A Study of Multienzyme in MOF for Biosensing

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Abstract. At present, diabetes has become a widespread disease between young people and the elderly. New methods to detect diabetes are required. Hence, enzyme biosensors (glucose biosensors) based on immobilization methods are invented to solve the problem. However, enzyme stability can not be guaranteed. Metal-organic-framework has indicated a bright future in improving stability. In this project, o-Phenylenediamine (OPD) coating is used as a coloring agent to detect glucose. As is proved by previous researchers, taking advantage of the cascade reaction of glucose oxidase (GOx) and horseradish peroxidase (HRP) enzymes, filter paper-based zeolitic imidazole framework-L (ZIF-L) thin film can be used to detect glucose considering its high acid stability and ease of fabrication. The results reveal that ZIF-L/GOx&HRP was linearly sensitive in the range of glucose concentrations (1, 2, 3, 4, and 5mM). After optimization by enzyme concentration and synthesis, 0.75mol/mL GOx&HRP with 30 minutes synthesis time illustrates the best performance.

Keywords: Biosensors, Metal-organic-framework, Zeolitic Imidazole Framework-L, Enzymes.

1. Introduction

In recent years, more and more people are suffering from diabetes, and the population tends to be younger. Thus, biosensors that can detect diabetes more precisely are required. Biosensors are analytical devices that combine biological components with physical and chemical detectors and chemicals. Its main components are the molecular recognition part (sensitive element) and the conversion part (transducer). Also, converting bioactive signals into electrical signals through biosensors is capable. Additionally, the biosensor is a high-tech infiltrated by various disciplines that combine biology, chemistry, physics, and electronic technology. Due to its high selectivity, high sensitivity, fast analysis speed and low cost, especially advanced levels of automation, integration, and other characteristics, it has been obtained in recent decades and developed very rapidly. In 1967, Updike and Hicks discovered the first generation of enzyme-based sensor [1]. The report claimed that using van der Waals forces, ionic bonds, or covalent bonds to absorb enzyme is an immobilization method for enzyme biosensors. Oxidoreductase, polyphenol oxidase, peroxidase, and amino oxidase occupy the most popular enzymes. Furthermore, Diviès implements the first microbial or cell-based sensor [2]. Then the first tissue-based sensor was reported by Rechnitz [3]. These Organelle-based sensors are made from membranes, chloroplasts, mitochondria, and microparticles detect the amino acid arginine. However, such biosensors have higher stability but longer detection time and lower specificity. Therefore, the research of biosensors has become a new focus of the world's science and technology development, as well as playing an important role in many fields.

Biosensors mainly have three classification naming methods based on molecular recognition components, signal converters, and interaction between the objective and the molecular recognition element [4].

Among the biosensors categories mentioned above, glucose biosensors are one of the most important. Clark and Lyon proposed the first glucose biosensor in 1962, depending on the electrochemical method using Glucose Oxidase (GOx) [5]. This biosensor provides a high level of sensitivity due to the electrochemical method and its relatively low manufacturing cost. Unlike other enzymes (such as hexokinase and glucose 1-dehydrogenase), this enzyme-based biosensor is highly sensitive to glucose and can withstand changes in pH and temperature.

Moreover, metal-organic frameworks occupy an important position in the field of biosensors. They are coordination networks with organic ligands that contain potential voids [6]. Under some special circumstances, pores are stable to eliminate guest molecules, solvents normally, and some other

compounds may be refilled during the procedure. Because of this property, MOF is important for storing gases such as hydrogen and carbon dioxide. Additionally, gas purification, gas separation, catalysis, conductive solids, and supercapacitors can be considered as other possible applications of MOF [7].

The properties of MOF play an important role in the research of network chemistry as well as its synthesis methods. Compared to MOF, the covalent organic skeleton (COF) has an extended structure that is completely made of light elements [8]. Due to the increase of MOF types and the gradual development of MOF synthesis, MOF will have immeasurable application prospects. In terms of gas adsorption and separation aspect, the synthesis of MOF with high adsorption ability can be used for hydrogen storage, adsorption, and separation of toxic and harmful gases. Some severe environmental problems faced by people will also be alleviated or solved. In the catalysis field, using different metal mixtures to construct synthetic MOF with high catalytic performance will further improve the catalytic efficiency. Otherwise, synthetic MOF materials can be used for the extraction and isolation of target proteins in complex systems over biomedical field. Due to the characteristics of small pore size and good biocompatibility, nanoscale MOF materials also have great prospects in drug transport and metabolism.

In addition to the applications mentioned above, enzyme-MOF can be considered one of the most potential materials for biosensing applications. Over the past decades, immobilizing soluble enzymes on MOF have been the target of intense research by scientists from many fields. By immobilizing the enzyme on the MOF, the properties of the enzyme, such as storage, chemical stability and affinity with the substrate, can be greatly improved. As the enzyme performance improved, high enzyme loading was also observed when using MOF as an immobilized support. Recently, taking advantage of metal-organic framework (MOF) for an enzyme in situ biomineralization studies has shown great promise to improve enzyme stability without damaging enzyme activity and property. Most enzyme-MOF studies emphasize synthesizing enzyme-MOF complexes in free particle systems that are mixed with analytes and then filtered out of solution before analysis [9]. In the following table 1, the justification for choosing the MOF material will be indicated. Based on previous research, these methods are adapted to synthesize enzyme@MOF thin film [10].

According to the research, a simple preparation method of enzyme-MOF membranes for enzyme-based biosensors was developed, as well as research the biosensors' characteristics, stability and properties. This approach not only has low cost, but also reduces the operating conditions of the field equipment. The sensitivity of the complex to inhibitors indicates that more research is needed to confirm the spatial arrangement of the enzyme in the ZIF-L matrix and its role. ZIF-L/GOx and HRP in situ biosensors have good selectivity, high sensitivity, and wide range of linear response performance. The sensitivity results show that the ZIF-L structure in ZIF-L/GOx and HRP could improve the linear sensitivity by inhibiting diffusion. In summary, using ZIF-L is a good choice. In this study, since filter paper is used as a substrate containing abundant functional groups, surface modification is not necessary.

