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Letter

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Cholesterol Oxidase/Triton X-100 Parked Microelectrodes for the Detection of Cholesterol in Plasma Membrane at Single Cells

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Abstract. The classic electrochemical analysis of plasma membrane cholesterol at single cells utilizes cholesterol oxidase modified microelectrode that oxidizes local cholesterol efflux from plasma membrane to generate hydrogen peroxide for the electrochemical quantification. In this letter, a mixture of cholesterol oxidase and Triton X-100 was filled in the micro-capillary that could park at Pt layer coated tip due to slow hydrodynamic flow. During the contact of the tip with cellular membrane, Triton X-100 at the tip permeabilized the contacted membrane to release cholesterol for the reaction with cholesterol oxidase. As compared with the linkage of cholesterol oxidase at the electrode surface, the oxidase parked in aqueous solution at the tip had a higher turn-over rate resulting in larger electrochemical signal for single cell analysis. More charge collected at acyl-coA: cholesterol acyltransferase (ACAT) inhibited cells supported that this novel detection stragety could monitor the flunctation of membrane cholesterol at single cells. The sucessful detection of plasma membrane cholesterol at single cells using oxidase parked microelectrode will provide a special stragety for the fabrication of biosensor that permits the integration of more molecules without function groups at the electrode to measure active and inactive molecules in plasma membrane. Moreover, the larger electrochemical signals collected could further increase the spatial resolution for single cell electrochemical analysis.

Plasma membrane is an important compartment of cells, which functions as the permeability barrier to maintain trans-membrane electrical potentials and provide a suitable environment for organelles.[1] Lipids and many proteins are the main components in plasma membrane to play key roles in many physiological processes. Among these components, cholesterol is an essential molecule to associate lipids and membrane proteins. The binding of cholesterol and lipids at most membrane regions is strong resulting in low escape tendency of cholesterol, termed as "inactive cholesterol".[2] Recently, lipid rafts that are specialized membrane domains enriched in certain lipids cholesterol and proteins are observed in cell membranes.[3] In these rafts, cholesterol has weak binding with lipids owing with high escape tendency, termed as "active cholesterol".[4-8] The presence of active membrane cholesterol has been known to contribute to many functions, such as cholesterol efflux, endocytosis and signal transduction.[9,10] Therefore, the detection of total and active cholesterol in plasma membrane at single living cells is critical for the understanding of cholesterol function in cellular biology.

Fluorescence imaging, oxidase based assay and electrochemical analysis are main strategies for the measurement of plasma membrane cholesterol at single cells.[11-15] Although fluorescence imaging using either fluorescent cholesterol analog or cholesterol specific binding molecule (flipin) is popular to visualize the local distribution of membrane cholesterol, the usage of the analog or binding molecule might not reflect the natural information of membrane cholesterol.[11,12] In the past years, our group has introduced cholesterol oxidase into the solution to react with active cholesterol from outer leaflet at plasma membrane for the generation of hydrogen peroxide, which is detected using electrochemiluminescence.[13, 16-18] However, oxidase based assay provides the amount of active membrane cholesterol at single cells only. Moreover, the local information of membrane cholesterol is missing.

To achieve the local detection of membrane cholesterol at single living cells, Burgess's group used cholesterol oxidase modified microelectrodes to detect cholesterol efflux from plasma membrane in a micrometer region. In that method, an oxidase modified Pt microelectrode (4 μm diameter) was contacted with cellular plasma membrane, in which cholesterol efflux from plasma membrane was reacted with oxidase to generate hydrogen peroxide for electrochemical analysis. The experimental results exhibited that the currents associated with cholesterol efflux were correlated with the fluctuation of membrane cholesterol at single cells.[14,15] However, the oxidase

covalently or physically immobilized oxidase at the electrode surface has a limited turn-over rate resulting in weak current, which is difficult to be collected. Also, no direct quantification of total membrane cholesterol, including active and inactive cholesterol, is achievable. This missing information results in the difficulty to correlate total and active membrane in the study of cholesterol homeostasis.[19] To realize the analysis of total membrane cholesterol, the detergent to permeabilize cellular membrane is needed to release cholesterol during the local detection. However, the modification of the detergent and oxidase at the microelectrode surface is challenging.

