Miniatuized-Screen printed electrode of Electrochemical Biosensor for Monitoring Insulin in Diabetic Patients

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ABSTRACT

The present work deals with the development of miniaturized electrochemical biosensor, an enzyme electrode for monitoring insulin. The purpose of study was to develop miniature enzyme electrode with good sensitivity, micro sampling, and medical application and cost effectiveness. The present study comprised of 90 subjects categorized into three groups: group 1- normal individual, group 2 - non-insulin dependent diabetic mellitus patients (NIDDM), group 3- insulin dependent diabetic mellitus patients (IDDM). Insulin levels were monitored on three different transducers using screen printed enzyme electrode. The enzyme electrode was prepared by immobilization of red blood cells (RBC) with the covalent cross linker glutaraldehyde and along with the signalization with 3-aminopropyltriethoxysilane. The signal was monitored on three different transducers i.e. polarograph, amperometer and photofluorometer. The current response for the electrode was proportional to the insulin concentration and the response time was 25 mins. The lower detection limit of insulin was 0.06 nM. The stability of the immobilized biocomponent on screen printed electrode (SPE) was for 24hrs at 4º C. The developed enzyme electrode had been applied for determination in normal subjects, non-insulin dependent and insulin dependent diabetic mellitus patients.

Key words: SPE, Insulin biosensor, Transducer, NADPH oxidation, Red blood cells.

Abbreviations: SPE-Screen printed electrode, RBC- Red blood corpuscles, DME- Dropping mercury electrode, NIDDM- Non-insulin dependent diabetic mellitus patients, IDDM- Insulin dependent diabetic mellitus patients, PBS- Phosphate buffer saline, NADPH- Nicotinamide dinucleotide phosphatedehydrogenase, IRMA- Immunoradiometric assay, ELISA-Enzyme linked immune-sorbent assay, TCNO-7,7,8,8-tetracyanoquinoindimethane.

1. INTRODUCTION

Diabetes mellitus is a chronic hyperglycemic condition in which blood glucose level increases above the normal level and there are disturbances in carbohydrates, fats, proteins metabolism associated with absolute or relative deficiency in insulin secretion or insulin action. According to 2012 update of statistical analysis, in youth app.186000 people<20 years of age have diabetes (www: American Heart Association). In diabetes both glucose and insulin monitoring are important. So insulin monitoring for diabetic patients has its own significance for clinical diagnostics. The analysis of insulin is done by Biological and immunological assays. Insulin is estimated by ELISA and IRMA. These methods are no doubt precise but suffer from the drawback of more time response, high cost and laboratory bound. Biosensors on the other hand have the advantages of specificity, fast response times, low cost, portability, ease of use and a continuous real time signal. The previous methods are hazardous as radioactive isotopes such as I125 or I131 is used for assay of insulin (Talwar et al. 2005).

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The micro-technology is better than the conventional methods. The best developed biosensors are undoubtedly within the field of clinical medicine, where glucose biosensors play a vital role in the measurement of blood glucose that is necessary for the management of diabetes. On a widespread scale this miniaturized technique had been adopted and much work had already done on it. The technology was shifted towards miniaturization for the benefit of less time consuming, micro-sampling, cost effectiveness and sensitivity. Such miniaturized devices are commonly based on enzymatic oxidation of the enzymes. Much work had been done on glucose by the immobilized enzyme coupled to amperometer detection of the liberated hydrogen peroxide (Bilitewski et al. 1992, Bilitewski et al. 1991, Koopel et al. 1994, Motta et al. 1994).

In 2001, a paper was presented in international conference on micro technologies (screen printed electrode) in medicine and biology, SPE were used for miniaturizations of electrochemical optical choline biosensor.These were recommend to use either as disposable devices or as reusable devices after buffer wash. It covered a linear range $4 \times 10^{-4}$ M to $2 \times 10^{-3}$ M (Leca et al. 2001). In the same year, an insulin sensitive RuOx-modified electrode was developed for simultaneous amperometric measurement of glucose and insulin. The developed amperometric sensor was linear up to 1000 mM insulin and 14 mM glucose (Wang and Zhang, 2001). In 2002, glucose biosensor based on glucose oxidase and prussian blue (PB) bulk modified carbon screen-printed electrode was developed which exhibited limit of detection of 0.2m M and linear range up to 4m M. The main benefit of this developed biosensor was its total insensitivity to oxygen, ascorbate, urate and paracetamol (Pravda et al. 2002). In the similar type of study, screen-printed carbon electrodes (SPCEs) were used for acetylcholinesterase covalently bonded directly to its surface. Screen printed electrode a micro technology, have become important in view of cost effectiveness and micro sampling (Hong et al. 2002). In the mini review article, Hart described the design and fabrication of electrochemical sensor/biosensors based on screen printed technology and its application in various streams i.e. pharmaceutical, biomedical, environmental and industrial analysis (Hart et al., 2004). A comparison between several acetylcholine immobilization procedures was studied on the 7, 7', 8, 8'-tetracyanoquinodimethane (TCNQ)-modified graphitic working electrodes (Nunes et al. 2004). Another type of electrochemical sensor i.e polargraphic sensor was developed for hydrogen peroxide measurement in which hemoglobin was exploited which acted as a catalyst. No doubt, the results were precise, but it had a drawback that disposal of mercury is hazardous to mankind (Sun et al. 2005).

