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Detection of Riboflavin by Localized Surface Plasmon Resonance Biosensor with Titanium Nitride and Nanostructure

by

CHUNG Yui Cheung

April 2019
Detection of Riboflavin by Localized Surface Plasmon Resonance Biosensor with Titanium Nitride and Nanostructure

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Project Supervisor : Prof. Lawrence C M WU
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Abstract

Surface plasmon resonance (SPR) is the oscillation of coherent delocalized electrons (plasmons) at the interface between a metal film and a dielectric medium excited by incident light. It is well known that plasmon resonance is very sensitive to the change of the medium in contact with the metal layer. Therefore, SPR has been adopted as biosensors in some applications such as protein detection owing to its high sensitivity as well as the label free and real time response. Typically, gold is chosen as the SPR material because it exhibits good plasmon resonance in the middle of the visible range and its excellent resistance to oxidation. Also, some nanostructures of SPR material have been found useful for plasmonic sensing. Recently, titanium nitride (TiN) has been considered as an alternative plasmon material for gold. In this dissertation, an inductive study was conducted to illustrate and understand the feasibility and effect of titanium nitride and nanostructures as an alternative material for plasmonic biosensing.

Riboflavin, an essential vitamin for human, was proposed to be the biosubstance to be sensed in this dissertation due to the expected bioaffinity towards TiN. Traditionally, there are several methods for riboflavin detection. Yet, the majority of methods have drawbacks. In particular, many of these methods require extensive preparation before detection test, especially the requirement of bio-labelling. Therefore, a label-free, direct and simple plasmonic biosensing system of riboflavin with TiN and nanostructures was studied in this dissertation.

The plasmonic material, TiN film, was synthesized by radio-frequency magnetron sputtering. Also, the fabrication of TiN nanostructures was done by a three-step method to understand the effect of nanostructures on the plasmonic sensing. Atomic force microscopy and ultraviolet–visible spectroscopy were performed to characterize the parameters and topographies of the plasmonic materials. A common path interference phase detection method was used to perform the whole sensing experiment.

Direct adsorption tests of 10000 ppb riboflavin on different plasmonic substrates were firstly done. The results showed that there was adsorption of riboflavin on both TiN
nanoholes and TiN film. This is the first time ever positive result of riboflavin detection with TiN and nanostructures. The response from the TiN nanoholes was higher than the response from TiN film due to signal amplification from the nanostructures. As TiN nanoholes provided significant response in direct adsorption, it was further tested with various concentrations of riboflavin solution. It was discovered that there was a concentration-dependent response of direct adsorption of riboflavin on TiN nanoholes from 100 ppb to 10000 ppb. The higher the concentration, the larger the response. Saturation test of riboflavin on TiN nanoholes was also conducted to understand the binding events between riboflavin and TiN. It was found that the phase responses reduced when testing with the same piece of TiN nanoholes chip. The binding sites on the surface were indicated to be saturated due to the reduction in adsorption response.

Moreover, a novel functionalization method with biotinylated riboflavin binding protein on TiN film was introduced to improve the selectivity and detection response of riboflavin on TiN film. It was found that there was an improvement in the detection of functionalized TiN film for 10000 ppb riboflavin, compared with bare TiN film.

In conclusion, an investigation in plasmonic detection system of riboflavin has been successfully designed and conducted. The discoveries in this dissertation have shown a great potential for the detection of riboflavin with TiN nanostructures. Moreover, by employing the biotinylation method, TiN film should be applicable for a broad range of plasmonic biosensing.
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Last but not least, I would like to thank Miss Christy CHAN and Miss Tiffany Pang, the language advisers in ELC for the writing advice on the report.
1 Introduction and Objectives

Riboflavin, known as vitamin B2, is a vitamin that is mandatory to a wide variety of cell activities. It is a water-soluble vitamin that can be found in different foods. Since the human body cannot produce and store riboflavin, it is vital to get riboflavin from dietary sources [1]. Various studies have been investigating on riboflavin deficiency, showing that an adequate amount of riboflavin is required for human body in order to maintain healthy metabolism. Therefore, the development of detection system for riboflavin is important [2].

Traditional methods for riboflavin detection have several limitations. For example, the microbiological assay has poor reproducibility, long preparation and analysis time. While, on the other hand, Surface Plasmon Resonance (SPR) sensors offer real-time and label-free measurement. SPR is the oscillation of coherent delocalized electrons (plasmons) at the interface between a metal film and a dielectric medium stimulated by incident light. SPR sensors determine the analyte content by measuring the change in refractive index due to the present of analyte molecules. It is a highly attractive sensing technique for determination of riboflavin but the existing SPR detection based on gold substrates is indirect and complicated [3].

Instead of using gold as the SPR material, titanium nitride (TiN) could be an alternative. A study has found that the stacking formation of both N-Ti and O-Ti bonds via and carboxyl (COOH) and carbonyl (C=O) functional group would provide strong binding affinity towards TiN [4]. It is expected that riboflavin molecules would have a reasonable bioaffinity towards TiN due to the existence of carbonyl and amine groups in riboflavin. In this work, direct adsorption tests of riboflavin on TiN were done.

