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Pt-decorated MWCNT-ionic liquid composite-based hydrogen peroxide sensor to study microbial metabolism using scanning electrochemical microscopy

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Abstract

Hydrogen peroxide (H₂O₂) is a highly relevant metabolite in many biological processes, including the oral microbiome. To study this metabolite, we developed a 25 μ m diameter, highly sensitive, non/enzymatic H₂O₂ sensor with a detection limit of 250 nM and a broad linear range of 250 nM to 7 mM. The sensor used the synergistic activity of the catalytically active Pt nanoparticles on a high surface area multiwall carbon nanotube and conducting ionic liquid matrix to achieve high sensitivity (2.4 ± 0.24 mA cm⁻² mM⁻¹) for H₂O₂ oxidation. The unique composite allowed us to miniaturize the sensor and couple it with a Pt electrode (25 μ m diameter each) for use as a dual scanning electrochemical microscopy probe. We could detect 65 ± 10 >M of H₂O₂ produced by *Streptococcus gordonii* (Sg) biofilms at a 50 μ m above its surface in the presence of 1 mM glucose and artificial saliva solution (pH 7.2 at 37°C). Because of the high stability and low detection limit, the sensor showed a promising chemical image of H₂O₂ produced by Sg biofilms. We were also able to detect 30 μ M of H₂O₂ at 50 μ m above the biofilm in the presence of the H₂O₂-decomposing salivary lactoperoxidase and thiocyanate, which would not otherwise be possible by existing H₂O₂ assay. Thus, this sensor can potentially find application in the study of other important biological processes in a complex matrix where circumstance demands a low detection limit in a compact space.

Graphical Abstract

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Supporting Information

Sg gel biofilm CFU based viability tests, Material characterization: FTIR of f-MWCNTs, EDAX recorded on Pt-MWCNTs and cyclic voltammogram obtained at 25 μ m, Pt/MWCNT-IL/packed sensor in 0.5 M H₂SO₄, Table: Data obtained from calibration curves recorded at 0.5 V vs Ag/AgCl at sensors made with various compositions in phosphate buffered saline and artificial saliva solution at 37°C, Response time of the optimized 10:90 wt% Pt-MWCNT-IL-packed sensor, SECM probe approach curves recorded on the surface of the Sg hydrogel biofilm matrix made in PDMS and on the surface of PDMS using a 25 μ m Pt in a dual (H₂O₂ sensor/Pt) SECM probe.

Keywords

Non-enzymatic hydrogen peroxide sensor; ultramicroelectrode sensor; SECM

INTRODUCTION

Hydrogen peroxide (H_2O_2) is of great importance in regulating diverse and crucial biological processes in eukaryotes, such as intracellular signaling, phagocytosis, antibacterial defense, and stem cell proliferation.^{1–9} Furthermore, numerous microbial studies have demonstrated the importance of H_2O_2 in modulating bacterial ecology and signaling including antagonizing competitors in the oral biofilm.^{10–13} For example the commensal oral species *Streptococcus gordonii* (Sg) produces H_2O_2 to control other pathobionts¹³ thus supporting a healthy oral microbiome. H_2O_2 is produced in the presence of oxygen while carbohydrates are metabolized, inhibiting acid-producing bacterial species such as *S. mutans* implicated in the development of caries.^{13,14–18} The inhibitory effect of H_2O_2 extends also towards periodontal pathogens such as *Aggregatibacter actinomycetemcomitans* (Aa).¹⁹ However, the interspecies interaction between Aa and Sg seems to be more intricate since non/lethal concentrations of H_2O_2 favor survival of Aa against the host innate immunity by inducing a specific transcriptional response²⁰ tailored towards complement evasion. This indicates that the concentration of H_2O_2 in the context of a biofilm determines its potential as antimicrobial or signaling component. This fact is complicated by the presence of powerful detoxifying enzymes in saliva such as salivary lactoperoxidase (LPO), which beside diffusion limitation and metabolic variation can change the concentration of H_2O_2 produced by biofilms dramatically. Thus the landscape of H_2O_2 production in high resolution with a wide range of concentration is impossible to determine with conventional H_2O_2 detection methods.

A major limitation of current approaches is that almost all studies used colorimetric assays²¹ to determine the concentration of H_2O_2 . In recent study Kreth et al., were unable to detect any H_2O_2 produced by Sg in the presence of salivary LPO and thiocyanate (SCN^-).²² Typically, these assays require the collection of an aliquot of samples at a certain time point and thus lack temporal and spatial resolution. This becomes more evident when attempting to measure H_2O_2 close to the biofilm before it is decomposed by relevant H_2O_2 -decomposing agents including salivary LPO and thiocyanate SCN^- . Thus, a new analytical technique is needed to quantitatively measure local H_2O_2 concentration and its distribution in 3D space surrounding the biofilm to determine whether any H_2O_2 is present under biological relevant conditions. This will greatly advance the biological findings about the importance of H_2O_2 -producing Sg in maintaining a healthy oral microbiome.

