Evaluation of microalbuminuria using a novel biosensor

by

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Declaration

I certify that this work does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

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Abstract

Chronic kidney disease (CKD) is the progressive loss of kidney function over time, and it is a common disorder, which is associated with the increasing risk of kidney failure as well as cardiovascular disease. Moreover, CKD has become a critical and increasing public health issue among the world. However, due to the asymptomatic features of CKD in the early stage, most patients can only realize this disorder until it has been developed into an advanced stage.

Albumin is the preferred urinary protein for the early detection of CKD. The increased urinary excretion of albumin can be considered as the earliest manifestation of CKD. Besides, albuminuria can accompany related kidney diseases as well, and microalbuminuria can be regarded as a critical sign for the development of CKD. In this study, a novel method utilising fluorescence for measuring the levels of albumin in the range of microalbuminuria was evaluated. The biosensor with aggregation inducted emission (AIE) feature called TC-426 was used. When TC-426 is in aggregation state, due to the strong π to π interaction force, the planar structure of molecule piles up together, leading to the quenching of fluorescence. However, when it interacts with albumin molecules, the hydrophobic phenyl rings of TC-426 were prompted to enter the hydrophobic cavities of albumin, inducting the emission of fluorescence. This study evaluated the optimal working conditions of TC-426 including the incubation time with albumin solution and the ratio to albumin solution. Moreover, the correlations between the concentration of albumin solution and its corresponding fluorescence intensity in different environments including deionised (DI) water, artificial urine and real urine sample were evaluated as well. Furthermore, the interference of creatinine was characterized. It was found that TC-426 is an outstanding biosensor with sensitivity and selectivity for the detection of albumin.

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Table of Contents

List of Figures

List of Tables

Chapter 1: Introduction

Kidney failure has become a public health issue in worldwide which comes with continuously increased incidence and prevalence. Patients with kidney failure require a replacement therapy, which is expensive and always with poor outcomes (Eknoyan et al., 2004). However, the chronic kidney disease (CKD) has a substantially higher prevalence, with adverse outcomes such as cardiovascular disease (CVD), loss of kidney function and even premature death (Webster et al., 2017). It is an urgent problem that requires the global effort to improve the outcomes of CKD (Levey et al., 2005). Early diagnosis based on the presence of albuminuria is able to permit intervention to manage and monitor CKD in the early stage (James et al., 2010). Moreover, retain albuminuria can be considered as an important marker for kidney damage. To be specific, the level of urine albumin to creatinine ratio (UACR) of patients with CKD is twice or three times than the normal value, high level is not frequent in people under 40 years old, and is related closely to adverse outcomes (Levey et al., 2005). However, there are limitations on the detection of albuminuria in the tradition way, such as the quantities of albumin in urine samples are too small to be detected by the standard dipstick (Glassock, 2010) and the inaccuracy of using immunoassays (Chen et al., 2017).

The novel biosensors based on luminescence materials have attracted people's focus because of their sensitivity, selectivity and efficiency (Chen et al., 2017). In this thesis, a fluorescence-based biosensor is used to evaluate albuminuria by the quantification of albumin levels.

1.1 Project Background

Human serum albumin (HSA) is an important protein in human body for its capacity in maintaining the intravascular osmotic pressure, buffering capacity, wound healing and so on. The structure of albumin is shown in Figure 1.1.

Figure 1.1 Structure of HSA

As shown in the figure, HSA is a heart-shaped protein, which is formed by the assembly of three homologous domains. There are two subdomains within each homologous domain, which are considered as the common structural motifs. There are hydrophobic cavities in subdomains IIA and IIIA, and they function as the principal regions of ligand binding to albumin. In a healthy human body, the level of albumin is controlled in the interstitial spaces of organs, intercellular and body fluids. Therefore, the level of albumin in biological fluid can be considered as an indicator of the health condition of human body, and the abnormal level of albumin can be a mark of various diseases. For example, in patients with CKD, HSA cannot be filtered in kidney due to the lesions of glomeruli and tubules, therefore, albumin and can be excreted with urine. The presence of albumin in urine is regarded as albuminuria when the level of albumin is above 30 mg/L, and by regular monitoring of the albumin level in urine, it is possible for the early diagnosis of CKD.

As people pay more and more attention to CKD, there are already different methods for detecting the albumin levels such as colourimetric methods, capillary electrophoresis and Bromocresol purple assays; however, these methods have drawbacks in one way or another in terms of accuracy, selectivity, cost efficiency and efficiency. This thesis applies a novel fluorescence-based method which takes the advantages of sensitivity, selectivity and time efficiency.

Fluorescence is a phenomenon that happens in the molecular level, the luminophore becomes electronically excited and emits light after absorbing the incident light. Fluorescence intensity is a value used to quantify fluorescent absorption and emission. However, a phenomenon called 'aggregation caused quenching (ACQ)' always happens in high concentration solution, which makes the luminescence weakened or quenched. The structural reason of ACQ effect is that most luminophores contain planar aromatic rings which leads the luminophores to pile up together in high concentration, thereby the luminescence is weakened or quenched. To address the ACQ effect, aggregation induced emission (AIE) effect which is exactly opposite to ACQ effect came out. The basic theory is to change the planar structure to non-planar structure so that when the luminophores are in aggregation state, the fluorescence intensity has a dramatic increase. In this thesis, an AIE-based biosensor called TC-426 was used to detect the levels of albumin within the range of microalbuminuria. The chemical structure of TC-426 is shown in Figure 1.2.

Figure 1.2 Chemical structure of TC-426

As a new developed AIE-based biosensor, the possible mechanism of the interaction between albumin and TC-426 is that due to the phenyl rings of TC-426 are hydrophobic, the luminogenic molecules were prompted to enter into the hydrophobic cavities of albumin and aggregate together, and by utilising the AIE nature of TC-426, the fluorescence intensity is enhanced.

If a suitable ratio of albumin to TC-426 can be determined, as the increase of albumin concentration, more luminogenic molecules are able to enter into the hydrophobic cavities and the AIE effect will be more significant, which results in the increase of the fluorescence intensity. Therefore, by measuring the fluorescence intensity of the complex of albumin and TC-426, the detection and quantification of albumin can be achieved.

1.2 Project Objectives

The objective of this thesis is to evaluate the albumin levels within the range of microalbuminuria by using the fluorescence-based biosensor TC-426. The first aim is to determine the optimal working conditions for TC-426 to deliver a reasonable performance. Then, by applying the optimal working conditions, evaluate the correlation between albumin concentration and its fluorescence intensity. Moreover, by changing the environment of solvent, evaluate the performance of detecting albumin levels in DI water, artificial urine and real urine sample. At last, investigation of the interference of the presence of creatinine will be conducted.

Chapter 2: Literatures review

This review is of current literature on chronic kidney disease, including its background knowledge, effects on the patient's body and the detection and monitoring techniques in the development of its early stage. It is consisted of two parts. In the first part, a brief introduction of CKD will be given in terms of its clinical symptoms and the effects on the human body. Moreover, the importance of the detection and monitoring of the early stage of CKD will be demonstrated. Then, current techniques for detection and monitoring CKD will be introduced along with their advantages and drawbacks. Furthermore, in the second part, the fluorescence-based technology will be introduced. Then, the aggregation-caused quenching (ACQ) effect and the aggregation-induced emission (AIE) will be outlined. Next, as a novel biosensor, the applications related to AIE-based biosensor will be demonstrated. Finally, the AIE-based biosensor for detecting albumin will be reviewed.

2.1 Chronic kidney disease

2.1.1 Global increased health problem

Chronic kidney disease is the progressive loss of kidney function over time, and it is a common disorder which is associated with the increasing risk of kidney failure as well as cardiovascular disease (James et al., 2010). CKD and kidney failure have increased in prevalence globally due to the aging population and the worldwide rising of diabetes (James et al., 2010). It has become a critical and increasing public health issue among the world. It is reported that, in over 20 million Americans, one in nine adults has CKD and there are another huge group of people at increased risk of getting CKD (Couser et al., 2011). Furthermore, in Australia, it is reported that more than 30% of Australian tend to develop CKD or at high risk of developing CKD and for the grope of people those are over 75 years old, almost half of them have certain indicators of CKD. As a

result, every year in Australia, there are more than two thousand patients that are newly diagnosed are in the need of dialysis or even kidney transplantation (White et al., 2010). Additionally, it has shown that patients with CKD have two- or three-times higher risk of cardiac death comparing to the same age and gender group. Thus, it is the kind of disease that the mortality rate increases over time. It is a tough public health problem to be addressed.

2.1.2 Clinical symptoms and criteria for definition of CKD

The worst outcome of CKD can be kidney failure, and the symptoms are normally related to the complications of the gradual loss of kidney function. CKD can be defined either by using biopsy, imaging and urinalysis if there is evidence of abnormal structure or function or defined by a sustained decline in the rate of glomerular filtration.

Levey et al. (2005) have demonstrated the criteria for definition of CKD. To be specific, the duration of the disorder can be an indicator of CKD. Typically, the duration of CKD is over three months which is different to the acute kidney disease.

Then, the second criteria for definition of chronic kidney disease is the glomerular filtration rate (GFR), which is the most effective indicator of kidney function. It can be measured by equations related to serum albumin and serum creatinine. According to the range of GFR, five stages were specified by the National Kidney Foundation's Kidney Disease Outcome Quality Initiative (KDOQI) (Levey et al., 2005). Normally, GFR in healthy adults is around 125, and kidney failure can be defined when GFR is lower than 15, (Levey et al., 2011). Except the criteria by GFR, the abnormal structure and function can also define CKD. As examples, for pathological abnormalities, clinical diagnosis of glomerular diseases including diabetes and systemic infections; vascular diseases including atherosclerosis, vasculitis and hypertension; and cystic disease such as polycystic kidney disease can define CKD or kidney damage (Levey et al., 2011).

Additionally, kidney damage can also be defined by imagining abnormalities through medical imaging technologies, such as CT, MRI, ultrasound and more. Certain

symptoms of the kidney damage can be diagnosed such as polycystic kidneys, renal artery stenosis and small and echogenic kidneys (Levey et al., 2011).

Last but not least, as a critical marker of kidney damage, albuminuria is a sign that indicates the kidney disease, which means there is excess albumin in people's urine. Normally, it is found that the albumin exists in blood as a type of protein and due to the glomerular filtration and retraction of tubules, albumin would not exist in the urine from people with healthy kidney. However, when the kidney is damaged to a certain extent, the glomerular permeability will increase accordingly, which causes the albumin leaks into urine (Levey et al., 2011). Currently, a few studies have reported that albuminuria can predict chronic kidney disease independently and accurately (Johnson et al., 2012). To evaluate the albuminuria, urinary albumin-to-creatinine ratio (UACR) is utilized. The normal range of UACR is below 10mg/g and when it is in the range of 30 to 300, kidney disease is possibly present. Finally, when UACR is over 300, it can be regarded as macroalbuminuria or clinical proteinuria and the kidney has been damaged into an advanced stage (Levey et al., 2005). Table 1 is the thresholds for abnormal albumin to creatinine ration.

Table 1 (Levey et al., 2005) Thresholds of albuminuria

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2.1.3 Early detection of CKD

The National Kidney Disease Education Program (NKDEP) emphasised that early detection of CKD is critical. One of the important objectives is to prevent

cardiovascular disease in CKD (CVD). It is reported that patients with CKD are under higher risk of CVD than the generals, even they are only at the early stage of CKD. Moreover, early detection can manage and arrest the progression effectively (Narva, 2008). They also pointed out that most people are asymptomatic until CKD has developed into the advanced stage. In this case, the damage to the kidney would be difficult to treat with, and the loss of kidney function is impractical to get recovered through the conventional treatment.

Additionally, Chadban et al. (2003) also pointed out that most patients are unaware of the situation that their kidney is getting worse over time because of the asymptomatic early stage of CKD. It is reported that, before any symptoms appear, an individual can loss up to 90% of their total kidney function (White et al., 2010). Early detection and management of CKD is vital since it can reduce up to 50% of the progression of kidney failure and its complications (Chen et al., 2017).

As mentioned, it is difficult to recognise CKD in the early stage because of its asymptomatic nature. In Australia, around 25% of the patients started their dialysis just after less than 90 days when they presented to the nephrologist, and this number is presenting without improvement with time. The early reorganization of CKD permits the preventive measures to be taken in certain stage of the treatment to favorably affect the clinical outcomes.

The early-stage-detection of CKD depends on the result of tests on urine and blood, to be specific, the albumin or protein exist in urine and serum creatinine exists in blood. By combining the results together with the blood pressure measurement, this method is commonly used as clinical measurement of kidney damage and it can also classify into stages by severity (Levey et al., 2005). It was demonstrated that the urine testing for albumin presents reasonable sensitivity and superior specificity. The utilisation of serum creatinine concentration and estimated GFR has certain limitations, but more concerns have been payed to improve the performance of the related methods. Furthermore, the clinical action pathways based on evidence of CKD in various stages have been published as shown in Table 2.

Table 2 (Levey et al., 2005) Clinical action in various stages of CKD

2.1.4 Screening and monitoring techniques on CKD

In the USA, Australia and Europe, the clinical practice guidelines have recommended that the urine albumin to creatinine ratio (UACR) is supposed to use for early detection of CKD. It can be cooperated with testing estimated glomerular filtration rate (eGFR) to screen for CKD and the related prognostic grading (Narva, 2008). Human serum albumin (HSA) is one of the amplest proteins with the concentration of 35-50 g/L (Berg and Lane, 2011). Normally, there is no albumin in human urine due to the effects of glomerular filtration, active tubular secretion and reabsorption. However, in the case of patients with kidney damage, a small amount of albumin can leak into the urine, this condition was defined as albuminuria. It can raise the albumin level by up to 300 mg/L in urine (Chen et al., 2017). Therefore, albuminuria testing is typically applied as an initial screening technique for kidney disease.

As Martin (2011) reported, the immunoassays are the clinical routine measurement method for urine albumin at present. It has the limitation of detection at 2-10 mg/L. Moreover, it tends to underestimate the amount of albumin due to the chemical structure of albumin. To be specific, albumin has more than five antigenic sites; the

urine environment is complex, which makes it difficult for the antibodies to detect the forms of albumin. Therefore, the immunoassays are considered as an inaccurate method for testing microalbuminuria.

Moreover, Delanghe et al. (2017) have reported a dye-binding based test strips for albuminuria analysis. In his study, test strips Meditape UC-11A were used. They contain reagent pads for ordinal scale assessments of creatinine, pH, bilirubin, relative density and more. The low reflectance value, which is corresponded to a high concentration, expressed in the format of percentage from 0% to 100%. Besides, when the reflectance value is 0%, the strip is black, and when it goes up to 100%, the colour turns to white, which means with the increase of the concentration of the analyte, the colour of the strip becomes shallow.

Furthermore, a specific algorithm is employed to rate the colour. The principle of albumin test applies the pH indicator based on the protein error of tetrabromophenol blue. This indicator is greatly sensitive to albumin with the range up to 150 mg/L. However, it has a poor selectivity to albumin due to there are other proteins that have same size as albumin. In their study, they applied the strips on patients with kidney damage for albuminuria testing. Although it presents a relative reasonable performance over a wide rage, in severely ill patients, the amount of detected albumin is rather small due to the glycation and carbamylation, which causes the underestimation of albuminuria.

Recently, the fluorescence-based technology takes the advantages of sensitivity, time-efficiency and selectivity to become a popular candidate in biomedical field.

2. 2 Fluorescence-based technology

2.2.1 Fluorescent technique on biomedicine

Fluorescence has attracted increasing attention in acting as a novel biosensor technique; it can play the role in visualizing cell biology in variety levels from molecules

to the whole organism. At the beginning, fluorescence was mostly applied by attaching on specific antibodies to the target protein. Then, the developed fluorophores can track the organ cells and ions directly (Giepmans et al., 2006). Recently, fluorophores have allowed non-invasive living cells imaging as well as delivering dynamic biochemical signals. Situ et al. (2016) have reported a biocompatible biosensor tetraphenylethene pyridinium (TPE-PyN3). In this study, this fluorogen presents high affinity to mitochondria. Moreover, it allows long-term observation of the specific cell imaging with outstanding image-stability. Besides, due to the sensitivity to the membrane potential of the mitochondria, the cell apoptosis is able to be detected.

Additionally, Liow et al. (2017) have demonstrated the development of a self-indicating drug delivery system with excellent fluorescence emission for real-time screening of agent concentration in local. It was designed for drug tracking system in local in vivo, it has high affinity to the target tumour and indicates the change of local drug concentration according to the change of local temperature. This fluorescence-based thermogel owns greater biocompatibility comparing to the commercial tracker. It is reported that this fluorescence-based thermogel tracking system is much more superior as it has better selectivity, long time lasting and non-toxic to patients.

Furthermore, fluorophores can also act as bioprobes for quantitative measurement with proven sensitivity and selectivity (Vazquez-Romero et al., 2013). The biopolymers can be quantified or recognised by the increase of the luminescence emission or spectral shifts after the processes of complexation or conjugation with target proteins (Hong et al., 2010). In addition, Liu et al. (2015) have reported the design and synthesize of an 8-hydroxyquinoline-based derivative as a fluorescence-based bioprobe, which has excellent selectivity and sensitivity to formaldehyde in aqueous solution. It can achieve quantitative measurement of formaldehyde in a certain range of concentration. Moreover, this fluorescence-based bioprobe is suitable for serving as a preliminary method in commercial food.

Moreover, in biomedical research, fluorescence-based bioprobe also plays a critical role. Peng et al. (2011) demonstrated a fluorescence-based bioprobe that can

quantitatively detect the Hydrogen Sulfide (H_2S) in blood. H_2S is considered as a biological indicator in a wide range of physiological activities. It is reported that the cardiovascular system is affected by H_2S in a certain extent as it can play a role like the channel opener for K-ATP. Therefore, monitoring the amount or concentration of H_2S S is helpful for managing certain disorders. In his research, Dansyl was selected to synthesize the sulfide-sensitive agent. As a result, this fluorescence-based bioprobe has proven selectivity for detecting H_2S both in aqueous solution and blood. Also, it is a low-cost, rapid and high efficiency detection method for sulfide.

As a conclusion, the common applications of fluorescence in biomedicine are fluorescence-based sensors for real time cells imagine, fluorescent labelling for tracking target proteins or cells and bioprobes for quantitative measurement.

2.2.2 Aggregation-caused quenching (ACQ)

Although fluorescence-based biosensors have attracted increasing attention, there is a thorny problem related to the emission quenching of typical fluorescent molecules in physiological buffer or aqueous medium. This phenomenon of reduced fluorescence intensity caused by the aggregation of fluorescent molecules is called aggregation-caused quenching (ACQ) (Hong et al., 2011).

As Hong reported, as an example shown in Figure 1, the dilute solution of DDPD in tetrahydrofuran (10 mM) is highly luminescent. However, after adding water into the solution, its emission is weakened as the proportion of water in the solution increases. The reason is that DDPD is insoluble in water, adding water will increase the local luminophore concentration, thus DDPD molecules is forced to aggregated. Moreover, as shown in Figure 2.1, when the water content is over 60 vol %, most of the DDPD molecules get aggregated. As a result of the formation of DDPD molecules aggregation, the light emission is fully quenched.

Figure 2.1 Fluorescence photographs of solutions/suspensions of DDPD (10 mM) in THF/water mixtures with different water contents (Hong et al., 2011).

Furthermore, from the molecular level of view, as shown in Figure 2.2, there is a disc-like perylene core structure in the planar luminescent molecules, when the luminescent molecules are in aggregation state, the disc-like rings tend to pile up together, which is caused by the intense π to π stacking interactions between the aromatic rings. Thus, the light emission of the luminescent molecule is completely quenched. Conversely, for the hexaphenylsilole (HPS) as an example of non-planar luminescent molecules, because of restriction of intramolecular rotation, when it is in aggregation state, the emission is actually enhanced. To conclude, ACQ effect is caused by the structures of conventional luminescent molecule, which are typically consist of planar aromatic rings, they tend to pile up together in high concentration.

Figure 2.2 Planar luminophoric molecules. (B) Non-planar luminogenic molecules (Hong et al., 2011).

Typically, the ACQ effect is regarded as detrimental from the practical applications point of view. For instance, the dilute solution always emits weakly, which causes an inferior sensitivity in fluorescence screening systems. However, a higher concentration

of the fluorophore cannot improve the sensitivity due to the ACQ effect. As thus, the small amount of fluorophore molecule can be rapidly photobleached when the excitation light source applies an intense laser beam (Hong et al., 2009). As a thorny problem, many research groups have spared efforts to find the way to overcome the obstacles of the ACQ effect.

2.2.3 Aggregation-induced emission (AIE)

Aggregation-induced emission (AIE) effect is exactly opposite to the ACQ effect, which allows the luminogenic molecules to enhance the emission through the aggregation process rather than working against the process (Hong et al., 2011). Hexaphenylsilole (HPS) can be regarded as the archetypal AIE luminogen, since it is different to the conventional luminophores in term of the molecule structure. To be specific, as shown in Figure 2.2.2.2, comparing to the conventional luminophores which have the disc-like planar perylene, HPS is a non-planar molecule and has a shape similar to the propeller. It is believed that the structural difference that leads to the different emission behaviour (Hong et al., 2011). Moreover, the properties of the propeller-shaped non-planar structure caused the restriction of intramolecular motion (RIM) which leads to the emission of HPS molecules in the aggregate state (Hong et al., 2009). As a conclusion, the RIM process is the main cause of AIE effect. Up to now, many research groups have utilized the RIM process to explore new AIE systems.

