INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

www.ijabpt.com ISSN : 0976-4550

Volume-6, Issue-1, Jan-Mar-2015, Coden IJABFP-USA, Copyrights@2015

Received: 11th Oct-2014 *Revised: 29th Oct-2014* *Accepted: 1st Nov-2014*

Review article

BIOSENSORS: A SENSITIVE STRATEGY TO DETECT GLUCOSE IN BODY FLUIDS

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ABSTRACT

Over the past decades diabetes is one of the leading causes of mortality and morbidity in the world, thus inventing glucose biosensors with accurate continuous monitoring is of growing concern amongst the scientists worldwide. This manuscript reviews the development of glucose biosensors over the last 50 years since the invention of the first glucose sensing electrode and various approaches considered to develop accurate and modern techniques of glucose sensing. This review provides brief introduction to principles of various glucose biosensors with systemization and classification of glucose monitoring principles. Thus the main aim of this manuscript is to check history of glucose biosensors, comment on their current status and commercial aspects, and examine future challenges.

Key words: Biosensors, Glucose Biosensors, Glucose monitoring, Glucose oxidase enzyme

INTRODUCTION

Diabetes is a metabolic disorder caused due to insulin deficiency and hyperglycemia (C.C. Cowie *et al*. 2010) and is reflected by blood glucose concentrations higher or lower than the normal range of 80-120 mg/dL. It has been estimated that 2.8 % of the world population was affected by diabetes in 2000, which accounted for approximately 171 million people. By 2030 these numbers have been projected to more than double, with around 366 million people suffering from the diabetes globally (S. Wild *et al*.2004). Biosensors are basically analytical tools that detect any targeted analyte by combining it with a biological component and physicochemical detector. It consists of two components, one is a bioreceptor i.e. any sensitive biological element (e.g. Enzyme, antibodies, DNA etc) that recognizes the targeted analyte and second is a transducer which is used to convert the biochemical signal resulting from the interaction of analyte and bioreceptor into the physical(majorly electronic) signal. These biosensors are used *in-vivo* meaning inside the body, *in-vitro* i.e. inside test tubes, at-line means can be used at production line where a sample can be taken at regular intervals and tested and in-line i.e. it can be permanently fixed in the production line to continuously monitor the analyte. Biosensors show their importance in environment and healthcare industries. They serve the healthcare industry by measurement of biomolecules like glucose, ethanol, lactates, choline, folic acid etc. and in case of environment the widely studied analytes are Biological Oxygen Demand (BOD), river water pH and heavy metal ions content, herbicides, toxic substances etc. Work of the development of biosensors for detecting glucose was initiated as early as in 1960s. It started with the development of first glucose biosensing electrode in 1962 by Clark and Lyons (E. Wilkins and P. Atanasov, 1996). This first glucose biosensor was an electrode coated with thin layer of Glucose Oxidase (GOx) enzyme i.e. bioreceptor and then further entrapped by a semi permeable membrane which allowed passage of glucose towards the enzyme. The measurement of glucose was done on the bases of consumption of oxygen resulted due to enzyme catalyzed reactions equations 1 and 2.

$$\text{Glucose} + O_2 \xrightarrow{\text{GOx}} \text{Gluconic acid} + H_2O_2 \qquad \text{Equation 1}$$

$$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O \qquad \text{Equation 2}$$

In the above case there was problem of electro-active interference, which was corrected by the use of two electrodes. One electrode was covered with the enzyme GOx and the other measured the current based on the production of hydrogen peroxide. G.G. Guilbault and G.J. Lubrano (G.G. Guilbault and G.J. Lubrano, 1973) invented a glucose biosensing electrode in 1973 based on amperometric measurement of hydrogen peroxide product. This biosensor worked with reactions 1 and 3.

