UNIVERSITI PUTRA MALAYSIA

ENCAPSULATED MINI PROTEIN 20 IN ZEOLITIC IMIDAZOLATE FRAMEWORK-8 FOR DEVELOPMENT OF URIC ACID ELECTROCHEMICAL BIOSENSOR

SITI FATIMAH NUR BINTI ABDUL AZIZ

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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June 2022

Chairman : Assoc. Prof. Shahrul Ainliah Alang Ahmad, PhD
Faculty : Science

A biological sample with an abnormal level of uric acid (UA) can result in human health problems. Thus, it is important to evaluate UA concentration in the physiological samples. To measure UA, novel mini protein with 20 amino acid mimicking uricase (mp20) was designed and further used as bioreceptor. Despite the efforts to explore the stability and activity of mp20 in the construction of new biosensing devices, their "fragile" nature under suboptimal storage conditions is a major issue that hinders their extended use as bioreceptors that may lose the binding affinity of substrate, creating to false positives and false negatives. Thus, the encapsulation of mp20 within zeolitic immidazolate frameworks-8 (mp20@ZIF-8) enhance their stability without losing their intended functions is a promising prospect. In this research, synthesis work of mp20@ZIF-8 was carried out using biomineralization approach and optimized by varying organic linker ratios and reaction time. Physicochemical structures of mp20@ZIF-8 were characterized by powder X-ray Diffraction (PXRD), Ultraviolet -Visible (UV-Vis), Fourier transform infrared (FTIR), thermogravimetric analysis (TGA), and Brunauer-Emmett-Teller (BET). Differential pulse voltammetry (DPV) was utilized to monitor the electrochemical performance of mp20@ZIF-8 modified on a reduced graphene oxide/screen printed carbon electrode (rGO/SPCE). Surface characterization of the fabricated electrode surface was assessed via field emission scanning microscopy (FESEM). In accordance to the preliminary findings, the biocomposite synthesized at a Zn/HmIm molar ratio of 1:8 for 0.5 h with a rhombic dodecahedron morphology and particle size of 170 nm larger than 100 nm of ZIF-8 observed via High-resolution transmission electron microscope (HRTEM) exhibited the highest peak current for uric acid detection. The representative cyclic voltammogram for the mp20@ZIF-8/rGO/SPCE exhibited a quasi-reversible behaviour with low redox peaks current, indicating the poor conductivity of the mp20@ZIF-8, which is also supported by the larger semicircle arc in the Nyquist plot upon fabrication of the rGO/SPCE surface with the biocomposite.

The optimization of the mp20@ZIF-8 biosensor was further investigated utilizing a central composite design-response surface methodology (CCD-RSM). In the presence of the same fold concentration of interfering species, the constructed biosensor demonstrated excellent selectivity with a detection limit of 0.27 μM. The biosensor's suitability for detecting uric acid in human serum and urine samples was verified using HPLC and a commercial uric acid meter. An attempt to address the mp20 limitation, which is its inactive catalytic properties, was conducted by introducing a metal cofactor in assay. Initially, the mini protein and potential metal cofactor were subjected to computational study via MIB (metal ion-binding site prediction and docking server), a web server employed to predict and locate the metal ion-binding sites in proteins. Following that, a series of experimental designs based on uricase enzymatic assay were conducted to investigate the efficacy of metal cations towards mp20 activation. The results of the colorimetric assay demonstrated that the presence of copper(II) ions stimulates the production of quinoid, which is consistent with previous findings. In comparison with the free mp20, the mp20@ZIF-8, which is further employed for colorimetric detection of uric acid, possesses excellent stability, selectivity, and sensitivity properties owing to the availability of superior porous host protection. The potential of the Cu(II) activated mp20@ZIF-8 as an electrochemical enzymatic biosensor was also assessed. The fabricated biosensor shows an increased detection signal with LOD of 0.21 μM, which is lower than the uric acid threshold level in physiological samples under optimum conditions acquired through the RSM method. Further exploration into the applicability of the constructed mp20@ZIF-8 as a portable electrochemical device was also executed, considering the feasibility of the selected approach and the outstanding stability of the detection signal over time. As a conclusion, the findings indicate that a facile, sensitive, selective, and low-cost biocomposite, mp20@ZIF-8, may offer a versatile strategy for both non-enzymatic and enzymatic detection of uric acid in biological samples.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

