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Detection of ferricyanide as a probe for the effect of hematocrit in whole blood biosensors

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Measurement of the concentration of an analyte in whole blood can be influenced by a range of factors; the red cell content or hematocrit (Hct) of the sample, the distribution and rate of movement of analyte between red cells and plasma, the amount of protein in solution, the viscosity of the sample and fouling of the sensor. The effect of the red cells is the major factor that must be taken into account. Using the analyte molality rather than the analyte molarity, the theoretical response for a range of analytes which are found in plasma and in the red cells can be calculated. For an analyte which is found in plasma alone, the effect of hematocrit is significant, with a bias of −1% per %Hct; if the analyte can freely and rapidly diffuse between the red cells and plasma, this bias is reduced to zero. Using ferrocyanide as a model analyte, the effects of fouling and reduced sample viscosity were measured to be –0.2% per %Hct, giving an overall bias of −1.2% per %Hct, a level of bias which is not clinically acceptable. This bias can be negated by measuring the hematocrit separately and incorporating it into the measurement algorithm. Such a correction is essential for the correct measurement of the concentration of an analyte in whole blood.

Introduction

The last two decades have seen a huge effort devoted to developing biosensors for an array of analytes which can measure the concentration of an analyte at the point of sampling. The advantages of these sensors can include relatively low cost, ease of use, and the ability to perform testing at the point of need.[1,2] However despite intense efforts only one type, that of glucose biosensors (albeit in a variety of formats), has been commercially successful on a large scale. A range of factors ensured the commercial viability of glucose biosensors.[1,2] These include: (i) a demonstrable need for such testing (due to the complications caused by diabetes[3]); (ii) a sensor that is relatively easy to use and which requires a small sample volume of 5 μl or less (the smaller the volume the less painful the test); (iii) a disposable, single use sensor that uses whole blood, requiring no dilution or addition of reagents; (iv) a stable and cheap biorecognition element (glucose oxidase); (v) a reasonably high analyte concentration (*ca.* 5 mM); and (vi) the ability to mass produce the sensors at low cost on a large scale (10^6 units per day).

The results obtained with glucose biosensors provide acceptable results when compared to established methods. However, they do not provide the accuracy or the precision that are available with established methods. The American Diabetes Association recommends that readings be within 5% of the reference, plasma based, value.[4] A recent study[5] showed that approximately 50% of readings (of a total of 1987) on a range of glucose meters gave readings within the recommended range, though this was a significant improvement on the results obtained with earlier meters.[6]† The main problem facing such sensors arises from the use of blood as the sample matrix. Blood is a complex fluid[7] containing red cells [44 ± 5% (male), 39 ± 5% (female) v/v‡)] proteins (mainly albumin, 7% m/v) and lipids (0.3% m/v). The response of a sensor to an analyte in a sample of blood can be radically different to that obtained with a sample of buffer.[8,9] Understanding the effect of blood on the response of a sensor is essential in order to obtain accurate readings in whole blood.

For an electrochemical glucose biosensor based on the use of a mediator such as ferrocene or ferricyanide (Scheme 1), the effect of blood can be described by five factors, namely: (i) volume displacement, primarily by red cells and to a lesser degree by plasma proteins, such as albumin, and by lipids; (ii) the concentration of analyte in red cells and whether or not the analyte can freely diffuse out of the red cell; (iii) fouling of the electrode; (iv) a decrease in the diffusion coefficient of the mediator, D_m; and (*v*) the presence of interfering species such as ascorbate and acetaminophen.

In this paper the effect of blood on the response of an electrochemical sensor is examined with an emphasis on factors (i)–(iv). Bias due to interfering species will not be examined as it can be accounted for *via* incorporation of a second electrode to measure the signal due to such species.[9,10] Volume displacement is primarily a function of the red cell content of the sample, the contribution of proteins and lipids being significantly smaller.[7] Electrode fouling and changes in D_m can occur in the presence of red cells and plasma proteins. By performing calibration measurements in plasma, the effect of whole blood on the measurement is confined to the red cells. If this effect can be quantified, the response in whole blood can be compared to that in plasma, the sample type primarily used in clinical laboratories and the type of measurement to which clinicians are accustomed to using. The theoretical results

$$\text{Glucose} + \text{GOx(FAD)} \longrightarrow \text{Gluconolactone} + \text{GOx(FADH}_2\text{)}$$
$$\text{GOx(FADH}_2\text{)} + \text{M}_{ox} \longrightarrow \text{GOx(FAD)} + \text{M}_{red}$$

Scheme 1

† It should be noted that while the accuracy of these meters does not match that of laboratory methods, the meters provide scientifically untrained diabetics with the means of monitoring their blood sugar levels with a degree of accuracy sufficient to significantly reduce the health complications associated with the condition.