Furthermore, since the strong affinity of biotin towards TiN is well studied, functionalization of biotinylated riboflavin binding protein (RBP) on TiN film was also employed in this report. Biotinylated RBP is acted as a biorecognition element which is immobilized on the TiN film. The biotinylated RBP would bind to riboflavin molecules when riboflavin is in contact with TiN surface. A layer of medium of higher refractive index would be created. This would cause a change in phase response, which would be measured to help quantifying the amount of riboflavin present.
Localized surface plasmon resonance (LSPR) was also employed because it would enhance the detection response near the surface due to the result of nanostructures of the plasmonic substrate. A methodology of fabricating TiN nanostructures was introduced to perform the LSPR detection of riboflavin.

By utilizing the riboflavin bioaffinity towards TiN and the functionalization of biotinylated RBP on TiN, direct and simple detection of riboflavin was studied in this dissertation.
2 Literature Review

2.1 Surface Plasmon Resonance (SPR) Sensors

Surface Plasmon Resonance (SPR) Sensors are optical sensors based on the excitation of surface plasmons [5]. In the early 1980s, the study of process of SPR at the surface of metals [6] and sensing of gases [7] was first demonstrated. Since then, SPR sensors have become a significant tool for characterizing and quantifying bimolecular interaction. It is widely applied in different areas such as material characterization, food quality, medical diagnostics and environment monitoring. SPR is a real-time label-free technique which eliminates the usage of labelling chemicals and enables real time response. As a result, SPR sensors outperform many traditional biosensing techniques [5].

Surface plasmons were first observed by Wood in 1902 who reported anomalies in the spectrum of light diffracted on a metallic diffraction grating [8]. They are the coherent delocalized electrons (plasmons) that oscillate at the interface between a metal film and a dielectric medium [9]. The metal and dielectric should be a semi-infinite metal with permittivity \( \varepsilon_m = \varepsilon'_m + i \varepsilon''_m \) and a semi-infinite dielectric with permittivity \( \varepsilon_d = \varepsilon'_d + i \varepsilon''_d \), where \( \varepsilon' \) is the real part and \( \varepsilon'' \) is the imaginary part [5].

2.1.1 Excitation of surface plasmons

The traditional way for optical excitation of surface plasmons is prism coupling as shown in Figure 1. When light passes through the higher refractive index prism, it is totally reflected at the base of prism in contact with the metal film. An evanescent wave is then generated to penetrate through the metal film. Coupling of evanescent wave and the surface plasmons resonance can be done by adjusting the incidence angle of light [5].
Another approach to excite the surface plasmons is based on a grating coupler as shown in Figure 2. When light is incident from the dielectric medium on the metal film grating, the light wave is diffracted in different angles. If the momentum of diffracted light lies in the plane of the grating (plane y-z) is equal to the propagation constant of surface plasmons, the coupling between diffracted light and surface plasmons can occur [10].

The third way to excite surface plasmon is waveguide coupling as shown in Figure 3. When a light wave travels in a waveguide in form of guided modes, a portion of the electromagnetic field propagates as an evanescent wave and penetrate through the metal film to excite the surface plasmon at the outer boundary of the metal film. The coupling between the guided mode and the surface plasmon can be fulfilled if the two waves are having the same value of propagation constant [5].
By the optical excitation of a light wave, the surface plasmon propagates along the metal thin film and its evanescent wave probes the sample medium in contact with the metal film. A change in refractive index of the dielectric occurs [5]. The propagation constant of the surface plasmons wave along the metal-dielectric interface is highly sensitive to the change in the refractive index of the dielectric [11] [12]. The change in propagation constant alters the coupling between the light wave and the surface plasmons [13]. By measuring different characteristic of the light wave interacting with the surface plasmons, SPP sensors can be classified into five systems with intensity, angular, wavelength, phase or polarization modulation [10].

2.1.2 Modulation methods of SPR

The most commonly used modulation system is angular modulation. A convergent monochromatic light wave is used to excite the surface plasmons at different incident angles. The angle of incidence generating the strongest coupling is measured and used as the sensor output [14].

For wavelength modulation, a polychromatic light beam is used to excite the surface plasmons at a single angle of incidence. The wavelength generating the strongest coupling is measured and used as the output [15].

For intensity modulation, a monochromatic light beam is used to excite the surface plasmons at single angle of incidence. The intensity of the coupling between the light wave and the surface plasmons is measured and used as the sensor output [7].

For phase modulation, a monochromatic light beam is used to excite the surface plasmons at a single angle of incidence. The shift in phase of the light wave coupling with the surface plasmons is measured and used as the sensor output [16].

For polarization modulation, a monochromatic light beam is used to excite the surface plasmons at a single angle of incidence. The change in polarization of the light wave coupling with the surface plasmons is measured and used as the sensor output [17].
2.1.3 Surface Plasmon Resonance Affinity Biosensors

There are two types of SPR sensors, either direct or indirect. For direct SPR sensors, the measured quantity (mostly refractive index) modulates the light characteristics directly. For indirect SPR sensors, the measured quantity modulates an intermediary quantity of the characteristics of light indirectly. A classic example of indirect SPR sensor is the SPR affinity biosensor [13].

