Moreover, a change in TBPB – BSA solution could also observed by naked eyes, as shown below. Therefore, this calibration curve was used for quantitation of albumin.

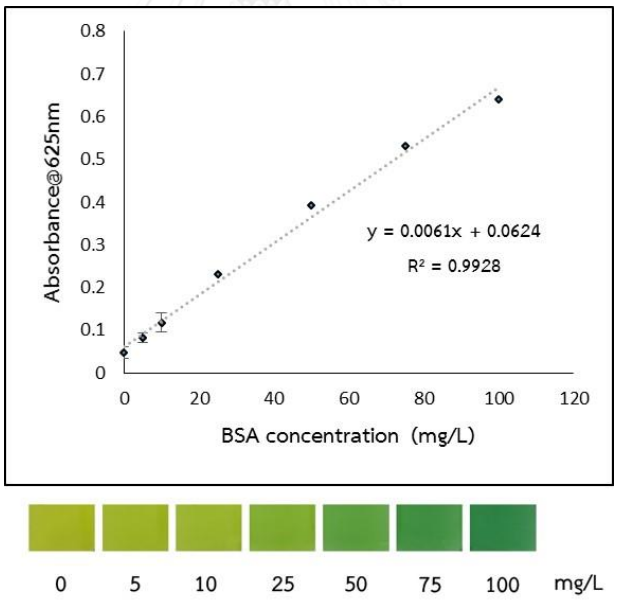

Figure 4.10 The calibration curve and color chart for albumin determination in dropping method.

In this work, BSA prepared in synthetic urine ranged from 10 – 30 mg/L was diluted 4-fold with 5 mM phosphate buffer pH 7.0. BSA was extracted onto the modified membrane by filtering and detected with dye binding reagent. After

detection, the amount of BSA was calculated from the calibration curve as mention above. The results in Table 4.2 showed the amount of BSA after extraction process is slightly increased and the observed color of TBPB-BSA solution is clear, indicating the interfering substance may be reduced.

Table 4.2 The amount of BSA in synthetic urine determined by our method.

Amount of BSA before extraction (mg/L)	Amount of BSA after extraction by the proposed method (mg/L)	Observed color by naked eyes
2.50	9.56 ± 0.05	
3.75	15.41 ± 0.72	
5.00	23.15 ± 0.48	
6.25	30.54 ± 0.41	
7.50	36.13 ± 1.32	

Furthermore, BSA solutions that were not extracted by the proposed method was investigated. The BSA solutions were prepared in synthetic urine in the concentration range of 10 – 30 mg/L, and then the pH of solution was adjusted to pH 3 using 1.0 M HCl solution. After that, 2.0 mL of the solution was mixed with 0.1 mL of dye binding reagent. The results were found that the observed color of TBPB – BSA solution without extraction process could not be detected due to the effect of urine matrix as illustrated in Table 4.3. The amount of of BSA without extraction was compared with BSA after extraction by the proposed method. The results confirmed that our developed method could be extracted BSA from urine matrix.

Table 4.3 The amount of BSA in synthetic urine determined by dropping method without the extraction process.

Amount of initial BSA in synthetic urine (mg/L)	Amount of BSA without extraction by the proposed method (mg/L)	Observed color by naked eyes
10	8.09 ± 0.59	
15	8.35 ± 0.46	
20	10.28 ± 0.79	
25	10.95 ± 0.39	
30	14.09 ± 0.68	

4.3 Method performance for albumin determination

In this section, the proposed method was validate. The 10 – 30 mg/L BSA standard solutions were prepared in synthetic urine and diluted 4-fold with 5 mM phosphate buffer pH 7. The 30 mL of diluted sample was filtered using the PDADMAC/PSS modified membrane with the constant flow rate (1 mL/min). The modified membrane filter was immersed in 2.5 mL of 10 mM pH 3 citric-citrate buffer solution for 10 minutes. This solution was mixed with 0.1 mL of 0.9 mM TBPB in 6.0 % (w/v) Triton X-100 and measured the absorbance of TBPB-BSA complexes by using a UV-Visible spectrophotometer at a wavelength of 625 nm. The relationship between the BSA concentration and the absorbance intensity, accuracy and precision were evaluated for the method performance.

4.3.1 The relationship between the BSA concentration and the absorbance intensity

To calculate the amount of BSA in human urine, the relationship between the BSA concentration and the absorbance intensity was studied at the

concentration in the range of 2.5 – 7.5 mg/L under the optimal condition. The working linear range of proposed method was performed by plotting between the absorbance of TBPB – BSA complexes at 625 nm (A_{625}) after extraction versus the concentration of BSA before extraction.

From the results, the graph in Figure 4.11 illustrated a good linear relationship in the range of 2.5 – 7.5 mg/L with the linear regression equation of y = 0.0327X + 0.0404 and correlation (R^2 value) of 0.9965. Moreover, the color of TBPB – BSA solution could be observed by naked eyes, as shown below. The presence of albumin in urine could change the color from yellow to green. However, it's difficult to distinguish the color of TBPB – BSA solution between the concentration 5.00 and 6.25 mg/L by naked eyes.

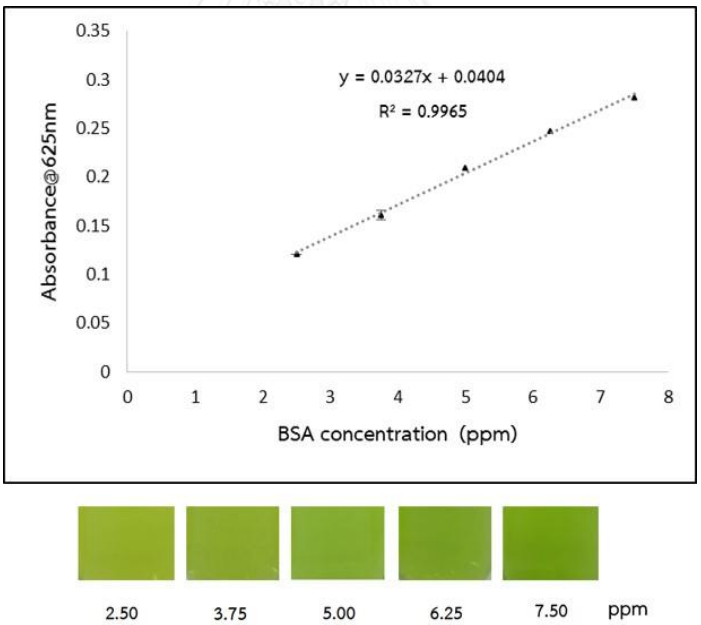

Figure 4.11 The linear relationship between the BSA concentration and the absorbance intensity after extraction and color chart for albumin determination (BSA solution were 4-fold diluted).

