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Detection of Bacterial Rhamnolipid Toxin by Redox Liposome Single Impact Electrochemistry

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Abstract: The detection of Rhamnolipid virulence factor produced by *Pseudomonas aeruginosa* involved in nosocomial infections is reported by using the redox liposome single impact electrochemistry. Redox liposomes based on 1,2-dimyristoyl-sn-glycero-3-phosphocholine as a pure phospholipid and potassium ferrocyanide as an encapsulated redox content are designed for using the interaction of the target toxin with the lipid membrane as a sensing strategy. The electrochemical sensing principle is based on the weakening of the liposomes lipid membrane upon interaction with Rhamnolipid toxin which leads upon impact at an ultramicroelectrode to the breakdown of the liposomes and the release/electrolysis of its encapsulated redox probe. We present as a proof of concept the sensitive and fast sensing of a submicromolar concentration of Rhamnolipid which is detected after less than 30 minutes of incubation with the liposomes, by the appearing of current spikes in the chronoamperometry measurement.

Pathogenic bacteria secrete various virulence factors as their biochemical weapons to kill the target host cells. They directly interact with the outer lipid bilayer membrane of eukaryotic cells, leading to the premature cell death by apoptosis or necrosis. Such virulence factors are highly involved in the bacterial infection processes, hence identifying them early is the key for the success in bacterial detection purposes.^[1,2] *Pseudomonas aeruginosa* secretes glycolipidic biosurfactant named Rhamnolipid (RL) which have shown a broader cytolytic activity against human macrophages, keratinocytes and lung epithelial cells.^[3-5] Therefore assays identifying RL toxin at a very early stage are of great importance for the specific detection of *Pseudomonas aeruginosa* which is involved in nosocomial infections.^[4,6] The amphiphilic nature of RL biosurfactant allows to partition into biological and artificial membranes altering their biophysical properties.^[6] Based on the RL interaction properties with the lipid membrane, we designed redox 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) liposomes containing 0.5 M potassium ferrocyanide as an encapsulated electrochemical active probe. Redox DMPC liposomes allowed to detect a submicromolar concentration of RL in aqueous solution in less than 30 minutes thanks to the electrochemistry of single impacts at a Pt ultramicroelectrode (UME).

The single-entity impact method is a very sensitive and powerful electroanalytical tool for biological and environmental purposes such as electrochemical detection of pathogens.^[7-10] The versatility of ultramicroelectrodes (UMEs) gives unique and essential properties for electroanalytical sensing owing to their small size, high sensitivity, fast steady-state response, low double-layer charging current and minimal ohmic loss.^[11] The observation of these stochastic events can provide information on various single nanoparticles contrary to ensemble (bulk) measurements.^[12-15] The main advantage of studying collisions of single entities is the low limit of detection (in principle, one single species) inherent to this electroanalytical method and the ability to study single entities (cells, viruses, nanoparticles, etc....) in real time (dynamic measurement).^[16-27]

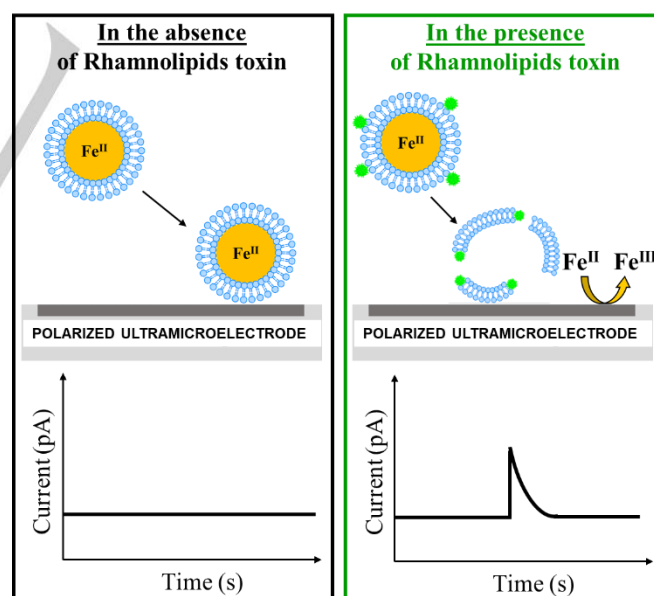


Figure 1. Principle of the bacterial Rhamnolipid toxin detection by redox liposome single impact electrochemistry