2.2.4 Applications of AIE-based biosensors

In the aggregated state, the AIE luminogen is highly emissive which makes it a superior candidate for real-world applications. D-glucose is a fundamental and indispensable substance for living organisms and it is a critical source that provides energy for physical activities. Its level in blood and urine has been regarded as a critical biomarker for certain disorders. Hu et al. (2014) have developed an AIE-based sensor for detecting the D-glucose in aqueous solution. The hydrogen peroxide is formed by the

glucose oxidase oxidized, which further oxidized the arylboronic pinacolester group of the AIE-based sensor to allow an emissive but insoluble tetraphenylethene (TPE) derivative.

Moreover, heparin is a kind of anticoagulant which plays a critical role in preventing the formation of blood clots. It is found that in the period of surgery, monitoring the level of heparin can efficiently prevent haemorrhage or thrombosis. (Shi et al., 2012) have developed a fluorescence based AIE bioprobes for monitoring heparin. A feature of this probe is that it does not emit in aqueous solution, but when with the existence of heparin, it starts to emit strongly. Meanwhile, there is no effect on the bioprobe while chondroitin-4-sulfate (ChS) and hyaluronic acid (HA) exist, which makes the bioprobe a selectivity and sensitively candidates for monitoring heparin level.

2.2.5 AIE-based biosensors for human serum albumin (HSA)

As the most abundant protein in human plasma, HAS plays multiple roles in human body such as the main modulator of fluid distribution between body compartments, reflecting the main determinant of plasma oncotic pressure (Fanali et al., 2012). The normal range of HAS in plasma is 35~ 50 g/L, and abnormal level of HSA can be considered as the abnormal physiological indicators in the human body, and is also an early sign of cardiovascular diseases, liver diseases, diabetes and kidney diseases (Liu et al., 2019). HSA consists of three spherical structural domains and a total of six structural subdomains (IA, IB, IIA, IIB, IIIA, IIIB). In these subdomains, there are two drug sites, of which the drug site 1 is located in the IIA subdomain, and its hydrophobic cavity can combine with many common micro-molecule drugs to deliver to certain sites of human body. The drug site 2 is located in the IIIA subdomain, and its internal cavity is not only hydrophobic, but also rich in donors and receptors of hydrogen bond (Liu et al., 2019).

Anees et al. (2014) designed a squaraine dye, i.e., Sq for detecting HSA. Generally, squaraine dyes tend to form aggregates and undergo self-assembly under aqueous

conditions which cause strong quenching of emission. However, after adding HSA, the disassembly of the dye was inducted, which promotes the encapsulation of the dye in the hydrophobic cavities of HSA. This reaction triggers the reaction between the dye and HSA, which results in the generation of a new chromophore that can emit strong green fluorescence. The dye Sq demonstrated a promising selectivity for the quantitative measurement of HSA in human blood samples.

Moreover, Hong et al. (2010) developed a non-emissive TPE derivative called BSPOTPE for HSA detection and quantitation. While this biosensor takes the advantages of a broad working range (0-100 nM), low limitation of detection (down to 1nM) and an excellent selectivity to HSA, it can also be utilized as a superior protein stain for HSA visualization. The hydrophobic phenyl rings of BSPOTPE leads the luminophores to enter the hydrophobic cavities and pile up together, which enhances the emission of fluorescence. Furthermore, BSPOTPE was used to investigate the unfolding process of HSA.

Furthermore, Yu et al. (2016) constructed a nanoscale molecule for evaluating site-specific recognition and conformational changes of HSA. This biosensor is a TPE derivative called TPE-red-COOH. This biosensor was self-assembled into nanospheres with an average diameter of 34 nm, and there was hardly any fluorescence emitted. After adding HSA, the nanospheres was disassembled to 6 nm within 5 min, and TPE-red-COOH entered the Cleft 1 of HSA and emitted red light signal at 610 nm under the irradiation of ultraviolet light. The whole process occurred rapidly, and the detection limit was 2.7 nmol.

Chapter 3: Methodology

3.1 General Methods

3.1.1 Materials

The AIE based biosensor TC-426 used in this thesis was provided by Mr Tze Cin Owyong from Melbourne University through the collaboration. Other chemicals used in this thesis were purchased from Sigma-Aldrich, Australia and used without further treatment.

3.1.2 Sample preparation

As suggested, TC-426 was dissolved in dimethyl-sulfoxide (DMSO) solution, the stock solution was made to 5 mM and kept at -20℃ in the dark for long term storage. Moreover, the recommended working solution of TC-426 was prepared daily by suitable dilution of the stock solution with DMSO to 10 μM. Furthermore, artificial urine was prepared according to the procedure previously published (Chutipongtanate and Thongboonkerd, 2010). Besides, according to the recommendation of urinalysis, the urine sample was prepared freshly and by the first morning void (Martin, 2011).

3.1.3 Measurement of fluorescence intensity

To record the fluorescence intensity emission of samples, a Cary Eclipse Fluorescence Spectrophotometer was employed. There is a scan device located inside the spectrophotometer which can excite the sample through ultra-violet (UV) light and records the emission fluorescent light in a configured range of wavelength. Moreover, the fluorescence intensity is correlated to the number of luminous molecules.

To measure the excitation and emission wavelengths of a sample, the excitation and emission wavelength were evaluated by using “zero order” function. To be specific, for

10 μM TC-426 dye, in the excitation model, firstly set the emission wavelength as zero order, then scanned the sample, and the wavelength of the peak value in the returned excitation spectrum was the excitation wavelength of the sample. Then, in the emission model, set the excitation wavelength and scanned the sample, and the wavelength of the peak value in the returned emission spectrum is the emission wavelength of the sample.

To make the results reliable and repeatable, every sample was poured into three cuvettes, and the amounts of sample in each cuvette were same. Scanned each cuvette and took the mean value as result.

3.2 Evaluate the range of albumin concentration

It is crucial to measure the albumin in a meaningful range for the detection of microalbuminuria. The main purpose of selecting a proper range of albumin concentration is to: first, evaluate the measuring limitation of this biosensor with albumin; second, evaluate the correlation between albumin concentration and its fluorescence intensity; third, set proper concentration gradient of albumin to evaluate the sensitivity of this biosensor.

From the literature review chapter, it is known that when the albumin concentration is above 20 mg/L and urine albumin to creatinine ratio (UACR) is above 30 mg/g, it can be defined as albuminuria. Therefore, the albumin concentration range can be configured between 100 mg/L to 1000 mg/L to evaluate the limitation and the overall correlation between albumin concentration and its fluorescence intensity. The concentration gradient can be set as 100 mg/L, just to roughly observe the shape of the correlation curve. Then the concentration range can be reduced to 20 mg/L to 200 mg/L. The main purpose is to, first, evaluate the sensitivity of the biosensor; second, it is the threshold range of microalbuminuria which is more meaningful for early detection of chronic kidney disease; accordingly, the concentration gradient of albumin can be reduced to 25 mg/L.

3.3 Evaluate the biosensor's working conditions

To get the best performance of the biosensor, the working conditions of the biosensor should be optimised. The definition of the "best performance" is for a given series of albumin solution with various concentrations, the biosensor can turn out different fluorescence intensity with certain correlation, and there should be a significant difference between the lowest and highest concentrations. Moreover, the correlation between different concentrations and corresponding fluorescence intensity should have certain correlation and is best to be linear. This requires evaluating the following conditions: first, investigate the incubation time of the biosensor and albumin solution; second, optimise the biosensor to albumin solution ratio; third, the changing of working conditions caused by different environments.

3.3.1 Incubation time

The main reason of the evaluation of the reaction of biosensor and albumin solution was to fix the incubation time so that further results can be reliable. To do this, the biosensor is added into the highest concentration (1000 mg/L) of albumin solution at the ratio of 1:1. After adding the biosensor, place the container on the vortex mixer, mix the solution for 10 seconds to accelerate the incubation process, then transform the mixed solution into a cuvette. Then, use the kinetic model of spectrophotometer to continuously record the emitted fluorescence intensity. The scanning period can be set as 40 minutes, once it finished, the amount of time taken for the fluorescence intensity remaining stable can be known from the result.

3.3.2 Biosensor to albumin solution ratio

To evaluate the optimal biosensor to albumin solution ratio, the highest and lowest concentrations of albumin solution were prepared. There was a total of five groups of albumin samples with the highest and lowest concentrations of albumin. Then five

ratios of biosensor to albumin solution were used to evaluate the effect that different amounts of biosensor to albumin solution would affect the detection. The biosensor was added into each group with ratio to albumin solution of 1:1, 1:3, 1:5, 3:1 and 5:1. For this evaluation, the various biosensor to urine ratios and the fixed variables used are shown in Table 3. The ratio that delivers the greatest difference of fluorescence intensity between the highest and lowest concentrations would be taken as the optimal ratio.

Table 3 Evaluation of biosensor to albumin ratio

Variable	Description
Ratio of biosensor to albumin solution	➢ 1:1 ➢ 1:3 ➢ 1:5 ➢ 3:1 ➢ 5:1
Fixed variables	➢ Concentration of albumin solution: highest (1000 mg/L) and lowest (100mg/L) ➢ Incubation time: used the incubation time evaluated in 3.3.1 ➢ Slit

3.3.3 Chose the proper slit of spectrophotometer

The fluorescence intensity can be amplified or reduced physically by adjusting the slit of the spectrophotometer. The important factor that determines the size of slit is the fluorescence intensity of the highest concentration of the sample. The maximum measurement range of the spectrophotometer is 1000 a.u.; if the fluorescence intensity of the highest concentration is beyond 1000 a.u., the result becomes unreliable. In this case, it is supposed to decrease the size of the slit. By contrast, because there may be background source in the solution, the fluorescence intensity can be disturbed and becomes weak. If the fluorescence intensity of the highest concentration of the sample is weak, but still has a relative great difference to the

sample with the lowest concentration, the size of slit can be increased to amplify the fluorescence intensity. For adjusting the slit, the various size of the slit and the fixed variables used are shown in Table 4. The slit that can bind the fluorescence intensity in a wide and valid range would be taken as the optimal slit.

Table 4 Investigation of the slits of spectrophotometer

Variable	Description
Size of slit (Excitation wavelength* Emission wavelength)	➢ 5nm* 5nm ➢ 5nm* 10nm ➢ 10nm* 10nm
Fixed variables	➢ Concentration of albumin solution: highest (1000 mg/L) and lowest (100mg/L) ➢ Ratio of biosensor to albumin solution (evaluated in 3.3.2) ➢ Incubation time: used the incubation time (evaluated in 3.3.1)

3.4 Evaluate in different environments

This thesis evaluated the biosensor in different environments. Started from evaluating in DI water since it has the least amount of distractions, then in artificial urine and lastly in real urine sample. In DI water and artificial urine, the evaluation basically follows the process as shown in Figure 3.1.

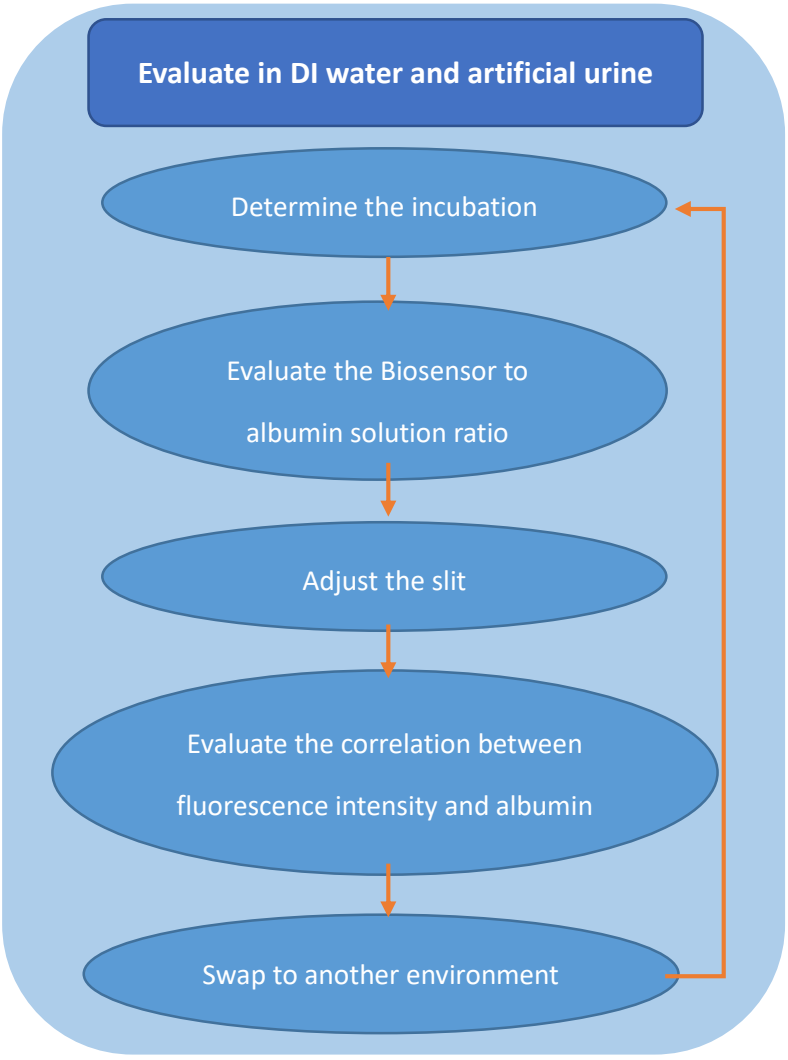

Figure 3.1 Evaluation process in DI water and artificial urine

In the real urine sample, as the consideration of the stronger interference of background source, the urine sample may need to be diluted. Therefore, the dilutions of urine ratio need to be investigated. After determining the incubation time in urine sample, the highest and lowest concentrations of albumin solution (albumin resolved

in urine) were taken, then diluted the solution with the different portion of DI water and measure the fluorescence intensity. For this evaluation, the various dilutions of urine ratio and the fixed variables used are shown in Table 5. The dilute ratio of urine sample that turns out the greatest difference of fluorescence intensity between the highest and lowest concentrations would be taken as the optimal dilute ratio.

Table 5 Evaluation of the urine dilution ratio

Variable	Description
Dilution of urine ratio (with DI water)	➢ Undiluted ➢ 1:1 ➢ 1:3 ➢ 1:5 ➢ 1:7 ➢ 1:9
Fixed variables	➢ Concentration of albumin solution: highest and lowest concentrations ➢ Ratio of biosensor to albumin solution (1:1, hypothetically) ➢ Incubation time: used the incubation time (evaluated in 3.3.1) ➢ Slit

Therefore, the evaluation in real urine sample can follow as Figure 3.2 shows.

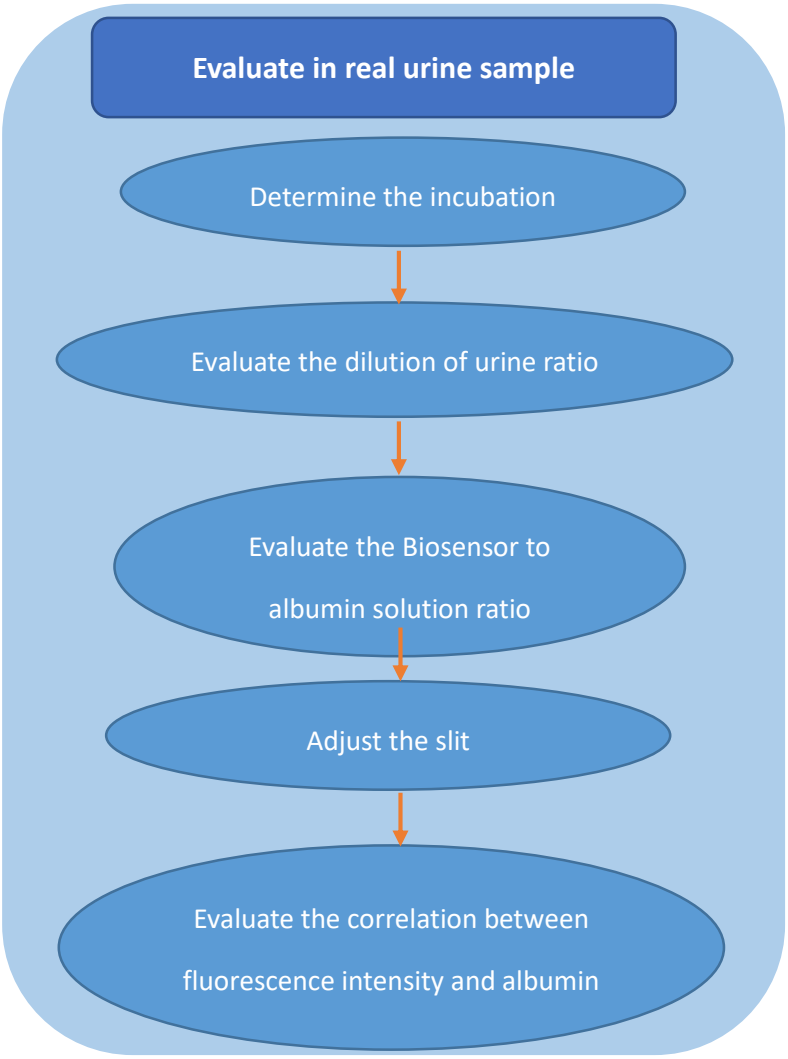

Figure 3.2 Evaluation process in DI water and artificial urine

3.5 Evaluate the interference of creatinine

Creatinine is also an important marker for the detection of chronic kidney disease; therefore, evaluating the interference of creatinine to this biosensor became necessary. The evaluation of the interference of creatinine is to add certain amount of creatinine into different concentrations of albumin solution and compare the change of the fluorescence intensity to the corresponding albumin solution without adding creatinine. The amount of creatinine added into the albumin solution was calculated according to the threshold value of the definition of microalbuminuria. The deductive process is shown below:

1) When the albumin concentration ranges from 20 mg/L to 200 mg/L, and the urine albumin to creatinine ratio (UACR) ranges from 30 mg/g to 300 mg/g, it can be considered as microalbuminuria.
2) This thesis wanted to evaluate the interference of creatinine to the biosensor within the range of microalbuminuria.
3) Therefore, to ensure the amount of added creatinine is eligible for (1) and (2), the lowest concentration of albumin solution should add the amount of creatinine that makes UACR greater than the minimum threshold value; the highest concentration of albumin solution should add the amount of creatinine that makes UACR less than the maximum threshold value.
4) To do this, took 10 mL of each albumin solution sample with different concentrations; therefore, the amount of albumin in the solution can be known mathematically by the equation:$\mathrm{m} = \mathrm{c} * \mathrm{v}$. For example, 10 mL of 20 mg/L albumin solution contain $10 * 10^{-3}L * 20mg/L = 0.2\mathrm{mg}$, and once again, it can be figured out that 10 mL of the highest concentration albumin solution contains 2 mg albumin.
5) The amount of creatinine added into the albumin solution should meet the conditions: $\frac{\mathrm{albumin_{lowest}}}{creatinine} > 30mg/g$ and $\frac{\mathrm{albumin_{highest}}}{creatinine} < 300mg/g$. Finally, it turns out that $\frac{2\mathrm{mg}}{300mg/g} < creatinine < \frac{0.2mg}{30mg/g}$, therefore, the amount of added creatinine can be taken as 0.007g.

For this evaluation, the variables used are shown in Table 6.

Table 6 Evaluation in different environments

Variable	Description
Fixed variables	➢ albumin solution with creatinine: 10 mL of each albumin solution with different concentrations and 0.007g creatinine.

Environment	➢ albumin solution: 10 mL of each albumin solution with different concentrations ➢ Ratio of biosensor to albumin solution (evaluated in 3.3.2) ➢ Incubation time: used the incubation time (evaluated in 3.3.1) ➢ Slit ➢ Artificial urine ➢ Real urine sample

Chapter 4 Results and discussion

This chapter presents the experimental results and the related discussions. Since the biosensor was evaluated in different environments including DI water, artificial urine and the real urine sample, the results of each environment will be discussed separately, and finally, a comparison between the three environments will be given. Moreover, the results will be presented in the order referring to methodology chapter, from incubation time, biosensor to albumin solution ratio, and finally to the correlation curve of albumin concentration and fluorescence intensity. Furthermore, the result of the evaluation of the interference of creatinine was presented.

4.1 Biosensor with albumin in DI water

4.1.1 Incubation time

1 mL TC-426 was added into 1 mL 1000 mg/L albumin solution (resolved in DI water) in a closed tube, then the mixed solution was transferred into a cuvette. Used the excitation wavelength 510 nm to excite the mixed solution, it was found that the peak fluorescence intensity was at 535 nm, and the slit was set as 5* 5 nm. Measured the emission fluorescence spectrum by every 30 seconds. The result is shown in Figure 4.1.

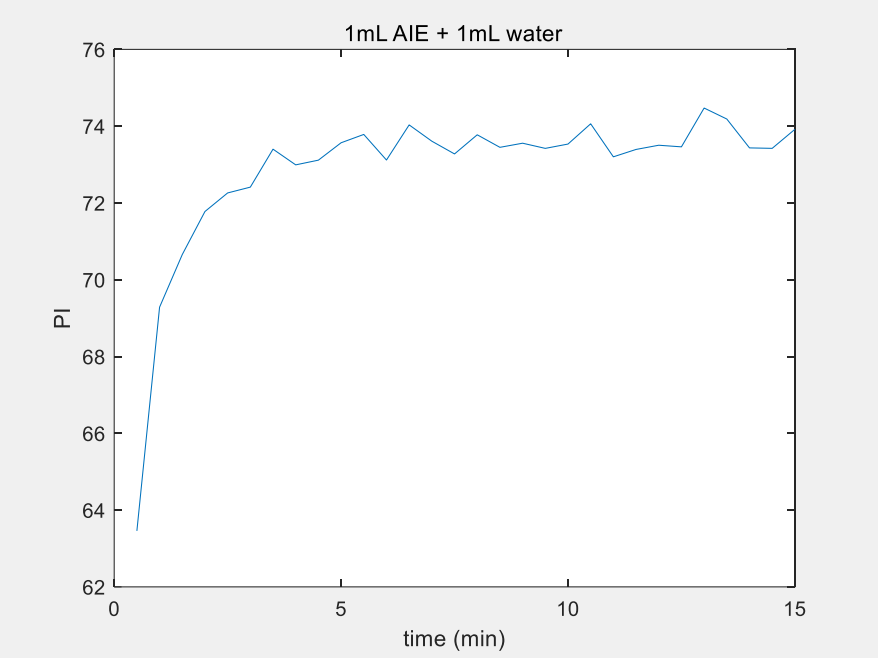

Figure 4.1 Incubation time in DI water

From the result, it was found that after 5 minutes, the peak fluorescence intensity became stable. Although it still has slight fluctuations, 5 minutes can be considered as the incubation time of TC-426 with albumin in DI water.