A biorecognition element is needed for a SPR affinity biosensor to recognize and interact with the chosen analyte. The biorecognition elements are immobilized on the solid surface of the metal film. When a liquid sample containing the selected analyte molecules is in contact with the metal surface, the biorecognition elements bind to the selected analyte molecules as shown in Figure 4. A layer of medium with higher refractive index is produced at the metal-dielectric interface. The change in refractive index alters the propagation constant of the surface plasmons. This change alters the coupling between the light wave and the surface plasmons [10] [13].

![Figure 4 Binding event on metal surface](image)

In fact, the concentration and properties of selected analyte molecules at the metal surface give rise to the change in refractive index on the metal surface. The concentration of captured molecules can be calculated from equation

\[
\Delta n = \frac{dn}{dc} \Gamma
\]

where \( \Delta n \) is the change in refractive index, and \( dn/dc \) is the refractive index increase depending on the change in concentration of analyte molecules within a thin layer at
the surface of thickness h. The value of dn/dc is mostly from 0.1-0.3 mLg\(^{-1}\). \(\Gamma\) is the surface concentration in mass/area [5].

### 2.2 Localized surface plasmon resonance (LSPR)

Localized surface plasmon resonance (LSPR) is an enhanced electromagnetic field effect near the surface due to the result of metal nanostructures and nanoparticles. Different from traditional SPR metal thin film, conversion of photon into plasmonic resonance is utilized by the nanostructures and nanoparticles [18]. The local electromagnetic field is confined within the vicinity of the nanostructures and nanoparticles which have a much smaller size than the wavelength of the excitation light. The coherent localized plasmonic oscillation generated is called LSPR [19]. The oscillation field causes the conduction electrons to vibrate coherently and polarization charges accumulate on the surface of the nanoparticles when the radius of nanoparticles is less than one-tenth of the wavelength of the excitation light [20]. The intensity of electric field close to the plasmonic nanoparticles is improved and it drops abruptly away from the nanoparticles surface [21].

Studies have shown that there is a considerable enhancement of the electric field induced by light on an individual isolated nanoparticle [22]. When the distance between nanoparticles is less than a few nanometres, it enlarges the amplitude of the confined electric field in the interval between the nanoparticles. The electromagnetic field in between the nanoparticles is then overlapped and hybridized. Therefore, several orders of magnitude of improvement is given out, compare to the propagating plasmon effect [23]. It is known that a regular array of gold nanostructures is extremely sensitive to the local refractive index change. As a result, LSPR is an ideal choice for detection of small biomolecules such as vitamins [24]. The regular array such as dots, hole and pillar can be made by lithography methods. However, it is hard to carry out mass production of the LSPR sensor due to the high production cost [25]. Instead, a simple two-step deposition-annealing technique was reported to make self-assembly (SAM) gold nanoislands of random array [26]. Successful examples have
been reported for the application of SAM on biosensor [27]. Owing to the higher sensitivity and the label-free real-time response, LSPR is an ideal sensing technique for biosensing applications [22].

2.2.1 Methodology for Self-assembly (SAM) gold nanoislands (AuNIs)
The SAM AuNIs sample can be made by sputtering gold thin film onto a BK7 glass slide and further thermal annealed at 550°C for 9 hours in air [22]. The gold thin film is usually metastable in the as-deposited state and undergoes agglomeration (solid-state dewetting process) to form arrays of nanoislands as shown in Figure 5 [28].

![Figure 5 Schematic fabrication process of SAM gold nanoislands](image)

2.2.2 Titanium Nitride as an alternative material for LSPR
Typically, gold is chosen as the LSPR material because it exhibits good plasmon resonance in the middle of visible range and excellent resistance to oxidation, and its non-toxicity [4]. Recently, Titanium nitride (TiN) has been considered as an alternative plasmon material for gold. TiN shows strong properties in different fields like hardness, thermal stability, corrosion resistance and chemical stability [29]. Also, TiN thin film has shown an adjustable plasmonic absorption in middle visible range and great SPR sensing performance under both wavelength and phase modulation in common-path sensing system [30].

To carry out LSPR, nanostructure is necessary. A study by Guler et al. demonstrated that the strength of LSPR mode for TiN nanoparticles is similar to the LSPR mode for gold nanoparticles [31]. For the light in near infrared region, TiN nanoparticles are more efficient than gold nanoparticles as shown in Figure 6 [32]. Therefore, TiN is a prospective material for LSPR applications particularly in near infrared region.
Figure 6 Quality factor ($Q_{\text{LSPR}}$) and near field intensity efficiency ($Q_{\text{NF}}$) for Au (r=57 nm), TiN (r=74 nm) and ZrN (r=46 nm) nanoparticles [32]

2.2.3 Fabrication of TiN nanohole array

Self-assembly (SAM) gold nanoislands are needed for TiN nanohole fabrication [33]. A thin film of TiN is sputtered on the glass slide with SAM gold nanoisland. AFM topographical study indicated that TiN films are mechanically strong [34]. The removal of the gold nanoislands with iodine is the last step and the TiN film remains on the glass would have nanohole array. The schematic fabrication process is shown in Figure 7.

