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Amperometric Glucose Biosensor Based on Activated Carbon/5-Methyl 1, HydroxyPhenazine/Glucose Oxidase Matrix

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Abstract

Recently, advance bio-sensing devices have given considerable importance, because of their capability to identify the target compounds promptly. In this context,2nd generation glucose biosensor has been fabricated using natural redox compound, 5-methyl 1, hydroxyphenazine (5-MHP)obtained from *P. aeruginosa*. Amperometric detection was based on interaction of glucose with the working electrode loaded with activated carbon (AC), 5-MHP and glucose oxidase (GOx), AC/5-MHP/GOx. Working electrodes prepared at three different temperatures 4°C, 25°C and 47°C, sensitively detect glucose andshowed the linear ranges of R^2 = 0.98, R^2 = 0.98 and R^2 = 0.99 respectively with detection limit of 0.3µM at signal-to-noise ratio (S/N)=3. It was found that GOx immobilization temperature directly influence the long term efficiency of glucose biosensor. Electrode fabricated at 4°C exhibited greater operational stability i.e. 74% followed by 25°C (68%) and 47°C (48%). Furthermore, theresponse timewith eachglucoseconcentration (2.0 to 26.0mM) was relatively less i.e. 2s, for enzyme electrode fabricated at 4°C whereas it was 4s and 5s for working electrodes prepared at 25°C and 47°C respectively.

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Introduction

Electrochemical biosensors are the most suitable devices that enablethe rapid quantitative determination of glucose with miniaturization, portability, sensitivity and simplicity(7). The idea of most glucose biosensors is based on reaction catalyzed by glucose oxidase (GOx) enzyme that produces peroxide (H_2O_2).The transduction signal is generated by electrochemical oxidation of peroxide thereby quantifyingthe glucose concentration in test sample(29). However, peroxide produced hasdetrimental effecton GOxactivity that limits the biosensor performance(4). Moreover, sensor is also subjected to interference by presence of certain electroactive species such as, ascorbic acid and uric acid which oxidize atthe same potential as H_2O_2, generating false positive results.A promising solution to overcome theselimitations is to replace the molecular oxygen (O_2) as a natural electron acceptor of GOxbyelectro active redox compounds.In addition, the use of mediating molecules facilitate the rapid electron tunneling from the $FADH_2$ of GOx to electrode surface because direct electron conduction between enzyme and electrode is too slow as $FADH_2$ is embedded inside the glycoprotein at a depth of about 13–15 Å(27).

Previously, severalredoxmediators have been reported to be used in biosensor such as, tetrathiafulvalen, adenines(26), viologen(13), quinolic(12), potassium hexacyanoferrate(21), osmium bipyridine and ferrocene(6, 8). Among the various redox mediators,phenazines are considered the molecules of choice in bio-electrochemical sensors because of their ability to undergo rapid bio-electrocatalytic transformations and to form a relatively stable electro-conductivematrix(1).Phenazines have been identified to be strongly incorporated inside the nafion film through hydrophobic interactions(16). With this in mind, highly sensitive glucose biosensor was constructed utilizing endogenous metabolite of*Pseudomonas aeruginosa*, 5-methyl 1,hydroxy phenazine (5-MHP) as redox mediator.5-MHP has a redox potential of −40 mV vs. standard hydrogen electrode (SHE) at pH 7 (28).

This potential is sufficiently high to oxidize the $FADH_2$at GOx active site while low enough to effectively transfer electrons to electrode (5). In addition, activated carbon (AC) was loaded on the surface of working electrodeto amplify the sensor output signal through increased surface area and improved electrical conductivity (10, 15).

Besides, enzyme leaching from electrode surface is another major issue encountered in long term performance of glucose biosensors (17). Immobilization process dynamics and molecular conformation of proteins contribute to the enzyme layer stability over the extended period of time (25). Nevertheless, little is known about the effectiveness and specifics of enzyme immobilization (18).The stability of enzyme electrode is strongly dependent on the enzyme immobilization conditions and techniques(23).In this study, the effect of casting enzyme immobilization temperature on apparent operational stability and reusability of enzyme electrode was investigated.