Table 1. The justifying criteria for choosing MOF material

Criteria	Item	Justification
Technique	Solvothermal	Facile and flexible. Most commonly used method
Substrate/ support	Polypropylene membrane	Acts as a support for the film. Cheap hydrophobic material.
Substrate modification	PDA/PEI	Provide hydrophilic attachment for enzyme@MOF in-situ growth. Widely studied and understood.
Enzymes	Glucose oxidase and hydrogen peroxidase	Coupled enzyme reaction to produce detection of glucose. Average stability in room temperature.
MOFs	ZIF-8	Repeat experiment to validate results. Acts as a control experiment. A good benchmark to study the effect of MOF thickness to enzyme activity.
	ZIF-L	Provide different thickness and shape/size of pores. It was shown ZIF-L has higher acid stability than ZIF-8 [11].
	ZIF-90	Provide hydrophilic environment to enzymes. It was shown that hydrophilic environment can increase enzyme activity [12].

2. Material and Apparatus

Material: Filter paper, Zinc nitrate (Zn (NO3)2), 2-Methylimidazole (HMIM), Glucose oxidase (GOx), horseradish peroxidase (HRP), Phosphate-buffered saline (PBS), o-Phenylenediamine (OPD) were all purchased from Sigma-Aldrich.

Apparatus: FTIR spectrometer and UV-visible spectrophotometer.

3. Methods

3.1. ZIF-L enzyme synthesis

3.1.1. ZIF-L fabrication

$Zn(NO_3)_2 \cdot 6H_2O$ (0.1352 g) and HMIM (0.5968 g) are dissolved separately in 20 mL water, and then two aqueous solutions are mixed together. HRP (9 mg) and Gox (6 mg) are added into the solution. Filter paper is soaked in the solution and stood still for 1 h. Then use solution to rinse the filter paper for several times. Last, make the filter paper dry and cut it into strips.

3.1.2. Eye detection

OPD (10mg) is dissolved in 10 mL water and then the solution is coated on the strips. Wait for 10~15 minutes and observe the colour change.

3.2. Enzyme loading

3.2.1. Bradford assay

The Bradford method was used to measure the concentration of the enzyme after immobilization for the purposes of investigating the immobilization efficiency and enzyme loading capacity of nanoparticles.

Before reading the data, the Carry 100 UV-visible spectrophotometer is calibrated at wavelength of 595 nm. Researchers have proved that using UV-visible spectrophotometer at 595nm has minimum interference of oligodopa solution and arginine solution. Hence, the reading data will be more precise. This experiment was done by mixing a small amount of sample (0.05 mL volume) with 1.5 mL

Bradford Reagent to measure the enzyme concentration in each sample. Specific results will be illustrated in discussion part.

3.2.2. Calculation of Loading Capacity and Enzyme Immobilisation Efficiency

The enzyme loading capacity (μg enzyme /mg nanoparticles) and the enzyme immobilization efficiency were calculated using equations 1 and 2 below based on the enzyme concentration obtained by Bradford method at each immobilization stage.

Equation 1 Loading Capacity of Enzymes on Nanoparticles:

$$\text{Loading capacity} = \frac{M_{immobilised}}{M_{nanoparticles}}$$

Equation 2 Immobilisation Efficiency of Enzymes on Nanoparticles:

$$\text{Immobilisation Efficiency (\%)} = \frac{M_{immobilised}}{M_{added}} x100$$

Where $M_{immobilised}$ is the mass of enzyme immobilised on the particles, $M_{nanoparticles}$ is the mass of nanoparticles formed, and M_{added} is the mass of enzyme added in the initial solution.

3.3. Sensitivity test

After ZIF-L fabrication, five different concentrations of glucose solution for 20 mL are made (1, 2, 3, 4 and 5 mM). 10 mg OPD are weighed to combine with five different concentrations of glucose solution respectively in sequence. Then ZIF-L is put into the solution with stirrer and time was recorded every 2 minutes and 30 seconds for 10 minutes (4 times). Meanwhile, UV-visible spectrophotometer was used to test the absorbance for each solution on the base of timing.

3.4. Optimization experiment

3.4.1. Changing enzyme concentration

After the first experiment by using 5 mg enzymes, the results did not show a good graph. Thus, enzyme amount was changed from 5 mg to 10 mg and 15 mg. The operation process is still in accordance with the above steps.

3.4.2. Changing ZIF-L synthesis time

ZIF-L was made by the same approach above but synthesis time are changed into 30 minutes, 1 hour and 2 hours. The performance was tested by UV-visible spectrophotometer.

3.5 Eye-based detection

OPD (61.2 mg) dissolved in glucose solution (10 mL 5mM) is coated on the surface of filter paper based ZIF-L thin film. After 10 to 15 minutes, observe the colour change on samples.

4. Results and discussions

4.1. Characterization

4.1.1. Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) is an essential surface characterization technique that is used. Electrons are used by scanning electron microscopes to form images. This allows a greater depth of field, so that a larger number of samples can be focused at a time. SEM also produces high-resolution images. ZIF-L surface SEM is shown below (Fig. 1).

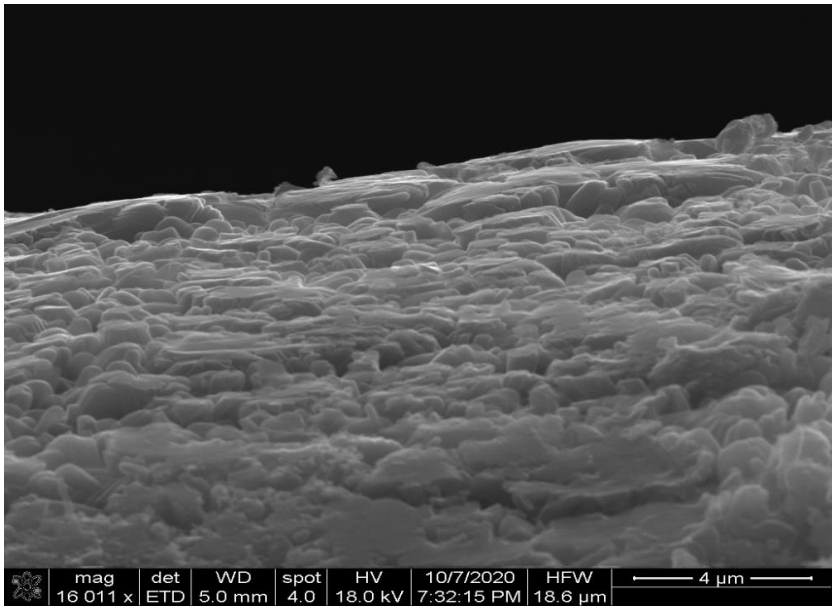

Figure 1. Filter paper ZIF-L SEM

4.1.2. Fourier Transform Infrared Spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) is normally used to obtain infrared spectra that absorbs and emits solids, liquids or gases [13]. High-resolution spectral data can be obtained by the FTIR spectrometer in a wide spectral range at the same time. The Fig. 2 below indicates the comparison of MOF with different synthesis times.