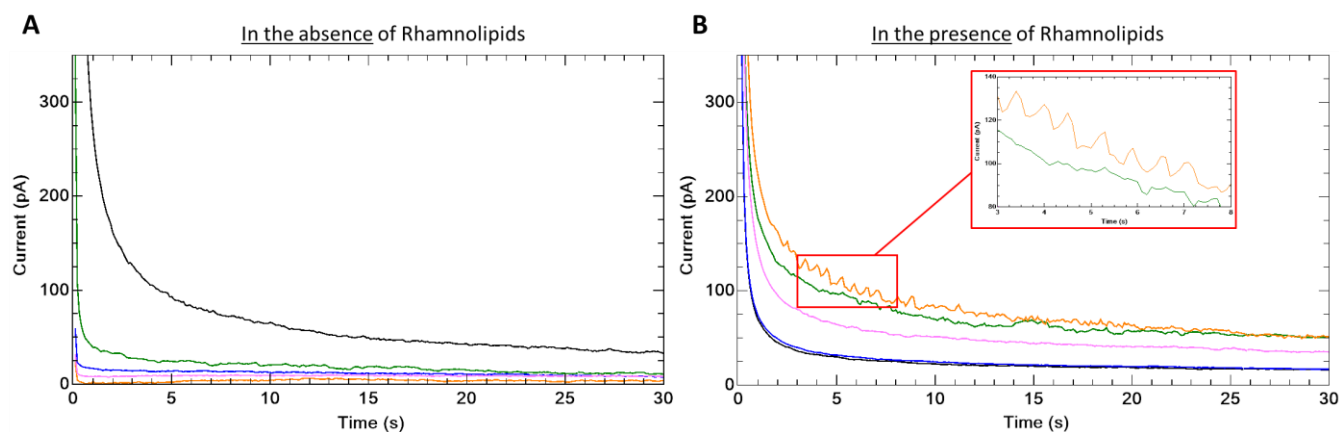


Figure 2. Chronoamperometry measurements recorded at +0.7 V vs Ag/AgCl on 10 μm Pt UME in **A.** 0.1 M PB at pH 7.4 (black line, left) and **B.** 500 nM RL and 0.1 M PB at pH 7.4 (black line, right) after addition of 3 pM redox DMPC liposomes at 0 min (blue line), 15 min (orange line), 30 min (green line) and 50 min (pink line).

Electrochemistry of single redox liposome impacts at an UME consists in detecting the electrolysis of a redox probe encapsulated inside a liposome when it is released at the UME after impact (or collision). The UME is polarized at the oxidation or reduction potential of the encapsulated redox probe and a concentration of several pico-molar of redox liposomes added to the aqueous buffer electrolyte is enough to detect "current spikes" in the chronoamperometry ($i-t$) curve corresponding to discrete collision events.^[23,28–36] The electron transfer does not readily occur across a lipid bilayer, thus the electrolysis of the liposome redox active content after collision and membrane rupture or opening at the UME surface led to many studies dealing with the membrane permeation mechanism.^[23,28,30–40]

Here, our work is based on the previous results where no current spike was observed in the chronoamperometry curve because the redox DMPC liposomes did not break during impact (or collision) onto the UME surface.^[23,28] Hence, the electrochemical sensing principle is based on the weakening of the liposomes lipid membrane upon interaction with a destructive bacterial virulence factor which leads upon impact at an UME to the breakdown of the liposomes and the release/electrolysis of its encapsulated redox probe, as previously reported.^[1,6,41] In the presence of RL toxin in solution (acting like a surfactant in the lipid membrane), current spikes corresponding to the electrolysis of the encapsulated redox probe released from weakened liposomes are detected (Figure 1). Thanks to the redox liposome single impact electrochemistry, the highest detection limit of RL toxin (500 nM) has been reached in comparison to several micromoles per liter previously reported.^[1]

Chronoamperometry measurements were recorded at +0.7 V vs Ag/AgCl (3 M NaCl) onto a 10 μm diameter disk Pt UME in 0.1 M phosphate buffer (PB) at pH 7.4 as aqueous electrolyte in the absence and presence of commercial RL (R90). The chosen concentration of RL (500 nM) presented in this work has been evaluated being the most appropriate for the proof of concept. Indeed, the toxin's detection with the redox liposomes single impact electrochemistry has to follow strict parameters such as a short time of detection (less than 1 hour after inoculation with liposomes) in order to propose a fast and sensitive sensing method.