The major focus of this study was to develop a durable and highly sensitive glucose biosensor for continuous monitoring of blood glucose level.It aims to establish aneconomical bio-sensing system based on AC/5-MHP/GOxmatrix to overcome the issues of intermittent contact between bio-sensing element and probing electrode, reduced response and enzyme sheddingencountered previously.The optimum temperature of enzyme immobilization was investigatedto ensure the long term performance of biosensor. The effect of different enzyme immobilization temperature i.e. 4°C, 25°C and 47°C on the response time, sensitivity, reusability and storage stability of working electrodeswas evaluated.Additionally the detailed spectroscopic and microscopic analysis of working electrode to understand the fabrication and performance of glucose biosensor was carried out.

Materials and methods

Materials

Culture media and chemicals used were of analytical grade, obtained from Sigma Chemicals Corporations, Oxoid company UK, DIFCO Laboratories (Michigan, USA), Gas Hub Pte Ltd, Du Pont Company, Fluka chemicals corporations (UK), Merck (United States). Digital multimeter (model: UT33B; UNI-T) was obtained from GlobalmediaPro (New Zealand).

Electrodes fabrication

Graphite wafers (0.6cm×0.6cm) with surface area of 2.4cm² were used as electrodes. Prior to surface modification electrode material was polished with sand paperand subsequently washed with acetone, isopropyl alcohol and deionized water. The working electrode was loaded with activated carbon, 5-methyl 1, hydroxyphenazine, glucose oxidase and nafion jacket. Nafion casing was layered to prevent the enzyme loss from electrode. The counter electrode was made electro-catalytically active by coating with platinum catalyst.

Activated carbon loading

Activated carbon was deposited on bare electrode to increase the surface area for biomolecules adherence. AC coating was performed by electrode dipping method. For electrodes fabrication 4% solution of activated carbon was prepared in absolute ethanol. A 5% solution of Nafion 115 (Gas Hub Pte Ltd, Du Pont Company) was mixed with AC solution in ratio of 1:2 to make a slurry mix. The resultant mixture was vortexed for 20 seconds to allow the complete mixing of AC powder. Graphite slabs connected to copper wire were dipped in AC/nafion solution for about 5 seconds. Immediately after dipping, carbon electrodes were dried in oven at 300°C for 30 min. Heating at high temperature completely fixed the carbon particles to the electrode surface.

Mediator binding

A purified 5-methyl 1, hydroxyphenazine was prepared from *P. aeruginosa*(14). A 5M aqueous solution of 5-MHP mediator was dissolved in 5% nafion ethanol solution in the ratio of 2:1. AC coated electrodes were dipped in mediator nafion solution for half an hour and dried in fume hood.

Enzyme preparation

Glucose oxidase(GOx) was produced from *P. aeruginosa*by incubating in minimal salt medium (MSM) (Table.1) at 37°C and250rpm for 16 to 18 h. The cells were separated by centrifugation at 10,000rpm for 20 min. The cell paste obtained was washed with ice-cold deionized water and centrifuged at 10,000rpm for 10 min. This washing and centrifugation step was carried out twice. For each gram of wet cell mass 2ml of deionized water was added. The resultant suspension was subjected to sonic treatment at 9kc oscillator for 1 h. Sonication was performed in chilled environment by circulating ice cubes through apparatus. Supernatant was separated from cell debris by centrifugation at 10,000 rpm for 1 h at 4°C. The cell free crude extract contained GOx. Enzyme was purified through liquid-liquid reversed micelle method (19).

GOx assay

The enzymatic activity of glucose oxidase was determined by spectrophotometric measurement at 270nm based on conversion of *p*-benzoquinone to hydroquinone. One unit of enzyme activity is the amount of enzyme that catalyzes the oxidation of 1 µmole of glucose to gluconic acid and H_2O_2 in 1min at 25°C. H_2O_2 produced reduce benzoquinone to hydroquinone. Therefore, one unit of enzyme activity is defined as quantity of enzyme that reduces 1 µmole of *p*-benzoquinone to hydroquinone. 1ml of glucose solution (1M), 0.5ml of *p*-benzoquinone (0.5ml) and 0.45ml of sodium citrate buffer (0.1 M pH 5.5) were pre-incubated for 5min and used as blank. Reaction occurred by addition of 50µl of enzyme solution. After 1min incubation at 25°C increase in absorbance was measured on spectrophotometer at 270nm. Enzyme activity was calculated according to formula,Enzyme activity = $Vs \times V/I \times A_{270} \times \mu$

Where, Vs is absorbance of sample, V is volume of reaction mixture, I is length of pathway i.e 2cm, A_{270} is molar coefficient of hydroquinone i.e. 2.31, µ is microliters of GOx.

