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# Amperometric Glucose Sensors for Whole Blood Measurement Based on Dehydrogenase Enzymes

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Additional information is available at the end of the chapter

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## 1. Introduction

Self-monitoring blood glucose (SMBG) is an important component of modern therapy for diabetes mellitus. SMBG has been recommended for people with diabetes and their health care professionals in order to achieve a specific level of glycaemic control and to prevent hypoglycaemia. The goal of SMBG is to collect detailed information about blood glucose levels at many time points to enable maintenance of a more constant glucose level by more precise regimens. It can be used to aid in the adjustment of a therapeutic regimen in response to blood glucose values and to help individuals adjust their dietary intake, physical activity, and insulin doses to improve glycaemic control on a day-to-day basis.

SMBG can aid in diabetes control by: (<http://www.diabetes.co.uk/blood-glucose/blood-glucose-self-monitoring.html>)

- facilitating the development of an individualized blood glucose profile, which can then guide health care professionals in treatment planning for an individualized diabetic regimen;
- giving people with diabetes and their families the ability to make appropriate day-to-day treatment choices in diet and physical activity as well as in insulin or other agents;
- improving patients' recognition of hypoglycaemia or severe hyperglycaemia;
- enhancing patient education and patient empowerment regarding the effects of lifestyle and pharmaceutical intervention on glycaemic control.
- helps to determine which foods or diet are best for one's control
- helps inform the patient and doctor about how well the medication regime is working
- reduces anxiety about, and increases understanding of, hypoglycaemia. This is because if untreated hypoglycaemia can result in coma for diabetic patients and is therefore a

condition that diabetics try to avoid through proper action. To this end, regular testing can predict dangerous drop in blood glucose concentration which can lead to hypoglycaemia.

- is important for undertaking dangerous tasks which could be influenced by high or low blood sugar, such as driving and handling dangerous machinery

Disadvantages are mainly seen when either the patient lacks motivation to test or does not have sufficient education on how to interpret the results to make sufficient use of home testing equipment. Where this is the case, the following disadvantages may outweigh the potential benefits:

- anxiety about one's blood sugar control and state of health
- the physical pain of finger pricking
- expense to the NHS or other medical body

Numerous trials have been carried out to determine the true impact of SMBG on glycaemic control. Some, including randomized, controlled trials, have demonstrated the efficacy of SMBG. Among patients with type 1 diabetes, SMBG has been associated with improved health outcomes. <sup>[1]</sup> Specifically, increasing frequency of SMBG was linearly correlated with reductions in HbA1c among type 1 patients in Scotland. <sup>[2]</sup> Among patients with type 2 diabetes, a higher frequency of SMBG was associated with better glycaemic control among insulin-treated patients who were able to adjust their regimen. <sup>[3]</sup>

SMBG works by having patients perform a number of glucose tests each day or each week. The test most commonly involves pricking a finger with a lancet device to obtain a small blood sample, applying a drop of blood onto a reagent strip (typically an enzyme electrode), and determining the glucose concentration by inserting the strip into an electronic meter for an automated reading. Test results (a measure of the glucose concentration in the blood sample) are then recorded either in a logbook or stored in the glucose meter's electronic memory. People with diabetes can be taught to use their SMBG results to correct any deviations out of a desired target range by changing their carbohydrate intake, exercising, or using more or less insulin.

The frequency with which patients with diabetes should monitor their blood glucose level varies from person to person. Most experts agree that insulin-treated patients should monitor blood glucose at least four times a day, most commonly fasting, before meals, and before bed. In addition, patients using insulin can benefit by obtaining postprandial blood glucose readings to help them more accurately adjust their insulin regimen. A positive correlation between frequency of SMBG and glycaemic control among patients with insulin-treated type 1 or type 2 diabetes has been demonstrated. <sup>[1-3]</sup> Patients treated with intermediate, short-acting, or rapid-acting insulin may benefit from SMBG data to make adjustments in their regimen.

For patients with type 2 diabetes, optimal SMBG frequency varies depending on the pharmaceutical regimen and whether patients are in an adjustment phase or at their target for glycaemic control. If a patient is on a stable oral regimen with HbA1c concentration

within the target range, infrequent SMBG monitoring is appropriate. In such cases, patients can use SMBG data as biofeedback at times of increased stress or changes in diet or physical activity.<sup>[4,5]</sup>

It is important to point out however that this debate is not straightforward, however. One factor that is often overlooked is that the numbers obtained by testing are only one part of the picture, which requires additional data to be complete. For example, it is important to relate the number to what and when the patient last ate. The patient's exercise regime must also be considered, as well as when and how much medication has been taken. If this were not complicated enough, the patient (and physician) need to consider factors such as whether the patient has recently been ill, or even subjected to high levels of stress, which can distort the picture.

Blood glucose meters which utilise an enzyme electrode (a term first coined by Updike and Hicks in the late sixties<sup>[6]</sup>) as the glucose sensing element are particularly suitable medical devices for SMBG.

All the commercial electrochemical meter systems typically comprise two components. The meter - applies potential differences in a programmed sequence to the sensor, collects current data and analyses the current time response of the sensor, records and displays results. The enzyme electrode (or biosensor) test strip - collects the blood sample, the sample undergoes an enzymatic chemical reaction followed by an electrochemical detection step. The patient simply inserts the enzyme electrode into the meter and applies a small drop of blood to the sensor. After a short delay (typically 5 seconds) the blood glucose value is displayed by the meter in mg/dL or mmol/L.

The advantages offered by biosensors in SMBG arise for the following reasons. Blood is a complex fluid and glucose levels vary widely over time in a single patient, many factors besides glucose vary in blood from healthy, patients (haematocrit, oxygen levels, and metabolic by-products) therefore great specificity is a prime requirement. In addition, patients with diabetes may have a wide range of other medical problems creating even greater variation in their blood. Finally, biosensors can be used directly in the blood without requiring major modifications to the biological sample (increased temperature or pressure, dramatic pH changes, addition of highly reactive chemicals, etc).

The enzyme electrodes commonly used in SMBG can be defined as a combination of any electrochemical probe with a thin layer of enzyme based reagent that is selective for glucose (*or other important analytes such as  $\beta$ -hydroxybutyrate*). In these devices, the function of the enzyme is to provide selectivity by virtue of its biological affinity for a particular substrate molecule. For example, an enzyme is capable of catalysing a particular reaction of a given substrate even though other isomers of that substrate or similar substrates may be present.

Typically, the progress of the enzyme reaction (which is related to the concentration of analyte) is monitored by the rate of formation of product or the disappearance of a reactant. If either the product or reactant is electroactive, then the progress of the reaction can be monitored directly using amperometry. In this technique, current flow is measured in

response to an applied voltage. The resultant current is monitored by the meter and then interpolated into an accurate measurement of glucose using on-board software algorithms giving the user a concentration value in typically less than 7 seconds.

The final method of analysis used will ultimately depend on several properties of the enzyme. The main considerations are;

1. does the enzyme contain redox active groups
2. are the products of the biochemical reaction electroactive
3. is one of the substrates or cofactors electroactive
4. what is the required speed of response
5. what will be the final application of the sensor

The answer to the first three criteria will depend largely on the system under investigation. The answer to the latter three depends on the requirements and application of the sensor under consideration. If the enzyme does not contain any redox groups, then the method of analysis will be restricted to monitoring either the release of products or the consumption of substrate by their reaction at the transducing electrode. The current produced can then be related to the concentration of analyte. Of particular relevance to this article, are the devices that incorporate nicotinamide adenine dinucleotide (NAD), flavin adenine dinucleotide (FAD) or pyrroloquinoline quinone (PQQ) dependent dehydrogenase enzymes.

## 2. Electrochemical oxidation of NAD(P)H

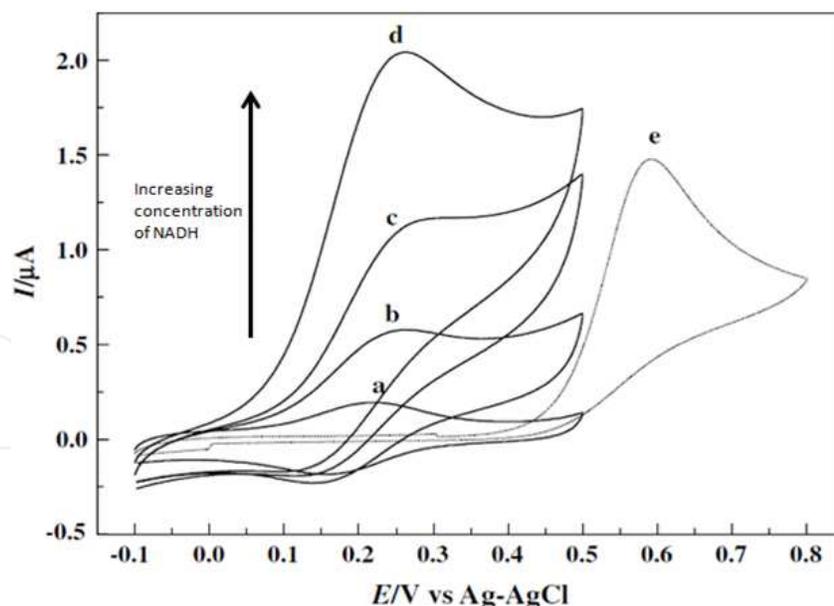
Given that the nicotinamide coenzymes are electron carriers, and therefore by definition electroactive, it would appear at first sight (points 2 and 3 above) that these systems would be ideal candidates for commercial enzyme electrode devices. The electrochemical oxidation of NADH to NAD<sup>+</sup> is however both chemically and kinetically complicated at common electrode surfaces such as gold, platinum or glassy carbon.

The electrochemical detection of NADH has generated great interest because the pyridine nucleotides NAD<sup>+</sup> and NADP<sup>+</sup> are ubiquitous in all living systems and are required for the reactions of more than 450 oxidoreductases.<sup>[7]</sup> Although the formal potential of NADH/ NAD<sup>+</sup> couple in neutral pH at 25 °C is estimated to be 0.56 vs. SCE<sup>[8, 9]</sup> significant over-potential is often required for the direct oxidation of NADH at bare electrodes.<sup>[10]</sup> Unlike in nature where the oxidation of NADH occurs as a 1-step hydride transfer, on bare electrodes the reaction has been shown to occur via a different and higher energy pathway which produces biologically inactive NAD· radicals as intermediates. The large amount of energy required to produce these intermediates is the origin of the large overpotential (typically 1 Volt) required at bare electrodes. As a result, the direct electrochemical oxidation of NADH has been shown to produce a mixture of products including biologically active NAD<sup>+</sup>, (NAD)<sub>2</sub> dimers and products from the side reactions of the electrogenerated NAD· radicals.

In addition, the direct oxidation of NADH is often accompanied by electrode fouling due to the polymerisation oxidation products on the electrode surface.<sup>[11]</sup> The fouling of the electrode surface can occur by two mechanisms. First, the NAD· radicals interact directly with oxide

functionalities on the surface of the electrode and second the reaction product,  $\text{NAD}^+$ , adsorbs onto the electrode surface. Both of these processes are irreversible and result in the gradual blocking of the electrode during continued oxidation. It is this electrode fouling that results in the irreproducibility of the analytical signal from bare electrodes. In addition, if this method were to be used in commercial glucose sensors for SMBG, the high over potentials required would result in the response being a combination of signals from the oxidation of both NADH and common interferents, e.g. ascorbic acid, uric acid, paracetamol etc. found in blood.

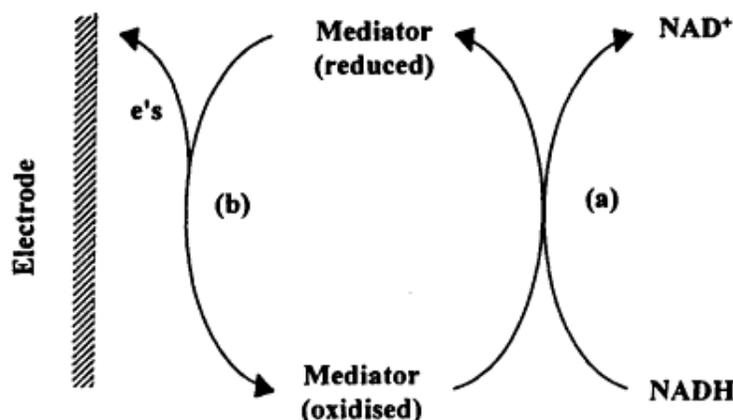
The problems mentioned above can be overcome by using small catalytic molecules called mediators. These molecules can transfer charge from the reduced coenzyme directly to the electrode thereby bypassing the direct oxidation of NADH. The use of this approach has three potential advantages when compared to the direct oxidation of the reduced nicotinamide. First, by judicious choice of the mediator problems associated with electrode fouling or competing reactions can be avoided (especially true if the chosen mediator has a site for hydride transfer). Virtually 100% of the oxidation product via this mechanism is biologically active NAD. Second, the rate of electron transfer between the NADH molecule and the mediator can be enhanced resulting in a more stable increased signal. The rate at which NADH is chemically oxidised will be governed to a large extent by the potential difference between the formal redox potential of the two species. Finally, a mediator can be selected with a redox potential that will limit the effects of interference. This is particularly relevant in cases where the bio-analyte is present in blood at low concentrations as in the case of beta-hydroxybutyrate (0.0 – 0.3mEq/L). The electrocatalytic oxidation of NADH at a modified electrode is shown schematically in Figure 1. Of particular



**Figure 1.** Cyclic voltammograms of the o-AP modified GC electrode in phosphate buffer (a) in the absence and in the presence of increasing concentrations of NADH/mM: (b) 0.1, (c) 0.3 and (d) 0.5; and (e) 0.6 at bare GC in phosphate buffer (0.1 M, pH 7.0). Scan rate = 10 mV/s. Note, the peak oxidation for NADH at the bare electrode occurs at 0.6 Volts. When the electrode is modified, in this case with o-amino phenol the oxidation of NADH is concomitant with the oxidation of the immobilised redox couple (traces b – d). *Figure and data adapted from reference 11.*

interest are voltammograms a and e. These show the direct oxidation of NADH (e) at the glassy carbon electrode and the cyclic voltammogram (a) of the immobilised species. Addition of NADH to the solution causes an increase in 1. Note that the signal due to the oxidation of NADH now occurs at the potential of the mediator resulting in a decrease in the operating voltage of approximately 0.3 V.