PROTEIN MINI 20 YANG DIKAPSULKAN DALAM KERANGKA IMIDAZOLAT ZEOLITIK-8 UNTUK PEMBANGUNAN BIOPENDERIA ELEKTROKIMIA ASID URIK

Oleh

SITI FATIMAH NUR BINTI ABDUL AZIZ

Jun 2022

Pengerusi : Prof. Madya Shahrul Ainliah Alang Ahmad, PhD
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Sampel biologi dengan tahap asid urik (UA) yang tidak normal boleh mengakibatkan masalah kesihatan pada tubuh manusia. Oleh itu, ini adalah penting untuk menilai kepekatan UA dalam sampel fisiologi tersebut. Untuk mengukur UA, protein mini novel dengan 20 acid amino menyerupai urikase (mp20) telah direkabentuk dan selanjutnya digunakan sebagai bioreseptor. Walaupun pelbagai usaha dijalankan untuk menyelidik kestabilan dan aktiviti mp20 dalam pembinaan peranti biopenderia baharu, sifat dasarnya yang rapuh pada keadaan penyimpanan suboptimum menjadi masalah utama menghalang lanjutan kegunaannya sebagai bioreseptor yang berkemungkinan hilang keupayaan mengikat substrat secara afiniti, dan menghasilkan positif palsu dan negatif palsu. Oleh itu, pengkapsulan mp20 dalam kerangka immidazolat zeolitik-8 (mp20@ZIF-8) meningkatkan kestabilan mereka tanpa kehilangan fungsi yang dikehendaki merupakan satu prospek yang memberangsangkan. Dalam kajian ini, kerja mensintesis mp20@ZIF-8 telah dijalankan menggunakan kaedah biomimetik mineralisasi dan dioptimakan melalui pempelbagaian nisbah penghubung organik dan masa reaksi. Struktur fizikokimia mp20@ZIF-8 telah dicirikan melalui pembelauan sinar-X serbuk (PXRD), spektroskopi ultraungu/boleh nampak (UV-Vis), spektrometer inframerah transformasi Fourier (FTIR), analisis termogravimetri (TGA), pencirian kawasan permukaan Brunauer-Emmett-Teller (BET). Voltammetri denyut pembeza (DPV) telah digunakan untuk memantau prestasi mp20@ZIF-8 yang difabrikasi pada grafin oksida terturun/elektrod karbon bercetak skrin (rGO/SPCE). Pencirian pada permukaan elektrod yang telah difabrikasi juga ditaksir melalui mikroskopi medan pancaran pengimbasan elektron (FESEM). Selaras dengan penemuan awal kajian, biokomposit yang disintesis pada nisbah kepekatan Zn/HmIm 1:8 selama 0.5 jam dengan morfologi dodekahedron rombus dan zarah bersaiz 170 nm lebih besar daripada ZIF-8 yang bersaiz 100 nm diperhatikan melalui resolusi tinggi mikroskopi elektron transmisi (HRTEM) telah menunjukkan arus puncak tertinggi untuk pengesanan asid urik.