4.3.2 Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD and LOQ were studied by filtering the diluted 4-fold synthetic urine without the albumin in 10 replicates. The LOD and LOQ values of proposed method obtained from a UV-Vis spectrophotometer were found to be 0.47 and 0.68 mg/L, respectively.

4.3.3 Accuracy and precision

The spike method was used to examine the accuracy of the propose method. Fresh urine samples were collected and diluted 4-fold with 5 mM phosphate buffer solution at pH 7. Then, the standard solution with a concentration of 2.50 and 3.75 mg/L was spiked into the urine sample. Then, the albumin determination was performed following the dropping method. The accuracy and precision was assessed in the terms of %recovery and %RSD, respectively.

The recovery of albumin from spiked sample was found in the range of 81.2 – 106.9%. The relative standard deviation (%RSD) from the proposed method were obtained in the range of 0.81 – 3.2%. The %RSD and %recovery are accepted for Association of Official Chemists, AOAC (%RSD < 11 and %recovery 80-110), respectively as shown in Table 4.4.

Table 4.4 The recovery of albumin in urine sample, as determined by the proposed colorimetric method (N = 3).

Sample*	Amount added (mg/L)	Proposed method			Paper color	Naked eyes detection
		Found (mg/L)	Recovery (%)	RSD (%)		
Urine 1	0	2.35 ± 0.07	-	-		2.50-3.75
	2.50	4.67 ± 0.04	92.9 ± 0.8	0.8		5.00
	3.75	6.36 ± 0.20	106.9 ± 3.2	3.2		6.25-7.50
Urine 2	0	1.69 ± 0.03	-	-		2.50
	2.50	3.72 ± 0.06	81.2 ± 1.6	1.6		3.75
	3.75	6.30 ± 0.07	103.4 ± 1.0	1.0		6.25-7.50

***Samples were 4-fold diluted

The amount of BSA evaluated by naked eyes was in agreement with the amount of BSA determined by a UV-Vis spectrophotometer. However, it's difficult to distinguish the color of TBPB – BSA solution between the concentration 3.75 and 5.00 mg/L by naked eyes.

4.3.4 Repeatability

The repeatability and %RSD study of our developed method were investigated (n = 5). The results found good repeatability from our developed method with %RSD of 1.86%, which is accepted for AOAC as illustrated in Table 4.5.

Table 4.5 The repeatability of our developed method.

Number of PDADMAC/PSS modified membrane filter	Amount of BSA detected by our developed method (mg/L)
1	6.65
2	6.85
3	6.96
4	6.76
5	6.93
Average amount of BSA detected by our developed method (mg/L)	6.83 ± 0.13 (%RSD = 1.86%)

4.4 Creatinine determination

This work presents a method to extract and determine creatinine in sample solution using the modified membrane filter. The CA membrane (25 mm in diameter) was modified with PDADMAC/PSS, on which PSS is the outermost layer. Before extraction by the membrane, creatinine solution was diluted with HCl solution to convert creatinine to its protonated form [73]. Consequently, creatinine can be extracted onto the membrane via electrostatic interaction between negatively charged PSS and positively charged creatinine. After creatinine extraction, Jaffé reaction was performed to determine the amount of creatinine by dropping 0.2 mL of alkaline picrate solution onto the membrane. The presence of creatinine resulted in a change of the membrane filter color from yellow to dark yellow or brown that could be observed by naked eyes. To quantify the content of creatinine, a photo of the membrane filter was taken and subjected to Image J software to measure the color

intensity in gray scale mode. Moreover, this method was applied for creatinine detection in human urine sample.

4.4.1 The determination of creatinine in standard solution

This study was performed to find the linear relationship between the creatinine concentration and the color intensity in gray scale mode or mean gray values observed by the Image-J software. When creatinine concentration increased, the color on the membrane changed from yellow to dark yellow and brown and the color intensity in gray scale deceased. The color change of membranes related to creatinine concentrations was clearly observed by naked eyes as shown in Figure 4.12. The linear relationship was obtained in the concentration range from 0 – 100 mg/L due to the limit of the membrane capacity.

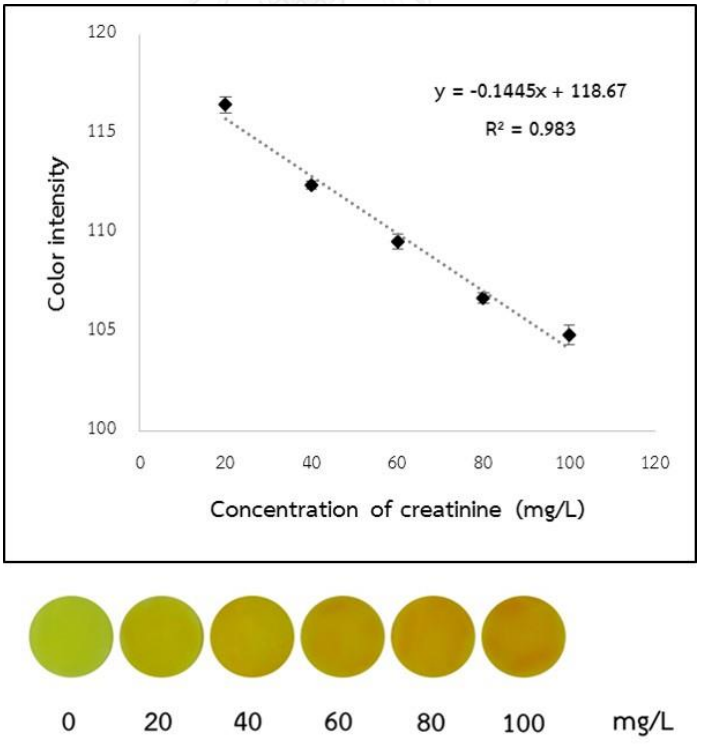

Figure 4.12 The color intensity and chart of membrane color observed in the detection of creatinine in standard solutions (reaction time = 5 minutes).