Among the mediators used so far are quinones, diimines, ferrocene, thionine oxometalates, polymetallophthalocyanines, ruthenium complexes, pyrroloquinoline quinone, fluorenones, and quinonoid redox dyes such as indamines, phenazines, phenoxazines and phenothiazines.<sup>11</sup> A generalised reaction schematic for the mediated oxidation of NADH is shown below in Figure 2.



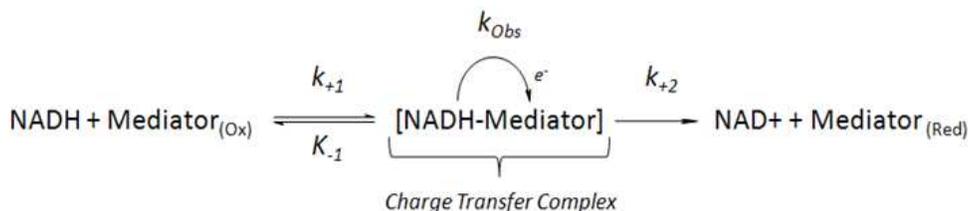
**Figure 2.** Scheme showing the mediated oxidation of NADH. In this context, the mediator reacts with NADH in a chemical step to oxidise it (a) and is itself reduced. The mediator is then itself reoxidised at the electrode surface (step b). The chemical oxidation of NADH by this mechanism bypasses the problems associated with the direct oxidation at a bare electrode surface.

To design an NADH sensor, the mediator is normally immobilized on the electrode surface or within the electrode material. Mediators can be immobilized by chemisorption by covalent attachment directly electrode surface or by electrochemical polymerisation of the mediators at the electrode surface or, alternatively via covalently attached/physically entrapment in polymers, incorporation in carbon paste grown at electrode surface or deposited on the electrodes by drop coating. The method that is ultimately chosen to produce the modified electrode depends upon the method of mass production (for commercial sensors) and the materials used in the device. For example, in sensors that utilise screen printed carbon based electrodes (*screen-printing technology is a kind of low-cost thick film technology which allows to deposit thick films, a few to hundreds micrometers and is well suited for mass production and portable devices. Such a micro fabrication route offers high-volume production of extremely inexpensive and yet highly reproducible disposable enzyme electrodes – this will be discussed further in the text*) it is convenient to incorporate the mediator directly into the carbon ink particularly if the mediator contains delocalised aromatic rings, as found in quinine and phenoxazine dye based mediators, which form strong chemisorbed bonds with the carbon and graphite plates. The mediator loading, activity stability etc can all be investigated using conventional electrochemical techniques such as DC cyclic voltammetry.

It is important that modified electrodes designed for the reoxidation of NADH are stable over their stated shelf-life, respond only to NADH and not to any other species present in the blood sample satisfy the following kinetic requirements; i) the reaction between the NADH molecule and the mediator is fast, ii) the transfer of charge within the mediating layer is fast and iii) the electron transfer between the reduced mediator and the electrode is also fast.

### 3. Kinetic modelling of NADH oxidation at chemically modified electrodes.

In the scientific literature, NADH oxidation at chemically modified electrodes is most commonly suggested to occur via a two-step reaction mechanism. In the first step, NADH forms a charge transfer complex with the oxidised form of the mediator bound to the electrode surface. In the second step, electron exchange takes place and the complex breaks down producing  $\text{NAD}^+$  and a reduced mediator site. Because the electrode is polarised, the reduced mediator site is reoxidised in a non-rate limiting electron exchange to the bulk electrode material. This scheme is shown in Figure 3. The important kinetic constants are also represented.



**Figure 3.** Two-step mechanism commonly proposed for the oxidation of NADH at chemically modified electrodes. The mediator and NADH form a charge transfer complex that dissociates to give rise to the reduced mediator and biologically active  $\text{NAD}^+$ .

For this type of mechanism, the catalytically limiting current ( $i_k$ ) observed under controlled hydrodynamic conditions can be expressed as:  $i_k = nFAK_{\text{cat}}\Gamma C_{\text{NADH}}/K_M$  where  $C_{\text{NADH}}$  is the bulk concentration of NADH and  $\Gamma$  represents the concentration of binding sites in the immobilised film. This type of model assumes that the rate of electron transfer between the mediating species in the film and the NADH is sufficiently fast so as not to be rate limiting, the NADH freely diffuses into the film whereupon it adsorbs to the catalytic site and it undergoes oxidation to  $\text{NAD}^+$ . Also, the expression for  $i_k$  is valid only for thin films where the concentration of NADH is insufficient to saturate the binding sites.<sup>[12]</sup>

Due to the formation of the charge-transfer complex, this reaction scheme is commonly analysed using Michaelis-Menten kinetics. From figure 3 it is possible to construct the following kinetic argument.

This is now a straight line plot. From the slope of such a plot, values of  $k_{+2}$  can be calculated. By extrapolation of zero NADH concentration, i.e. the intercept, values of  $K_M$  can be estimated. Values for  $k_{\text{Obs}}$  can be obtained from Koutecky-Levitch plots under steady state

$k_{+1}$  and  $k_{-1}$  represent the formation and dissociation rate constants for the complex

$k_{+2}$  represents the catalytic rate constant for the break down of the complex

$K_M$ , which is analogous to the Michaelis – Menten constant, is defined as  $K_M = \frac{k_{-1} + k_{+2}}{k_{+1}}$

The second order reaction rate,  $k_{obs}$ , for any given concentration of NADH can thus be expressed as

$$k_{obs} = \frac{k_{+2}}{K_M + [NADH]} \quad (\text{eq 1})$$

Inverting eq. 1 gives the following expression;

$$\frac{1}{k_{obs}} = \frac{K_M}{k_{+2}} + \frac{[NADH]}{k_{+2}} \quad (\text{eq 2})$$

#### Scheme 1.

oxidation conditions as described by Compton and Hancock.<sup>[12]</sup> Typically, values of  $k_{obs}$  tend to be in the range  $10^{-3}$  to  $10^{-1}$   $\text{cm s}^{-1}$ . Thus it is possible using such laboratory techniques to ensure that the surface coverage of mediator (moles/ $\text{cm}^2$ ) is optimised to achieve favourable measurement linearity and speed of response.

## 4. Commercial sensors for SMBG incorporating NAD(P)-linked dehydrogenase enzymes

Commercial examples of this type of device are the “Abbott Optium Xceed glucose and  $\beta$ -ketone test strips” (<http://www.abbottdiabetescare.com/precision-xtra-blood-glucose-and-ketone-monitoring-system.html>). These strips make use of the  $\text{NAD}^+$  dependent glucose dehydrogenase (EC 1.1.1.47) and  $\text{NAD}^+$  dependent  $\beta$ -hydroxybutyrate dehydrogenase (EC 1.1.1.30). Exogenous  $\text{NAD}^+$  is incorporated into the reagent ink which is deposited onto the individual electrode by screen printing. The mediator molecule that is used to recycle the reduced form of the coenzyme is phenanthroline quinone (*Manufacturers own vial insert data sheet*). Advantages often cited with this type of chemistry include good selectivity and no reaction with oxygen.

## 5. Screen printing as a means of mass-manufacturing enzyme sensors

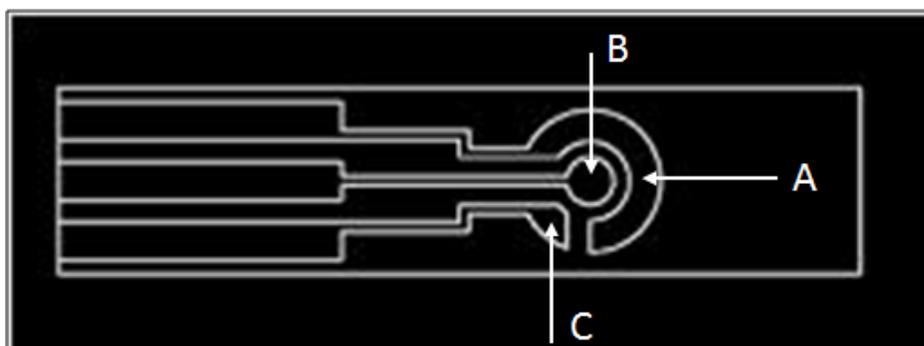
Screen printing is arguably the most versatile of all printing processes. It can be used to print on a wide variety of substrates, including paper, paperboard, plastics, glass, metals, fabrics, and many other materials including paper, plastics, glass, metals, nylon and cotton. Some common products from the screen printing industry include posters, labels, decals, signs, and all types of textiles and electronic circuit boards. The advantage of screen printing over other print processes is that the press can print on substrates of any shape, thickness and size.

A significant characteristic of screen printing is that a greater thickness of the ink can be applied to the substrate than is possible with other printing techniques. This allows for some

very interesting effects that are not possible using other printing methods. Because of the simplicity of the application process, a wider range of inks and dyes are available for use in screen printing than for use in any other printing process. The major chemicals used include screen emulsions, inks, and solvents, surfactants, caustics and oxidizers used in screen reclamation.

Screen printing consists of three elements: the screen which is the image carrier; the squeegee; and ink. The screen printing process uses a porous mesh stretched tightly over a frame made of wood or metal. Proper tension is essential to accurate colour registration. The mesh is made of porous fabric or stainless steel mesh. A stencil is produced on the screen either manually or photochemically. The stencil defines the image to be printed in other printing technologies this would be referred to as the image plate.

Screen printing ink is applied to the substrate by placing the screen over the material. Ink with a paint-like consistency is placed onto the top of the screen. Ink is then forced through the fine mesh openings using a squeegee that is drawn across the screen, applying pressure thereby forcing the ink through the open areas of the screen. Ink will pass through only in areas where no stencil is applied, thus forming an image on the printing substrate. The diameter of the threads and the thread count of the mesh will determine how much ink is deposited onto the substrates. Figure 4 shows an example of an image for an electrochemical cell consisting of a working, a counter and a reference element. This type of structure can be easily produced by screen printing.



**Figure 4.** Example of a screen printed electrochemical cell (screen image) showing the Reference element (A), the working electrode (B) and the counter electrode (C). Commercially available screen printed electrodes for research purposes can be obtained from DropSens, Edificio CEEI - Parque Tecnológico de Asturias - 33428 Llanera (Asturias) Spain. [http://www.dropsens.com/en/screen\\_printed\\_electrodes\\_pag.html](http://www.dropsens.com/en/screen_printed_electrodes_pag.html)

Many factors such as composition, size and form, angle, pressure, and speed of the blade (squeegee) determine the quality of the impression made by the squeegee. At one time most blades were made from rubber which, however, is prone to wear and edge nicks and has a tendency to warp and distort. While blades continue to be made from rubbers such as neoprene, most are now made from polyurethane which can produce as many as 25,000 impressions without significant degradation of the image.

If the item was printed on a manual or automatic screen press the printed product will be placed on a conveyor belt which carries the item into the drying oven or through the UV

curing system. Rotary screen presses feed the material through the drying or curing system automatically. Air drying of certain inks, though rare in the industry, is still sometimes utilized.

The rate of screen printing production was once dictated by the drying rate of the screen print inks. Due to improvements and innovations the production rate has greatly increased. Some specific innovations which affected the production rate and have also increased screen press popularity include:

- Development of automatic presses versus hand operated presses which have comparatively slow production times.
- Improved drying systems which significantly improves production rate.
- Development and improvement of U.V. curable ink technologies
- Development of the rotary screen press which allows continuous operation of the press. This is one of the more recent technology developments.

There are three types of screen printing presses. The flat-bed (probably the most widely used), cylinder, and rotary. Flat-bed and cylinder presses are similar in that both use a flat screen and a three step reciprocating process to perform the printing operation. The screen is first moved into position over the substrate, the squeegee is then pressed against the mesh and drawn over the image area, and then the screen is lifted away from the substrate to complete the process. With a flat-bed press the substrate to be printed is positioned on a horizontal print bed that is parallel to the screen. Rotary screen presses are designed for continuous, high speed web printing. The screens used on rotary screen presses are seamless thin metal cylinders. The open-ended cylinders are capped at both ends and fitted into blocks at the side of the press. During printing, ink is pumped into one end of the cylinder so that a fresh supply is constantly maintained. The squeegee is a free floating steel bar inside the cylinder and squeegee pressure is maintained and adjusted by magnets mounted under the press bed.

Screen printing inks are moderately viscous inks which exhibit different properties when compared to other printing inks such as offset, gravure and flexographic inks though they have similar basic compositions (pigments, solvent carrier, toners, and emulsifiers). There are five different types of screen ink to include solvent, water, and solvent plastisol, water plastisol, and UV curable.

### **5.1. UV curable inks**

UV curable inks consist of liquid pre-polymers, monomers, and initiators which upon being exposed to large doses of U.V. Radiation instantly polymerize the vehicle to a dry, tough thermosetting resin. They also require less energy, overall, to dry or "cure" compared to gas or electric driers. The down side of UV inks is they can cost as much as three times that of regular inks and must be handled differently than conventional inks due to safety issues. Additionally, solvents are required for clean-up which results in some VOC emissions.

## 5.2. Plastisol Inks

Plastisol inks (both solvent and water based) are used in textile screen printing. Plastisol ink is a PVC (polyvinyl chloride) based system that essentially contains no solvent at all. Along with UV ink used in graphic screen printing, it is referred to as a 100% solid ink system. Plastisol is a thermoplastic ink in that it is necessary to heat the printed ink film to a temperature high enough to cause the molecules of PVC resin and plasticizer to cross-link and thereby solidify, or cure. The temperature at which most plastisol for textile printing cures at is in the range of 149 °C to 166 °C (300 °F to 330 °F). Plastisol inks are commonly used for printing graphics on articles such as tee shirts.

## 5.3. Solvent inks & water inks

Solvent and water based screen printing inks are formulated with primarily solvent or water. The solvent evaporates and results in VOC emissions. Water based inks, though they contain significantly less, may still emit VOC's from small amounts of solvent and other additives blended into the ink. The liquid waste material may also be considered hazardous waste. Water-based inks are a good choice when a "soft hand" is desirable. Water-based inks also have the advantage of being an excellent ink system for high speed roll-to-roll yardage printing. Such printing is done on large sophisticated equipment that has very large drying (curing) capacity. Finally, because of the fragility of the components used in the manufacture of enzyme electrodes, e.g. enzymes, co-enzymes and mediators water based inks tend to be the ink of choice when formulating the reagent ink component of the device.

