VIP Electrochemistry Very Important Paper

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# Electrochemical Red Blood Cell Counting: One at a Time

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***Abstract:*** *We demonstrate that the concentration of a red blood cell solution under physiological conditions can be determined by electrochemical voltammetry. The magnitude of the oxygen reduction currents produced at an edge-plane pyrolytic graphite electrode was diagnosed analytically at concentrations suitable for a point-of-care test device. The currents could be further enhanced when the solution of red blood cells was exposed to hydrogen peroxide. We show that the enhanced signal can be used to detect red blood cells at a single entity level. The method presented relies on the catalytic activity of red blood cells towards hydrogen peroxide and on surface-induced haemolysis. Each single cell activity is expressed as current spikes decaying within a few seconds back to the background current. The frequency of such current spikes is proportional to the concentration of cells in solution.*

The growing need for replacement of conventional medicinal diagnostics and biosensing methods with practical devices is at the heart of modern technology.[1,2] For example, replacing red blood cell counting by imaging (with optical microscopes) with faster, more accurate, and automated techniques can provide a fertile ground for the realization of a point-of-care test devices. These can be potentially used for a rapid and cost-effective diagnostic of blood-related diseases such as anaemia,[3] diabetes,[4] acatalasemia,[5] and vitamin E deficiency.[6] The applications of electrochemical methods for these purposes are advantageous mostly because of the practical characteristics of electrochemical devices notably fast response, rapid diagnostic, and cost-effective materials which can be used in situ and often at point of care. Recently, few reports have suggested that red blood cells (RBC) can be electrochemically detected directly on various electrodes.[7–10] Herein, an analytical method for a fast count of red blood cells is presented, under conditions suitable for a point-of-care test. Further, importantly and newly, we exploit the catalytic response of RBC towards hydrogen peroxide which is hitherto yet to be explored electrochemically. The fast response of the cells towards the decomposition of hydrogen peroxide together with the use of a state-of-the-art nano-impact technique allowed us to detect the individual cells near a microelectrode. Using this technique, simultaneous information on unknown RBC concentrations can be gained as well as cell activity towards $H_2O_2$. We first investigated the electrochemical response arising from RBC immobilized on an edge-plane pyrolytic graphite (EPPG, $R = 1.5$ mm) serving as a working electrode. EPPG is compatible with biological molecules[11] thus eliminating complexity from additional supporting materials such as nafion,[7,8] or graphitized mesoporous carbon.[12] Specifically, facile electron transfer of the FeIII/FeII redox couple of haemoglobin on EPPG was demonstrated by Toh at el.[13] In all measurements reported here, a platinum wire and a saturated calomel electrode (SCE) were used as a counter electrode and a reference electrode, respectively. To mimic a clinical condition environment, a 20 mM PBS solution at pH 7.4 under no degasification (solution of air atmosphere was present) was used in all measurements (see the Supporting Information for further details). In all experiments, the 100% solution of sheep red blood cells (Patricell) was diluted at least to 25% in order to decrease the solution viscosity, avoid agglomeration and to obtain a homogeneous film for drop-cast experiments. The electrochemical response of various concentrations of sheep red blood cells drop-casted on an EPPG electrode is shown in Figure 1 a. The bare electrode showed a small electrochemical

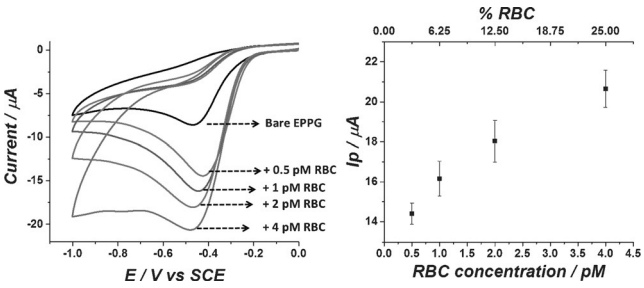


***Figure 1.*** a) Voltammetry of a 5 μL RBC drop-cast on an EPPG electrode, from solutions with designated initial concentration. b) The peak current (absolute values) as a function of initial RBC concentration drop cast. For all measurements a 50 $mVs^{-1}$ scan rate was used.