The effect of reaction time on creatinine detection

After solutions containing creatinine in the concentration range of 0 – 100 mg/L were extracted by the modified membranes, the content of creatinine on the membrane was detected with standard Jaffé reaction. The effect of reaction time in the time period of 2 – 10 minutes was evaluated by observing the membrane color. The results in Figure 4.13 show that the color intensity in gray scale slightly deceased when reaction time increased. The fully color development on modified membrane could be observed within 5 minutes at room temperature. A longer reaction time did not yield significant color change or improve the sensitivity of the detection, indicating that the reaction between creatinine and alkaline picrate was completed. To achieve short analysis time, the reaction time of 5 minutes was selected.

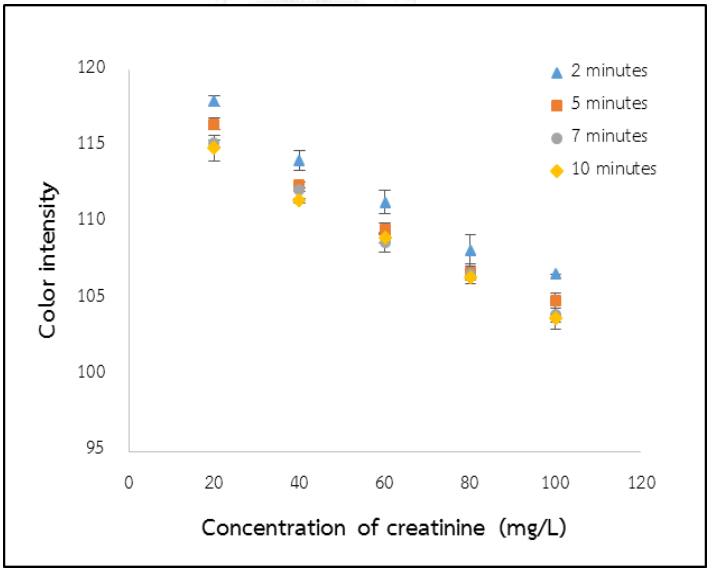

Figure 4.13 The effect of reaction time on the color intensity of creatinine complex on membrane after extraction of creatinine in standard solution in the concentration range of 0 – 100 mg/L.

4.4.2 The determination of creatinine in synthetic urine

4.4.2.1 The effect of urine matrix on creatinine extraction

To evaluate the applicability of the method in the detection of creatinine in human urine sample, the method was first applied to determine creatinine in synthetic urine. The synthetic urine has the same composition as human urine such as urea, NaCl, and Na_2SO_4. The working linear range (0 – 100 mg/L) obtained from the determination of creatinine in standard solutions was adopted. In creatinine standard solutions, 1 mM HCl solution was used to convert creatinine to its cationic form. However, due to a high ionic strength of synthetic urine and the presence of different compounds, a higher concentration of HCl was required to maintain the acidic condition for creatinine extraction. In this work, we proposed to study creatinine in the concentration range of 0 – 3000 mg/L which is the normal level of creatinine in human urine sample. The linear range obtained from section 4.4.1 was found in a range form 0 – 100 mg/L. Thus, the creatinine in synthetic urine solutions was diluted 30-fold with 5 mM HCl to minimize the effect of matrix and achieve the linear range of creatinine determination.

In extraction process, the membrane modified with PDADMAC/PSS was submerged in 2.00 mL synthetic urine solution for 10 minutes. Then, the amount of extracted creatinine was detected by dropping 0.20 mL of alkaline picrate solution onto the modified membrane. The recorded photos were analyzed with Image J program. The results of the detection of creatinine in DI water and in synthetic urine are compared as shown in Figure 4.14.

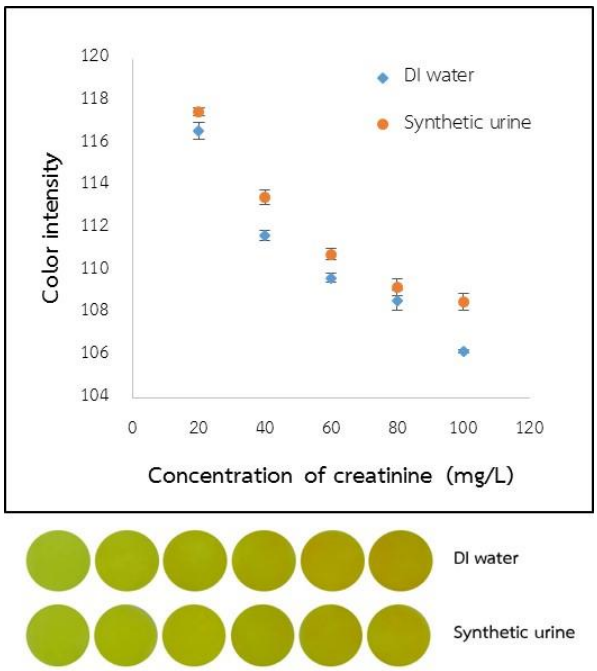

Figure 4.14 The color intensity and chart of membrane color observed in the detection of creatinine in DI water and in synthetic urine (reaction time = 10 minutes).

Under the same analytical condition, the observe color on membranes after the detection of creatinine in synthetic urine was less intense than those used in DI water, indicating the lower extent of creatinine extracted on membranes. It is probably because some cationic species in urine matrix competed with creatinine in the extraction on membrane resulting in a lower content of extracted creatinine. Despite of sample dilution, the urine matrix exhibited interfering effect on the detection. Therefore, the calibration curve for creatinine determination will be constructed by using matrix matched standards.

Furthermore, to enhance a color change and hence, improve the method sensitivity, the volume of creatinine solution was increased from 2.00 mL to 4.00 mL. The effect of reaction time was once again investigated.

