Voltammetry in sheep's blood: membrane-free amperometric measurement of O_2 concentration

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Abstract

An amperometric method was applied for the electroanalytical measurement of oxygen content in sheep's blood. This method was based on a bare platinum microdisc electrode coupled with the use of chronoamperometry. A linear relationship between the chronoamperometric current and the oxygen concentration was observed in both saline solution and sheep's blood. The developed method was able to measure the oxygen percentage with an error of ca. 1.3% in sheep's blood. In addition, this article presents the first study on direct voltammetry in sheep's blood and a dissociative CE process was proposed to explain the electrochemical behaviour of oxygen reduction in blood on a platinum electrode in which the 'free' oxygen was first dissociated from oxyhaemoglobin prior to electron transfer with the magnitude of the observed current controlled by the diffusion of oxyhaemoglobin to the electrode where for sufficiently large electrodes (greater than ca. 1 micron in radius) the dissociation proceeds to completion on the voltammetric timescale allowing quantitative measurements.

Key words:

Oxygen sensor; amperometric sensor; voltammetry; sheep's blood; chronoamperometry; membrane free

1. Introduction

Gas sensing is of great importance in diverse contexts such as industrial safety, environmental monitoring and medical applications for the detection of various gases including carbon monoxide, carbon dioxide, ammonia and oxygen.[1-4] The measurement of the oxygen content of blood, either as a concentration or equivalently as a partial pressure, is critically important and valuable in clinical diagnostics. Normal arterial oxygen pressure is approximately 75 to 100 mmHg. A number outside of this range indicates an abnormal condition possibly triggering further attention or medical treatment. Techniques used in oxygen detection include coulometric sensing[5], paramagnetic sensing[6, 7] and electrochemical sensing[7, 8]. Among the techniques used for the gas detection, the advantages offered by electrochemical sensors are good selectivity, high sensitivity and low cost.

Amperometric sensors, one category of electrochemical sensors, are presently competitive in the gas-sensing market. Commercial gas sensors have been brought to the market from diverse sensing companies such as Honeywell (sps.honeywell.com), Alphasense (www.alpha-sense.com), Dräger (www.draeger.com) and Figaro (www.figarosensor.com) amongst others. The development of this type of sensors dates from the introduction of the Clark-type sensor for blood oxygen in 1956 in which the amperometric measurement of dissolved oxygen in body fluid[9] was facilitated by a membrane-covered platinum macroelectrode to avoid possible biological materials adsorbing onto the electrode surface with the oxygen reduction current found to be proportional to the partial pressure of oxygen. The mode of operation of such membrane-covered sensors requires the diffusion of dissolved oxygen within the sample up to and then through the protective membrane to the surface of the working electrode held under potentiostatic control where the oxygen is reduced to water via a four electron transfer process due to the well-known strong electro-catalytic activity of platinum towards the oxygen reduction reaction (ORR). Such membrane-covered electrodes are the basis of many modern gas sensors both in vivo and in vitro.[10] Beyond membrane covered electrodes, N. Holmstrom *et al.* developed an implantable amperometric blood oxygen sensor employing double potential step chronoamperometry where a gold working electrode was directly in contact with venous blood for use in pacemaker applications in animals, giving an observed unchanged high sensitivity for up to 4 years.[11] S. Dong *et al.* established an electrochemical sensing platform with the design of a working electrode modified with carbon nanotubes and ionic liquid, providing a mediator-free oxygen sensor for use in erythrocytes separated from human blood samples.[12] More recently, a micro-needle implantable platinum-based sensor for oxygen detection in vascular blood (ex-vivo) was developed by M. Mir *et al.* where a layer of Nafion was coated onto the electrode to reduce the electrode poisoning due to the adsorption of biological materials within the blood.[13]