In recent decades, scanning electrochemical microscopy (SECM) has proved to be an appropriate noninvasive tool to study various^{17,23,24} live biological systems, including the measurement of the local concentration of bacterial metabolites. In general, a Pt or Au electrode of 10 or 25 μm diameter was used^{17,24–26} as an H_2O_2 sensor in the SECM/ bacterial experimental setup. Abucayon et al.²⁴ reported measuring the rate of decomposition of H_2O_2 by a catalase enzyme present in *Vibrio fischeri* biofilm, and Liu et al.¹⁷ used a similar 25 μm Au electrode to quantify the H_2O_2 produced by Sg bacterial biofilm. Both reported sensors, however, showed a detection limit in the micromolar range. The 25 μm diameter Au UME for SECM has also been modified with prussian blue to function as a H_2O_2 sensor.²⁷ However, no application in high sodium content complex biological relevant media has been reported using this type of sensor.^{28,29} An alternative approach was the use of SECM and scanning ion-conductance microscopy (SICM) combined, as reported by Connell et al.³⁰ In that approach, one of the two barrels was used as an SICM probe to position it w.r.t the substrate, whereas the other barrel was modified with electrochemically deposited Pt on a pyrolytically deposited carbon electrode. Although high-resolution topographical and chemical images of the Au nanoparticles were described, no working calibration curve for the H_2O_2 sensor was reported so as to quantify the amount of H_2O_2 produced by these Au nanoparticles. Recently, graphene has been used as an attractive electrode material to immobilize noble metal nanoparticles, which can be used as an electrochemical H_2O_2 sensor.³¹ Sun et. al.³² used graphene/supported PtPb nanoparticles to detect H_2O_2 produced by RAW 246.7 cells. This macro/sized, 3 mm diameter PtPb/G nanocomposite sensor showed a broad linear range of 2 nM to 2.5 mM with a low detection limit of 2 nM. Similarly, Liu et al.³³ used a 3 mm diameter sensor composed of Ag nanoparticles-porous graphene and horseradish peroxidase enzymes to detect H_2O_2 produced by PC12 cells. In their study, they reported a 0.0267 nM limit of detection and a linear range of 7 orders of magnitude. The common feature of these reported highly sensitive H_2O_2 sensors³³ is the large surface area fabricated on a 3 mm glass carbon electrode or they are used in PBS buffer or similar clean solution.

The main challenges that remain lie in designing and fabricating a highly sensitive micrometer-sized H_2O_2 sensor for SECM that can perform an approach curve and at the same time has the capability to detect nanomolar to low micrometer H_2O_2 levels in a complex biological system such as biofilm. In the present study, we report a novel method for fabricating a 25 μm , H_2O_2 -selective, dual SECM probe to map the 3D microenvironment above the Sg bacteria biofilm in the presence of artificial saliva solution. The unique catalytic properties of Pt nanoparticles (Pt-NPs), stabilized on multiwall carbon nanotubes (MWCNTs) and a conducting ionic liquid (IL) matrix, assisted us in achieving a highly sensitive 25 μm diameter H_2O_2 sensor with a nanomolar limit and a broad working range in artificial saliva (AS) solution. Using our newly developed sensor with SECM, we were able to measure the local H_2O_2 concentration produced by Sg biofilms under complex salivary conditions, i.e. in the presence of relevant salivary LPO and SCN^- , and thus demonstrated its application for studying microenvironment/dependent bacterial metabolic activity in a complex live system.

MATERIALS AND METHODS

Materials:

Non-graphitized MWCNTs were procured from US Research Nanomaterials, Inc. (Houston, TX, USA). Dihydrogen hexachloroplatinate (IV) hexahydrate, 1-butyl-4-methylpyridinium hexafluorophosphate (IL), and H₂O₂ (30%) were purchased from Alfa Aesar, Sigma-Aldrich, and Macron Fine Chemicals, respectively. Lactoperoxidase (LPO) was obtained from Spectrum Chemical. Potassium thiocyanate (KSCN) was purchased from Alfa Aesar. Alginate sodium salt was purchased from Sigma Aldrich. Brain heart infusion (BHI) medium and agar were obtained from BD.

Artificial saliva (AS) solution was freshly prepared by mixing 0.70 mM CaCl₂, 0.43 mM MgCl₂, 4 mM KH₂PO₄, 20 mM HEPES, 30 mM KCl in deionized (DI) water (18 MM) and pH of solution was adjusted to 7.2. Phosphate buffered saline (PBS) solutions (pH 7.2) was prepared in DI water (18 MM) and stored at room temperature.

Bacterial strains and growth conditions:

Streptococcus gordonii DL1³⁴ was initially grown on BHI agar plates in 5% CO₂ at 37 °C. Prior to the experiments, bacteria were inoculated in BHI liquid medium at 37 °C under anaerobic conditions and harvested when reaching logarithmic growth (A₆₀₀ ~ 0.6) to fabricate bacteria-alginate biofilms.