Moreover, it can be observed that although the highest concentration of albumin solution was used, the peak fluorescence intensity was relatively low. Therefore, except adjusting the ratio of TC-426 to albumin solution, the size of the slit should be increased properly, such as increasing to 5* 10 nm.

4.1.2 Biosensor to albumin solution ratio

The slit was changed to 5* 10 nm. Proper amount of TC-426 was added into albumin solutions (1000 mg/L and 100 mg/L respectively) in a tube with different ratios, i.e., 2:1, 5:1, 7:1, 1:2, 1:5, 1:7. Then, each sample was poured equally into three cuvettes, used the excitation wavelength of 510 nm to measure the emission fluorescence spectrum.

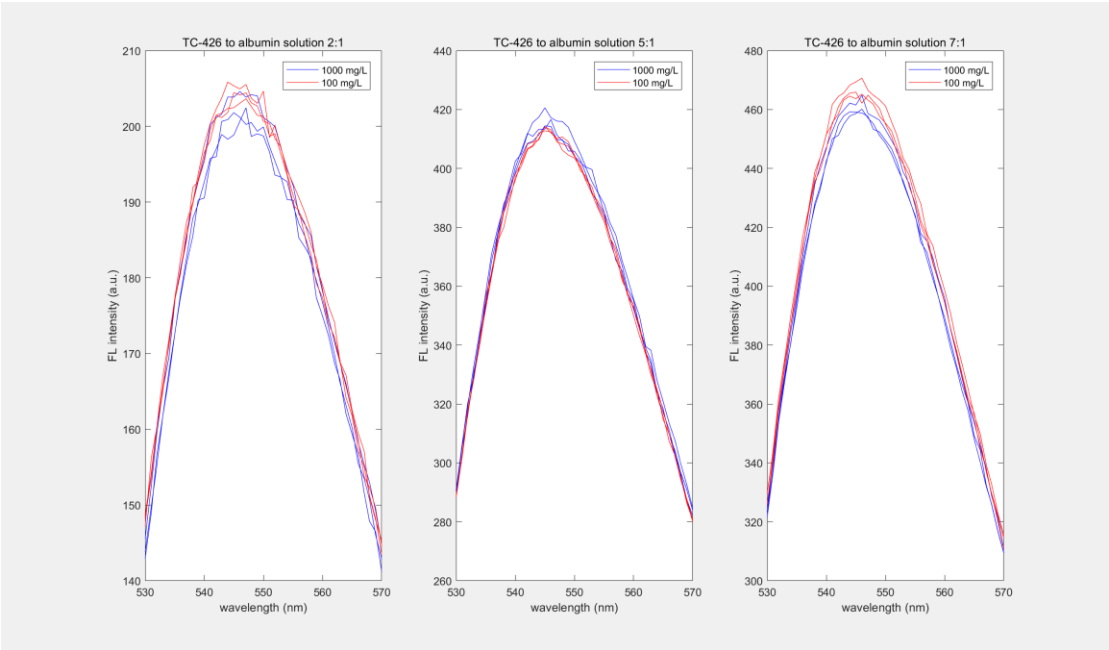

Figure 4.2 Comparison between the results of ratio 2:1, 5:1 and 7:1

From Figure 4.2, there were no significant differences of the fluorescence intensity between the high and low concentrations of albumin solution; however, as the portion of TC-426 increased, the peak fluorescence intensity increased correspondingly. In the mixed solution, when the amount of TC-426 is greater than the amount of albumin, TC-426 cannot distinguish the different concentrations of albumin solution because the fluorescence intensity is in 'luminophores domain'. In another words, change of fluorescence intensity caused by the change of the amount of TC-426 was much greater than change of albumin concentration; therefore, the change of fluorescence intensity caused by different albumin concentration became negligible. In this case, it

is hard to use TC-426 to measure the fluorescence intensity of albumin solution with different concentrations.

Then, changed the ratio of TC-426 to albumin solution to 1:2, 1:5 and 1:7 (Figure 4.3).

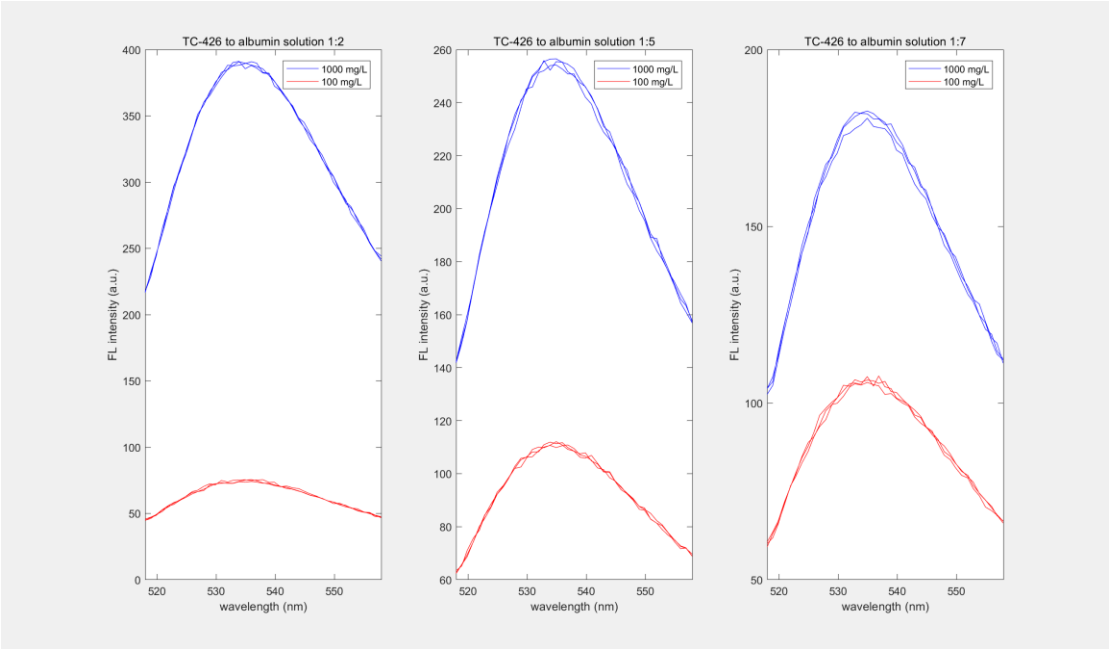

Figure 4.3 Comparison between the results of ratio 1:2, 1:5 and 1:7

It is obvious that when the amount of TC-426 is less than the amount of albumin solution, there is significant difference of fluorescence intensity between the high and low concentration of albumin solutions. Moreover, the wavelength of peak fluorescence intensity shifted from 545 nm to 535 nm. It is known that the emission wavelength of TC-426 is at 545 nm, when the amount of TC-426 was greater than the amount of albumin, the luminophore domains the emission. On the contrary, when the amount of albumin was greater than TC-426, more luminophores can get into the hydrophobic pocket of albumin. The response of different ratios was examined by plotting the peak fluorescence intensity versus the ratio of albumin to TC-426 (Figure 4.4).

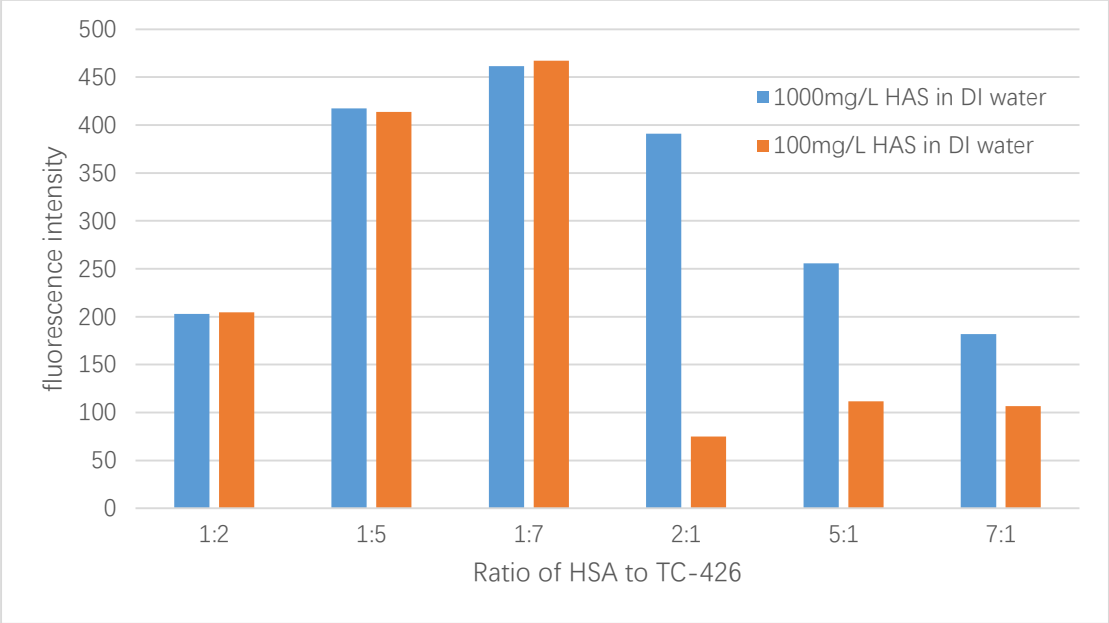

Figure 4.4 Results of different ratios of albumin to TC-426 in DI water

It is clear from the result that when the ratio of albumin solution to TC-426 is 2:1, the difference of the peak fluorescence intensity between 1000 mg/L albumin solution and 100 mg/L albumin solution is the greatest. Therefore, 2:1 can be considered as the optimal ratio of albumin solution to TC-426 in DI water.

4.1.3 Correlation between albumin concentration and its fluorescence intensity

Made adequate amounts of albumin solution with ten different concentrations, i.e., 100 mg/L, 200 mg/L, 300 mg/L… 1000 mg/L. Then, added the correct amount of TC-426 into the albumin solution with the ratio of albumin solution to TC-426 2:1. Incubated the samples for 5 minutes and poured each sample equally into three cuvettes. Used 510 nm as the excitation wavelength and set the slit as 5* 10 nm. Measured the emission fluorescence spectrum of each sample (Figure 4.5).

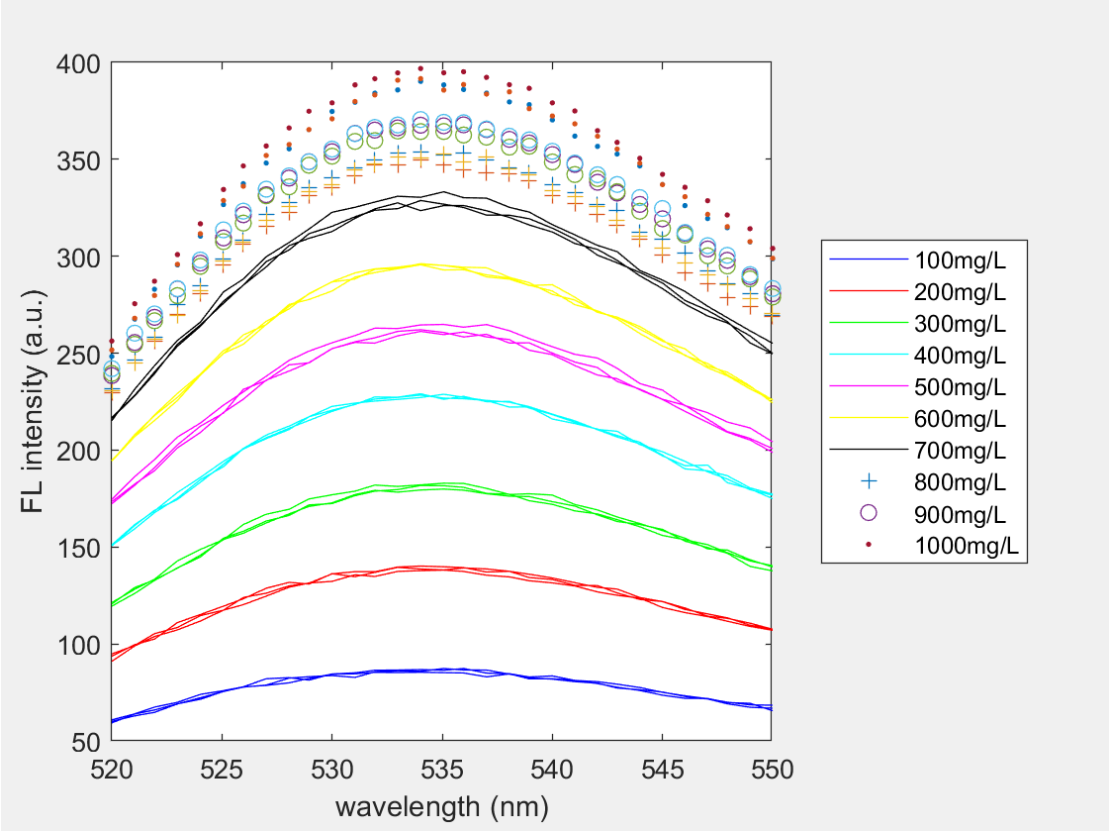

Figure 4.5 Results of albumin with different concentrations in DI water

Take the mean value of the peak fluorescence intensity of each sample, to plot the correlation curve in terms of the albumin concentration and its corresponding fluorescence intensity (Figure 4.6).

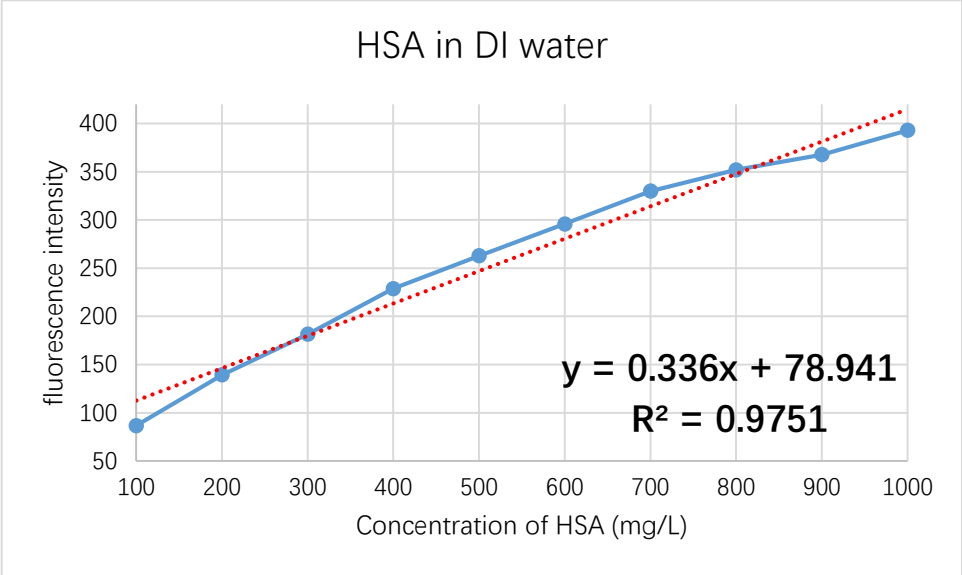

Figure 4.6 Correlation curve of albumin concentration and peak FL intensity in DI water

The fit curve can be represented by the equation: $y = 0.336x + 78.941$, with $R^2 = 0.9751$. The correlation between the albumin concentration and its corresponding fluorescence intensity is highly linear. Moreover, for further evaluation of the sensitivity of TC-426, the concentration gradient of albumin solution was reduced to 25 mg/L. 36 samples of albumin solution were made, i.e., 125 mg/L, 150 mg/L, 175 mg/L, 1000 mg/L. However, it was found that TC-426 ran out, more TC-426 solution was made. To double-check the incubation time does not change, evaluated the incubation time again using slit 5*10 nm, excitation wavelength, and the ratio of albumin solution to TC-426 2:1. Moreover, this time a more accurate kinetic model was applied. The result is shown in Figure 4.7.

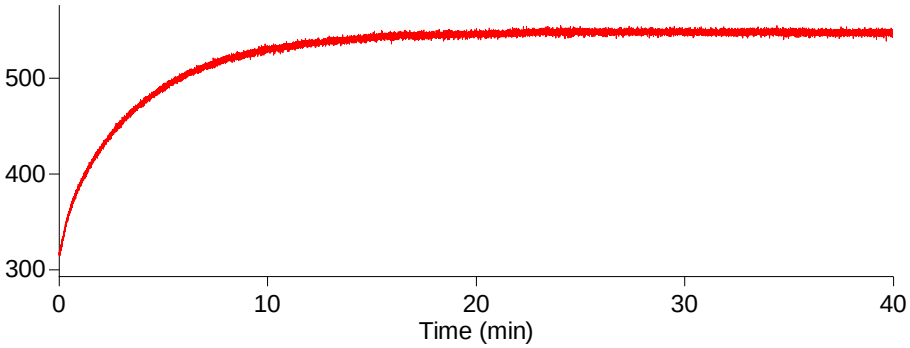

Figure 4.7 Incubation time in DI water

From the result, it was found that the incubation time increased to 20 minutes. In this case, a possible reason is that the previous evaluation of incubation time did not apply the mixing process of TC-426 and albumin solution, which may have caused the inadequate reaction between the two solutions and constrained the maximum fluorescence intensity; and high frequency vibration can promote TC-426 to enter the hydrophobic pocket of albumin. As shown in Figure 4.8, 1000 mg/L albumin solution was mixed with TC-426 at a ratio of 2:1, the peak fluorescence intensity kept stable around 530 a.u., which increased about 130 a.u. comparing to the result in Figure 4.7. Therefore, the incubation time of TC-426 with albumin solution in DI water should be changed to 20 minutes after mixed the solution on the vortex mixer for 10 second. The responses of TC-426 were examined by plotting the peak fluorescence intensity versus the HSA gradient (Figure 4.8).

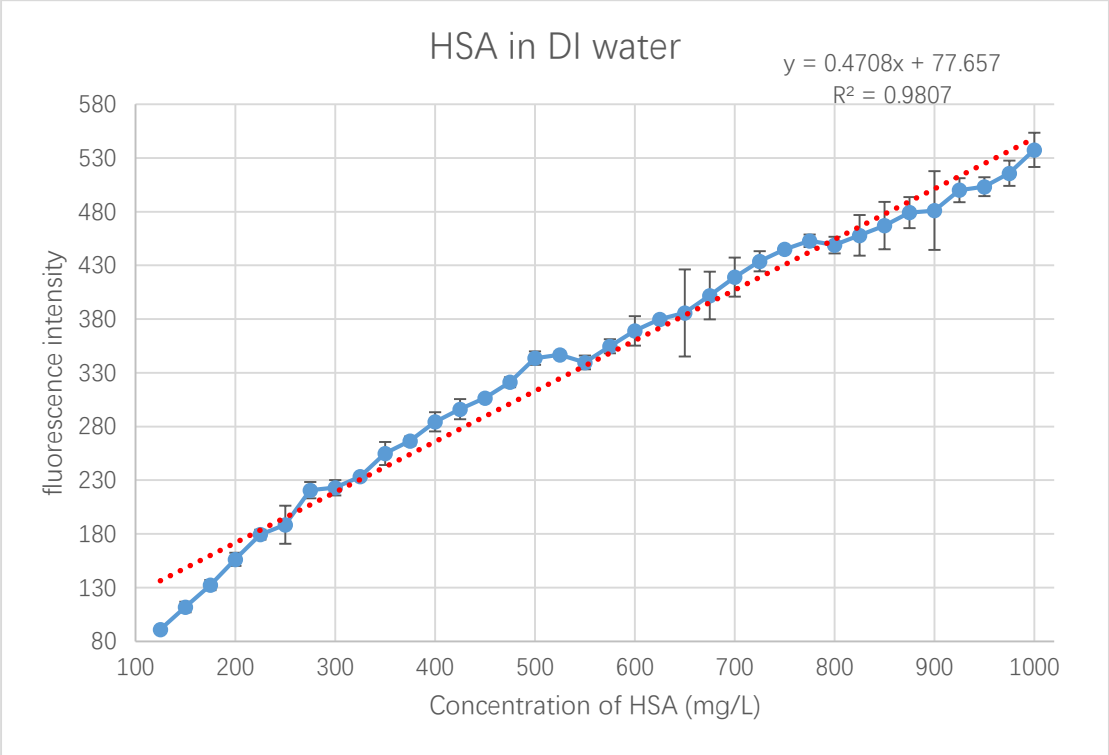

Figure 4.8 Correlation curve of albumin concentration and peak FL intensity in DI water

The fit curve can be represented by the equation: $y = 0.4708x + 77.657$, with $R^2 = 0.9807$. From the result, it can be concluded that in DI water, by using TC-426 as a

biosensor, the fluorescence intensity of albumin solution was positively correlated with its concentration. Moreover, four linear dynamic ranges were found, i.e., 125 mg/L~ 300 mg/L, 300 mg/L~ 500 mg/L, 550 mg/L~ 800 mg/L, and 800 mg/L~ 1000 mg/L. As the concentration increases, the slope of the four ranges decreased, which reflects the fact that TC-426 is more sensitive in the low concentration range. Furthermore, in the last two ranges, the deviation of results increased significantly, especially when the albumin concentration was 650 mg/L, the lowest measured value was at the same level as in 500 mg/L, while the highest measured value was at the same level as in 700 mg/L. The possible reason of the large deviation of the results in high concentration is the increasing instability of the interaction between the luminophores and albumin caused by the increasing albumin molecules

4.2 Biosensor with albumin in artificial urine

In this part, albumin was resolved in artificial urine which was prepared according to the procedure previously published (Chutipongtanate and Thongboonkerd, 2010). The formula of artificial urine is listed in Table 7.