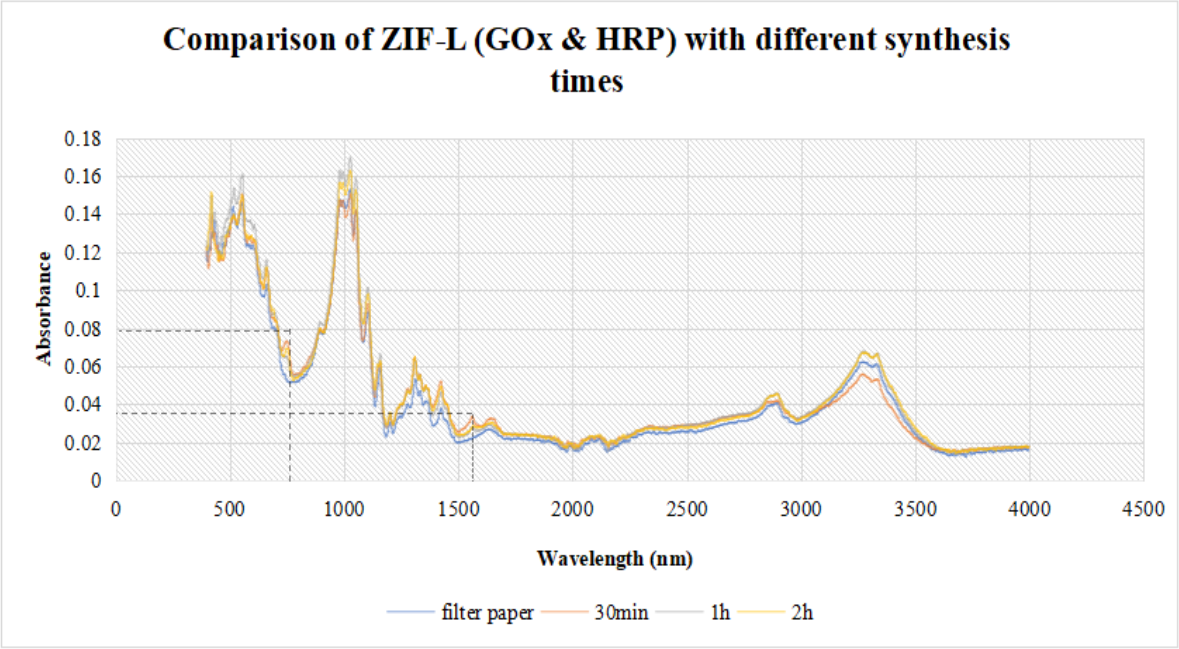

Figure 2. Comparison of MOF with different synthesis times graph

From Fig. 2, there exists a peak at about 750 nm and 1544 nm on enzyme MOF. This illustrates the MOF has C-H bending (750 nm) and N-O stretching is contributed by the presence of enzyme (1544 nm).

4.2. Enzyme loading

As is mentioned in methods part, Bradford Assay is used to calibrate ZIF-L. The synthesis method is 15 mg enzymes with 1 hour synthesis time. The calibration curve is shown in Fig. 3.

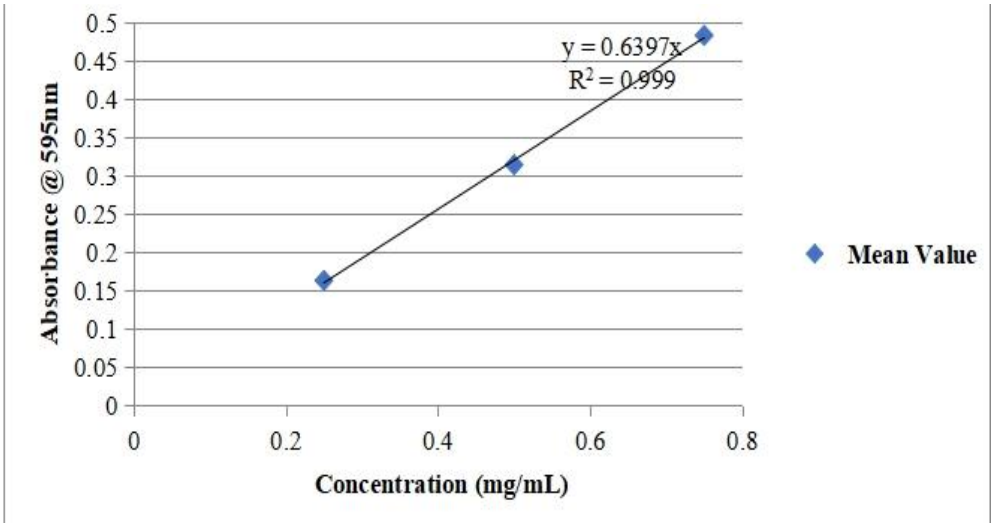

Figure 3. Calibration curve for ZIF-L

Based on the calculation of Loading Capacity and Enzyme Immobilisation Efficiency, the enzyme loading efficiency is 38.4% which means ZIF-L is efficient.

4.3. Sensitivity Test

4.3.1. Benchmark Test

Benchmark test is implemented to validate the experimental procedure and confirm that there is no interference from other components at target absorbance value. The Fig. 4, Fig. 5, Fig. 6 and Fig. 7 blew show the whole process.