In this work, cholesterol oxidase/Triton X-100 parked microelectrodes were designed to achieve higher electrochemical signal for the detection of plasma membrane cholesterol in single living cells, as illustrated in Figure 1. The solution of cholesterol oxidase and Triton X-100 was filled in a hollow capillary with a 1 μm diameter tip. A ring of Pt was prepared at the tip to electrochemically oxidize hydrogen peroxide generated from the reaction of cholesterol and cholesterol oxidase. When the capillary was immersed into the solution, a high level of the solution inside the capillary induced a slow hydrodynamic flow towards the tip. Due to the extremely slow rate, a thin aqueous layer with cholesterol oxidase and Triton X-100 could be assumed to park at the tip. During the contact of the tip with plasma membrane, Triton X-100 permeabilized the local cell membrane so that total membrane cholesterol, including active and inactive, in inner and outer leaflets of plasma membrane was free to be reacted with cholesterol oxidase for the generation of hydrogen peroxide. Cholesterol oxidase was present in an aqueous layer at the contact region, and thus, a high turn-over rate was guaranteed providing larger electrochemical signals.

The micro-capillary with a ring of Pt at the tip was prepared following our previous protocol.[20] More details were shown in the experimental section in the supporting information. To fabricate a ring of Pt layer at the tip of capillary, the outer wall of the capillary was sputtered with a 70 nm Pt layer. Scanning electron microscopy (SEM) image in Figure S1A and B (supporting information) showed the inner and outer diameters of the tip to be ~ 1.5 and 1.7 μm, respectively. After painting with wax, Pt layer at the tip was not covered with wax that was used as the electrode surface for the oxidation of hydrogen peroxide. SEM image in Figure S1C and D (supporting information) exhibited that the outer diameter of the tip increased to ~ 2 μm.

To achieve the analysis of membrane cholesterol, cholesterol oxidase and 1% (V:V) Triton X-100 dissolved in 10 mM phosphate buffer saline (PBS, pH 7.4) was filled into the capillary. When the capillary was immersed into the solution, the liquid level inside the capillary was ~ 5 mm higher than that of bulk solution to initial hydrodynamic flow towards the tip. Since our capillary was not the classic cylindrical capillary, the hydrodynamic flow at the tip was estimated following the equation for hydrodynamic flow in the capillary.[21]

$$\frac{V}{t} = \frac{\Delta P \pi d^4}{128 \eta L} \qquad (1)$$

where V/t is the volume egressed from the tip per time, ΔP is the pressure difference between liquid levels inside the capillary and of the bulk solution, d is the diameter of tip (1 μm for our capillary), η is the buffer viscosity and L is the capillary length (2 cm for our capillary). The amount of the solution egressed out of the capillary was calculated to be ~ 60 $\times 10^{-18}$ L/s, which suggested a layer of 6 nm/s layer at the tip. Assuming the detection procedure was completed in 180 s, the thickness of the aqueous layer with cholesterol oxidase was ~ 1 μm. Therefore, it could be assumed that cholesterol oxidase and Triton X-100 parked at the tip in a limited time for the reaction with cholesterol.

The detection of aqueous cholesterol was initialed to characterize the sensing ability of the prepared microelectodes. After the immersion of the capillary in 10 mM PBS (pH 7.4) with 50 mM methyl- β -cyclodextrin (MβCD), the background charge was collected from the micro-ring electrode under the potential of 600 mV, as shown in Figure 2A (trace a). Then, 100 μM cholesterol was injected into the solution, and the charge was collected again, as shown in Figure 2A (trace b). More charge was observed, as illustrated in Figure 2B, exhibited the reaction of aqueous cholesterol with cholesterol oxidase to generate hydrogen peroxide and the following electrochemical oxidation to contribute the charge. The removal of cholesterol oxidase from the capillary did not induce an obvious charge increase, as shown in Figure S2 (supporting information), which supported the detection of aqueous cholesterol using cholesterol oxidase/Triton X-100 parked microelectrodes.