The applied potential for the chronoamperometry measurements presented in Figure 2 is +0.7 V vs Ag/AgCl, corresponding to the steady-state current of $\text{K}_4\text{Fe}(\text{CN})_6$ encapsulated redox probe at a Pt UME determined by cyclic voltammetry (Figure S1). The Pt UME was gently polished using a wetted fine grid silicon carbide paper from Struers (4000-grid SiC) before each electrochemical experiment. The variation of the background current (baseline) observed in the different recorded $i-t$ curves (Figure 2) could be due to the partial covering of the Pt UME surface by liposomes and/or lipids and/or toxins. As expected, no current spike is detected in Figure 2A in all $i-t$ curves recorded in the absence of RL, confirming that redox DMPC liposomes do not break during the impact onto Pt UME and no redox probe is released and electrolyzed.^[23,28] In Figure 2A, only an increase of the background current noise is observed, probably related to the redox DMPC liposome single impacts onto Pt UME surface, in agreement with the electrochemical collision experiments by the "blocking effect" method (Figure S2). Also, in the absence of redox DMPC liposomes, in 500 nM RL aqueous solution (Figure 2B, black curve), no current spike is observed over a 30 s polarization time. The same behaviour (no current spike) is reported for the $i-t$ curve recorded immediately after addition of an appropriate concentration of the redox DMPC liposomes solution (Figure 2B, blue curve). This suggests that an inoculation time is necessary for the interaction of RL with the liposomes lipid bilayer before to observe current spikes related to the single impact events, which is in agreement with the previous results reported in literature.^[1] Indeed, the time required for observing the Rhamnolipid's effect which weak the liposomes in order to they break and/or release their content during the impact onto UME is not well understood at this stage of the study and probably dependent on both liposome and toxin concentrations. Significant current spikes appear in the $i-t$ curve after only 15 min of incubation in RL solution (Figure 2B, orange curve), suggesting that RL affect the redox DMPC liposomes membrane leading to the breakdown during the collision onto Pt UME surface, as previously observed with Triton X-100 surfactant.^[23,28] The shape of current spikes is quite broad for single collisions of 300 nm diameter liposomes containing 0.5 M ferrocyanide whose the electrolysis is fast. This is probably due to the interaction of RL toxin with the liposome lipid bilayer which leads to a slow release

of the liposome redox content during the collision at the UME surface. After 30 min of redox DMPC liposomes incubation in the 500 nM RL aqueous solution (Figure 2B green curve), the current spikes are no longer clearly visible and after 50 min (Figure 2B, pink curve), the $i-t$ curve became flat again as initially. A possible explanation of this effect is that the inoculation time is became too much high, leading to the destruction of all redox DMPC liposomes in solution in the presence of RL, hence no possible impact. Therefore the ideal time of incubation in our collisions experiment is between 10 and 30 min after addition of redox liposomes in RL solution where the current spikes are clearly distinguished in the $i-t$ curves (Figures 2B and S3, orange curves). As a control experiments, chronoamperometry measurements were performed in the same experimental conditions but using non-redox DMPC liposomes (with only encapsulated phosphate buffer) and no current spike was observed in all $i-t$ curves recorded over 1 hour (Figure S4).

In order to confirm that the current spikes observed in the orange $i-t$ curve (after 15 min of incubation) presented in Figure 2B are related to the redox liposome single impacts, the charge passed during the individual electrolysis was determined by integration of each current spike (Figure S5). To take into consideration that only current spikes of at least three times the background current noise (600 ± 100 fA) were considered for integration. From the measured charge of each current spike, the liposome hydrodynamic diameter was calculated using Faraday's law, as indicated in Equation 1 (Figure S5).

$$d_{lip} = 2 \sqrt[3]{\frac{3Q}{4\pi n_e F C_{redox}}}$$

Equation 1

In Equation 1, d_{lip} is the liposomes hydrodynamic diameter, Q is the measured charge, n_e is the number of electrons transferred during the electrolysis, $n_e = 1$, F is Faraday's constant, and C_{redox} is the concentration of redox species encapsulated in the liposomes, $C_{redox} = 0.5$ M. Here, we assume that the initial concentration (0.5 M) of encapsulated ferrocyanide is the same in each liposome and also that all of the contents is oxidized at the UME surface during the liposome collision.