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \qquad \text{Equation 3}$$

This principle was then transferred to Yellow Spring Instrument (YSI) Company, which introduced the first glucose biosensors in 1975 that measured glucose in blood samples. These biosensors offered good accuracy and precision. Vast variety of enzyme biosensors based on different enzymes, different electrode materials, different ways of immobilizing enzyme on electrodes and various semi permeable membrane compositions have since been demonstrated. Use of HRP i.e. Horseradish Peroxidase enzyme was also suggested to improve the oxidation rate of generated hydrogen peroxide. In 1980s more attention was paid to the development of mediator based glucose biosensors which marked the invention of second generation of electrochemical glucose biosensors. During this decade in 1987 the first commercial self monitoring of blood glucose (SMBG) strips were launched. During 1990s major concern of the scientist working in this field was to establish proper and enhanced electrical communication between glucose and its redox centre. In early 2002, first noninvasive glucose biosensor was launched which was a wearable watch. During the last decade more work in glucose biosensors is regarding the development of commercially available enzyme free glucose biosensor.

Generations of Glucose Biosensors

There are total three generations of electrochemical glucose biosensors. These three geneartions of glucose biosensors basically deferred in electron transfer mediator from redox center to the lectrode. The difference between all the three generations of glucose biosensors can be easily understoood with the help of Figure 1.

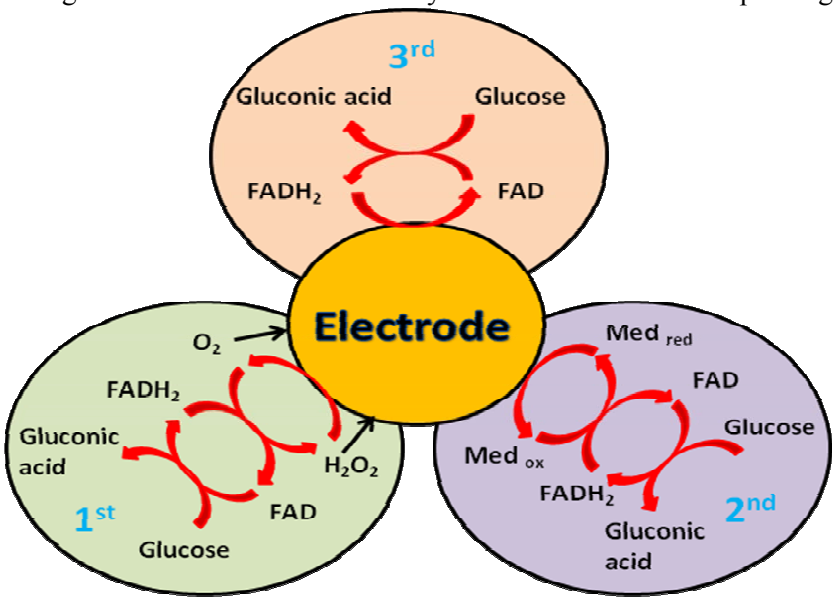

Figure 1 Three generations of Glucose Biosensors

In all three of these the redox center is same i.e. GOx (FAD). Here FAD is flavin adenosine diphophate. In all three generations of them glucose gets oxidised to gluconic acid and the FAD group of redox center gets reduced to $FADH_2$.

The first generation of glucose biosensors worked on following reactions 4 and 5.

$$\text{Glucose} + \text{GOx(FAD)} \rightarrow \text{Gluconic acid} + \text{GOx}(FADH_2) \qquad \text{Equation 4}$$

$$\text{GOx}(FADH_2) + O_2 \rightarrow \text{GOx(FAD)} + H_2O_2 \qquad \text{Equation 5}$$

The produced hydrogen peroxide is then oxidized as per reaction equation 3 and generates electrons which accounts for the electric current and thus glucose detection. This first generation glucose biosensors showed two major diasadvantages (J. Wang,2008).

a) One of them was electro-active interference. The measurement of hydrogen peroxide at an electrode needs relatively high potential which resulted at times in the interference due to other species like ascorbic acid, uric acid and drug molecules like acetaminophen. This species contributed to the current and thus led to less accuracy and also affetcted the selectivity of the GOx enzyme. To ovecome this problem some methods were tried, one of them was to coat the electrode with single or multilayers of membranes or polymers like oxidized polpyrrole, cellulose acetate films, electropolymerized films of polyphenylendiamine etc. which on the principle of size exclusion allowed only glucose molecules to cross through it and reach the redox center and thus blocking other electro-active species.

b) The second method attempted was to operate the electrode at optimum potential which needs HRP to facilitate oxidation of hydrogen peroxide or use of other elecrodes that allowed strong preferential electrocatalytic detection of generated hydrogen peroxide. The second disadvantage of first generation was its oxygen dependence. As it depends on oxygen availability there are fluctuations in the results obtain based on this.One of the method to overcome this problem was to use other enzyme that does not need oxygen, the particular enzyme used widely was Glucose dehydrogenase (GDH) (K.M. Narayan *et al*, 2006). GDH does not require oxygen but it needs PQQ(Pyrroloquinoline Quinone) cofactor. The quinoprotein recognition element of GDH used PQQ factor and also GDH-PQQ is an eficien enzymatic sysytem with very fast electron tranfer rate.