Instrumentation:

All electrochemical measurements were performed by using a bipotentiostat and a scanning electrochemical microscope (model 920D) (CHI, Austin, TX, USA) with a standard three-electrode system: sensor or Pt ultramicroelectrode (UME) (working electrode), Pt wire (counter electrode), and Ag/AgCl (1 M KCl) (reference electrode).

Pt-decorated MWCNT (Pt MWCNT) fabrication and characterization:

Non-graphitized MWCNTs were first functionalized with carboxylic acid (–COOH) groups.^{35,36} In brief, 60 mg of MWCNTs was sonicated for 1 h in concentrated (16 M) nitric acid by using a sonic bath and then refluxed at 80 °C for 72 h. Modified MWCNTs were then separated from supernatant acid, diluted with DI water, and filtered with Whatman filter paper (grade 42). The sample was further washed with a copious amount of DI water and then dried at 60 °C for 5–6 h. The –COOH functionalization of MWCNTs was confirmed and quantified by Fourier transform infrared (FTIR) spectroscopy and acid-base titration. For Pt-NP deposition, 16 mg of dihydrogen hexachloroplatinate (IV) hexahydrate was added to 40 mg of –COOH- functionalized MWCNTs (f-MWCNTs) dispersed in 40 mL of a 3:2 v/v ethylene glycol:water mixture and then refluxed at 110 °C for 8 h.³⁷ The Pt-decorated MWCNTs were washed with DI water and dried in a vacuum oven at 60 °C for 5–6 h. The schematics for the –COOH functionalization and Pt decoration of MWCNTs is shown in Figure 1A. The sample was later characterized by scanning electron microscopy (SEM) and energy dispersive X-ray analysis (EDAX).

Fabrication and characterization of micro-sized dual H₂O₂ sensor:

A theta pipette with 1.5 mm OD and 1 mm ID was used to make the dual SECM probe. Two 25 μ m diameter Pt wires (Goodfellow Cambridge Ltd.) were sealed in a vacuum into each barrel of the pulled theta pipette by using a nichrome wire (Omega NI60-032-200) heating coil. Electrical connections were made by copper wires and silver epoxy. The electrode was polished to expose the Pt surface, and an RG (ratio of insulation glass diameter to conducting Pt diameter) value of 8 was used for all experiments. For peroxide sensor fabrication, one Pt electrode was etched in a solution of 60:36:4 v/v Ca:H₂O:HCl to make a 10 μ m cavity by applying 5 V across a graphite rod.³⁸ The etched cavity was cleaned by sequential sonication of the tip in water and absolute ethanol, and it was dried under a stream of nitrogen gas. The cavity on the UME was packed with a catalyst: w/w % of Pt-MWCNTs and 1-butyl-4-methylpyridinium hexafluorophosphate (room temperature IL). The extra paste sticking to the glass surface of the UME was wiped out with a wet Kimwipe (Figure 1B). The packing was confirmed by observing the UME under an optical microscope. The top view of the optical micrograph of the fabricated dual (H₂O₂ sensor/Pt) probe is shown in Figure 1C. Electrochemical characterization of the dual (H₂O₂ sensor/Pt) probe was performed by recording voltammograms in 1 mM ferrocene methanol in AS (pH 7.2) solution. Various combinations of the Pt-MWCNT-IL mixture were tested to optimize the lower detection limit and sensitivity for H₂O₂ detection. Later, the sensor was calibrated by applying +0.5 V vs Ag/AgCl (oxidation potential of H₂O₂) and recording the i-t curve in a PBS solution and an AS solution (pH 7.2) with the addition of a known concentration of H₂O₂ at 23 °C and 37 °C. In addition, we fabricated f-MWCNT-IL-packed and hydrogen peroxidase-immobilized dual probe UME sensors and tested them for H₂O₂ detection under similar experimental conditions in order to compare their efficiency with the Pt-MWCNT-IL UME sensor. The sensors were characterized by EIS (galvanostatic mode) by using a three-electrode system with a Biologic Science Instruments galvanostat/potentiostat (SP 200) with EC-Lab V11-02 software. EIS tests were carried out in 1 mM ferrocyanide/ferricyanide in 0.1 M KCl solution in the range of 3000 kHz to 0.1 Hz. Data is presented in the form of a Nyquist plot.

Sg-alginate gel biofilm preparation:

To prepare the Sg-alginate gel biofilm substrate, bacterial cells were harvested from logarithmic growth phase ($A_{600} \sim 0.6$) in BHI by centrifugation (centrifuge settings and temperature needs to be mentioned) and suspended in 10 mM PBS (pH 7.2) to an $A_{600} \sim 2.0$. One mL of suspension was mixed with 1 mL of 4% sterile alginate solution. Next 2 mM CaCl₂ was added to this mixture to form a soft gel. The temperature of all solutions was maintained at 4 °C. 11 μ L of the soft gel was added to a PDMS cavity (diameter 2.5 mm, depth 1200 μ m) mounted on a glass slide. The substrate was kept undisturbed for 20 min to settle in the cavity and then the gel was cured at 4 °C with 1% CaCl₂ solution for 1 h.