Enzyme immobilization

GOx was immobilized on modified graphite surface by physical adsorption method. Immobilization was performed electrostatically at three different temperatures i.e. 4°C, 25°C and 47°C to determine the efficiency of adherence. AC-phenazine coated carbon wafers were dipped in enzyme solution. 15Units/ml of GOx was prepared in deionized water. Electrodes were kept at different temperatures of 4°C, 25°C and 47°C for 24 h. After enzyme stacking, graphite slabs were dried at room temperature in hood for about 30min. The enzyme was entrapped within phenazine polymer layer. Finally electrode surface was covered with nafion coating (3% nafion-ethanol solution) to prevent the loss of enzyme. The electrodes were placed in 5cm glass cell (Fig.1). Enzyme electrodes were stored at 4°C in dry state when not in used.

Electrochemical measurements

Amperometric studies were performed at room temperature in sterile auxiliary electrolyte, phosphate buffer (0.1M pH 7.2). Response time, sensitivity and operational stability;reusabilityand storage stability of electrodes fabricating at different temperature (4°C, 25°C and 47°C) were determined by sensingcurrent across 10 ohms (Ω) resistor through precision multimeter (UT33B; UNI-T).The glucose solution was prepared 24 h before experiment for stabilization of L and D isomers. Increasing concentrations i.e. 2to 26mM of sterile glucose solutions were added to electrochemical cell after stable voltage was achieved. The effects of enzyme immobilization temperature on operational stabilities of working electrodes were recorded using 26mM glucose solution. The detection limit was calculated based on signal to noise ratio by formula,LOD=3×σ/s Where σ is standard deviation of blank measurements, and s is slope calibration curve of standard. Each observation was conducted in triplicates and recordings were subjected to statistical analysis.

Results

Amperometric response of AC/5-MHP/GOx electrode

Amperometric detection was performed separately with each electrode prepared at 4°C, 25°C and 47°C. Response current generated corresponded to the glucose concentration in solution.

Table 1. Chemical composition of minimal salt media (MSM) used for production of glucose oxidase from *P. aeruginosa*

SL	Chemicals	Percent composition
1	Glucose	8%
2	$MgSO_4$	0.05%
3	KH_2PO_4	0.125%
4	K_2HPO_4	0.001%
5	$NaNO_3$	0.5%,
6	Yeast extract	0.1%
7	KCL	0.05%
8	$FeSO_4$	0.001%
9	$CaCO_3$	0.3%

*pH 7.2.

The strength of signal generated at working electrode is directly related to concentration of substrate in sample. The current response generated following the addition of different concentrations of glucose was plotted in calibration curve (Fig.2A).

The peak current detected subsequent the additions of glucose dilutions were recorded.

The linear responses were observed over the glucose concentration of 2.0 to 26.0mM with correlation coefficients of R^2 = 0.98, R^2 = 0.98 and R^2 = 0.99 at 4°C, 25°C and 47°C respectively (Fig.2A). The response of sensors to glucose concentrations is shown by linear increase in current density up to 22mM. The linearity of calibration curve is reasonably acceptable which implies that it can be used for quantification of glucose.

The detection limit was 0.3µM at S/N=3 (signal-to-noise ratio of 3). Gluconic acid is produced as by-product of glucose oxidation in enzyme catalyzed reaction. It was demonstrated that production of gluconic acid in glucose biosensor directly indicated the substrate utilization in sample. Maximum gluconic acid concentrations of 1.10µg/ml, 1.07µg/ml and 1.23µg/ml were produced from 26mM glucose solution by 4°C, 25°C and 47°C adapted electrodes respectively (Fig 2B). The respective regression coefficients of glucose-gluconic acid curves were R^2 = 0.991 (4°C) R^2 = 0.992 (25°C) and R^2 = 0.993 (47°C) (Fig.2B).