4.4.2.2 The effect of reaction time

The creatinine standard solutions were prepared in synthetic urine and diluted 30-fold with 5 mM HCl to achieve the concentration range 0 – 100 mg/L. The modified membranes with PDADMAC/PSS were dipped in 4.00 mL of prepared creatinine solutions for 10 minutes. After extraction process, the detection of creatinine was performed by using Jaffé reaction. The color development was monitored in the time period ranging from 2 – 15 minutes as shown in Figure 4.15.

The obtained results show that the color intensity at high concentration of creatinine was significantly lower when reaction time of longer than 5 minutes was applied. It indicated that the creatinine complex could be formed in higher extent. A reaction time greater than 10 minutes did not yield a significant change in the color intensity implying that the reaction between creatinine and alkaline picrate was completed. To reduce the analysis time, the reaction time of 10 minutes was selected for further study.

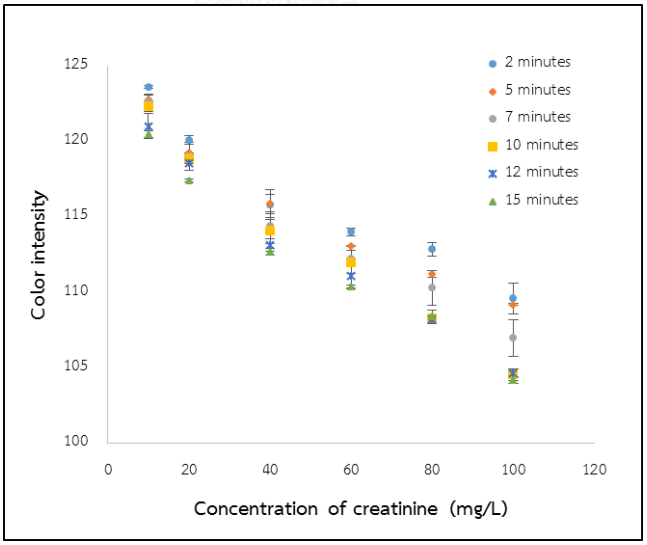

Figure 4.15 The color intensity of creatinine complex on membrane after extraction of 4.00 mL of creatinine in synthetic urine with various time (2-15 minutes); the concentration of creatinine standard solution in the range of 0 – 100 mg/L.

4.5 Method validation for creatinine determination

The proposed method was validated under the optimum condition described as followed. 4.00 mL of sample or standard creatinine solution was extracted by using PDADMAC/PSS membrane filter for 10 minutes and detected with 0.20 mL of 0.04 M alkaline picrate solution for 10 minutes. The intensity of membrane color was collected by taking a photo of the membrane and subjected the photo to Image J software. The linear range, the limit of quantitation, accuracy, and precision of the proposed method were evaluated. The method was applied to urine sample analysis and compared with the standard Jaffé method.

4.5.1 Calibration curve and linearity

Under the optimum conditions, the linear relationship between the color intensity and concentration of creatinine was obtained in the concentration ranging from 0 – 100 mg/L as shown in Figure 4.16. From the naked eyes detection, the color of membranes changed from yellow to dark yellow and brown when the concentration of creatinine solution increased.

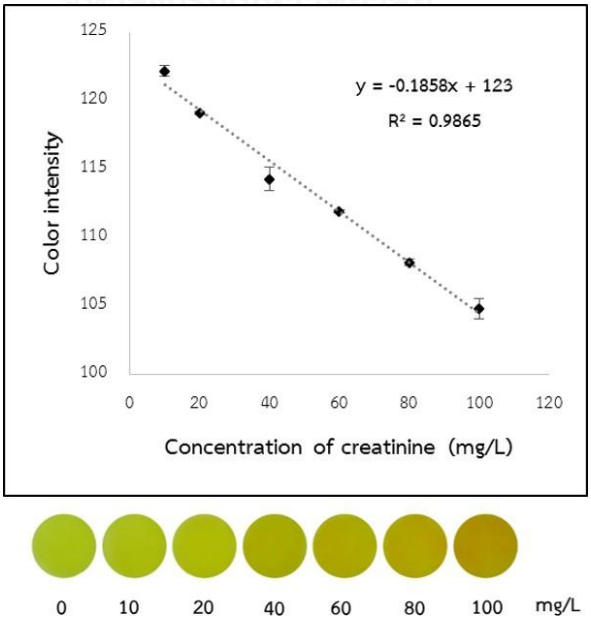

Figure 4.16 The calibration curve for creatinine determination.

4.5.2 Limit of quantitation (LOD) and limit of quantitation (LOQ)

The LOD and LOQ were determined by immerging the PDADMAC/PSS membrane into 4.00 mL of blank synthetic urine without creatinine. The extraction and detection procedure were performed in 10 replicates. The LOD and LOQ values of proposed method obtained from Image J were found to be 3.53 and 22.47 mg/L, respectively.

4.5.3 Accuracy and precision in urine sample analysis

The spiked method was used in this study to evaluate the accuracy of the method. The creatinine standard solution was added into diluted urine samples to obtain a concentration of 20 or 40 mg/L. The accuracy and precision of the method was reported in term of percent recovery and %RSD, respectively.

Freshly collected urine samples were diluted 30-fold with 5 mM HCl solution. The amount of creatinine was determined with the proposed method and compared with the standard Jaffé method as shown in Table 4.6. The recovery of creatinine from spiked sample analyzed by the proposed method was found in the range of 102.2 – 103.3% and 95.2 – 110.4% for the standard Jaffé method. Moreover, the performance of the proposed method for creatinine determination was comparable to the standard Jaffé method in terms of the method accuracy. It was found that the results obtained from both methods were not significantly different at 95% confidence level. The relative standard deviation (RSD) of the analytical results was less than 7.6% which indicated an acceptable precision as set by Association of Official Analytical Chemists (AOAC) (%RSD < 11 and %recovery 80-110).

Table 4.6 The amount of creatinine determined by the proposed method and Jaffé method (N = 3).