From the perspective of optimising electroanalysis in blood, membrane free measurements may offer greater sensitivity whilst measuring full current – voltage curves ('voltammetry') can give a better indication of the 'quality' of the measurement than recording the current at a single potential since the effects of electrode fouling are much more obvious. Analytical voltammetry in which the potential is swept has been proved to be a powerful technique in both research and real applications which include but are not limit to drug quality evaluation in pharmaceuticals[14, 15], and the quantification of antioxidants (e.g. piperine[16], vanillin[17], chilli[18]) in food. Considerable research has been done to voltammetrically study biological samples either in vivo or ex vivo.[19-21] A convenient method using cyclic voltammetry (CV) for the quantitation of the antioxidant capacity of low molecular weight antioxidants in blood plasma and tissue homogenates was developed by S. Chevion *et al.*; the results obtained from CV were proved to be reliable by comparison with the results from HPLC-electrochemical detection.[20, 21] K. V. Derina *et al.* used voltammetry for the determination of cholesterol in human blood serum using a modified carbon-containing electrode with a renewable surface, giving a limit of detection of 0.01 μM.[22] Clinically, CV has been used previously to determine the total reducing power (TRP) of tissues and body fluids which represented the total anti-oxidant capacity of an examined substance; this capacity plays an important role withstanding the destruction and inflammation caused by reactive oxygen species with body fluids.[23, 24] The peak potential from the cyclic voltammogram distinguishes the type of low molecular weight anti-oxidants and the magnitude of anodic current allows the determination of concentration.[23, 24] With the advantages of voltammetry in mind, it is surprising to find that little research has been attempted to directly obtain voltammograms for oxygen reduction in whole blood with measurements in blood plasma or serum usually preferred due to the complicated chemical composition of blood.

In this work, the direct voltammetry of oxygen reduction in sheep's blood is obtained using a membrane-free platinum microdisc electrode. The ORR is first studied at a bare Pt macroelectrode in both saline solution and sheep's blood as a function of variable oxygen content. A platinum microdisc electrode is then applied as the working electrode in combination with chronoamperometry for direct oxygen measurement. Such experiments are conducted both in saline solution (0.1 M NaCl; having a similar chloride concentration to that in blood[25]) and in sheep's blood . A linear calibration curve is obtained in sheep's blood in the O_2 percentage range from 0 to 100% (balanced with N_2). To the best of our knowledge, this is the first ORR study performed directly in whole blood and enables us to measure the oxygen content in sheep's blood samples from different batches saturated with air with an error of around 1%.

2. Material and Methods

2.1 Chemical reagents

Potassium chloride (KCl; Aldrich; 99%), hexaammineruthenium(III) chloride ($[Ru(NH_3)_6]Cl_3$, Aldrich, 98%), sodium chloride (NaCl; Aldrich; 99%), and defribrinated sheep blood (VWR International Ltd, UK) were used as purchased without further purification. Nitrogen (N_2, BOC, high purity oxygen free), oxygen (O_2, BOC, 99.5% purity) and air (BOC, 21% ± 0.5% oxygen, balance nitrogen) were used directly and bubbled into solutions of interest using a gas mixing pump (Wosthoff oHG, Bochum, Germany). Solutions were prepared using deionised water (Milipore) with a resistivity of 18.2 MΩ cm at 298 K.

2.2 Instrumentation

All electrochemical measurements were conducted with a µAutolab Type III potentiostat using a standard three-electrode setup in an optimised thermostatted electrochemical cell inside a Faraday cage. The design of this homemade potentiostat is described in detail in the literature.[26] A platinum microdisc electrode (5.05±0.05 µm in radius, Goodfellow Cambridge Ltd., Cambridge) and platinum macroelectrode (1.60±0.01 mm in diameter, BASi, USA) were used as working electrodes. A saturated calomel electrode (SCE saturated KCl; BASi, Japan) and a platinum wire were used as reference electrode and counter electrode, respectively. The platinum working electrode was cleaned by polishing using alumina of decreasing size (1.0, 0.3 and 0.05 µm, Buehler, IL, U.K.) on a polished pad, washed with deionized water and then dried with nitrogen. The size of the electrode was calibrated from the steady-state current ($I_{s.s}$) of the one electron reduction of 1 mM $[Ru(NH_3)_6]Cl_3$ with 0.1 M KCl at 25 °C. A literature value for the diffusion coefficient[27] of $[Ru(NH_3)_6]Cl_3$ (8.43×10^{-10} m^2 s^{-1}) was used to calculate the radius of electrode (r) using Equation 1 which is exclusively for microdisc electrodes; the value of which was averaged from three repeated measurements.