1. SAM gold nanoislands

2. Sputtering of TiN

3. Removal of gold nanoislands

![Fabrication steps for TiN nanoholes arrays](image)

Figure 7 Fabrication steps for TiN nanoholes arrays
2.3 Riboflavin

Riboflavin, known as vitamin B2, is a water-soluble vitamin that can be found in different foods. It is important in many activities of cells such as electron transfer, nucleic acid repair and cell apoptosis in the respiratory chain. Since riboflavin is unable to be made by the human body, sufficient dietary intake is necessary in order to prevent skin and mucosal disorders, angular cheilitis, anemia [35]. A deficiency of riboflavin can even cause cancer [36]. Riboflavin deficiency is common in many areas around the world especially for the less developed countries. The section of more developed countries shows a low intake of dairy products and meat [37]. There is a large group (10%-27%) of elderly with a noticeable deficiency of riboflavin in the United States [38].

2.3.1 People at High Risk of Riboflavin Deficiency

Riboflavin deficiency is relatively usual when dietary intake is inadequate since it is a water-soluble vitamin that cannot be stored in human body and continuously excreted in the urine [39]. Pregnant and lactating women and their babies are groups of people with high risk of riboflavin deficiency. Pregnant or lactating women who seldom eat meats or consume dairy products, such as vegetarians, are at risk of riboflavin inadequacy which brings harmful influence to both mothers and their babies [40]. For pregnant women, preeclampsia can be induced more likely and it may increase the risk of poor prognosis for both mothers and their babies [41]. A study showed that the foetal growth in weight and length is correlated positively with maternal intake of riboflavin [42].

Infant formula is a supplement to breast milk to support the normal growth of infants. In China, there is a standard amount of vitamin B2 in different nutrient food. According to “GB 14880-2012 National Food Safety Standard for the Use of Nutritional Fortification Substances in Foods”, the amount of riboflavin for infant milk powder and pregnancy formulas should be 8-14 mg/kg and 4-22 mg/kg respectively [43]. During the first six months of life especially for preterm or low-weight baby, a proper
amount of vitamin B2 is crucial in the baby formula [44]. Therefore, it is important and necessary to detect the concentration of vitamin B2 in food or supplement for quality control.

2.3.2 Limitations of conventional method for Riboflavin detection
The traditional ways to detect riboflavin, for instance microbiological assay [45], spectrophotometric [46], high-performance liquid chromatography (HPLC) [47], immunoassay [48] and capillary electrophoresis [49] are having some limitations. For example, the microbiological assay has some disadvantages such as poor reproducibility, long preparation and analysis time. The incubation time for “VitaFastVitamin B2”, a microbiological vitamin test kit, is around 44 hours. As mentioned above, these existing detection methods are unfavourable in time-consuming operation, and high cost equipment. Instead, SPR sensors offer greater simplicity, faster response and lower cost. The SPR system is easy to use due to the automatic mixing and injection of the sample solution. A quick and precise measurement can be given since the vitamin B2 concentration in the specimen is measured and computed by the software directly [3]. As a result, SPR is a highly attractive sensing technique for determination of riboflavin.

2.3.3 Existing SPR detection of Riboflavin based on gold chip
A SPR based detection of riboflavin was done by Caelen et al. in 2004. The results showed that the limit of detection (LOD) was 70ng/mL and the limit of quantification (LOQ) was 234 ng/mL which are equal to 70 ppb and 234 ppb respectively. As mentioned above, the standard amount of riboflavin in infant milk powder should be 8-14mg/kg, which is equal to 8000-14000 ppb. Although the gold-based SPR detection provides reasonable sensing performance which can perform well in quality control, it is not an attractive choice for bustling routine laboratories due to the complicated procedures.

This gold-based SPR detection is an indirect detection by measuring the free excessive riboflavin binding protein after complexation with riboflavin. Gold will not be able to
bind with riboflavin naturally since riboflavin does not have any chemical groups that can bind with the gold surface. An organic synthesis of the riboflavin derivative has to be performed [50]. The riboflavin derivative has to be covalently immobilized on the surface first and riboflavin protein is added to riboflavin specimen for complexation of proteins. Lastly, the riboflavin specimen is injected to the film surface and the free excessive riboflavin binding protein binds to the chip surface with riboflavin derivative. The indirect assay and complicated procedures are the limitations of this SPR-based detection [3]. Instead, by utilizing the riboflavin bioaffinity towards TiN, a direct and simple detection of riboflavin can be performed.

It is expected that the LSPR detection of riboflavin with TiN would have good plasmonic sensing performance to meet the expected low LOD and fast detection due to the bioaffinity towards TiN films.

2.3.4 Riboflavin adsorption on TiN

Riboflavin belongs to the class of B vitamins that play critical roles in cell metabolism. B vitamins include 8 vitamins like thiamine (B1), riboflavin (B2) and biotin (B7) [51].

A study has shown that biotin has a stronger binding affinity towards titanium nitride films than gold films in experiment. Computational simulations of adsorption mechanism of biotin done by Qiu et al. have found that the stacking formation of both N-Ti and O-Ti bonds via and carboxyl (COOH) and carbonyl (C=O) functional group may be the reason for the strong binding [4].