Voltammogram berkitar bagi mp20@ZIF-8/rGO/SPCE mempamerkan sifat seakan-balik dengan arus puncak redoks yang rendah, menunjukkan kekonduksian yang lemah oleh mp20@ZIF-8 yang turut disokong lengkungan semibulatan yang lebih besar di dalam plot Nyquist semasa fabrikasi permukaan rGO/SPCE dengan biokomposit tersebut. Pengoptimuman biosensor mp20@ZIF-8 telah dikaji menggunakan metodologi reka bentuk komposit tengah-kaedah tindak balas permukaan (CCD-RSM). Dengan kehadiran kadar kepekatan spesies bendasing yang sama, biosensor yang dibangunkan menunjukkan selektiviti yang bagus dengan had pengesanan (LOD) iaitu 0.27 μM. Kesesuaian biosensor untuk mengesan asid urik dalam serum manusia dan sampel air kencing telah disahkan menggunakan HPLC dan meter asid urik komersial. Percubaan untuk menangani kekurangan mp20 iaitu ketidakaktifan ciri pemangkinannya telah dijalankan dengan memperkenalkan kofaktor logam dalam assai. Pada permulaan, protein mini dan kofaktor logam yang berpotensi ditakhluk kepada kajian pengkomputeran menerusi MIB (jangkaan tapak pengikat-ion logam dan pelayan dok), iaitu satu pelayan web yang digunakan untuk meramal dan mencari tapak pengikat ion logam dalam protein. Berikutan itu, satu siri reka bentuk eksperimen berdasarkan ujian enzim urikase telah dilakukan untuk menyiasat keberkesanan kation logam terhadap pengaktifan mp20. Keputusan ujian kolorimetrik menunjukkan bahawa kehadiran ion kuprum(II) merangsang pembentukan quinoid, iaitu selari dengan penemuan sebelumnya. Berbanding dengan mp20 bebas, mp20@ZIF-8, yang selanjutnya digunakan untuk pengesanan asid urik secara kolorimetrik, mempunyai sifat kestabilan, selektiviti dan kepekaan yang sangat baik dengan kehadiran perlindungan perumah berliang unggul. Potensi Cu(II) teraktif mp20@ZIF-8 sebagai biosensor elektrokimia enzimatik juga turut dinilai. Biosensor yang difabrikasi menunjukkan peningkatan isyarat pengesanan dengan had pengesanan (LOD) iaitu 0.21 μM, iaitu lebih rendah daripada paras asid urik dalam sampel fisiologi di dalam keadaan optimum yang diperoleh melalui kaedah RSM. Penerokaan lanjut mengenai kebolehgunaan mp20@ZIF-8 yang dibina sebagai peranti elektrokimia mudah alih turut dilaksanakan, dengan mempertimbang kebolehlaksanaan pendekatan yang dipilih dan kestabilan isyarat pengesanan yang bagus dalam satu jangka masa. Sebagai kesimpulan, kesemua hasil dapatan menunjukkan bahawa biokomposit mp20@ZIF-8 yang mudah, sensitif, selektif dan berkos rendah boleh menawarkan satu strategi serba boleh untuk pengesanan asid urik secara bukan-enzimatik dan enzimatik dalam sampel biologi.

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%	Percentage
(FESEM)	Field Emission Scanning Microscopy
(HRTEM)	Field Emission High-Resolution Transmission Electron Microscope
(POCTs)	Point-of-care-tests
(*R*ct)	Charge Transfer Resistance
°C	Degree Celcius
º	Degree
2θ	2 Thea / 180º
3D	Three Dimensional
4-AAP	*4-aminoantipyridine*
Å	Armstrong
ANOVA	Analysis of Variance
APS	Ammonium Per Sulphate
ASN	Asparagine
ASP	Aspartic Acid
BET	Brunauer-Emmett-Teller
BSA	Bovine serum albumin
C.V	Coefficient of Variation
CCD	Central Composite Design
cm^3/g	Gram per cubic centimetre
CV	Cyclic Voltammetry
CYS	Cysteine
DET	Direct Electron Transfer
DFT	Density Functional Theory