Table 7 Component of artificial urine used in this study (Chutipongtanate and Thongboonkerd, 2010)

	Concentration (mM)	Molar mass
Ammonium chloride	15	53.49
Calcium chloride	3	110.98
Creatinine	varies	113.12
Magnesium sulfate	2	246
Monosodium phosphate	3.6	119.98
Potassium chloride	30	74.5513
Sodium bicarbonate	2	84.007
Sodium chloride	54	58.44

Sodium citrate	5	294.1
Sodium oxalate	0.1	133.999
Sodium phosphate dibasic	0.4	141.96
Sodium sulfate	9	142.04
Urea	200	60.06
Uric acid	1	168.11

At first, all the experimental conditions expect the environment did not change to evaluate the influence of changing environment. Then, the optimal working conditions of TC-426 was evaluated.

4.2.1 Incubation time

To evaluate the incubation time, 1000 mg/L albumin solution (HSA resolved in artificial urine) and TC-426 were mixed in a tube as the ratio of 1:1. Placed the tube on the vortex mixer and mixed the solution for 10 seconds at its highest level. Then, poured the mixed solution immediately into a cuvette, used 510 nm as the excitation wavelength and 535 nm as the emission wavelength, set the size of slit to 5*10 nm, used the kinetic model to record the emission fluorescence intensity by time (Figure 4.9).

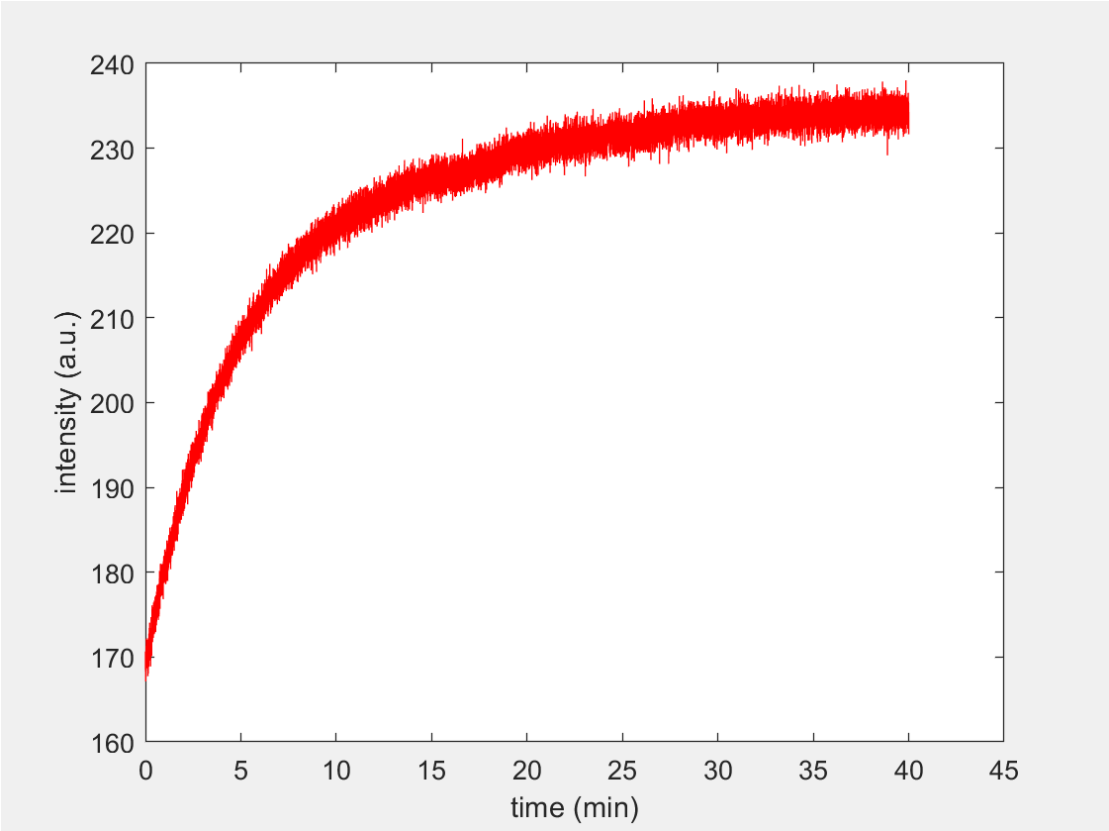

Figure 4.9 Incubation time in artificial urine

From the result, it can be known that the incubation time of TC-426 and albumin solution in artificial urine can take 20 minutes, which is same as in DI water. For comparing the influence caused by different environments, the environment was considered as the only variable, and other conditions were kept unchanged. The experimental conditions were listed in Table 7.

Table 8 Experimental conditions in DI water and artificial urine

Environment	Incubation time	Optimal ratio of albumin to TC-426	Slits of spectrophotometer
DI water	20 minutes	1:2	5 nm* 10 nm
Artificial urine	20 minutes	1:2	5 nm* 10 nm

4.2.2 Comparison of the fluorescence intensity between albumin in DI water and artificial urine

Resolved proper amount albumin in artificial urine to make albumin solution with different concentrations, i.e., 100 mg/L, 200 mg/L, 300 mg/L, 1000 mg/L. Applied the experimental conditions listed in Table 7, the emission fluorescence spectrums of each samples were plotted (Figure 4.10).

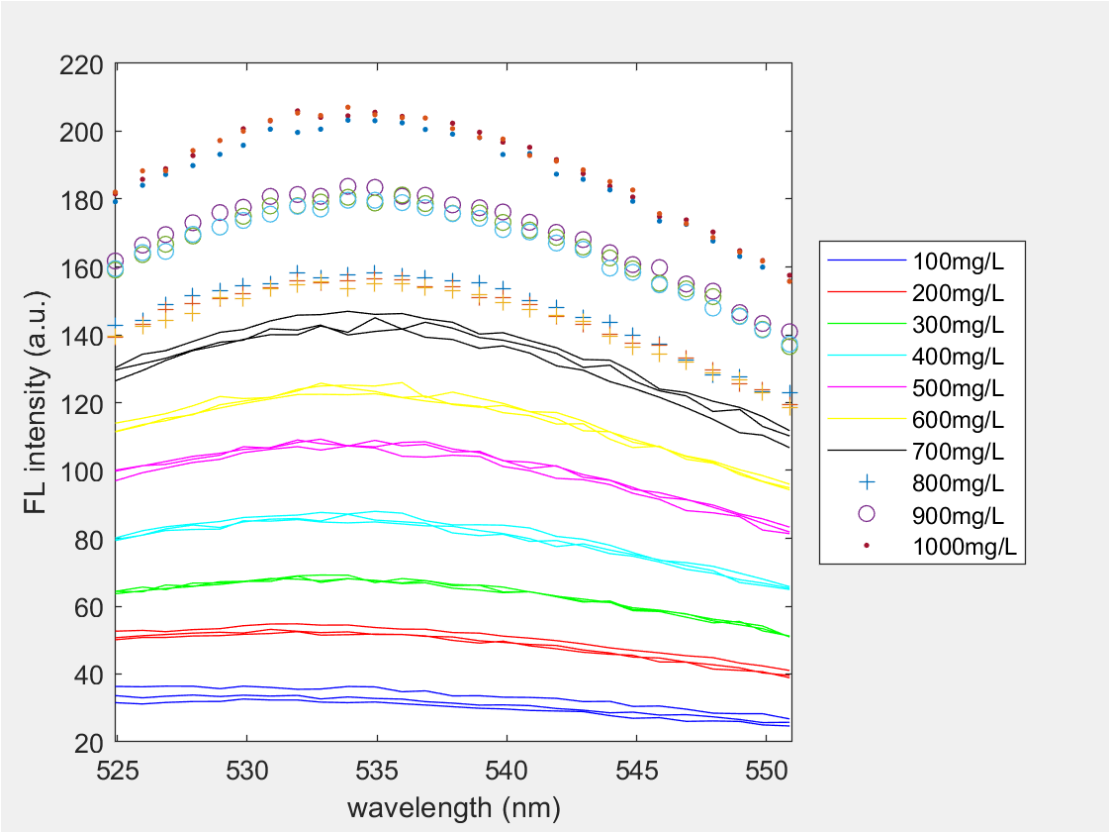

Figure 4.10 Results of albumin with different concentrations in artificial urine with same working conditions as in DI water

The response of TC-426 to different concentration albumin solutions was examined by plotting the peak fluorescence intensity versus the albumin concentration gradient (Figure 4.11).

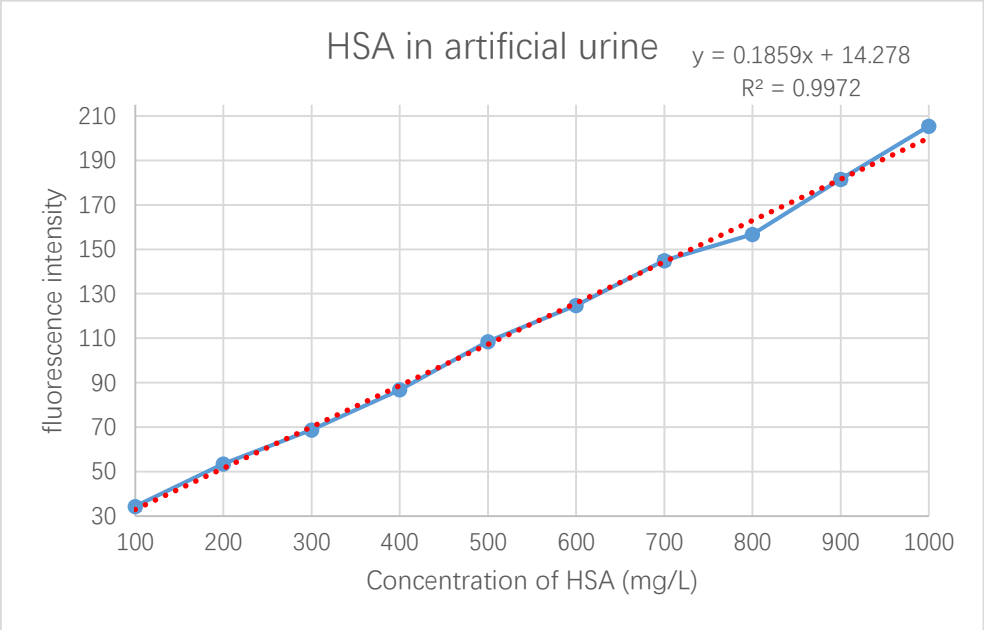

Figure 4.11 Correlation curve of albumin concentration and peak FL intensity in artificial urine

From the result, it can be known that the correlation between different concentrations of albumin solution and its corresponding peak fluorescence intensity is linear. To compare the influence caused by changing environment from DI water to artificial urine, plotted the two curves together as Figure 4.12 shows.

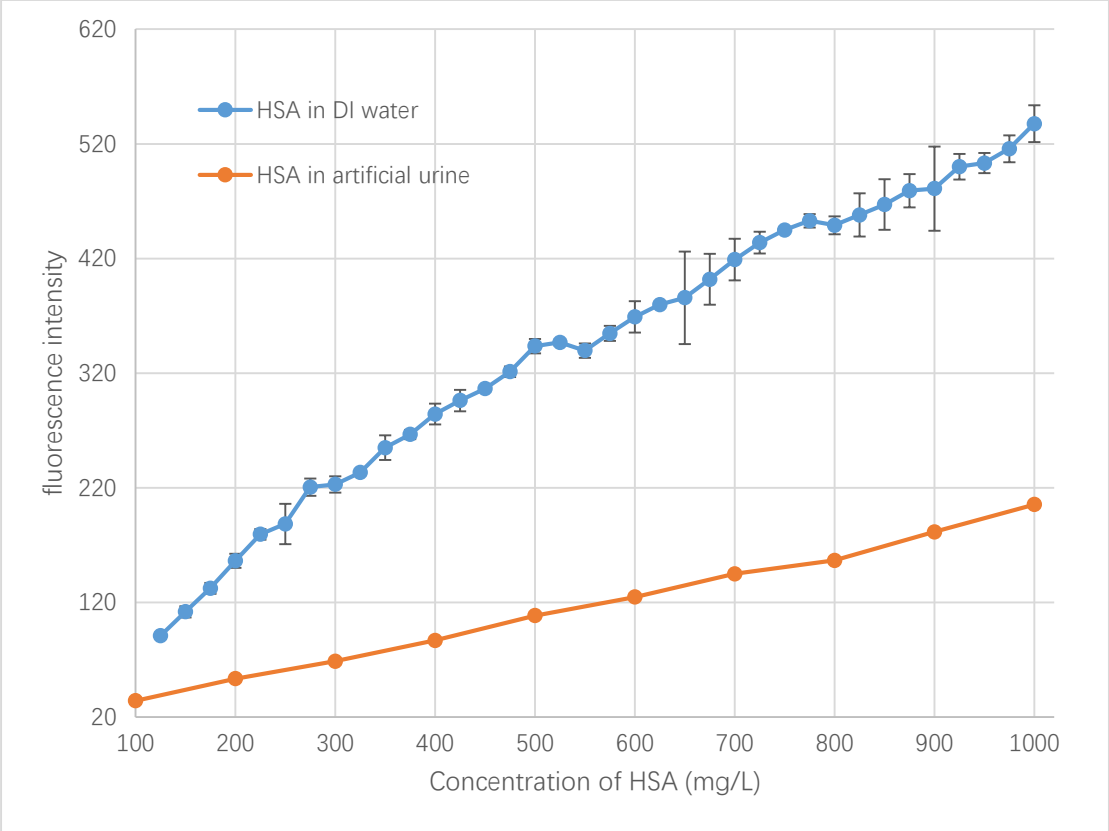

Figure 4.12 Comparison of the results in DI water and artificial urine

There are two major differences between DI water and artificial urine. First, in artificial urine, the overall fluorescence intensity had a significant drop, which means TC-426 was interfered by certain substance in artificial urine, resulting in a decrease of fluorescence intensity. Second, the slop of the correlation curve in artificial urine has better linearity. To be specific, the correlation curve in artificial urine is closer to a straight line, while DI water presented four ranges with different slopes. Besides, the deviation in artificial urine is small in the whole range.

4.2.3 Increased the size of slit

From Figure 4.11, although the result presented a linear correlation curve, the overall fluorescence intensity had a significant drop, especially when the concentration of albumin solution was below 200 mg/L which is the threshold value of microalbuminuria, the fluorescence intensity became less than 100 a.u.. In this case, it would be hard to evaluate the fluorescence intensity in the range between 20 mg/L and 200 mg/L. Therefore, the fluorescence intensity should be amplified by increasing the size of slit. Repeated the experiment in section 4.2.2, changed the slit from 5*10 nm to 10*10 nm and kept other conditions unchanged. The emission fluorescence spectrums are shown in Figure 4.13.

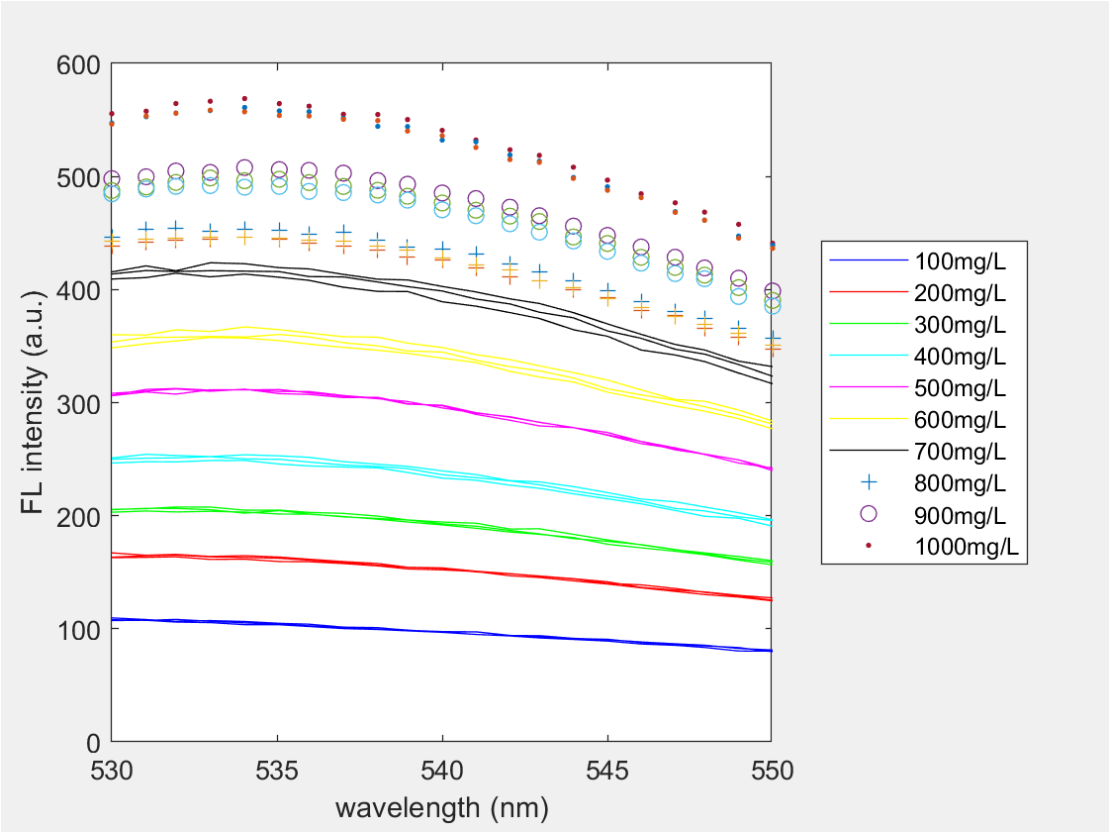

Figure 4.13 Results of albumin with different concentrations in artificial urine with increased slits

The response of TC-426 to different concentration albumin solutions was examined by plotting the peak fluorescence intensity versus the albumin concentration gradient (Figure 4.14).

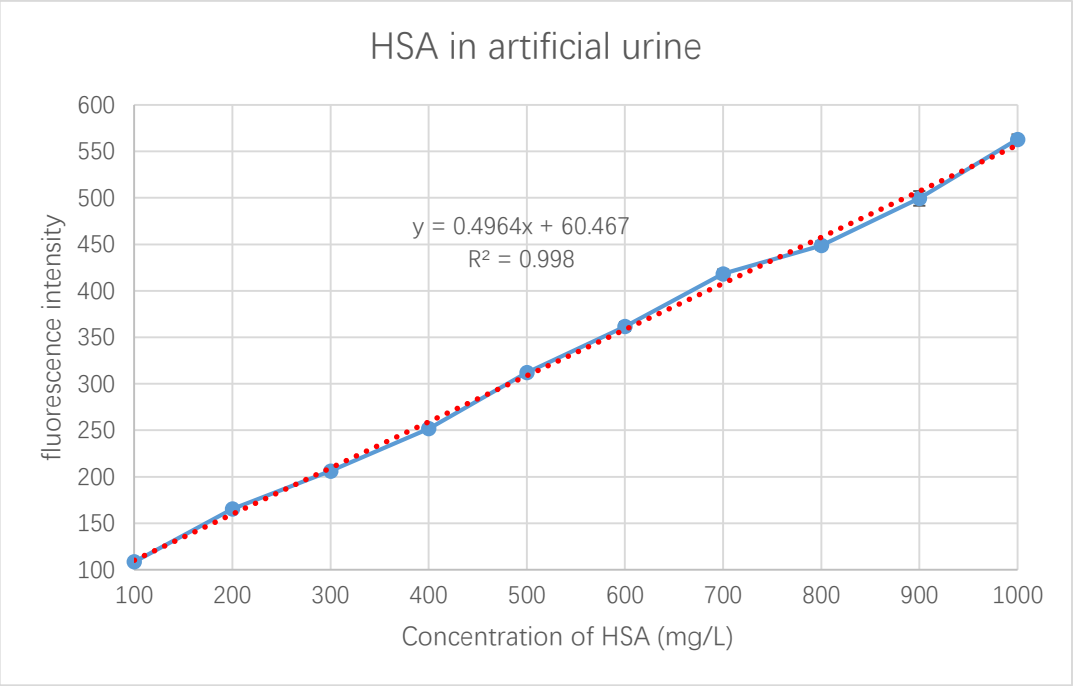

Figure 4.14 Correlation curve of albumin concentration and peak FL intensity in artificial urine

From the result, there was a significant increase of the overall fluorescence intensity. Moreover, R^2 is equal to 0.998 which is close to the result in section 4.2.2, which means the linear correlation has good repeatability. However, the experiment was taken under the ratio of albumin solution to TC-426 as 2:1, the optimal ratio for artificial urine have not been investigated. Furthermore, the fluorescence intensity in low concentration (100 mg/L to 200 mg/L) had been amplified above 100 a.u., therefore, it is able to evaluate the correlation between fluorescence intensity and albumin concentration in the threshold range of microalbuminuria.