$$I_{s.s} = 4nFDCr \qquad \text{Equation 1}$$

where n is the number of electrons transferred, F is the Faraday constant (96485 C mol^{-1}), D is the diffusion coefficient (m^2 s^{-1}) and C is the concentration (mol m^{-3}).

3. Results and Discussion

The electrochemical behaviour of dissolved oxygen at Pt macroelectrodes and Pt microelectrodes was examined in saline solution (0.1 M NaCl) and sheep's blood; the solutions were bubbled with gases containing variable oxygen content (0% to 100% O_2 balanced with N_2) using a gas mixing pump for at least 15 minutes prior to experiments. All experiments were conducted at 25 °C unless otherwise stated.

3.1 Oxygen reduction reaction at a Platinum macroelectrode

First, the voltammetric reduction of dissolved oxygen under quiescent conditions was studied at a Pt macroelectrode in oxygen-saturated saline solution and sheep's blood at different scan rates at 25 ^{o}C. High purity oxygen gas was bubbled into the solution of interest for at least 15 minutes before experiments. The Pt working electrode was polished prior to batches of experiments in saline solution whilst the polishing procedure was applied between each scan for experiments in sheep's blood to keep the electrode surface active. The chloride anion is known to adsorb onto the platinum surface and the formation of platinum oxides and platinum-chloride complexes occurs at potentials more positive than ca. 0.4 V.[28] Consequently, the cyclic voltammetry was swept catholically from 0.1 V to -0.6 V to get an oxygen reduction peak using a freshly polished electrode for each voltammogram recorded. Figure 1 shows the comparison of the cyclic voltammetric responses at a Pt macroelectrode in O_2-saturated saline solution (blue curve) and sheep's blood (red curve) at 0.05 V s^{-1}. Oxygen reduction peaks were observed at ca. -0.25 V and -0.47 V at 0.05 V s^{-1} in saline solution and sheep's blood respectively however, a clear difference in the voltammetric behaviour between these two solutions was evidenced where the oxygen reduction peak obtained in sheep's blood was broader than that in saline solution with a lower peak current and the peak potential shifted to a more negative position in sheep's blood. Both systems were further investigated using cyclic voltammetry at variable scan rates; the corresponding voltammograms are presented in SI Sections 1 and 2 where the peak currents increased with scan rate and the peak potential for oxygen reduction shifted to a more negative value as the scan rate increased in both systems. In saline solution, well-defined oxygen reduction peaks were observed at all scan rates and a linear relationship was obtained between the cathodic peak current and the square root of scan rate, suggesting a diffusion-controlled electrode process for oxygen reduction in saline solution. The observed oxygen reduction peak is consistent with literature in both peak potential and peak current under oxygen-saturated condition.[28, 29] Qualitatively different electrochemical behaviour of oxygen reduction was however, observed in sheep's blood in that the oxygen reduction peaks obtained were too broad to find the precise peak potential which limits the use of a macroelectrode for the oxygen measurement. The difference in the magnitude of the current reflects in part the different solubility of oxygen in the two systems (0.0576 mL and 0.0276 ml O_2 per mL of solution at a partial pressure of 760 mmHg in 0.1 M NaCl and sheep's blood at 25 ^{o}C respectively[30],[31]) while the change in peak potential likely indicates a change in electron transfer mechanism and/or kinetics in sheep's blood where the oxygen is predominantly bound to haemoglobin[32, 33] compared to that in aqueous saline solution. This phenomenon has been observed in literature[13] but has not been quantitatively studied yet. Note that the haemoglobin concentration in sheep's blood varies from 1.9-3.1 mM.[34, 35]

The broadness of the oxygen reduction peak in sheep's blood limits the analysis of the electrode kinetics as well as the possibility of using a Pt macroelectrode to directly measure oxygen content in sheep's blood. Accordingly a Pt microdisc electrode was then employed as the working electrode in the following sections.

