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Glucose Oxidase Entrapment in an Electropolymerized Poly(tyramine) Film with Sulfobutylether- β -Cyclodextrin on Platinum Nanoparticle Modified Boron-Doped Diamond Electrode

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Glucose oxidase (GO) was entrapped in an electrodeposited poly(tyramine) film together with a negatively charged sulfobutylether- β -cyclodextrin (SBCD) on the active area of a platinum (Pt) nanoparticle modified boron-doped diamond (BDD) electrode. Electrodeposition of tyramine and simultaneous entrapment of GO/SBCD were performed in 50 mM phosphate buffer, pH 7 containing 0.1 M tyramine, 1750 units GO, and 10 mM SBCD. Atomic force microscopy (AFM) imaging revealed the presence of semicircular nanofibers with a height of 40 nm and an averaged length of 795 nm throughout the electropolymerized film surface. The combined film of poly(tyramine) and SBCD served as an excellent matrix polymer for the GO immobilization with high stability, selectivity, and reproducibility. Sensitive and selective detection of H₂O₂ was realized at +0.4 V vs 3 M Ag/AgCl, since the poly(tyramine)/SBCD film was capable of preventing the passage of electroactive uric and ascorbic acids to the electrode. The glucose biosensor exhibited a remarkably selective response to glucose with a detection limit of 10 μ M, linearity up to 110 mM, and a response time of 2 s. Glutaraldehyde cross-linking of the film with entrapped GO completely eliminated electroactive interference caused by uric and ascorbic acids.

Introduction

Immobilization of a biosensing probe in an electropolymerized polymer matrix allows for precise deposition of the biomolecule on any conducting surface regardless of dimension and geometry.^{1,2} As electropolymerization only occurs on the electrode surface, the recognition biomolecule is virtually entrapped in proximity to the electrode. This is an important feature for the fabrication of microelectrodes and microelectrode arrays. In this procedure, the amount of the immobilized probe can be easily manipulated by changing its concentration or by adjusting the film thickness through the applied potential and/or the deposition time. Compared to conducting counterparts such as polypyrrole and its derivatives,³ the electropolymerization of nonconducting polymers often generates considerably thinner membranes (~10–100 nm) due to their self-limiting nature.⁴ Such nonconducting films also display fast response, excellent permselectivity, and high reproducibility. Among various electropolymerizable monomers, tyramine can be considered as one of the most promising monomers for producing a strongly adhering polymer film.⁴ Indeed, poly(tyramine) (PTy) has considerable advantages over other electrodeposited polymers with respect to reproducibility and the ability to screen out interferences when used in complex samples.^{5–8} With the amino group separated from the phenolic ring by two methylene groups, only the phenol moiety participates in polymerization. The electropolymerization of tyramine can be conducted under basic,^{5–8} acidic,⁹ or neutral pH with a controllable thickness.

The boron-doped diamond (BDD) thin-film electrode has attracted considerable interest due to its superior features such

as high current density, wide potential window, low background current, extreme electrochemical stability, and high resistance to fouling.¹⁰ However, because of its chemical inertness, it is very difficult to modify the BDD electrode to impart novel electrocatalytic properties. Nevertheless, the BDD electrode can be photochemically modified with appropriate solutions of a terminal alkene to yield monolayer coverage of the surface.¹¹ BDD electrodes can also be modified with copper,¹² *o*-aminobenzoic acid,¹³ and Pt nanoparticles, carbon nanotubes, and enzymes.¹⁴ A random distribution of palladium nanoparticles supported on a BDD electrode or a palladium plated BDD microelectrode array can serve as a sensing platform for the electrocatalytic detection of hydrazine.¹⁵ Pt nanoparticles have been deposited on nanostructured BDD surfaces with larger electrochemical area for methanol anodic oxidation.¹⁶ By both sputtering and electrodeposition, Wang and Swain have achieved the deposition of Pt nanoparticles on BDD films.¹⁷ Notice also that Sine et al.¹⁸ have described microemulsion-synthesized Pt/Ru/Sn nanoparticles on BDD for alcohol electrooxidation. However, the stability and reproducibility of the modified BDD electrode are still problematic and have not been fully addressed.

