Electrochemical impedance spectroscopy to study physiological changes affecting the red blood cell after invasion by malaria parasites

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\textbf{A B S T R A C T}

The malaria parasite, \textit{Plasmodium falciparum}, invades human erythrocytes and induces dramatic changes in the host cell. The idea of this work was to use RBC modified electrode to perform electrochemical impedance spectroscopy (EIS) with the aim of monitoring physiological changes affecting the erythrocyte after invasion by the malaria parasite. Impedance cell-based devices are potentially useful to give insight into cellular behavior and to detect morphological changes. The modelling of impedance plots (Nyquist diagram) in equivalent circuit taking into account the presence of the cellular layer, allowed us pointing out specific events associated with the development of the parasite such as (i) strong changes in the host cell cytoplasm illustrated by changes in the film capacity, (ii) perturbation of the ionic composition of the host cell illustrated by changes in the film resistance, (iii) releasing of reducer (lactic acid or heme) and an enhanced oxygen consumption characterized by changes in the charge transfer resistance and in the Warburg coefficient characteristic of the redox species diffusion. These results show that the RBC-based device may help to analyze strategic events in the malaria parasite development constituting a new tool in antimalarial research.

1. Introduction

The malaria parasite, \textit{Plasmodium falciparum}, invades human erythrocytes and induces dramatic changes in membrane fluidity, permeability, deformability, and adhesiveness of the host cell. The 48-h developmental cycle in the erythrocyte can be divided into three distinct stages. The first one (ring to trophozoite, 0–24th hour) corresponds to the preparation of the first nuclear division. The communication and degradation pathways are slowly settled by the parasite and haemoglobin degradation begins. The second phase corresponds to the first nuclear division (early schizont, 24–30th hour), while haemoglobin degradation is increased, leading to hemozoin production by haem biocrystallization. The third phase (old and segmented schizont, 30–48th hour) corresponds to the next nuclear division and leads to erythrocyte membrane disruption releasing 16–32 free merozoites ready to invade more intact red blood cells. \textit{In vitro}, \textit{P. falciparum} cultures can be synchronized for short periods (4 h) by the combination of selective concentration and/or lysis. Such a synchronization has allowed the analysis of various events during the cycle (differential expression of mRNA, hemozoin production, transient chemicals production, etc.). As the erythrocyte is a non-nucleated cell, a considerable number of the signals recorded in parasitized red blood cells is imputable to the parasite.

The aim of the work was to use a red blood cell-based device to monitor changes affecting the cells caused by invasion of \textit{Plasmodium}. In a previous paper, we described a strategy to immobilize red blood cells onto a gold electrode (Ribaut et al., 2008b) in a reproducible manner. The idea was to use the modified electrode to perform electrochemical impedance spectroscopy (EIS). Impedance cell-based sensor arrays that were first described by Giaever and Keese (1984), are potentially useful to give insight into cellular behavior, to detect morphological changes (Arndt et al., 2004; Yang et al., 2007), to study cell adhesion (De Blasio et al., 2004) attachment and spreading (Xiao et al., 2002; Luong et al., 2001; Ehret et al., 1997), alterations of the physiological state or to test the efficiency of drugs (Otto et al., 2004) or effectors (Tilili et al., 2003; Tiruppathi
et al., 1992; Nguyen et al., 2004; Xiao and Luong, 2003) (environmental sensing). The physical background to the technique is based on the electrically insulating effect of cell membranes at low frequencies. The cellular sensing method allows real-time monitoring of cells, avoids the use of labelled molecules and does not interfere with cell metabolism in vitro.

In this paper, we apply for the first time, electrochemical impedance spectroscopy (EIS) to the red blood cell (RBC) with the aim of monitoring the physiological changes affecting the cell after invasion by the malaria parasite. The first part of this paper briefly describes the immobilization process whereas the second part concerns the differentiation between healthy and parasitized red blood cells by EIS.

2. Materials and methods

2.1. Materials

11-Mercaptoundecanoic acid (MUA), 6-mercapto-1-hexanol (MH), N-hydroxysuccinimide (NHS), N-ethyl-N′-(dimethylamino)propyl)-carbodiimide (EDC), phosphate buffered saline and protein G solution were purchased from Sigma–Aldrich. The anti-D (IgG) antibody Lorisix was supplied by Eurobio and absolute ethanol from Fisher Scientific. Ultrapure water was obtained using a Milli-Q water system (Simplicity, Millipore). RPMI 1640 was purchased from BioWhittaker. Red blood cells (RBC) and human serum albumin G solution were purchased from Sigma–Aldrich. The anti-D (40/IL9262) was purchased from the EFS (Etablissement Français du Sang, Toulouse, France) and Heps were from Lonza.