DPV	Differential Pulse Voltammetry
EIS	Electrochemical Impedance Spectroscopy
FTIR	Fourier Transform Infrared
g/mol	Gram per mole
GLN	Glutamine
GLU	Glutamic Acid
GO	Graphene Oxide
h	Hour
H_2O_2	Hydrogen Peroxide
HIS	Histidine
HmIm	2-methylimidazole
HPLC	High Performance Liquid Chromatography
HRP	Horseradish Reroxidase
Hz	Hertz
IPTG	Isopropyl-β-D-thiogalactopyranoside
kDA	Kilo Dalton
LB	Luria Bertani
LOD	Limit of Detection
LOQ	Limit of Quantification
LSV	Linear Sweep Voltammetry
M	Molar
MET	Mediated Electron Transfer
mg	Milligram
MIB	Metal ion-binding site prediction and docking server
Min	Minutes
mL	Millilitre

MOFs	Metal Organic Frameworks
mV/s	Millivolt per second
nm	Nanometer
OFAT	One-factor-at-a-time
P/P_0	Relative Pressure
PBS	Phosphate Buffer Saline
pH	Exponential of the concentration of hydrogen ion
PRESS	small prediction error of sum of squares
PXRD	Powder X-ray Diffraction
rGO	Reduced Graphene Oxide
rpm	Rotation per minute
RSD	Relative Standard Deviation
RSE	Standard Error Percentage
RSM	Response Surface Methodology
s	Seconds
SDS PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SPCE	Screen Printed Carbon electrode
SWV	Square Wave Voltammetry
TEMED	N, N, N',N'-tetramethylethane-1,2-diamine
TGA	Thermogravimetric Analysis
THR	Threonine
U	Unit
U/mL	Unit per millilitre
UOX	Urate Oxidase
UV-Vis	Ultraviolet -Visible

V	Voltage
w/v	Weight per volume
ZIFs	Zeolitic Immidazolate Frameworks
μA	Microampere
μL	Microlitre
μM	Micromolar
Ω	Ohm

CHAPTER 1

INTRODUCTION

1.1 Background study

Unhealthy lifestyle, such as poor calorie restriction, dietary changes, irregular purine-rich intake (e.g., seafood, meat sugar-sweetened beverages), metabolic syndrome, diuretic usage, and hectic schedule are among multivariate factors that contribute to an elevation of serum uric acid. This has been associated with multiple disorders, including gout and hyperuricemia (Mohd *et al.*, 2011) (Islam *et al.*, 2018). Studies on the epidemiology and therapy of gout patients revealed the most prevalent risk factors for gout patients related to urate crystal formation. This includes ischemic heart disease (83.3%), diabetes mellitus (75.9%), hyperlipidemia (53.7%), hypertension (44.4%), family history of gout (22.2%), and alcohol intake (9.3%), followed by ageing (> 40 years old), which contributes to the multifactorial causality and leads to the prevalence of hyperuricemia (Sulaiman *et al.*, 2019). Hyperucemia is diagnosed when higher serum uric acid levels (>416.4 μM for men and >386.7 μM for women) or urinary uric acid levels (> 700 mg/day) are traced (Islam *et al.*, 2018b). Additionally, the condition may impair renal function by causing glomerular damage and renal arteriolar alterations, resulting in chronic kidney disease (Siu *et al.*, 2006; Chonchol *et al.*, 2007). According to the Global Burden of Disease (GDB) Chronic Kidney Disease Collaboration reports from 2017, chronic renal disease was responsible for around 4.6% of all deaths globally (Webster *et al.*, 2017; Cockwell *et al.*, 2020). Therefore, urinary or serum uric acid (sUA) concentration has been used as an indicator to monitor several health conditions, including renal failure.