SECM-biofilm experiments:

To study the metabolic activity of bacteria with and without LPO and SCN⁻, we prepared a Sg-alginate gel biofilm substrate as described above and placed it on the SECM stage (see Figure 5A and 5B). We added 1 mM ferrocene methanol in AS solution to the sample after it

was triple washed with AS solution. The dual SECM probe (H_2O_2 sensor/Pt) was then attached to the holder, and the Pt electrode of the dual probe was used to fix the probe-substrate distance ($50\text{ }\mu\text{m}$) by performing an approach curve on the hydrogel-biofilm substrate. Ferrocene methanol was used as a redox mediator in this experiment, as it demonstrated to be non/toxic to bacteria according to our CFU based viability tests (Figure S1). Later the solution was replaced by AS solution only and the temperature was raised to $37\text{ }^\circ\text{C}$. The dual SECM probe was then switched to H_2O_2 sensor mode by applying $+0.5\text{ V}$ to the Pt-MWCNT-IL-packed electrode. We then added 1 mM of glucose to the solution to measure the H_2O_2 generated by the biofilm w.r.t. time. The X-directional H_2O_2 concentration profile above the biofilm was recorded by moving the H_2O_2 sensor in x direction ($60\text{ }\mu\text{m}$ above). Z direction H_2O_2 concentration profile was also recorded by moving sensor in Z direction from a distance of 50 to $1000\text{ }\mu\text{m}$ above the Sg-hydrogel substrate. The chemical image of H_2O_2 produced by Sg was obtained by the H_2O_2 sensor at a constant distance of $50\text{ }\mu\text{m}$ above the substrate at $37\text{ }^\circ\text{C}$. Similarly, an Sg-hydrogel substrate morphological image was obtained by using the Pt electrode of the dual SECM probe with ferrocene methanol as a redox mediator at $23\text{ }^\circ\text{C}$.

To study the effect of the LPO enzyme and SCN^- on the local concentration of H_2O_2 above the biofilm, we performed a similar set of fixed-distance ($50\text{ }\mu\text{m}$ above the biofilm) and z-direction scan experiments by using the H_2O_2 sensor of the dual SECM probe. First, the LPO enzyme ($10\text{ }\mu\text{g/mL}$) and then potassium thiocyanate (KSCN, 1 mM) was added after we observed H_2O_2 production by the Sg biofilm.

RESULTS AND DISCUSSION

Characterization of Pt decorated MWCNTs (Pt MWCNTs):

Because of their unique structure, MWCNTs have a high surface-to-volume ratio^{39,40} and thus provide an ideal template to grow catalytic Pt-NPs. However, pristine MWCNTs as acquired from the supplier do not provide enough defect sites for growing nanoparticles. Therefore, we refluxed the MWCNTs in concentrated nitric acid (16 M) for 72 h at $80\text{ }^\circ\text{C}$ to increase the number of defect sites and the $-\text{COOH}$ functionalization of the MWCNTs, thus enhancing their dissolution in aqueous solution mixture for further treatment. We obtained $6.5 \pm 1.0\%$ of $-\text{COOH}$ functionalization of MWCNTs, as determined by acid-base titration. Functionalization of MWCNTs was further confirmed by recording the FTIR spectrum of the COOH-MWCNTs (Figure S2 A), which showed a sharp peak at $\text{ca. } 1608\text{ cm}^{-1}$ and a broad band at 3359 cm^{-1} due to carbonyl in carboxylic acid and $-\text{OH}$ stretching.⁴¹ Carboxylic moieties and defects on the surface of the MWCNTs provided nucleation sites for the formation of stable Pt-NPs on this surface (Figure 1A). These functional groups also provided sites for the covalent linking of enzymes for enzymatic sensor fabrication. f-MWCNTs showed almost endless entangled bundles of tubular structures with an average diameter of $\text{ca. } 65 \pm 10\text{ nm}$ in SEM (Figure 2A). SEM images recorded on the Pt-MWCNTs showed the formation of Pt-NP clusters on the surface of f-MWCNTs (Figure 2B), and the loading of Pt on MWCNTs was estimated to be $1.11\text{ at. wt.}\%$ in EDAX analysis (Figure S2 B). X-ray diffraction (XRD) data recorded on Pt-MWCNTs showed strong reflections at 39.7° , 67.4° , and 81.2° due to Pt(111), Pt(220), and Pt(311) planes of polycrystalline

platinum with graphitic (002), (100), (004), and (110) reflections⁴¹ (Figure 2C). These values confirmed the formation of crystalline Pt-NP-modified MWCNTs (Pt-MWCNTs) for H₂O₂ sensor development.