Different enzyme immobilization procedures were performed on screen printed electrode for detection of anticholinesterase pesticides. ACHE immobilization with glutaraldehyde produced best results with good sensitivity (Givanda et al. 2004). A similar study was done by Pohankal and his co-workers in 2007 and recommended cross linking by glutaraldehyde as an important part of biosensor preparation which yielded optimal results (Pohankal et al. 2007). In 2010, another type of biosensor was developed for estimation of arsenic based on screen printed electrode technology (Mendez et al. 2010). In 2011, Keow et al. developed screen printed histamine biosensor, fabricated from the entrapment of diamine oxidase. The histamine biosensor showed response time of < 50 s with a linear response range from 0 to 60 ppm histamine. The sensitivity of the biosensor was 5.56 nA ppm-1, with the detection limit of 0.65 ppm histamine. In our previous study, we had developed electrochemical biosensor based on carbon paste electrode for monitoring insulin in normal individuals and diabetic mellitus patients. On similar footsteps, electrochemical biosensor was developed, but based on micro technology. The developed biosensor has been applied for monitoring insulin in normal individual, diabetic patients – non-insulin dependent and insulin dependent diabetes mellitus patients (Kaur and Verma, 2012).

The aim of carrying out this research was shifting our approach towards miniaturization and develops miniaturized electrochemical screen printed enzyme electrode systems. The red blood cells have been immobilized coently in glutaraldehyde (Jager and Bilitewski, 1994) and the response of the immobilized biocomponents has been seen on different transducers i.e. amperometer, polargraph and photoflurometer. This developed biosensor comes under third generation biosensor and has been applied for monitoring insulin in normal individual, diabetic patients – NIDDM and IDDM.

### 2.1. Materials

#### 2.1.1. Subjects

The present study comprised of 90 patients from government and private hospital, Patiala. The consent from the patient was taken who were enrolled for study and approval from ethics committee of the institute. The insulin levels were assayed by the developed miniaturized biosensor by three different transducers i.e. amperometer, polargraph and photoflurometer.

#### 2.1.2. Blood Sample

The serum sample was used for insulin determination.

#### 2.1.3. Reagents and Chemicals

As it is an approach towards miniaturization, the screen printed base electrode were used (33.5 mm x 101.5 mm) for immobilization of the red blood cells. The base electrodes have been procured from kind courtesy by Dr. Joseph Wang, New Mexico State University, USA. NaCl solution , phosphate buffer saline (K2HPO4/KH2PO4), NaOH , Highly Purified Porcine Insulin (40U/ml), glucose , NADP Solution, KCl, glutaraldehyde solution, 10% amidriethoxysilane (ATS) were of analytical grade and were procured from SRL chemical Ltd.,Torrent pharmaceuticals Ltd., S.D fine chemicals, Fluka-Germany and Himedia. The 3 ml sized glass cells (i.e. for reaction mixture) were fabricated from Central Scientific Instruments Organization, Chandigarh.

#### 2.2. Methodology

##### 2.2.1. Fabrication of Screen Printed Electrode

The red blood cells after being treated with phosphate buffer of pH 7.4 were used for immobilization on screen-printed base electrodes. This immobilization has been done with the covalent cross linker, glutaraldehyde (Bilitewski et al, 1991). Further for good fixation of biocomponent, sialanization with 10%, ATS (amidetriethoxysilane) has been done. The fabricated electrode was applied in the cell with a 2 ml of reaction mixture on three transducers. The stability of the biocomponent was checked. The immobilized biocomponents had a shelf life of 24 hours if stored in PBS of 7.4 pH in the refrigerator at 4-8 ºC.

##### 2.2.2. Experimental Part

The experiments were run with the blank, standard and unknown samples. The oxidation curve was observed at 0.32V which is the oxidation potential of NADPH and for the blank, the oxidation potential curve was observed at 0.27V. The volume mixture of 40ml was reduced to 2ml and microsampling was the unique feature of this developed miniaturized electrode. Optimization of various parameters for production of NADPH was studied which included temperature, reaction time, glucose concentration, NADP+ and RBC (for immobilization). The red blood cells have been immobilized in glutaraldehyde (Jager and Bilitewski, 1994) and the response of the immobilized biocomponents has been seen on different transducers i.e. amperometer, polargraph and photoflurometer. This developed biosensor comes under third generation biosensor and has been applied for monitoring insulin in normal individual, diabetic patients – NIDDM and IDDM.