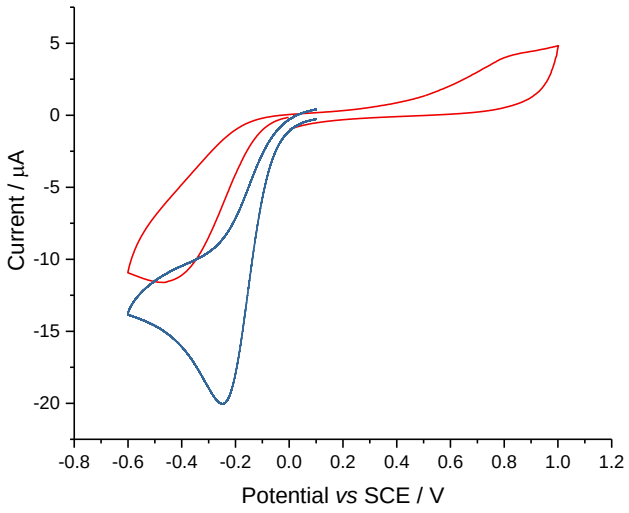

Figure 1 Cyclic voltammograms at a Pt macroelectrode (d=1.6 mm) in oxygen-saturated saline solution (0.1 M NaCl) (blue) and sheep's blood (red) at 0.05 V s^{-1} at 25 °C.

3.2 Oxygen reduction reaction at a Pt microdisc electrode

3.2.1 Cyclic voltammetry at a Pt microdisc electrode

Oxygen reduction reaction was first studied at a Pt microdisc electrode (radius of 5.05 µm) in both saline and sheep's blood with variable oxygen percentages using cyclic voltammetry. The corresponding cyclic voltammograms under oxygen-saturated condition at a scan rate of 0.02 V s^{-1} are presented in Figure 2 where in saline solution (Figure 2a) clear steady state voltammetric cathodic currents for oxygen reduction were obtained due to convergent mass transport and at long times in the chronoamperometry. Figure 2b), in contrast shows that the voltammetric oxygen reduction signals were less well defined in sheep's blood most likely due to the electrode fouling from biological materials. To minimize the effects of fouling over prolonged exposure to blood we used potential step chronoamperometry immediately following the application of a potential required to establish the limiting current, providing the advantages of short time scale, quick response with minimized fouling and allowing the use of small sample volume. Chronoamperometry at a Pt microdisc electrode (radius of 5.05 µm) at a potential of -0.65 V was thus employed to quantitatively determine the oxygen content in both saline solution and sheep's blood at 25 °C.