‡ Mean values ± 1*s*. Values can vary depending on the patient, *e.g.*, neonatal blood has a hematocrit content of 53 ± 6%, smokers and people living at high altitudes also have elevated hematocrit levels.

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expected from direct reading whole blood sensors (involving no sample pre-treatment) were calculated and compared to those obtained using plasma samples. The detection of ferricyanide in plasma and blood was then examined to demonstrate the effects of fouling and viscosity in addition to the volume displacement effect.

Experimental

All reagents were obtained from Sigma. Water (18 MΩ) was obtained from an Elga-Stat water purification system. Blood was collected from a single, healthy, volunteer, using EDTA as anti-coagulant. This procedure was carried out under the approval of the Ethics Committee of the University of Limerick. Solutions of varying hematocrit were prepared by separating the red cells from plasma and reconstituting the latter with the appropriate volume of red cells. The hematocrit content was then determined using a Hawskley centrifuge and hematocrit reader. Viscosity measurements were performed using an Ostwald viscometer (Schott). Phosphate buffer (0.01 M, pH 7.3) containing 0.1 M NaCl was used throughout. Electrochemical experiments were performed using a CH-802 potentiostat (CH Instruments). The platinum working electrode (1.6 mm diameter), Ag/AgCl reference electrode and platinum wire counter electrode were obtained from BAS. All potentials are reported with respect to Ag/AgCl. Conductivity measurements were performed using a WPA CM 35 conductivity meter and probe.

Results and discussion

In blood, displacement of water by red cells makes it necessary to consider the mass of H_2O present in a sample rather than the normally used sample volume. As discussed by Fogh-Andersen and others,[8,9] it is the molality (mol kg^{-1}) rather than the molarity of an analyte that is important. The response of a sensor in plasma and in whole blood is therefore determined by the analyte molality and this is calculated below. It is important to note that in developing a sensor of the type discussed here (Scheme 1), the analyte molality and molarity in a solution of aqueous buffer are, to all intents and purposes, equivalent. This is not necessarily the case when samples of whole blood are used.

Plasma

The lowered water content of plasma ensures that the molality of an analyte will always be higher than the molarity by a factor of 1.00/0.93, where 0.93§ is the mass of water (kg) per litre of plasma.[7] The mass of water in the sample (denoted plasma water volume, PWV) is given by:

$$\mathrm{PWV} = 0.93 \times V_S(1 - \mathrm{Hct}) \quad (1)$$

where V_S is the sample volume (and in this case is equal to V_P, the volume of plasma since Hct, the hematocrit content, equals zero). The molality of the analyte in plasma, $[A]^m_P$, is:

$$[A]^m_P = [A]_P/0.93 \quad (2)$$

Whole blood

It is assumed that the analyte of interest is always found in plasma. While this is not always the case, *e.g.*, lead is found mainly in the red cells,[12] detection of such analytes requires lysis of the red cells and will not be considered here (virtually all biosensors developed to date detect an analyte which is found in plasma and/or the red cells). Given this assumption, two scenarios then occur with whole blood, depending on whether or not the analyte is found in the red cells as well as in plasma. The equation describing the general case will be derived first.

The molality of analyte in the red cells is given by:

$$[A]^m_{RBC} = [A]^m_P K \quad (3)$$

where K is the partition function and is equivalent to the ratio of the molality in the interior of the red cells to that in plasma. The water mass in the red cells,[13] RBCV, is:

$$\mathrm{RBCV} = 0.71\ V_S\ \mathrm{Hct} \quad (4)$$

The water mass in a sample of whole blood, m_{WB}, is the sum of eqns. (2) and (4):

$$m_{WB} = 0.93 \times V_S(1 - \mathrm{Hct}) + 0.71\ V_S\ \mathrm{Hct} \quad (5)$$