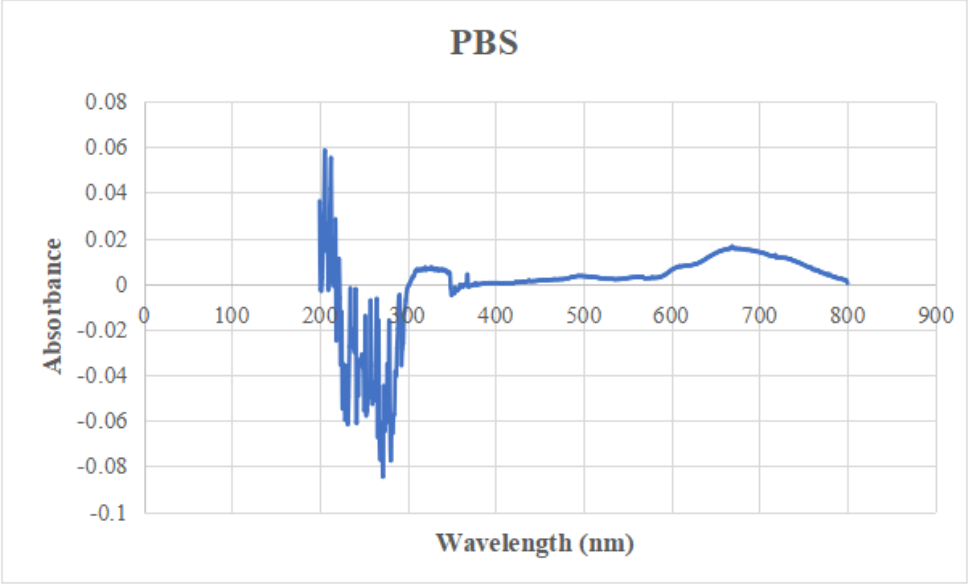

Figure 4. PBS absorbance graph

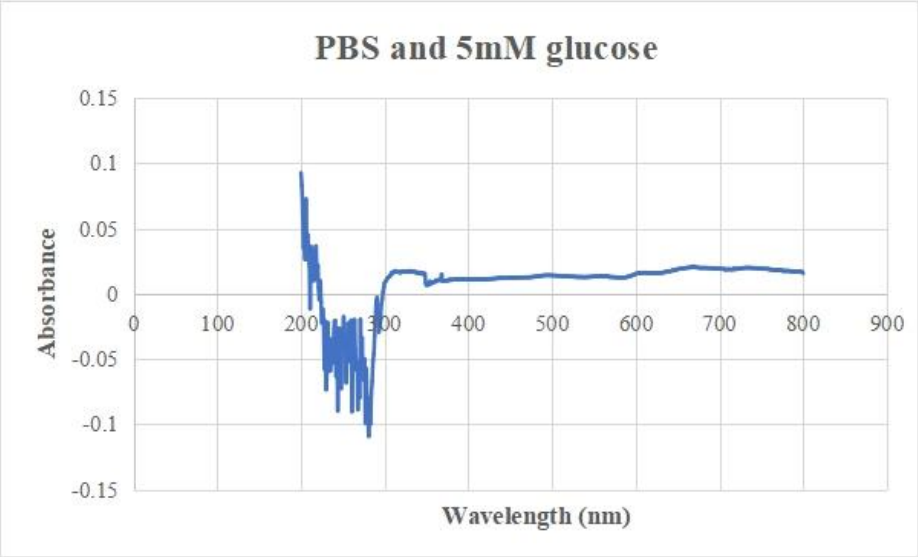

Figure 5. PBS and 5mM glucose absorbance graph

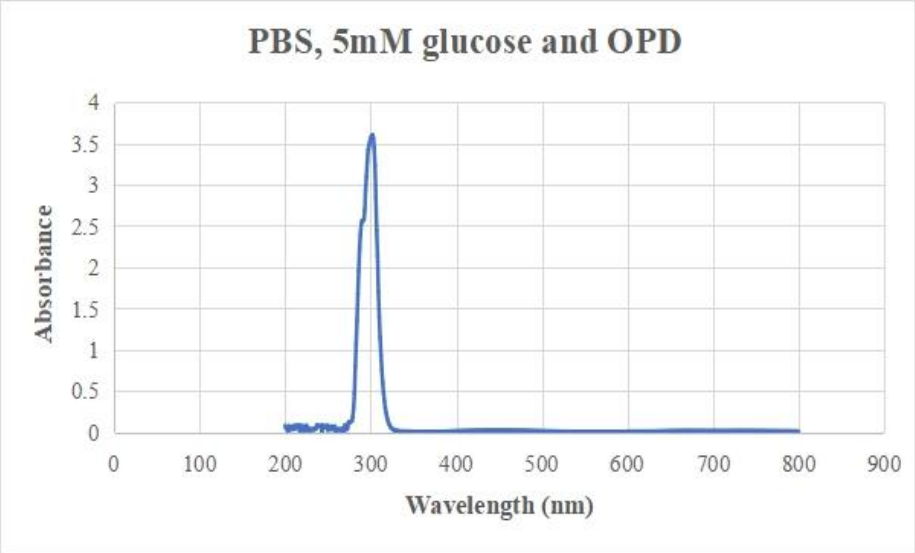

Figure 6. PBS and 5mM glucose with OPD absorbance graph

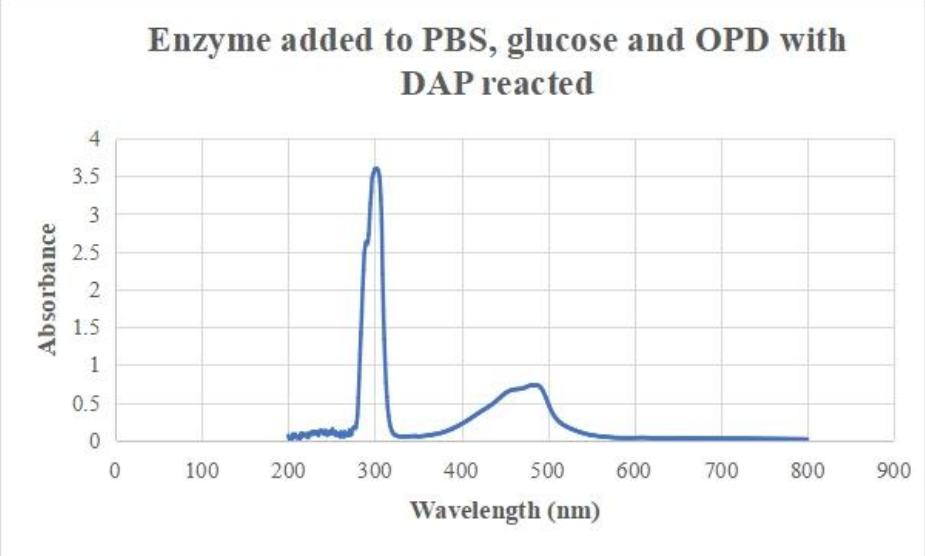

Figure 7. Enzyme added to PBS, glucose and OPD with DAP reacted absorbance graph

As is shown from Fig. 4, only PBS is tested illustrating a relatively smooth curve. Then 5mM glucose is added into PBS, it also shows a similar curve which means there does not exist many changes. We can observe from Fig. 7 that there is a peak at about 450 nm. This phenomenon illustrates that as enzyme is added into the PBS, glucose and OPD mixture, the OPD is converted to DAP which can be detected at 450 nm wavelength.

4.3.2. Response Time @ 5mM