The drawback of first generation glucose biosensors i.e. oxygen dependency led to the invention of second generation glucose biosensors.This generation of glucose biosensors eliminated the need of natural electron mediator i.e. oxygen by using synthetic electron mediator. Mediators used were ferrocene derivatives, ferricyanides, pheno-thiazine compounds, quinone compunds (J. Wang, 2000) etc. They worked on reaction equation 4 and following reactions 6 and7.

$$GOx(FADH_2) + 2Mox \rightarrow GOx(FAD) + 2Mred + 2H^+ \qquad \text{Equation 6}$$

$$2Mred \rightarrow 2Mox + 2e^- \qquad \text{Equation 7}$$

In above equations M is electron mediator used. By using these mediators the drawback of oxygen dependency was solved and also as these mediators need less potential to operate, as the electro-active interference due to other species was minimized. Second generation glucose biosensors almost eliminated the drawbacks of first generation glucose biosensors but they had their own disadvantages such as some of the mediators proved to be toxic for longer and continuous use. Also, as mediators are small and diffusive molecules, maintaining their presence near electrode over a long duration was found to be difficult.

These drawbacks of second generation glucose biosensors led to the increasing demand of inventing a glucose biosensors that was mediator free (both natural and synthetic). This led to the the invention of third generation glucose biosensors. These biosensors were designed so as to get direct electrical communication between the enzyme redox center generated electrons and electrode. Various attempts were made to obtain this generation of glucose biosensors. One of them is development of mesoporous electrodes that entraps the enzyme on the porous surface of the electrode leading to direct electron transfer between enzyme and electrode. The other attempt was the use of Indium Tin Oxide (ITO) electrode. Zinc Oxide (ZnO) nanorod were formed on the ITO electrode and then GOx was adsorbed on the surface of these nanorods. This adsorption was then secured with the help of Nafion film. As GOx is present very near to the electrode surface, when glucose gets oxidized the electrons are transferred easily to the electrode surface due to the ZnO nanorods. The other approach to make third generation glucose biosensor was to use conducting organic salt electodes. These electrodes worked on the principle of charge transfer complexes such as tetrathiafulvalene-tetracyanoquinodimethane (TTF-TCNQ). Various mechanisms of electron transfer at electrode surface due to these complexes have been suggested by various authors but it is still controversial. Based on these three generations of glucose biosensors there were many advances that were carried out in this field later in the 20th and 21st century.

New advances in glucose biosensors field

Carbon Nano Tubes (CNTs)

CNTs are basically allotropes of carbon with cylindrical nano sized structure. They can be single walled (SWCNT) or multi walled (MWCNT). There are numerous ways in which CNTs are explored for the use in glucose sensing. One of them is covalent attachment of GOx enzyme to the CNTs. First the electrode was coupled to SWCNT with the help of a coupling reagent. Then redox center i.e. GOx (FAD) was covalently attached to CNT again with the help of coupling reagent. Thus the glucose gets oxidized at the redox center and direct electron transfer was obtained across the CNTs to the electrodes. The other method in which CNTs are used for glucose biosensing is encapsulation (K. Balasubramanian and M. Burghard, 2006) of GOx enzyme in sol-gel matrix. By this method basically a Glass Carbon Electrode (GCE) was first modified by coating it with MWCNTs and then the coated CNTs was covered with sol-gel or hydrogel matrix containing GOx. Yet another method of using CNTs for glucose monitoring is electropolymerization. Now in this method GOx is mixed with monomer and this monomer is electropolymerized on the GCE surface. This is how enzyme gets embedded into the polymer matrix. Uses of CNTs as individual nano-electrode and also as CNT nano fiber for glucose sensing have been tried. However there is one major drawback of using CNTs commercially for glucose sensing, i.e. its cost.