Screen (or image transfer) preparation includes a number of steps. First the customer provides the screen printer with objects, photographs, text, ideas, or concepts of what they wish to have printed. The printer must then transfer a "picture" of the artwork (also called "copy") to be printed into an "image" (a picture on film) which can then be processed and eventually used to prepare the screen stencil. Once the artwork is transferred to a positive image that will be chemically processed onto the screen fabric (applying the emulsion or stencil) and eventually mounted onto a screen frame that is then attached to the printing press and production begins. Screen mesh refers to the number of threads per inch of fabric. The more numerous the threads per inch the finer is the screen. Finer mesh will deposit a thinner ink deposit. This is a desirable affect when printing a very fine detail and halftones. Typically a fabric should be 200-260 threads per inch. Water based inks work best on finer mesh. These are generally used in graphic and industrial printing. Course mesh will deposit a heavier ink deposit. This type of screen is used on flatter, open shapes. Typically a course screen mesh will be 160-180 threads per inch. These are generally used in textile printing. An example artwork for a multi-layered screen printed device. Each colour represents a different layer requiring different screens and artworks.

Enzyme electrodes are normally built up in layers using different art works and different inks. The inks range from; conducting carbon and silver inks (to generate the conducting pathways and/or the reference half cell of the device), inks containing the various chemical and biochemical components of the device (enzyme, buffer salts, mediator, stabilisers etc)

and inks that are used to define structural components of the device (insulating inks to define the electrode areas and geometries, adhesive inks to provide three dimensional elements such as capillary spacers and coloured inks to provide branding and product identification.) Each of these individual layers normally requires a separate screen and is carried out at a separate printer/dryer station.

The blood monitors (the systems that the user receives) are made up of three main parts: the Optium meter, the Optium Plus blood glucose electrodes, and the Optium blood  $\beta$ -Ketone electrodes. When the blood sample is applied to the electrode, the glucose or ketone ( $\beta$ -hydroxybutyrate) in the blood reacts with the chemicals on the electrode. This reaction produces a small electrical current that is measured and the result is displayed by the sensor. Optium Xceed monitors are designed for testing blood obtained from a finger prick, but you can also use it to test blood from other, less painful, sites such as the base of the thumb, forearm or upper arm. A recent study published in *Clinical Chemistry and Laboratory Medicine* [13] concluded that the Optium Blood glucose test strip had a within-run imprecision coefficient of variation (CV) of 4.2%. Good response linearity was found in glucose concentrations in the range 31–444 mg/dL (1.7–24.7 mmol/L). In the concentration range studied, the glucose meter error was 5.14% and the linear regression equation was  $y = 0.91x + 6.2$  ( $r=0.984$ ) against a Modular P clinical analyzer. The Passing-Bablok agreement test indicated good concordance of results. However, for glucose concentrations  $<100$  mg/dL (5.6 mmol/L) ( $n=69$ ) the error was 6.82% with regression equation  $y=0.86x+5.9$  ( $r=0.757$ ). Between-lot differences amounted to 0.7%–18.2%. The authors concluded that meter had good precision and accuracy when compared to the laboratory method and met the quality recommendations of the National Committee of Clinical Laboratory Standards (NCCLS, currently the Clinical Laboratory Standards Institute), the National Academy of Clinical Biochemistry (NACB) and the International Organization for Standardization (ISO).

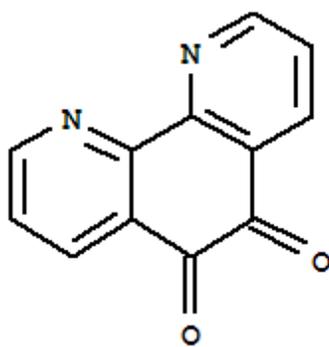
## 6. Ketone sensors which incorporate a NAD-linked dehydrogenase

In addition to glucose, another important analyte which is of particular relevance to diabetic patients is the ketone body,  $\beta$ -hydroxybutyrate. Diabetic ketoacidosis (DKA), a condition where the level of ketone bodies in the blood are elevated, is a problem that occurs in people with diabetes. It occurs when the body cannot use sugar (glucose) as a fuel source because there is no insulin or not enough insulin. Fat is used for fuel instead. People with type 1 diabetes do not have enough insulin, a hormone the body uses to break down sugar (glucose) in the blood for energy. When glucose is not available, fat is broken down instead.

As fats are broken down, acids called ketones build up in the blood and urine. In high levels, ketones are poisonous. This condition is known as ketoacidosis. Blood glucose levels rise (usually higher than 300 mg/dL) because the liver makes glucose to try to combat the problem. However the cells cannot pull in that glucose without insulin. Diabetic ketoacidosis is often the first sign of type 1 diabetes in people who do not yet have other symptoms. It can also occur in someone who has already been diagnosed with type 1 diabetes. Infection, injury, a serious illness, or surgery can lead to diabetic ketoacidosis in people with type 1

diabetes. Missing doses of insulin can also lead to ketoacidosis in people with diabetes. People with type 2 diabetes can develop ketoacidosis, but it is rare. It is usually triggered by a severe illness. In ketoacidosis, the body fails to adequately regulate ketone production causing such a severe accumulation of keto acids that the pH of the blood is substantially decreased. In extreme cases ketoacidosis can be fatal.<sup>14</sup> Despite considerable advances in diabetes therapy, key epidemiological figures related to DKA remained nearly unchanged during the last decades at a global level. Prevention of DKA – especially in sick day management – relies on intensive self-monitoring of blood glucose and subsequent, appropriate therapy adjustments. Self-monitoring of ketone bodies during hyperglycemia can provide important, complementary information on the metabolic state. Both methods for self-monitoring of ketone bodies at home are clinically reliable and there is no published evidence favouring one method with respect to DKA prevention.

Ketone sensitive test strips can be manufactured using NAD-linked enzyme,  $\beta$ -hydroxybutyrate dehydrogenase and chemically modified electrodes like the ones described above. It is interesting to note however that in the case of this particular enzyme representatives of the common classes of quinoid NADH redox mediator, including Meldola Blue, 4-methyl-1,2-benzoquinone, 1-methoxy phenazine methosulphate and 2,6-dichloroindophenol, were shown to inhibit the NAD-dependent enzyme  $\beta$ -hydroxybutyrate dehydrogenase, severely limiting their utility in the construction of a stable biosensor electrode for the ketone body  $\beta$ -hydroxybutyrate.<sup>[14]</sup> The authors speculated that this was due to 1,4-nucleophilic addition with enzyme amino acid residues such as cystine present on the enzyme. Consequently, this mode of inhibition is overcome through the use of mediators such as 1, 10-phenanthroline quinone (Figure 5.)



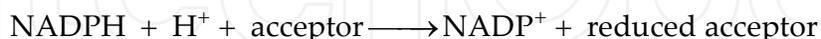
**Figure 5.** Chemical structure of 1,10 Phenanthroline quinone (oxidised form). The oxidation potential of this molecule is 0.1 Volt vs Ag/AgCl. The electrochemical reduction of 1,10-phenanthroline-5,6-quinone, like other quinones, is reversible and occurs by 2e<sup>-</sup> transfer in a single step in aqueous solution and by two 1 e<sup>-</sup> transfer steps in aprotic media.

This technology resulted in the launch of the MediSense® Optium™  $\beta$ -Ketone electrode. The strip was stable, ( $\leq 10\%$  loss in response at 30 °C versus 4 °C) with a long shelf life of 18 months. Diabetics were able to determine their  $\beta$ -hydroxybutyrate level with good precision (0.43 mM 3-OHB, 10.5% CV; 1.08 mM, 5.9%; 3.55 mM, 3.2%; n = 20 per level) and accuracy (versus reference assay: slope = 0.98; intercept = 0.02 mM, r = 0.97, n = 120) over the range 0.0–6.0 mM in 30 s using a small volume of blood (5  $\mu$ l). The electrode had a low

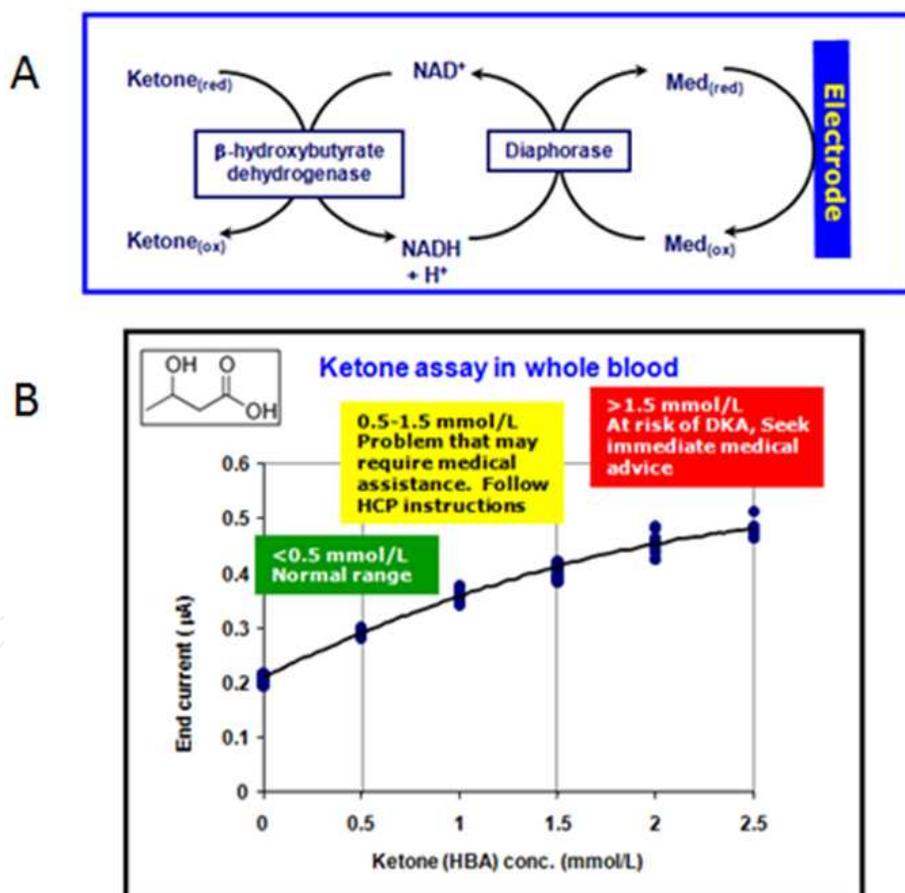
operating potential (+200 mV versus Ag/AgCl) such that the effect of electroactive agents in blood was minimised. [15-18]

## 7. Alternative approaches to ketone sensing using Diaphorase

The diaphorases (EC1.6.99.3) are a ubiquitous class of flavin-bound enzymes that catalyze the reduction of various dyes which act as hydrogen acceptors from the reduced form of di- and tri- phosphopyridine nucleotides, i.e. NADH, NADPH. They catalyse the following reaction:



Either NADH or NADPH may be used as reductants. However, no exchange of hydrogen between the coenzymes is catalysed. Typical acceptor molecules include dyes such as 2, 6- dichlorophenolindophenol and tetrazolium dyes and redox couples such as ferricyanide anions and ferricinium cations. The Expsy entry for diaphorase is <http://enzyme.expasy.org/EC/1.6.99.3>



**Figure 6.** Calibration curve (B) for the diaphorase based ketone sensor developed and manufactured in house (unpublished results). The calibration characteristics are shown over the physiologically relevant range 0 – 2.5 mmol/L  $\beta$ -hydroxybutyrate spiked into a whole blood sample. Each concentration determination is 16 repetitions. The reaction scheme for the sensor is shown in (A). Here, the mediator is the ferricyanide anion. HBA in the blood was measured using the Randox RX Monza Chemistry Analyser, <http://www.randox.com/rx%20monza.php>. The test time was 7 seconds.

In this configuration,  $\text{NAD}^+$ , Diaphorase, acceptor molecule and  $\beta$ -hydroxybutyrate dehydrogenase are all formulated together in the enzyme ink and laid down on the test strip using an appropriate manufacturing method. Data generated in-house (unpublished results) using this kind of prototype test strip (with ferricyanide as the acceptor) is shown below for the clinically relevant concentration range of  $\beta$ -hydroxybutyrate. The strip was manufactured by screen printing (*cf* above). Ferricyanide, buffer salts,  $\text{NAD}^+$ , binders, diaphorase and  $\beta$ -hydroxybutyrate dehydrogenase were mixed into a suitable enzyme ink and printed onto carbon electrodes. The operating voltage was 0.4 Volts.

## 8. Glucose test strips using PQQ linked and FAD-linked glucose dehydrogenase

PQQ-GDH (pyrrolo quinoline quinone glucose dehydrogenase) belongs to a class of enzymes called quinoproteins which require ortho-quinone cofactors to oxidize a wide variety of alcohols and amines to their corresponding aldehydes and ketones. The soluble quinoprotein Glucose Dehydrogenase, EC 1.1.99.17, (sGDH), systematic name D-glucose: (pyrrolo-quinoline-quinone)1-oxidoreductase, uses pyrrolo-quinoline quinone (PQQ) as a cofactor. sGDH is a strongly basic ( $\text{pI} = 9.5$ ) dimeric enzyme of identical subunits. One monomer (50 kDa, 454 residues) has been reported to bind one PQQ molecule and two  $\text{Ca(II)}$  ions. One of the  $\text{Ca(II)}$  ions is required for activation of the cofactor; the other is needed for functional dimerisation of the protein. sGDH oxidizes a wide range of mono- and disaccharides and is able to donate electrons to several neutral or cationic artificial electron acceptors, including short artificial ubiquinone homologues. The natural electron acceptor of PQQ-GDH is ubiquinone although the enzyme will react with a variety of artificial acceptors such as the ferricyanide anion ion and the ferricinium cation. The oxidised form of PQQ can be converted into the reduced form  $\text{PQQH}_2$  by the transfer of 2 electrons and two protons from the substrate molecule. [19-21]

There are two types of PQQ-GDH enzymes that can be considered for biosensor design. One is intracellular and soluble (sPQQ-GDH) whereas the other molecule is insoluble and firmly bound to the outer surface of the cytoplasmic membrane (mPQQ-GDH). mPQQ-GDH is very selective for glucose but has the disadvantage that it requires extensive solubilisation and stabilisation with detergent molecules<sup>[22]</sup>. For these reasons, mPQQ-GDH has not been successfully commercialised for biosensor application. It can oxidise a number of monosaccharides, in addition to glucose, such as maltose, mannose and lactose. Consequently, patients that have high levels of, for example maltose in the blood (which could result as a side effect of peritoneal dialysis) or have an inbred genetic disorder resulting in impaired carbohydrate metabolism, would obtain an inaccurate high reading when testing with glucose electrodes incorporating this enzyme. The increasing demand for dialysis and slower growth in capacity for haemodialysis has reinforced the need for an integrated approach to providing dialysis. Peritoneal dialysis is the preferred option for a proportion of patients with end stage renal failure. Peritoneal dialysis fluid usually contains glucose as an osmotic agent to enable water to pass across the peritoneum. Some patients

lose the osmotic effect of glucose quickly, but large icodextrin molecules, which are not easily transported across the peritoneal membrane, maintain an osmotic gradient. Icodextrin is not metabolised in the peritoneal cavity, but the polymer can move into the blood stream via the lymphatic system. During systemic circulation, icodextrin is mainly metabolised into maltose which accumulates due to a lack of circulating maltase. It is the accumulation in the systemic circulation of these metabolites of icodextrin that may lead to the disparity between finger stick and formal blood glucose measurement. Maltose interferes with glucose assays that use glucose dehydrogenase with cofactor pyrroloquinolinequinone (PQQ-GDH) leading to falsely increased readings.