reduction response because of oxygen in solution (black upper curve, $[O_2] = 0.25$ mM[14]), in good agreement with the expected theoretical value (see calculations in the Supporting Information, Figure S1). After drop-casting the red blood cells on an EPPG electrode, the oxygen reduction current was enhanced. As can be seen from Figure 1 b, the magnitude of current peak ($I_p$) was proportional to the concentration of the RBC drop-casted at a fixed volume of 5 μL. It is evident that a solution containing an unknown concertation of RBC can be analytically diagnosed using this electrochemical method.

The enhancement of the oxygen reduction currents is a result of excess oxygen inside the cell. This is consistent with

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previous results that demonstrated direct oxygen reduction using other electrodes modified with RBC or complete blood.[7,8,12,15] Here, we show that this is true for solutions containing high RBC concentrations as well. The RBC concentration in the human blood is around 50%[16] (ca. 8 pM), which supports the rational for using only lightly diluted samples (up to 10 times) for the purpose of fast analytical diagnostics.

The oxygen reduction signal can be further enhanced by the cells catalytic reaction towards hydrogen peroxide. The decomposition of hydrogen peroxide to oxygen is fast under high RBC concentrations and is caused mainly by catalase activity within each red blood cell [Eq. (1)].[17]

$$2\,H_2O_2 + RBC \rightarrow O_2 + 2\,H_2O \qquad (1)$$

The latter process is unexplored electrochemically and the produced oxygen can be tracked electrochemically.

Thus, we have performed another set of experiments in which RBC solutions were exposed to hydrogen peroxide. The EPPG electrode was inert to hydrogen peroxide reduction under this potential window and only produced a small reductive current caused by the atmospheric oxygen concentration in the solution (Figure 2a, black upper curve). The

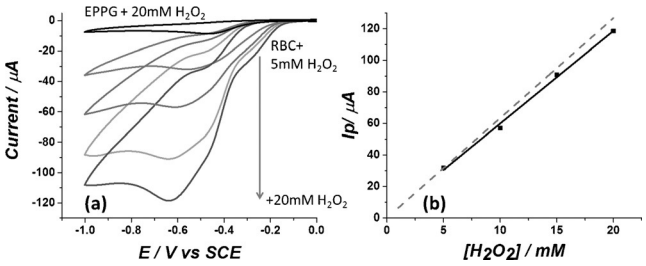


***Figure 2.*** a) Voltammetry of a solution containing 4 pM RBC (25%) with 5, 10, 15, and 20 mM $H_2O_2$ on an EPPG electrode. b) The peak current (absolute values) as a function of the hydrogen peroxide concentration (black squares). For all measurements a scan rate of 50 mV s$^{-1}$ was used. The dashed line corresponds to the theoretical peak current arising from an oxygen solution with a concentration of $[0_2] = {}^1/_2\,[H_2O_2]$. For the theoretical calculation a transfer coefficient of $\alpha = 0.4$[18] and oxygen diffusion coefficient of $D_{O2} = 1.8 \times 10^{-9}$ m$^2$ s$^{-1}$ were used.[19]

electrochemical response of a solution containing a fixed concentration of RBC in solution and various concentration of hydrogen peroxide is shown in Figure 2a. The peak currents (Figure 2b, black squares) are linear with respect to the hydrogen peroxide concentration and are consistent with the theoretical peak current arising from a solution with oxygen concentrations of $[0_2] = {}^1/_2\,[H_2O_2]$ (dashed line). The results presented here are complementary to the oxygen reduction currents produced by the cells (Figure 1) as they hold information both on the number and the activity of the cells.

