Simultaneous Multiselective Spectroelectrochemical Sensing of the Interaction between Protein and Its Ligand Using the Redox Dye Nile Blue as a Label

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A new binding assay for a protein and its ligand based on a spectroelectrochemical method was demonstrated using avidin–biotin and 17β-estradiol–antiestradiol antibody. The sensor consists of a selective film coated on an optically transparent electrode (OTE) consisting of indium tin oxide (ITO). Attenuated total reflection (ATR) was used for optical detection. The binding event of the ligand to the protein was detected using the ligand labeled with the electroactive dye Nile blue (NB). The spectroelectrochemical behaviors of NB and the labeled ligand were investigated using various ion-exchange films, such as perfluorosulfonated ionomer (Nafion), Nafion-silica, poly(acrylic acid) (PAA)-silica, poly(styrenesulfonic acid) (PSSA)-silica, and heparin-silica films, which were spin-coated onto the ITO electrode. The optical signal was monitored to follow the accumulation of labeled ligand in the film and its electrochemical modulation. The signal from the labeled ligand possesses three modes of selectivity based on charge-selective partitioning, the chosen electrolysis potential, and the particular wavelength for measuring absorbance. The interaction between the labeled ligand and its protein was observed by the decrease in the changes of optical response of the labeled ligand, indicating the specific binding of labeled ligand to the protein.

Spectroelectrochemistry has been used for over 4 decades to investigate a wide variety of inorganic, organic, and biological redox systems, in which its main purpose has been to study the mechanisms of electrode reactions. The primary advantage of spectroelectrochemistry is the cross-correlation of information attainable from simultaneous electrochemical and optical measurements.†‡ Therefore, if the information obtained from the spectroelectrochemical responses can be incorporated into the selective system of a sensor, the overall selectivity of the sensor would be significantly enhanced. However, only a few attempts have been made so far toward coupling electrochemistry with spectroscopy as the basis of chemical sensing. Although dual selectivity based on optical detection of electrochemically generated species® and the electrochemical detection of photochemically generated species† has been reported, the electrochemical potential merely functions to regenerate species to reuse these sensors.

The spectroelectrochemistry sensor concept combines electrochemistry, optical spectroscopy, and selective partitioning into a single device. Our group has been developing a new functional spectroelectrochemical sensor for some time based on this concept.†‡ The sensor consists of an optically transparent...
showed a substantial difference in molar absorptivity during electrochemistry. A competitive binding assay, which uses the specific interaction between a protein and its ligand, is one of the important analytical methods. Generally, the assays are based on a reaction in which an analyte displaces a labeled ligand. Binding assays using a nonisotopic label like an enzyme have increased dramatically in recent years. Electrochemical binding assays are a group of nonisotopic techniques that have been the subject of continued research and development. However, most of the assays require a separation or washing procedure to remove free labeled molecules from those bound to protein before any measurements can be made. The separation process not only complicates the operation and is time-consuming but may also cause a deviation in the reaction equilibrium between the protein and its ligand. We reported earlier on some simple electrochemical binding assays to detect a protein–ligand interaction, such as antibody–antigen, avidin–biotin, lectin–sugar, and receptor–estrogen. These methods do not require a separation procedure, since the protein–ligand interaction can be evaluated solely on the basis of changes in the electrode response of free ligand labeled with an electroactive compound. In these cases, the signal from the labeled ligand disappears or decreases on binding with protein. This principle for the detection of an interaction between a protein and its ligand is called "sequestration electrochemistry." 