In this paper, a BDD electrode is modified by electrodepositing platinum nanoparticles (PtNPs) by a multipotential step deposition technique to provide the most stable PtNPs. Tyramine and negatively charged sulfobutylether- β -cyclodextrin (SBCD) are co-electrodeposited on the PtNP-modified BDD electrode to form a permselective film against uric acid (UA) and ascorbic acid (AA). As a model system, glucose oxidase (GO) is also co-electrodeposited with tyramine and SBCD to form a stable and specific film for hydrogen peroxide and glucose detection. The analytical performance of the glucose biosensor with respect to sensitivity and selectivity is presented and discussed in detail.

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To our knowledge, this is the first stable glucose biosensor with remarkable selectivity for H_2O_2 and glucose using the PTy/SBCD/PtNP-modified BDD electrode.

Experimental Methods

Chemicals. Tyramine hydrochloride, ascorbic acid (AA), uric acid (UA), hydrogen peroxide (H_2O_2), glutaraldehyde, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), and other chemicals were purchased from Sigma-Aldrich (Dublin, Ireland). Sulfobutylether- β -cyclodextrin (SBCD) with a degree of substitution of 4 was obtained from Applied Biosystems Div. Perkin-Elmer (Foster City, CA) and used as received. Deionized water ($18.2 \text{ M}\Omega \cdot \text{cm}$) was obtained from a Milli-Q (Millipore, Bedford, MA) water purification system. All reagents were of analytical grade with highest purity.

Electrode Preparation. The boron-doped diamond electrode (BDD, 3 mm diameter, 0.1% doped boron, Windsor Scientific, Slough, Berkshire, U.K.) was polished with polishing paper (grid 2000, Hand American Made Hardwood Products, South Plainfield, NJ) and subsequently with alumina (Buehler, Markham, ON, Canada) until a mirror finish was obtained. After 5 min of sonication to remove the alumina residues, the electrode was immersed in concentrated H_2SO_4 for 3 min followed by thorough rinsing with water and ethanol. The electrode was then transferred to the electrochemical cell for cleaning by cyclic voltammetry between -0.5 and $+2.0 \text{ V}$ vs Ag/AgCl (3 M NaCl) at 100 mV s^{-1} in 50 mM phosphate buffer, pH 7 until a stable CV profile was obtained.

Electrodeposition of Platinum Nanoparticles. Pt nanoparticles (PtNPs) were deposited on the BDD electrode by electrodeposition of 2 mM H_2PtCl_6 in 0.5 M H_2SO_4 by a multipotential step deposition technique to provide the most stable PtNPs with controllable size and density.^{12,19} Activation by potentiostatic anodic polarization in 0.1 M H_2SO_4 at $+2.0 \text{ V}$ vs Ag/AgCl for 10 min was a prerequisite. As demonstrated by Sine,¹⁹ this treatment reduces the background current by eliminating adsorbed hydrogen and the sp^2 graphitic phase. As discussed later, the treated BDD electrode exhibited very low background current, within a few nA. Duration for this treatment was also very important, since prolonged polarization might passivate the BDD surface and inhibit the subsequent Pt electrodeposition. Detailed information of the treatment is well presented by Sine.¹⁹ PtNPs were then deposited on the pretreated BDD electrode by the multipotential step technique. The two-step potential program was performed for 15 cycles (1 s at the deposition potential followed by 5 s at the relaxation potential).

Electropolymerization of Tyramine. Tyramine hydrochloride (0.1 M) was dissolved in 50 mM phosphate pH 7, consisting of 10 mM SBCD. Electropolymerization, cyclic voltammetry (CV), and amperometric (I/t) measurements were performed using a CHI 1040A electrochemical workstation (CH Instruments, Austin, TX). All experiments were performed at room temperature ($22\text{--}24^\circ\text{C}$) using a three-electrode system consisting of a BDD electrode as working electrode, an Ag/AgCl electrode (3 M NaCl) as reference electrode (BAS), and a platinum wire as counter electrode. Enzyme solution was prepared by dissolving 10 mg/mL glucose oxidase (GO, Sigma-Aldrich, type X-S, *Aspergillus niger*, 175 000 U/g) in 50 mM phosphate buffer, pH 7 containing 0.1 M tyramine and 10 mM SBCD. Chronoamperometry was carried out in 50 mM phosphate buffer, pH 7. Different stock concentrations of anhydrous β -D-glucose (BDH, Toronto, ON, Canada) were prepared in 50 mM phosphate buffer, pH 7, and stored at 4°C (mutarotation was allowed for at least 24 h before use). Scanning electron

