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

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A B S T R A C T

The malaria parasite, *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, *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 dis-

ruption releasing 16–32 free merozoites ready to invade more intact red blood cells. *In vitro*, *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 *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 (Tlili et al., 2003; Tirupathi

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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'*-(dimethylaminopropyl)-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 AB⁺ were provided by the EFS (Etablissement Français du Sang, Toulouse, France) and Hepes 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 in PBS) and then a layer of anti-D (40 µg/ml) were deposited on the surface.

The RBC (healthy or parasitized), suspended in serum-free RPMI 1640 medium to approximately 50% haematocrit, were deposited on the modified gold electrodes and incubated for 1.5 h at 37 °C in an atmosphere containing 5% CO₂. The electrodes were then immersed in RPMI 1640, and immediately placed in the impedance electrochemical cell for measurements.

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 voltmaster 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 HEPES 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.

It is important to note that considerable differences in equilibrium potentials were recorded for healthy ($E_{eq} = 60 \text{ mV} \pm 20 \text{ mV}$) or parasitized ($E_{eq} = -140 \text{ mV} \pm 50 \text{ mV}$) 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. Dioxygen 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 dioxygen which is always present is the oxidizer.

In order to obtain information on the electrical properties of parasitized versus normal red blood cells, impedance plots have been fitted according to the model circuit presented in Fig. 1b. The electrochemical system is described by the classical Randles circuit: as usual R_s is the electrolyte resistance, Q_{dl} is the double layer capacity (here a constant phase element), R_{ct} is the charge transfer resistance and Z_w is the Warburg impedance under non-stationary conditions. R_{ct} traduces the easiness of electronic transfer whereas

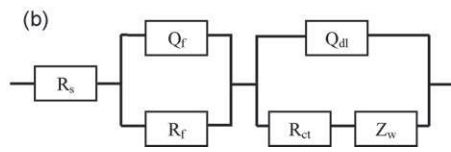
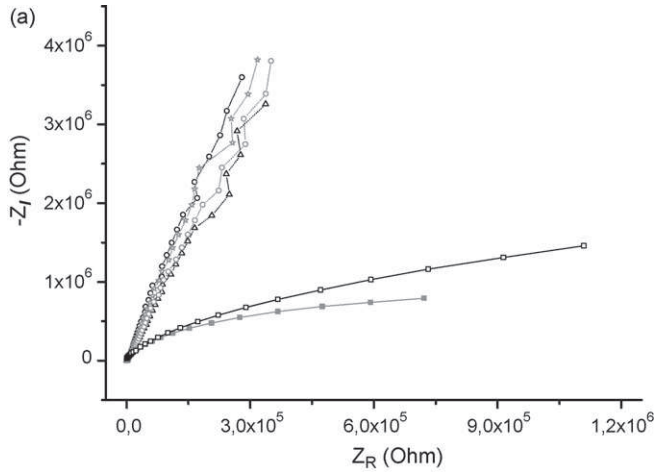


Fig. 1. (a) Nyquist plots of non-Faradic impedance spectra taken in RPMI in the frequency range 100 mHz to 50 kHz with (○) gold electrode, (★) step I: mixed SAMs modified gold electrode, (●) step II: addition of protein G on the thiol layer, (△) step III: blockage with anti-D, step IV: immobilization of normal (□) or parasitized (■) red blood cells. In the case of RBC layers, the symbols correspond to the experimental data (normal (□) and parasitized red blood cells (■)) and the line represents the simulated spectra with the parameters calculated by FRA software from the equivalent circuit model presented in (b), where R_s corresponds to the resistance of the solution, R_f to the resistance of the film, Q_f to the capacity of the film, Q_{dl} to the double layer capacity, R_{ct} to the resistance of charge transfer and Z_w to the Warburg impedance.

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_f , 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_s 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 $\mu\text{F s}^{n-1}$) and parasitized erythrocytes (4 $\mu\text{F s}^{n-1}$). From an electrical point of view, the capacity of the film can be considered as the sum in series of the capacity corresponding to each layer. The capacity of the film is given by the relation: $1/Q_{\text{film}} = 1/Q_{\text{SAM}} + 1/Q_{\text{PG}} + 1/Q_{\text{AD}} + 1/Q_{\text{RBC}}$. Taking into account that Q_{SAM} , Q_{PG} , Q_{AD} should be constant; a decrease in Q_{film} corresponds to a decrease in Q_{RBC} 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 mor-