Electrochemical characterization of the Pt-MWCNTs was performed by recording the cyclic voltammogram (CV) at a 25 μ m diameter Pt-MWCNT-IL packed sensor in 0.5 M H₂SO₄ (Figure S2 C). The CV showed the H and O₂ region, which is typically observed in the case of polycrystalline Pt in H₂SO₄ solution, confirming the presence of Pt-NPs in the composite matrix. The broad reduction peak observed at 0.44 V in the oxygen region was shifted towards the positive side because of the synergistic interaction of Pt-NPs with MWCNTs.⁴² The reduction current was stable over repeated cycling in 0.5 M H₂SO₄, indicating that there was no leaching of the Pt-NPs from the surface of the MWCNTs during measurement. In addition, the surface functional groups and the defects incorporated during acid treatment helped to improve the stability and electrocatalytic activity of the Pt-NPs, making this composite a suitable candidate for sensor application.⁴³

Optimization of Pt-MWCNT-IL composite to fabricate a 25 μ m diameter dual H₂O₂ sensor:

One of the major challenges in developing a micron-sized H₂O₂ sensor is to achieve high sensitivity and a low detection limit (~nanomolar to sub/micromolar) on an extremely small electroactive surface area without compromising the stability of the sensor. These criteria are even more critical when designing an SECM probe to quantitatively map H₂O₂ concentration, along with performing an approach curve to fix the probe/substrate distance. Therefore, we designed a dual SECM probe in which the two Pt electrodes were 25 μ m diameter each, one to perform the approach curve and the other modified to function as an H₂O₂ sensor (see Figure 1B). Although several studies used the bare Pt electrode as an H₂O₂ sensor,^{24–26} stability and sensitivity are still an issue, as confirmed in our studies (see Figure 3A). We thus etched and packed one of the two Pt electrodes with Pt-NP-decorated MWCNTs and with a conducting room temperature IL (1-butyl-4-methylpyridinium hexafluorophosphate), which significantly improved the sensitivity of the sensor by 40% in comparison to the bare Pt electrode (Figure 3A). The sensor comprising 10:90 wt. % of Pt-MWCNTs-IL composites showed the best sensitivity of 2.4 ± 0.24 mA cm⁻² mM⁻¹ with a lower detection limit of 250 nM and a broad working range of 250 nM to 7 mM (Table S1, Figure 4C and 4D). Although several previous reports described a graphite powder, carbon nanotube, IL-packed electrode to detect H₂O₂,^{44–46} the pyridinium hexafluorophosphate-based IL used in this study showed one of the lowest background currents without any heat pretreatment and was thus able to achieve a lower detection limit.

To further confirm our sensor performance, we conducted a detailed comparative study between different compositions (such as MWCNTs-paraffin oil, f-MWCNTs-IL, peroxidase-MWCNTs-IL, Pt-MWCNTs-IL) of H₂O₂ sensors in AS solution at 37 °C, as shown in Figure 3A. As reported earlier,⁴⁷ MWCNTs also showed a catalytic response for the detection of H₂O₂ with a lower detection limit of 500 μ M and a sensitivity of 6×10^{-6} mA cm⁻² mM⁻¹ in the concentration range of 500 μ M to 2.0 mM when packed with 15 wt. % paraffin oil (Table S1 Part a). This response was further enhanced with IL binder because of its thermal stability, ionic conductivity, and good electrochemical stability. The MWCNT-IL

sensor detected a concentration of H_2O_2 that was 5 times lower (LOD 100 μM) than that detected with the MWCNT-paraffin oil sensor, with a sensitivity of $0.87 \text{ mA cm}^{-2} \text{ mM}^{-1}$. The f-MWCNT-IL- and hydrogen peroxidase f-MWCNT-IL-packed sensors showed a lower detection limit of 10 μM with sensitivities of $1.56 \text{ mA cm}^{-2} \text{ mM}^{-1}$ and $1.67 \text{ mA cm}^{-2} \text{ mM}^{-1}$, respectively, but failed in terms of stability at 37 °C. This might be because only $6.5 \pm 1.0\%$ of $-\text{COOH}$ functionalization was achieved after acid treatment of MWCNTs, which limits maximum enzyme loading to obtain a reproducible response. The values obtained for a lower detection limit, a linear working range, and sensitivity for oxidation of H_2O_2 are summarized in Table S1. Thus, the data obtained from sensor screening tests demonstrated that the Pt/MWCNT/IL sensor showed the best performance in terms of detection limit, stability, and broad linear range for the detection of H_2O_2 at 37 °C.