microscopy (SEM) micrographs of the bare and modified electrodes were obtained using a Hitachi scanning electron microscope (S-2600 N, Tokyo, Japan), operated in high vacuum and variable pressure (15 kPa) mode at $4\text{--}24 \text{ kV}$ with a working distance of $4\text{--}20 \text{ mm}$. Atomic force microscopy (AFM) was used to estimate the thickness of the PTy/SBCD films by electrodeposition of tyramine and SBCD on a silicon wafer (0.5 mm) covered by a thin gold (100 nm) surface (Platipus Technologies, Madison, WI). AFM micrographs were obtained using a Nanoscope IV instrument (Digital Instruments, Veeco, Santa Barbara, CA) with a silicon tip operated in tapping mode.

Results and Discussion

The PTy/PtNP-modified BDD Electrode. As expected, the pristine BDD electrode exhibited no redox activity for H_2O_2 over most of the potential range whereas the electrodeposition of PtNPs (ranging from 80 to 170 nm in diameter, Figure 1) resulted in the sensitive detection of H_2O_2 . The distribution, size, and number of PtNPs deposited on the electrode surface during nucleation followed by particle growth were affected by platinum salt concentration, deposition time, and deposition potential. The optimization involved the multipotential step technique as described in the Experimental Methods section and the two-step potential program (deposition potential of -0.3 V vs Ag/AgCl for 1 s, followed by 5 s at the relaxation potential of $+1.3 \text{ V}$), which was performed for 15 cycles to attain the optimum signal toward H_2O_2 response. However, the stability of the PtNP-modified BDD electrode was very limited due to the weak interaction between BDD and PtNPs. The signal response for H_2O_2 continuously decreased after 20–30 repeated analyses (figure not shown). Electrochemical cleaning by cyclic voltammetry even at slow scan rates ($50\text{--}100 \text{ mV/s}$) also adversely affected its activity toward H_2O_2 , owing to the detachment of PtNPs. The cyclic voltammogram of the PtNP-modified BDD electrode was not stable, and the peak at -0.1 V , a feature of Pt electrochemistry, decreased with repeated scan, which confirmed the detachment of PtNPs (figure not shown). In addition, the signal response of AA and UA at their physiological levels (0.1 mM) was $\sim 50\%$ of H_2O_2 at the same concentration (data not shown). Tyramine was electropolymerized to retain PtNPs on the electrode with the resulting cyclic voltammogram (CV) being greatly dependent on pH. The CV profile obtained in 50 mM phosphate buffer, pH 7 revealed a continual current decrease with the number of deposition cycles (Figure 2A). Unlike the electropolymerization of tyramine in NaOH,^{5–8} the film formed at pH 7 was not self-limiting and could be up to $1 \mu\text{m}$, depending upon the deposition condition and the electrode surface.²⁰ As a phenol derivative, the electrooxidation of tyramine produced phenoxy radicals, which in turn reacted with a neighboring tyramine molecule to form a para-linked dimer. Further oxidation led to oligomers and the eventual formation of a thin poly(tyramine) thin film (PTy). The amino group is separated from the phenolic ring by two methylene groups; hence, only the phenol moiety is oxidized to perform the polymerization.^{4–9} Increasing the number of deposition cycles, reflecting increasing film thickness, decreased the signal response of H_2O_2 , whereas its permselectivity against AA and UA was significantly improved. Therefore, 30 deposition cycles were considered as the best compromise. The electrodeposited PTy film has played a dual role in improving selectivity and stability of the modified BDD electrode. Both UA and AA (0.1 mM) only provoked a 15% signal response compared to that of H_2O_2 at the same level. The PTy-modified BDD electrode thus exhibited considerable suppression ($\sim 3\text{--}$

Figure 1. Size analysis of Pt nanoparticles electrochemically deposited on the boron-doped diamond (BDD) electrode before their aggregation to form bigger clusters.