The charge increases in Figure 2B were linear exhibiting constant current responses from the electrochemical oxidation of hydrogen peroxide. This steady-state response suggested a faster replenishment rate of hydrogen peroxide at the microelectrode surface compared to the generation

rate of hydrogen peroxide from cholesterol reaction with cholesterol oxidase. Therefore, the gradual rise in the charge in Figure 2B likely reflected the real time oxidation of hydrogen peroxide during the cholesterol reaction. The slopes of the charge in Figure 2B were calculated to be 12- 126 pA for cholesterol with the concentration from 0.1 to 3 mM, which was ~ 10 fold larger than the current response from 4 μm oxidase modified microelectrode reported before.[14] As a result, this design of cholesterol oxidase parking in the aqueous layer at the electrode maintained high activity of oxidase that resulted in the significant enhancement in the sensitivity. Moreover, this design permitted the parking of the detergent at the electrode that was difficult to be modified to achieve the detection of molecules in cellular plasma membrane.

Figure 2C showed the relationship of charge increase with the concentration of aqueous cholesterol in the range of 0.1 – 3 mM. The positive correlation between the charge increase and cholesterol exhibited that our microelectrodes could monitor the fluctuation of cholesterol. The nonlinear relationship at relative high concentration might be complicated by the enzyme kinetic behavior and diffusional loss of hydrogen peroxide. The relative standard deviations from three electrodes were calculated to be less than 9.99% suggesting good reproducibility.

For single cell analysis, the microelectrode filled with cholesterol oxidase and Triton X-100 was attempted to be contacted with single living cells, as imaged in Figure 3A. The background charge was collected at the microelectrode under the potential of 600 mV that was positioned away from the cell, as shown in Figure 3B (trace a). After positioning the microelectrode to touch cellular plasma membrane, the potential of 600 mV was applied immediately at the electrode to record the charge in Figure 3B (trace b). The additional charge was collected, as shown in Figure 3C (trace a), suggested that plasma membrane cholesterol could be detected. The cell kept intact under bright-field observation after the touching of the electrode with the cell, which indicated that this small amount of Triton X-100 permeabilized the contact region only.

Based on the molecule area of cholesterol (38$Å^2$/molecule) at plasma membrane,[22] approx. 24 $\times 10^{-18}$ mole cholesterol was existed at the contact region that generated a charge of ~ 4.8 pC. Therefore, the charge collected in the first second was mainly contributed by cholesterol at the contact region, and the following gradual charge increase might be ascribed to the reaction of cholesterol in the contact region nearby through fast lateral diffusion. During the analysis, intracellular cholesterol might diffuse to plasma membrane that introduces the additional charge.

However, since cholesterol is hydrophobic and over 90% cholesterol is existed in plasma membrane, the possible contribution from intracellular cholesterol to the detection of membrane cholesterol should not be significant.

The removal of Triton X-100 from the solution in the capillary gave less charge increase in the analysis, as shown in Figure 3C (trace b), which should be attributed to the reaction of cholesterol efflux with cholesterol oxidase. Since cholesterol efflux was origined from active membrane cholesterol with high escape tendency,[14] the charge contributed from cholesterol efflux should be less than the value from the analysis of membrane cholesterol in presence of Triton X-100. The other control experiment was performed when cholesterol oxidase was removed from the capillary. The typical charge curves were shown in Figure S3 (supporting information). An average charge of 0.68 nC was observed that should be attributed to non-specific adsorption of lipids at the electrode surface during the contact. All these results suggested that cholesterol oxidase/Triton X-100 parked microelectrode could detect plasma membrane cholesterol that was not associated with its activity in plasma membrane.

