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Surface-Bound Lipid Vesicles Encapsulating Redox Species for Amperometric Biosensing of Pore-Forming Bacterial Toxins

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Pore-forming toxins (PFTs) are a group of bacterial protein toxins that exhibit their cytotoxicity by acting on the plasma membrane and permeabilizing cells.[1] The nonselective fluxes induced by the toxins cause osmotic imbalance and loss of enzymes and other important contents, leading ultimately to cell lysis and death. Streptolysin O (SLO) is a 61 kDa PFT[2] produced by *Streptococcus pyogenes* A and C, and it binds to cholesterol (CHO) in the target membranes. Bound toxins associate with each other to form arc- and ring-shaped clusters that insert into the bilayer to produce oligomeric transmembrane pores of up to 35 nm in diameter.[2a] Significantly, pore-formation by SLO occurs on both erythrocyte membranes and CHO-rich bilayer vesicles,[3] providing an unparalleled means to manipulate membrane materials toward some desirable functionality. Toxin−membrane interactions have attracted considerable interest recently,[4] and advances in new membrane techniques[5] further facilitate the studies. Bayley et al. have reported single-molecule stochastic sensors using staphylococcal α-hemolysin that produces an "on/off" response to analytes in the transmembrane electrical current.[6] Biosensing of gramicidin[7] and colicin N[8] has been demonstrated with ion-channel proteins.

The pores defined by α-hemolysin and ion-channel proteins are small and typically single-molecule based, allowing one targeted molecule to pass at a time.[6c,d] We set out to investigate larger pores on membranes in which diffusion of small molecules across the pores could be implemented in a detection scheme. In this communication we report the fabrication and characterization of redox-encapsulated supramolecular assemblies of bilayer vesicles on a gold surface. The permeability of the lipid membrane and release of redox content from the surface-bound vesicular sensing layer are assessed electrochemically as a function of SLO activity in pursuit of a novel detection method for the toxin.

The vesicles were assembled with four lipid components: phosphatidylcholine (**1**), cholesterol (**2**), diacetyl phosphate (**3**), and 1-octadecanethiol (**4**) with a molar ratio of 10:10:2:1. Compound **4** was used to ensure firm attachment of the vesicles on the gold surface. Redox probe $K_3Fe(CN)_6$ was encapsulated into the vesicles by probe sonication of the dried lipids in a buffer containing 50 mM $K_3Fe(CN)_6$, followed by 1 h incubation at 4 °C. To assemble vesicle layer on the electrode, an aliquot of 50 μL of vesicle solution was pipetted onto a gold disk electrode (1.6 mm i.d.), allowing vesicles to adsorb for an hour before excess vesicular particles and free $Fe(CN)_6^{3-}$ were rinsed off with a Tris buffer. A Teflon cell unit was mounted onto the electrode via an O-ring to define a 40-μL electrochemical cell volume.

Figure 1 shows the cyclic voltammetric response of toxin-induced release of $Fe(CN)_6^{3-}$ from surface-bound vesicles with respect to SLO concentration. The supporting electrolyte is 10 mM Tris buffer containing 0.15 M NaCl (pH 7.5). The samples were incubated for 30 min at 37 °C before the measurements were conducted. From

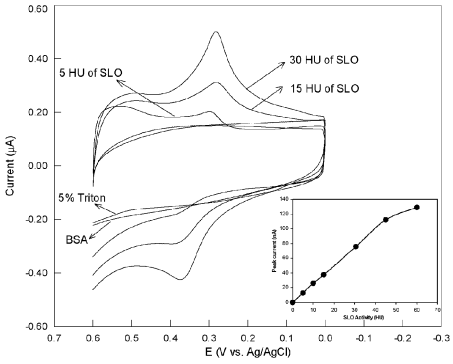

Figure 1. Cyclic voltammograms of $Fe(CN)_6^{3-}$ encapsulated vesicle sensor on a gold electrode for SLO, Triton and BSA. The scan rate is 50 mV/s. (Inset) Calibration curve for SLO obtained by DPV method.

Figure 1, the Faradaic current of $Fe(CN)_6^{3-}$ increases with the amount of SLO toxin in the electrochemical cell. The peak current for 30 HU[9] of SLO is 254 nA at the scan rate of 50 mV/s, indicating a relatively large current density. The inset shows the amperometric response curve for SLO obtained by differential pulse voltammetry (DPV) at 20 mV/s scan rate. A linear relationship was obtained for SLO concentrations ranging from 5 to 45 HU. The curve levels off at higher concentrations, suggesting possible saturation of pore formation on the bilayer membrane. The peak separation ΔE_p is 90 mV and the calculated $E^{\circ\prime}$ is 0.33 V. The control experiments were carried out with 0.227 mM BSA and 5% Triton-100. BSA was used to study nonspecific interactions without damaging the vesicles, while Triton was used to induce total vesicular disruption. No Faradaic current was detected for the vesicular sensor after 30 min incubation with BSA or Triton.

It is worth noting that the current peaks for $Fe(CN)_6^{3-}$ released from vesicles do not fit entirely into the shape of a typical semi-infinite diffusion process.[10] Instead, they exhibit some degree of symmetry (Figure 1). For comparison, the voltammetric behavior of 0.005 M $Fe(CN)_6^{3-}$ on a vesicle modified (no $Fe(CN)_6^{3-}$ encapsulated) gold surface was probed. Attachment of a vesicle layer on gold did not affect the response shape as expected. However, the peak current of $Fe(CN)_6^{3-}$dropped by 25% as compared to that on a bare Au electrode as a result of partial blockage of the electrode surface by the vesicles. The peak separation ΔE_p increased to 159 mV, suggesting much slower kinetics. These results show a different electrochemical behavior from the toxin-released $Fe(CN)_6^{3-}$. Together with the observation that vesicle disruption by Triton failed to generate voltammetric signals, it appears to us that toxin-released $Fe(CN)_6^{3-}$ must undergo a response mechanism that is distinct from the conventional semi-infinite diffusion model on bare and vesicle-modified electrodes.