Sample*	Amount added (mg/L)	Proposed method			Jaffé method		
		Found (mg/L)	Recovery (%)	RSD (%)	Found (mg/L)	Recovery (%)	RSD (%)
Urine 1	0	27.7±0.3	-	0.9	27.3±0.1	-	0.4
Urine 2	0	10.7±0.6	-	5.9	10.9±0.2	-	1.7
Urine 3	0	20.7±1.6	-	7.6	22.0±0.2	-	1.0
	20	41.4±0.7	103.3±1.7	1.7	41.0±0.6	95.2±1.4	1.4
	40	61.6±2.2	102.2±3.6	3.6	66.1±0.2	110.4±0.3	0.3

*Samples were 30-fold diluted

For naked eyes detection, the membrane color after sample analysis was compared with the standard color chart as previously shown in Figure 4.16. The results in Table 4.7 illustrated the color on membranes used to detect creatinine in urine samples. The amount of creatinine evaluated by naked eyes was in agreement with the amount of creatinine determined by the standard Jaffé method. However, it is difficult to detect creatinine at low level by naked eyes.

Table 4.7 The colorimetric detection of proposed method by naked eyes by comparing with the chart of color of creatinine.

Sample*	Membrane color	Concentration (mg/L)	
		Naked eyes detection	Standard method
Urine 1		20 - 40	27.3
Urine 2		10	10.9
Urine 3		10 - 20	22.0

*Samples were 30-fold diluted

From the method validation, this proposed method could be applied for creatinine determination in human urine by both colorimetric and naked eyes detection. No analytical instrument is required. Moreover, the analysis time is shorter than the standard Jaffé method.

4.6 Albumin to creatinine ratio

The range of linearity obtained from albumin and creatinine determination were used to calculate albumin to creatinine ratio (ACR). The albumin to creatinine ratio from the proposed method could be determined albumin to creatinine ratio at low level (10 – 30 mg/g) as shown in Figure 4.17.

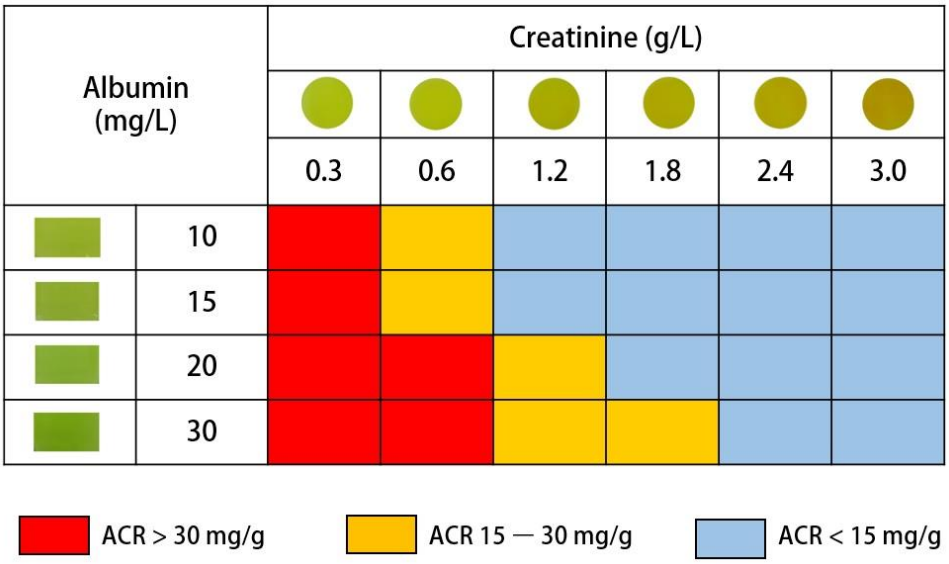

Figure 4.17 Albumin to creatinine ratio from the proposed method.

In this work, two urine samples (urine 1 and urine 2) were collected and measured both albumin and creatinine by the proposed method. From Table 4.4 and 4.6, the amount of albumin and creatinine of urine 1 and urine 2 was used to calculate the albumin to creatinine ratio. It was found that the albumin to creatinine of urine 1 and urine 2 were 11.5 mg/g and 21.1 mg/g, respectively. Therefore, the proposed method was successfully detected albumin to creatinine ratio below 30 mg/g. It is useful for screening test of renal failure in diabetes patient at an early stage, whereas the microalbumin urine strip test could not distinguish the albumin to creatinine ratio between 10 and 30 mg/g.

CHAPTER V
Conclusion

The cellulose acetate (CA) membranes were modified using the layer-by-layer self assembly technique to extract albumin and creatinine before detecting with the colorimetric method. The studied ranges of albumin and creatinine were 0 – 30 mg/L and 0 – 3000 mg/L, respectively. For the characterization of PEMs on membrane using ATR-FTIR and UV-Vis spectrophotometer, the results confirmed the successful of outer surface modification with PDADMAC for albumin determination and PSS for creatinine determination. TBPB in Triton X-100 was used as dye binding reagent to detect albumin, resulting in the color change from yellow to green. Jaffé reaction was used to detect creatinine, forming a yellow-orange complex.

For albumin determination, BSA in synthetic urine was diluted 4-fold with pH 7 5 mM phosphate buffer and 30 mL of the solution was filtered through the modified PDADMAC/PSS membrane for albumin extraction. The adsorbed BSA was desorbed from membrane and 0.90 mM TBPB and 6.0 % (w/v) Triton X-100 was dropped into the eluate. The suitable condition was applied to determine the amount of BSA in urine samples. The results from the proposed method showed a good linear relationship between the BSA concentration and the absorbance intensity in the range of 2.5 – 7.5 mg/L. The recoveries were obtained in the range of 81.2 – 106.9% and the relative standard deviation were obtained in the range of 0.81 – 3.2%, which are accepted for Association of Official Analytical Chemists, AOAC. The LOD and LOQ were found to be 0.47 and 0.68 mg/L, respectively.

For creatinine determination, creatinine in synthetic urine was diluted 30-fold with 5 mM HCl and the modified membrane was dipped into the solution to extract creatinine. The adsorbed creatinine was detected by dropping alkaline picrate onto membrane. The obtained results showed the linear range of the proposed method in the concentration range from 0 – 100 mg/L with the reaction time was 10 minutes.