23 cells were analyzed using our electrodes individually and these results were summarized in Figure 3D. The average charge increase collected was 1.32 nC with a relative standard deviation of 53.8%. After extracting the charge of 0.68 nC from non-specific adsorption, 0.64 nC remained suggested 3.2 fmole cholesterol in the plasma membrane reacted with cholesterol oxidase. Based on the molecule area of cholesterol and the lateral diffusion coefficient (15×10^{-12} m^2/s) in cellular membrane,[22,23] ~ 3.2 fmole cholesterol reacted in 180 s should come from a ~ 21 μm diameter region at plasma membrane. The size was larger than the contact dimension confirmed the lateral diffusion of membrane cholesterol during the analysis. The withdrawn and re-contact of the microelectrode with the cell at the same position did not give the detectable charge increase, which indicated the depletion of membrane cholesterol after the first contact/reaction period. Meanwhile, the large deviation suggested high heterogeneity of plasma membrane cholesterol at single cells, which was consistent with our previous result on total membrane cholesterol.[13,24]

To further validate our electrodes for the evaluation of membrane cholesterol at single cells, membrane cholesterol at single cells was elevated by the incubation of the cells with Sandoz 58-035, an Acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor, over 16 hours at 37 °C The

inhibition of intracellular ACAT stopped the esterification of cholesterol into cholesterol ester, and thus, the content of membrane cholesterol increased.[25] After collecting the charges from single ACAT inhibited cells before and after the contacting of the electrode, the charge increases were calculated. The charge increases from 28 single living cells were summarized in Figure 4A. This statistical data in Figure 4B exhibited that the charge increase from ACAT inhibited cells was larger than that from normal cells, which was consistent with the fluctuation of membrane cholesterol after the inhibition of intracellular ACAT. This result supported that our method could monitor the alteration of membrane cholesterol at single cells with different states.

In conclusion, cholesterol oxidase/Triton X-100 parked microelectrodes were designed successfully for the evaluation of membrane cholesterol in single living cells. The introduction of Triton X-100 at the electrode surface permeabilized the membrane in the contact region so that total cholesterol was detected. Moreover, cholesterol oxidase stayed in the aqueous solution between the cellular membrane and the electrode providing higher turn-over rate than that from oxidase modified at the electrode. The achievement in the analysis of membrane cholesterol using this new electrode provided a new strategy for the fabrication of biosensor, which could integrate more molecules without the function groups at the electrode to realize single cell analysis. The further work will focus on the development of smaller electrodes to achieve the analysis with higher spatial resolution. More investigation on the stability of aqueous oxidase layer and the reproducibility on the charge increase are needed so that mapping cholesterol distribution using this new microelectrode is feasible. Also, two sets of capillary electrodes loaded with cholesterol oxidase and cholesterol oxidase/Triton X-100, respectively, will be contact with the same positions at plasma membrane in serial for the determination of active and total membrane cholesterol at the contact region. The data obtained could provide the direct evidence whether active cholesterol is tightly regulated as a signaling agent whereas bound cholesterol varies between cells to a greater extent.

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Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

Experimental section and three additional figures about SEM characterization of capillary tip and more charge curves.

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Figures and Captions.

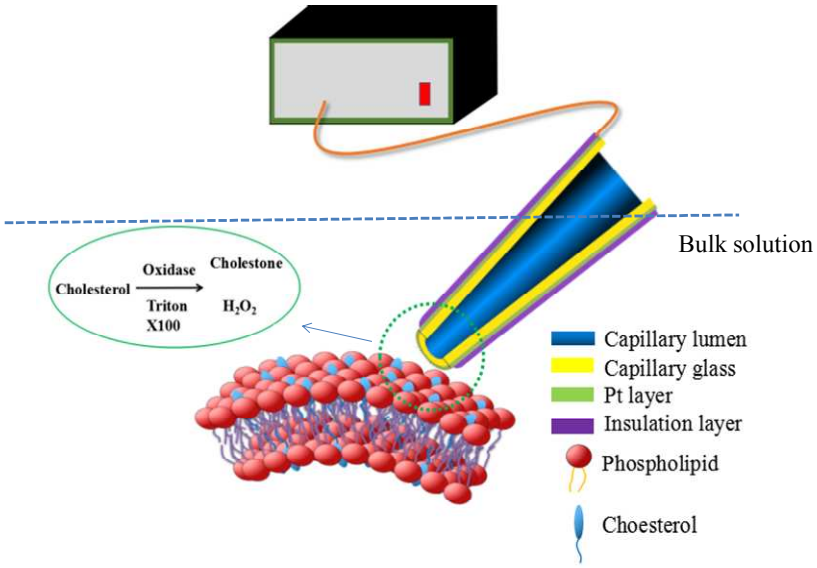
Figure 1. Schematic setup for cholesterol oxidase/Triton X-100 parked microelectrode for the detection of membrane cholesterol at single living cells.