In this experiment, we use 3.5mg OPD dissolved in 3.5mL 5mM glucose solution.

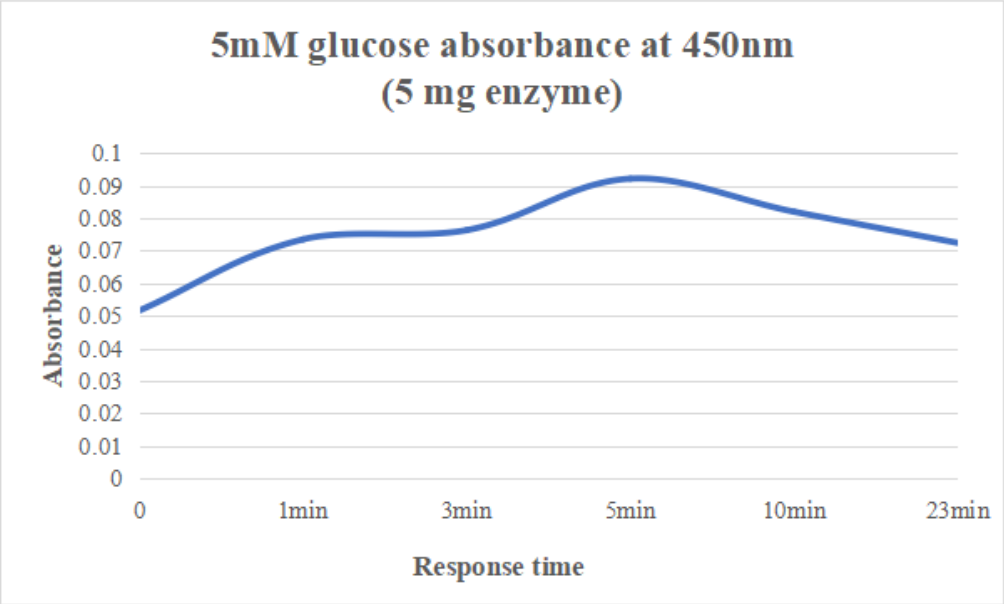

Figure 8. 5mM glucose absorbance against response time

The Fig. 8 shows a fluctuation and the maximum absorbance is obtained at about 5 minutes response time. The probable reasons are small amount of OPD and few particles on MOF.

4.3.3. Different Concentrations

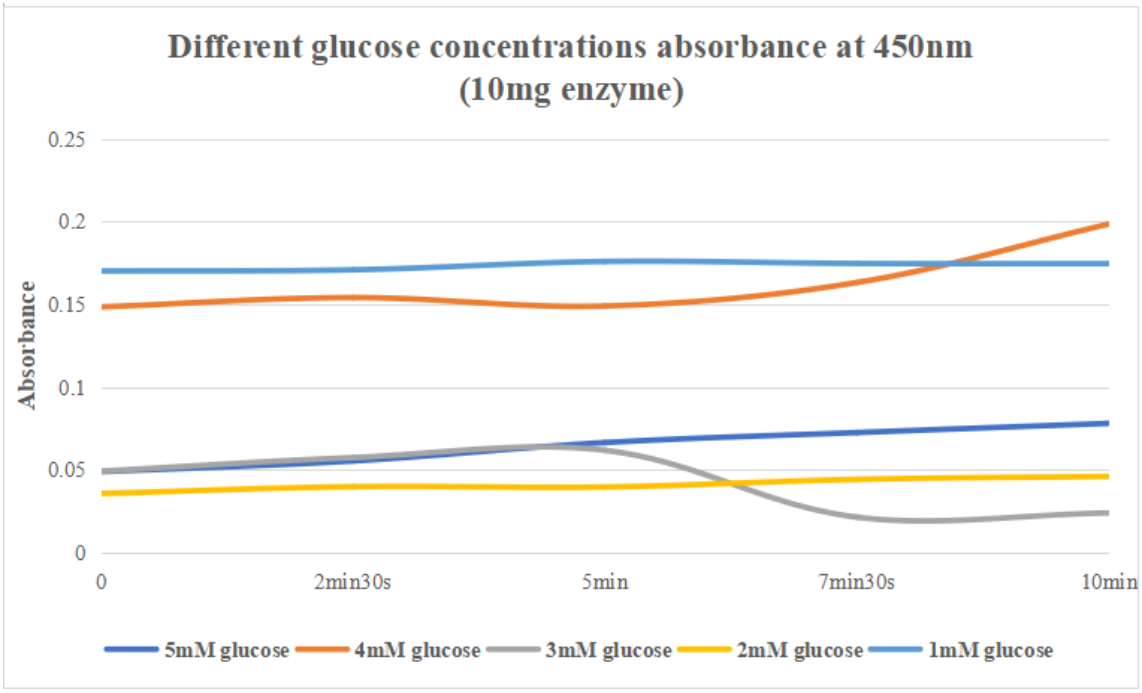

Figure 9. Different glucose concentrations absorbance at 450nm (10mg enzyme)

From previous paper, the maximum absorption is around 450 nm [4]. Five different concentrations of glucose with OPD are tested. The synthesis lasts for 1hour with 10 mg enzyme (GOx & HRP). 10 mg OPD are dissolved in 20mL glucose solution. The Fig. 9 below illustrates a clear comparison of different glucose concentrations absorbance at 450nm.