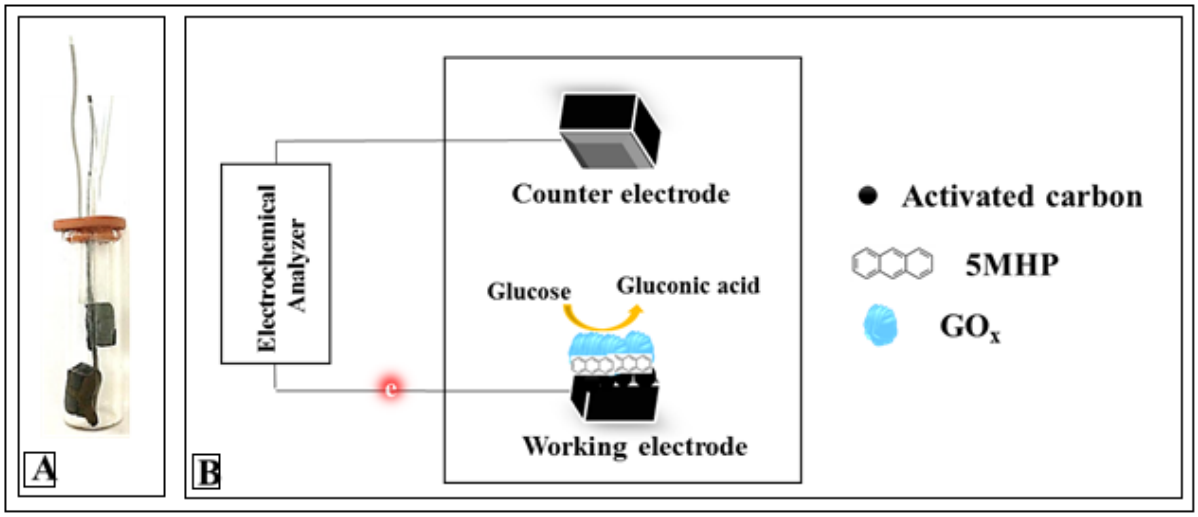

Fig. 1. An overview of glucose biosensor.

Response time

After introduction of glucose solution in biosensor, current started to increase instantly and reached the maximum value within few seconds. The maximum time needed to reach the 95% saturation level of enzyme represents response time. The minimum response time of 2s was observed with electrode fabricated at 4°C (Fig 3A) which meant that biosensor achieved 95% of steady state current in merely 2s. This signified faster electron exchange reaction between working electrode and electrolyte. However, longer response time of about 5s was examined for 47°C electrode whereas 25°C electrode showed the response after about 4s (Fig.3A). These measurements were carried out with various glucose concentrations ranging from 2 to 26mM.

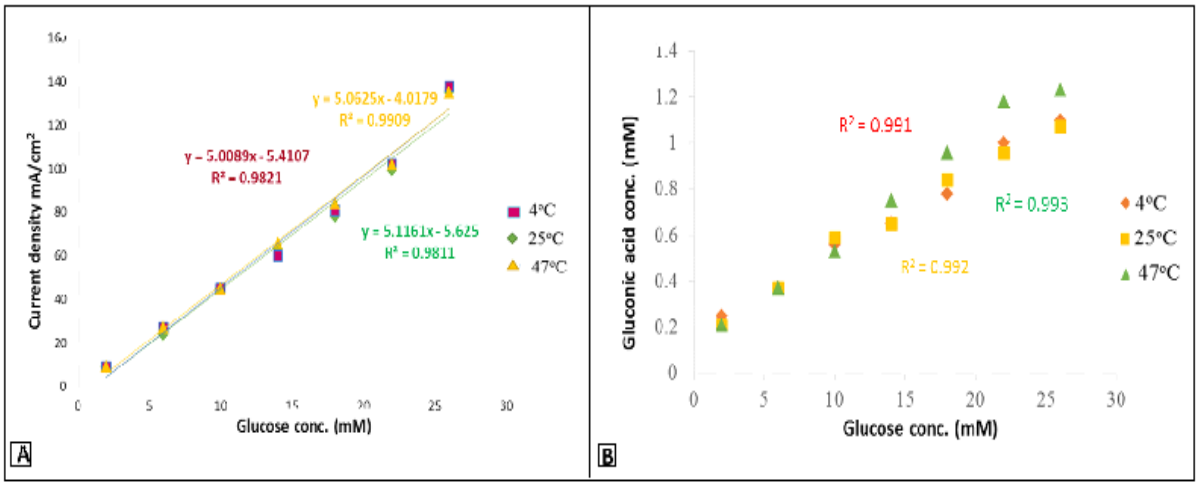

Fig. 2 (A). Amperometric response generated with different concentrations of glucose (B) gluconic acid produced with corresponding concentrations of glucose (2.0 to 26.0mM).

Sensitivity

It has been shown that sensitivity of biosensors increasewith increase in glucose concentration demonstrating electrocatalytic ability of working enzyme electrodes. This indicated that enzyme adsorption within mediator matrix is an efficient enzyme immobilization technique.