![Chemical structure of biotin](image)

Figure 8 Chemical structure of biotin [52]
Since the binding affinity of riboflavin towards TiN is still unknown, experimental or computational investigations are needed to understand the affinity. By comparing the chemical structure of biotin and riboflavin as shown in Figure 8 and Figure 9, both of them contain carbonyl (C=O) functional groups and riboflavin contains one more carbonyl group than biotin. Both of them contain amine (NH) groups but it may only provide less stable physisorption as estimated by DFT computation [4]. The excellent affinity of biotin towards TiN is due to the O-Ti bonds via carbonyl (C=O). Therefore, it is reasonable to hypothesize that riboflavin molecule would have a sensible bioaffinity towards TiN films.

2.3.5 Biotinylation of riboflavin binding protein (RBP)
Biotin is a small molecule found in all living cells. Biotin can covalently attach to a protein through the process of biotinylation. Since it is relatively small, it is unlikely to affect the biological activities of the conjugated proteins [54]. Biotin can bind with different reactive groups so as to attach to most proteins. The reactive groups can bind to the functional groups that are present in protein such as primary amines, carbohydrates, carboxyls, and sulfhydryls. N-hydroxysuccinimide ester (NHS) is one of the reactive groups which targets primary amines of the protein. Biotin N-hydroxysuccinimide ester (biotin-NHS) is able to bind most protein, therefore it is the most frequently used biotinylation reagent [55]. Figure 10 shows the chemical structure of Biotin-NHS. Figure 11 shows the chemical structure of RBP.
RBP could be biotinylated by treating RBP with biotin-NHS in dimethyl sulfoxide (DMSO) and incubating at room temperature [57].

As shown in Figure 12, biotinylated RBP is acted as a biorecognition element which is functionalized on the TiN surface. The biotinylated RBP could selectively capture the riboflavin molecules when riboflavin is in contact with TiN surface. A layer of medium of higher refractive index would be created. This would cause a change in phase response, which would be measured to help quantifying the amount of riboflavin present.
3 Methodology

3.1 Preparation of SAM gold nanoislands chip
A 35 mm x 25 mm with 2 mm thickness BK7 glass was cut and put into a glass-washing container with ethanol. The container was bathed in an ultrasonic cleaner for 15 minutes to remove contaminants. Then the glass piece was dried in an air dryer at 50°C.

An ion sputtering machine (JEOL JFC-1100E) was used to deposit gold on the glass piece. After putting the glass piece into the machine, the chamber was pumped down for 15 minutes to reach the vacuum state. The sputtering current was controlled at 10 mA for 70 seconds. The sputtering time was controlled to obtain the thickness of the gold film from 8.0 to 9.0 nm.

Then, the gold chips were put into an oven to anneal them. The time for the rise in temperature from room temperature (24°C) to 550°C was controlled at 30 minutes. Then, the chips were kept at 550°C for the next 9 hours. After 9 hours, the oven was automatically switched off and the chips were kept in the oven to cool down slowly to room temperature. After annealing, self-assembly gold nanoislands chips were formed. With the range of thickness from 8.0 to 9.0 nm mentioned before, the diameter of the gold nanoislands obtained after annealing would be controlled to be within 100 to 200 nm for the fabrication of TiN nanoholes structure.

For characterization, topographical scan of the gold nanoislands chip using tapping mode atomic force microscopy was performed. It is noted that by changing the sputtering and annealing condition, the size of the nanoislands could be adjusted.

3.2 Preparation of TiN thin film and TiN nanoholes structure
Two types of chips were used which were bare glass chips and gold nanoislands chip. The bare glass chips were used to synthesize TiN film while gold nanoislands chip were used to fabricate TiN nanoholes structure.
An SP530 radio frequency sputtering system was used to deposit TiN on the chips. After putting the chips into the system, the chamber was pumped down automatically to reach the desired base vacuum state, $5 \times 10^{-6}$ Torr. Since TiN was a ceramic insulator, the maximum sputtering power was kept at 60W and the power density was 3 W/$\text{cm}^2$. The gas flow rate was Ar at 10 SCCM with pressure 1-2 bar. The thickness of the TiN was controlled from 27 to 30 nm. The TiN sputtering rate was controlled at 0.78 nm/min. The sputtering time was 35 minutes. TiN thin film chips were produced in this method.

For the nanoholes chips, the thickness was also controlled from 27 to 30 nm because the height of the gold nanoislands was around 50 to 60 nm. Otherwise, the TiN would cover the gold nanoislands excessively and the gold removing procedure, which will be explained later, would not be able to perform. With the thickness range from 27 to 30 nm, the gold nanoislands could be exposed to the surface for the iodine gold leaching process.

Since the gold nanoislands could be leached in iodine solution while TiN would not react with iodine, the removal of gold nanoislands on the TiN chips could be carried out in iodine solution. The TiN-AuNIs chips were soaked in the iodine solution for about 10 hours. The gold nanoislands were dissolved in the solution. The TiN film remaining on the chip would then contain nanoholes structure. Since the TiN is mechanically very strong, wiping the chip with cotton swab could be performed to remove the gold remaining in the chip after iodine leaching.