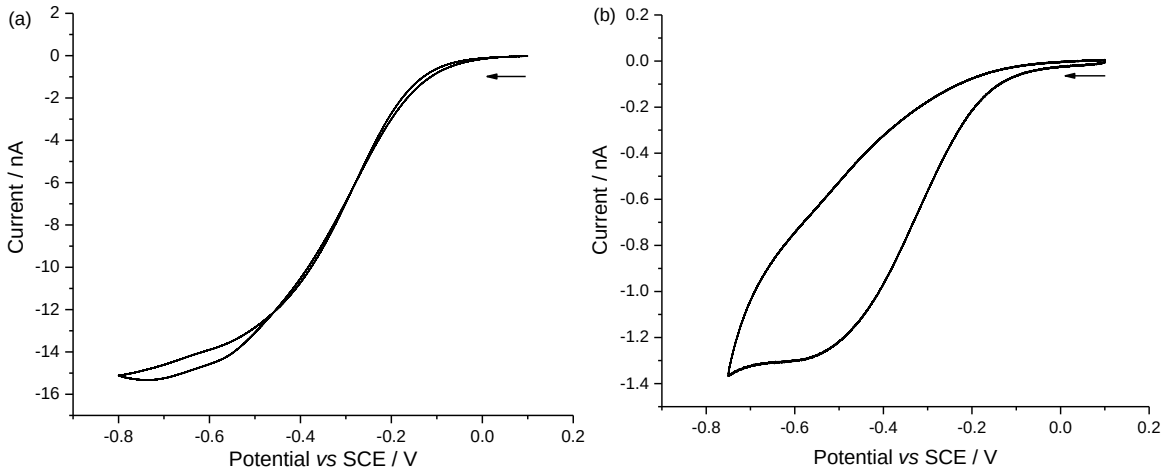

Figure 2 Cyclic voltammograms at a Pt microdisc electrode (r=5.05 µm) in O_2-saturated (a) saline solution and (b) sheep's blood at 0.02 V s^{-1} at 25 ^oC. The arrow shows the starting direction of the scan.

3.2.2 Chronoamperometric study in saline solution

For chronoamperometry in saline solution potentials steps from a potential corresponding to no current flow were made to an applied potential of -0.65 V *vs.* SCE, corresponding to a sufficiently negative potential for a diffusion limited reduction on the basis of the voltammetric behaviour. Figure 3a) shows the chronoamperometric response of a Pt microdisc electrode towards oxygen reduction over a time period of 1.5 seconds in saline solution with variable oxygen percentages (0% - red, 25% - blue, 50% - yellow, 75% - green, 100% - black). The chronoamperometric current increases with increasing oxygen percentage in saline solution. Currents recorded at 1.5 s were chosen for further analysis since at such times quasi-steady state currents are realised; the corresponding calibration curve is plotted in Figure 3b). From the slope of the linear fitting of the calibration curve (1.63×10^{-10}) combined with the diffusion coefficient of O_2 in 0.1 M NaCl ($D = 1.93 \times 10^{-9}$ m^2 s^{-1})[36], the apparent number of electrons transferred (n_{app}) in oxygen reduction was calculated to be ca. 3 based on Equation 1). Moreover, the magnitudes of the steady state currents for oxygen reduction obtained from chronoamperometry were shown to be consistent with those from voltammetry, such as in Figure 2(a).

The mechanism of oxygen reaction has been much investigated.[37, 38] It is known to proceed via either a two-electron transfer process to form H_2O_2 (Equation 2) or a four-electron transfer process to form H_2O (Equation 3) depending on the electrode material. For materials such as platinum with well-known strong electrocatalytic activity towards oxygen reaction, it is widely believed that oxygen can be directly reduced at macroelectrodes to water via a four electron transfer process.

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

$$O_2 + 4H^+ + 4e^- \rightarrow H_2O$$ Equation 3

However, it has been rigorously established by D. Pletcher and colleagues that the products formed in the oxygen reduction on platinum as well as the apparent number n_{app} of electrons involved depends on the mass transport conditions and the value of n_{app} decreases as the electrode becomes smaller.[39] In the case of a Pt microdisc electrode with a radius of 5 µm under neutral conditions, the value of n_{app} was found to be around 3[39], which is consistent with our experimental result.