The molality of analyte in whole blood is the sum of that in plasma and in the red cells:

$$[A]^m_{WB} = \frac{[A]^m_P V_S[0.93(1-\mathrm{Hct})]}{m_{WB}} + \frac{[A]^m_P V_S\, 0.71\, K\, \mathrm{Hct}}{m_{WB}} \quad (6)$$

It should be noted that an electrochemical sensor can only detect electroactive species that are present in plasma. Analytes in the red cells can be detected and measured only if they can freely diffuse out of the red cells into the plasma. The amount of analyte in the red cells that can be detected is a function of the nature of the analyte and of the actual test itself. If none of the analyte in the red cells is available or if the rate of transfer of the analyte out of the red cells is slow, the available analyte molality will decrease with increasing hematocrit as the plasma content of the blood decreases. To take the degree of availability into account it is necessary to multiply the red cell analyte molality by a factor F ($0 \leqslant F \leqslant 1$). Eqn. (6) then reduces to:

$$\frac{[A]^m_{WB}}{[A]^m_P} = \frac{0.93(1-\mathrm{Hct}) + 0.71 K F \mathrm{Hct}}{0.93(1-\mathrm{Hct}) + 0.71\mathrm{Hct}} \quad (7)$$

From eqn. (1), the plasma water volume decreases with increasing hematocrit. Using eqn. (7), it can be seen that if an analyte occurs only in plasma ($K = 0$), or if none is released from the red cells ($F = 0$), $[A]^m_{WB}$ will be strongly dependent on the hematocrit, decreasing with increasing hematocrit. If however the analyte can freely diffuse out of the red cells this hematocrit dependence can be greatly reduced. The rate of analyte efflux from red cells can be very rapid (on the time scale of the assay) with the result that a significant portion of the analyte is consumed in the test.[14–17] Fig. 1 shows a plot of $[A]^m_{WB}/[A]^m_P$ *versus* hematocrit for a range of values of F (at $K = 1$). The ratio decreases with Hct for $F < 1$, with slight deviations from linearity, as expected from eqn. (7). Similar results are obtained for a range of values of K at $F = 1$. When $K = F = 1$, there is no hematocrit bias, as all of the analyte is available for measurement. In this case, all of the analyte is detectable and the analysis is equivalent to performing a titration. For the case where the analyte occurs only in plasma ($K = 0$) or where analyte efflux from the red cells is slow ($F = 0$), there is a significant bias of −0.01 per %Hct. Under these circumstances, measurement of a sample containing 40% Hct will yield a reading which is 40% less than that obtained with a plasma sample.

§ The average mass of water in plasma and red cells is 0.93 and 0.71 kg dm^{-3}, respectively. These values can vary, *e.g.*, Kessler *et al.*[11] reported a red cell water content of 0.71± 0.01 kg dm^{-3} in 24 normal subjects. Higher values can occur, *e.g.*, patients with renal dysfunction[11] had a red cell water content of 0.71 ± 0.02 kg dm^{-3} (17 subjects).

In addition to the effects of analyte distribution between the red cells and plasma and the rate of analyte movement between plasma and the red cells, other factors have to be taken into account. In particular, in an electrochemical device, changes in diffusion coefficient and fouling of the electrode require consideration. In order to investigate these effects, ferricyanide was used as a model analyte, and the response of an electrode to ferricyanide was monitored in plasma and in whole blood. The ferro–ferricyanide couple displays reversible electrochemical behaviour and has been used in a number of commercially available glucose biosensors.[14–16] Ferricyanide does not cross the red cell membrane and is representative of an analyte found only in plasma ($K = 0$).¶

Fig. 2 shows a plot of the charge passed *versus* the molality of ferricyanide. In the presence of 7% BSA (m/m), the slope of the line decreased from 15.7 to 11.5 μC kg mmol^{-1}. As the molality of ferricyanide was identical in the presence and absence of BSA, the decrease in response must be due to other factors. The viscosity of phosphate buffered saline increased by 18% from 8.91×10^{-3} to 1.05×10^{-3} N s m^{-2} on addition of 7% BSA (m/m).[18] From the Stokes–Einstein relationship,[18] the diffusion coefficient, *D*, is inversely related to the viscosity, indicating that 66% of the decrease in the slope can be accounted for by a decrease in *D*. The remainder of the decrease can then be ascribed to electrode fouling, producing a decrease in the surface area of the electrode. The 27% decrease in the response on addition of BSA illustrates the substantial, detrimental influence that plasma proteins such as albumin can have on the measurement of an analyte. This decrease demonstrates the importance of calibrating sensors in plasma (or at least in solutions containing 7% albumin) rather than in buffer alone.