Currently, there are various well-validated techniques adopted to detect uric acid in clinical laboratories, such as chromatographic, spectrophotometric, and colorimetric approaches, which need costly analytical equipment, time-consuming, tedious sample pre-treatment, and highly trained personnel. Instead, electrochemical techniques have been widely implemented as they possess the merits of high simplicity, lower instrumental cost, fast response, and high sensitivity that could be applied to various physiological sample (Lakshmi *et al.*, 2011; Tang *et al.*, 2018; Yan *et al.*, 2020). Taking advantage of the exceptional attributes such as cost-effective, short time analysis, and capability of providing an easy-to-operate guideline for users, electrochemical techniques have been applied for the determination of various analytes in a great variety of matrices, including for uric acid recognition (Lakshmi *et al.*, 2011; Zhu *et al.*, 2015). Generally, in electrochemical methods, there are two types of approaches: non-ezymatic and enzymatic sensors. In a non-enzymatic approach, the uric acid detection could be performed via electrochemical oxidation of the substrate through the facile modification of the electrode surface with highly conductive organic and inorganic compounds. However, regardless of its easy-to-fabricate steps, the direct oxidation of electroactive species such as uric acid on the bare solid electrode interface results in slower electrode kinetics, high over-potential and interference from co-existing species, which affects the analytical performance (Chen *et al.*, 2014).

Thus, the poor selectivity of the non-enzymatic uric acid sensor, which results in unfavourable conditions likely in physiological samples, has led to the desire to construct an enzymatic interface. Enzymatic biosensor is widely used because of its selectivity, as it involves the use of uricase enzyme, which has a specific active site to bind with the targeted analyte and trigger the decomposition of uric acid (Lakshmi *et al.*, 2011). Nonetheless, despite the high specificity offered by enzyme electrodes, the performance is likely hindered by the complicated immobilization and stabilization-protocol, high-cost, ease of premature degradation, leaching of soluble mediator that reduces the charge transfer, electron transfer hindrance due to unreachable redox centers, and vulnerability at high temperatures and harsh conditions, thus limiting their use to wider applications (Yan *et al.*, 2020). Therefore, current efforts have mainly focused on utilizing new materials with outstanding properties to develop sensitive electrochemical uric acid sensors and biosensors. With remarkable progress in nanoscience and nanotechnology, nanomaterial-based electrochemical transducers possess great potential in enhancing electrochemical sensors and biosensors' sensitivity and selectivity (Zhu *et al.*, 2015).

The material, which is portrayed as a biorecognition element, plays an important role in the development of a high-performance electrochemical sensing interface for recognizing target molecules through various analytical principles. Apart from producing a synergistic effect on the conductivity, catalytic activity and biocompatibility, which accelerates the signal transduction, the nanomatrix is also responsible for amplifying biorecognition events with its specifically designed structure. Recent advances in nanoengineering have exposed materials that have different sizes, shapes (e.g., nanorods, nanotubes, nanowires, metal organic frameworks (MOFs)) and porosity that are widely used in designing biofunctionalized electrodes (Zhu *et al.*, 2015; X. Wang *et al.*, 2019; Liu *et al.*, 2019). Inorganic-organic composites such as ZnO (Ahmad *et al.*, 2015; Fu *et al.*, 2016), gold nanoparticles (Zhu *et al.*, 2017), graphene oxide and reduced graphene oxide (rGO) (Fu *et al.*, 2016; Bai *et al.*, 2017; Zhu *et al.*, 2017); Zhang D. *et al.*, 2017) and mesoporous silica (Walcarius, 2001; (Zhou *et al.*, 2014) are among the most common matrices reported in the construction of uric acid electrochemical sensor. For example, ZnO nanorods have been employed as a biomatrix to immobilize uricase for catalytic electrochemical detection of uric acid. However, the void of the material does not necessarily fit the biomolecule size (Zhang *et al.*, 2004; Ahmad *et al.*, 2015). The real challenge in introducing mesoporous silica as a support material is the leaching of biomolecules from the support and the surface charge, which could reduce the enzyme loading and promote the denaturation process due to the small repulsion between them, which eventually causes the instability and agglomeration of the enzyme (Lian *et al.*, 2017; Sigurdardóttir *et al.*, 2018).

Another type of growing nanomaterial, namely metal organic frameworks (MOFs), has attracted particular interest. Its tunable porosity size, topology, green synthesis approach, and biocompatibility features make it able to address the issue of enzyme stability and cost (Zhu *et al.*, 2015). Several studies have been found on the employment of MOF derivatives as sensing elements for the construction of non-enzymatic electrochemical uric acid sensors, whereas a limited number of reviews have reported on the enzymatic approach.