Graphene Based Biosensors

Graphene has also been employed for the purpose of glucose biosensing because of its electron transportation ability and speed, biocompatibilities, high thermal conductivity, high electrocatalytic activity towards hydrogen peroxide and excellent performance for direct electrochemistry of GOx. Many graphene based glucose biosensors have been demonstrated. One of them is the use of graphene nanosheets (Z. Zhu *et al*. 2012). Graphene nanosheets are synthesized and then covalently conjugated with the enzyme GOx. This modified graphene nanosheets are then immobilized on GCE which has already been treated with Polypyrrole. In this case the reactions are same as third generation glucose biosensors i.e. no mediator is needed for electron transfer. Chemically reduced graphene oxide electrodes have also been used for glucose sensing. Also graphene/metal nanoparticles based glucose biosensors are demonstrated which gave good accuracy and high precision.

Non-Enzymatic Glucose Biosensors

Though the enzymatic glucose biosensors have dominated the market, they suffer various drawbacks resulting from inherent stability issues of the enzyme itself. There are thermal and chemical stability issues with GOx i.e. it loses its activity outside the pH range of 2-8 and above temperature 40ºC. Also in enzymatic glucose biosensors there is a need to coat electrode with single or multiple layers of the enzyme which adds one more step to the manufacturing process of commercial glucose biosensors. The sensitivity of these enzymatic glucose biosensors widely depends on the enzyme that has been immobilized thus making it less reproducible. All these are reasons for the need of non-enzymatic glucose biosensors. Researchers are still working to obtain a non-enzymatic glucose biosensor that can be launched commercially. In recent years various materials have been tested for their electro-catalytic response to glucose oxidation, some of them are metals and metal oxides, graphene, nanotubes, polymers and composites (K.E. Toghill and R.G. Compton, 2010). Starting with metals and metal oxides, Copper Oxides (CuO) are widely preferred for direct electro-oxidation of glucose. CuO nanosheets (Z.H. Ibupoto *et al*. 2013) were used for this purpose. Cuprous Oxides (Cu_2O) were also tested for their biosensing activity. Various morphological structures of Cu_2O were tested out of which rhombic dodecahedral nanocrystals has shown promising results. The other metal widely tested is Nickel. Here also various morphological structures of Nickel oxides (NiO) were tested out of which flower like NiO morphology gave good results with more accuracy (V.A. Kumary *et al.* 2013). The major problem with NiO is its inability to work at physiological pH. Yet other metals widely tested for glucose biosensing are Platinum (Pt) electrode and gold electrodes. Platinum electrode showed good results but had major drawback of poisoning due to various species. All these non-enzymatic glucose biosensors based on metal oxides have also one common drawback i.e. the exact mechanism of glucose oxidation at its surface is still controversial. The second approach for development of enzyme free glucose biosensors is use of graphene based composite glucose biosensors. One method is electrodepositing copper nanoparticles on graphene nanosheets which are chemically synthesized. This gave better results and good accuracy over only graphene based glucose biosensors. Another method is use of composites of PtNi (platinum-Nickel) nanoparticles and graphene. Yet another approach for non enzymatic glucose biosensors is use of CNTs. As CNTs have shown direct and easy electron transfer across it, various metals are conjugated with CNTs and tested for biosensing activity without using enzyme for oxidation of glucose. Some of the examples are MnO_2, SnO_2 and CuS_2 etc.

Optical Glucose Biosensors

These biosensors use optical fibers which are nothing but small flexible glass wires. Various attempts have been made for incorporation of these optical fibers in glucose biosensing because of their fast and easy transmission of signals (D.L. Meadows and J.S. Schultz, 1993). One of them consisted of fluorescent chemical complex immobilized on hydrogel (biopolymer permeable to glucose) and quencher (responsive to glucose). In the presence of glucose fluorescence was obtained as quencher binds to glucose. In the absence of glucose quencher hinders fluorescence by binding to fluorescent chemical complex. This principle was once used in hospital as catheters for glucose biosensing. Other approach for optical glucose biosensors is coating the optical fibers with the GOx enzyme and then further coating it with oxygen sensitive coating. In this case glucose monitoring is done by measuring consumption of oxygen. The semi permeable oxygen sensitive coating is designed to ensure that the reaction proceeds with a stoichiometric excess of oxygen. A latest method of measuring glucose consists of sensing material, light source, optical fiber and detector. Here in this case optical fiber transmits signal and also acts as substrate for the sensing material used. As the reaction occurs between sensing material and glucose there are changes obtained in optical as well as physicochemical properties. The transduction mechanism generates optical signal that can be related to analyte concentration i.e. glucose concentration. This optical signal is sent to detector via optical fibers. To measure the optical signal difference between incident and output light is obtained and glucose concentration can be determined with the use of mathematical calculations. With advances in optical glucose biosensor there is one major disadvantage- slow response time.

