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Short communication

An electrochemical sensor concept for the detection of bacterial virulence factors from *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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ABSTRACT

This work presents an electrochemical sensor concept for bacterial toxin detection using biomimetic lipid vesicles containing potassium ferricyanide. The toxin mediated release of redox couples from the vesicles was quantified by measuring the redox current on screen printed electrodes, and the detection limits to rhamnolipid, delta toxin and bacterial supernatants from clinical wound pathogens were examined. Overall detection limits of both redox and carboxyfluorescein containing vesicles were one order of magnitude lower than the cytotoxic dose of studied toxins to T lymphocytes.

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1. Introduction

The early detection of bacterial pathogens is a key challenge in clinical diagnosis of skin and soft tissue infections, especially in patients with burn wounds [1,2]. The involvement of large wound areas and the host immunological complications following thermal injury make burn wounds vulnerable to infection. Early diagnosis of burn wound infections can be difficult, as classical symptoms such as pyrexia (high temperature) have limited utility due to damage to the patient's thermal regulation following a significant burn injury [1,3]. Although there have been advances in wound care and treatment, burn wound infection is still a primary cause of morbidity and mortality [3]. Recently, wound dressing technology has rapidly progressed and many types of dressings are available nowadays [4], but to our knowledge there is no wound dressing on the market, capable of informing clinicians/patients of the wound infection.

Electrochemical biosensors have demonstrated the bacterial detections [5,6]. Such detection schemes primarily utilize the electrodes modified with biological recognition elements such as enzymes to generate the electrochemical signal when attached elements react with the specific target analytes/bacteria. The assembly of receptor coupled-lipid mono/bilayer on electrode [7,8] and redox liposomes [9,10] triggered response by the bacteria/toxins were also studied. Recently there has been progress in discovering new infection markers for bacterial detection in wounds [11]. Monitoring the redox activity of pyocyanin and the uric acid level in wounds were demonstrated for the prediction of early wound infections [12,13]. Wound infection is the result of the systematic colonization of pathogens, and the associated virulence factors act as the weaponry of bacteria in damaging host tissues irreversibly. Therefore, identifying the associated virulence factors is the key for the success in bacterial detection schemes. Strains of *Pseudomonas aeruginosa* secretes biosurfactant rhamnolipids which have shown a broader cytolytic activity against human microphages, keratinocytes and lung epithelial cells [14], and therefore assays identifying rhamnolipids provide a novel method in specific detection of *P. aeruginosa* [15,16]. Likewise opportunistic human pathogen, *Staphylococcus aureus* secretes delta toxins which are responsible for the lysis of erythrocytes, organelles and a wide range of cells [17]. Some conceptual studies have already targeted bacteria/toxins [18,19], but more works has to be done, especially targeting a broader range of virulence factors.

This study examined the exotoxins secreted from the two selected strains of clinical wound pathogens: *S. aureus* and *P. aeruginosa*. The detection principle was based on the interaction of toxins with lipid bilayer and the concomitant breakdown of phospholipid vesicles by bacterial toxins triggered the release of encapsulated redox couples which were detected by the electrodes. The assay performance, in term of dose response and detection limit to two primary toxins and

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bacterial supernatants was studied. The results were compared with the response of carboxyfluorescein containing vesicles to the similar virulence factors.

2. Materials and methods

2.1. Materials and vesicle preparation

Phospholipids, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was supplied from Avanti Polar Lipids, USA. Cholesterol, 10,12-tricosadiynoic acid (TCDA), potassium ferricyanide (PFC) and 5,6-carboxyfluorescein were purchased from Sigma Aldrich. TCDA is a synthetic diacetylene polymer lipid used to improve the stability of vesicles by lateral polymerization [20]. HEPES and 1 mol dm^{-3} KCl buffers were prepared in high electrical resistant water (18.2 MΩ cm), sterilized and used in preparing PFC and carboxyfluorescein vesicles respectively. For encapsulation inside vesicles, 100 mmol dm^{-3} PFC in KCl and 50 mmol dm^{-3} carboxyfluorescein in HEPES were used. Luria Broth (LB) and Tryptic Soy Broth (TSB) were supplied from Fisher Scientific. The rhamnolipid (R-95) from *P. aeruginosa* was purchased from Sigma Aldrich. The delta toxin was synthesized by Biomatik (USA), using the protein sequence provided [17].

The preparation of vesicles was presented in previous work [20]. In short, 100 mmol dm^{-3} of lipids, cholesterol and TCDA in chloroform were mixed (55:20:25%V/V) and 600 μL of mixture was dried at 10^{-3} mbar for 60 min, before rehydrated in 5 mL of 100 mmol dm^{-3} PFC in KCl (50 mmol dm^{-3} carboxyfluorescein in HEPES was otherwise used in preparation of carboxyfluorescein vesicles). The solution was then heated at 75 °C for 10 min followed by three freeze–thaw cycles, before extruding 3 times through a porous polycarbonate membrane (200 nm, Whatman, USA) using a LF-50 extruder (Avestin, USA) heated at 55 °C. Vesicles were purified in Sephadex NAP-25 columns (GE Healthcare, U.K.). Finally vesicles were polymerized in 1 mL quartz vials with a UV dose of 90 mW cm^{-2} in a commercial flood exposure (254 nm UV source, Hamamatsu, Japan), after a storage of vesicles at 4 °C for a week.