3.3 SPR optical sensing system

The whole sensing experiment was performed in a common-path optical sensing system, as shown in Figure 13. It consists of a white light LED source (LedEngin, LZ1-00W00) with a temperature-stabilizer to produce light of wavelength ranging from 530 nm to 760 nm. Polarizer-1 was used to linearly polarize the light. The birefringent crystal was used to refract and divide the polarized light into two separate polarizations. It also produced a optical path difference between the two polarizations. A glass prism was used to perform total internal reflection of the light and excite
surface plasmon resonance at the substrate-analyte interface [58]. After the light passing through the probe cell, the path difference was changed. Then, polarizer-2 was used to recombine the two polarizations from the probe cell. Finally, the phase change information was collected by the spectrometer.

A peristaltic pump was used to bring the deionized water or analyte solution into contact with the TiN nanoholes chip as shown in Figure 14. TiN chip was mounted between the flow cell and the prism. The side of the glass piece with TiN was facing the flow cell.

Figure 13 Schematic diagram of the common-path optical sensing [22]

Figure 14 The TiN chip was mounted into the flow cell
3.4 Direct adsorption test of Riboflavin on TiN

Phosphate-buffered saline (PBS) buffer solution was firstly injected into the probe cell to set up the baseline for 400s. Then, the riboflavin dissolved in PBS were injected into the probe cell and detected by TiN films for 1800s. Finally, the PBS buffer solution was injected into the probe cell again to rinse the TiN chips for at least 1800s until the response was stabilized. The final phase response would indicate the affinity between riboflavin and TiN if it does not return to the baseline. The larger the difference between the final phase response and the baseline, the stronger the affinity. Sets of experiments were conducted as shown in Table 1. For comparison, adsorption test of riboflavin was also performed on gold film and gold nanoislands.

Table 1 Experimental sets for riboflavin adsorption with different concentrations

<table>
<thead>
<tr>
<th>Injecting orders:</th>
<th>PBS baseline</th>
<th>Riboflavin solutions</th>
<th>PBS rinse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Set 1</td>
<td>400s</td>
<td>1800s (10000 ppb)</td>
<td>1800s</td>
</tr>
<tr>
<td>Set 2</td>
<td>400s</td>
<td>1800s (1000 ppb)</td>
<td>1800s</td>
</tr>
<tr>
<td>Set 3</td>
<td>400s</td>
<td>1800s (100 ppb)</td>
<td>1800s</td>
</tr>
</tbody>
</table>

3.5 Preparation of Biotinylated of RBP

The most common biotinylation reagent is biotin-NHS as mentioned before. First, biotin-NHS powder was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 20 mg/mL. Then 10 mL of RBP in PBS was prepared with 1 mg/mL concentration. After that, 1 mL of biotin-NHS solution was mixed with 10 mL RBP with gentle stirring at room temperature for 2 hours in the dark. Finally, the biotinylated RBP solution was mixed with 0.1 mL of 0.5 M ethanolamine and incubated for 30 min [59].

3.6 Detection of riboflavin after functionalization with biotinylated RBP

For selectively capturing the target analyte, riboflavin, TiN surface was functionalized with biotinylated RBP. First, PBS buffer solution was injected into the probe cell to set up the baseline for 800s. Second, biotinylated RBP was injected to functionalize the
TiN surface. Then PBS was injected again to build a second baseline. After that, riboflavin solution was injected into the system for detection. Finally, PBS was injected again to flush the TiN surface. The final stabilized phase response was proportional to the concentration of riboflavin. The larger the difference, the higher the concentration of riboflavin. Set of experiment was conducted as shown in Table 2.

Table 2 Experimental set for detection of riboflavin with biotinylated RBP on TiN

<table>
<thead>
<tr>
<th>Injecting orders:</th>
<th>PBS baseline</th>
<th>Biotinylated RBP</th>
<th>Second PBS baseline</th>
<th>Riboflavin solution</th>
<th>PBS rinse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Set 1</td>
<td>800s</td>
<td>800s</td>
<td>1600s</td>
<td>3600s (10000 ppb)</td>
<td>800s</td>
</tr>
</tbody>
</table>
4 Results and Discussion

4.1 Chips characterization

4.1.1 Atomic force microscopy (AFM) analysis of SAM gold nanoislands chips

Gold nanoislands chips were prepared by using ion sputtering for 60, 65, 70, 75, 80 seconds at 10 mA followed by 9 hours of annealing at 550°C. Table 3 shows the relationship between sputtering time and the morphology of the AuNIs. It can be seen that the longer the sputtering time, the larger the AuNIs size. As shown in Figure 15 and Figure 16, for 70s of sputtering time, the nanoislands were evenly and randomly distributed on the glass substrate and they were similar in size.

Table 3 Statistical analysis of SAM-AuNIs parameters.

<table>
<thead>
<tr>
<th>Sputtering Time/s</th>
<th>60</th>
<th>65</th>
<th>70</th>
<th>75</th>
<th>80</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Au film thickness/nm</td>
<td>7.8</td>
<td>8.0</td>
<td>8.2</td>
<td>8.8</td>
<td>9.1</td>
</tr>
<tr>
<td>Mean AuNIs diameter/nm</td>
<td>119.3</td>
<td>135.5</td>
<td>153.9</td>
<td>174.8</td>
<td>178.6</td>
</tr>
<tr>
<td>Mean AuNIs height/nm</td>
<td>25.1</td>
<td>31.2</td>
<td>42.8</td>
<td>51.4</td>
<td>59.6</td>
</tr>
</tbody>
</table>

Figure 15 AuNIs distribution with 70s sputtering time under AFM (5μm x 5μm)
4.1.2 Ultraviolet–visible spectroscopy investigation on TiN chips

To show whether TiN formed on the glass substrate could have plasmonic response, Ultraviolet–visible (UV-Vis) spectroscopy was performed. The TiN continuous film chips showed peaks at around 320 nm as shown in Figure 17. The results had good agreement with reported values from previous studies [60].