##### 2.2.3. Instrument Measuring Procedure

The transducers used in this study were electrochemical sensors i.e. polargraph systronics-1632, polargraph modified to amperometer and photoflurometer-systronic-151. The response of NADPH was monitored by the oxidation of NADPH. The chemical experiments were...
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2.2.4. Protocol  
The working electrode SPE was applied in the cell of polarograph. The approach in this study was towards miniaturization. The reaction mixture was prepared in the following way: The reaction mixture was prepared in the special fabricated cell of 3ml volume.

HK  
Glucose + ATP $\rightarrow$ Glucose6P + ADP  
G-6-PDHG  
Glucose 6-P + NAD$^+$ $\rightarrow$ 6-Phosphogluconolactone + NADPH + H$^+$

Figure 1  
The enzymatic reaction

Figure 2  
Standard Curve of Insulin DME

Figure 3  
A Polarogram of IDDM patient: insulin = 0.6nM, Current = 1µA, Voltage =0.32V, SPE-RBC electrode

Figure 4  
Standard Curve of Insulin with Photofluorometer

Figure 5  
Standard Curve of Insulin using amperometer as transducer conducted using a 3 electrode configuration in the special fabricated cell of 3ml volume.

Figure 6  
An Amperogram of Normal individual using SPE, D.P=0.32V, Current =1mA, Insulin Level =162 nM
Biosensor for Monitoring Insulin in Diabetic Patients, IDDM and NIDDM Patients using SPE electrode.

The optimum time the response was observed. The electrochemical measurements were performed using a polarograph/ amperometer and the current produced was anodic and the formation of NADPH was monitored on photofluorometer by excitation at 360 nm and emission at 470 nm.

3. RESULT AND DISCUSSION

3.1. Bioassay in electrochemical study

The principle in the screen printed electrode a system is i.e. the oxidation of NADPH. The developed electrochemical insulin biosensor contains SPE-RBC electrode. The red blood cells contain enzymes hexokinase, glucose-6-phosphate dehydrogenase, ATP and insulin receptor. This enzymes machinery is used for insulin detection. The insulin facilitates the transport of glucose across rbc membrane by glucose permease transporter (Chang and Murray, 2009) which is converted to glucose-6-phosphate by hexokinase and further to 6-phosphogluconate by glucose-6-phosphat dehydrogenase with the concomitant generation of NADP. The reactions are shown in Fig.1. The NADP and glucose were added exogenously to drive the reaction in forward direction. The amount of NADPH produced is proportional to insulin concentration and signal was observed at oxidation potential of + 0.32.

3.2. Approach towards miniaturization

The polarograph instrument had a working cell size of 40 ml, in order to make more economical it was reduced to 3 ml only. The unique thing in this approach was the screen printed electrode inherits working electrode as well as the reference electrode and platinum electrode as auxiliary of polarograph was used in this system.

3.3. Disposable Nature

The reusability of the biocomponent on same screen printed base electrode was studied with fresh reaction mixture and no deflection and signal was observed on different transducer.

4. POLAROGRAPH AS TRANSUDER

The electrochemical measurements were performed using a polarograph in a three electrode configuration that included dropping mercury electrode (DME as working electrode) and reference of SPE and platinum wire electrode (auxiliary electrode) fitted in the special fabricated cell having working volume of 2 ml.

4.1. Insulin Standard Curve

Insulin standard curve ranging from (0.15-0.6) nM was constructed as shown in Fig.2.

4.2. Application of developed insulin Biosensor

The developed biosensor has been applied for determination of insulin in normal individual, IDDM and NIDDM patients. Normal range for healthy individual is 2-150 nM/mL (Kaplan, 1987). Table 1 shows the results and statistical analysis of normal individual (group-1), NIDDM (group-2) and IDDM (group-3) patients. The insulin levels of NIDDM were close to the normal individuals whereas the IDDM patients have lower insulin level than normal range.

The results were statistically significant by this developed method i.e. p value<0.0001. The history of the patients correlates with the results as they were taking anti diabetic drugs daily to maintain normal glucose level. Some IDDM patients had high normal insulin levels as those patients were taking insulin intravenously daily. Polarograph of one of the IDDM Patient is shown in Fig.3. This was the polar gram of IDDM patients which gave the response at 1μA as the insulin concentration was very low.