To determine the exact role of IL and Pt-NPs in a sensor composition and the effect of temperature on sensor performance, we calibrated one sensor made without Pt-NPs, i.e. a f-MWCNT-IL sensor, and one made with Pt-NPs, i.e. a Pt-MWCNT-IL sensor, at 23 °C and 37 °C (Figure 3B). Both sensors showed ~60% increment in sensitivity at 37 °C compared with that at 23 °C. This enhancement in sensor performance can be attributed to the intrinsic properties of IL, which improves its ionic conductivity at higher temperatures. However, the presence of Pt/NPs in the sensor composite provided catalytic sites for the oxidation of H_2O_2 and hence showed highest sensitivity at 23 °C ($0.99 \pm 0.12 \text{ mA cm}^{-2} \text{ mM}^{-1}$) and at 37 °C ($2.4 \pm 0.24 \text{ mA cm}^{-2} \text{ mM}^{-1}$) compared with that of the f-MWCNT-IL sensor. To explore this result, we recorded the electrochemical impedance spectra (EIS) at the sensors in 1 mM ferricyanide/ferrocyanide in 0.1 M KCl. Figure 3C shows the Nyquist plots recorded at the f-MWCNT-IL and Pt-MWCNT-IL sensors. Results indicate that there was a 20% decrement in the charge transfer resistance (R_{ct}) at 37 °C compared with the R_{ct} obtained at 23 °C for both sensors because of the improved ionic conductivity of IL. However, the Pt-MWCNT-IL sensor showed the lowest R_{ct} (24 k Ω at 23 °C and 20 k Ω at 37 °C), indicating that the Pt-NPs in the Pt-MWCNT-IL composite have good conductivity to facilitate the necessary electron transfer pathway, which explains the improved performance of the sensor at 37 °C.

Henceforth, we test the Pt-MWCNT-IL sensor for H_2O_2 oxidation by performing cyclic voltammetry. Figure 4A shows the CVs recorded at a Pt-MWCNT-IL sensor with an increasing concentration of H_2O_2 from 1 mM to 5 mM in AS solution (pH 7.2). After the addition of H_2O_2 , the cathodic current increased at -0.078 V because of the reduction of H_2O_2 to H_2O , and the anodic current increased at 0.5 V because of the oxidation of H_2O_2 to O_2 at the sensor surface. This suggests that there is minimal fouling at the Pt-MWCNT-IL sensor surface even in the presence of a high concentration (1 mM to 5 mM) of H_2O_2 . The Pt-MWCNT-IL composite sensor showed good catalytic activity for H_2O_2 oxidation/reduction reaction because of the presence of Pt-NPs. In addition, MWCNTs with a large basal plane/edge plane ratio enhance the conductivity and speed of electrode kinetics. In addition, we recorded the CVs at 25 μm Pt UME-, f-MWCNT-IL-, and Pt-MWCNT-IL-packed sensors in a 1 mM ferrocene methanol redox solution (Figure 4B). The steady-state current (I_{ss}) observed at the f-MWCNT-IL packed sensor was ~50% less than that at the Pt UME sensor, whereas the Pt/MWCNT/IL sensor showed an I_{ss} current that was comparable to that of Pt UME, which is also close to the theoretical current ($I_{\text{ss}} = 3.67 \text{ nA}$) calculated

from the steady/state equation of UME ($I_{ss} = 4nFD_0C_0^*$).⁴⁸ Therefore, the observed I_{ss} at the Pt-MWCNT-IL sensor was ascribed to the highly conducting matrix, which has electrochemical properties and geometry comparable to that of the 25 μm Pt UME and also Pt-MWCNT-IL sensor shows similar kinetics to that of Pt UME for the oxidation of redox species.

Figure 4C and 4D show typical calibration curves obtained with an optimized sensor: a 10:90 wt. % Pt-MWCNT-IL sensor in lower and higher concentration ranges of H_2O_2 at 37 °C in AS solution. The data were obtained from the I-t curves recorded at 0.5 V vs Ag/AgCl with the addition of a known concentration of H_2O_2 . The sensor showed a linear range from 10 μM to 500 μM with a sensitivity of $2.4 \text{ mA cm}^{-2} \text{ mM}^{-1}$ (Figure 4C), which is 3 times higher than the reported sensitivity value obtained at the H_2O_2 -selective Prussian blue-modified SECM probe.²⁷ The inset to Figure 4C shows the calibration curve obtained at a sensor with the addition of (lower concentration) 100 nM to 8 μM H_2O_2 . Our newly developed sensor showed a lower detection limit of 250 nM, which is almost 40 times lower than reported,²⁷ with a linear range from 250 nM to 8 μM . As there is only 10 wt % of Pt-MWCNTs in a catalyst composite, the observed background current at the sensor was less (0.1–1 pA), which is a key point in the detection of small signal changes for the nanomolar concentration of H_2O_2 . We have also calibrated an optimized sensor with the addition of a higher concentration, i.e. 2 mM to 10 mM H_2O_2 in AS solution (Figure 4D). The sensor showed a linear response from 2 mM to 7 mM with a sensitivity of $0.13 \text{ mA cm}^{-2} \text{ mM}^{-1}$. Thus, our newly developed sensor showed a broad working range from 250 nM to 7 mM H_2O_2 with minimal fouling of the sensor surface, as revealed in Figure 4A, 4C, and 4D. Here, the surface functionality incorporated in the MWCNTs helped in the self-cleaning of the Pt surface,^{41,49} and the IL provided a stable conducting matrix, which also provided mechanical stability for the sensor during lengthy time measurements.