In the present study, we developed a new binding assay for a protein and its ligand based on a spectroelectrochemical method. The assay incorporated the detection principle of sequestration electrochemistry that has three modes of selectivity, i.e., electrochemistry, spectroscopy, and chemical partitioning, which can operate simultaneously in a single device. Avidin–biotin and 17β-estradiol–antiestriadiol antibody were adopted as models for the protein–ligand system. The ligand labeled with the electroactive dye Nile blue (NB) was prepared so that the binding event between the protein and its ligand could be detected spectroelectrochemically. NB has a high molar absorption coefficient, and because it has an amino group, it can be coupled easily to a variety of commercially available reagents. The mechanism for the assay is illustrated in Figure 1. In the absence of protein, free ligand labeled with a dye can be accumulated into the chemically selective film coated on the OTE. The evanescent wave at the reflection point penetrates the film such that electrochemical events within the film can be monitored optically. In its operation, a potential is applied to the OTE to electrolyze the labeled ligand that has partitioned into the film. The quantitative sensing of the labeled ligand is based on the magnitude of the change in transmittance of the light propagated by ATR; that is, the signal of the labeled ligand is an optical change that occurs in response to an electrochemical event. However, the labeled ligand bound to the protein is not able to penetrate into the film, and consequently, the spectroelectrochemical modulation decreases. Therefore, the binding event between the ligand and its protein can be detected indirectly without a separation process. Initially, we have investigated the spectroelectrochemical behavior of NB and the labeled ligand using the ITO glass electrode coated with various ion exchange films and developed assays for avidin and antiestriadiol antibody.

**EXPERIMENTAL SECTION**

**Chemicals and Materials.** Nile blue chloride (NB), tetraethoxysilane (TEOS), and Nafion perfluorinated ion-exchange material (5 wt % solution in lower aliphatic alcohols and water) were obtained from Aldrich (St. Louis, MO). Sulfo-NHS-LC biotin, avidin, and 2-(N-morpholino)ethanesulfonic acid (MES) were from Pierce (Rockford, IL). Mouse anti-17β-estradiol (6-CMO-BSA) monoclonal antibody (anti-E2 Ab) was from Fitzgerald Inc. (Concord, MA). 17β-estradiol (E2), 17β-estradiol-6-[O-carboxymethyl] oxime, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and heparin sodium salt were from Sigma Chemical Co.
Poly(acrylic acid) sodium salt (PAA, MW 60 000, 35 wt % aqueous solution) and poly(styrenesulfonic acid) sodium salt (PSSA, MW 70 000) were from Polysciences Inc. (Warrington, PA). Tris-HCl buffer solution (50 mM Tris-HCl, pH 7.5; 50 mM NaCl; 0.02% sodium azide) was used as a solvent for incubating protein and as a supporting electrolyte for the spectroelectrochemical measurements. All other reagents were analytical grade, and water was deionized. All chemicals were used as received without further purification. ITO coated float glass (11–50 Ω/□, 150 nm thick ITO layer on 1.14 mm thick glass) was obtained from Thin Film Devices (Anaheim, CA) and cut into 1.3 cm × 7.6 cm slides. The ITO slide was cleaned before use with an A2 Harrick Ar plasma cleaner.

**Formation of Thin Films on ITO Glass Substrates.** Silica sol was prepared by mixing 2.0 mL of TEOS, 4 mL of water, and 0.1 mL of 0.1 M HCl and stirring at room temperature for 3 h. PAA (2 wt %), PSSA (10 wt %), and 10 wt % heparin aqueous solutions were made by dilution with deionized water. Nafion solution was used directly as purchased. The volume ratios of Nafion and other polyelectrolyte aqueous solutions to the silica sol were 3:1 and 1:1, respectively. Polyelectrolytes were incorporated into silica sol–gel processed materials by mechanically blending the sol with polyelectrolyte aqueous solutions. Nafion film and the polyelectrolyte sol–gel processed films were formed by spin-coating onto the ITO glass slides for 30 s at 3000 and 6000 rpm, respectively. The thickness of the spin-coated film was ~260 nm as measured by ellipsometry. Spin-coating was done with a 1-PM101DT-R485 Photo-Resist-Spinner (Headway Research Inc., Garland, TX). After spin-coating, all films on ITO glass were dried for 2 days at room temperature.