Electrodes fabrication temperature demonstrated a negligible effect on responsiveness to glucose. Nevertheless, a direct relation was observed between substrate concentrations and biosensors sensitivities to analyte. The maximum output response of working electrode prepared at 4°C was 27.6µA/cm², at 25°C (27.0µA/cm²) and at 47°C (27.08µA/cm²)(Fig.3B).

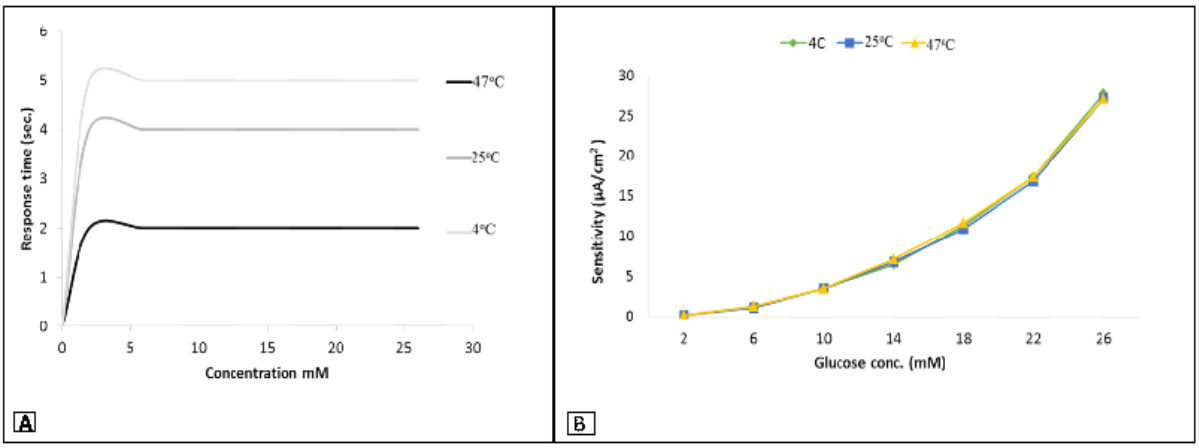

Fig. 3. (A)Response time dependence on immobilization temperature ofGOx(B) sensitivity of glucose biosensors as function of amperometric response with different concentrations of glucose.

Operational stability

Reusability

The operational stabilities of proposed biosensors were investigated by conducting electrochemical measurements under optimal conditions of pH 7, temperature and substrate concentration (26.0mM). The action of biosensors was estimated as maximum current generated at higher glucose concentration (26.0Mm) over the time.

To determine the reusability of enzyme electrodes, repeated measurements were carried out multiple times a day. After the initial five consecutive recordings, 4°C electrode retained 82% of its initial response whereas 25°C and 47°C electrodes maintained 75% and 59% of actual activity respectively (Fig.4A). However, response decreased further to 74% (4°C), 68% (25°C) and 51% (47°C) after about 10 recordings.