The proposed method for creatinine determination was applied to urine samples and the results were compared with the standard Jaffé method. The recoveries of the proposed method was found in the range of 102.2 – 103.3% and 95.2 – 110.4% for the standard Jaffé method. The relative standard deviation (RSD) of proposed method was less than 7.6% and the LOD and LOQ were found to be 3.53 and 22.47 mg/L, respectively.

From the all obtained results, the proposed method could be applied in human urine for determining albumin to creatinine ratio at low level. This could be useful as a simple and cost-effective detection kit for screening test of renal failure in diabetes patient at an early stage.

Suggestion for future research

Suggestion for the further work is to optimize volume and flow rate for albumin determination to achieve a higher amount of adsorbed BSA. Additionally, the proposed albumin and creatinine determination should be applied to another real sample and the albumin to creatinine ratio should be calculated.

REFERENCES

[1] Gao, B., Li, Y., and Zhang, Z. Preparation and recognition performance of creatinine-imprinted material prepared with novel surface-imprinting technique. J Chromatogr B Analyt Technol Biomed Life Sci 878(23) (2010): 2077-2086.

[2] Jafar, T.H., Chaturvedi, N., Hatcher, J., and Levey, A.S. Use of albumin creatinine ratio and urine albumin concentration as a screening test for albuminuria in an Indo-Asian population. Nephrol Dial Transplant 22(8) (2007): 2194-2200.

[3] Erman, A., et al. The urine albumin-to-creatinine ratio: assessment of its performance in the renal transplant recipient population. Clin J Am Soc Nephrol 6(4) (2011): 892-897.

[4] Sung, K.C., et al. Urine albumin/creatinine ratio below 30 mg/g is a predictor of incident hypertension and cardiovascular mortality. J Am Heart Assoc 5(9) (2016): 1-11.

[5] Woo, J., Floyd, M., Cannon, D.C., and Kahan, B. Radioimmunoassay for urinary albumin. Clinical chemistry 24(9) (1978): 1464-1467.

[6] Wu, T.-L., Chang, P.-Y., Li, C.-C., Tsao, K.-C., Sun, C.-F., and Wu, J.T. Microplate ELISA for urine microalbumin: reference values and results in patients with type 2 diabetes and cardiovascular disease. Annals of Clinical & Laboratory Science 35(2) (2005): 149-154.

[7] Layne, E. Spectrophotometric and turbidimetric methods for measuring proteins. Methods in Enzymology 3 (1957): 447-454.

[8] Abitbol, C., Zilleruelo, G., Frendlich, M., and Strauss, J. Quantitation of proteinuria with urinary protein/creatinine ratios and random testing with dipsticks in nephrotic children. The Journal of Pediatrics 116(2) (1990): 243-247.

[9] Free, H.M., Collins, G.F., and Free, A.H. Triple-test strip for urinary glucose, protein, and pH. Clinical Chemistry 6(4) (1960): 352-361.

[10] Barratt, J. and Topham, P. Urine proteomics: the present and future of measuring urinary protein components in disease. Canadian Medical Association Journal 177(4) (2007): 361-368.

[11] Kutter, D. Chemistry: A chemical test strip to determine low concentrations of albumin and creatinine in urine. Laboratory Medicine 29(12) (2015): 769-772.

[12] Delanghe, J.R. and Speeckaert, M.M. Creatinine determination according to Jaffe—what does it stand for? NDT Plus 4(2) (2011): 83-86.

[13] Borges, J. and Mano, J.F. Molecular interactions driving the layer-by-layer assembly of multilayers. Chem Rev 114(18) (2014): 8883-8942.

[14] Joseph, N., Ahmadiannamini, P., Hoogenboom, R., and Vankelecom, I.F.J. Layer-by-layer preparation of polyelectrolyte multilayer membranes for separation. Polymer Chemistry 5(6) (2014): 1817-1831.

[15] Yang, J.M., Tsai, R.-Z., and Hsu, C.-C. Protein adsorption on polyanion/polycation layer-by-layer assembled polyelectrolyte films. Colloids and Surfaces B: Biointerfaces 142 (2016): 98-104.

[16] Ghiggeri, G.M., Candiano, G., Delfino, G., and Queirolo, C. Electrical charge of serum and urinary albumin in normal and diabetic humans. Kidney International 28(2) (1985): 168-177.

[17] Paroni, R., Fermo, I., Cighetti, G., Ferrero, C.A., Carobene, A., and Ceriotti, F. Creatinine determination in serum by capillary electrophoresis. Electrophoresis 25(3) (2004): 463-468.

[18] Kinsella, J. and Whitehead, D. Proteins in whey: chemical, physical, and functional properties. Advances in food and nutrition research 33 (1989): 343-438.

[19] Boye, J.I., Alli, I., and Ismail, A.A. Interactions involved in the gelation of bovine serum albumin. Journal of Agricultural and Food Chemistry 44(4) (1996): 996-1004.

[20] Jeyachandran, Y.L., Mielczarski, E., Rai, B., and Mielczarski, J.A. Quantitative and qualitative evaluation of adsorption/desorption of bovine serum albumin on hydrophilic and hydrophobic surfaces. Langmuir 25(19) (2009): 11614-11620.

[21] Bernardi, M., Maggioli, C., and Zaccherini, G. Human albumin in the management of complications of liver cirrhosis. Critical Care 16(2) (2012): 211-217.

[22] Koroshi, A. Microalbuminuria, is it so important? Hippokratia 11(3) (2007): 105-107.

[23] Zunszain, P.A., Ghuman, J., Komatsu, T., Tsuchida, E., and Curry, S. Crystal structural analysis of human serum albumin complexed with hemin and fatty acid. BMC Struct Biol 3 (2003): 6-14.

[24] Barr, D.B., Wilder, L.C., Caudill, S.P., Gonzalez, A.J., Needham, L.L., and Pirkle, J.L. Urinary creatinine concentrations in the U.S. population: implications for urinary biologic monitoring measurements. Environmental Health Perspectives 113(2) (2005): 192-200.

[25] Arndt, T. Urine-creatinine concentration as a marker of urine dilution: reflections using a cohort of 45,000 samples. Forensic Sci Int 186(1-3) (2009): 48-51.