**Labeling Biotin and E2 with Nile Blue.** The labeled biotin (LB) was prepared by mixing 4 mM NB and 2 mM sulfo-NHS-LC biotin in 1 mL of 0.1 M phosphate buffer (pH 7.5)-DMF solution (7:3 v/v %). The labeled E2 (LE) was prepared by adding the mixture of 3 mM NB and 15 mM 17β-estradiol-6-[O-carboxymethyl]oxime in 0.3 mL of DMF to 0.7 mL of 0.1 M MES buffer solution (pH 4.7) containing 5 mM EDC. After incubating each solution in the dark for 1 day at room temperature, the reaction for labeling and the difference in the Rf value on the thin layer chromatography, the structures of the product are suggested as shown in Figure 2. The concentrations of LB and LE were determined by absorbance at 625 nm. The stabilities of LB and LE were examined 1 month later from measurements of their cyclic voltammograms and UV spectra.

![Chemical structures of biotin labeled with Nile blue (LB) and 17β-estradiol labeled with Nile blue (LE).](image)

**Spectroelectrochemical Measurement.** The instrumental setup for ATR spectroelectrochemistry is standard and consists of a He–Ne laser, λ = 633 nm (model no. 1135P, JDS Uniphase (Mauteca, CA)) coupled into the ITO glass slide of the spectroelectrochemical cell with a Schott SF6 coupling prism (Karl Lambrecht (Chicago, IL)). A high viscosity refractive index standard fluid (n = 1.517, Cargille Laboratories Inc. (Cedar Grove, NJ)) was used to span the prism/ITO glass gap. The angle of the incident light into the prism was adjusted to maximize the ATR throughput, as determined by measuring the intensity of the outcoupled light. Light propagated through the ITO glass slide was outcoupled with another SF6 coupling prism and focused on a photodiode detector (Photonic Detectors, Digi-Key PDB-V107). The ATR spectroelectrochemical cell made with black Delrin was equipped with a reference electrode, electrical contact with a counter electrode, and sample inlet–outlet holes as shown in Figure 3. The counter electrode (stainless steel foil, 0.05 mm thick) was sandwiched between 0.25 mm thick silicone gaskets and located between the Delrin spectroelectrochemical cell and ITO glass. The light path was 4.5 cm long. Electrochemical operation was done with a conventional three op-amp potentiostat built in our laboratory. All potential values were applied against an Ag/AgCl reference electrode (EE 008 miniature reference electrode, Cypress Systems (Lawrence, KS)).

Thin layer spectroelectrochemical measurements used a CV-27 potentiostat (BAS, West Lafayette, IN) and a Hewlett-Packard 8453 diode array spectrophotometer. The thin layer cell consisted of ITO glass coated with Nafion-silica film or bare ITO glass (4.5 cm x 1.0 cm) on one side and glass on the other side. A silicone sheet (0.25 mm thick) was used as a spacer. The counter electrode was Pt wire, and the reference electrode was Ag wire. The thin
layer cell and the other electrodes were dipped in a Petri dish (3.5 cm diameter × 1.2 cm high) containing the solution.

**Procedure of the Spectroelectrochemical Binding Assay.**

The labeled ligand and various concentrations of protein were incubated in the buffer solution with stirring for 30 min at room temperature to reach equilibrium, and then the spectroelectrochemical measurement was made. Sample solution (150 µL) was injected into the spectroelectrochemical cell, and 0.5 V was applied to the electrode for 15 min to accumulate the labeled ligand into the polyelectrolyte film coated on the ITO glass electrode surface. The transmittance and current changes caused by the specific binding of the labeled ligand with protein were recorded simultaneously during a double step potential between 0.5 and −0.8 V.

**RESULTS AND DISCUSSION**

**Spectroelectrochemical Behavior of NB on the Polyelectrolyte Coated ITO Electrode.** NB accumulated in the Nafion-silica film shows a redox couple with a well-defined, reversible cyclic voltammogram (not shown) with an anodic peak at 0 V and a cathodic peak at −0.3 V. Thus, NB in the film could be maintained in its parent form by applying a potential of 0.5 V and reduced by applying −0.8 V.