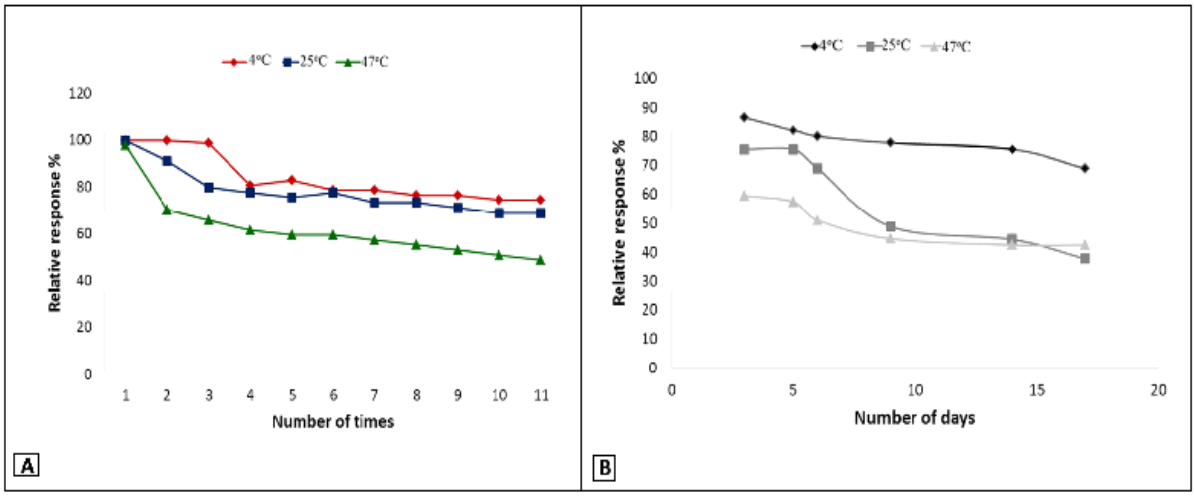

Fig. 4. (A)Storage stability (B) operational reusability of glucose biosensors over the extended period of time

Storage stability

The storage stability of enzyme electrodes was examined by determining amperometric response within 20 days. The stability of enzyme immobilized at different temperatures was investigated as process of time (days). Enzyme electrodes were kept at 4°C in dry state protected from light. The relative response decreased to 86.6% (4°C), 75.5% (25°C) and 59.5% (47°C) after 3 days of storage (Fig.4B). The decrease in amperometric signal continued and after 15 days biosensor at 4°C, 25°C and 47°C retained 75.5%, 44.4% and 42.5% of initial activity respectively (Fig.4B). These findings illustrated that stability of GOxenzyme adhered to electrode surface is attributed to immobilization temperature.