2.2. Methods

2.2.1. Electrode manufacturing process

The microelectrodes were designed using microelectronic mass fabrication processes as described in previous paper by metalization of pyrex wafer (Ribaut et al., 2008b). Hydrophobic PSX patterned micro-tanks were designed to prevent the spread of drops of aqueous solutions used for immobilization out of the sensitive surface.

2.2.2. Electrode modifications

The microelectrodes were prepared as previously described (Ribaut et al., 2008b). Briefly, the electrodes were modified by a self-assembled monolayer of mixed thiols (1 mM MUA/10 mM MH). After esterification of terminal groups by NHS and EDC (15 mM NHS/75 mM EDC), a layer of protein G (100 μg/ml) was deposited on the modified gold electrode. The same procedure was employed for grafting layers, whereas the impedance spectra of the cells are more curved. At first, the spectra of the layers show a high resistance of charge transfer (around 400 MΩ) which means that the exchange current at equilibrium was quite low and traduces the lack of redox couples. On the contrary, the spectra of the erythrocytes show a lower resistance of charge transfer. Furthermore, one can note that parasitized RBC present different properties in comparison with the normal ones.

It is important to note that considerable differences in equilibrium potentials were recorded for healthy (Eeq = 60 mV ± 20 mV) or parasitized (Eeq = −140 mV ± 50 mV) RBC. The equilibrium potential is a mixed potential between the different redox couples of the system electrode-medium: the most reducing and the most oxidizing species. Dioxgen O2 is always present and must be the oxidant while the reducer may be from the RBC because without the RBC there is no exchange current. The cathodic displacement of the free potential between healthy and parasitized RBC (pRBC) implies the appearance of a new more powerful reductant while dioxgen which is always present is the oxidizer.

2.2.3. Parasite culture and magnetic separation

Parasites were cultured according to the method described by Trager and Jensen (1976) with modifications described by Benoit et al. (1995). To enrich the in vitro cultures of Plasmodium, red blood cells were then transferred onto an LD-column placed in a Midi MACS magnet. This magnetic separation method allows an enrichment of up to 95% parasitemia (Ribaut et al., 2008a).

2.2.4. Electrochemical impedance spectroscopy

Electrochemical experiments were performed using a Voltalab 80 PGZ 402 and the voltamaster 4 software with a conventional three-electrode cell including a saturated calomel electrode (SCE) as the reference electrode, a gold electrode (0.255 cm2) as the counter electrode and the modified gold electrode (0.053 cm2) as the working electrode. The impedance spectra were recorded in culture medium (RPMI containing 12.5%, v/v of HEPS 1 M) in a frequency range from 50 kHz to 100 mHz at the free potential of the solution with an amplitude of the alternating voltage equal to 10 mV.

3. Results and discussion

3.1. Immobilization of red blood cells

As described in a previous paper (Ribaut et al., 2008b), a special method to graft red blood cells based on antigen/antibody cross-linking and a self-assembled monolayer (SAM) has been developed in our laboratory and has been used in these studies.

The immobilization of normal RBC was then simply performed by depositing a drop of medium containing red blood cells on the modified gold electrode. The same procedure was employed for parasitized red blood cells after a concentration step based on the magnetic properties of products generated by the infection (Ribaut et al., 2008a) since in normal cultures the parasitaemia does not exceed 8%, which was not sufficient to reveal differences between layers of healthy or parasitized RBC by impedance. The concentration process achieved a parasitaemia close to 95%, thus guaranteeing a signal characteristic of the infection. The electrodes modified by cellular layers of healthy or parasitized RBC were then used as working electrodes for electrochemical impedance spectroscopy.

3.2. Impedance spectroscopy

Impedance measurements were carried out in the frequency range 50 kHz to 100 mHz at the equilibrium potential at each stage of the modification process. Nyquist diagrams are presented in Fig. 1a. A significant difference in the impedance spectra is observed in the presence of normal or parasitized erythrocytes. Indeed, similar impedance spectra characterized by straight lines have been recorded for grafting layers, whereas the impedance spectra of the cells are more curved. At first, the spectra of the layers show a high resistance of charge transfer (around 400 MΩ) which means that the exchange current at equilibrium was quite low and traduces the lack of redox couples. On the contrary, the spectra of the erythrocytes show a lower resistance of charge transfer. Furthermore, one can note that parasitized RBC present different properties in comparison with the normal ones.