Figure 17 UV-Vis spectra of TiN films
4.1.3 AFM topographic analysis on TiN chips

As shown in Figure 18, the height is provided to show the surface morphology and nanostructures of TiN chips. The nanoholes chips were successfully fabricated with the help of gold nanoislands chips. LSPR could be performed with the nanoholes chips due to the presence of the nanostructures. Thickness of the film is measured to be around 28.9 nm.

![AFM topography](image)

*Figure 18 AFM topography (1μm x 1μm) of TiN continuous film (Left) and nanoholes chip (Right)*

4.2 Direct adsorption tests of riboflavin

4.2.1 Adsorption tests of riboflavin on different plasmonic substrates

Direct adsorption tests of riboflavin on Au film, AuNIs, TiN film and TiN nanoholes were performed with 10000 ppb riboflavin solution. The results are shown in Figure 19.
The phase response for riboflavin was the difference between the final response and the initial baseline. For the TiN nanoholes and TiN film, the phase responses were 0.60 rad and 0.14 rad, respectively, which indicated that there was adsorption of riboflavin on both TiN nanoholes and TiN film. For the AuNIs and Au film, the phase responses was less than 0.002, which indicated that there was virtually no adsorption.

The response from the TiN nanoholes was higher than the response from TiN film due to the signal amplification from the nanostructures. Since the size of riboflavin molecules is small, and that the TiN nanoholes nanostructure size matched that of the riboflavin molecules, high sensitivity local refractive index change occurred. That is, this was an LSPR effect that had provided a higher response to the riboflavin adsorption layer.

For the Au nanoislands and Au film, there was no adsorption since riboflavin does not have chemical groups that can bind with the gold surface.
4.2.2 Concentration-dependent response of riboflavin on TiN nanoholes

As TiN nanoholes provided significant response in direct adsorption, it was further tested with various concentrations of riboflavin solution. In Figure 20, the riboflavin solution was injected during 0s to 1800s. PBS rinsing was done during 1800s to 3600s. PBS solution was also tested as a reference response.

![Graph showing concentration-dependent response of riboflavin on TiN nanoholes](image)

Figure 20 Concentration-dependent response of direct adsorption of riboflavin on TiN nanoholes

For 100 ppb solution, the phase response increased to 0.13 radian during 900s to 1800s and stabilized after PBS rinsing at 0.11 radian. For 1000 ppb solution, the phase response kept increasing until PBS rinsing and stabilized after 2600s at 0.57 rad. For 10000 ppb solution, the phase response kept increasing and stabilized after 3200s at 0.64 rad.

All riboflavin samples produced increasing phase responses which indicated that a layer of medium with higher refractive index was produced on the TiN surface for each detection. All of the samples detected did not return to the PBS baseline after a long rinsing time. Due to the higher refractive medium was not flushed away by PBS rinsing, the phase response did not drop. It indicated that there were some binding events between riboflavin and TiN nanoholes surface.
For the higher concentration solutions, 1000 ppb and 10000 ppb, they produced increasing phase response during the PBS rinsing (1800s-3600s). One possible reason for this might be that the riboflavin molecules in higher concentration solutions were too dense. Molecules were stacking together which did not make good contact with the TiN surface. After PBS rinsing, the riboflavin molecules were being diluted and making better contact with the TiN surface. A layer of medium of closely packed riboflavin with higher refractive index was formed. Therefore, the phase response increased. Figure 21 shows a schematic drawing of the possible mechanism.

![Riboflavin diluting mechanism](image)

4.2.3 Saturation of riboflavin on TiN nanoholes

The saturation test was done by using the same piece of TiN chip to test with 1000 ppb riboflavin solution. The test was repeated for 4 times. In Figure 21, the riboflavin solution was injected during 0s to 1800s. PBS rinsing was done during 1800s to 3600s.
Figure 22 Phase response of showing the saturation of riboflavin on TiN nanoholes.

Test sequence: Test 1, Test 2, Test 3, then Test 4

The phase response of Test 1 kept increasing until PBS rinsing and stabilized at 0.55 rad. It was in good agreement with the direct adsorption of riboflavin in Figure 20 which was 0.57 rad.

For Tests 2, 3 and 4, the phase responses were reduced to 0.38 rad, 0.20 rad and 0.07 rad respectively. As mentioned before, there were some binding events between riboflavin and TiN surface. Since the binding sites on the TiN surface were occupied by the earlier arrived riboflavin, the binding sites for later riboflavin would be reduced. The change in refractive index of the layer of medium on the TiN surface would be reduced. Therefore, the phase responses for Tests 2 to 4 were decreased due to the saturation of the TiN surface.