Since we want to use this newly developed sensor as an SECM probe, it is important to observe the response time and stability of a sensor. A fast response time of 500 ms was observed at the optimized Pt-MWCNT-IL sensor (Figure S3), making it a suitable candidate to be used as an H_2O_2 -selective SECM probe for continuous real-time monitoring of released H_2O_2 from live biological samples. The sensor showed a stable response for the continuous detection of H_2O_2 without any surface fouling.

Application of an H_2O_2 microsensor as an SECM dual probe to study Sg biofilm metabolism in real time:

We use our newly developed compact H_2O_2 dual SECM probe to measure the H_2O_2 produced by Sg biofilms in real time. The compact size (25 μm diameter), higher sensitivity, and wide working range in biologically relevant AS solution gave us the unique ability to position our sensor very close (20 μm) to the Sg biofilm. This enabled us to quantify the H_2O_2 produced before it diffused in the bulk solution or was decomposed by the LPO enzyme. The SECM H_2O_2 probe was thus positioned at a known distance of 50 μm above the Sg biofilm (Figure 5B) with the aid of an approach curve, as shown in Figure 5C and Figure S4. We detected 50 μM of H_2O_2 at 50 μm above the 1 mm dia. Sg biofilm when it was exposed to 1 mM glucose at 37 °C (Figure 6A). The H_2O_2 reached a steady state after

30 min because of the diffusion limitation of glucose through the hydrogel matrix (similar to the biofilm matrix). The x-direction scan at a constant distance of 60 μm above the biofilm also confirmed an H_2O_2 concentration of 60 μM (Figure 6B). The H_2O_2 concentration profile was observed to be extended 500–700 μm on both sides of the biofilm, i.e. an Sg biofilm of 1 mm diameter is able to influence the surrounding environment up to a distance of 500 μm , which is significant in a multispecies biofilm where other species coexist within a few microns. The chemical image of distribution of H_2O_2 above the biofilm showed a unique microenvironment created by the Sg biofilm (Figure 6C). A corresponding morphological image of the biofilm-hydrogel matrix is shown in Figure 6D. Both the H_2O_2 and morphological mapping were performed at a constant distance of 60 μm above the surface using the SECM chemical imaging mode.

Effect of lactoperoxidase (LPO) and thiocyanate (SCN^-) on local H_2O_2 concentration:

We further extended our study in detecting H_2O_2 in the presence of LPO and SCN^- present in human saliva. Since this enzyme decomposes the H_2O_2 produced by bacteria, a traditional H_2O_2 assay is unable to detect any local H_2O_2 produced by bacteria in real time. Our highly sensitive miniaturized SECM-based H_2O_2 sensor gave us the unique capability to detect and quantify the local H_2O_2 concentration even in the presence of an H_2O_2 -decomposing agent. Figure 7A and 7B (black curve) reveal that, with the addition of 10 $\mu\text{g/mL}$ of LPO above the biofilm in AS, the local concentration of H_2O_2 was suppressed by $\sim 30\%$ and after the addition of KSCN, the local peroxide concentration dropped from 80 μM to 30 μM . Z-direction concentration mapping (Figure 7B (black curve)) indicated the formation of a low concentration gradient from the surface to only 500 μm above the biofilm as compared to the Z direction concentration profile observed in a separate set of experiments carried out without LPO and KSCN (red curve in Figure 7B). This finding is important, as it confirms that there is indeed an approximate micromolar concentration of H_2O_2 present in close proximity to the biofilm where most bacterial communication occurs. It thus verifies that Sg is beneficial in maintaining our healthy oral microbiome while LPO and SCN^- protect our oral cavity from damage by H_2O_2 .

CONCLUSIONS

We used the catalytic properties of Pt-NPs stabilized on high surface area MWCNTs and dispersed them in a conducting IL matrix to electrochemically detect H_2O_2 . The unique nature of this composite allowed us to miniaturize the sensor to a 25 μm diameter size. The optimized sensor composition (10:90 w/w % Pt-MWCNTs-IL) showed a sensitivity that was 3 times higher than that with the bare Pt UME or any similar size SECM probe, and it was stable for continuous measurement of H_2O_2 with a lower detection limit of 250 nM in AS solution at 37 $^\circ\text{C}$. The high-stability microsensor enabled us to quantitatively map the 3D distribution of H_2O_2 produced by Sg biofilm. Because of its small size and broad working range (250 nM to 5 mM), this sensor can be applied to the real-time monitoring of H_2O_2 release from live cells and bacteria and to the study the effect of oxidative stress in biological relevant environments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1:

Schematic representation of (A) preparation of Pt modified multiwall carbon nanotubes (Pt-MWCNTs) and (B) dual (H_2O_2 sensor/Pt) SECM probe: one 25 μm Pt electrode etched and packed with Pt-MWCNT-ionic liquid to make H_2O_2 sensor and second 25 μm Pt electrode used to fix the sensor-biofilm distance in SECM experiments. (C) Optical micrograph (top view) of the dual probe – the black colour depicts the H_2O_2 sensor packed with catalysts and the yellow disc represents the Pt probe.