Figure 2. (A) Cyclic voltammogram of the electrodeposition of 0.1 M tyramine in 50 mM phosphate, pH 7 on a PtNP-modified boron-doped diamond (BDD) electrode. (B) Cyclic voltammogram of the electrodeposition of 0.1 M tyramine and 10 mM SBCD in 50 mM phosphate buffer, pH 7 on a PtNP-modified BDD electrode. Cyclic voltammetry from -0.1 to $+1.7$ V, vs Ag/AgCl, for 30 cycles at 0.5 V/s.

to 4-fold) for both AA and UA. The detection limit for H_2O_2 ($S/N = 3$) was 30 nM with linearity up to 5 mM. Notice also that the PTy-modified BDD electrode completely blocked the diffusion of 5 mM $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ (figure not shown), indicative of a sieving feature of the PTy film. Such results implied very low porosity of the PTy film which in turn hindered the passage of AA and UA.

The PtNP/PTy/SBCD Modified Electrode. The experiment was then conducted to electropolymerize tyramine on the PtNP-modified BDD electrode in the presence of 10 mM SBCD (Figure 2B). Although the resulting CV profile was similar to that of the electrodeposition without SBCD, the current decrease after each deposition cycle was noticeably smaller. Tyramine with a pK_a of 9.74 – 10.52 is protonated at pH 7 to display ionic interaction and inclusion complexation with SBCD, resulting in stable complexes. It was also expected that SBCD with a much bigger chemical dimension compared to $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ would be retained on the electrode together with PtNPs. AFM could not be used to probe the morphology and the film thickness of the BDD electrode owing to its uneven surface and the oversized length (4 cm). Nevertheless, the thickness of the PTy-SBCD film electropolymerized on a thin gold surface was estimated as 17 ± 1.3 nm (at 95% confidence interval, $n = 10$) (Figure 3A). AFM imaging also revealed an interesting feature of the PTy/SBCD film which was not observed for the film formed in the absence of SBCD. The surface possessed several semicircular chains ranging from 646 to 1625 nm in length with a height of 40 nm (Figure 3B). Phenol has been known to form an inclusion complex with α -CD or β -CD with a binding constant of 87 and 214 M^{-1} , respectively, as determined by near-infrared spectroscopy.²¹ Similarly, phenol derivatives also display inclusion complexation with β -CD.²² Therefore, it was anticipated that tyramine formed an inclusion complex with SBCD which was then electropolymerized to form such nanochains. Work has been in progress to identify and characterize such nanochains and their properties.

The PTy/SBCD/PtNP-modified electrode exhibited a signal response of ~ 0.22 μA for 0.1 mM H_2O_2 , similar to the film formed in the absence of SBCD or 17-fold lower than that of the PtNP-modified electrode. However, the modified electrode, poised at $+0.6$ V was capable of rejecting 95% AA and 81% UA at 0.1 mM (Figure 4, curve A). Apparently, SBCD was capable of effectively rejecting the passage of negatively charged

Figure 3. (A) AFM imaging of the electrodeposition of 0.1 M tyramine and 10 mM SBCD (in 50 mM phosphate buffer, pH 7) on a thin gold electrode (film thickness measurement $20\ \mu\text{m} \times 20\ \mu\text{m}$ AFM tapping-mode imaging). (B) The AFM micrograph was performed for length measurement, $5\ \mu\text{m} \times 5\ \mu\text{m}$ AFM tapping-mode image. The length of the nanofibers ranged from 646 to 1625 nm.

Figure 4. Signal response of the PtNP-modified BDD electrode after co-electrodeposition of 0.1 M tyramine and 10 mM SBCD (referred to as the PTy/SBCD/PtNP-modified BDD electrode). The detecting electrode was poised (A) at +0.6 V vs 3 M Ag/AgCl and (B) at +0.4 V vs 3 M Ag/AgCl. (a) 0.1 mM H_2O_2 , (b) 0.1 mM AA, (c) 0.1 mM UA, and (d) 0.1 mM H_2O_2 .