[26] Chen, C.H. and Lin, M.S. A novel structural specific creatinine sensing scheme for the determination of the urine creatinine. Biosens Bioelectron 31(1) (2012): 90-94.

[27] Brandt, J.R., Wong, C.S., Jacobs, A., and Staples, A.O. Urine albumin excretion: Characterization of normal variability in healthy children. Open Journal of Pediatrics 03(02) (2013): 58-64.

[28] Amin, S.V., Illipilla, S., Hebbar, S., Rai, L., Kumar, P., and Pai, M.V. Quantifying proteinuria in hypertensive disorders of pregnancy. Int J Hypertens 2014 (2014): 1-10.

[29] Decher, G. Fuzzy nanoassemblies: Toward layered polymeric multicomposites. Science 277(5330) (1997): 1232-1237.

[30] Ariga, K., Hill, J.P., and Ji, Q. Layer-by-layer assembly as a versatile bottom-up nanofabrication technique for exploratory research and realistic application. Phys Chem Chem Phys 9(19) (2007): 2319-2340.

[31] Boudou, T., Crouzier, T., Ren, K., Blin, G., and Picart, C. Multiple functionalities of polyelectrolyte multilayer films: new biomedical applications. Adv Mater 22(4) (2010): 441-467.

[32] Yusoff, I.I., Rohani, R., and Mohammad, A.W. Pressure driven conducting polymer membranes derived from layer by layer formation and characterization : a review. Journal of Engineering Science and Technology 11(8) (2016): 1183-1206.

[33] Lutkenhaus, J.L. and Hammond, P.T. Electrochemically enabled polyelectrolyte multilayer devices: from fuel cells to sensors. Soft Matter 3(7) (2007): 804-816.

[34] Tang, Z., Wang, Y., Podsiadlo, P., and Kotov, N.A. Biomedical applications of layer-by-layer assembly: from biomimetics to tissue engineering. Advanced Materials 18(24) (2006): 3203-3224.

[35] De Koker, S., Hoogenboom, R., and De Geest, B.G. Polymeric multilayer capsules for drug delivery. Chemical Society Reviews 41(7) (2012): 2867-2884.

[36] Zhao, Q., An, Q.F., Ji, Y., Qian, J., and Gao, C. Polyelectrolyte complex membranes for pervaporation, nanofiltration and fuel cell applications. Journal of membrane science 379(1) (2011): 19-45.

[37] Sun, Q., Tong, Z., Wang, C., Ren, B., Liu, X., and Zeng, F. Charge density threshold for LbL self-assembly and small molecule diffusion in polyelectrolyte multilayer films. Polymer 46(13) (2005): 4958-4966.

[38] Ciferri, A. Ionic mixed interactions in macromolecules. Chemistry–A European Journal 16(36) (2010): 10930-10945.

[39] Decher, G. Layer-by-layer assembly (putting molecules to work). in Multilayer Thin Films, pp. 1-21: Wiley-VCH Verlag GmbH & Co. KGaA, 2012.

[40] Dubas, S.T. and Schlenoff, J.B. Factors controlling the growth of polyelectrolyte multilayers. Macromolecules 32(24) (1999): 8153-8160.

[41] Dubas, S.T., Limsavarn, L., Iamsamai, C., and Potiyaraj, P. Assembly of polyelectrolyte multilayers on nylon fibers. Journal of Applied Polymer Science 101(5) (2006): 3286-3290.

[42] Dubas, S.T., Farhat, T.R., and Schlenoff, J.B. Multiple membranes from "true" polyelectrolyte multilayers. Journal of the American Chemical Society 123(22) (2001): 5368-5369.

[43] Decher, G. Polyelectrolyte multilayers, an overview. Multilayer thin films: Sequential assembly of nanocomposite materials 2 (2003).

[44] Andrade, J. and Hlady, V. Protein adsorption and materials biocompatibility: a tutorial review and suggested hypotheses. Biopolymers/Non-Exclusion HPLC (1986): 1-63.

[45] Elgersma, A.V., Zsom, R.L.J., Norde, W., and Lyklema, J. The adsorption of bovine serum albumin on positively and negatively charged polystyrene latices. Journal of Colloid and Interface Science 138(1) (1990): 145-156.

[46] Kim, J. and Somorjai, G.A. Molecular packing of lysozyme, fibrinogen, and bovine serum albumin on hydrophilic and hydrophobic surfaces studied by infrared–visible sum frequency generation and fluorescence microscopy. Journal of the American Chemical Society 125(10) (2003): 3150-3158.

[47] Roach, P., Shirtcliffe, N.J., Farrar, D., and Perry, C.C. Quantification of surface-bound proteins by fluorometric assay: comparison with quartz crystal microbalance and amido black assay. The Journal of Physical Chemistry B 110(41) (2006): 20572-20579.

[48] Tantipolphan, R., Rades, T., McQuillan, A.J., and Medlicott, N.J. Adsorption of bovine serum albumin (BSA) onto lecithin studied by attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy. International Journal of Pharmaceutics 337(1–2) (2007): 40-47.

[49] Eisner, C., et al. Major contribution of tubular secretion to creatinine clearance in mice. Kidney international 77(6) (2010): 519-526.

[50] Mitchell, R.J. Improved method for specific determination of creatinine in serum and urine. Clinical chemistry 19(4) (1973): 408-410.

[51] Thermo Fisher, S. Thermo Scientific Pierce protein assay technical handbook. [South Bend, Ind.] :: Thermo Scientific, 2009.

[52] Geumann, C., Gronborg, M., Hellwig, M., Martens, H., and Jahn, R. A sandwich enzyme-linked immunosorbent assay for the quantification of insoluble membrane and scaffold proteins. Anal Biochem 402(2) (2010): 161-169.

[53] Pugia, M.J., Lott, J.A., Profitt, J.A., and Cast, T.K. High-sensitivity dye binding assay for albumin in urine. Journal of clinical laboratory analysis 13(4) (1999): 180-187.