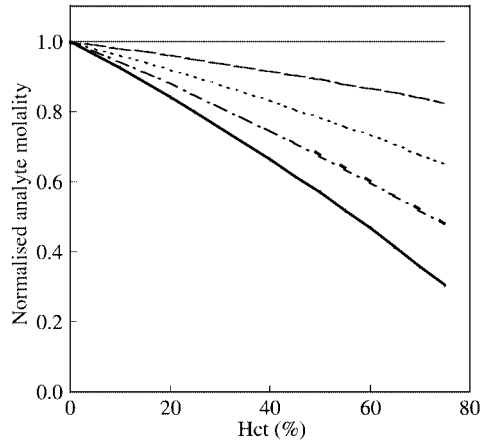

Fig. 1 Plot of normalised analyte molality *vs.* hematocrit using whole blood with $K = 1$ and $F = 0$ (——), 0.25 (– – – –), 0.5 (·····), 0.75 (- - - -), and 1 (——).

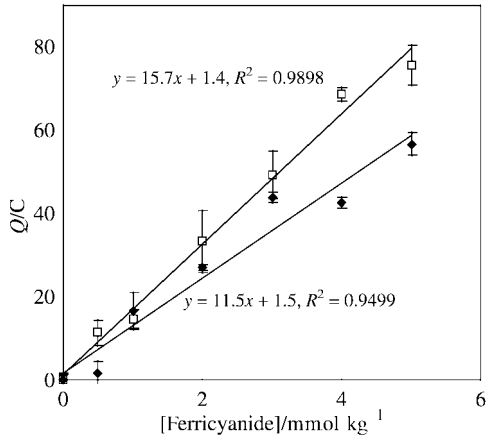

Fig. 2 Plot of charge passed (0–30 s) for the reduction of ferricyanide in buffer in the presence (◆) and absence (□) of 7% BSA (m/m). Error bars represent one standard deviation.

Cyclic voltammograms of plasma and whole blood displayed no discernible redox process at 0 V, indicating that it was feasible to detect ferricyanide at this potential in such solutions. In plasma and whole blood solutions containing ferricyanide, repetitive scanning of the electrode resulted in a decrease in the peak current and an increase in peak separation, indicating that it was essential to clean the electrode after each run (data not shown). Such results illustrate the difficulties of performing analysis in whole blood and underscore one of the advantages of using single use sensors.

Fig. 3 shows the current traces obtained with whole blood containing 5 mmol kg^{-1} ferricyanide at different levels of hematocrit. As the red cell content of the sample increases, the response decreases significantly. Fig. 4 shows that the response obtained for 5 mmol kg^{-1} ferricyanide in the hematocrit range 0–50% decreases linearly with Hct. On normalising the response to that of plasma, the charge decreases by 0.012 per %Hct. Volume displacement accounts for the majority, 0.01 per %Hct, of this decrease. As with albumin, increased solution viscosity and/or electrode fouling must account for the remainder. It was not possible to distinguish between the effects

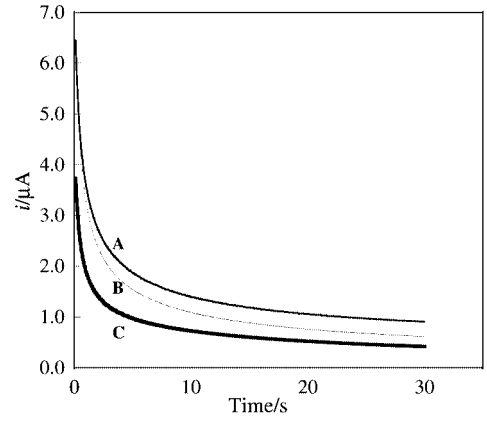

Fig. 3 Plot of charge passed (0–30 s) for the reduction of 5 mmol kg^{-1} ferricyanide in plasma (A), whole blood containing 31% Hct (B), and whole blood containing 50% Hct (C). The standard deviations for A, B and C are 2, 8 and 11%, respectively.