4.4. Optimization Result

4.4.1. The effect of different enzyme amount

In this project, we change the enzyme amount from 5 mg (Fig. 10) to 10 mg (Fig. 11) due to the strange data from 5 mg dosage. Then the amount is added to 15 mg (Fig. 12). Because the first experiment only testing 5 mM glucose solution, we make a comparison graph (Fig. 13) of all three enzyme amounts with 5 mM glucose solution.

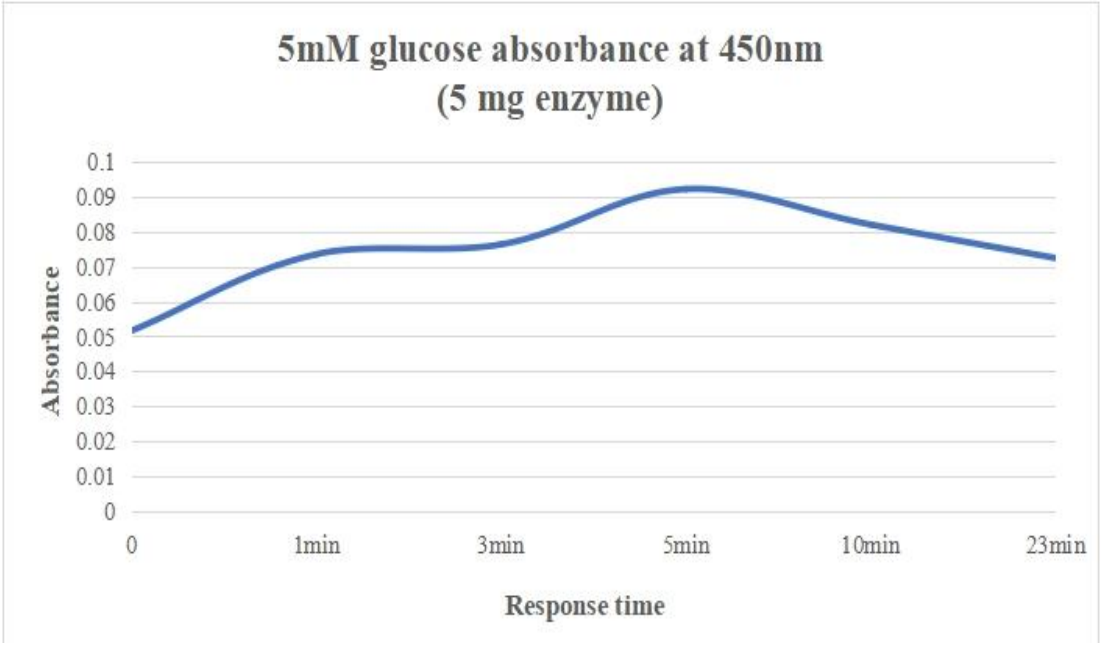

Figure 10. 5 mM glucose absorbance against response time

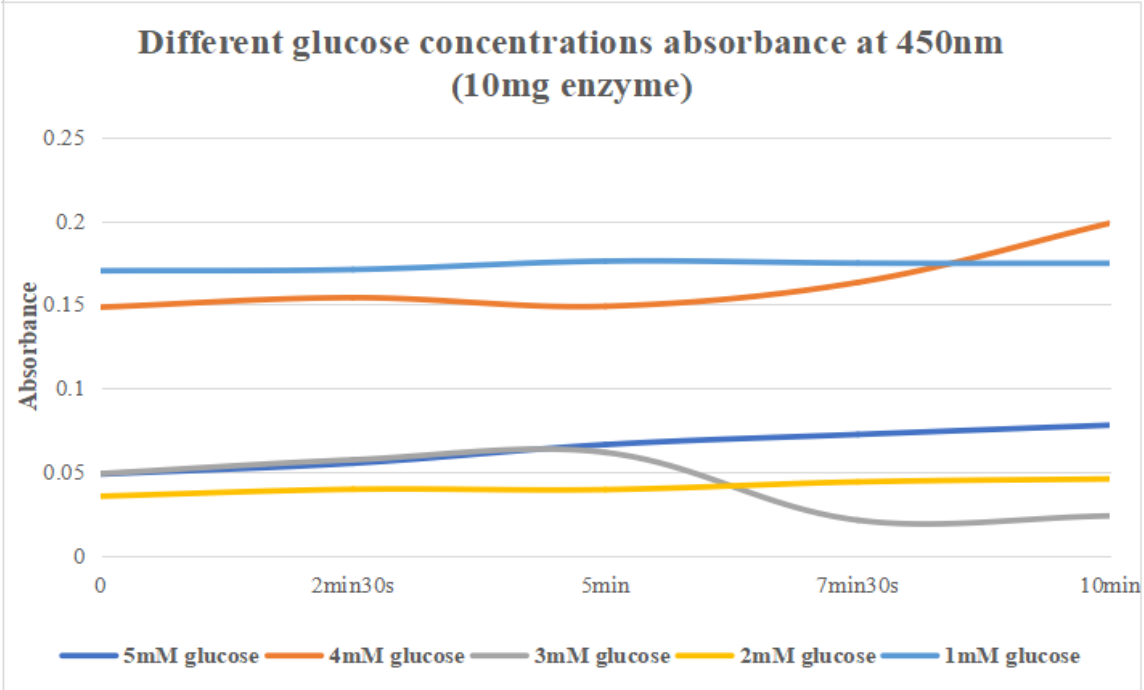

Figure 11. Different glucose concentrations absorbance at 450nm (10mg enzyme)