UA and AA from the active area of the electrode. AA with a $\text{p}K_{\text{a}}$ of 4.19 exists practically as the ascorbate anion at the physiological pH 7 ([ascorbate]/[ascorbic acid] = $10^{2.8}$ or 631). Although the $\text{p}K_{\text{a}}$ of UA is higher (5.8), the urate anion is still predominant at pH 7 compared to the free acid form ([urate]/[uric acid] = $10^{1.2}$ or 15.6). Nevertheless, a very small fraction of UA (about 6%) in the neutral form would form the inclusion complex with the hydrophobic cavity of SBCD via hydrophobic–hydrophobic interaction and/or hydrogen bonding. Consequently, the PTy-SBCD film was able to prevent the passage of UA and AA to the electrode surface, particularly for hydrophobic UA. Notice that when the modified BDD electrode was poised at +0.4 V, the signal response to H_2O_2 was reduced $\sim 25\%$. However, both UA and AA provoked no significant interference at this applied potential (Figure 4, curve B). This permselectivity was an important finding, since the AA can be easily oxidized even at +0 V where the current peak of AA oxidation is +230 mV even with glassy carbon electrode.²⁰ AA is a very flat molecule with $\text{p}K_{\text{a}1} = 4.19$ and $\text{p}K_{\text{a}2} = 11.57$, and at pH 7 only one acidic hydroxy group should be dissociated. Its three remaining hydroxyl groups then

interacted with SBCD via hydrogen bonding. More hydrophobic UA would reside inside the hydrophobic cavity of SBCD. UA has four ionizable hydrogen ions (positions 1, 3, 7, and 9); however, only the hydrogen ion on position 9 ($\text{p}K_{\text{a}} = 5.8$) is ionizable at physiologic pH. Thus, it was also able to bind strongly with the hydroxyl groups of SBCD via hydrogen bonding to strengthen the UA–SBCD inclusion complex. Increasing the concentration of SBCD from 5 to 15 mM displayed no effect on the signal response for H_2O_2 or the permselectivity against UA and AA. Therefore, 10 mM SBCD was used in all subsequent experiments. With linearity up to 7 mM, the detection limit of the PTy/SBCD/PtNP-modified BDD electrode (10 nM) was favorably compared to Pt-implanted BDD electrodes (30 nM) using ion implantation,²¹ a procedure that requires special equipment and instrumentation for implanted Pt using Pt rods as targets followed by annealing at 850 °C.

The modified BDD electrode exhibited a 4-fold lower detection limit compared to the electrochemical scheme using PtNPs assembled in poly(diallyldimethylammonium chloride), 42 nM.²² It also compared well with the glassy carbon electrode modified with single walled carbon nanotubes and platinum nanoparticles (25 nM).²⁵ In addition, electropolymerization used in this study would allow reproducible, precise, uniform, and thickness-controlled polymer coating without any limitation of the size, area, and geometry of the surface.¹ The biosensor using horseradish peroxidase with gold nanoparticles²⁶ provides a detection limit of 650 nM for H_2O_2 , whereas a mesoporous Pt ultramicroelectrode only possesses a detection limit of 4.5 μM .²⁷

Analytical Performance of the Glucose Biosensor. As a proof of concept, a biosensor for glucose was constructed by electrodeposition of glucose oxidase (GO) from a solution consisting of 0.1 M tyramine, 10 mM SBCD, and 1750 U GO on the PTy/SBCD/PtNP-modified BDD electrode. For the detection of hydrogen peroxide ($\text{H}_2\text{O}_2 \rightarrow 2\text{H}^+ + \text{O}_2 + 2\text{e}^-$), a coproduct formed during the enzymatic oxidation of glucose, the working electrode was poised at +0.4 or +0.6 V vs 3 M Ag/AgCl. Glucose oxidase is a homodimer glycoprotein with a

Figure 5. Amperometric response of the PTy/SBCD/PtNP-modified BDD electrode to 1 mM glucose in 50 mM phosphate buffer, pH 7. The electrode was poised at +0.4 V vs 3 M Ag/AgCl. Inset: (upper) detection limit and (lower) calibration plot.