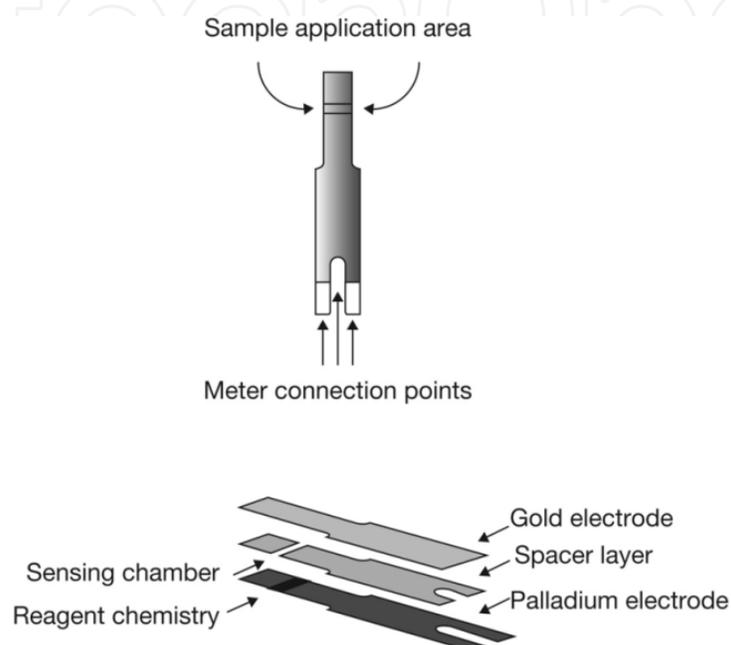
Because of the oxygen insensitivity of PQQ-GDH however, there is much commercial interest in producing a mutant form of the enzyme that retains its non-reactivity to oxygen but improving its specificity with respect to D-glucose. Recently, Roche have reported the successful production of a mutant strain of PQQ-GDH which shows no cross-reactivity with maltose. [23]

Finally, it is worth mentioning that PQQ containing proteins lend themselves to an interesting electrode configuration. Whilst flavoproteins such as glucose oxidase exchange electrons with an electrode surface via small molecular weight mediators such as ferrocene, ferricyanide etc, PQQ containing enzymes can exchange electrons via cytochrome b562. This cytochrome will exchange electrons directly with the electrode surface without the need for any mediator molecule. [24] Information on PQQ-GDH can be found on the EXPASY Proteomics server at: <http://expasy.org/enzyme/1.1.5.2>.

A new enzyme on the market which has just recently become commercially available to the biosensor industry is the flavo-protein FAD-GDH [25] (Toyobo Develops FAD-GDH Enzyme, produced by *Aspergillus* for SMBG <http://www.toyobo.co.jp/e/press/press31072009.htm>). As its name suggests, the enzyme catalyses the oxidation of glucose but does not utilise dioxygen as a co-reactant. It can react with a number of artificial electron acceptors such as the ferricyanide anion and the ferricinium cation. The enzyme, which is isolated from *Aspergillus terreus* shows good thermal stability and high selectivity for glucose. Its absorption spectrum is typical of flavoproteins showing two distinctive peaks corresponding to the oxidised flavin cofactor at 465nm and 385 nm. In the presence of glucose the enzyme is bleached and these characteristic absorption bands disappear. [26] According to the data sheet from the manufacturer (Toyobo, Japan) the enzyme has a pH optimum of 7.0 and a KM for glucose of 67.6mM. The information on the EXPASY proteomics server relating to this enzyme is at: <http://expasy.org/enzyme/1.1.99.10>.

This enzyme is currently used in a number of glucose test strips including the OneTouch Verio family of test strips produced and supplied by LifeScan. The design of the OneTouch Verio BG test strip is shown in Figure 7. The test strip incorporates gold and palladium electrodes which are orientated in a co-facial manner. The dimensions of the two electrodes are defined and controlled during the manufacturing process by a die-punch process. The electrodes are separated from each other by a thin plastic spacer that has a nominal thickness of 95  $\mu\text{m}$ . The glucose-sensitive reagents, citraconate buffer salts, potassium

ferricyanide mediator and flavo-protein glucose dehydrogenase (FAD-GDH), are laid down on the 'bottom' palladium electrode. FAD-GDH enzyme was selected for its high substrate-specificity and non-reactivity towards oxygen. The strip may be defined as a 'side-fill strip' because blood may be applied to the 400 nL sample chamber from either the left or the right side of the test strip (Figure 7). The levels of glucose in the sample are determined within 5 seconds, the BG value being shown on the meter display in geographically appropriate units (mg/dl or mM).



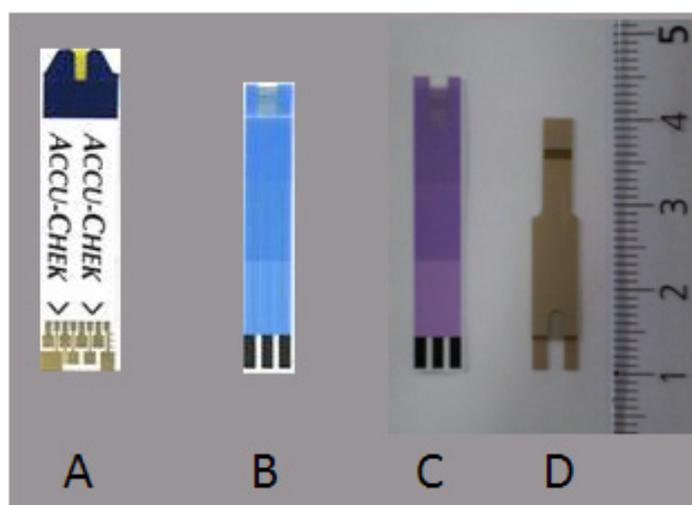
**Figure 7.** Architecture of the OneTouch Verio glucose test strip. The test strip incorporates gold and palladium electrodes that are orientated in a cofacial manner. The dimensions of the two electrodes are defined and controlled during the manufacturing process by a die-punch process. The electrodes are separated from each other by a thin plastic spacer that has a nominal thickness of 95  $\mu\text{m}$ . The glucose-sensitive reagents, citraconate buffer salts, potassium ferricyanide mediator, and flavoprotein glucose dehydrogenase, are laid down on the "bottom" palladium electrode. The user can apply blood to the test strip either from the right hand side or the left hand side. The glucose level is reported within 5 seconds of the start of the test procedure.

Manufacturing process controls and built-in signal processing compensation mechanisms eliminate the need for user calibration coding, thus reducing the potential for user error. The meter uses a novel multi-pulse signal and has an improved glucose-Hct-temperature-antioxidant compensation algorithm for higher accuracy and precision over a wide range of blood samples. The OneTouch Verio BG test strip is designed to work with meters that are technically equivalent but have different user interfaces. Currently available meters include OneTouch Verio, OneTouch VerioPro, and OneTouch VerioIQ. The performance characteristics of the OneTouch VerioPro BGMS are summarized as follows: plasma equivalent calibration; 0.4  $\mu\text{l}$  sample size; FAD-GDH enzyme; 20–600 mg/dl glucose range; 20–60% Hct range; 10–90% humidity range (non-condensing); 6–44°C temperature range; and up to 3048 m altitude use. A summary of the dehydrogenase enzymes currently used in commercial enzyme electrodes is shown in Table 1.

Enzyme	Reacts with O <sub>2</sub> ?	Reacts with maltose?
PQQ-Glucose Dehydrogenase	No	Yes (Not Genetically Engineered variant <sup>[23]</sup> )
NAD-Glucose Dehydrogenase	No	No
NAD-β-hydroxybutyrate dehydrogenase	No	No
FAD-Glucose Dehydrogenase	No	No

**Table 1.** Commonly used dehydrogenase enzymes for Commercial Self testing Biosensors

Shown below are examples of commercially available test strips for home use which incorporate dehydrogenase based enzymes.



- The Roche Accu-Check Aviva test strip. This test strip uses PQQ-GDH as the enzyme of choice. <http://www.roche.com/products/product-details.htm?type=product&id=2>
  - The Abbott Optium Xceed glucose test strip. This strip uses NAD-Glucose dehydrogenase in the sensing chemistry. <http://www.abbott-diabetescare.com/AU/ProductDetail.aspx?product=57>
  - The Abbott Optium Xceed ketone test strip. This strip uses NAD<sup>+</sup> linked β-hydroxybutyrate dehydrogenase. Because it uses the same mediator chemistry as B, and hence operates at the same potential, the two strips can be used in the same meter. <http://www.abbott-diabetescare.com/AU/ProductDetail.aspx?product=57>
  - The LifeScan OneTouch Verio test strip. This strip incorporates FAD-GDH in the strip chemistry to measure glucose in whole blood. <http://www.lifescan.co.uk/ourproducts/teststrips/onetouchverio>
- (Photograph courtesy of Mr Christopher Leach, LifeScan Scotland Ltd., Inverness, UK).

**Figure 8.** Examples of commercially available test strips for self-testing utilising dehydrogenases.

## 9. Biosensors using dehydrogenase enzymes for continuous monitoring

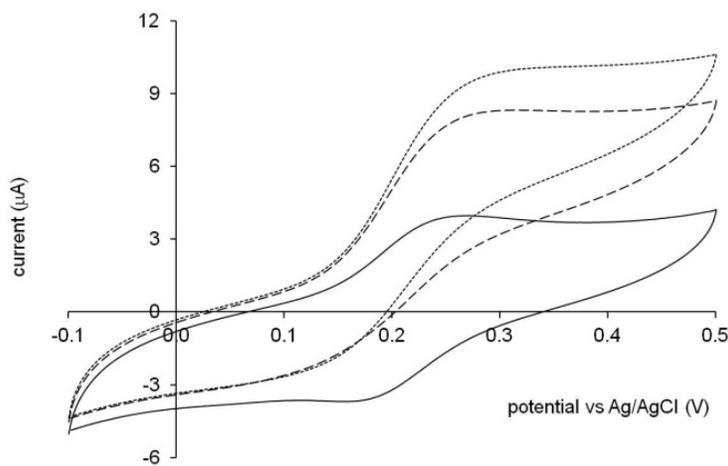
### 9.1. FAD-GDH biosensor for continuous glucose monitoring

It has been well established that many of the diabetic complications leading to both chronic and acute health problems, such as adult blindness, end-stage renal disease, lower-limb amputations, and heart disease or stroke, can be reduced or even prevented through intensive

blood glucose control. [27-29] Effective glycaemic control requires frequent measurements of blood glucose in order to take necessary therapeutic interventions. Such an approach is exemplified by the use of so-called 'continuous glucose monitoring' (CGM) apparatus and methodologies that are used by a growing number of patients to monitor their diabetes condition. Such systems are composed of a probe that is inserted into the body such that it contacts glucose containing liquids in the body, such as interstitial fluid. These CGM systems are designed to operate over extended periods of time, typically over a number of days or longer. In reality CGM is a misnomer, inasmuch as the device samples in an episodic manner, but on a sufficiently high frequency to distinguish such devices from single measurement episodic systems. Nevertheless, compared with episodic self-monitoring blood glucose (SMBG), CGM follows blood glucose dynamics and hence, provides patients and healthcare professionals with not only current blood glucose levels, but also real-time rate and direction of changes. Blood glucose thresholds can be set to alert for possible dangerous trends, for instance rapid blood glucose descents that may lead to hypoglycemia. Advances in CGM research and development are also critical to realize "artificial pancreas" of a closed-loop system in conjunction with an insulin pump.