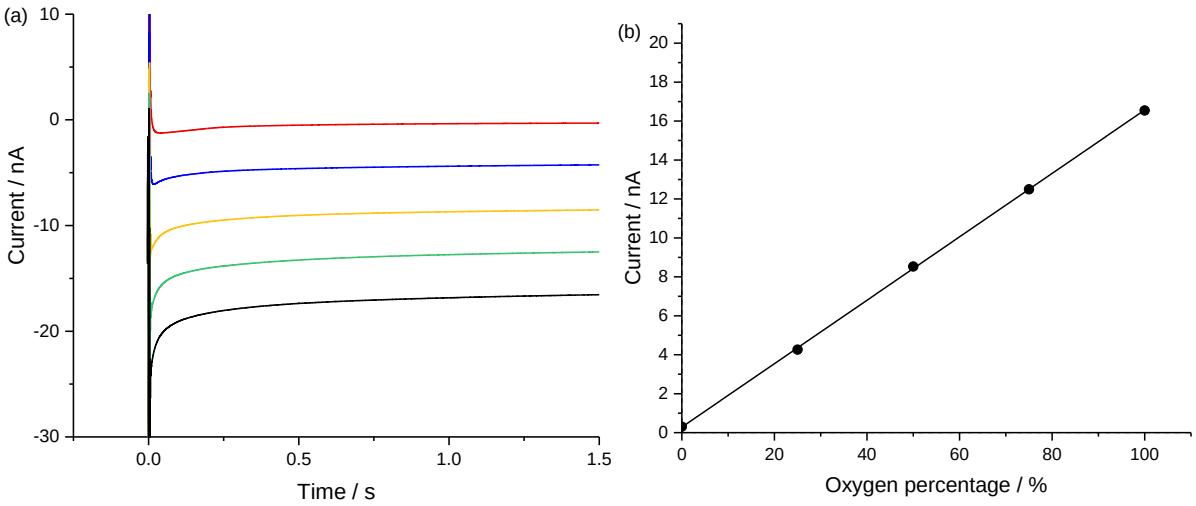

Figure 3 (a) Chronoamperometric responses at a Pt microdisc electrode (r=5.05 µm) in 0.1 M NaCl with various O_2 percentages at 25 °C (0% - red, 25% - blue, 50% - yellow, 75% - green, 100% - black). The applied potential used was -0.65 V. (b) Linear fitting of the magnitude of chronoamperometric current at 1.5s from (a) as a function of oxygen percentage in 0.1 M NaCl. The slope of the linear fitting is 1.63×10^{-10} A per percent of oxygen.

3.2.3 Chronoamperometric study in sheep's blood

Similar experiments were conducted at a Pt microdisc electrode in sheep's blood with variable oxygen percentages at 25 °C. In particular a potential of -0.65 V was applied to the electrode for a time duration of 1.5s. The resulting chronoamperometric responses are shown in Figure 4a) where the steady state currents increased with increasing oxygen percentage. Figure 4b) depicts the correlation between the cathodic steady state current at 1.5s and the oxygen content of the sheep's blood; a linear relationship was observed across the whole range studied. The linear relationship indicates this developed method is potentially analytically useful and reliable for direct oxygen measurement with a 'naked' electrode in whole blood without requiring the use of protecting layer on the electrode surface as has been usually employed in most blood gas sensors[9, 13].