Commercial Perspective of Glucose Biosensors

There are over 40 glucose biosensors in the market for self monitoring of blood glucose and they account for over 85% of world's total biosensors market (J. Wang, 2008). Table 1 explains principles of some commercially available electrochemical systems for self-monitoring of blood glucose.

Table 1- Principles of some commercial glucose biosensors (J. Wang, 2000 and E. Wilkins, 1996)

Manufacturer	Brand	Mechanism
Bayer	Ascensia Contour®	GDH-FAD
Life Scan	One Touch® UltraLink™	GOx
Roche	Accu-Chek® Aviva	GDH-PQQ
Roche	Accu-Chek® Active	GDH-Ferrocene
Bayer	Elite®	GOx- Ferricyanide
Medtronic	MiniMed®	GOx
Life Scan	SureStep®	GOx-Ferricyanide

Recently an advanced and non-invasive technique has been explored. The product that uses this technology is GlucoWatch. It's a wrist watch that continuously displays blood glucose level. It works on the principle of reverse iontophoresis. Iontophoresis is the principle widely used in the cosmetic industry. Iontophoresis (S. Park, 2006) works by combination of two mechanisms, one is electro- migration and the other is electro-osmosis. Electro-migration is movement of ions in the skin in response to the current applied, for example when a small positive current is applied it drives positively charged molecules deeper inside the skin and tissues due to repulsion of like charges on the molecules and when negative current is applied, it drives the negatively charged ions deeper into the skin again same due to the repulsion of like charges on the molecules. The second mechanism is electro-osmosis which is the main principal transport mechanism of uncharged molecules here mainly glucose and of high-molecular-weight cations. The skin is negatively charged at physiologic pH and thus acts as a semi permeable membrane to cations (A. Sieg *et al*, 2004) This preferential passage of counter ions due to electro-migration induces an electro-osmotic solvent flow that may carry neutral molecules in the anode-to-cathode direction. GlucoWatch works on the reverse iontophoresis principle. It consists of two electrodes, AutoSensor and hydro gel containing enzyme. When some little current that is produced by the batteries of the watch is applied to the skin it drives negatively charged molecules away from the skin and in return sodium ions (Na^+) go towards the cathode. Along with positively charged sodium ions neutral molecules like glucose goes towards the cathode. This glucose gets oxidized due to the enzyme present in the hydro gel and hydrogen peroxide produced is used to calculate the amount of glucose present. This GlucoWatch gives continuous reading of glucose in blood but is less accurate compared to the other invasive glucose biosensors. Thus it cannot replace the invasive glucose biosensors but it can reduce the number of times patient has to use the invasive glucose biosensor per day also the other disadvantage of requirement of warm-up period (approximately 2hours) limits the usage of GlucoWatch.

CONCLUSION

Diabetes is leading health problem worldwide needing continuous and accurate blood glucose monitoring. Over the past 50 years tremendous progress has been made in development of electrochemical glucose biosensors. Despite impressive progress in glucose biosensors there are still many challenges awaiting researcher's attention which will lead to stable, accurate, reproducible and less paining glucose biosensors. There is still the need to determine the exact mechanism at the metal electrodes in case of non-enzymatic glucose biosensors so that non-enzymatic glucose biosensors can be made commercially available. Also in the case of noninvasive glucose biosensors, further study is required to completely replace the noninvasive glucose biosensors in place of invasive ones. To face all these challenges and get success, understanding of biochemistry, electro chemistry, surface chemistry, material chemistry and physiology is a must. Thus despite over five decades of research and available and wide use of glucose biosensors there is still an opportunity for some innovative invention to obtain glucose biosensors which are accurate, quick, painless and the most importantly economic.

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