Enzyme catalysed electrochemical biosensors have been the most successful technology for the commercialized SMBG products (as discussed in the preceding part of this chapter). For CGM, enzymes are also employed as the means of target analyte recognition, coupled to electrochemical transduction methods for determination of the analyte of interest. Correspondingly, such systems have been so far limited to the use of a redox enzyme, such as glucose oxidase (GOx) in which the prosthetic group is intimately associated with the enzyme, such that it cannot diffuse or leach away over the duration of sensor operation.<sup>[30]</sup> Recently commercialized flavin adenine dinucleotide-dependent glucose dehydrogenase (FAD-GDH, EC 1.1.99.10), where the FAD moiety forms an integral part of the enzyme, has attracted great attention for blood glucose monitoring because of its advantages over GOx in terms of insensitivity to molecular oxygen. However, like many redox enzymes, direct electron transfer between FAD-GDH and electrode surface cannot occur because the active centre FAD is insulated by the large proteins. Therefore, mediators are usually employed to shuttle electron between the FAD moiety and electrode surface. An example of such mediators is potassium ferricyanide used for LifeScan OneTouch Verio family of SMBG products. However, use of small molecular mediators in biosensors faces tremendous technical challenges for CGM which requires essentially all the reactive reagents immobilized without leaching out from the electrodes to achieve long-term measurement stability and to meet biocompatibility requirements, in particular for in-vivo applications. An attractive approach to tackle this challenge is to use a polymeric mediator which has mediator moieties chemically attached to polymer chains. Because of its large molecular size, the polymeric mediator can be co-immobilized with enzyme at electrode by various means, including surface grafting, [31-34] layer-by-layer surface adsorption, [35-37] retention behind semi-permeable dialysis membranes, [38-40] physical entrapment [41-43] or cross-link in hydrogels, [44-47] entrapment in electropolymerized [48,49] or chemically formed layers [50] or in inorganic layers, [51,52] and blend in carbon pastes. [53]

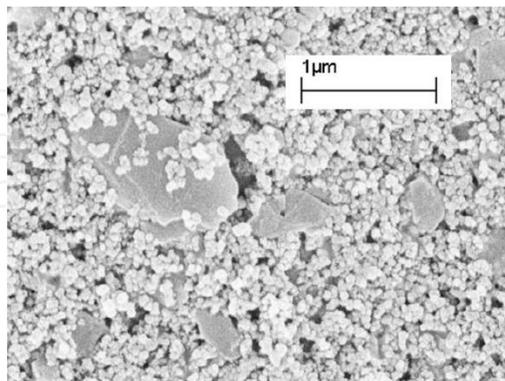
At LifeScan Scotland Limited, we have synthesized a ferrocene polymeric mediator which is a copolymer of vinylferrocene, acrylamide and 2-(diethylamino)ethyl methacrylate.<sup>[54]</sup> Like small molecular mediators, an effective polymeric mediator for biosensors should be able to exchange electrons with enzyme prosthetic group and then be re-oxidized/re-reduced at electrode in a reversible manner at a sufficient low potential to avoid or minimize interferences resulting from oxidation/reduction of other components in the sample fluid, such as uric acid, ascorbic acid etc. in a bodily fluid. Figure 9 shows cyclic voltammograms of the ferrocene polymeric mediator and FAD-GDH (from Toyobo Co. Ltd., Japan) in phosphate buffered saline (PBS). In the absence of glucose, cyclic voltammetry shows almost symmetric anodic and cathodic peaks which are attributed to oxidation and reduction of the ferrocene moieties of the polymeric mediator. After adding 2.5 mM glucose, the cyclic voltammogram changed dramatically, with a large increase in the anodic peak and a significant decrease in the cathodic peak. This was a typical phenomenon of enzyme-dependent catalytic reduction of ferrocenium generated during the oxidation half-cycle in the presence of glucose. The cathodic peak increased further as glucose concentration increased to 5.0 mM. These results clearly indicated that the ferrocene polymeric mediator exhibited preferential redox properties with respect to transfer of electrons from the reduced enzyme prosthetic group FADH<sub>2</sub> to the glassy carbon electrode surface. The ferrocene moieties are reduced to ferrocenium moieties upon accepting the electrons from FADH<sub>2</sub> and then are re-oxidized on transfer of electrons to the electrode.



**Figure 9.** Cyclic voltammograms: 1.5mg/mL FAD-GDH, 9 mg/mL ferrocene polymeric mediator in 0.01M pH7.4 PBS, scan rate 5 mV/s, without (solid line), with 2.5 mM glucose (broken line) & 5.0 mM glucose (dotted line)

We have also developed a technique for co-immobilization of FAD-GDH and the ferrocene polymeric mediator in modified electrodes which were fabricated by screen-printing a water-based carbon ink containing both the enzyme and the ferrocene polymeric mediator. The ink contained graphite particles as conductive pigments and a pH sensitive copolymer as a binder.<sup>[55]</sup> The copolymer binder was water-soluble in the presence of ammonium hydroxide and hence, the ink was miscible with dissolved FAD-GDH and the ferrocene polymeric mediator during the ink formulation. As ammonia evaporated upon drying a screen-printed ink pad, the

binder copolymer became water-insoluble and the ink layer evolved to form a 3-dimensional nano-porous structure which effectively entraps the large molecules of the enzyme and the ferrocene polymeric mediator whilst allows free diffusion of glucose and water molecules. [54] Figure 10 shows scanning electron microscopy image of the electrode surface.



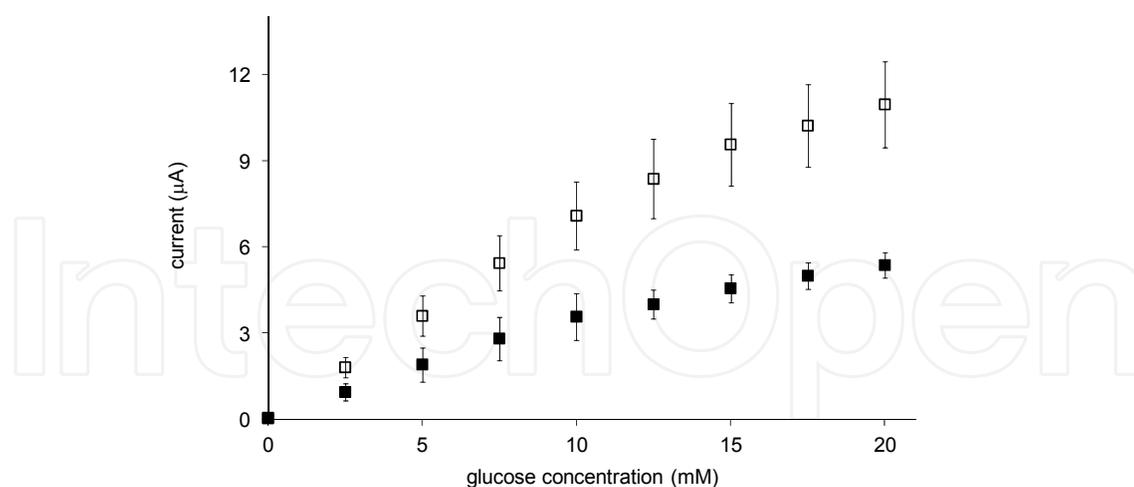
**Figure 10.** Scanning electron microscopy image of an electrode with immobilized FAD-GDH and the ferrocene polymeric mediator

The main advantages of these electrodes are

- The enzyme and ferrocene polymeric mediator are co-immobilized in vicinity of the electrode graphite particles, which is beneficial for fast electron transfer.
- The 3-dimensional nano-porous structure enables higher loading of the redox species and provides larger electrode surface area than a planar electrode with an enzyme layer.
- No chemical reactions are involved throughout the immobilization and hence, potential enzyme denaturing is avoided.
- The screen-printing technique suits mass production and has low manufacturing cost.

Because of the presence of ammonium hydroxide, the water-based carbon ink was basic in nature. FAD-GDH stability in the wet ink was studied by comparing glucose sensitivity of the modified electrodes fabricated from the same batch of formulated ink with varied stand-out time. The glucose sensitivity of the electrodes was assessed by testing current responses of a 3-electrode setup to glucose in a concentration range of 0 to 20 mM. The test conditions and results are presented in Figure 11. It is clearly seen that 2-day stand-out of the wet ink significantly reduced glucose calibration slope of the test. During the stand-out period, the wet ink containing FAD-GDH and the polymeric mediator was kept in a fridge at 4 °C and negligible changes in ink rheology and solid content were detected. For comparison, the stand-out was also investigated for an ink using GOx as an enzyme and insignificant effect on glucose calibration slope was found. Therefore, the reduction in glucose sensitivity of the FAD-GDH working electrodes is attributed to decrease in the enzyme activity probably resulting from the enzyme denaturing in the basic wet ink.

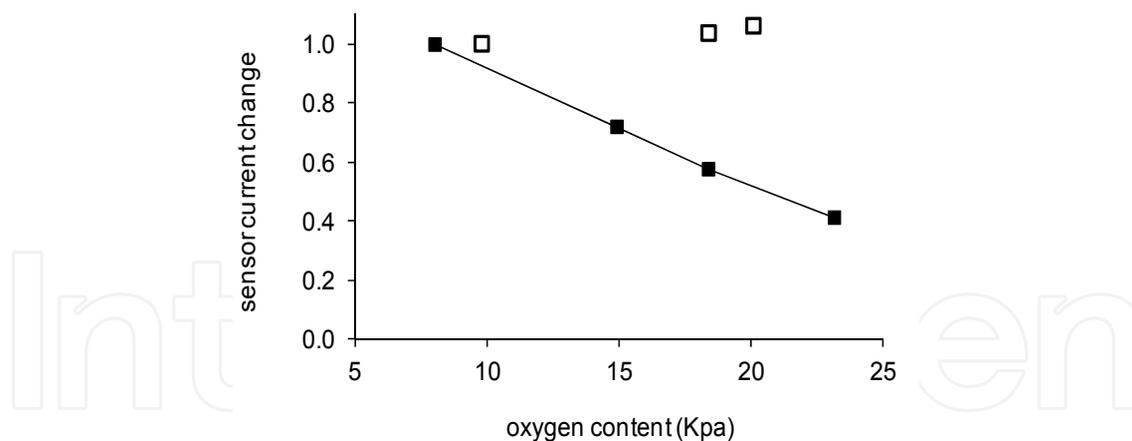
Since Clark and Lyons reported the first enzymatic electrode for glucose measurement in 1962, [56] molecular oxygen has been involved in the enzymatic redox reactions as an electron acceptor for the first generation of biosensors. However, this type of biosensors is based on measuring generation of hydrogen peroxide or depletion of oxygen and hence, exhibits



**Figure 11.** Calibration plots for screen-printed electrodes prepared by using the ink printed after ink formulation without delay (□) and the ink printed 2 days (■) after ink formulation, tested in 0.01M pH7.4 PBS by using an Ag/AgCl reference electrode and a platinum counter electrode, 0.3 V potential, room temperature, the error bars are first standard deviation of 7 electrodes

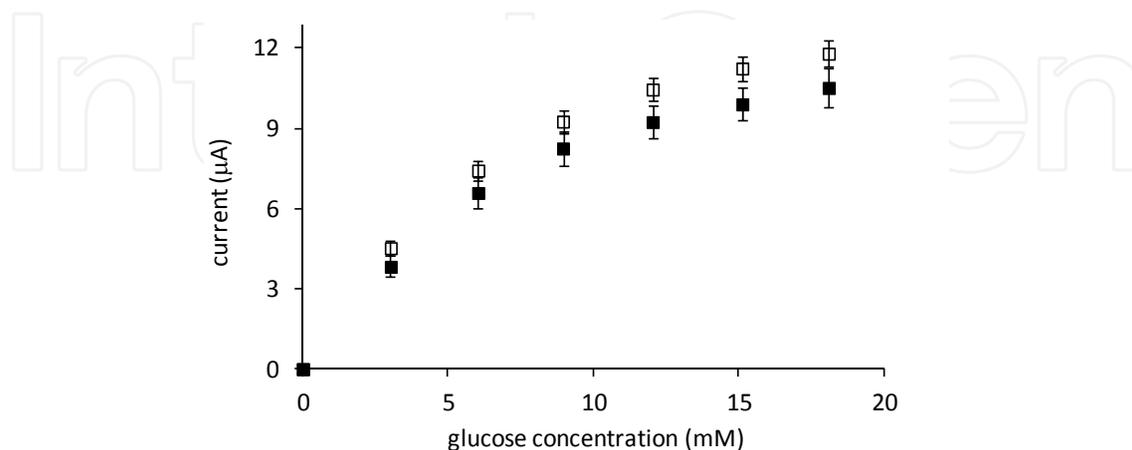
fundamental limitations. Measurement of hydrogen peroxide requires relatively high operational potential (normally  $>0.45V$ ) and suffers from significant interference resulting from oxidation of other substances in the bodily fluid. [57, 58] For oxygen measurement, the test result is sensitive to the variations in oxygen supply and test conditions. [59] The second generation biosensors use mediators to shuttle electrons between the enzyme prosthetic groups and electrodes. Typically, mediators have the attractive property of being selected for a particular desirable redox potential at which the mediators readily undergo redox reactions at the electrode whilst the redox reactions of the interferents are insignificant. However, for some enzymes, such as GOx, oxygen can compete with the mediators to accept electrons from the reduced prosthetic group  $FADH_2$  to form hydrogen peroxide which cannot be oxidized at the electrode under the applied potential for the mediator re-oxidation. As a result, the biosensor response to the analyte is dampened in the presence of oxygen. As shown in Figure 12, tested in PBS with a 3-electrode setup, the current response to 5 mM glucose for the screen-printed electrode modified with GOx and the ferrocene polymeric mediator decreased almost 60% as blood oxygen content increased from 8 Kpa to 23.14 Kpa. In contrast, the screen-printed electrode modified with FAD-GDH and the ferrocene polymeric mediator had little change in the current response in a range of the blood oxygen content from 9.8 Kpa to 20.1 Kpa.

The oxygen insensitivity of FAD-GDH makes the enzyme very attractive for the development of CGM biosensors. This is because the biosensors using an oxygen-sensitive enzyme in general has more profound response dampening effect at low glucose concentrations due to the generation of hydrogen peroxide, which can contribute to significant accuracy error for the CGM and impose critical challenges for CGM to provide patients and healthcare professionals with a true picture of ambient glycemia profile which is critical for reliable detection and/or prediction of hypoglycemia, an important step to good diabetes management.



**Figure 12.** Effect of oxygen content on current response to 5 mM glucose in 0.01M pH7.4 PBS for screen-printed electrodes containing FAD-GDH (□) and GOx (■), tested by using an Ag/AgCl reference electrode and a platinum counter electrode

Like many medical devices, CGM biosensors normally need sterilization, in particular for *in vivo* or *ex-vivo* applications to eliminate any harmful contaminants such as fungi, bacteria, viruses, and spore forms etc. There are different established methods for sterilizing medical devices. Some of them involve the use of high energy means which can cause damages to materials in certain circumstances. It is essential that the key redox species, including FAD-GDH and the ferrocene polymeric mediator in the biosensor are not subject to any significant damages during a chosen sterilization process. Figure 13 shows comparison of glucose calibration plots for the same batch electrodes fabricated by screen-printing the water-based carbon ink containing FAD-GDH and the ferrocene polymeric mediator before and after 25 KGy e-beam sterilization. The results indicate 10-16% reduction in steady state current in the tested range of glucose concentrations. A sample of the ferrocene polymeric mediator also went through the sterilization process alongside the electrodes.  $^1\text{H}$  nuclear magnetic resonance and size exclusion chromatography indicated no change to the composition and molecular weight of the ferrocene polymeric mediator after the e-beam sterilization. The reduction in the current responses to glucose is probably attributed to e-beam induced decrease in the enzyme activity.