Figure 2. (A) The charges of cholesterol oxidase/Triton X-100 parked microelectrode exposed to the buffer (10 mM PBS with 50 mM MβCD) (curve a), with 0.1 (curve b), 0.5 (curve c), 1 (curve d), 2 (curve e) and 3 mM cholesterol (curve f); (B) the differences in the charges collected in 0.1 (curve a), 0.5 (curve b), 1 (curve c), 2 (curve d) and 3 mM cholesterol (curve e) after extracting the background charge; (C) the correlation between the charge increases and the cholesterol concentrations at 0.1, 0.5, 1, 2 and 3 mM. The charge was collected at the time of 180 s. The error bar presented the standard deviation from three electrodes.

Figure 3. (A) The bright-field image of cholesterol oxidase/Triton X-100 parked microelectrode in contact with single living Hela cell; (B) the charges collected before (curve a) and after (curve b) the contact of the cholesterol oxidase/Triton X-100 parked electrode with the cell; (C) the charge increases collected from cholesterol oxidase/Triton X-100 parked microelectrode (curve a) and cholesterol oxidase parked microelectrode (curve b); (D) the charge increases collected from 23 single cells using cholesterol oxidase/Triton X-100 parked microelectrode. The charge was collected at the time of 180 s.

Figure 4. (A) The charge increases collected from 28 single ACAT inhibited cells using cholesterol oxidase/Triton X-100 parked microelectrode. The charge was collected at the time of 180 s. (B) the statistical analysis of charge increases from normal and ACAT inhibited cells. The error bar presented the standard deviation from the measurements at these cells.

Figure 1.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Capillary lumen
Capillary glass
Pt layer
Insulation layer
Phospholipid
Choesterol

Figure 2.

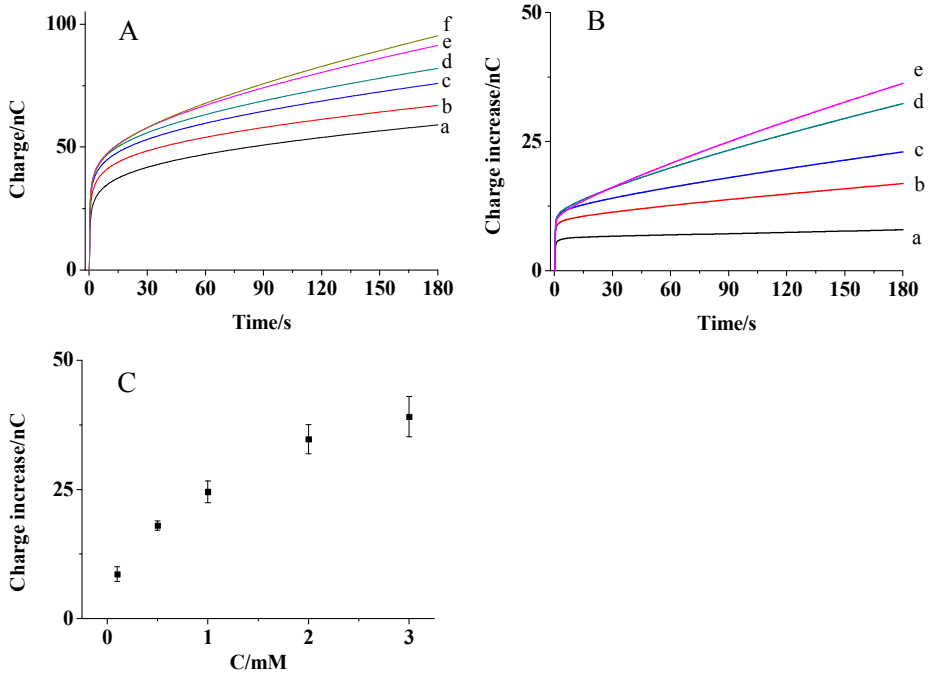
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Figure 3.