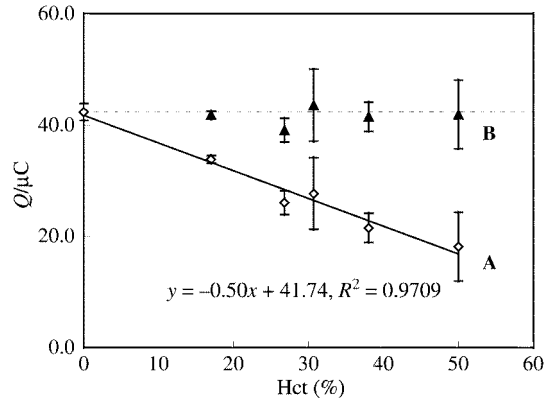

Fig. 4 Plot of charge passed (0–30 s) for the reduction of 5 mmol kg^{-1} ferricyanide (plasma) as a function of hematocrit (A), data in (A) corrected for hematocrit (B). Error bars represent one standard deviation. The dashed line represents the plasma charge.

¶ Verification that ferricyanide does not cross the membrane was demonstrated by examining the red cells before and after exposure to ferricyanide (after washing with isotonic saline). Entry of ferricyanide to the red cells would cause rapid oxidation of hemoglobin, changing the colour from red to dark brown. No such colour change was observed.

of increased viscosity and electrode fouling as blood is a non-Newtonian fluid.[19] For example the viscosity of blood of 50% Hct is three times higher than that of blood of 17% Hct (5.6×10^{-3} *vs.* 1.8×10^{-3} N s m^{-2}), indicating that the effect of viscosity is significantly less than that described by the Stokes–Einstein equation.

The average bias of the data in Fig. 4 is 39%, a level of hematocrit bias that is clinically unacceptable.‖ Measurements to within 5% of the reference value are not possible with the model system described here. However, given the linear hematocrit bias displayed by the electrode, measurement of the hematocrit content of the sample should make it possible to take into account some or all of the effect of the red cells and improve the accuracy of the measurement. The conductivity of whole blood was measured over the hematocrit range used (17–50%). A linear response was obtained (Hct_{calc} = (−1.55 Hct + 100) mS, R^2 = 0.9799). The calculated charge, Q_{calc}, was then obtained using eqn. (8):

$$Q_{calc} = Q_{exp} - Hct_{calc}/(-0.5) \quad (8)$$

where Q_{exp} represents the experimental charge and −0.5 is the slope of the plot of charge *versus* hematocrit. The corrected data in Fig. 4 show that there is a significant improvement in the data, with Q_{calc} being independent of Hct and the average bias decreasing to 3%. These results demonstrate that accurate measurement of the molality of an analyte can be performed in whole blood, provided that the calibration procedure includes data obtained over a range of hematocrit, and that a method of measuring the hematocrit is incorporated into the sensor.

It should be noted that all clinical tests are based on plasma samples and use molarity rather than molality as it is neither practical nor convenient to measure the water mass of a sample. The results described here demonstrate that a test can be performed in whole blood, and yield a measurement which is equivalent to plasma molality. The analyte molarity can then be obtained by multiplying the molality by 0.93.[7] The detection of lactate, troponin I, and glucose in whole blood will be discussed as representative examples of clinically important tests. Measurement of troponin I is important as a cardiac marker,[20] while lactate[21] and glucose[8,9] are important metabolites. Troponin I is found only in plasma (K = 0) while lactate[21] and glucose[8] are also found in the red cells. Transport of lactate out of the red cells is slow on the time scale of 20–30 s[21] ($F \approx 0$) while transport of glucose occurs much more quickly ($F \approx 1$).[14–17]

For troponin and lactate, only analyte in plasma can be detected by a whole blood sensor, making these analytes similar to ferricyanide. Therefore in comparison to the response of a plasma analyser, a sample containing 40% Hct will yield a measurement that is 48% lower. This level of bias is clearly not acceptable in a clinical setting. For example, with troponin, such a level of bias may result in a sensor indicating that a cardiac infarction had not occurred when in fact it had.