The spectral property changes of NB that accompany the electrochemical reaction were first investigated by transmission thin layer spectroelectrochemistry. Figure 4 shows the absorption spectra of NB that had partitioned into a Nafion-silica coated ITO electrode. This film is optically transparent above 500 nm.\(^{(39)}\) Prior to measurements, the Nafion-silica coated ITO electrode was incorporated into a thin layer cell that was filled with 10⁻⁴ M NB solution for 30 min to accumulate NB within the Nafion-silica on the ITO electrode. The NB solution in the thin layer spectroelectrochemical cell was replaced by Tris-HCl buffer (pH 7.5). The spectral measurements were made by first applying a potential of 0.5 V to obtain an initial spectrum of NB in the film (curve a) and then stepping to −0.8 V (curves b−g) to monitor the reduction of NB. The absorbance decreased significantly with increasing time at −0.8 V as the NB that had been accumulated in the Nafion-silica film was reduced. This result shows that NB undergoes a strong optical change due to the electrochemical reaction. Thus, NB is a good candidate spectroelectrochemical probe for the ligand in the protein−ligand spectroelectrochemical assay based on the concept proposed in this study.

A critical component of the sensor is the selective film into which the labeled ligand partitions for detection by spectroelectrochemical modulation. For optical applications, the film must be transparent within the wavelength range of NB as a labeling compound. Furthermore, it is preferred that the labeled ligand can be accumulated highly and selectively from solution into the film. We chose the optimal film for this binding assay by investigating the spectroelectrochemical behavior of NB using some cation-exchange polyelectrolytes: Nafion, Nafion-silica, PAA-silica, PSSA-silica, and heparin-silica thin films spin-coated on ITO. It has been reported that Nafion enables large molecular weight hydrophobic cations to accumulate, preferably to small hydrophilic ones.\(^{(40)}\) Chen et al. measured hemoglobin electrochemically using methylene blue (MB) incorporated in a Nafion modified electrode.\(^{(41)}\) They showed that methylene blue can be highly accumulated within the Nafion film (extraction coefficient = ~110). Thus, it is expected that NB, which has a similar structure to MB, can also be accumulated into the Nafion film. We have also reported some polyelectrolyte-containing silica composite materials prepared by sol−gel processing.\(^{(39, 42)}\) These films, consisting of the sol−gel derived material, possess properties suited for a spectroelectrochemical sensor, such as nanoscale porosity, high optical transparency, ion-exchangeability, variable thickness (0.1−3.0 µm), and physicochemical stability. Moreover, the films prepared by the spin-coating method have a uniform surface and adhere tightly to ITO glass. These properties are important for the operation of the ATR sensor system.

The spectroelectrochemical modulation of NB accumulated in the candidate films is compared in Figure 5, which shows the change in percent transmittance (%) at 633 nm for Nafion, Nafion-

\[(\text{Figure 3. Schematic of the spectroelectrochemical cell: (a) sample inlet, (b) sample outlet, (c) reference electrode (Ag/AgCl), (d) SF6 coupling prisms, (e) electrical contact with counter electrode, (f) counter electrode (stainless steel foil), (g) gasket, (h) working electrode (ITO glass), and (i) cell base.})\]

\[(\text{Figure 4. Absorbance spectra of NB within Nafion sol−gel film on the ITO electrode. The measurements were done using transmission thin layer spectroelectrochemistry by applying a potential at (a) 0.5 and then at −0.8 V for (b) 1, (c) 3, (d) 5, (e) 10, (f) 20, and (g) 30 min.)}\]
Figure 5. Optical signal (%T) modulation at 633 nm for $10^{-5}$ M NB incorporated within (a) Nafion, (b) Nafion-silica, (c) PAA-silica, (d) PSSA-silica, and (e) heparin-silica film on the ITO electrode. The cyclic potential was scanned between 0.5 and $-0.8$ V for 20 times at a scan rate of 15 mV/s.