2.2. Screen printed electrodes

Electrodes were supplied from Gwent Ltd., U.K. Each polyester sheet comprises of three planar electrodes printed with gold polymer (working electrode), silver/silver chloride (reference electrode) and carbon/graphite pastes (counter electrode). Differential Pulse Voltammetry (DPV) measurements were performed at room temperature using μAutoLab (type III) potentiostat (Metrohm, U.K.). The electrode was preconditioned at 0 V for 1 min prior to each measurement. Initial potential was 50 mV vs. Ag/AgCl reference electrode and the final potential was 400 mV with a scan rate of 3 mV s^{-1}. Modulation amplitude was set to 50 mV with modulation and interval times of 0.04 s and 0.1 s respectively. Each voltammogram was fitted using Autolab software to define the peak current at 200 mV (reduction potential of PFC) [21].

2.3. Toxins and bacteria culture

Supernatant from three strains, *S. aureus* (USA300), *P. aeruginosa* (PAO1) and an attenuated strain of *Escherichia coli* (DH5α), as a control, were used to study their lipolytic activity on PFC vesicles. USA300 is a clinical bacterial isolate which carries both the mecA gene conferring methicillin resistance (MRSA) and the PVL gene which codes for the panton valentine leukocidin toxin. Bacteria from a single colony was picked and enriched in respective LB/TSB media in a shaking incubator at 37 °C for 18 h, before being centrifuged at 5000 rev min^{-1} for 15 min, followed by filtering using 0.22 μm membrane filters. These supernatants were diluted in KCl buffer and vesicles, to result the desired concentration (%V) for each measurement. Rhamnolipid and delta toxins were prepared in phosphate buffer saline.

2.4. Electrochemical characterization

The electrode was prewashed in 0.1% TWEEN 20 for 3 min before the background currents in KCl and then in vesicles (100 μL) were measured. Vesicles were mixed with supernatants/toxins to a final volume (260 μL) on the electrode and were consistently used on each electrode for dose response measurements. For each mixture, one measurement in every 5 min was recorded for an hour. For the data presentation, the average and standard deviation of the three final peak currents were used to plot for each measurement. Peak currents against the respective toxin doses were plotted and the detection limit for each toxin was estimated by the linear fitting. Detection limit is defined as the concentration corresponding the concentration of toxins equivalent to three time standard deviation of control (no toxins).

2.5. Fluorescent assay

Vesicles containing carboxyfluorescein were incubated with toxins and the fluorescent responses were measured to compare the sensitivity and detection limit with PFC vesicles. Fluorescent assay was performed with Omega microplate reader (BMG Labtech, U.K.) using excitation and emission wavelengths of 485 ± 12 and 520 nm respectively. 96-well micro titre plate was used with a fluorescent gain at 600. In a fluorescent measurement the same volume of vesicles/toxin combination was used as in study of PFC vesicles mentioned in Section 2.4.

3. Results and discussion

3.1. Evaluation of PFC on screen printed electrodes

Initial screenings were first carried out to examine the minimum detectable limit of the redox couples. A range of PFC (from 1 μmol dm^{-3} to 10 mmol dm^{-3}) prepared in KCl was used to measure the peak current at 200 mV, and the minimum PFC providing a measurable peak current was found to be 50–100 μmol dm^{-3} (Fig. 1). The enclosed volume of vesicles was calculated using the vesicle parameters (Section 2.1). Assuming the vesicles have a bilayer thickness of 5 nm and a uniform diameter of 200 nm, it was calculated that 100 μL of vesicles containing 100 mmol dm^{-3} PFC provided a maximum PFC concentration of 1.5 mmol dm^{-3} if all the vesicles were lysed in a final volume of 260 μL. This is expected to be 15–30 times above the detectable limit of the system under study.

3.2. Dose responses of PFC vesicles to exotoxins

Initial experiments involved measuring time-dependent current response of PFC vesicles to the lipolytic activity of rhamnolipid and delta toxin. The measurements indicated that the peak current generally stabilized within 60 min of toxin inoculation with vesicles (Fig. 2). Rhamnolipid mediated release of PFC from vesicles was clearly observed and the response was concentration dependent (Fig. 2A). The redox current in absence of exotoxins was possibly due to residual probes remaining outside the vesicles. It was noted that the relative increase in peak current induced by 75 μmol dm^{-3} rhamnolipid was about 0.9 μA, which was only one third of an estimated value (Section 3.1). This result suggests two possible scenarios. First, not all vesicles may lyse by the highest dose of rhamnolipid. Although the PFC release from vesicles is a function of toxin concentration, the quantity of toxin required to break emptying a single vesicle is not known. Second, the electrochemical response solely depends on mass transport. The natural diffusional effect of PFC may also be responsible for an exponential decay of peak current response with time. The similar responses were observed in tests with delta toxin (Fig. 2B). The detection limit of rhamnolipid and delta toxin deduced from dose response graphs were 11 and 2.9 μmol dm^{-3} respectively (Fig. 2C).

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