In the case of glucose, the situation is different since the analyte can diffuse out of the red cells. If there is a sufficiently large diffusion gradient** (provided by consuming a significant amount of glucose), the rate of glucose efflux can be very rapid[14–17] and a value of $F \approx 1$ is assumed. The effect of this movement of glucose is to increase the concentration of mediator in the plasma. If all of the glucose diffuses out of the red cells, the molality of ferrocyanide in plasma (generated *via* the oxidation of glucose by glucose oxidase, Scheme 1) will increase with increasing hematocrit. This increased response can be seen in Fig. 5, where the response of solutions containing 5 mmol kg^{-1} ferricyanide (in plasma and in red cells) is plotted against hematocrit. The response increases initially on going from 0 to 17% Hct and then decreases with decreasing hematocrit. The initial rise from 42.2 to 46.2 μC occurs on account of the higher concentration of ferricyanide in the plasma portion of the sample, *e.g.*, in 17% Hct, the concentration of ferricyanide in plasma is actually 6.0 mmol kg^{-1} (5.0 mmol kg^{-1} in the whole sample), since there is none in the red cells. In the absence of red cells the charge should have increased by 20% to 50.4 μC. However, the increase in the plasma molality of ferricyanide with increasing hematocrit is offset by the increased viscosity of the solution and by fouling of the electrode, with the result that the response is significantly less than 50.4 μC and starts to decrease above 17% Hct. From the data in Fig. 5, the response of the electrode decreases linearly with Hct in the range 17–50% Hct. Eqn. (8) can again be used to account for the hematocrit dependence (using −0.24 as the slope of the plot of charge *versus* hematocrit). The corrected data is independent of Hct, with the average bias being reduced from 16 to 2%. Note that the charge calculated for plasma is significantly higher than the experimentally measured value (48.9 *vs.* 42.2 μC). This difference is a result of higher levels of ferricyanide present in plasma and is in reasonable agreement with the calculated value of 50.4 μC. The data again show that there is a significant hematocrit bias which has to be taken into account in order to perform clinically accurate measurements.

In the above analysis it is assumed that $F \approx 1$ for a glucose sensor. This is not necessarily the case. With normal levels of glucose (5 mM) analysis is complete within 30 s and the current measured at 30 s is identical to that obtained at 120 s.[22] With higher glucose concentrations (23 mM), it is necessary to wait for 90 s for all of glucose to be oxidised. These results demonstrate that glucose sensors will exhibit a hematocrit bias[23] when the analysis time is reduced to less than 30 s and/or high concentrations of glucose are present.[22]

Conclusion

The major difficulty with performing measurements with whole blood is a result of the red cell content of the sample. Significant biases can result when using whole blood, demonstrating that it is not sufficient to develop a sensor and report results obtained in solutions of buffer alone, the performance of the sensor in whole blood has to be measured and compared to that in plasma. The electrochemical detection of ferricyanide as a model system

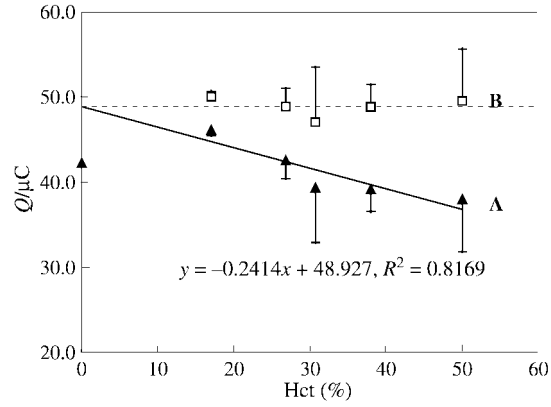

Fig. 5 Plot of charge passed (0–30 s) for the reduction of 5 mmol kg^{-1} ferricyanide (whole blood) as a function of hematocrit (A), data in (A) corrected for hematocrit (B). Error bars represent one standard deviation (only one shown for clarity). The dashed line represents the calculated plasma charge.

‖ Bias was calculated by taking the mean of the absolute value of (Q_{HCT}–Q_{plasma}) × 100/Q_{plasma} over the range of hematocrit. The value of Q_{plasma} was that calculated from the plot of Q *vs.* Hct.

** A large diffusion gradient can be generated by using high levels of glucose oxidase (2000 units ml^{-1}) and of ferricyanide (300 mM).[13] These levels are sufficient to catalyse the oxidation of a significant portion of the glucose and the reduction of ferricyanide within about 20 s.[13]

yields a significant measurement bias of −1.2% per %Hct in whole blood. Such a bias makes it essential that measurement of the sample hematocrit be incorporated as part of the sensor in order to counteract the detrimental effect of the red cells on the response, enabling direct comparison of the results obtained in whole blood with those obtained in plasma.

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