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In order to obtain information on the electrical properties of parasitized versus normal red blood cells, impedance plots have been fitted according to the classical Randles circuit: as usual Rs is the electrolyte resistance, Qdl is the double layer capacity (here a constant phase element), Rd is the charge transfer resistance and Zw is the Warburg impedance under non-stationary conditions. RsRd traduces the easiness of electronic transfer whereas
Z_w represents the resistance to the mass transfer, i.e. to the diffusion. The multilayer structure of the electrode is taken into account by addition in a serial configuration of R_i, resistance of the film, and Q_f, capacitance of the dielectric of the film. In fact, this multilayer structure could be considered as the sum of the characteristics of each layer. For simplicity, the model shown in Fig. 1b was chosen.

The fitted plots of RBC layers are presented as a line in Fig. 1a. The corresponding electrical values obtained for experiments performed the same day with 4 modified electrodes (4 with healthy and 4 with parasitized cells) are summarized in Table 1.

The resistance R_i of the electrolyte (470 Ω) is logically constant independently of the RBC state. The imperfect capacity of the film Q_f decreases between healthy (8 µF s⁻¹) and parasitized red blood cells (4 µF s⁻¹). From an electrical point of view, the capacity of the film Q_f, resistance of the film R_f, and capacity of the dielectric of the film Q_d should be constant; a decrease in Q_dim corresponds to a decrease in Q_BBC for parasitized RBC compared with healthy ones. The decrease originates from changes induced by the RBC infection, since after invasion, *P. falciparum* initiates a series of morphological and biochemical rearrangements within the host cell cytoplasm. The mature erythrocyte becomes among other things, as shown in Fig. 2a and b, a sack of haemoglobin with no endogenous protein export or protein synthesis machinery (Tillyea and Hanssen, 2008). The strong changes in the host cell cytoplasm and membrane may induce changes in the dielectric constant of the cellular layer, thus changing its capacity.

The resistance of the film R_i decreases for parasitized RBC compared with healthy ones from 22 to 12 KΩ. This evolution results from dramatic changes in the host cell membrane induced by the infection. To survive within a red blood cell, the malaria parasite alters the permeability of the host’s plasma membrane to accomplish nutrient uptake and disposal of waste products. The pathogen permeabilizes host erythrocytes for a large variety of solutes (Ginsburg et al., 1983; Kutner et al., 1982), including organic and inorganic anions (Ginsburg and Kirk, 1998; Kirk, 2001) cations (Duranton et al., 2003; Kirk et al., 1994; Staines et al., 2000, 2001), carbohydrates, amino acids, nucleosides and small peptides. In mammalian erythrocytes infected with malaria parasites there is a marked perturbation of the normal Na⁺/K⁺ levels (Saliba et al., 2006; Staines et al., 2007) in the cytosol. As every cell, the erythrocyte maintains a high intracellular K⁺ and low intracellular Na⁺ concentration. The Na⁺/K⁺ pump generates substantial opposing concentration gradients for both ions leading to a steady-state cytoplasmic [Na⁺]-to-[K⁺] ratio. Malaria parasites induce a marked perturbation of the normal Na⁺/K⁺ levels in mammalian erythrocytes, with amongst other things, a progressive increase in the Na⁺ concentration all over the cycle. The perturbation by the parasite of the ionic composition of its host cell could explain changes in the film resistance, rendering the cellular layer less insulating.