4.2.4 Biosensor to albumin solution ratio

From literatures, it is known that the normal range of albumin is less than 20 mg/L, and between 20 mg/L and 200 mg/L is the threshold of microalbuminuria. Therefore, adjusted the range of albumin concentration to the range from 20 mg/L to 200 mg/L. Then, prepared albumin solutions with different concentrations, i.e., 25 mg/L, and 200 mg/L. Furthermore, mixed with proper amount of TC-426 with different ratios to albumin solutions, i.e., 1:1, 1:3, 1:5, 3:1, 5:1.

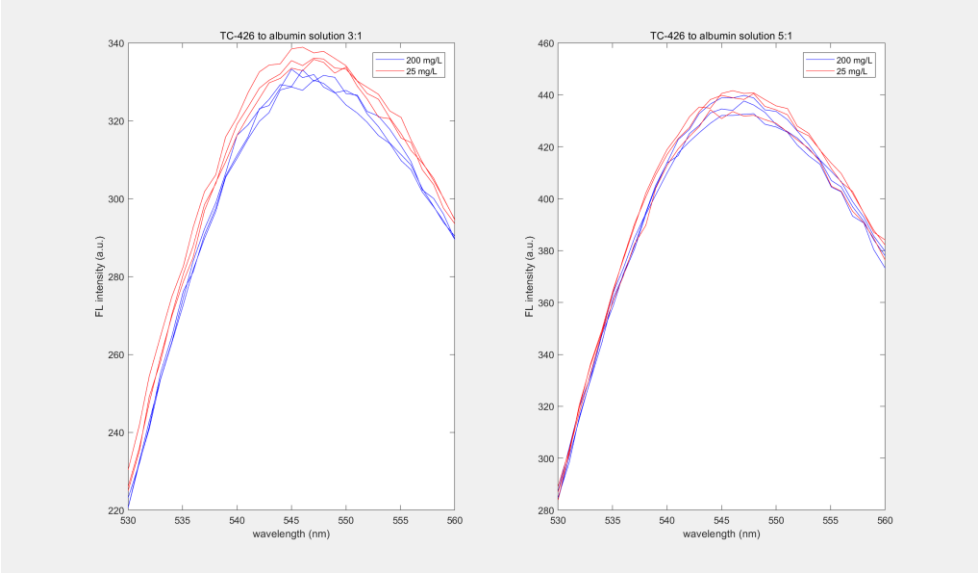

Figure 4.15 Result of TC-426 to albumin ratio 3:1 and 5:1 in artificial urine

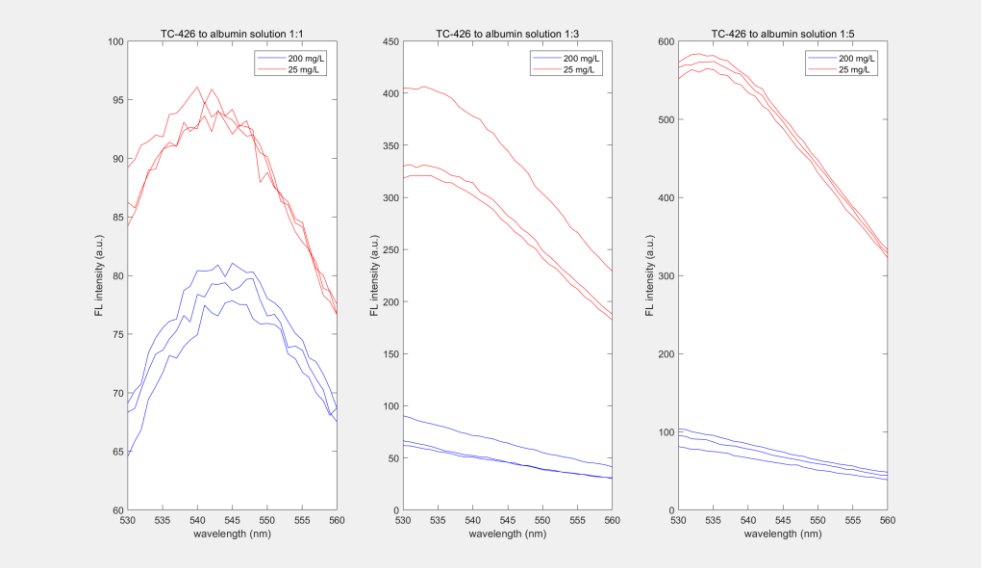

Figure 4.16 Result of TC-426 to albumin ratio 1:1, 1:3 and 1:5 in artificial urine

To make the results more intuitive, took the value of peak fluorescence intensity of each emission fluorescence spectrum (Figure 4.17).

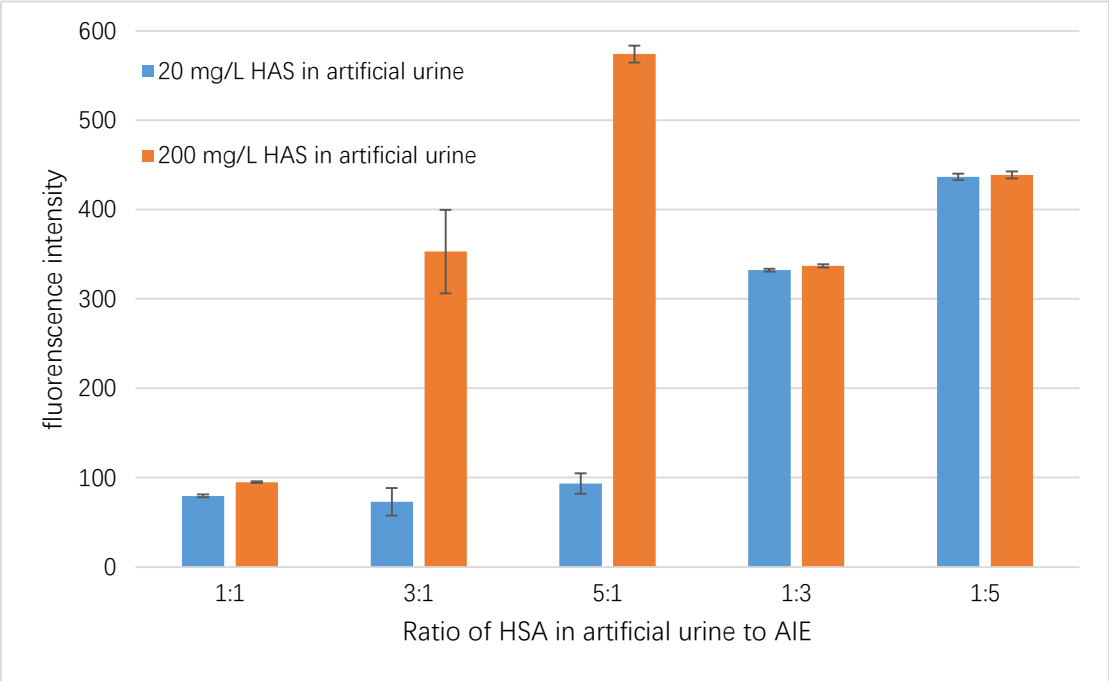

Figure 4.17 Results of different ratios of albumin to TC-426 in artificial urine

From the result, when the ratio of albumin solution to TC-426 was 5:1, the difference of the peak fluorescence intensity between 200 mg/L and 20 mg/L albumin solution is the greatest. Moreover, from the emission fluorescence spectrums, it was found that when the amount of TC-426 was greater than the amount of albumin solution, i.e., ratio of TC-426 to albumin solution is 3:1 and 5:1 respectively, the peak fluorescence intensities of 200 mg/L and 20 mg/L albumin solution were almost same (Figure 4.15), and the wavelength of the peak fluorescence intensity was at 545 nm. Furthermore, when the amount of TC-426 was equal to the amount of albumin solution, a slight difference of the peak fluorescence intensities between the two different concentrations of albumin solution appeared (Figure 4.16), and the peak fluorescence intensity had a dramatic decrease. Besides, the wavelength of the peak fluorescence intensity shifted to 540 nm. Finally, when the amount of TC-426 was less than the amount of albumin solution, i.e., ratio of TC-426 to albumin solution is 1:3 and 1:5 respectively, the significant differences of the peak fluorescence intensities between

the two albumin solutions can be observed (Figure 4.2.4.2), and the wavelength of the peak fluorescence intensity was at 535 nm, this phenomenon also happened in the previous experiments in section 4.1.

One possible reason can be considered as that when the amount of TC-426 was greater than the amount of albumin solution, the fluorescence was emitted by TC-426 itself (the complex of TC-426 and albumin molecules was negligible since the albumin is insufficient), therefore the peak fluorescence was at 545 nm, and the fluorescence intensity increased as the amount of TC-426 increased (increased from 3 parts to 5 parts). When the amount of TC-426 was equal to the amount of albumin solution, the fluorescence was emitted by part of TC-426 and part of the complex of TC-426 and albumin molecules, and the peak fluorescence intensity shifted to 540 nm. Finally, when the amount of TC-426 was less than the amount of albumin solution, the fluorescence was emitted completely by the complex of TC-426 and albumin molecules, the peak fluorescence intensity shifted to 535 nm, and the peak fluorescence intensity increased as the amount of albumin solution increased (increased from 3 parts to 5 parts). Another possible reason is that changing environment promotes to shift the spectrum. TC-426 was ordinally resolved in DMSO since it is consisted of phenyl rings that are insoluble in water; therefore, adding water or artificial urine can cause the blue shift.

4.2.5 Correlation between albumin concentration and its fluorescence intensity

Prepared albumin solutions with different concentrations i.e., 25 mg/L, 50 mg/L, 75 mg/L, 200 mg/L. Then, added the correct amount of TC-426 into the albumin solution as 5:1 is the ratio of albumin solution to TC-426. Incubated the samples for 20 minutes and poured each sample equally into three cuvettes. Used 510 nm as the excitation wavelength and set the slit as 10* 10 nm. Measured the emission fluorescence spectrum of each sample (Figure 4.18).

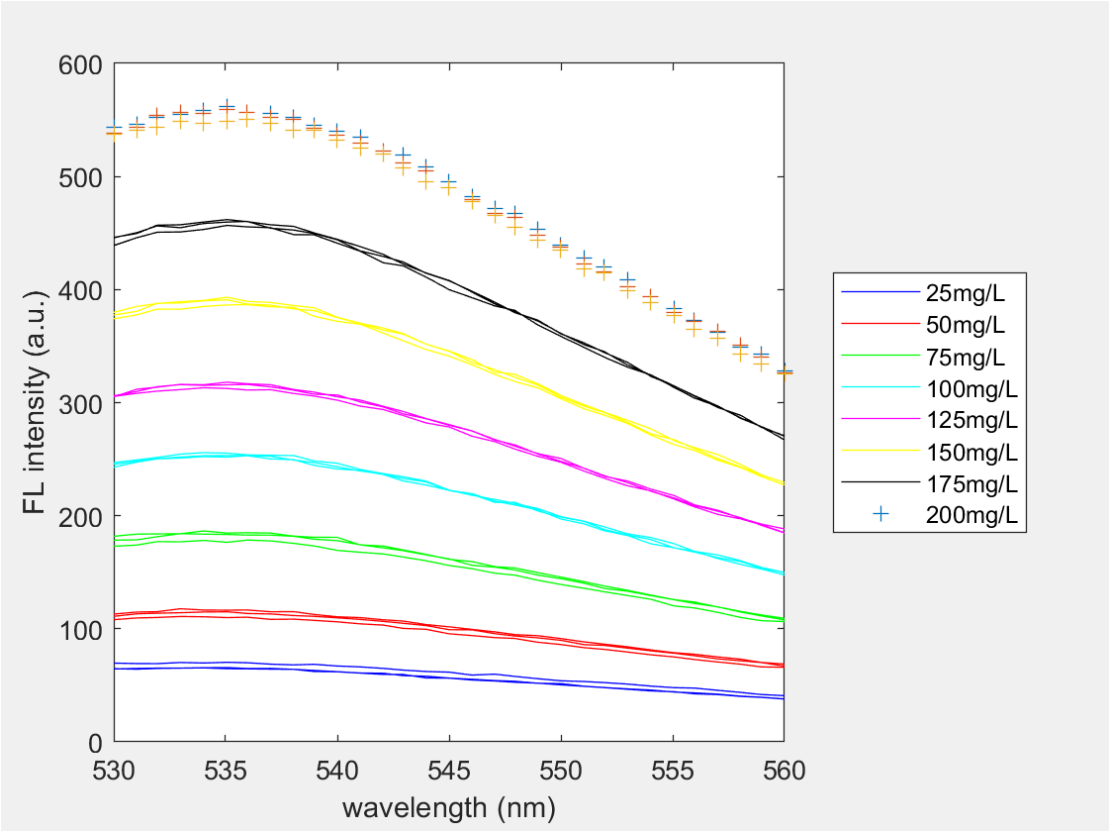

Figure 4.18 Results of albumin with different concentrations in artificial urine

Same process as in section 4.1.3, took the mean value of the peak fluorescence intensity of each sample, plot the correlation curve in terms of the albumin concentration and its corresponding fluorescence intensity, the result is shown in Figure 4.19.

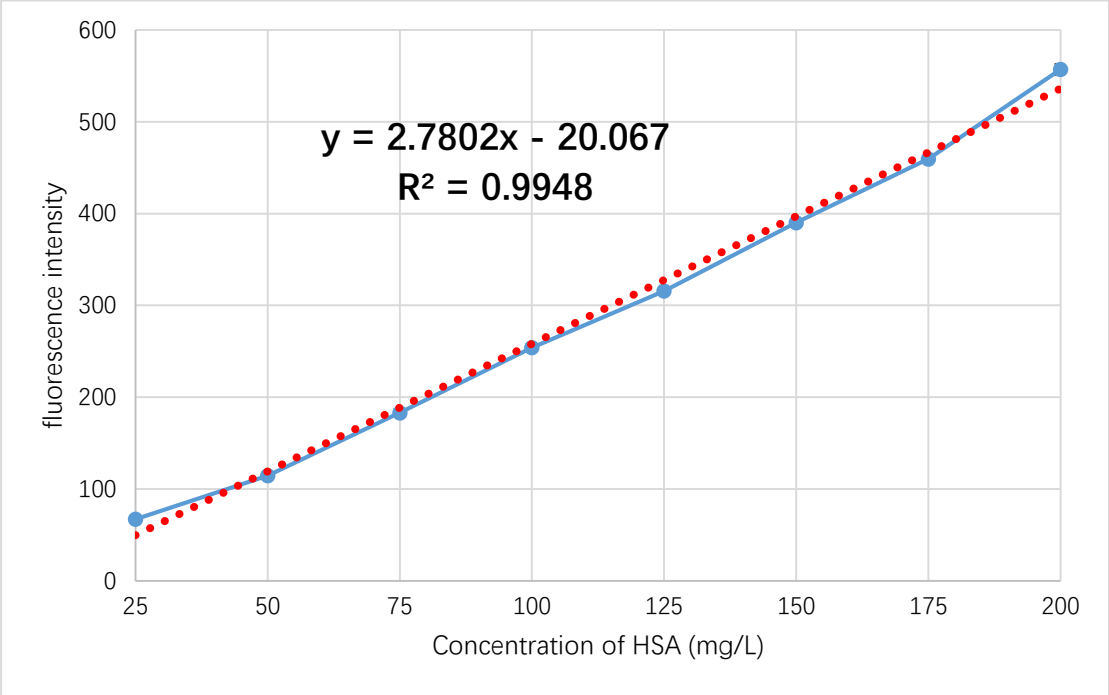

Figure 4.19 Correlation curve of albumin concentration and peak FL intensity in artificial urine

From the result, the threshold range of microalbuminuria is within the linear dynamic range with $R^2 = 0.99$. It presents an excellent sensitivity to the albumin in artificial urine.

4.2.6 Interference of creatinine

To evaluate the interference of the presence of creatinine, correct amount of creatinine (0.007 g as calculated in section 3.5) was added into 10 mL of each albumin solution with different concentrations. Mixed the albumin solution with creatinine thoroughly and poured each sample into a tube, added correct amount of TC-426 to make albumin solution to TC-426 ratio as 5:1. Placed each sample on the vortex mixer and mixed for 10 seconds respectively, and incubated the samples for 20 minutes. Then, poured each sample equally into three cuvettes, and scanned the emission fluorescence spectrums (Figure 4.20).

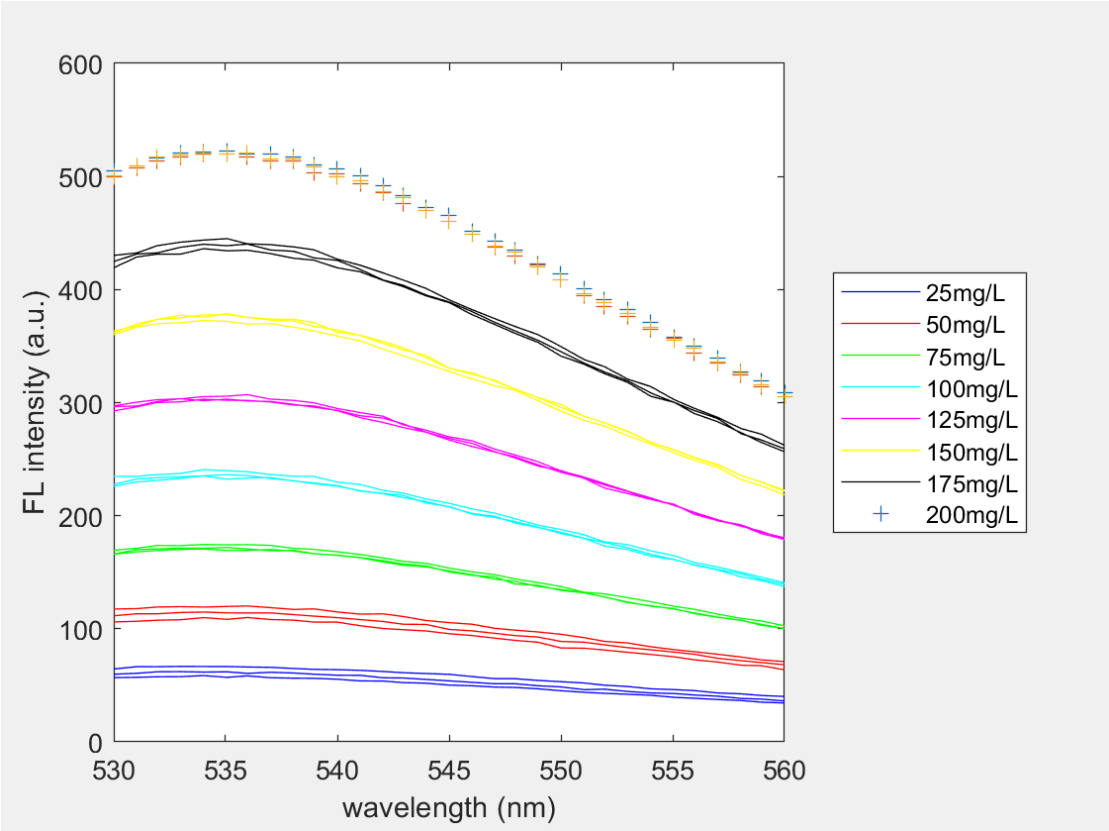

Figure 4.20 Results of albumin with different concentrations in artificial urine with the presence of creatinine

Took the mean value of the peak fluorescence intensity of each sample, plot the correlation curve in terms of the albumin concentration and its corresponding fluorescence intensity, the result is shown in Figure 4.21.

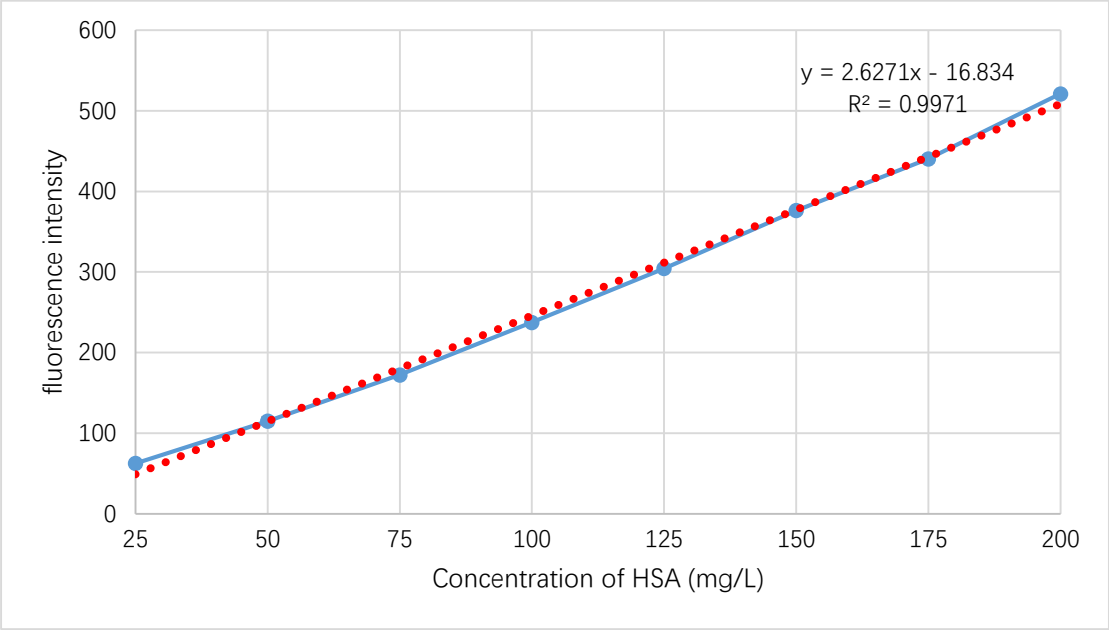

Figure 4.21 Correlation curve of albumin concentration and peak FL intensity in artificial urine with the presence of creatinine

From the result, it can be found that after adding creatinine, the correlation curve is still linear. To find out the interference caused by creatinine, plotted the result with the result in section 4.2.5 together as Figure 4.22 shows.