[54] Korenaga, T., Izawa, M., Takahashi, T., and Stewart, K.K. Development of capillary stream sensor based on FIA for the determination of proteins in biological samples with high precision and sensitivity. Bunseki kagaku 40(9) (1991): 481-487.

[55] Kanchana, W.-i., Sakai, T., Teshima, N., Katoh, S., and Grudpan, K. Successive determination of urinary protein and glucose using spectrophotometric sequential injection method. Analytica Chimica Acta 604(2) (2007): 139-146.

[56] Jung, K., Nickel, E., and Pergande, M. A microalbuminuria assay using bromphenol blue. Clinica chimica acta 187(2) (1990): 163-172.

[57] Ratanawimarnwong, N., et al. Simultaneous injection effective mixing flow analysis of urinary albumin using dye-binding reaction. Talanta 96 (2012): 50-54.

[58] James, G.P., Bee, D.E., and Fuller, J.B. Proteinuria: accuracy and precision of laboratory diagnosis by dip-stick analysis. Clinical chemistry 24(11) (1978): 1934-1939.

[59] สถิระพจน์, บ. Proteinuria: A Comprehensive Approach to Diagnosis. เวชสารแพทย์ทหารบก (Royal Thai Army Medical Journal) 64(3) (2013): 10.

[60] Pandey, P.C. and Mishra, A. Novel potentiometric sensing of creatinine. Sensors and Actuators B: Chemical 99(2) (2004): 230-235.

[61] Isildak, I., Cubuk, O., Altikatoglu, M., Yolcu, M., Erci, V., and Tinkilic, N. A novel conductometric creatinine biosensor based on solid-state contact ammonium sensitive PVC–NH 2 membrane. Biochemical Engineering Journal 62 (2012): 34-38.

[62] Liotta, E., Gottardo, R., Bonizzato, L., Pascali, J.P., Bertaso, A., and Tagliaro, F. Rapid and direct determination of creatinine in urine using capillary zone electrophoresis. Clinica Chimica Acta 409(1) (2009): 52-55.

[63] Zinellu, A., et al. Plasma creatinine and creatine quantification by capillary electrophoresis diode array detector. Analytical biochemistry 342(2) (2005): 186-193.

[64] Sittiwong, J. and Unob, F. Detection of urinary creatinine using gold nanoparticles after solid phase extraction. Spectrochim Acta A Mol Biomol Spectrosc 138 (2015): 381-386.

[65] Husdan, H. and Rapoport, A. Estimation of creatinine by the Jaffe reaction. Clinical Chemistry 14(3) (1968): 222-238.

[66] Pandey, P.C. and Mishra, A.P. Novel potentiometric sensing of creatinine. Sensors and Actuators B: Chemical 99(2) (2004): 230-235.

[67] Grabowska, I., Chudy, M., Dybko, A., and Brzozka, Z. Determination of creatinine in clinical samples based on flow-through microsystem. Analytica Chimica Acta 540(1) (2005): 181-185.

[68] Debus, B., Kirsanov, D., Yaroshenko, I., Sidorova, A., Piven, A., and Legin, A. Two low-cost digital camera-based platforms for quantitative creatinine analysis in urine. Anal Chim Acta 895 (2015): 71-79.

[69] Wei, Y.-j., Li, K.-a., and Tong, S.-y. The interaction of bromophenol blue with proteins in acidic solution. Talanta 43(1) (1996): 1-10.

[70] Harmoinen, A., Sillanaukee, P., and Jokela, H. Determination of creatinine in serum and urine by cation-exchange high-pressure liquid chromatography. Clinical chemistry 37(4) (1991): 563-565.

[71] Kaneko, E., Yasuda, H., Higurashi, A., and Yoshimura, H. Spot test of urinary protein using Erythrosin B and a membrane film. Analyst 135(7) (2010): 1564-1568.

[72] Martinez, A.W., Phillips, S.T., Butte, M.J., and Whitesides, G.M. Patterned paper as a platform for inexpensive, low-volume, portable bioassays. Angewandte Chemie International Edition 46(8) (2007): 1318-1320.

[73] Sittiwong, J. and Unob, F. Paper-based Platform for Urinary Creatinine Detection. Analytical Sciences 32(6) (2016): 639-643.

[74] Brooks, T. and Keevil, C.W. A simple artificial urine for the growth of urinary pathogens. Lett Appl Microbiol 24(3) (1997): 203-206.

[75] Benavidez, T.E. and Garcia, C.D. Potential-assisted adsorption of bovine serum albumin onto optically transparent carbon electrodes. Langmuir 29(46) (2013): 14154-14162.

[76] Tjipto, E., Quinn, J.F., and Caruso, F. Layer-by-layer assembly of weak-strong copolymer polyelectrolytes: A route to morphological control of thin films. Journal of Polymer Science Part A: Polymer Chemistry 45(18) (2007): 4341-4351.

[77] Tjipto, E., Quinn, J.F., and Caruso, F. Layer-by-layer assembly of weak-strong copolymer polyelectrolytes: A route to morphological control of thin films. Journal of Polymer Science Part A: Polymer Chemistry 45(18) (2007): 4341-4351.

[78] Zhou, W., He, J., Cui, S., and Gao, W. Studies of electrospun cellulose acetate nanofibrous membranes. Open Materials Science Journal 5 (2011): 51-55.

[79] Salloum, D.S. and Schlenoff, J.B. Protein adsorption modalities on polyelectrolyte multilayers. Biomacromolecules 5(3) (2004): 1089-1096.

APPENDIX

VITA

Miss Lalitphan Hongtanee was born on November 20, 1991 in Roi-et, Thailand. She graduated with high school degree from Mahasarakham University Demonstration School, Mahasarakham in 2009. She graduated with a Bechelor's degree of Science from Khonkaen University in 2013. Since 2014, she has become a graduate student at the program in Analytical Chemistry, Faculty of Science, Chulalongkorn University. She had a great opportunity to present her work in poster session in the topic of "Development of urinary albumin and creatinine detection kit" (Poster session) in Pure and Applied Chemistry International Conference 2017 (PACCON 2017) at Centra Government Complex Hotel & Convention Center Chaeng Watthana, Bangkok, Thailand. She finished her Master's degree of Science in 2017.