5. PHOTOFLUOROMETER AS TRANSUDER

The electrochemical measurements were performed using photofluorometer. The SPE which inherited both the electrodes (working and reference) was applied in the cell. The reaction mixture was prepared in the same manner and after optimum time the response was observed. The formation of NADPH was monitored by Photofluorometer by excitation at 360 nm and emission at 470 nm.

5.1. Insulin Standard Curve Using Photofluorometer as Transducer

Insulin standard curve ranging from (0.6-6.0) nM was constructed as shown in Fig.4.

5.2. Comparative Analysis of Normal Individuals, Insulin Dependent and Non-Insulin Dependent Patients Using Photofluorometer as Transducer

The Comparative Analysis of Normal Individuals, Insulin Dependent and Non-Insulin Dependent Patients are shown in Table 2 with the results and statistical analysis of normal individual, IDDM and NIDDM patients. The insulin levels of NIDDM were close to the normal individuals whereas the IDDM patients have lower insulin level than normal range. The results were statistically significant by this developed method i.e. p value<0.001 of NIDDM and <0.0001 of IDDM.

6. AMPEROMETER AS TRANSUDER

Here polarograph was modified to amperometer, so before starting with the experiment the apparatus was checked for its accuracy by running the usual dummy check.

6.1. Insulin Standard Curve

Insulin standard curve ranging from (0.06-0.48) nM was constructed as shown in Fig.5 using Screen printed base electrode.

6.2. Application of the Developed Biosensor Using Amperometer as Transducer

The screen printed electrodes were used as the sensing electrode on amperometer. The comparative analysis of normal individuals, NIDDM and IDDM was done. The Table 3 shows the insulin levels of normal individual, which were comparable to the non insulin dependent patients where as insulin level of IDDM was lower than both groups. The

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Table 1 Comparison of Insulin Concentration in Normal individuals, IDDM and NIDDM Patients using SPE-RBC electrode on Polarograph

<table>
<thead>
<tr>
<th>Normal Individual N = 30 (group-1)</th>
<th>NIDDM N=30 (group-2)</th>
<th>IDDM N =30 (group-3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean 83.4</td>
<td>85.8</td>
<td>114</td>
</tr>
<tr>
<td>S.D 13.8</td>
<td>23.4</td>
<td>9</td>
</tr>
<tr>
<td>SEM 2.52</td>
<td>4.2</td>
<td>1.6</td>
</tr>
<tr>
<td>p &lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Table 2 Comparison of Insulin Concentration in Normal individuals, IDDM and NIDDM Patients using SPE-RBC electrode on Photofluorometer

<table>
<thead>
<tr>
<th>Normal Individual N = 30 (group-1)</th>
<th>NIDDM N=30 (group-2)</th>
<th>IDDM N =30 (group-3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean 66.8</td>
<td>63.6</td>
<td>24</td>
</tr>
<tr>
<td>S.D 16.1</td>
<td>34.8</td>
<td>12</td>
</tr>
<tr>
<td>SEM 3.3</td>
<td>6.1</td>
<td>2.2</td>
</tr>
<tr>
<td>p 0.02</td>
<td>0.02</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

In our research, the developed method is better than the conventional analytical technique. The old method used is no doubt precise but it is time consuming and mostly lab bound whereas the biosensor approach has the advantage of specificity, fast response time, simplicity of construction of SPE-RBC electrode. It exhibits great prospects in the field of biosensor. By miniaturization technique, the detection limit of insulin has been found to be 0.06 n M. The approach in this strategy was towards miniaturization with respect to reduced reaction cell as well as sample volume. So, the developed biosensor is fast, sensitive, cost effective and eco-friendly as compared to existing methods.

### 7. RELIABILITY

The reliability of the developed electrochemical biosensor was checked by comparing the same reaction mixture on different transducers. The insulin level was measured electrochemically on polarograph, amperometer and then its deflection was monitored on photofluorometer by excitation at 360nm and emission at 470nm. The insulin level monitored by different transducers came comparable to each other which have made the electrochemical sensor more reliable.

### 8. CONCLUSION

To conclude, in this paper, screen printed enzyme electrode, an electrochemical biosensor has been developed for detection of insulin concentration. The developed biosensor had been applied on normal subjects, non-insulin dependent and insulin dependent diabetic mellitus patients and exhibits excellent sensitivity.

### SUMMARY OF RESEARCH

1. The miniaturized approach has been developed which is better than the conventional analytical technique which gives better detection limit than old methods.
2. This developed method has more importance for the Insulin dependent patients as the detection limit of insulin has been found to be 0.06 n M.

### FUTURE ISSUES

1. Reducing the response time of the developed method, by use of mediator.
2. Collaboration with industry for commercialization.

### DISCLOSURE STATEMENT

The work was carried out in the Department of Biotechnology, Punjab University, Patiala, with their full support for productive research work.

### ACKNOWLEDGEMENT

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