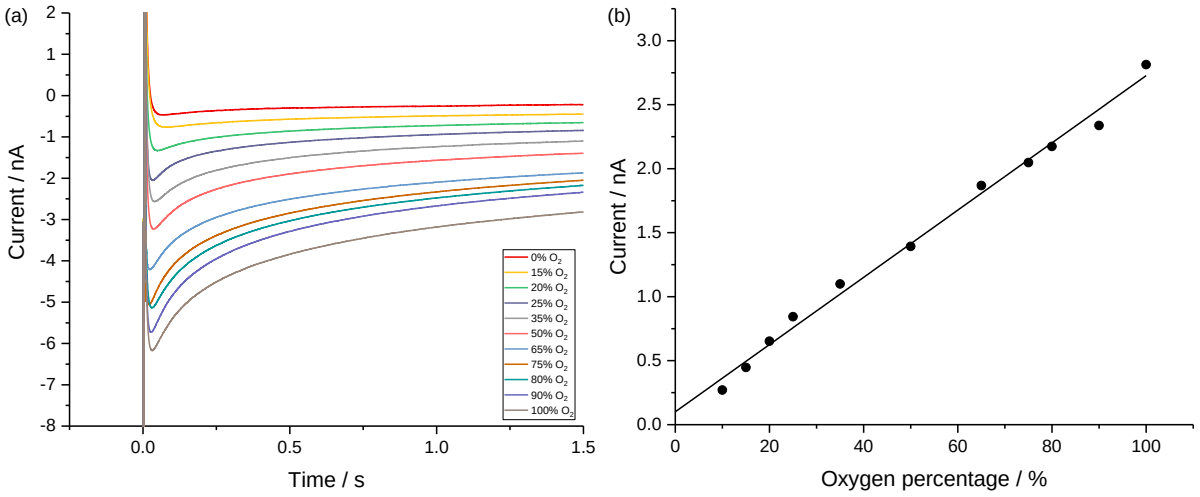

Figure 4 (a) Chronoamperometric responses at a Pt microdisc electrode (r=5.05 μm) in sheep's blood with various O_2 percentages at 25 °C. The applied potential was -0.65 V. (b) Linear fitting of the magnitude of chronoamperometric current at 1.5s from (a) as a function of oxygen percentage in sheep's blood. The slope of the linear fitting is 2.63×10^{-11}A per percent of oxygen.

The diffusion coefficient of free oxygen in whole blood is reported in the literature to be 1.60×10^{-9} m^2 s^{-1}.[40] However in blood almost all the oxygen is reversibly bound to haemoglobin, so called oxyhaemoglobin with a reported diffusion coefficient of 1.7×10^{-11} m^2 s^{-1}, only ca. 2% of oxygen is 'freely' dissolved in blood plasma and red blood cell water.[34, 40]

To understand the magnitude of the currents in Figure 2b), two limiting cases were considered assuming the total concentration of oxygen in O_2-saturated sheep's blood is 1 mM based on its reported solubility in blood with an averaged haemoglobin content of 15.5 g per 100 mL[30]: 1) all the oxygen in the blood is hypothetically 'free' (i.e. not combined to haemoglobin) and the reductive current measured assumed to come from 'free' oxygen, giving a calculated value of n_{app} was 0.84±0.01 ; 2) if all oxygen in the blood was combined to haemoglobin to form oxyhaemoglobin which diffused to the interface and was directly reduced, the value of n_{app} was calculated to be 79.4±0.9. The unrealistically low value of the first estimate and the absurdly high value of n_{app} from the latter coupled with the known speciation of O_2 in blood as being bound to haemoglobin indicates the likely chemical process (Equation 4) controls the currents flowing where the oxyhaemoglobin dissociates to form 'free' oxygen which is then reduced at the electrode surface with the oxyhaemoglobin, Hb_4O_8, assumed not to be electroactive. It is reported that each haemoglobin unit can bind up to four oxygen molecule and the dissociation of the first bound oxygen is shown in Equation 4:

$$Hb_4O_8 \underset{k'}{\overset{k}{\rightleftharpoons}} \mathrm{Hb}_4O_6 + O_2, k'/k = K \qquad \text{Equation 4}$$

where k is the dissociation rate constant (s^{-1}), k' is the re-combination rate constant (M^{-1} s^{-1}) and K is the corresponding equilibrium constant. The kinetics of dissociation has been extensively studied