Figure 2: Scanning electron microscopic images recorded on (A) -COOH functionalized MWCNTs (f-MWCNTs) and (B) Pt Nps modified MWCNTs (Pt-MWCNTs), (C) X ray diffraction spectra recorded on a multiwall carbon nanotube (MWCNTs) and a Pt-MWCNTs.

Figure 3:

(A) Screening of sensors made with various compositions of f-MWCNTs and Pt-MWCNTs with binders on the basis of sensitivity values obtained for the detection of H_2O_2 in the concentration range 10 μM to 500 μM (B) Screening of sensors made with 10:90 wt % of F-MWCNTs and Pt-MWCNTs with room temperature ionic liquid at 23 °C and 37 °C. (C) Impedance spectra recorded at the sensors at 23 °C and 37 °C in the frequency range of 3000 kHz to 0.1 Hz in 1 mM ferrocyanide/ferricyanide in 0.1 M KCl solution.

Figure 4:

(A) Cyclic voltammograms recorded with optimized sensor in 1 mM, 3 mM and 5 mM H_2O_2 in artificial saliva (AS) solution (pH 7.2). Scan rate is 50 mVs^{-1} . (B) Steady state voltammograms obtained with a 25 μm Pt disc and H_2O_2 sensor (in dual probe) in 1 mM ferrocene methanol in AS, pH 7.2. Scan rate is 1 mVs^{-1} . (C) Calibration curve obtained with a sensor made with 10: 90 w/w % Pt-MWCNTs-IL in AS solution (pH 7.2) with addition of 10 μM to 500 μM H_2O_2 . Sensitivity, $2.4 \text{ mA cm}^{-2} \text{mM}^{-1}$, inset shows the calibration curve obtained in 100 nM to 8 μM H_2O_2 with sensitivity $2.07 \text{ mA cm}^{-2} \text{mM}^{-1}$. (D) Calibration curve obtained in high concentration range from 2 mM to 10 mM H_2O_2 with a sensitivity of $0.13 \text{ mA cm}^{-2} \text{mM}^{-1}$ for a linear range of 2 mM to 7 mM.

Figure 5:

(A) Schematics of the scanning electrochemical microscopy (SECM) set-up used for the detection of local H₂O₂ concentrations released by Sg biofilms in physiological condition. (B) Schematics of the simulated biofilm substrate and the dual (H₂O₂ sensor/Pt) SECM probe used in SECM experiments. The sensor was fixed 50 μm above the Sg gel biofilm embedded in the PDMS cavity. The H₂O₂ mapping was performed in artificial saliva at 37 °C in presence of glucose with and without lactoperoxidase (LPO) and thiocyanate (KSCN) and (C) Representative probe approach curve recorded on the petri dish using the dual SECM probe (25 μm Pt electrode).

Figure 6:

Detection of local H_2O_2 concentrations above 1 mm dia. Sg gel biofilm using SECM with a 25 μm Pt-MWCNTs-IL packed sensor in dual (H_2O_2 sensor/Pt) SECM probe. +0.5 V was applied to the sensor for the detection of H_2O_2 . (A) Fixed distance (50 μm above) measurements of H_2O_2 generation, (B) x-directional H_2O_2 profile recorded by the sensor after addition of 1 mM glucose in pH 7.2 artificial saliva (AS) solution at 37 $^\circ\text{C}$., (C) represents a H_2O_2 chemical image recorded at a constant distance of 50 μm above the Sg gel biofilm surface in 1 mM glucose and AS solution at 37 $^\circ\text{C}$., and (D) morphology of the biofilm recorded 50 μm above the Sg gel biofilm surface in the presence of 1 mM ferrocene methanol in AS (pH 7.2) with Pt UME in dual SECM probe at 23 $^\circ\text{C}$.

Figure 7:

(A) H_2O_2 concentration measured at a 50 μm distance above the *S. gordonii* biofilm after addition of glucose, lactoperoxidase (LPO, 10 $\mu g/mL$) and potassium thiocyanate (KSCN, 1 mM). (B) Z-directional H_2O_2 concentration profile recorded above the biofilm after fixed distance H_2O_2 measurement. Black line indicates Z-direction H_2O_2 profile in the presence of LPO and KSCN corresponding to data shown in (A). The red line shows a Z-directional H_2O_2 profile without LPO and KSCN (data obtained from separate set of experiments). All experiments were carried out using Pt-MWCNTs-IL packed sensor in dual (H_2O_2 sensor/Pt) SECM probe at 37°C in the solution of 1 mM glucose and artificial saliva (pH 7.2).