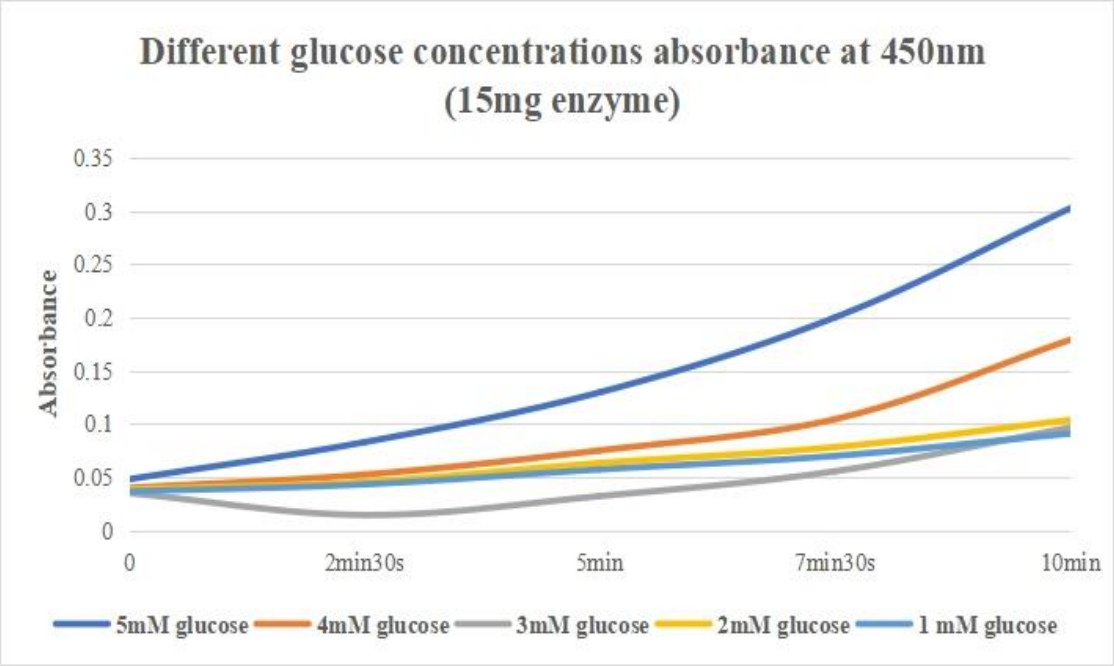

Figure 12. Different glucose concentrations absorbance at 450nm (15mg enzyme)

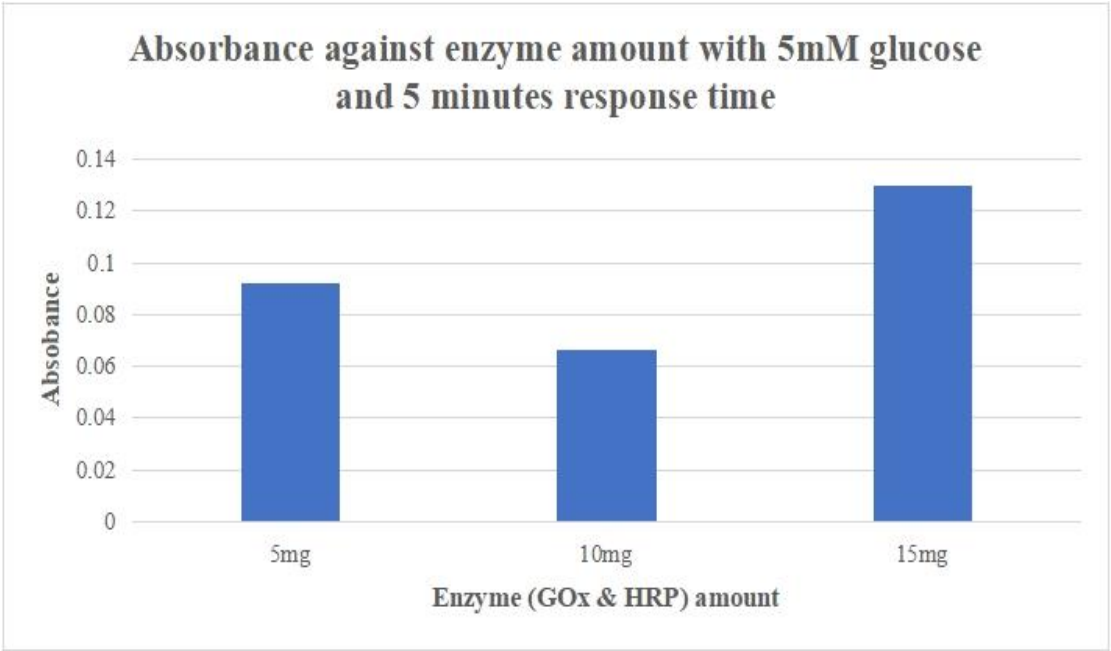

Figure 13. Absorbance against enzyme amount with 5mM glucose and 5 minutes response time

Based on the figures above, it is obvious that Fig. 12 shows the best linear graph. The conclusion can be obtained that 5mM glucose with 15 mg enzyme (GOx&HRP) is the best formula for application.

4.4.2. The effect of ZIF-L (GOx & HRP) synthesis time

The synthesis time is varied by taking out the filter paper at a certain time following a rinsing procedure. Based on Fig. 14, the performance of the glucose detection at 5 minutes response time is similar for three samples with 30 minutes, 1 hour and 2-hour synthesis time. However, as is shown in Fig. 15, after 5 minutes response time, the synthesis method with the lowest amount of time has the highest, while the longer the synthesis time, the lower its absorbance. This could be due to the enzyme denatured in the synthesis solution at room temperature after a certain time. Therefore, 30 minutes synthesis time can be recommended for future applications.