Since the response of the RBC towards hydrogen peroxide enhances substantially the oxygen reduction current magnitude, we have examined the possibility of detecting individual red blood cells. For this we have used the emerging nanoimpact technique,[20–23] a carbon fibre wire as a working electrode and a home-made potentiost.[23] Here, in addition to the voltammetric measurements, we have used "chronoamperometry" (current as a function of time at a fixed potential) as a way to detect transient events in an ultrasensitive fashion. We replaced the EPPG macroelectrode with a carbon wire ($l = 1$ mm, $R = 3.5$ µm) in order to sufficiently decrease the noise level of the currents effected by the electrode area.[24,25] As can be seen in Figure 3a, a solution containing RBC

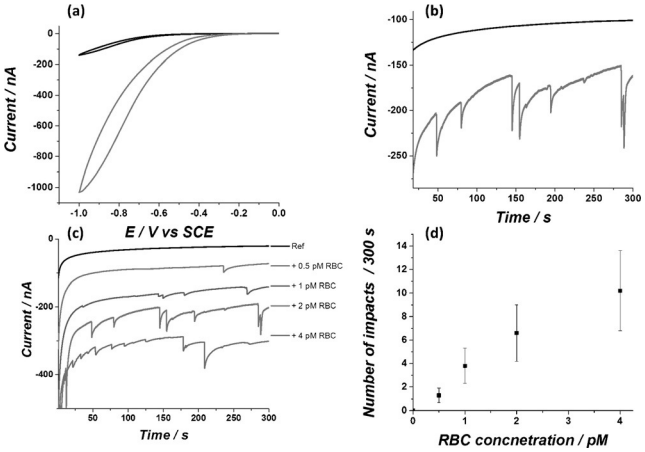


***Figure 3.*** a) Voltammetry of a solution containing 20 mM $H_2O_2$ (black) and with 2 pM RBC (gray) on a carbon wire. Voltammograms were performed under a 50 mV s$^{-1}$ scan rate. b) Chronoamperograms of a 20 mM hydrogen peroxide solution (black curve) and with 2 pM RBC (gray curve) under an applied potential of −0.8 V versus SCE. c) Chronoamperograms of 20 mM hydrogen peroxide with various concentrations of RBC in solution under an applied potential of −0.8 V versus SCE. The y axis was offset for clarity. d) Average spike frequencies during a 300 second scan as a function of RBC concentration.

produced larger currents from oxygen reduction (lower curve, $\approx -1$ µA at −1 V), compared to a solution without RBC (upper curve). This is consistent with the results shown in Figure 2a and is a direct cause of the fast decomposition of $H_2O_2$ into oxygen, by the RBC in solution. The higher oxygen concentration in the solution is manifested by the enhanced oxygen reduction currents produced under a reductive scan. The chronoamperometric measurements of a 20 mM $H_2O_2$ solution without RBC showed a smooth, featureless chronoamperogram (Figure 3b, upper black curve) under applied potential of −0.8 V versus SCE. However, when a 20 mM $H_2O_2$ was injected into RBC solution, large current spikes were detected (Figure 3b, lower curve). Each individual event showed a fast decrease in the current magnitude followed by a long decay to the background current. We have verified that the nature of the spikes is not caused by oxygen bubbles or from a solution containing equivalent concentration of oxygen (see Figure S2). We also verified that under insufficient reductive potential or without hydrogen peroxide, no current spikes are seen (see Figure S3). Therefore, we can ascribe each current spike to an individual RBC catalytic activity when colliding with the electrode. A catalytic cycle is formed by the hydrogen peroxide produced locally at the electrode which is further decomposed by the RBC located at

  

close proximity to the electrode to form oxygen and water. In order to reinforce our interpretation we have performed the same experiments under various concentrations of RBC in solution, with a fixed concentration of $H_2O_2$. It is evident from Figure 3c, that the frequencies of the current spikes increased when the concentration of the RBC in the solution were increased. The average spike frequencies observed over a 300 second scan under various RBC concentrations are shown in Figure 3d.