The biomolecules-MOFs composites were primarily studied for different kinds of applications such as transesterification (Cheong *et al.*, 2017), catalysis (Q. Wang *et al.*, 2017a), drug delivery (Zhuang *et al.*, 2014; Shu *et al.*, 2018) and sensing via other analytical approaches (Qu *et al.*, 2019) and different substrates (e.g. hydrogen peroxide (Zhang C. *et al.*, 2017), glucose (Ma *et al.*, 2013; Wu *et al.*, 2015).

1.2 Problem statements and research motivation

In recent years, there has been an urge to develop a simple device that is able to detect and quantify targeted uric acid with high accuracy. Current clinical diagnostics rely on several methods such as high performance liquid chromatography (HPLC), spectrophotometry, chemiluminescence, and electrochemical uric acid sensors. Based on the reported reviews, spectrophotometric techniques have been widely implemented for clinical usage since decades ago. The method generally involves a colorimetric enzymatic reaction where uric acid is oxidized in the presence of a specific enzyme, uricase, to form hydrogen peroxide, allantoin, and carbon dioxide. Nonetheless, the technique includes complex sample preparation, an erroneous detection signal due to coagulated blood particles from the blood serum sample, and strict predisposal (Lakshmi *et al.*, 2011; Liu *et al.*, 2019). Another method of detection was performed using flow injection spectrophotometry. The analysis was based on the reduction of potassium ferricyanide to potassium ferrocyanide in the presence of uric acid (Waseem *et al.*, 2011). Despite its sensitivity, the methods are sophisticated and expensive. Since the implementation of conventional approaches is facing limitations including time constraints, highly skilled personnel, and tedious sample pre-treatment, the electrochemical uric acid sensor has been given more attention due to its high sensitivity, simplicity, low cost, and easy miniaturization, which can be expanded to point-of-care-tests (POCTs) construction for self-monitoring purposes.

In an electrochemical approach, non-enzymatic sensors involve the detection of chemical or biological species through their redox activity with improved sensitivity. However, the sensors experience an interference effect in biological samples, which commonly occurs at a higher potential due to the presence of common electroactive species, most likely dopamine and ascorbic acid, due to their near oxidation potential to be separated at common electrodes, and also require high overpotentials as electrooxidation occurs at an inert metal surface, contributing to fouling and poor reproducibility due to accumulated oxidized product (Lakshmi *et al.*, 2011; Vinoth *et al.*, 2016). The limitation leads to the growth of enzymatic sensors. The utilization of native enzyme uricase for biosensor construction is preferable as the biorecognition element is able to provide specific binding sites and recognize the targeted molecule. Commercially available uric acid POCTs also depend on the catalytic activity of immobilized uricase on electrodes. However, there are two major drawbacks to using native enzymes in the system: (i) the high cost of larger-scale production and (ii) non-stability under inappropriate conditions such as high temperature, unfavourable pH, and organic solvents, which cause the enzyme structure to be disrupted.

To highlight, Puri *et al.* reported the detection of uric acid with a limit of detection (LOD) up to 0.0084 mM based on the modification of uricase with 3-aminopropyltriethoxysilane on a tin oxide microelectrode array (Puri *et al.*, 2013). However, the fabricated enzyme electrode tends to lose its activity by about 40% due to instability of the biomolecule over the silane matrix over a period of time. Another review reported by Piermarini *et al.*, represents the use of uricase based on screen-printed electrode modified with prussian blue coupled with portable instrumentation acquired a LOD of 0.01 mM towards uric acid detection with a decreasing 10% of initial activity after 15 days of storage (Piermarini *et al.*, 2013). Poor long-term stability was also discovered in Zhao *et al*.'s research, where the uricase immobilized zinc oxide micro/nanowires biosensor reported a LOD of 25.6 µM, losing 14% of activity after 120 h of preparation (Zhao *et al.*, 2013). Due to this insight, immobilization on a solid and porous surface becomes a favourable approach for biomolecules to exhibit prolonged biological functioning or turnover rate as compared to their free state.