Moreover, for Tests 2 to 4, there were also increasing phase responses during the PBS rinsing (1800s - 3600s). Since there was saturation on the TiN surface, there were many densely populated riboflavin molecules. This also supports the mechanism mentioned in Figure 21. Densely populated riboflavin could be rinsed to make better
contact with the TiN surface. Therefore, the phase responses increased during the PBS rinsing.

4.3 Detection of riboflavin after functionalization with biotinylated RBP

It is remembered that there was small adsorption of riboflavin on TiN film. Here, the detection of functionalizing the TiN film with biotinylated RBP was carried out. The functionalization of biotinylated RBP on TiN was done by injecting PBS, biotinylated RBP solution and PBS in the sequence as shown in Figure 23.

![Figure 23 Phase response of functionalization of TiN film](image)

The phase response increased to 2.1 rad when biotinylated RBP was injected into the TiN sensing chamber. Then, the sensing chamber was injected with PBS solution to remove the excess biotinylated RBP. After the PBS flushing, the phase response stabilized at 1.76 rad. It indicated that the TiN film surface had been successfully functionalized with biotinylated RBP.
The functionalized TiN film was then tested with 10000 ppb riboflavin to compare the sensing performance with bare TiN film. The results are shown in Figure 24.

![Figure 24 Phase response of 10000 ppb riboflavin on biotinylated RBP functionalized TiN film and TiN film](image)

The phase response of riboflavin detection on functionalized TiN film was 0.31 rad. For the bare TiN film, it was only 0.13 rad. This shows that there was an improvement in functionalized TiN film detection for 10000 ppb riboflavin.

There were some possible reasons for the improvement. Since the TiN surface was functionalized with biotinylated RBP, the binding events of riboflavin were no longer happened on the TiN surface. Instead, the riboflavin would bind to the biotinylated RBP, which was a few tens of nanometers above the TiN film due to the chain length of the biotin N-hydroxysuccinimide ester and RBP. Since the riboflavin binding events were moved away from the film surface, bulk effects of SPR response were utilized. Therefore, the phase response was improved.

Moreover, since RBP is the specific binding agent of riboflavin, it might also provide stronger and more selective bindings towards riboflavin. There could be more binding events. As a result, the phase response could be improved.
As mentioned above, biotinylated RBP TiN film has the capability to detect riboflavin. The ability to detect riboflavin with biotinylated RBP functionalized TiN nanoholes would be worthwhile to explore. Nevertheless, it is expected that the response will not be significant because the electromagnetic field of surface plasmon resonance is confined within the vicinity of the nanostructures. That is, the phase response signal will decay rapidly away from the plasmonic nanostructures surface, as the sensing volume is only around 30 nm above the nanostructures surface, and that the riboflavin binding event is a few tens of nanometers above the nanostructures surface. As a result, the detection of riboflavin with functionalized TiN nanoholes would be difficult.
4.4 Future developments

To further study the surface plasmon resonance biosensing of riboflavin with titanium nitride, several investigations can be done.

First, the detection range of concentration of riboflavin can be increased, in particular, for direct detection with TiN nanoholes. In this report, the range of detection was 100 ppb to 10000 ppb. In order to obtain the limit of detection of this system, the detection of riboflavin can be carried out over the range from 1 ppb to 100000 ppb.

Second, the computational investigation of riboflavin adsorption on TiN can be done. The adsorption energy via each functional group of riboflavin adsorbing onto the TiN surface can be computed, for example, via density functional theoretical (DFT) at the ground state using an appropriate DFT package [61]. It can help with understanding the adsorption mechanism and the relationship between adsorption and concentration of riboflavin.

Third, detection of riboflavin with functionalized TiN nanoholes can also be done. Although the detection is expected to be poor, it would be good to validate the expectation by conducting further experiments.

Last but not least, the feasibility of making different nanostructures combined with different functionalization methods can be further examined to develop a better plasmonic sensing system.
5 Conclusions

Riboflavin is mandatory to a wide variety of cell activities. The label-free detection of riboflavin with SPR and LSPR sensing based on TiN substrates and nanostructure have been successfully investigated in this report. This is the first time ever result of riboflavin detection with TiN. A direct and simple method of detection has been accomplished. It was found that TiN nanoholes were the best performed plasmonic substrate in direct adsorption test among four different plasmonic substrates due to the bioaffinity of riboflavin towards TiN and the signal amplification from the nanostructure.

TiN nanoholes were tested in detail with various concentrations of riboflavin. The work demonstrated the concentration-dependent response of riboflavin on TiN nanoholes. In addition, functionalization of TiN film with biotinylated RBP has improved the riboflavin detection signal with TiN film as the bulk effects of SPR response were utilized when the riboflavin binding events were moved away from the film surface.

This dissertation demonstrated the feasibility of a new concept of riboflavin detection. The objectives of this dissertation were successfully achieved. Although the report findings are not yet ready for real life implementation, it shows clearly the potential for riboflavin detection with TiN and nanostructures due to its excellent mechanical properties and chemical stability over the conventional gold materials. Moreover, by employing the biotinylation method, TiN film should be applicable for a broad range of plasmonic biosensing such as the detection of antigens and DNA.
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