Based on the calculated hydrodynamic diameters, the size distribution evaluated from the electrochemical collision experiments was compared to the one obtained from dynamic light scattering (DLS) data (Figure 3).

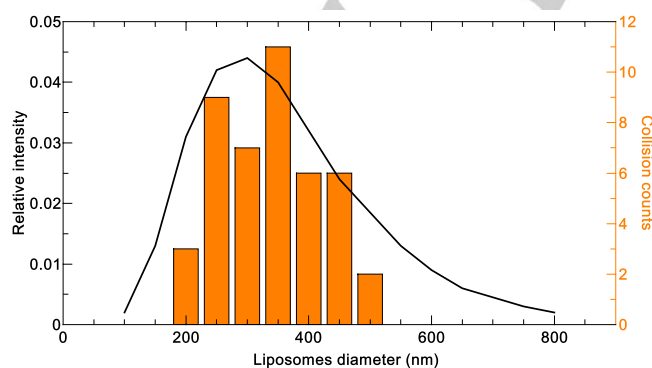


Figure 3. Size distributions from DLS data (black line) and from charge data by integrating current spikes of $i-t$ curve recorded at +0.7 V vs Ag/AgCl on 10 μ m Pt UME in 500 nM RL 0.1 M PB at pH 7.4 at 15 min after addition of 3 μ M redox DMPC liposomes (orange bar).

According to the overlay presented in Figure 3, the size distribution calculated from electrochemical data (orange bar) is in good agreement with the size distribution obtained from DLS data (black line). Indeed, the DLS data indicate that the peak diameter is 275 ± 120 nm (Figure S6) while the one calculated from electrochemical data is 335 ± 160 nm (Figure S5). The higher value obtained from electrochemical data can be due to the limited sensitivity of the collisions experiments regarding the background current noise which not allow to count the smallest current spikes in the $i-t$ curve. Hence, the calculated liposomes diameter is probably overestimated in the hypothesis where the smallest liposomes (below than 200 nm of diameter) are not detected. In addition, the DLS data indicate that the redox DMPC liposomes solution is quite polydisperse (PDI = 0.19), leading to a quite large size distribution (Figure S6). The results presented in Figure 3 confirm that the current spikes observed in the orange $i-t$ curve in Figure 2 are related to redox liposomes single impacts onto Pt UME. The destructive interaction of RL as a biosurfactant with the liposomes DMPC bilayer is necessary for leading upon impact onto Pt UME to the breakdown of the liposomes and/or the release/electrolysis of its encapsulated redox content.

In conclusion, a first proof of concept based on the electrochemistry of redox liposome single impacts for the bacterial virulence factors sensing is proposed with the detection of the lowest concentration of Rhamnolipid (500 nM) in less than 30 minutes. The next step of this study is to optimize the experimental setup such as the electrode and liposomes sizes according to the Rhamnolipid concentration in order to improve the sensitivity of the electrochemical signal and do a statistical analysis relating the time of inoculation to the toxin and liposome concentrations. This work should be extended to other toxins and types of liposomes in order to reach a selective detection which is a crucial parameter for biosensor applications. In the near future, this electrochemical biosensor concept should be used for the detection of virulence factors from the supernatant of pathogenic bacteria.

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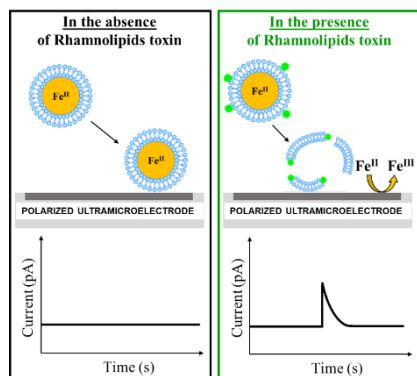
Keywords: bacterial toxins sensing • electrochemical single impacts • redox liposomes • lipid membrane • ultramicroelectrode

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Entry for the Table of Contents

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A proof of concept showing that the electrochemistry of redox liposomes single impacts is a powerful and efficient analytical technique for sensing target toxins produced by pathogenic bacteria such as Rhamnolipid from *Pseudomonas aeruginosa* which interacts destructively with the lipid membrane of liposomes. A high sensitivity of sensing has been reached with a submicromolar concentration of Rhamnolipid detected in less than 30 minutes.

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