Herein, in addressing the challenges, a previously constructed mini protein 20 (mp20) mimicking uricase is used in the present study as a replacement for the native structure. It is a small molecule of 20 amino acids that is able to interact with uric acid, with similar spectroscopic properties to its native counterpart, and is more stable and economical to produce and use. The stability of mini protein 20 can be improved by encapsulating the mp20 within a metal organic framework (MOF). Due to its porous materials and high loading capacity, MOF is an attractive support for enzymes. MOF is able to protect the enzyme from denaturation under non-biological conditions and improve the enzymatic activity and stability. In this work, zeolitic imidazolate frameworks-8 (ZIF-8), a sub-class of MOFs, are used as support for mp20 (mp20@ZIF-8). The biorecognition of uric acid with mp20@ZIF-8 using electrochemical techniques requires additional conductive material to improve the sensitive detection.This is due to non-conductive properties of ZIF-8 that limit the sensitivity of the developed biosensor. The fabrication of the high-conductive reduced graphene oxide layer on the screen-printed carbon electrode was purposely performed to enhance the electron transfer within the interface. Besides, the implementation of the mp20@ZIF-8 as a biorecognition element that could oxidize uric acid electrochemically in its inactive form and enzymatically in its activated state with the presence of a metal cofactor was the first strategy reported. Without understanding the mp20's nature as a binding receptor for sensing elements, it is difficult to propose and develop a highly selective and sensitive electrochemical biosensor for uric acid detection.

1.3 Novelty of research

The utilization of mini protein with 20 amino acids that mimic uricase (mp20) encapsulated in ZIF-8 microstructure decorated on reduced graphene oxide on SPCE is the first attempt being employed in the construction of a uric acid biosensor as a biomatrix interface. Moreover, the role of the encapsulated biorecognition element is found to be more stable compared to the native enzyme and more economical to produce as an enzymatic and non-enzymatic uric acid biosensor was first discovered. The developed biomatrix could be used as a potential replacement for the existing uricase biosensor used in several point-of-care tests (POCTs) products based on the acquired sensitivity that surpasses the normal range of uric acid excretion in the biological fluids.

1.4 Objectives of the study

The aim of this study is to develop a simple, selective, and sensitive biosensor based on encapsulated mini protein 20 mimicking uricase (mp20) into a zeolitic immidazolate framework-8 (ZIF-8) for the detection of uric acid in biological samples. The following specific objectives were designed to fulfil this aim:

1. To encapsulate mp20 into ZIF-8 (mp20@ZIF-8) microstructure via biomimetic mineralization method and its characterization.
2. To evaluate stability of mp20@ZIF-8 biocomposite against various temperatures and pH conditions and its potential for uric acid determination by a colorimetric assay.
3. To evaluate performance of mp20@ZIF-8 biocomposite using electrochemical technique in the presence of uric acid under optimal conditions and conduct comparable studies with conventional methods.
4. To validate the optimized modified electrode as portable electrochemical uric acid sensor.

1.5 Scope and limitation

In this study, the utilization of encapsulated micro protein mp20 into ZIF-8 fabricated on rGO/SPCE platform is shown to be selective for uric acid detection, either as an enzymatic or non-enzymatic sensor. However, there is a limitation in terms of acquiring a higher detection current density due to the non-conductive nanoporous ZIF properties and the need of a metal cofactor in the activation of the mp20 active site in order for the biosensor to function as an enzymatic biosensor and catalytically oxidize uric acid. Additionally, proper sealing and storage at 4 ºC are required to preserve the performance of this designed mp20@ZIF-8/rGO/SPCE electrode, which has a six-month lifetime.