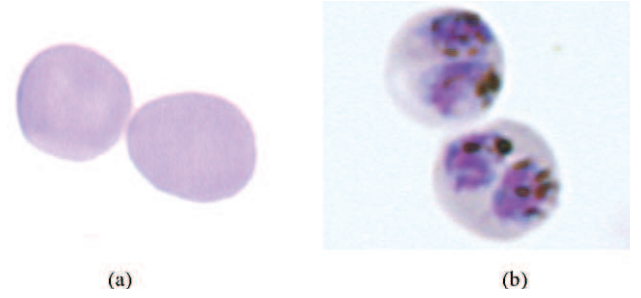


Fig. 2. Images ($\times 2000$) of normal (a) and parasitized (b) red blood cells both stained with Giemsa.

phological 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 (Tilleya 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_f decreases for parasitized RBC compared with healthy ones from 22 to 12 $\text{k}\Omega$. 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 $[\text{Na}^+]$ -to- $[\text{K}^+]$ 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_{dl} is the same for healthy and parasitized RBC (0.65 $\mu\text{F s}^{n-1}$) whereas the resistance of the charge transfer R_{ct} decreases from 2 to 1 $\text{M}\Omega$. As $i_0 = RT/nFR_{ct}$ (Bard and Faulkner, 2001), a reduction of the charge transfer resistance implies an increase in the exchange current i_0 with $i_0 = i_{\text{ox}} = -i_{\text{red}}$ and $i_{\text{ox}} + i_{\text{red}} = 0$ at equilibrium potential. Thus, an increase in the i_0 is necessary 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,

Table 1

Values of electrical components deduced from the modelling of Nyquist diagrams (Fig. 1a) according to the circuit model presented in Fig. 1b, R_s being the resistance of the solution, Q_f the capacity of the film, n the roughness coefficient related to the capacity, R_f the resistance of the film, Q_{dl} the double layer capacity, R_{ct} the charge transfer resistance and σ the Warburg coefficient.

R_s (Ω)	Q_f ($\mu\text{F s}^{n-1}$)	n	R_f ($\text{k}\Omega$)	Q_{dl} ($\mu\text{F s}^{n-1}$)	n	R_{ct} ($\text{M}\Omega$)	σ ($\Omega \text{s}^{1/2}$)
Healthy RBC (4 electrodes)							
470 \pm 1	8 \pm 0.8	0.9 \pm 0.09	22 \pm 10	0.65 \pm 0.04	0.95 \pm 0.004	2 \pm 0.5	2.9 \pm 1.7 $\times 10^5$
Parasitized RBC (4 electrodes)							
470 \pm 1	4 \pm 2	0.9 \pm 0.05	12 \pm 5	0.65 \pm 0.02	0.9 \pm 0.03	1 \pm 0.6	5.7 \pm 3 $\times 10^5$

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_{ox} 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 a 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 σ , 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 σ originates from the decrease of the concentration of the oxidant or/and reducer species.

$$\sigma = \sigma_{ox} + \sigma_{red} = \frac{RT}{n^2 F^2 A \sqrt{2}} \left(\frac{1}{C_o D_o^{1/2}} + \frac{1}{C_R D_R^{1/2}} \right) \quad (1)$$

In order to evaluate the contribution of the oxidant and the reducer on the Warburg coefficient σ , σ_{ox} was calculated. Considering that $[O_2] = 2.5 \times 10^{-4} M$ (solubility of O_2 in water at 20 °C) with a diffusion coefficient equal to $2 \times 10^{-5} cm^2/s$ (in water at 20 °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), $\sigma_{ox} = 2 \times 10^5 \Omega s^{1/2}$ for normal RBC. As $\sigma = 2.9 \times 10^5 \Omega s^{1/2}$ 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 predomi-

nantly controls the mass transfer. This result explains the increase of σ with RBC infection that would originate from an increase in the O_2 consumption for parasitized RBC (39) and thus, a decrease in the oxygen concentration in the medium as mentioned above.

4. Conclusion

This study demonstrates for the first time the feasibility of performing EIS measurements on RBC adsorbed onto gold electrodes for the characterization of the physiological changes affecting the cells after invasion by *Plasmodium falciparum*. By using parasitized erythrocytes at the last stage of development, specific events taking place after the invasion, such as the permeation of the cell membrane as well as the release of reducing species and enhanced oxygen consumption seem to have been demonstrated by this technique. Integration of this device on a multiparametric sensor including ions and oxygen sensors could confirm interpretation proposed in this paper and help to the comprehension of the mechanisms taking place after invasion.

The stage is now set to examine the following applications of this new RBC-based device: (i) differentiation of parasitized versus healthy RBC at each stage of the parasite development, (ii) comparison of cell responses in presence of classical or new antimalarial drugs on healthy and infected cells, (iii) understanding the strategic events occurring during parasite development when antimalarials having well-known mechanisms of action are added. Such analyses are currently under development in our laboratory.

The results are very encouraging, since in the long-term, such cell-based device may be useful to probe other cellular models and diseases (e.g. inflammation, immunology, etc.).

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