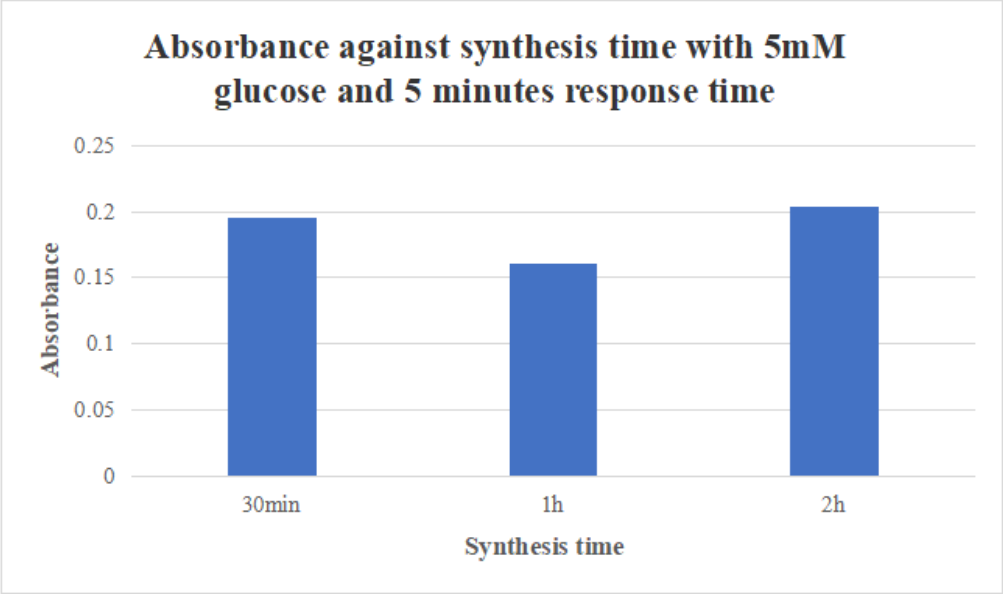

Figure 14. Absorbance against synthesis time with 5mM glucose and 5 minutes response time

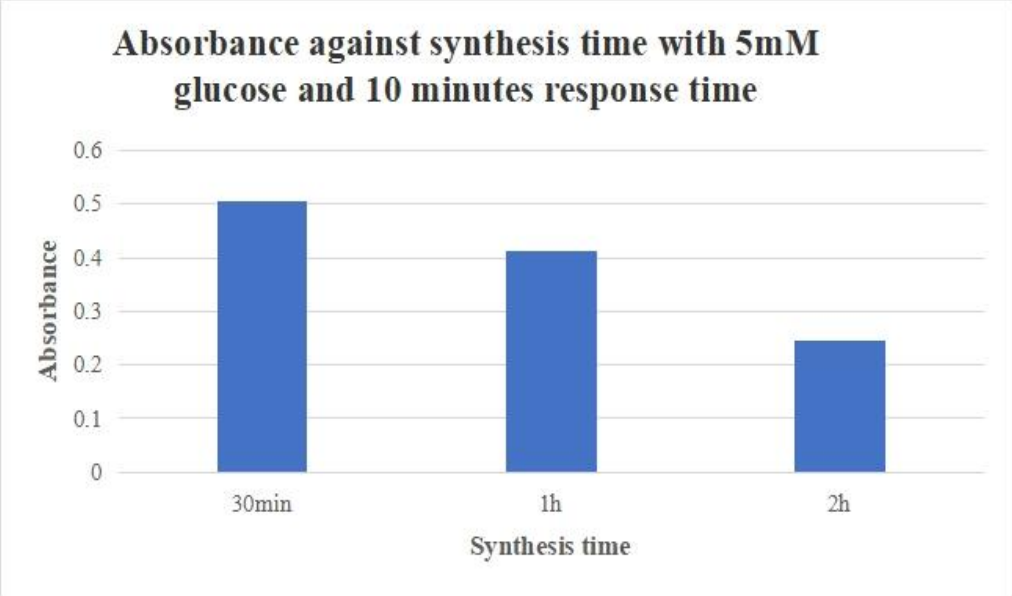

Figure 15. Absorbance against synthesis time with 5mM glucose and 10 minutes response time

4.5. Eye-based Detection

Five concentrations of glucose with OPD are coated on the ZIF-L surface. Fig. 16 below indicates the comparison between initial samples and color-changed samples from 1mM to 5 mM. The left-hand side shows a control.

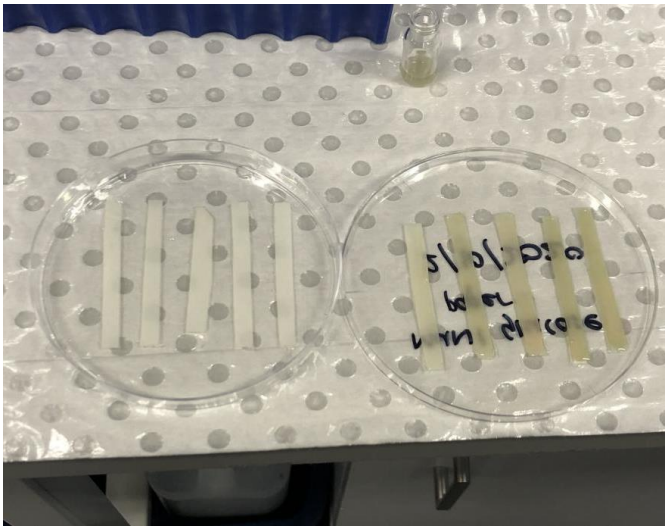

Figure 16. Filter paper based ZIF-L coated by OPD

OPD has a color change based on yellow, which the naked eye can detect. This has great potential for diabetes detection. However, the differences between each different concentration are not clear. This area will need improvement to be used as an eye-based detection.

5. Conclusion

Over the years, many people have been suffering from diabetes. Enzyme biosensors (glucose biosensors) based on immobilization methods have great potential to detect the disease by eye-based detection. Hence, manufacturing biosensors using metal-organic frameworks (MOF) as identification or transducer elements show good application prospects. This paper aims to find the best conditions for MOF on biosensing applications. The filter paper-based zeolitic imidazole framework-L (ZIF-L) thin film is chosen to test the selectivity and sensitivity as well as eye-based detection. According to the study, ZIF-L can have high sensitivity and color change when 15 mg enzymes (GOx & HRP) are added with 30 minutes of synthesis time. In the future, different types of MOF functionality for biosensing applications and their feasibility to be synthesized as MOF-MOF hybrid can be explored. We can also measure thickness related to the diffusion of substrate glucose. In the later stage, it is expected that the characteristics and performance of the MOF-on-MOF biosensor in relation to its synthesis parameters. In addition, improving the sensitivity of biosensors by increasing thickness can be considered. Moreover, enhancing selectivity by the enzyme location using MOF with functional groups is also a meaningful direction.

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