1.6. Thesis outline

Chapter 1 discussed the significance of addressing the most convenient technique for monitoring uric acid in light of the reported risk factor of its high level in human physiological samples as a result of excessive consumption of purine-based products. Moreover, the limitations of several methods used to detect uric acid in clinical laboratories were highlighted. Thus, the shortcomings serve to emphasize the relevance and main goal of this research, which is to design a simple and sensitive biosensor for sensing uric acid. The use of a mini protein 20 mimicking native uricase enzyme (mp20) previously encapsulated in a porous inorganic framework named ZIFs as a biocomposite for constructing uric acid biosensors was proposed due to the exceptional stability exhibited.

Next, **Chapter 2** focused on the background of the uric acid substrate and the development of conventional uric acid diagnostic methods. Since the main objective of this research was to study the analytical performance of the developed biosensor using a facile electrochemical technique, reviews on the various electrochemical approaches used for uric acid sensing were included. Additionally, the chapter discussed the commercially available uric acid testing meters for uric acid monitoring. Furthermore, the background of Metal Organic Frameworks (MOFs) and its subclass Zeolitic Immidazolate Frameworks (ZIFs) was also discussed, as well as the biocompatibility of the ZIF-8 as a protective host for preserving numerous biological components through immobilization and encapsulation approaches.

The experimental steps designed to fulfill the goal of this study was addressed in detail in **Chapter 3**. Overall, the procedure included the following steps: the expression and purification of mp20, biomimetic mineralization of mp20@ZIF-8 biocomposite, prediction and docking of metal cofactor onto the inactive mini protein 20 via the MIB web server, determination of mp20 and mp20@ZIF-8 enzymatic activity, as well as their stability and selectivity, preliminary study on the electrochemical response of the encapsulated mp20 onto ZIF-8 at different synthesized parameters, and electrochemical study on the electrochemical behaviour, optimization using OFAT and CCD/RSM approaches, analytical performance, interference, stability and reproducibility of the constructed mp20@ZIF-8/rGO/SPCE and the Cu(II) activated mp20@ZIF-8/rGO/SPCE surfaces towards the presence of uric acid. The procedure for method validation and real-time analytical detection utilizing portable electrochemical device, which were developed on the basis of the optimized mp20@ZIF-8/rGO/SPCE, was also included in this chapter.

Discussion on the expression and purification of mini protein 20 results, physicochemical characterization of the encapsulated mp20@ZIF-8 at different parameters, and preliminary study for determination of the optimal as-synthesized mp20@ZIF-8 at different parameters which was performed via evaluation of its electrocatalytic response due to its inactive virtue were covered in **Chapter 4**.

The development of the mp20@ZIF-8 modified onto reduced graphene oxide (rGO)/screen printed carbon electrode (SPCE) interface, or mp20@ZIF-8/rGO/SPCE surface in sensing uric acid via electrochemical technique followed by disclosure on the real sample detection and validation with the conventional approach and commercialized uric acid testing meter were described. Following the investigation of the catalytic properties of mp20 through metal cofactor activation, the prediction of metal ion-mini protein binding was evaluated computationally using a web server, called MIB, and the binding score prediction was disclosed. The results of this evaluation were presented in the form of binding score predictions. To validate the theoretical result, experimental study was conducted on the activation of the mini protein 20 in the presence of a metal cofactor using the uricase enzymatic assay. Following the confirmation of the activation study which was consistent with the computational result, an additional study comparing the naked mp20 and mp20@ZIF-8 biocomposite stability by the colorimetric technique was performed. Additionally, the analytical performance and applicability of an optimized Cu(II) activated mp20@ZIF-8/rGO/SPCE were investigated, including the selectivity and analytical performance of a biocomposite toward the targeted substrate. The last sub-chapter addresses the results of the construction of a portable electrochemical device based on the optimized mp20@ZIF-8/rGO/SPCE surface for the purpose of monitoring uric acid in physiological samples, including the interface's reproducibility over selected concentrations.

Chapter 5 includes the research summary, conclusions, and recommendations for future work on this project.

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