The typical current transient seen in Figure 4a is asymmetric and reflects a sharp current response that decays into

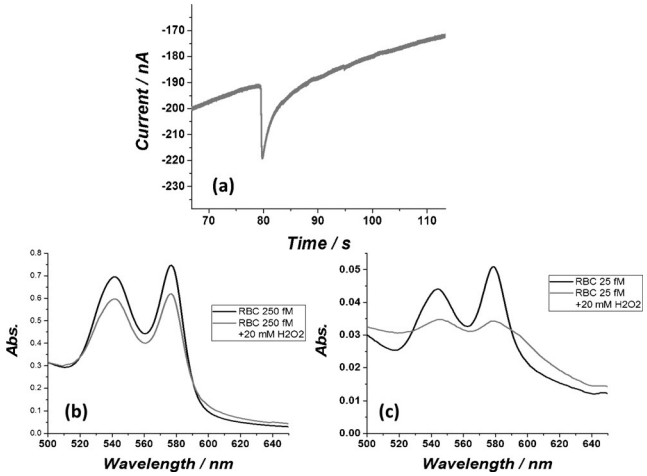


***Figure 4.*** a) Representative chronoamperometric spike produce from a RBC solution with hydrogen peroxide. UV/Vis spectra of b) 250 fM and c) 25 fM RBC solution with (gray) and without (black) 20 mM hydrogen peroxide. The measurements were taken two minutes after injection.

the background current in few seconds. The origin of the spike shape can be explained by a surface induced haemolysis of the cell when impacting the electrode. A probable cause could be the high concentration of hydrogen peroxide produced locally near the electrode surface. This reason is further reinforced by the UV/Vis absorption of RBC after exposure to 20 mM $H_2O_2$. As can be seen in Figure 4b,c. A haemolytic process occurs, expressed by attenuation of the typical absorption peaks around 575 and 540 nm. The attenuation of the absorption peak corresponds to degradation of oxyhemoglobin within the cell.[26] The haemolytic process is more pronounced when the cell concentration is decreased. This observation is consistent with previous reports which showed that the $H_2O_2$:RBC molarity ratio is a dominant parameter for causing cell lysis upon reaction with hydrogen peroxide.[26]

A clear evidence for hydrogen-peroxide-induced haemolysis was achieved in real time using an optical microscope. Images were obtained with an Axio examiner A1 microscope and ZEN 2 software (Carl Zeiss). The RBC (see Figure S4 for SEM images) were stable with no apparent haemolysis when mounted on a microscope slide (video 1a). However, upon exposure of low concentration RBC solution (0.5%, ca. 80 fM) to 20 mM $H_2O_2$ in PBS, a clear disappearance of the cells with time is seen, reflecting the haemolytic process (see video 1b). In our case, the high concentration of hydrogen peroxide is decomposed rapidly to form a supersaturated oxygen solution. Upon applying a sufficient reductive potential, a high concentration of $H_2O_2$ is reproduced locally near the electrode surface. Hence, only cells that are in close proximity to the electrode are prone to lysis and can release hydrogen peroxide scavengers near the electrode which eventually diffuse away.

In conclusion, a simple method for fast electrochemical counting of red blood cells is presented. A solution with an unknown concentration of cells can be drop cast on an electrode and the oxygen reduction current produced can be analytically diagnosed to give the number of cells in the solution with high accuracy. The conditions used here are suitable for particle clinical needs and do not require any additional components. In addition, we investigate for the first time the electrochemical response of a RBC solution to hydrogen peroxide. Using current–time measurements we show that single-cell catalytic activity can be seen. The single cell activity towards hydrogen peroxide may be further exploited to produce information on the concentrations of enzymes responsible for hydrogen peroxide catalysis on a single cell level. The method presented here hold promise for a number of practical biomedical uses and can be combined with recent artificial functionalities of RBC.[27]