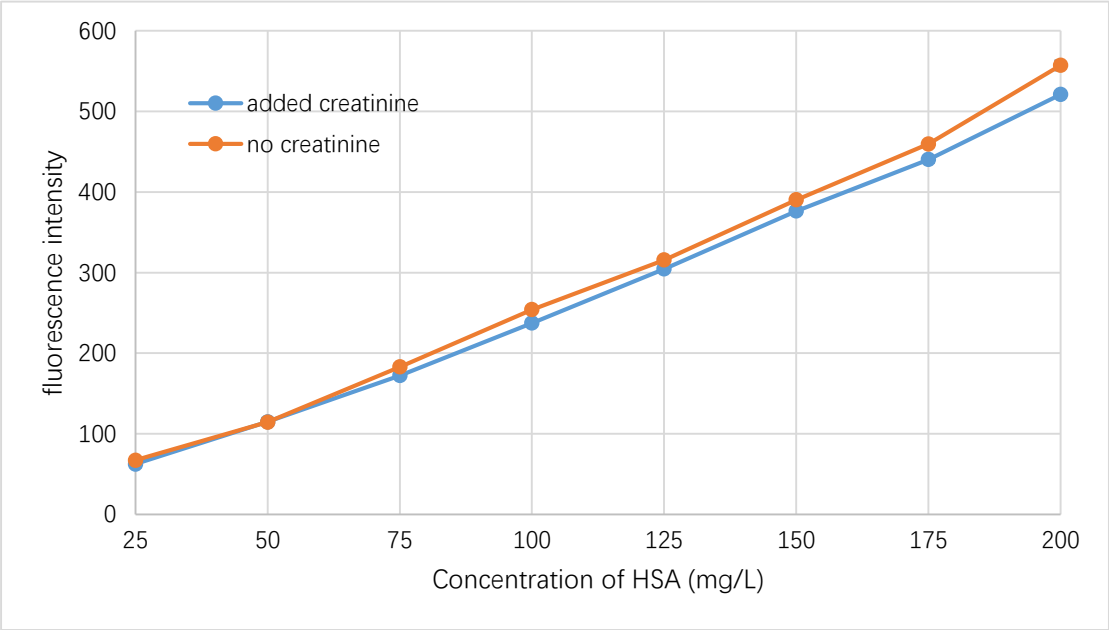

Figure 4.22 Comparison between the results of correlation curve with and without creatinine

When added creatinine into the albumin solution, the overall peak fluorescence intensity of albumin solution with different concentrations decreased. However, it was found that when the albumin solution was low (for example below 50 mg/L), the change of peak fluorescence intensity was negligible. Moreover, as the concentration of albumin increased, the difference between the peak fluorescence intensities of albumin solution with and without creatinine increased correspondingly, and when the albumin solution went to the highest level at 200 mg/L, the difference was the greatest. The mean value of the peak fluorescence intensity of the 200 mg/L albumin solution without creatinine is 557 a.u. while the peak fluorescence intensity of the 200 mg/L albumin solution with creatinine is 521 a.u., the difference is 56 a.u. which is about 10% of the maximum fluorescence intensity. As a conclusion, the presence of creatinine poses certain interference to the measurement of fluorescence intensity of albumin solution. However, the interference is about 10% of the maximum fluorescence intensity. Moreover, the amount of creatinine was calculated according to the threshold value of microalbuminuria, for macroalbuminuria, the urine albumin to creatinine ratio would be higher which means the amount of creatinine can be less comparing to the amount of albumin.

4.3 Biosensor with albumin in real urine sample

4.3.1 Incubation time

Resolved correct amount of albumin in real urine sample to make 1000 mg/L albumin solution. The reasons of taking the concentration of 1000 mg/L of albumin solution rather than 200 mg/L are: first, the incubation time of higher concentration of albumin solution takes longer since there are more molecules to interact with TC-426, therefore, the incubation time of a higher concentration of albumin solution is sufficient for lower concentration of albumin solution. Second, in real urine sample, the environment became more complex comping to in DI water and artificial urine and the background

source increased, using low concentration of albumin solution may be hard to observe the fluorescence emission. Therefore, using high concentration of albumin solution can make the fluorescence more significant. Then, took equal amount of albumin solution and TC-426 into a tube, placed the tube on the vortex mixer and mixed for 10 seconds. Then, poured the mixed solution into a cuvette and used the kinetic model of spectrometer to get the emission fluorescence intensity. The configurations of the spectrometer used 510 nm as the excitation wavelength and 535 as the emission wavelength, and the size of slit was 10*10 nm. The result is shown in Figure 4.23.

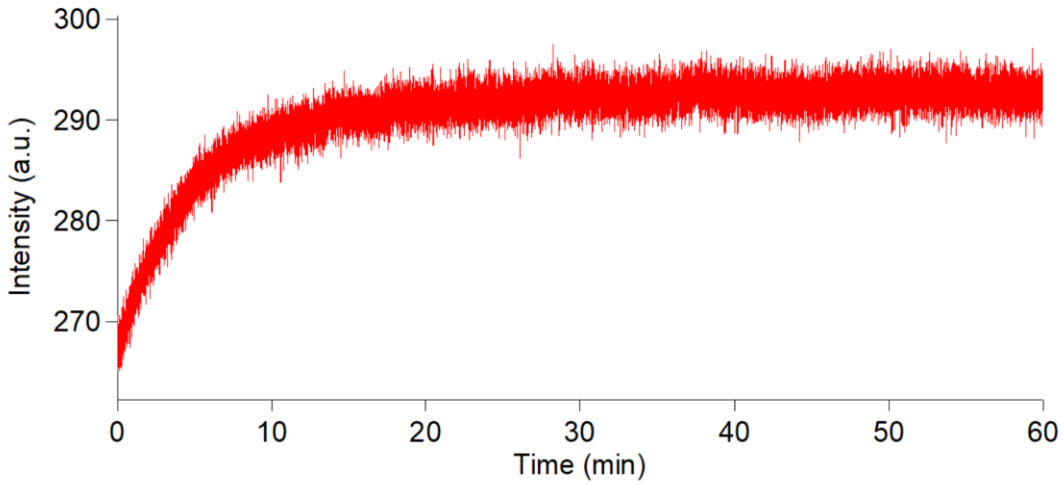

Figure 4.23 Incubation time in real urine sample

From the result, it was found that after 20 minutes, the fluorescence intensity became stable. Therefore, the incubation time can take 20 minutes.

4.3.2 Dilution of urine ratio

From the previous experiment in section 4.2.4, it was known that, in artificial urine, 5:1 is the optimal ratio of albumin solution to TC-426. Therefore, tentatively employed 5:1 as the ratio of albumin in real urine sample to TC-426. Then, diluted 200 mg/L and 25 mg/L of albumin solution (in urine) with DI water into different dilution ratio. Then, used the same procedures to measure the emission fluorescence spectrums of each

sample. The configurations were: excitation wavelength: 510 nm, size of slit: 10 nm* 10 nm, incubation time 20 minutes, albumin solution to TC-426 ratio: 5:1.

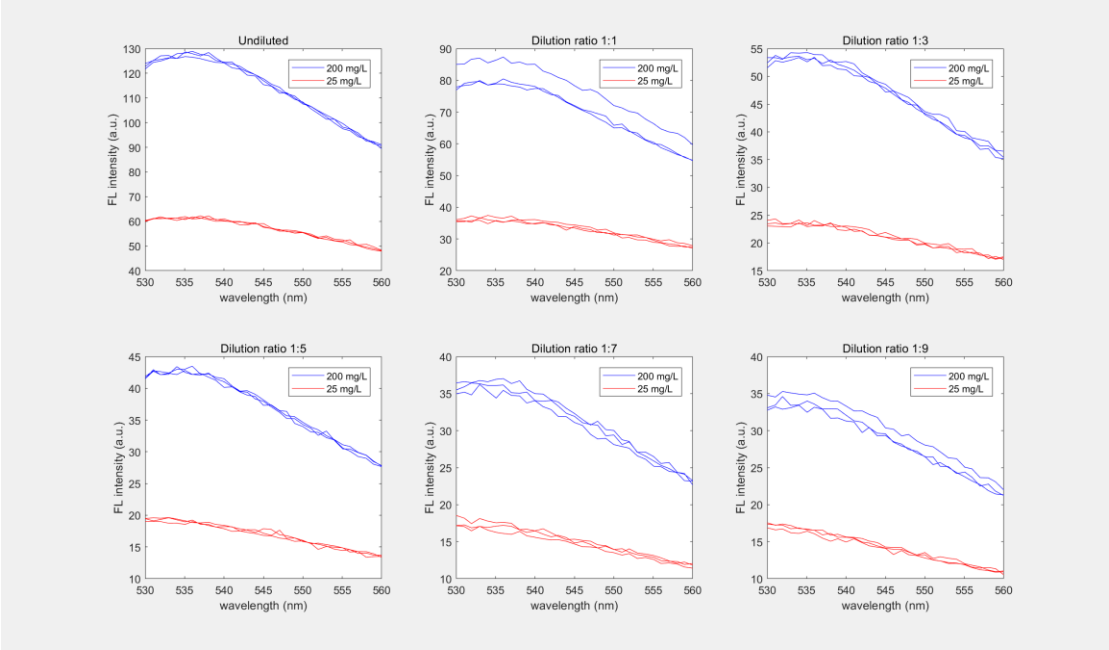

Figure 4.24 Spectrums of different dilution ratios

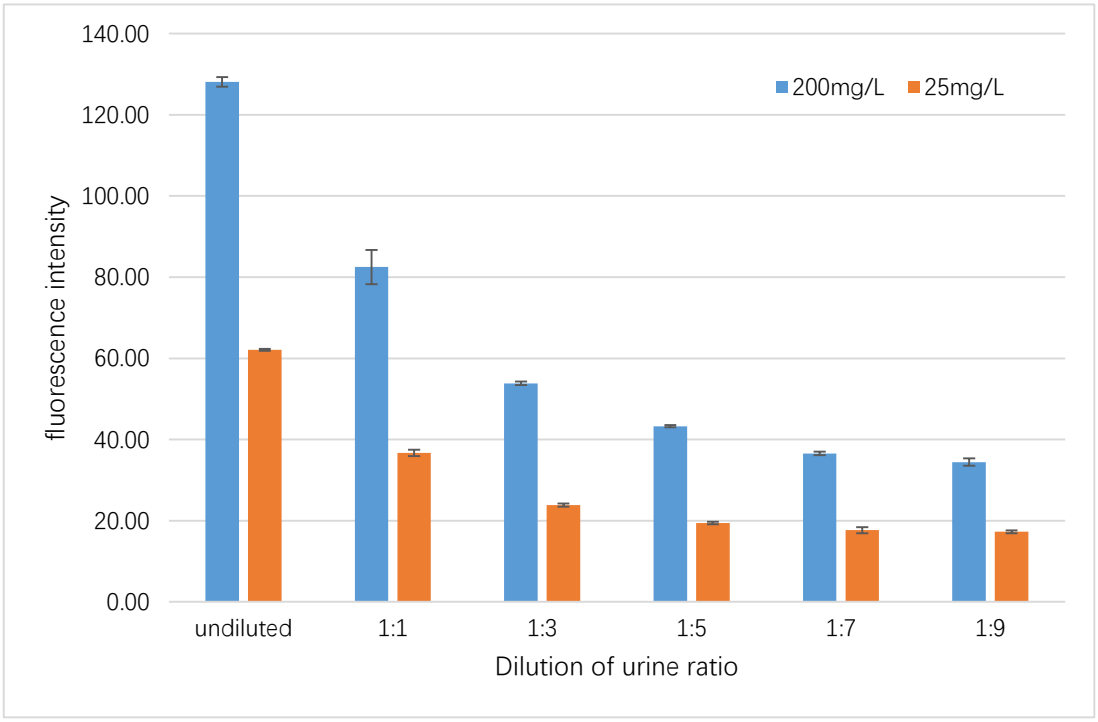

Figure 4.25 Result of each dilution ratio of urine

It can be found that the undiluted sample had the best performance since the difference of the peak fluorescence intensities between 200 mg/L and 25 mg/L albumin solution was the greatest. Therefore, there is no need to dilute the albumin solution.

In the evaluation of dilution of urine ratio, there are two different ways to evaluate the influence caused by diluting the urine sample. First, resolved albumin in the undiluted urine to make albumin solution with certain concentration, then diluted the albumin solution with different portions of DI water. Second, diluted the urine with different portions of DI water, then resolved albumin in the diluted urine to make albumin solution with certain concentration. In this thesis, the experiment adopted the first method. Because from the practical application point of view, this biosensor was designed to detect the albumin level in human urine, which essentially measures the albumin that is already in the urine rather than adding extra albumin into urine. However, the second method can be considered as further evaluation of the interference caused by the background source of urine sample.

4.3.3 Biosensor to albumin solution ratio

Prepared 200 mg/L and 20 mg/L albumin solutions, added correct amount of TC-426 to make different albumin solution to TC-426 ratio, i.e., 1:1, 1:2, 1:5, 2:1, 5:1. Placed the mix solution on the vortex mixer and mixed for 10 seconds, then incubated each sample for 20 minutes. Finally poured each sample equally into three cuvettes and measured the fluorescence emission spectrums. The excitation wavelength was 510 nm, and the size of slit was 10 nm* 10 nm. The result of albumin solution to TC-426 ratio 1:1 is shown as Figure 4.26.

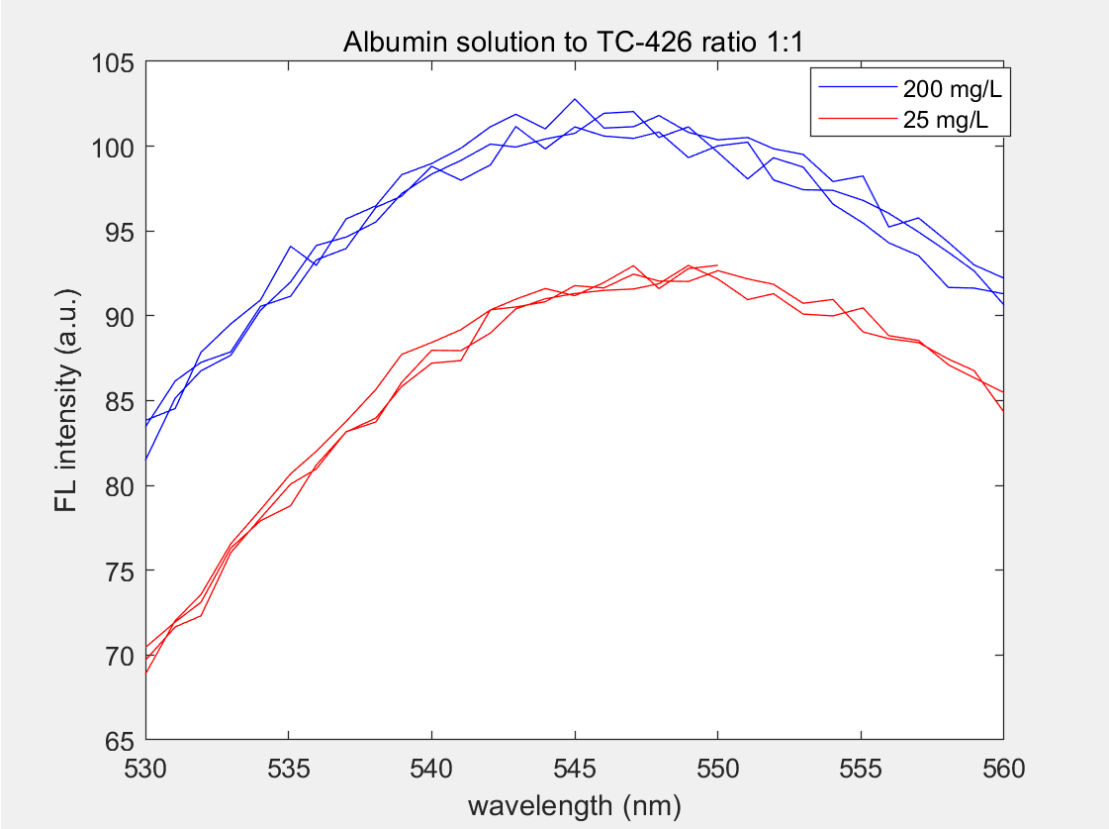

Figure 4.26 Result of TC-426 to albumin ratio 1:1 in real urine sample

The result of albumin solution to TC-426 ratio 1:2 and 1:5 are shown in Figure 4.27.

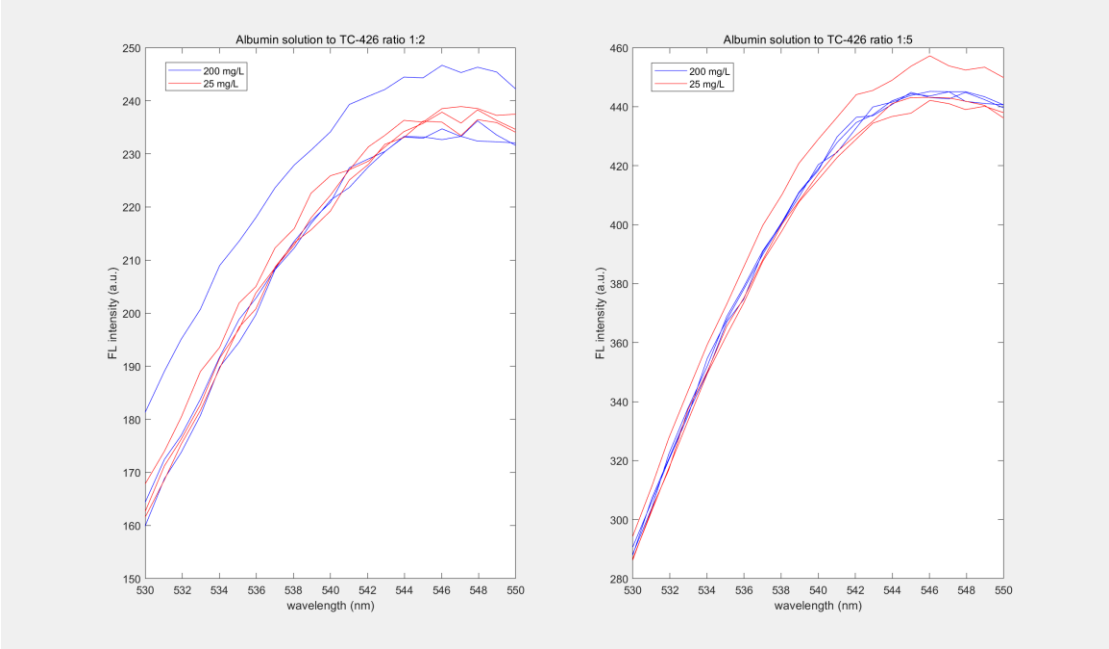

Figure 4.27 Result of TC-426 to albumin ratio 1:2 in real urine sample

The result of albumin solution to TC-426 ratio 2:1 is shown as Figure 4.28.

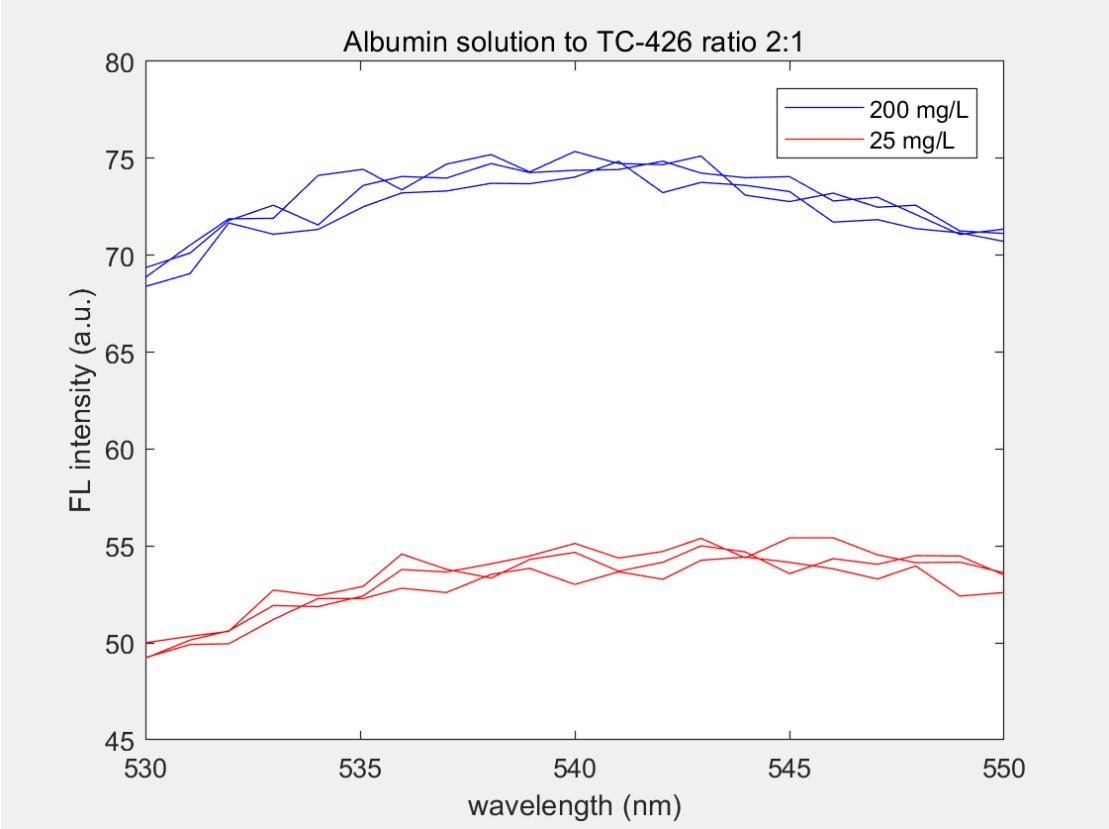

Figure 4.28 Result of TC-426 to albumin ratio 2:1 in real urine sample

The result of albumin solution to TC-426 ratio 5:1 is shown as Figure 4.29.