**Figure 13.** Calibration plots for screen-printed electrodes before (□) and after (■) 25 KGy e-beam sterilization, tested in 0.01M pH7.4 PBS by using an Ag/AgCl reference electrode and a platinum counter electrode, the error bars are first standard deviation of 7 electrodes

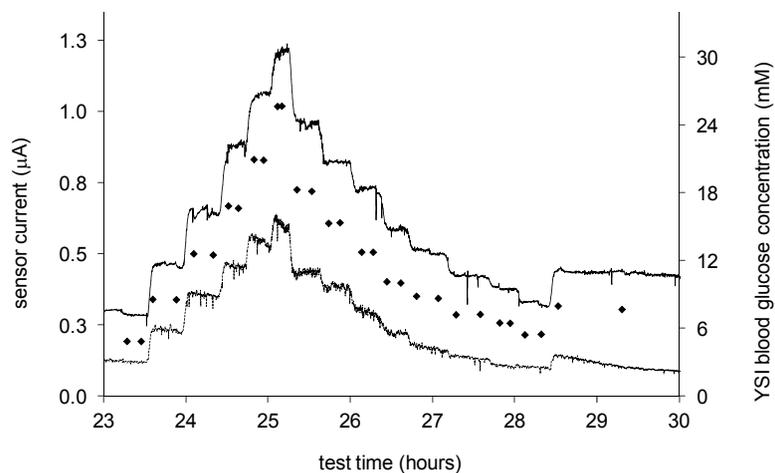
CGM biosensors were fabricated by sequentially screen-printing and drying a carbon ink, an Ag/AgCl ink and the water-based carbon ink containing FAD-GDH and the ferrocene polymeric mediator on the same plane of a plastic foil to form two carbon tracks, a pseudo-reference electrode and a working electrode, respectively. Then the plastic foil was heat-laminated onto a plastic disc which had pre-formed micro-channels (typical channel dimension: width  $\times$  depth = 0.3  $\times$  0.3 mm) in a way that the micro-channel runs over the two electrodes with the working electrode upstream of the pseudo-reference electrode. Surface areas exposed to the micro-channel are 0.3  $\times$  2.0 mm and 0.3  $\times$  5.5 mm for the working electrode and the pseudo-reference electrode, respectively. <sup>[54]</sup> For comparison, biosensors with GOx were also fabricated in the same way except for replacing FAD-GDH with GOx in the water-based carbon ink. Loadings of the two types of enzymes in the water-based carbon inks were identical in weight.

The two types of biosensor discs were tested side-by-side at room temperature. Human blood samples were collected on the same day for the test. Upon testing, a continuous flow of the human blood samples were pumped from blood sample reservoirs through the channels of each biosensor at 15  $\mu$ L per minute by using a peristaltic pump (Ismatec). Sensor current responses to blood glucose were recorded by using a potentiostat (Uniscan Instruments) operated at 0.3 V potential. Step changes of blood glucose concentration were realized by switching the blood sample reservoirs which were open to the atmosphere and under gentle magnetic agitation. Low glucose blood samples were prepared by standing the blood sample reservoirs in a 37 °C water-bath to accelerate glucose consumption by blood cells, whilst high glucose blood samples were prepared by spiking with a 20% wt/wt glucose stock (from Sigma-Aldrich). YSI measurements (YSI 2300 STAT Glucose Analyzer) of glucose concentrations were performed for the blood samples as references. In order to minimize error caused by blood mixing in the disc channel upon changing glucose concentration, the YSI measurements were performed by taking a sample from the reservoir when the previous blood sample in the biosensor micro-channel was completely depleted indicated by steady sensor current. Given the slow decrease in blood glucose concentration with time at room temperature, two YSI measurements were normally performed for each blood sample during the period it flowed through the biosensor disc channel.

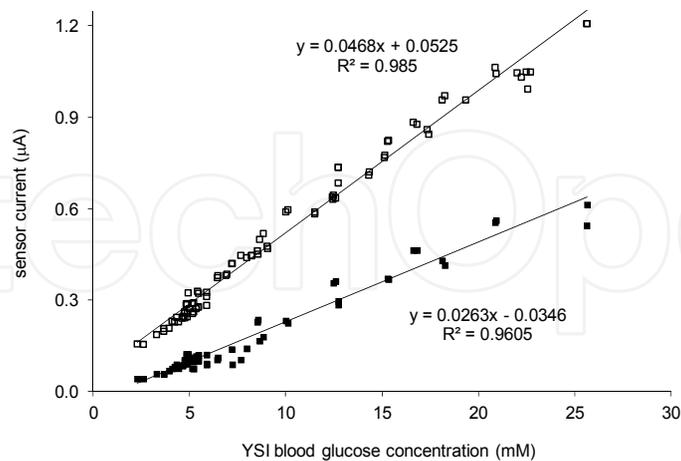
In a typical test, the two types of biosensors were tested continuously over 54 hours with daily changes of blood samples from different donors. The disc channels were flushed with PBS (0.01 M, pH7.4) prior to the blood changes. Figure 14 shows typical recorded current responses of one FAD-GDH and one GOx biosensors to the same blood samples with varied glucose concentrations in a range of 2.29 to 25.64 mM between 23 and 30 hour test time. YSI measured blood glucose concentrations are also shown. For both biosensors, the currents clearly followed the step-changes in blood glucose concentration and reached “steady-state” in 3 to 5 minutes. It is expected that the real response time of the biosensors to the blood glucose concentration changes is shorter than 3 to 5 minutes because the recorded steady-state currents can only be obtained after complete depletion of the “old” blood samples from the disc channels upon the reservoir switching. By a close look, the current response of the GOx biosensor clearly drifted whilst the current response of FAD-GDH sensor did not between 28.5

and 30 hour test time. This is probably due to the oxygen effect on the GOx biosensor as oxygen content of the blood sample gradually increased under continuous agitation.

The steady-state current responses of the two biosensors over the 54-hour test are plotted against the YSI measured blood glucose concentration in Figure 15. For both of the biosensors, the test results demonstrated good linear correlations between the current responses and blood glucose concentration over the tested glucose concentration range. However, the FAD-GDH biosensor had significantly higher current responses than the GOx biosensor, leading to higher sensitivity to glucose concentration variations and hence, potentially better measurement accuracy. This suggests that FAD-GDH has higher activity than GOx given that the enzyme loadings were the same for the two types of biosensors.



**Figure 14.** Current response variations of sensors with screen-printed working electrodes containing FAD-GDH (solid line) and GOx (dotted line) and Ag/AgCl reference electrode, with varied blood glucose concentrations ( $\blacklozenge$ ), tested with continuous flow of human blood at a flow rate of 15  $\mu\text{L}/\text{min}$



**Figure 15.** Calibration plots of sensors with screen-printed working electrodes containing FAD-GDH ( $\square$ ) and GOx ( $\blacksquare$ ) respectively and Ag/AgCl reference electrode, tested with continuous flow of human blood at a flow rate of 15  $\mu\text{L}/\text{min}$

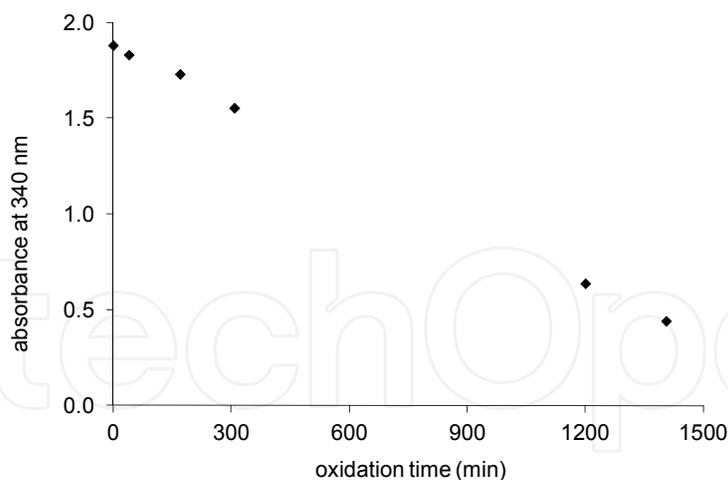
Because the blood cells continuously consumed glucose and result in gradual decrease in blood glucose concentration at room temperature, direct assessment on measurement

stability of the biosensors by recording sensor response change with time at a fixed glucose concentration proved to be unreliable. Nevertheless, closely aligned data points to the linear regression line in Figure 15 illustrate stable biosensor response during the 54-hour test, suggesting good FAD-GDH stability during the continuous measurement.

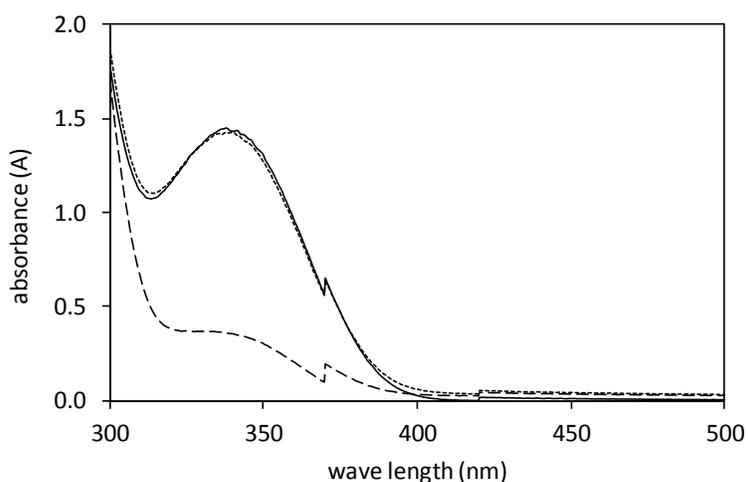
## 9.2. NAD and ferrocene polymeric mediator redox couple

As a coenzyme for hundreds of oxidoreductases, NAD has attracted great attention for biosensor development. Because its direct oxidation at electrode surface requires high applied potential and involves side reactions leading to the formation of electrochemically inactive by-products (see the preceding section), NAD is normally coupled with mediators for its application in biosensors. In the literature, ferrocenium and its derivatives have been reported as mediator for oxidation of NADH to NAD<sup>+</sup>.<sup>[60-63]</sup> As mentioned in the preceding section, small molecular mediators are not normally suitable for continuous monitoring biosensors. The effective immobilization of the ferrocene polymeric mediator in the screen-printed carbon electrode developed at LifeScan Scotland Limited holds potentials for development of continuous monitoring biosensors using NAD-dependant oxidoreductases. To investigate electrochemical communication between NAD and the immobilized ferrocene polymeric mediator, 0.3 V potential (ECOChemie PGStat Autolab, Type III) was applied to an electrochemical cell containing 3.0 mL 0.01M pH7.4 PBS and equipped with a screen-printed carbon electrode (approximately dimension length x width = 10 x 5 mm) with immobilized ferrocene polymeric mediator (about 12.5% wt/wt), an Ag/AgCl reference electrode and a platinum counter electrode to oxidize the ferrocene moieties of the polymeric mediator to ferrocenium moieties. After the current of ferrocene oxidation reached a steady level, PBS in the cell was replaced with 0.3 mM NADH (from Sigma-Aldrich) in 3.0 mL 0.01M pH7.4 and a step increase in current was detected, which indicated oxidation of NADH by the ferrocenium moieties of the polymeric mediator. The NADH oxidation was followed by measuring absorbance of the solution at 340 nm wavelength by UV/vis spectroscopy (Cecil Instruments, CE9500) at different time intervals. The result shown in Figure 16 illustrates gradual decrease in NADH concentration ( $E_{340} = 6,330$ ) with the oxidation time, which can be attributed to continuous oxidation of NADH by the ferrocene polymeric mediator immobilized in the carbon electrode.

0.65 mL above oxidized NADH solution was collected and mixed with 0.25 mL 99.5% ethanol (EtOH, Sigma-Aldrich) in a UV cuvette ( $l = 1\text{cm}$ ). UV spectra of the solution in the cuvette were recorded before and after addition of 0.4 mg alcohol dehydrogenase (ADH, Sigma-Aldrich). As shown in Figure 17, the addition of EtOH and ADH increased the absorbance peak at around 340 nm to almost the same level as the control solution which contained 0.3 mM NADH in 0.01M pH7.4 PBS and did not subject to the oxidation process. This suggests that NADH oxidation by the immobilized ferrocene polymeric mediator predominantly, if not completely produced electrochemically active NAD<sup>+</sup> that was reduced to NADH by EtOH in the presence of ADH.