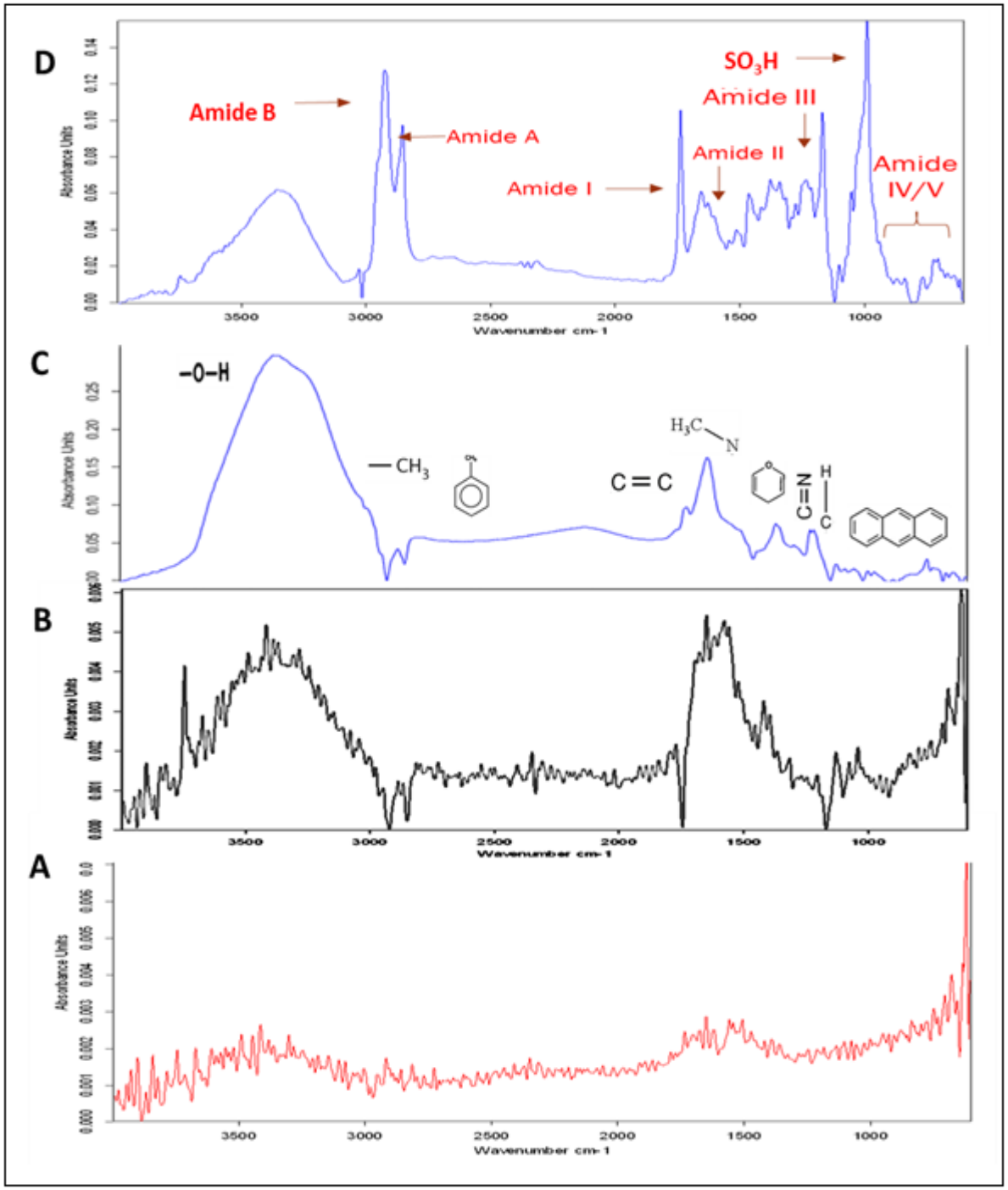

Fig. 5. FT-IR analysis of working electrode (A) bare graphite, (B) AC loading, (C) 5-MHP coating, (D) GOx immobilization.

FT-IR analysis of enzyme electrode

Enzyme electrode was subjected to FT-IR analysis before (Fig.5A) and after surface modification with activated carbon particles and biomolecules (5-MHP and GOx). The peak area in spectrum of activated carbon ranging between 3700–3100cm^{-1} was allocated to the vibrational movements of hydroxyl group (O-H) and C–H group. Aromatic C=C stretching was displayed in the band area of 1700–1500cm^{-1}. The band at 1000-1300 cm^{-1} was marked to the fingerprint region of oxidized carbon associated with C–O stretching in ether, phenols, alcohols and acids (Fig.5B). 5-MHP demonstrated major peaks at wavelengths of 3380.9 cm^{-1}(OH), 2882.17 cm^{-1} (-CH_3), 2805cm^{-1}(aromatic ring), 1641.73 cm^{-1}(C=C), 1391.19 cm^{-1} (-NH_3)and 653.61cm^{-1} (phenazine group).

Enzyme coating on graphite was confirmed through IR spectroscopy. In FT-IR spectrum glucose oxidase presented bands in the wavenumber region ranging between 4000–600 cm^{-1}(Fig.5D). The peptide groups of protein exhibited the characteristic peaks at 3300 cm^{-1} (amide A), 3100 cm^{-1} (amide B), 1600–1690 cm^{-1} (amide I), 1480–1575 cm^{-1} (amide II), 1300 cm^{-1} (amide III), 625–770 cm^{-1} (amide IV) and at 640–800 cm^{-1} (amide V).The band at 3366 cm^{-1} represented amide A group and was mainly due to stretching vibration of N–H group. The peak at 1655cm^{-1} region of spectrum was due to C=C vibration indicating amide B peptide group of glucose oxidase. The coupled vibrations of C=O at 1513cm^{-1} and C-N bonds at 1464cm^{-1} marked up amide I absorption band. Amide II region resulted from both bending vibrations of N–H as well as stretching vibrations of C–N group. The complex band at 1234cm^{-1} region illustrated amide III group. The peak area between 766cm^{-1}–616cm^{-1} was related to amide IV and amide V groups. The absorption peaks at 1377cm^{-1} and 1053cm^{-1} were assigned to CF_2 backbone and SO_3H (sulfonic group) of nafion covering on enzyme (Fig. 5D).

SEM analysis of working electrode

The morphology of AC coated graphite with immobilized 5-MHP and GOx was examined via scanning electron microscopy (SEM). The cross section of enzyme electrode was shown; immobilized GOx appeared as lumps on mediator matrix (Fig. 6). SEM imaging provides the evidence of glucose determination through reaction with GOx by glucose biosensor.

Discussion

The success of glucose biosensor relies on the efficient performance over extended time period and cost benchmarks worldwide. The present study established theeconomical bio-sensing systemto overcome the interference effectsexperienced in earlierapproaches. Amperometric determination of glucose is based on electrochemical response generated by reaction of glucose with immobilized GOx. 5-MHP matrix assisted in the rapid electron shuttling fromredox center of GOxto theenzyme electrode(11, 24).Whereas, AC film increased the surface area (500 m^2/g) of probing electrode that subsequently fortified the electrical connection between biological components and electrode interface. This resulted in enhanced electrons conduction and amplified output response (2). Various enzymes immobilization techniques have been applied so far. Immobilization of GOxby covalent bonding, entrapment and cross linking techniques cause the conformational changes in active site of enzyme that affect the catalytic activity of enzyme consequently, reducing accessibility of glucose (3). Physical adsorption method of protein immobilization is preferred for wide applications, since it enables the high enzyme loading and offers little or no conformational change(9). In this study,GOx was physical adsorbed within AC/5-MHP matrix. Adsorption of protein was mainly through salt linkages, Van der Waal's forces and hydrogen bonds that rendered enzyme relatively less disruptive(22).