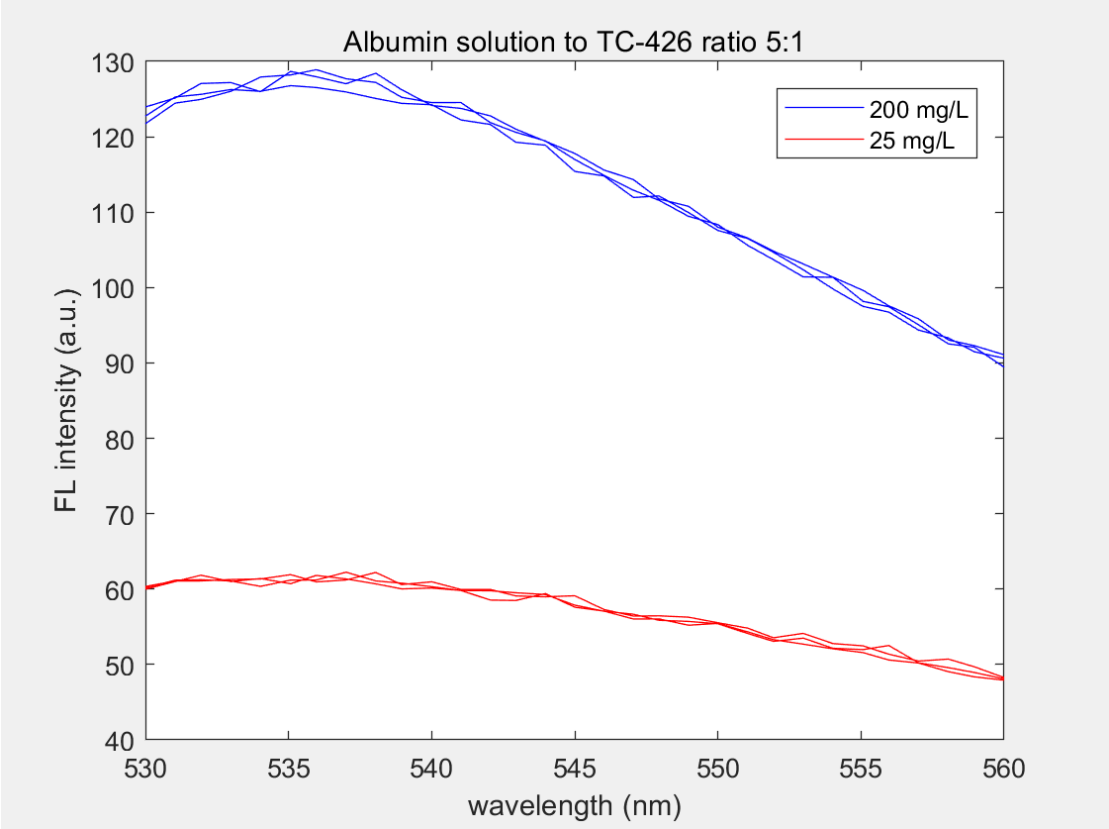

Figure 4.29 Result of TC-426 to albumin ratio 5:1 in real urine sample

From the emission fluorescence spectrums, it was found that the results were similar to the results in section 4.2.4. When the amount of TC-426 was greater than the amount of albumin solution, the peak fluorescence intensity was at 545 nm, and as the amount of TC-426 increased, the fluorescence intensity had a significant increased. But the difference of the fluorescence intensities between 200 mg/L and 25 mg/L albumin solutions was negligible. Moreover, when the amount of TC-426 was equal to the amount of albumin solution, a difference of the fluorescence intensities between 200 mg/L and 25 mg/L albumin solutions appeared. Furthermore, when the amount of TC-426 was less than the amount of albumin solution, the difference of the fluorescence intensities between 200 mg/L and 25 mg/L albumin solutions increased, and as the amount of albumin solution increased, the difference increased accordingly. Besides, when the ratio of albumin solution to TC-426 went to 5:1, the peak fluorescence intensity shifted to 535 nm.

Took the mean value of the peak fluorescence intensities of each sample, the result is shown in Figure 4.30.

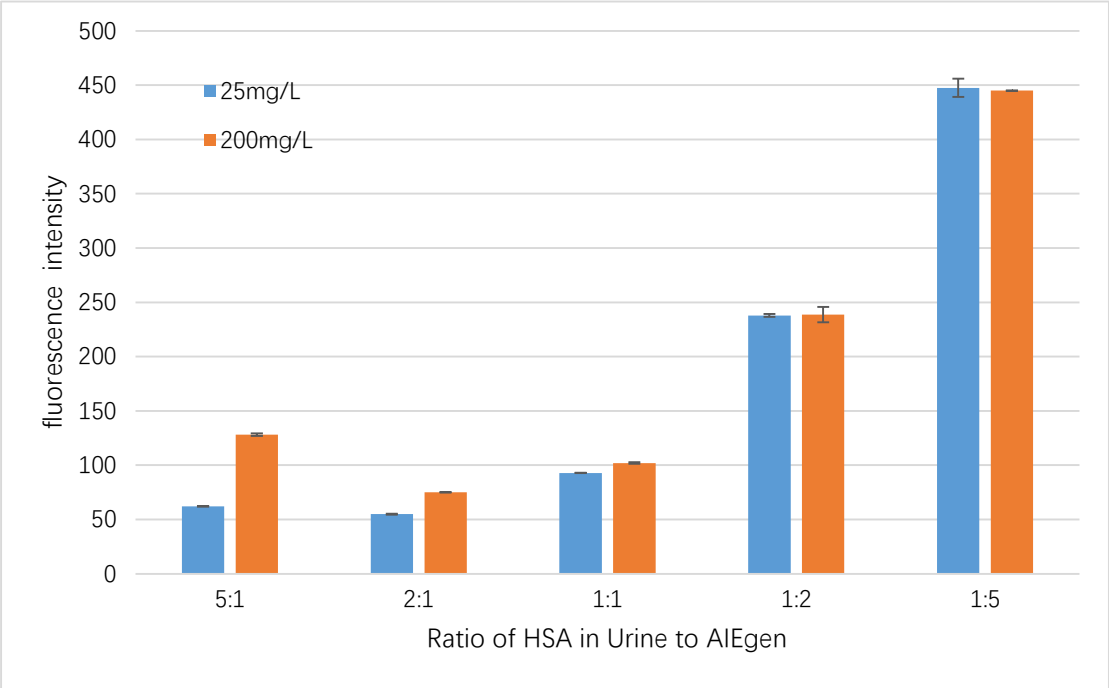

Figure 4.30 Result of different ratios of albumin to TC-426

From the result, when the amount of TC-426 was greater than the amount of albumin solution, although the peak fluorescence intensities were high, the difference of the fluorescence intensity of the two albumin solutions was negligible. However, when the ratio of albumin solution to TC-426 was 5:1, there was a significant difference between 200 mg/L and 25 mg/L albumin solutions. Therefore, in these 5 different ratios, 5:1 is the optimal ratio.

4.3.4 Correlation between albumin concentration and its fluorescence intensity

Prepared adequate amounts of albumin solutions with different concentrations (25 mg/L, 50 mg/L, 75 mg/L, 200 mg/L). Then, added the correct amount of TC-426 into the albumin solution as 5:1 is the ratio of albumin solution to TC-426. Incubated the samples for 5 minutes and poured each sample equally into three cuvettes. Used 510 nm as the excitation wavelength and set the slit as 10* 10 nm. Measured the emission fluorescence spectrum of each sample. The results for each sample were shown in Figure 4.31.

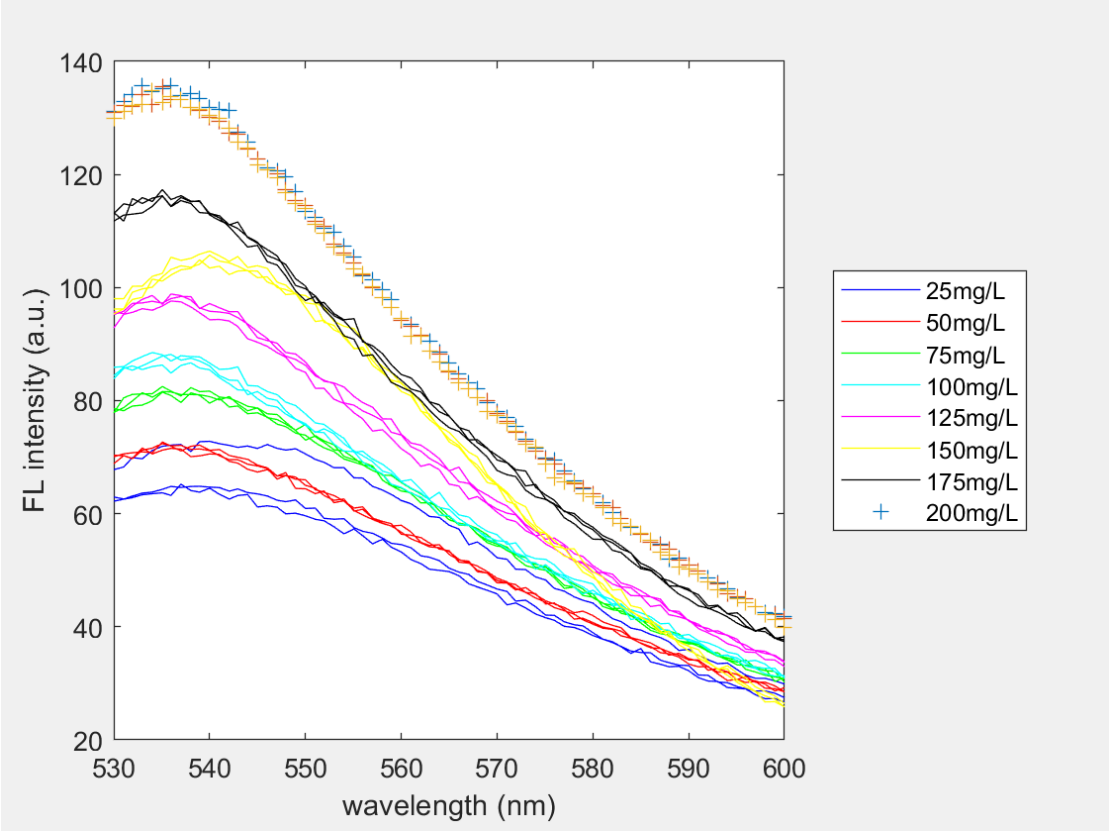

Figure 4.31 Results of albumin with different concentrations in real urine sample

Took the mean value of the peak fluorescence intensities of each sample, the result is shown in Figure 4.32.

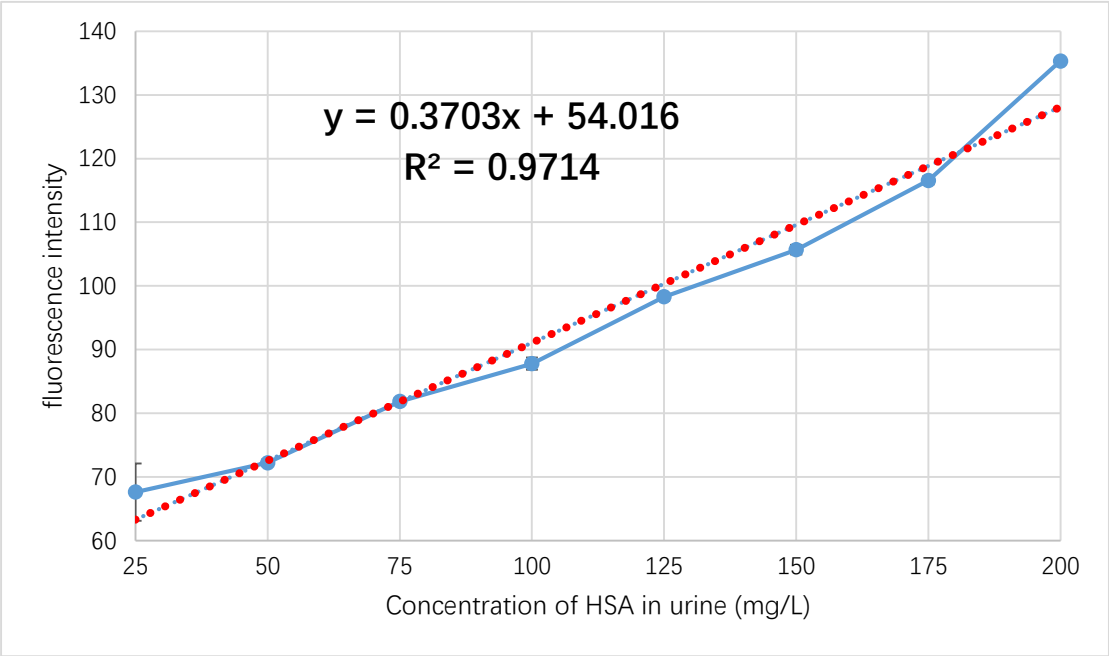

Figure 4.32 Correlation curve of albumin concentration and peak FL intensity in real urine sample

From the result, it presents a linear correlation curve with R^2 = 0.9714 and minor deviation, as the concentration of albumin solution increased, the peak fluorescence intensity increased accordingly. Comparing to artificial urine (Figure 4.33), it is obvious that when the environment changed from artificial urine to real urine sample, the fluorescence intensity dropped significantly. This might cause by the interference from the stronger background source in the urine sample, and certain substances in urine may interact with TC-426 molecules, which can restrain them to enter the hydrophobic pocket of albumin. Additionally, in real urine sample, there may be certain amount of albumin and creatinine, which can interference the result. Furthermore, the slope of the correlation curve in real urine sample decreased comparing to in artificial urine, leading a decrease in the sensitivity of TC-426. However, it still presented a linear correlation between the albumin concentration and the peak fluorescence intensity, which means in the real urine sample, TC-426 can still function in a reasonable level.

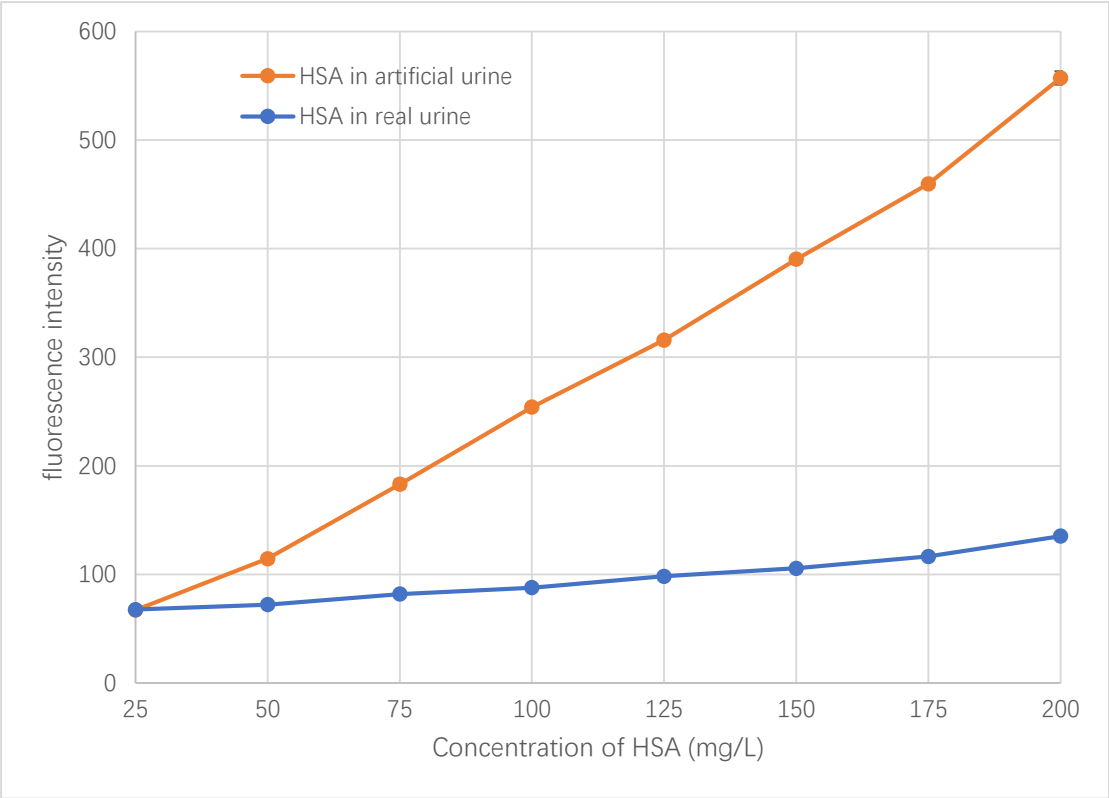

Figure 4.33 Comparison of the results in DI water and artificial urine

4.3.5 Interference of creatinine

To evaluate the interference of creatinine in real urine sample, added 0.007 g creatinine into 10 mL of each albumin solution with different concentrations. Mixed the albumin solution with creatinine thoroughly and pored each sample into a tube, added correct amount of TC-426 to make albumin solution to TC-426 ratio as 5:1. Placed each sample on the vortex mixer and mixed for 10 seconds respectively, then incubated the samples for 20 minutes. Then, poured each sample equally into three cuvettes, and scanned the emission fluorescence spectrums. The result of each sample is shown in Figure 4.34.

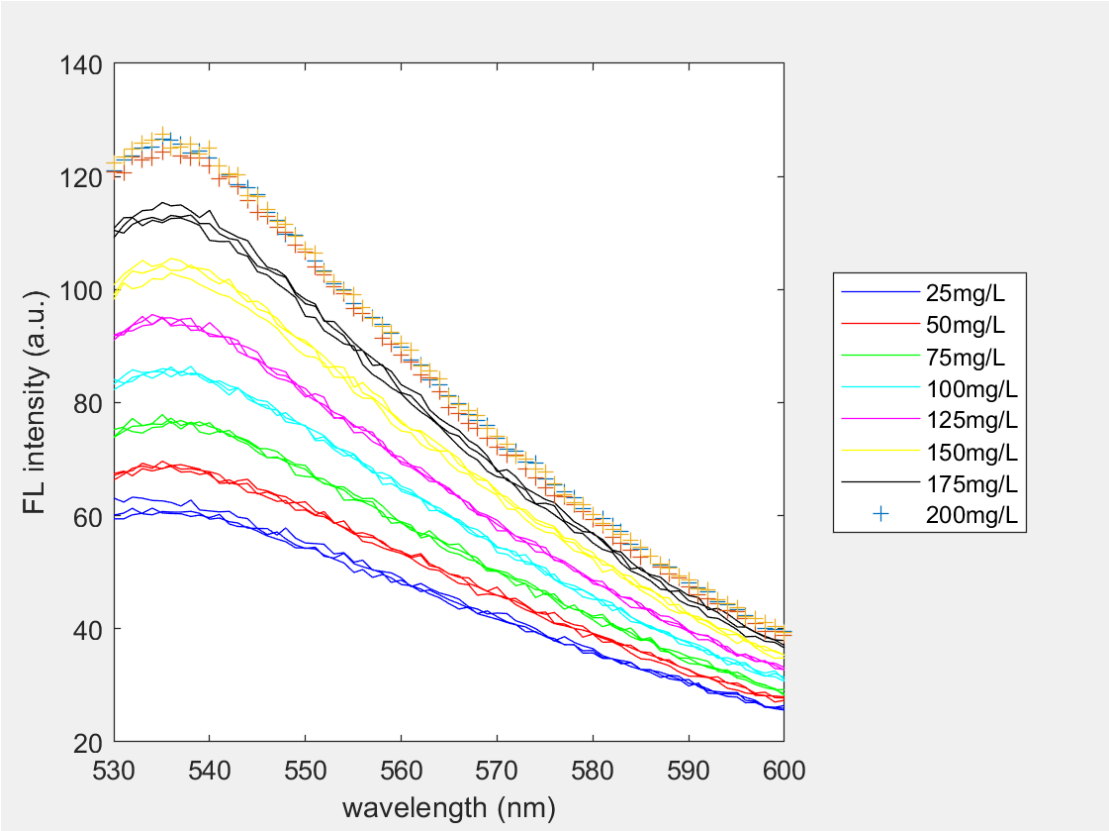

Figure 4.34 Results of albumin with different concentrations in real urine sample with the presence of creatinine

Took the mean value of the peak fluorescence intensity of each sample, the result is shown in Figure 4.35.