molecular weight of 160 kDa and 16% carbohydrate content.²⁸ Besides entrapment, GO with COOH groups of aspartic and glutamic acid, NH₂ of lysine and arginine, or other O–H groups should be able to bind to the PtNPs.²⁹ The resulting glucose biosensor was shown in an electrochemical test to maintain activity for H₂O₂ oxidation and glucose oxidation, respectively. The performance of the glucose biosensor is illustrated in Figure 5 for one representative of the prepared biosensors. The response time was very fast at 2 s (Figure 5), with the detection limit estimated as 10 μ M ($S/N = 3$) and linearity up to 110 mM glucose (Figure 5, inset). Both UA and AA at the physiological level (0.1 mM) exhibited no noticeable interference, particularly when the detecting electrode was poised at +0.4 V. The glucose biosensor was stable for several repeated analysis, and no attempt was made to study the stability or selectivity of the biosensor since the enzyme has been known to be very stable and specific. It should be noted that the PTy film is also extremely versatile and offers new opportunities to link bio-functional molecules via its abundance of NH₂ with NH₂ or COOH functional groups of the biomolecules via glutaraldehyde activation or carbodiimide cross-linking. The results obtained in this study, however, have confirmed that GO entrapped in the PTy matrix was very stable and retained on the electrode due to the low porosity of the electropolymerized film and its binding to PtNPs. When stored at 4 $^{\circ}$ C, the glucose biosensor was stable for at least several weeks without losing its initial activity for glucose or permselectivity against UA and AA. Among seven electrodes prepared under the same condition, their response to hydrogen peroxide only varied within $\pm 5\%$ from one electrode to another electrode. Similarly, the response for glucose obtained from one electrode to another electrode was very comparable (within $\pm 6\%$).

A series of experiments was also conducted to assess the analytical performance of the entrapped GO after its subjection to 0.2% glutaraldehyde overnight at 4 $^{\circ}$ C. As a bifunctional cross-linking agent, glutaraldehyde was expected to cross-link the amino group of PTy with that of the enzyme. However, glutaraldehyde might also intra or inter cross-link two PTy chains or the enzyme.³⁰ As shown in Figure 6, at +0.4 V vs 3 M Ag/AgCl, the biosensor after glutaraldehyde activation exhibited good responses to glucose with linearity above 100 mM glucose. UA and AA at 0.1 mM provoked no noticeable response, and even at 1 mM UA and AA only provoked a signal response of 3% and 5% compared to that of glucose at 5 mM, the normal level of glucose in blood. Apparently, the cross-linking of the enzyme with PTy affected the porosity and properties of the electropolymerized film while the excellent linearity and detection limit were retained. No noticeable improvement in permselectivity was noted when the entrapped

Figure 6. Amperometric response of the PTy/SBCD/PtNP-modified BDD electrode after glutaraldehyde cross-linking: (a) 5 mM glucose, (b) 0.1 mM AA, (c) 0.1 mM UA, and (d) 5 mM glucose. The detection electrode was poised at +0.4 V vs 3 M Ag/AgCl in 50 mM phosphate, pH 7. Inset: signal response to 1 mM glucose at +0.4 V vs Ag/AgCl in the same electrolyte.

enzyme was subject to 15 mM EDC in an attempt to cross-link the COOH group of the enzyme with the amino group of the PTy backbone.²⁹

Conclusions

In brief, the BDD electrode modified by PtNPs followed by the GO/PTy/SBCD electrodeposition has led to the fabrication of a stable biosensor for glucose with a low detection limit and rapid response time. This is an attractive procedure because the enzyme immobilization can be controlled closely, which is advantageous for microarray fabrication. Because the electropolymerized film is also permselective for H₂O₂ over UA and AA, the biosensor described here is very useful for assays of clinical significance. Together with thin film (BDD) electrodes, this immobilization procedure opens up new opportunities for the construction of various biosensors using oxidases based on the selective and sensitive detection of H₂O₂. The detection of H₂O₂ is of interest and importance to many other fields in analytical, environmental, and biomedical chemistry as well as food monitoring. H₂O₂ is necessary for the metabolism of proteins, carbohydrates, fats, vitamins, and minerals. Besides its essential role for the body's production of estrogen, progesterone, and thyroxine, H₂O₂ helps regulate blood sugar and the cellular energy production. Oxidative damages in the body are caused by the cellular H₂O₂ imbalance as this chemical plays an important role in cell signaling and communication.

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