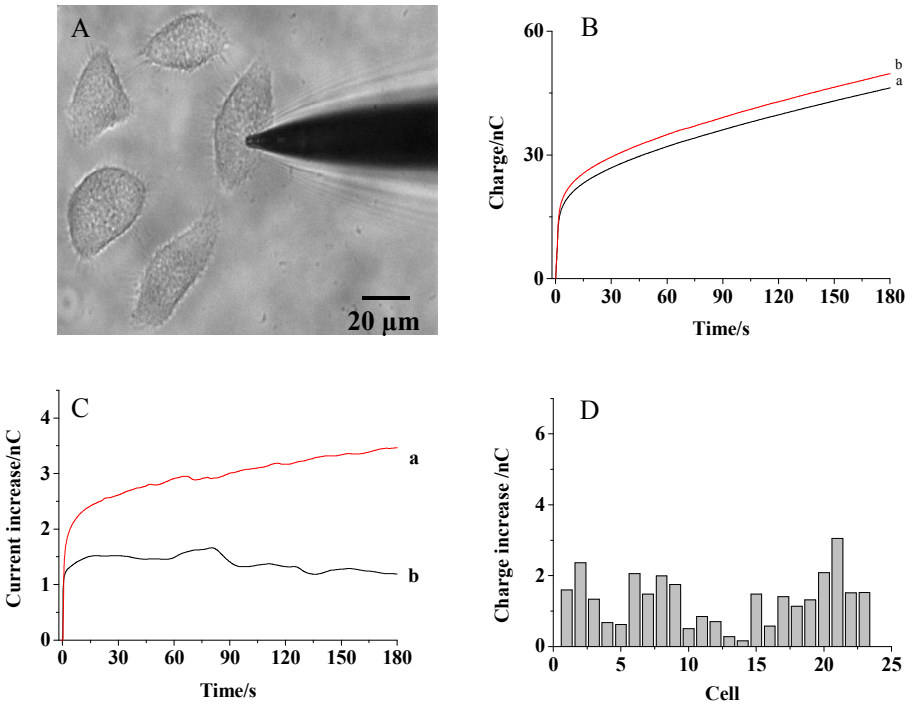
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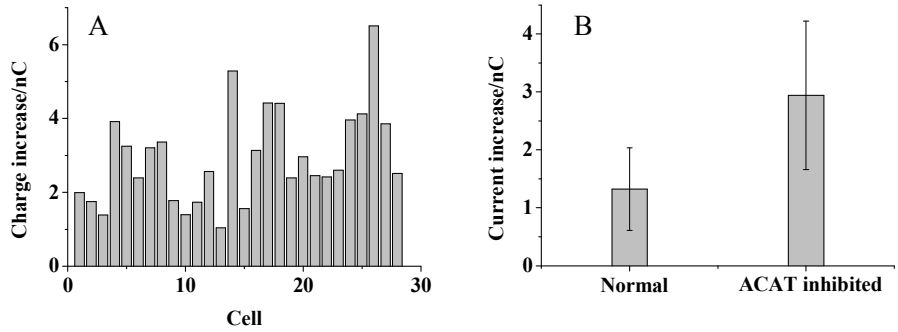
Figure 4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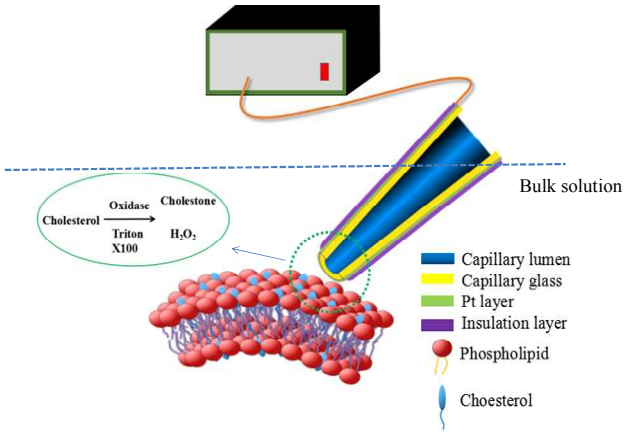
Bulk solution
Oxidase
Cholesterol
Cholestone
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H_2O_2
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