revealing that the value of k is essentially pH independent over the range of 7.1 to 9.1.[32, 33] The average values of k and K were measured to be about 30 s^{-1} and 1×10^6 at pH 7.1 at 19 °C, respectively and the corresponding k' was ca. 3×10^7 M^{-1} s^{-1} when the concentration of haemoglobin in blood was 2.5 mM.[32, 33] Thus the majority of oxygen in blood exists in the form of oxyhaemoglobin whilst the direct reduction of oxyhaemoglobin at a Pt electrode seemed impossible considering the extremely high and implausible value of n_{app} (79.4±0.9). The electrochemical behaviour of oxygen reduction at a Pt electrode however, is best explained by a 'CE' process as mentioned above that the dissociation of oxyhaemoglobin is necessary before the electrochemical reduction of the freed oxygen; the existence of the preceding chemical reaction is reflected in the higher overpotential needed for oxygen reduction in sheep's blood compared to that in saline solution at a Pt macroelectrode (Figure 2b). Under this scenario the current is controlled by the diffusion coefficients of both free oxygen and oxyhaemoglobin with the linear calibration (Figure 4b) suggesting that the dissociation proceeds quantitatively at the electrode used with 5 micron in radius (r). This is consistent with the effective diffusional rate constant of ca. 1 s^{-1} for D/r^2 which is slow compared to the value of 30 s^{-1} mentioned above for the rate of dissociation of oxygen from oxyhaemoglobin. Note the implication that for very small microelectrodes, below, one micron in size the measured currents may become kinetically controlled. Future work will address the dissociate CE process for species with markedly different diffusion coefficients.

3.3 Validation of direct oxygen measurement in sheep's blood

This developed method was then validated in sheep's blood samples from three different batches. During the validation process, each sample was air-saturated by purging air for at least 20 minutes prior to measurements. A potential of -0.65 V was then applied to the working electrode for 1.5s and the corresponding chronoamperogram was then recorded for each sample. The value of measured current at 1.5s was averaged from three individual measurements and the corresponding error was also calculated and tabulated in Table 1. According to the calibration curve extracted in the previous section, the oxygen percentages in sheep's blood samples was then calculated the values of which were consistent with the one given by the supplier (21%±0.5% oxygen, balance with nitrogen). The observed consistency indicates the possibility of using a bare Pt microelectrode for direct oxygen sensing in whole blood. However, the chronoamperometric current dropped by ca. 30% if a second chronoamperogram was immediately recorded probably due to the adsorption of protein from the blood onto the electrode surface (SI section 3). Therefore, the method is best employed in the form of a point-of-use 'disposable' renewable oxygen sensor.

Table 1 Experimental chronoamperometric current and their calculated O_2 percentages in sheep's blood samples from three different batches. All samples were saturated with air prior to measurement. Measurement for each sample was repeated three times. The applied potential during chronoamperometry is -0.65 V which was hold for 1.5s.

Sheep's blood	I(E_{chrono}=-0.65V, t=1.5s) / nA	Calculated O_2 percentage
Bottle 1	0.68 ± 0.06	21.1 ± 1.3%
Bottle 2	0.64 ± 0.03	20.4 ± 1.4%
Bottle 3	0.65 ± 0.04	20.8 ± 1.1%

*Value given by the supplier for the compressed air composition: 21%±0.5% oxygen, balance with nitrogen.

4. Conclusions

An amperometric sensor based on a bare Pt microdisc electrode for oxygen measurement in sheep's blood was developed without the need of protective membrane on the electrode surface which has been commonly used in traditional gas sensors. The Pt microelectrode coupled with the use of chronoamperometry was investigated in both saline solution and sheep's blood, showing a linear current response and this developed method was validated in different batches of air-saturated sheep blood samples, giving an error in oxygen percentage of ca. 1.3%. More importantly, the electrochemical behaviour for oxygen reduction in sheep's blood was investigated which was believed to be the first 'direct' voltammetry study. A CE process (chemical-electrochemical process) was proposed for the oxygen reduction in blood in which the oxyhaemoglobin dissociates to form free oxygen prior to the electrochemical reduction, resulting in a higher overpotential required for oxygen reduction in sheep's blood than that in saline solution. To summarise, this work presents an 'membrane-free' amperometric sensing method with a good accuracy which is applicable for the point-of-use oxygen sensor; the proposed CE mechanism for oxygen reduction in blood provides fundamental but vital knowledge for any further study in blood. Further theoretical understanding of the CE mechanism is under investigation with the help with machine learning.

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