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[1] L. Bissonnette, M. G. Bergeron, *Clin. Microbiol. Infect.* **2010**, *16*, 1044–1053.
[2] P. B. Luppa, C. Müller, A. Schlichtiger, H. Schlebusch, *TrAC Trends Anal. Chem.* **2011**, *30*, 887–898.
[3] A. Karnad, T. R. Poskitt, *Arch. Intern. Med.* **1985**, *145*, 1270–1272.
[4] A. V. Buys, M.-J. Van Rooy, P. Soma, D. Van Papendorp, B. Lipinski, E. Pretorius, *Cardiovasc. Diabetol.* **2013**, *12*, 25.
[5] D. L. Góth, P. Rass, A. Páy, *Mol. Diagn.* **2004**, *8*, 141–149.
[6] M. Miki, H. Tamai, M. Mino, Y. Yamamoto, E. Niki, *Arch. Biochem. Biophys.* **1987**, *258*, 373–380.

  

[7] Y. Ayato, K. Sakurai, S. Fukunaga, T. Suganuma, K. Yamagiwa, H. Shiroishi, J. Kuwano, *Biosens. Bioelectron.* **2014**, *55*, 14 – 18.
[8] R. J. Toh, W. K. Peng, J. Han, M. Pumera, *Sci. Rep.* **2014**, *4*, 6209.
[9] A. Doménech-Carbó, E. Villamón, I. Luna, D. Ramos, C. Doménech-Casasús, G. Cebrián-Torrejón, *Sens. Actuators B* **2016**, *226*, 419 – 428.
[10] Y.-X. Ci, H.-N. Li, J. Feng, *Electroanalysis* **1998**, *10*, 921 – 925.
[11] C. F. Blanford, F. A. Armstrong, *J. Solid State Electrochem.* **2006**, *10*, 826 – 832.
[12] K. Amreen, A. S. Kumar, *Analyst* **2016**, *141*, 2145 – 2149.
[13] R. J. Toh, W. K. Peng, J. Han, M. Pumera, *RSC Adv.* **2014**, *4*, 8050 – 8054.
[14] J. E. Sherwood, F. Stagnitti, M. J. Kokkinn, W. D. Williams, *Int. J. Salt Lake Res.* **1992**, *1*, 1 – 6.
[15] C. Ribaut, K. Reybier, B. Torbiero, J. Launay, A. Valentin, O. Reynes, P.-L. Fabre, F. Nepveu, *IRBM* **2008**, *29*, 141 – 148.
[16] C. Starr, B. McMillan, *Human Biology*, 11th ed., Cengage Learning, Boston, MA, USA, **2015**.
[17] G. F. Gaetani, A. M. Ferraris, M. Rolfo, R. Mangerini, S. Arena, H. N. Kirkman, *Blood* **1996**, *87*, 1595 – 1599.
[18] M. Gara, R. G. Compton, *New J. Chem.* **2011**, *35*, 2647 – 2652.
[19] L.-K. Ju, C. S. Ho, *Biotechnol. Bioeng.* **1985**, *27*, 1495 – 1499.
[20] M. Pumera, *ACS Nano* **2014**, *8*, 7555 – 7558.
[21] N. V. Rees, *Electrochem. Commun.* **2014**, *43*, 83 – 86.
[22] W. Cheng, R. G. Compton, *TrAC Trends Anal. Chem.* **2014**, *58*, 79 – 89.
[23] C. Batchelor-McAuley, J. Ellison, K. Tschulik, P. L. Hurst, R. Boldt, R. G. Compton, *Analyst* **2015**, *140*, 5048 – 5054.
[24] J. Yao, K. D. Gillis, *Analyst* **2012**, *137*, 2674 – 2681.
[25] J. Ellison, C. Batchelor-McAuley, K. Tschulik, R. G. Compton, *Sens. Actuators B* **2014**, *200*, 47 – 52.
[26] M. Mino, Y. Nishida, K. Murata, M. Takegawa, G. Katsui, Y. Yuguchi, *J. Nutr. Sci. Vitaminol.* **1978**, *24*, 383 – 395.
[27] Z. Wu, T. Li, J. Li, W. Gao, T. Xu, C. Christianson, W. Gao, M. Galarnyk, Q. He, L. Zhang, et al., *ACS Nano* **2014**, *8*, 12041 – 12048.

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