It has been demonstrated in this study that casting enzyme immobilization temperature affects the long term operational stability of enzyme electrode(Fig.4B). Greater stability of 86.6% was observed after 3 days of operation by electrode fabricated at low temperature (4^{o}C) than at high temperature (47^{o}C) (Fig. 4A, B). Highest current density of 27.6µA/cm^2 was recorded at greater enzyme substrate reaction.

Moreover,working electrode prepared at 4°C showed lesser response time i.e. 2s, retained greater operational stability and reusability than at 25°C and 47°C (Fig.4A,3A). This might be due to leakage of weakly attachedGOx from electrode surface.

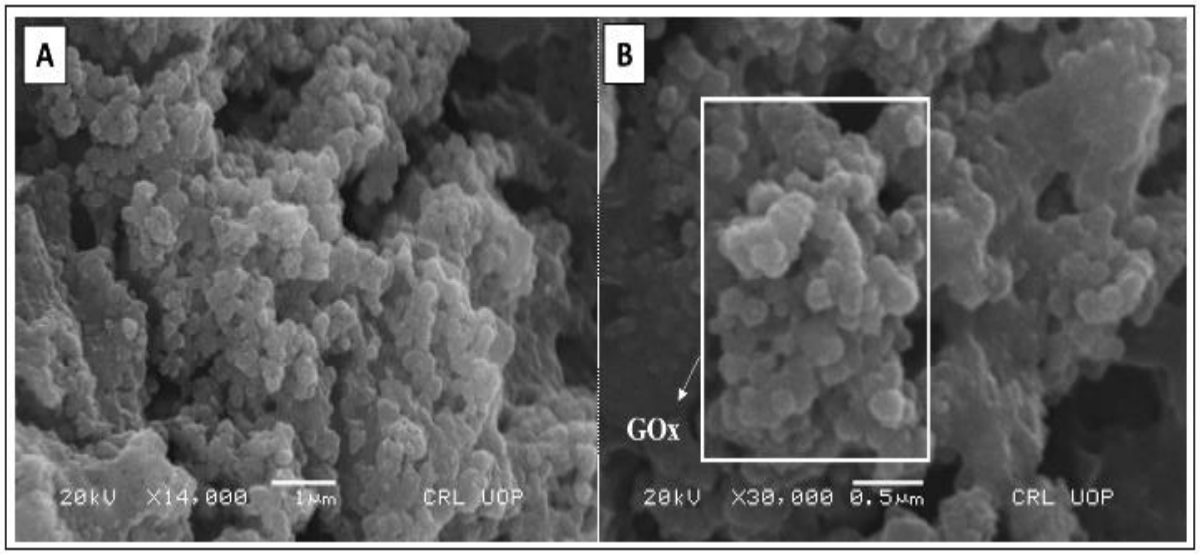

Fig. 6. Scanning electron microscopy (SEM) of working enzyme electrode.

The response time observed with these biosensors was considerably lower than electrochemical sensors (8, 20). However, GOximmobilization temperature (4°C, 25°C and 47°C) showed slight effect on the sensitivity of biosensors (Fig.3B). The sensitivities of biosensorto different concentrations of glucose were indicated by ascending lines demonstrating increased responsiveness with increase in concentrations of glucose.

Conclusion

In this study 2nd generation glucose biosensors were fabricated utilizingAC and natural redox mediator i.e. 5-MHP obtained from *P. aeruginosa*.GOx was successfully immobilized on the following matrix by physical adsorption method at three different temperatures i.e. 4°C, 25°C and 47°C. 5-MHP proved to be an efficient redox compound in glucose biosensors.The introduction of 5-MHP resulted in amplified output signal, lesser response time, good sensitivity and greater operational stability. Furthermore, the study demonstrated that enzyme was more firmly attached at low temperature (4°C) than at high temperature (47°C). Ithas been establishedthrough experimental results that GOximmobilized at 4°C retained greater operational stability and reusability (74%) than at 25°C and 47°C.

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