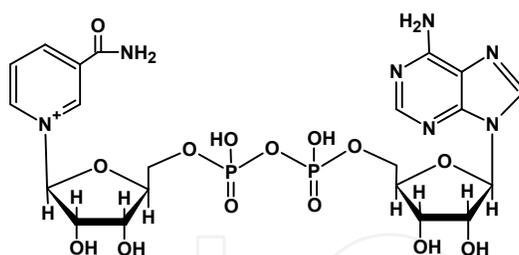
**Figure 16.** Variation of UV absorbance at 340 nm of 0.3 mM NADH in 0.01 M pH7.4 PBS by carbon electrode with immobilized ferrocene polymeric mediator at 0.3 V potential, by using an Ag/AgCl reference electrode and a platinum counter electrode, at room temperature



**Figure 17.** UV spectroscopy monitoring reduction of oxidized NADH in the presence of ADH and EtOH, 0.65ml NADH (0.2 mM) + 0.25ml EtOH (99.5%) + 20ul ADH (control) (solid line), 0.65ml oxidized NADH + 0.15mL EtOH (before reduction) (broken line), 0.65ml oxidized NADH + 0.25ml EtOH + 20 ul ADH (4mg/ml) (dotted line)

For continuous monitoring, use of native NAD as a coenzyme apparently is not ideal due to its low molecular weight and high water-solubility. Direct immobilization of free NAD at electrode has been a long-standing challenge. <sup>[64, 65]</sup> There are a large number of publications in the literature concerned with NAD retention for various purposes. In the field of biosensors, one promising approach is covalently attaching NAD moieties to polymer chains which can be immobilized at electrode by different means. This can be achieved by directly coupling NAD to an electrode modified with a polymer bearing functional groups,<sup>[66]</sup> entrapping polymeric NAD in semi-permeable membranes <sup>[67, 68]</sup> or synthesizing a charged polymeric NAD which was then physically adsorbed at electrode surface in conjunction with counter-charged polymer(s) in a manner of layer-by-layer. <sup>[69]</sup> At LifeScan Scotland Limited, we intend to develop continuous monitoring biosensors using NAD-dependent enzymes by immobilizing all the redox species, i.e.

an enzyme(s), the ferrocene polymeric mediator and a polymeric NAD in an electrode with the 3-dimensional nano-porous structure (see Figure 10). A polymeric NAD is normally synthesized by two routes. One is coupling NAD or NAD analogue with a polymer bearing functional groups. [66, 70-73] The other route involves synthesis of a NAD monomer and then its copolymerisation with another co-monomer(s). One of the challenges for NAD immobilization is to keep NAD coenzymic activity while achieving effective retention at electrode. Yamazaki *et al* synthesized three NAD monomers (N<sup>6</sup>-[N-(6-methacrylamidohexyl)carbamoylmethyl]-, N<sup>6</sup>[N-[2-[N-(2-methacrylamidoethyl)carbamoyl]ethyl] carbamoylmethyl]-, and N<sup>6</sup>-[N-[N-(2-hydroxy-3-methacrylamidopropyl) carbamoylmethyl] carbamoylmethyl]-NAD) and then copolymerized them with various co-monomers (acrylamide, N-(2-hydroxyethyl)-, N-ethyl-, N,N-diethyl-, and N,N-dimethylacrylamide, acrylic acid, and 6-methacrylamidohexylammonium) by free radical polymerisation to form a series of polymeric NADs. [74] Their studies revealed that hydrophilicity and length of the spacers linking NAD moieties and the polymer backbone had the most important effects on coenzymic activity of the polymeric NADs. This suggests that keeping mobility of the NAD moieties covalently attached to a polymer chain is critical to NAD coenzymic activity. Chemically modification to native NAD is required to tailor chemical properties of the spacers. N<sup>6</sup>-amino group on the adenine ring of NAD (see Figure 18) is normally selected as the site for this purpose. [75, 76] Lindberg *et al* reported alkylation of NAD<sup>+</sup> with iodoacetic acid followed by alkaline rearrangement to give N<sup>6</sup>-carboxymethyl-NAD<sup>+</sup>. [75] However, the reaction between NAD<sup>+</sup> and iodoacetic acid took 10 days in the dark at room temperature. We successfully synthesized N<sup>6</sup>-carboxymethyl-NAD<sup>+</sup> by a modified method with a dramatic reduction in the reaction time.



**Figure 18.** Chemical structure of NAD<sup>+</sup>

#### 9.4. Synthesis of N<sup>1</sup>-carboxymethyl-NAD<sup>+</sup> (compound 1 in Figure 19)

1.0g NAD<sup>+</sup> (1.51mmol) was dissolved in 3.5mL 0.1M pH 7.0 sodium phosphate buffer in a 5ml Biotage microwave reaction tube. Then, 1.5 g (8.06mmol, 5.34eq) iodoacetic acid was added and pH was adjusted to 7.0 by using 5.0M NaOH aqueous solution. The reaction vessel was sealed and the mixture was heated to 50°C for 10 minutes by using microwave irradiation. After that, the pink solution (c.a. 5.0 mL) was acidified to pH3.0 using 5M HCl aqueous solution before being poured into 25 mL pre-cooled (-5°C) mixture of acetone/IMS (1:1). The resulting precipitate was filtered, washed first with 5.0 mL IMS, then 15 mL dry diethyl ether before air drying under dry nitrogen for 10 minutes. Further drying overnight in a desiccator over fused CaCl<sub>2</sub> gave 1.62 g crude N<sup>1</sup>-carboxymethyl-NAD<sup>+</sup> as a pink amorphous solid.

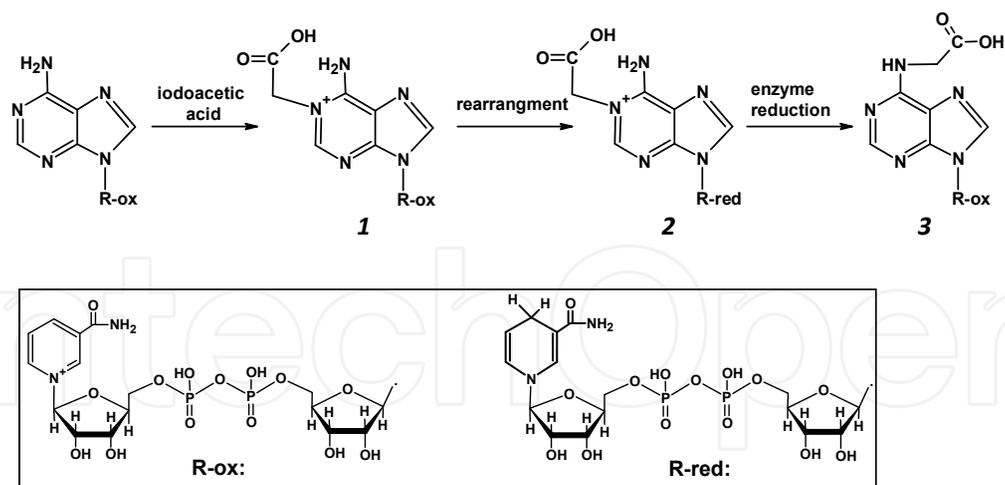
### 9.5. Synthesis of N<sup>6</sup>-carboxymethyl-NADH (compound 2 in Figure 19)

9.1g (c.a. 10.57mmol) above prepared crude N<sup>1</sup>-carboxymethyl-NAD<sup>+</sup> was dissolved in 1.3% w/v NaHCO<sub>3</sub> in 450 mL aqueous solution and the solution was deoxygenated by sparging with nitrogen for 10 minutes. 3.5 g (20.1mmol) sodium dithionite was added in one portion and the mixture was stirred at ambient temperature to affect reduction of the nicotinamide moiety. After 1.0 hour, the solution colour changed from pink to yellow. The solution was then sparged with air for 10 minutes to destroy any excess dithionite and the pH was brought to 11.0 by using 5M NaOH aqueous solution. The mixture was heated at 70°C for 90 minutes, to promote Dimroth rearrangement to N<sup>6</sup>-carboxymethyl-NADH, before cooling to 25°C. Thin-layer chromatography (silica gel, isobutyric acid/water/32% NH<sub>4</sub>OH (aq), 66/33/1.5 by volume) showed no evidence for the presence of N<sup>1</sup>-carboxymethyl-NADH.

### 9.6. Oxidation of N<sup>6</sup>-carboxymethyl-NADH to N<sup>6</sup>-carboxymethyl-NAD<sup>+</sup> (compound 3 in Figure 19)

The reaction mixture containing N<sup>6</sup>-carboxymethyl-NADH was treated with 17.5 mL 3M Tris buffer (pH7.0) and the pH was adjusted to 7.5 using 5M HCl aqueous solution. 3.5 mL acetaldehyde (62.6mmol) was added, immediately followed by 10.5 mg yeast alcohol dehydrogenase (*saccharomyces cerevisiae*) (~300U/mg) before allowing agitating at ambient temperature to deoxidize the nicotinamide moiety. After 18 hours, the reaction mixture (c.a. 485 mL) was concentrated *in vacuo* (30°C/10-15bar) to approximately 1/3 volume and poured into 1800 mL pre-cooled (-5°C) mixture of acetone/IMS (1:1). The fine slurry was left to age for 18 hours at 3°C. The resulting precipitate was collected by centrifugation and washed on a glass sinter with 40 mL IMS then 120 mL dry diethyl ether before air-drying under dry nitrogen for 10 minutes. Further drying overnight in a desiccator over fused CaCl<sub>2</sub> afforded 3.99g crude N<sup>6</sup>-carboxymethyl-NAD<sup>+</sup> as a tan coloured hygroscopic solid.

1.0 g of the above-prepared crude N<sup>6</sup>-carboxymethyl-NAD<sup>+</sup> was taken up in 20 mL water and passed through a Sephadex G10 gel filtration column (2x10cm, 20 mL). All eluted fractions containing UV active material were combined (60 mL total volume) and added to a column of Dowex 1-X2 ion exchange resin (Cl<sup>-</sup>, 4x50cm, 200 mL) which had been pre-equilibrated with water. A linear gradient of 0-50 mM LiCl (buffered to pH 3.0), at 10 mL per minute over 65 minutes, was applied using "Presearch Combiflash Companion" chromatography equipment. The fractions eluted between 25-35 mM were combined (c.a. 100 mL), neutralized to pH 7.0 with 5M LiOH and evaporated to approximately 1/3 volume and poured into 300 mL pre-cooled (-5°C) mixture of acetone/IMS (1:1). The fine slurry was left to age for 18 hours at 3°C. The resulting precipitate was collected by centrifugation and washed on a glass sinter with 30 mL IMS then 50 mL dry diethyl ether before air-drying under dry nitrogen for 10 minutes. Further drying overnight in a desiccator over fused CaCl<sub>2</sub> afforded 0.307g purified N<sup>6</sup>-carboxymethyl-NAD<sup>+</sup> as a cream coloured hygroscopic solid.



**Figure 19.** Synthesis of N<sup>6</sup>-carboxymethyl-NAD<sup>+</sup>

The synthesized N<sup>6</sup>-carboxymethyl-NAD<sup>+</sup> is an important intermediate for NAD immobilization at electrodes for continuous monitoring biosensors. An extension of this work could involve synthesis of various polymeric NADs with tailor-made chemical properties to meet biosensor requirements for continuous monitoring of different analytes.

## 10. Conclusions

In this article we have outlined various strategies for the electrochemical exploitation of dehydrogenase enzymes in sensor devices. The techniques used ultimately depend upon the class of dehydrogenase enzyme used. For enzymes that are NAD(P)<sup>+</sup> linked it is essential to develop a base transducer (a modified electrode) that efficiently reoxidises the reduced coenzyme. Alternatively, sensing schemes can be designed which utilize Diaphorase thereby facilitating the biochemical oxidation of reduced coenzyme. With FAD and PQQ dependent enzymes the most successful strategy has been to utilize mediator molecules such as the ferricyanide anion to couple the enzymatic activity to the electrode. Although not yet exploited commercially, dehydrogenase enzymes could also have a role in continuous monitors. With the NAD<sup>+</sup> dependent enzymes there is the additional complication of immobilizing the chemical components of the sensor to prevent drift in the device over time. This also includes the coenzyme molecule and we have illustrated how the synthesis of the N<sup>6</sup>-carboxymethyl derivative of NAD can be an important intermediate in achieving this. Attachment of suitable ligands at this position, with sufficient flexibility, should allow the development of stable reagents which will facilitate the development of continuous devices.

The electrochemistry of NADH oxidation has been well researched over the last 30 years. Also, over 250 enzymes use this ubiquitous coenzyme so schemes which utilize NAD dependent enzymes should allow for the measurement of a range of analytes in blood. In spite of this, it is interesting to note that with the exception of Abbott, none of the other major biosensor manufacturers have embraced this technology. The reason for this could be due to the fact that glucose SMBG is still the largest biosensor market worldwide and it is adequately served by enzymes such as glucose oxidase, FAD-GDH and PQQ-GDH, none of

which require any exogenous coenzyme to function. This makes the manufacture of the sensor relatively straight forward compared to those that require a modified electrode, an enzyme and in addition exogenous coenzyme added to the enzyme ink. In addition, switching to a NAD-dehydrogenase system may be incompatible with the manufacturing equipment currently used by manufacturers and thus prevent the adoption of this technology.

## Author details

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## 11. References

- [1] Evans JMM, Newton RW, Ruta DA, MacDonald TM, Stevenson RJ, Morris AD (1999) Frequency of blood glucose monitoring in relation to glycaemic control: observational study with diabetes database. *BMJ* 319:83–86,
- [2] Benjamin EM (2002) Self-Monitoring of Blood Glucose: The Basics. *Clinical Diabetes* January. 20:45-47
- [3] Franciosi M, Pellegrini F, De Bernardis G, Belfiglio M, Nicolucci A (2001) The impact of blood glucose self-monitoring on metabolic control and quality of life in type 2 diabetic patients. *Diabetes Care*. 24:1870–1877
- [4] Diabetes Education Study Group of the European Association for the Study of Diabetes (2003) Type 2 diabetes patient education basics. Blood glucose monitoring: a must in diabetes management.
- [5] Peel E (2004) Blood glucose self-monitoring in non-insulin treated Type 2 diabetes: a qualitative study of patients' perspectives. *British Journal of General Practice*. 54:183-8
- [6] Updike SJ, Hicks GP (1967) The Enzyme Electrode. *Nature*. 214:986 - 988
- [7] White HB (1982) Evolution of coenzymes and the origin of pyridine nucleotides, in: Everse J, Anderson B, You K-S, editors. *The Pyridine Nucleotide Cofactors*, Academic Press, New York, 1982, pp. 1 -17
- [8] Rodkey FL (1955) Oxidation-reduction potentials of the diphosphopyridine nucleotide system, *J. Biol. Chem.*, 213:777–786
- [9] Rodkey FL (1959) The effect of temperature on the oxidation-reduction potential of the diphosphopyridine nucleotide system. *J. Biol. Chem*. 234:188 - 90
- [10] Blaedal WJ, Jenkins RA (1975) Study of the electrochemical oxidation of reduced nicotinamide adenine dinucleotide. *Anal. Chem*. 47:1337–1343
- [11] Nassef HM, Radi A-E, O'Sullivan CK (2006) Electrocatalytic sensing of NADH on a glassy carbon electrode modified with electrografted o-aminophenol film *Electrochemistry Communications*. 8:1719–1725
- [12] Compton RG, Hancock G (1999) *Comprehensive Chemical Kinetics, Vol 37, Application of Kinetic modelling* (eds), Elsevier, pp.49 -85