The effect of SLO action on vesicles was further characterized by transmission electron microscopy (TEM) and dynamic light scattering (DLS) where the change of particle size of the vesicles

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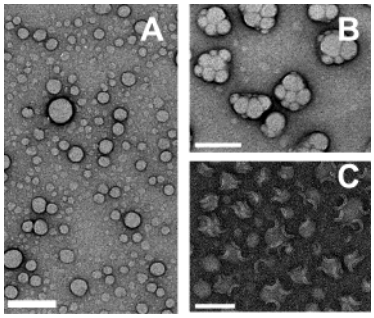

Figure 2. Images of various vesicles used in SLO sensors. (A) Vesicles consisting of three constituent lipids **1**, **2**, and **3**. Bar = 500 nm. (B) Vesicles that contain compound **4**. Bar = 300 nm. (C) Vesicles after incubated with 45 HU SLO. Bar = 80 nm.

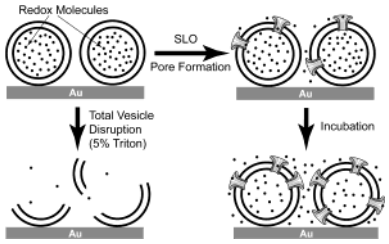

Figure 3. The proposed mechanism for electrochemical SLO toxin sensor.

upon composition and toxin binding was investigated. DLS measurements show that the vesicles composed of three components (**1**, **2**, and **3**, molar ratio 10:10:2) have an average size of 86.5 nm. When compound **4** was added to form the adsorption-enabled vesicles, the measured particle size split into two size domains. The smaller domain has an average size of 46 nm, while the large one is 162 nm. TEM revealed the existence of clusters of vesicles that likely constitute the larger domain (Figure 2B), while in the three-component system clustering of vesicles did not occur (Figure 2A). SLO incubation does not alter the existence of two size domains but slightly increases the particle size (63 and 192 nm, respectively). The size increase can be attributed to the structural change of the vesicles as a result of pore formation, which was further confirmed by TEM (Figure 2C). It must be noted that pore formation by SLO changes the vesicle size but does not affect the overall integrity of the vesicles. In contrast, when 5% Triton was used for incubation, no light-scattering signal could be detected, and no vesicles were observed by TEM, suggesting total disruption of the vesicles.

We therefore propose a controlled-release mechanism for the vesicle-based SLO sensor in which a thin-layer bulk electrolysis of $Fe(CN)_6^{3-}$ is accountable for the observed electrochemical behavior (Figure 3). SLO toxins interact with the cholesterol in the bilayer membrane and oligomerize to form pores, allowing $Fe(CN)_6^{3-}$ in the vesicles to leak out through a "gated" diffusion process. The pore-formation process does not damage the integral feature of the intact vesicles. As a result, the flux of $Fe(CN)_6^{3-}$ is controlled by the density of pores formed in the bilayer. Upon incubation, more $Fe(CN)_6^{3-}$ is released from the vesicles, eventually reaching its maximum concentration on the electrode surface before ultimately fading away into the solution bulk. A thin layer of electroactive species is thus formed during toxin-induction process with a thickness comparable to the vesicle size. It is remarkable that incubation at 37 °C for 30 min still allows for observation of large redox currents. The pore-bearing vesicles apparently serve as a shielding layer that significantly reduces mass transport of $Fe(CN)_6^{3-}$, as similarly observed in thin-layer coatings.[11] Triton, on the other hand, works in a different manner (Figure 3). The total disruption of the vesicular structure by Triton leads to rapid dilution of $Fe(CN)_6^{3-}$ into the bulk. Assuming a full vesicle coverage on the electrode, the total amount of $Fe(CN)_6^{3-}$ encapsulated in the surface-bound vesicles turns out to be about 8 pmol. Given the electrochemical cell volume of 40 μL, this translates to a 0.2 μM concentration, which is well below the limit of voltammetric measurement on this electrode.

The peak current for thin-layer bulk electrolysis in potential sweep mode is directly proportional to the scan rate (regardless of reversible or nonreversible reactions). In a semi-infinite diffusion process, this current is proportional to the square-root of the scan rate.[10] We plotted the logarithm of peak current verse the logarithm of the scan rate. A linear relationship was obtained, yielding a slope of 0.85 ± 0.05. This value falls between 1.0 (thin-layer electrolysis) and 0.5 (semi-infinite diffusion), but leans strongly toward a thin-layer-controlled process. The result clearly supports a thin-layer model stemming from pore-formation of SLO in the membranes of the vesicles adsorbed on the electrode surface. It should be pointed out that it is difficult to precisely define the redox layer's outer boundary. The variation of the slope from 1.0 indicates nonideality of the thin layer, which may result from the relatively wide size distribution of the vesicles and the concentration gradient due to diffusion.

In conclusion, we describe a new method to signal the presence of SLO toxin using a bilayer vesicle-based sensor on a gold surface. The controlled release of redox species from the surface-bound vesicles is mediated by the pore-formation functionality of SLO, allowing amperometric detection of the targeted toxin. The method could have wide application for the detection of functionally similar protein toxins.

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Supporting Information Available: Experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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