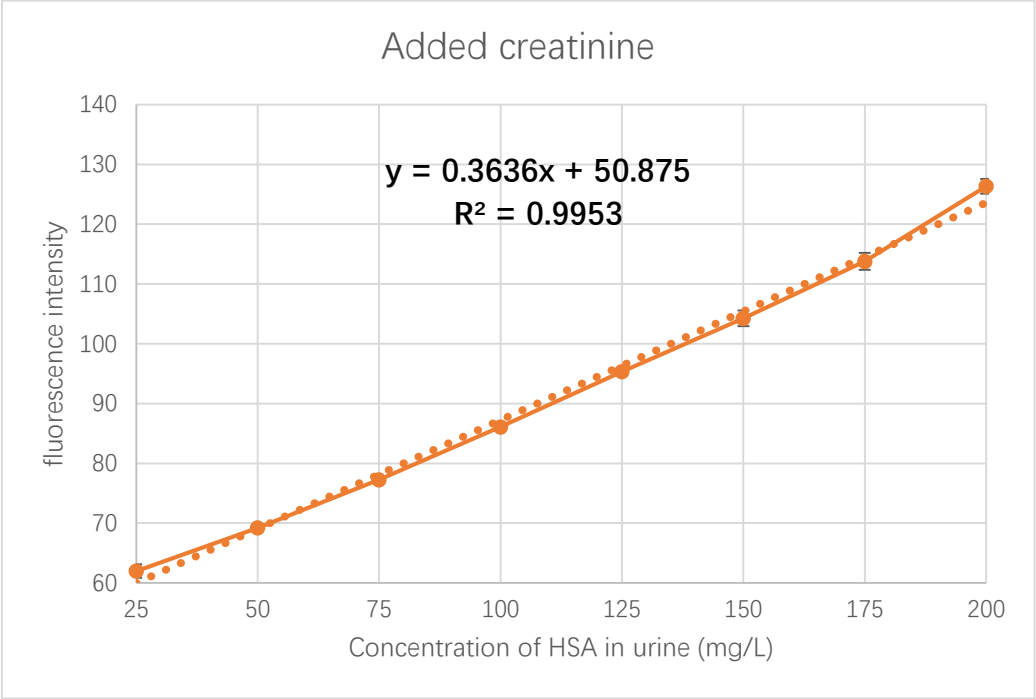

Figure 4.35 Correlation curve of albumin concentration and peak FL intensity in artificial urine with the presence of creatinine

It was found that after adding creatinine, the linearity of the correlation curve increased, with R^2 = 0.9953, TC-426 became more sensitivity. Plot with the result in Figure 4.3.4.2 together for comparison, the result is shown in Figure 4.36.

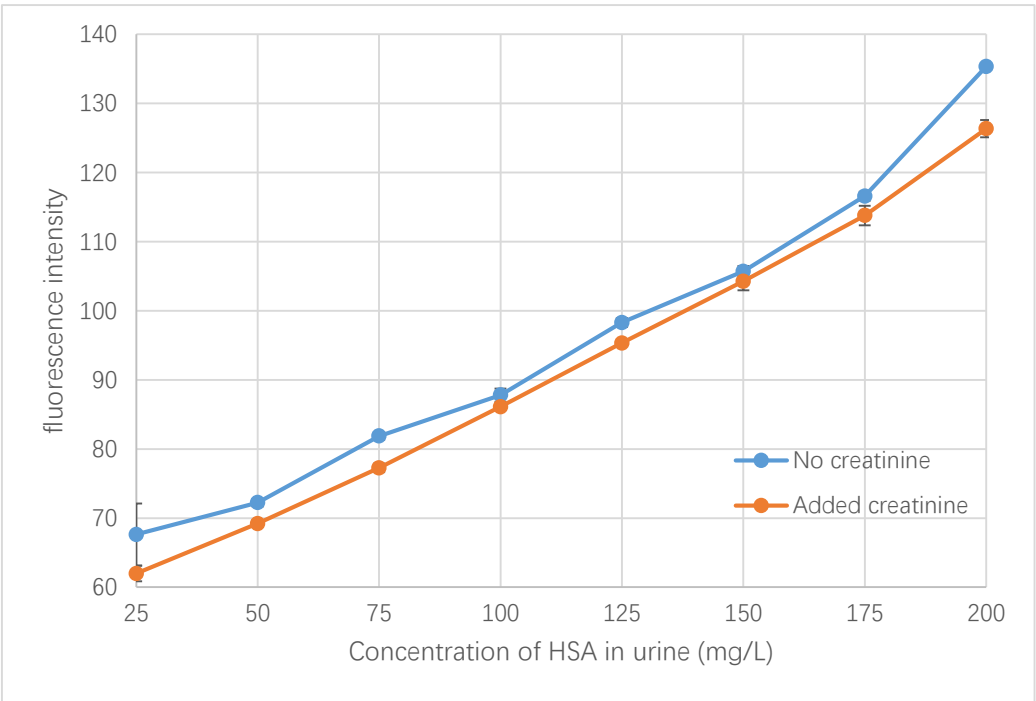

Figure 4.36 Comparison between the results of correlation curve with and without creatinine

From the result, when added creatinine into the albumin solutions, the fluorescence intensity had a slight decrease, which is similar to the result in artificial urine. However, when the albumin was in lower than 50 mg/L, unlike in artificial urine, the peak fluorescence intensity decreased after adding creatinine. In artificial urine, the fluorescence intensity tended to decrease after adding creatinine, and the fluorescence intensity decreased more as the albumin concentration increased. The interference needs to be further evaluated especially in the concentration below 50 mg/L. Because after adding creatinine, the slope in the range of 25 mg/L to 50 mg/L increased, which made the fluorescence intensity of 50 mg/L albumin solution with creatinine decrease to the same level of 25 mg/L albumin solution without creatinine; therefore, the accuracy of the result will decrease.

4.4 Comparison of the correlation of albumin concentration and fluorescence intensity in different environments

The correlation of albumin concentration and fluorescence intensity was evaluated in DI water, artificial urine and the real urine sample respectively. All of them presented a linear correlation. Particularly, as the albumin concentration increased, the fluorescence intensity increased correspondingly. Moreover, the incubation time was same in the three different environments; however, different environments altered the result in certain extend. To be specific, it was found that in DI water, the fluorescence intensity was the strongest, and the fluorescence intensity was the weakest in real urine sample. This phenomenon is due to the complexity of substances in the environments and the background source of the solvent. To the naked eye, DI water is clear and transparent, artificial urine is less transparent and is turbid, and the real urine sample has increased turbidity, and its colour became pale yellow.

Furthermore, the peak fluorescence intensity shifting phenomenon was found in all environments. For example, in artificial urine, when the amount of TC-426 was greater than the amount of albumin solution, the peak fluorescence intensity was at 545 nm, which is the emission wavelength of TC-426 itself. When the portion of TC-426 decreased to the same level as albumin solution, the peak fluorescence intensity shifted from 545 nm to 540 nm; then, when the amount of albumin solution was greater than TC-426, the peak fluorescence intensity shifted from 540 nm to 535 nm. This shifting phenomenon happened in real urine sample as well; however, the shift of the peak fluorescence intensity became ‘slower’. To be specific, the peak fluorescence intensity was at 545 nm all the time until the ratio of TC-426 to albumin became 1:5. This peak fluorescence shifting phenomenon can help with the understanding of the interaction between albumin solution and TC-426 to a certain extend. 535 nm can be considered as the emission wavelength of the complex of albumin molecule and TC-426 molecule. Moreover, the shifting processes is worth

further investigation, because when the peak fluorescence intensity is at 535 nm, the amount of TC-426 is less than the amount of albumin solution; it is unknown whether the amount of TC-426 is adequate to interact with albumin molecules; therefore, the threshold ratio that makes the peak fluorescence intensity shift needs to be evaluated to optimise the performance.

In addition, the sensitivity of TC-426 is strongest in DI water, and weakest in real urine sample, as the slope of the correlation curve in DI water is the greatest, and in real urine sample is the weakest. It can due to the environmental complexity. For example, the composition of urine is complex and uncertain, it contains the physiological waste of body including albumin, creatinine, or other substances that can disturbs the tracing of albumin. The interaction between albumin and TC-426 is thus interfered with, resulting in reduced sensitivity. Moreover, the pH value in the three environments may be different, the interference caused by pH value need to be further investigated.

4.5 Comparison with other biosensors for detecting albumin

Tu et al., (2019) designed a class of water-soluble tetrazolate-functionalized derivatives called TPE-4TA for the quantification of albumin (Figure 4.37).

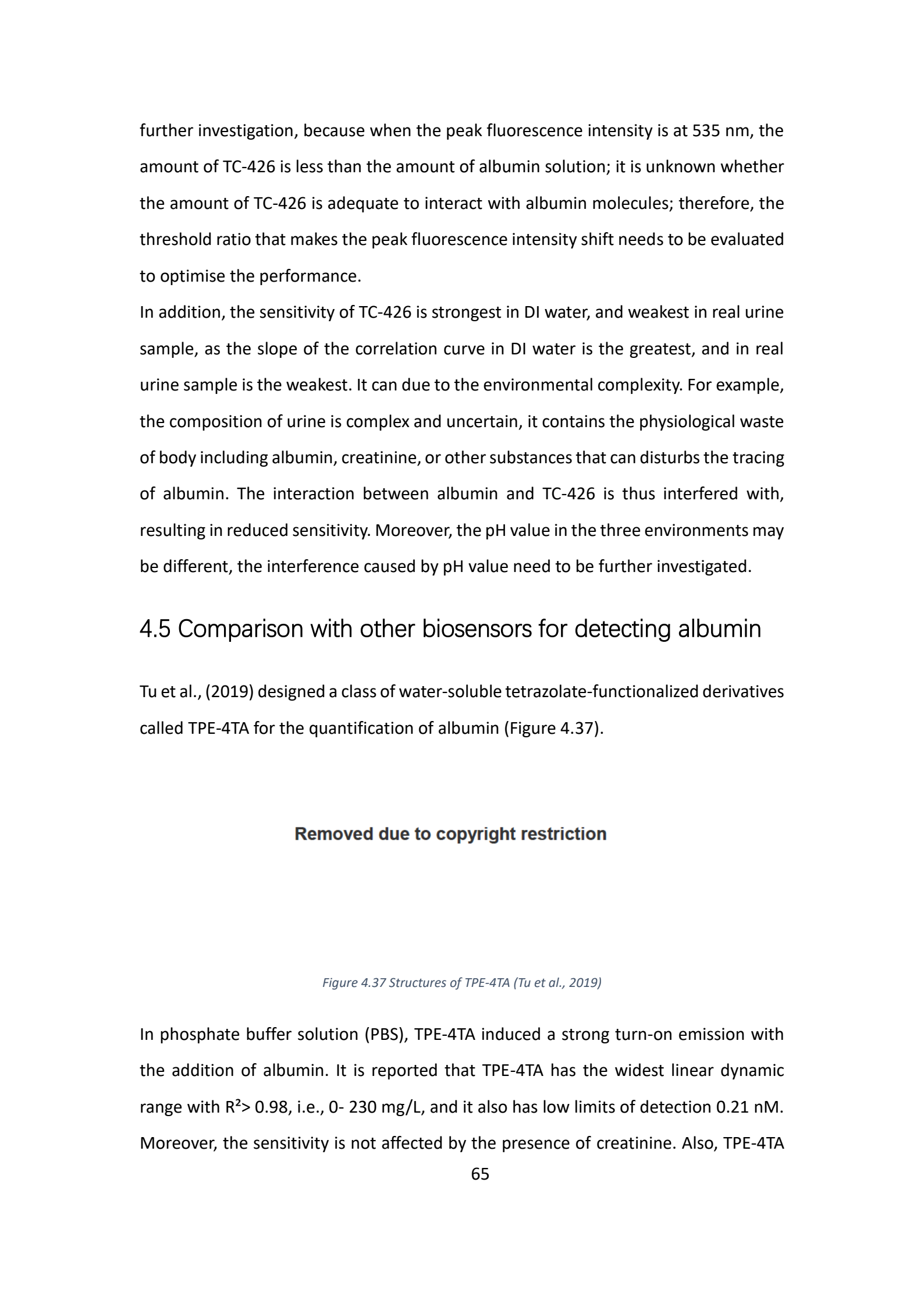

Figure 4.37 Structures of TPE-4TA (Tu et al., 2019)

In phosphate buffer solution (PBS), TPE-4TA induced a strong turn-on emission with the addition of albumin. It is reported that TPE-4TA has the widest linear dynamic range with $R^2 > 0.98$, i.e., 0- 230 mg/L, and it also has low limits of detection 0.21 nM. Moreover, the sensitivity is not affected by the presence of creatinine. Also, TPE-4TA

was examined for quantification of urinary albumin, in the microalbuminuria range, the linear correlation coefficient was 0.99.

Comparing to TPE-4TA, TC-426 has a wider linear dynamic range, in this thesis, the linear dynamic range includes the range between 100- 1000 mg/L in DI water, 25 mg/L-200 mg/L in artificial urine and real urine sample, with $R^2 > 0.98$. Moreover, the linear correlation coefficient in the quantification of urinary albumin within the microalbuminuria range is also 0.99. However, from the evaluation of the interference of creatinine, it was found that the sensitivity of TC-426 can be slightly influenced by the addition of creatinine.

Furthermore, Chen et al., (2017) evalauated another AIE-based biosensor BSPOTPE (Figure 4.38) for the quantification of albumin.

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Figure 4.38 Structure of BSPOTPE (Chen et al., 2017)

This biosensor demonstrates a shorter incubation time as 5 minutes, and also presents a linear correlation curve (Figure 4.39). They evaluated the correlation in artificial urine with the prescence of creatinine in a wider range. Comparing to the result in section 4.2.6, both of the two biosensors are tend to be influenced by the prescence of creatinine. In this thesis, the measured range of albumin was between 25 mg/L to 200 mg/L, i.e., 0.38 μM to 3.01 μM, the difference between the curves were not significant when the concentration of albumin was low, the slop of the correlation curves gradually decreased as the concentration of albumin increased, which is similar to BSPOTPE.

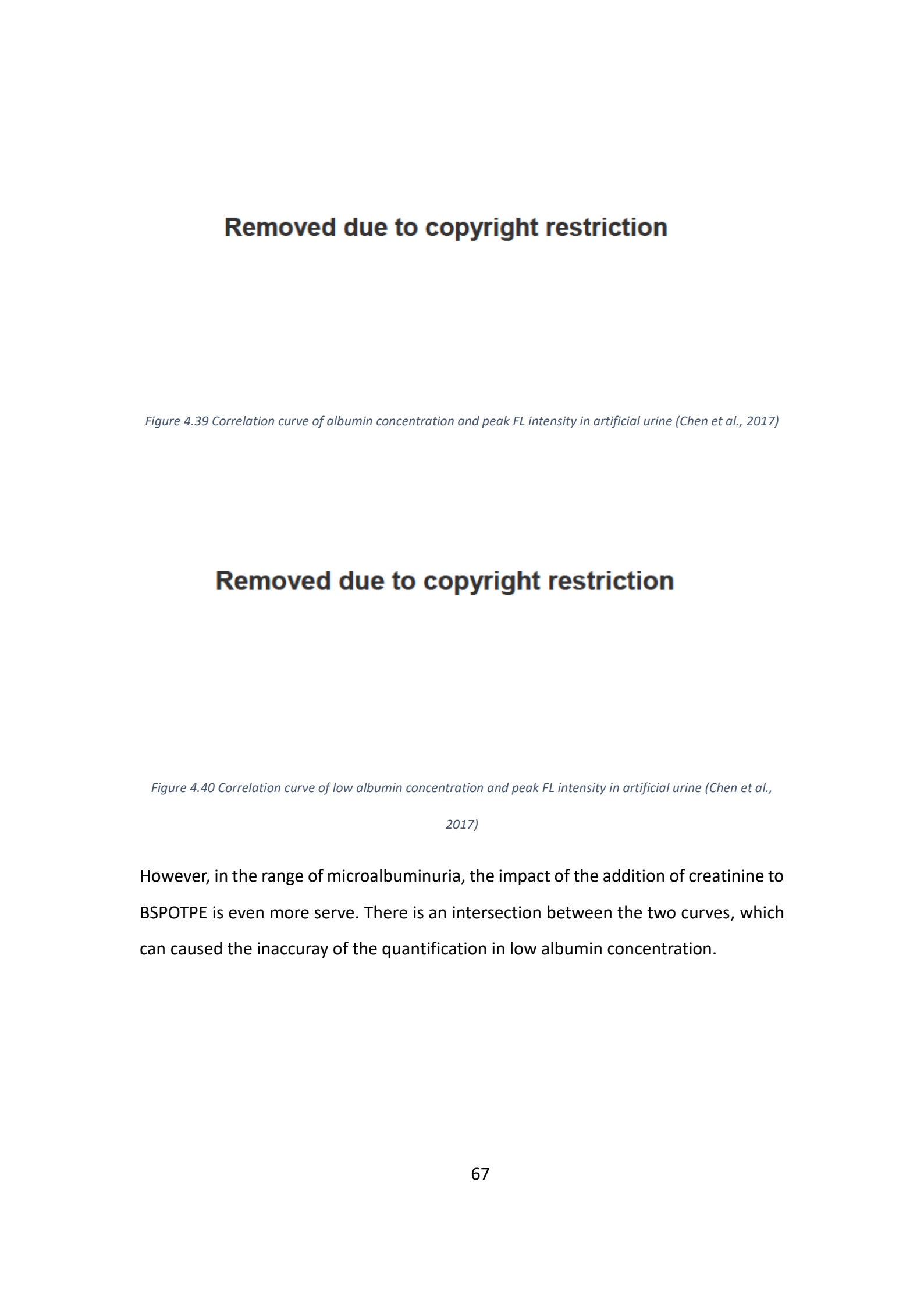

Figure 4.39 Correlation curve of albumin concentration and peak FL intensity in artificial urine (Chen et al., 2017)

Figure 4.40 Correlation curve of low albumin concentration and peak FL intensity in artificial urine (Chen et al., 2017)

However, in the range of microalbuminuria, the impact of the addition of creatinine to BSPOTPE is even more serve. There is an intersection between the two curves, which can caused the inaccuray of the quantification in low albumin concentration.

Chapter 5 Future work

TC-426 has demonstrated a superior capacity on detecting albumin in different environments; further investigations to optimise the performance are necessary.

5.1 Further evaluation of the optimal ratio of albumin to biosensor

As discussed in Section 4.4, the optimal ratio of albumin to biosensor need further investigation. In this thesis, the ratios 1:1, 1:2, 1:3, 1:6, 1:7 of albumin to TC-426 or TC-426 to albumin were evaluated. However, the threshold ratios that caused the shifting of peak fluorescence intensity in different environments have not been confirmed; therefore, the interaction state between TC-426 and albumin is uncertain, for example, is there unreacted albumin or TC-426 remaining in the solution? The evaluation of the optimal ratio can make the reaction more thorough; it is possible to increase the fluorescence intensity further and enhance the linearity of the correlation between albumin concentration and fluorescence intensity.

5.2 Evaluate the concentration of biosensor

It was found that when the environment was changed from DI water to artificial urine, the fluorescence intensity dropped significantly, and in real urine sample, the fluorescence intensity even decreased further. In this thesis, the fluorescence intensity was amplified in two ways. First, increased the size of the slits of the spectrophotometer, which is regarded as physically amplifying the fluorescence intensity. Second, adjusted the ratio of albumin to TC-426, which made the interaction between albumin and TC-426 more adequately. However, the first method of amplifying the fluorescence intensity did not solve the problem fundamentally. Because as the size of slits increased, more background source may come in which can

influenced the sensitivity of the biosensor. Furthermore, although by adjusting the ratio of albumin to TC-426, there was a difference in fluorescence intensity between low concentration albumin solution and high concentration albumin solution, the fluorescence intensity was still in a relative low level in real urine sample comparing to in artificial urine. Therefore, the influence on the fluorescence intensity by the concentration of TC-426 is worth to evaluate. In this thesis, the concentration was 10 μM as suggested by the provider. To increase the fluorescence intensity, the concentration of TC-426 can be increased for the evaluations of both artificial urine and real urine sample.

5.3 Evaluate in urine samples from different individuals

This thesis evaluated the levels of albumin in real urine sample by using TC-426, however, the urine sample was taken from only one individual. For the evaluation in the future, the urine samples can be taken from multiple individuals. It is possible to take urine samples from individuals with different physical fitness, genders or groups of age to compare the differences of results and obtain valuable conclusions.

5.5 Evaluate the influence of pH value

It was found that in different environments, i.e., DI water, artificial urine, and real urine sample, the sensitivity of TC-426 is different, pH value can be considered as one of the influence factors. Therefore, investigating the influence of different pH value can help improving the performance of TC-426.

5.5 Cooperation with creatinine biosensor

As known, UACR is an important ratio that can be used to estimate 24-hour urine albumin excretion. By applying UACR, 24-hour collection and timed specimens become unnecessary. Therefore, cooperation with a superior biosensor for detecting creatinine

can be meaningful to evaluate UACR. Moreover, the potential mutual interference of creatinine and albumin during detection and quantitation is also necessary to evaluate.

Chapter 6 Conclusion

With the increasing focus on chronic kidney disease (CKD), early detection on CKD has attracted more and more attention in the throughout the world. Microalbuminuria is a disorder with the presence of albumin in urine, which can be considered as the earliest manifestation of CKD. This thesis evaluated the levels of albumin within the range of microalbuminuria in different environments by using a fluorescence-based biosensor TC-426.

The optimal working conditions in different environments were investigated and are listed in table 9.

Table 9 Conclusion of working conditions

Environment	Incubation time	Optimal ratio of albumin to TC-426
DI water	20 minutes	2:1
Artificial urine	20 minutes	5:1
Real urine sample	20 minutes	5:1

Under the optimal working conditions, a linear correlation between fluorescence intensity and albumin concentrations in DI water, artificial urine, real urine sample was presented. Moreover, it was found that in DI water, the fluorescence intensity was the strongest, and in the real urine sample, the fluorescence intensity was the weakest. In the evaluation in the real urine sample, it is unnecessary to dilute the urine sample for detecting albumin since the undiluted urine sample presented the best performance. Furthermore, the interference of the presence of creatinine was evaluated; it was found that after adding a certain amount (according to the threshold value of UACR for microalbuminuria) of creatinine, the fluorescence intensity decreased slightly. Additionally, it was known that TC-426 emits strongly at 545 nm, when interacts with albumin, the emission wavelength shifts towards 535 nm. Promising results were

achieved and further evaluation of TC-426 is necessary since it is an excellent biosensor for detecting albumin.

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