- [13] Bogdan S, Jerzy N, Wojciec G (2008) Analytical evaluation of the Optium Xido blood glucose meter. *Clinical Chemistry and Laboratory Medicine*. Vol 46, Issue 1, pp.143–147
- [14] Eisenbarth GS, Polonsky KS, Buse JB (2008) Type 1 Diabetes Mellitus. In: Kronenberg HM, Melmed S, Polonsky KS, Larsen PR. *Kronenberg: Williams Textbook of Endocrinology*. 11th ed. Philadelphia, Pa: Saunders Elsevier; chapter 31, S13 – S61
- [15] Forrow NJ, Sanghera GS, Walters SJ, Watkin JL (2005) Development of a commercial amperometric biosensor electrode for the ketone  $\beta$ -3-hydroxybutyrate. *Biosensors and Bioelectronics*. 20:1617-1625
- [16] Vanelli M (2003) The direct measurement of 3-beta-hydroxybutyrate enhances the management of diabetic ketoacidosis in children and reduces time and costs of treatment. *Diabetes Nutrition and Metabolism*. 16:312-316
- [17] Guerci B (2003) Accuracy of an electrochemical sensor for measuring capillary blood ketones by fingerstick samples during metabolic deterioration after continuous subcutaneous insulin infusion interruption in type 1 diabetic patients. *Diabetes Care*. 26:1137-1141
- [18] Bektas F (2004) Point of care blood ketone testing of diabetic patients in the emergency department. *Endocrine Research*. 30:395-402
- [19] Duine JA, Frank J, van Zeeland JK (1979) Glucose dehydrogenase from *Acinetobacter calcoaceticus*: a 'quinoprotein'. *FEBS. Lett*. 108:443 – 446 Yamada M, Sumi K, Matsushita K, Adachi O, Yamada Y (1993) Topological analysis of quinoprotein glucose dehydrogenase in *Escherichia coli* and its ubiquinone-binding site. *J. Biol. Chem*. 268:12812 – 12817
- [20] Yamada M, Sumi K, Matsushita K, Adachi O, Yamada Y (1993) Topological analysis of quinoprotein glucose dehydrogenase in *Escherichia coli* and its ubiquinone-binding site. *J. Biol. Chem*. 268:12812 –12817
- [21] Oubrie A, Rozeboom HJ, Dijkstra BW (1999) Active-site structure of the soluble quinoprotein glucose dehydrogenase complexed with methylhydrazine: a covalent cofactor-inhibitor complex". *Proc. Natl. Acad. Sci. U. S. A*. 96:11787–11791
- [22] Dewanti AR, Duine JA (1998). Reconstitution of membrane-integrated quinoprotein glucose dehydrogenase apoenzyme with PQQ and the holoenzyme's mechanism of action. *Biochemistry*. 37: 6810–6818
- [23] Yamaoka H, Sode K (2007) SPCE Based Glucose Sensor Employing Novel Thermostable Glucose Dehydrogenase, FADGDH: Blood Glucose Measurement with 150nL Sample in One Second *J Diabetes Sci Technol*. 1:28–35
- [24] Ramanavicius K, Habermüller E, Laurinavicius V, Schuhmann W (1999) Polypyrrole entrapped quinoxaline protein alcohol dehydrogenase. Evidence for direct electron transfer via conducting polymer chains. *Anal. Chem*. 71: 3581–3586
- [25] Fraeyman A, Claeys G, Zaman Z (2010) Effect of non-glucose sugars and haematocrit on glucose measurements with Roche Accu-Chek Performa glucose strips *Ann Clin Biochem* September. 47:494–496
- [26] Tsujimura S, Kojima S, Ikeda T, Kanon K (2009) Potential-step coulometry of D-glucose using a novel FAD-dependent glucose dehydrogenase. *Analytical and Bioanalytical Chemistry*. 386:645-651

- [27] DCCT (1993) The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *New England Journal of Medicine*. 329:977-986
- [28] DDCI/EDCI (2005) Intensive diabetes treatment and cardiovascular disease in patients with type 1 diabetes. *New England Journal of Medicine*, 353:2643-2653
- [29] American diabetes association (2002) Implications of the United Kingdom prospective diabetes study. *Diabetes Care*. 25:528-532
- [30] Liu, Z, Cardosi M (2011) Electrochemical measurement of blood glucose for diabetic patients. In: Berhardt, Leon V editor, *Advances in Medicine and Biology*, vol 25, pp.1-39
- [31] Raitman OA, Katz E, Buckmann AF, Willner I (2002) Integration of Polyaniline/Poly(acrylic acid) Films and Redox Enzymes on Electrode Supports: An in Situ Electrochemical/Surface Plasmon Resonance Study of the Bioelectrocatalyzed Oxidation of Glucose or Lactate in the Integrated Bioelectrocatalytic Systems, *J. American Chemical Society*. 124:6487-6496
- [32] Yoon HC, Hong M-Y, Kim H-S (2000) Functionalization of a Poly(amidoamine) Dendrimer with Ferrocenyls and Its Application to the Construction of a Reagentless Enzyme Electrode, *Anal. Chem*. 72:4420-4427
- [33] Zhang S, Yang W, Niu Y, Sun C (2004) Multilayered construction of glucose oxidase and poly(allylamine)ferrocene on gold electrodes by means of layer-by-layer covalent attachment, *Sensors and Actuators B*. 101:387-393
- [34] Boguslavsky L, Kalash H, Xu Z, Beckles D, Geng L, Skotheim T, Laurinavicius V, Lee HS (1995) Thin film bienzyme amperometric biosensors based on polymeric redox mediators with electrostatic bipolar protecting layer, *Analytica Chimica Acta*. 311:15-21
- [35] Hoshi T, Sagae N, Daikuhara K, Takahara K, Anzai J (2007) Multilayer membranes via layer-by-layer deposition of glucose oxidase and Au nanoparticles on a Pt electrode for glucose sensing, *Materials Science and Engineering: C*. 27:890-894
- [36] Zheng H, Okada H, Nojima S, Suye S, Hori T (2004) Layer-by-layer assembly of enzymes and polymerized mediator on electrode surface by electrostatic adsorption, *Science and Technology of Advanced Materials*. 5:371-376
- [37] Gao Q, Guo Y, Zhang W, Qi H, Zhang C (2011) An amperometric glucose biosensor based on layer-by-layer GOx-SWCNT conjugate/redox polymer multilayer on a screen-printed carbon electrode, *Sensors and Actuators B*. 153:219-225
- [38] Bohm S, Pijanowska D, Olthuis W, Bergveld P (2001) A flow-through amperometric sensor based on dialysis tubing and free enzyme reactors, *Biosensors & Bioelectronics*. 16:391-397
- [39] Higson SPJ, Vadgama PM (1993) Diamond-like carbon coated microporous polycarbonate as a composite barrier for a glucose enzyme electrode, *Anal. Chim. Acta*, 271:125-133
- [40] Eisele S, Ammon HPT, Kindevater R, Grijbe A, Gopel W (1994) Optimized biosensor for whole blood measurements using a new cellulose based membrane, *Biosens & Bioelectron*. 9:119-124

- [41] Jiménez C, Bartrolí J, Rooij NF de, Koudelka-Hep M (1995) Glucose sensor based on an amperometric microelectrode with a photopolymerizable enzyme membrane, *Sensors and Actuators B: Chemical*. 27:421-424
- [42] Chen X, Hu Y, Wilson GS (2002) Glucose microbiosensor based on alumina sol-gel matrix/electropolymerized composite membrane, *Biosensors & Bioelectronics*. 17:1005-1013
- [43] Vilkanauskyte A, Erichsen T, Marcinkeviciene L, Laurinavicius V, Schuhmann W (2002) Reagentless biosensors based on co-entrapment of a soluble redox polymer and an enzyme within an electrochemically deposited polymer film, *Biosensors & Bioelectronics*. 17:1025-1031
- [44] Heller A (2006) Electron-conducting redox hydrogels: design, characteristics and synthesis, *Current Opinion in Chemical Biology*. 10:664-672
- [45] Himuro Y, Takai M, Ishihara K (2009) Poly(vinylferrocene-co-2-hydroxyethyl methacrylate) mediator as immobilized enzyme membrane for the fabrication of amperometric glucose sensor, *Sensors and Actuators B: Chemical*, 136:122-127
- [46] Feldman B, Brazg R, Schwartz S, Weinstein R (2003) A Continuous Glucose Sensor Based on Wired Enzyme™ Technology-Results from a 3-Day Trial in Patients with Type 1 Diabetes, *Diabetes Technology & Therapeutics*. 5:769-779
- [47] Kan S, Bae YH (2003) A sulfonamide based glucose-responsive hydrogel with covalently immobilized glucose oxidase and catalase. *J of Controlled Release*. 86:115-121
- [48] Lange U, Roznyatovskaya NV, Mirsky VM (2008) Conducting polymers in chemical sensors and arrays , *analytica chimica acta*. 614 :1-26
- [49] Pan D, Chen J, Yao S, Nie L, Xia J, Tao W (2005) Amperometric glucose biosensor based on immobilization of glucose oxidase in electropolymerized o-aminophenol film at copper-modified gold electrode, *Sensors and Actuators B*. 104:68-74
- [50] Njagi J, Andreescu S (2007) Stable enzyme biosensors based on chemically synthesized Au-polypyrrole nanocomposites, *Biosensors and Bioelectronics*. 23:168-175
- [51] Cosnier S, Le Lous K (1996) A new strategy for the construction of amperometric dehydrogenase electrodes based on laponite gel-methylene blue polymer as the host matrix. *J. Electroanal. Chem*. 406:243- 246
- [52] Mousty C (2004) Sensors and biosensors based on clay-modified electrodes—new trends. *Applied Clay Science*. 27:159- 177
- [53] Saito T, Watanabe M (1998) Poly(vinylferrocene-co-hydroxyethyl methacrylate) for use as electron mediator in enzymatic glucose sensor, *Reactive & Functional Polymers*. 37:263-269
- [54] Liu Z, Cardosi M, Rodgers J, Lillie G, Simpson L (2010) Synthesis and study of copolymer of vinylferrocene, acrylamide and 2-(diethylamino)ethyl methacrylate as a polymeric mediator for electrochemical biosensors, *Reactive & Functional Polymers*. 70:715-725
- [55] Rodgers J, Liu Z, McNeilage A, MacLennan M, Moffat J, Lillie G, MacDonald M (2008) Water-miscible conductive ink for use in enzymatic electrochemical-based sensors. US Patent 7465380

- [56] Clark LC Jr., Lyons C (1962) Electrode systems for continuous monitoring in cardiovascular surgery. *Ann. N. Y. Acad. Sci.* 102:29–45
- [57] Wang J (2008) Electrochemical glucose biosensors. *Chem. Rev.* 108:814–825
- [58] Dzyadevych SV, Arkhypova VN, Soldatkin AP, El'skaya AV, Martelet C, Jaffrezic-Renault N (2008) Amperometric enzyme biosensors: Past, present and future. *ITBM-RBM.* 29:171
- [59] Cardosi MF, Turner APF (1987) The realization of electron transfer from biological molecules to electrodes. In: Turner, A.P.F., Karube, I., Wilson, G.S. (Eds.), *Biosensors: Fundamentals and Applications*. Oxford University Press, London and New York, pp. 257–275
- [60] Serban S, Murr NE (2006) Redox-flexible NADH oxidase biosensor: A platform for various dehydrogenase bioassays and biosensors. *Electrochimica Acta.* 51:5143–5149
- [61] Serban S, Murr NE (2004) Synergetic effect for NADH oxidation of ferrocene and zeolite in modified carbon paste electrodes New approach for dehydrogenase based biosensors. *Biosensors and Bioelectronics* 20:161–166
- [62] Bu H, Mikkelsen SR, English AM (1998) NAD(P)H Sensors Based on Enzyme Entrapment in Ferrocene-Containing Polyacrylamide-Based Redox Gels. *Anal. Chem.* 70:4320–4325
- [63] Kato D, Iijima S, Kurita R, Sato Y, Jia J, Yabuki S, Mizutani F, Niwa O (2007) Electrochemically amplified detection for lipopolysaccharide using ferrocenylboronic acid. *Biosens. Bioelectron.* 22:1527–1531
- [64] Mano N, Kuhn A (2005) Molecular lego for the assembly of biosensing layers. *Talanta* 66:21–7
- [65] Maines A, Prodromidis MI, Tzouwara-Karayanni SM, Karayannis MI, Ashworth D, Vadgama P (2000) Reagentless enzyme electrode for malate based on modified polymeric membranes. *Anal Chim Acta.* 408:217–24
- [66] Suye S, Aramoto Y, Nakamura M, Tabata I, Sakakibara M (2002) Electrochemical reduction of immobilized NADP<sup>+</sup> on a polymer modified electrode with a copolymerized mediator. *Enzyme Microb. Technol.* 30:139–144
- [67] Montagné M, Marty JL (1995) Bi-enzyme amperometric d-lactate sensor using macromolecular NAD<sup>+</sup>. *Anal Chim Acta.* 315:297–302
- [68] Leca B, Marty JL (1997) Reusable ethanol sensor based on a NAD<sup>+</sup> dependent dehydrogenase without cofactor addition. *Anal Chim Acta.* 340:143–8
- [69] Suye S, Zheng H, Okada H, Hori T (2005) Assembly of alternating polymerized mediator, polymerized coenzyme, and enzyme modified electrode by layer-by-layer adsorption technique. *Sensors and Actuators B.* 108:671–675
- [70] Wykes JR, Dunnill P, Lilly MD (1972) The preparation of soluble high molecular weight NAD derivative active as a cofactor. *Biochimica Biophysica Acta.* 2686:260–268
- [71] Nakamura Y, Suye S, Kira J, Tera H, Tabata I, Senda M (1996) Electron-transfer function of NAD<sup>+</sup>-immobilized alginic acid. *Biochimica Biophysica Acta.* 1289: 221–225
- [72] Zapelli P, Pappa R, Rossodivita A, Re L (1977) Preparation and Coenzymic Activity of Soluble Polyethyleneimine-Bound NADP<sup>+</sup> Derivatives. *Eur. J. Biochem.* 72:309–315

- [73] Fuller CW, Rubin JR, Bright HJ (1980) A simple procedure for covalent immobilization of NADH in a soluble and enzymically active form. *Eur J Biochem.* 103:421–30
- [74] Yamazaki Y, Maeda H (1981) The Synthesis of New Polymer Derivatives of NAD by Radical Copolymerization and Their Coenzymic Activity. *Agric. Biol Chem.* 45:2277-2288
- [75] Lindberg M, Larsson P, Mosbach K (1973) A New Immobilized NAD<sup>+</sup> Analogue, Its Application in Affinity Chromatography and as a Functioning Coenzyme. *Eur. J. Biochem.* 40:187-193
- [76] Muramatsu M, Urabe I, Yamada Y, Okada H (1977) Synthesis and Kinetic Properties of a New NAD<sup>+</sup> Derivative Carrying a Vinyl Group. *Eur. J. Biochem.* 80:111-117