In the case of the electrochemical impedance of the Randles circuit, the imperfect capacity of the double-layer Q_d is the same for healthy and parasitized RBC (0.65 µF s⁻¹) whereas the resistance of the charge transfer R_t decreases from 2 to 1 MΩ. As \( i_{0} = \frac{k_{t}}{nF_{R}RC_{t}} \) (Bard and Faulkner, 2001), a reduction of the charge transfer resistance implies an increase in the exchange current \( i_{0} \) with \( i_{0} + i_{ox} = -i_{red} \) and \( i_{ox} + i_{red} > 0 \) at equilibrium potential. Thus, an increase in the \( i_{0} \) is necessarily linked to an increase in \( i_{ox} \) and \( i_{red} \). As explained above, the reduction current \( i_{red} \) comes from the reduction of oxygen. The increase in \( i_{red} \) is linked to the cathodic displacement of the free potential for parasitized RBC. However,
according to the Tafel law, a displacement of 200 mV of the equilibrium potential should induce an increase of $i_0$ by a factor of 40 for O$_2$ whereas in our case the current increases by only twofold, which implies that the oxygen concentration of the medium decreases for parasitized RBC compared with healthy ones. This result is in accordance with literature since Murphy et al. (1997) demonstrated that oxygen consumption was equal to 0.1 nmol/min for normal erythrocytes, whereas it reached 2.2 nmol/min in the case of Plasmodium infected cells. As far as $i_{red}$ is concerned, a displacement of the equilibrium potential towards the cathodic potential implies that, on one hand, the reducing species responsible for $i_{red}$ is different for parasitized and normal erythrocytes, and on the other hand, the oxidation of this reducer takes place at a lower potential. This new reducer has to be found in the molecular species released by the parasite or consequent to the RBC infection. This could be lactic acid that can be oxidized into pyruvic acid. The infected erythrocyte has 100-fold more intensive glycolytic activity than the healthy RBC to fulfil its substantial energy requirements (Elliott et al., 2001), ultimately converting glucose to lactic acid. Depending on the stage of development, parasitized red blood cells produced between 5 and 100 times more lactic acid than uninfected erythrocytes when cultured under identical conditions (Zolg et al., 1984). This enhanced activity would lead to an accumulation of lactic acid within the parasite cytosol and would threaten the osmotic stability of the cell (decrease in intracellular pH). For this reason the parasite has an efficient means of clearing lactic acid from its cytosol, leading to an increase in the lactic acid concentration in the erythrocyte. Given that measurements have been carried out for parasites at their latest stage of development, i.e. the schizont stage preceding membrane rupture, one can imagine that the extracellular medium contains low amounts of lactic acid.

Another source of reducing species released by the parasite is the haem (Fe(II)). To sustain its rapid development, the parasite digests host haemoglobin. While hydrolysis of haemoglobin makes amino acids available for parasite development, this process also releases the lipophilic prosthetic group haem, which is extremely toxic to the parasite. 75% of the toxic haem is detoxified by the parasite converting into an insoluble crystalline material called hemozoin (Pagola et al., 2000; Egan et al., 2002). The remainder appears to be degraded by a non-enzymatic process which leads to an accumulation of iron in the parasite. Even if alternative detoxication pathways, including haem degradation (Loria et al., 1999) or reaction with glutathione (Ginsburg et al., 1998; Garavito et al., 2007) or oxygen peroxide may also contribute to haem detoxification, one can imagine that a small amount escapes the neutralisation processes and could be detected as a reducing species.

The Warburg coefficient $\sigma$, which is related to the inverse of the Warburg impedance and corresponds to the mass transfer, increases for infected cells compared with normal ones. As illustrated in Eq. (1) given for a simple redox couple, an increase of $\sigma$ or/and reducer species originates from the decrease of the concentration of the oxidant and or/reducer species.

$$\sigma = \sigma_{ox} + \sigma_{red} = \frac{RT}{n^2 F^2 A \nu_0} \left( \frac{1}{C_p D_{ox}^{1/2}} + \frac{1}{C_p D_{red}^{1/2}} \right)$$

In order to evaluate the contribution of the oxidant and the reducer on the Warburg coefficient $\sigma$, $\sigma_{ox}$ was calculated. Considering that [O$_2$] = 2.5 × 10$^{-4}$ M (solubility of O$_2$ in water at 20 $^\circ$C) with a diffusion coefficient equal to 2 × 10$^{-5}$ cm$^2$/s (in water at 20 $^\circ$C) and that, as mentioned in a previous study, the working surface is only 2% of the naked electrode surface (surface coverage equal to 98%) (Ribaut et al., 2008b), $\sigma_{ox} = 2 \times 10^3 \Omega^{1/2}$ s$^{1/2}$ for normal RBC. As $\sigma = 2.9 \times 10^3 \Omega^{1/2}$ s$^{1/2}$ in this case and taking into account that the calculation has been performed assuming that culture medium is equivalent to water, it seems that the reduction of O$_2$ is predominant.