silica, PAA-silica, PSSA-silica, and heparin-silica film coated electrodes that were first immersed in $10^{-5}$ M NB for 15 min. The measurements were done in the spectroelectrochemical cell shown in Figure 3. The repetitive potential scans between 0.5 and $-0.8$ V modulate the optical signal. The magnitude of change in the optical signal shows the amount of NB accumulated within the film. The highest modulation amplitude of NB is obtained with the Nafion-silica and PSSA-silica films. However, the width of the optical modulation peaks obtained at Nafion-silica is slightly greater than for the PSSA-silica. Optical modulation is not observed in the PAA-silica film because NB does not penetrate into the film. However, Nafion and heparin-silica films show smaller modulation peaks. This is attributed to both the thickness of film, i.e., the capacity of Nafion to retain is smaller than that of Nafion-silica film, and the difficulty to accumulate NB into the heparin-silica film. Moreover, at the heparin-silica film, the percent transmittance increases with decreasing amplitude of modulation peaks as time increases. This may be due to heparin molecules eluting from the silica film. All the following experiments were done using the Nafion-silica film.

Spectroelectrochemical Behavior of LB at the Nafion-Silica Coated ITO Electrode. The electrochemical and spectroelectrochemical characteristics of LB at the Nafion-silica film coated on the ITO electrode were investigated. Figure 6A shows progressive cyclic voltammograms of $5 \times 10^{-5}$ M LB in Tris-HCl buffer (pH 7.5) obtained by scanning the potential 20 times with a scan rate of 15 mV/s. The voltammograms have well-defined oxidation and reduction peaks corresponding to the LB redox couple. The cyclic voltammograms are identical to those of NB. Therefore, this confirms that the electrode reaction of the NB portion of LB was not affected by labeling. The increasing peak currents with each scan indicate the time-dependent accumulation of LB into the Nafion-silica film. The optical signal modulation of LB that accompanies repetitive potential scanning is shown in Figure 6B. The modulation amplitude also increases with time as LB loads into the film until equilibration between the film and the solution is reached as shown in Figure 6A. Under the same conditions, optical signal modulation of LB produced no detectable change in transmittance at a bare ITO electrode. The change in the optical modulation amplitude plotted vs the number of cycles during the incorporation of different LB concentrations loaded into the film is shown in Figure 6C. All three different concentrations of LB reach equilibrium in less than five cycles (less than 20 min). However, the amplitude of optical modulation at $5 \times 10^{-5}$ M LB begins to decrease slightly after eight cycles (curve a). Although the reason for this small loss of LB after reaching maximum loading is unknown, it is not caused by Nafion eluting from the silica film, because when a lower concentration of LB loads into the film, the maximum amplitude of optical modulation is stable for at least 20 cycles (curves b and c). In addition, the electrochemical and optical responses of LB are higher than for NB, which may be attributed to the increase in hydrophobicity, and hence partitioning constant, caused by labeling. That is, the labeling converted the ionic amino group of NB into a hydrophobic moiety.

The changes in peak current and percent transmittance at different potential sweep rates were measured using an electrode coated with the Nafion sol−gel film equilibrated with $10^{-5}$ M LB. Figure 7 shows the plots of peak current and percent transmittance vs scan rates for 10−200 mV/s. The peak currents for LB increase linearly with scan rate, as shown in Figure 3. This indicates a typical thin-layer process.43 However, the changes in percent transmittance measured simultaneously with cyclic voltammetry decrease with increasing scan rate (curve b). At higher scan rates, the optical signal modulation is attenuated because of incomplete electrolysis of LB in the film. At these faster scan rates, only those LB molecules that are near the ITO surface within the film are cycled through the redox process. At the slow scan rates, the diffusion layer adjacent to the electrode surface exceeds the thickness of the optical path for ATR as defined by the evanescent wave and complete electrochemical redox cycling occurs. As the scan rate increases, the electrochemical diffusion layer decreases and its thickness becomes less than the optical path.

Spectroelectrochemical Binding Assays for Biotin−Avidin and 17β-Estradiol−Antiestriadiol Antibody. The spectroelectrochemical binding assays for avidin−biotin and E2−anti-E2 Ab systems were investigated. Spectroelectrochemical measurements were done with a double-potential step, as follows: (a) The potential was first held at a value that maintained NB in its colored state to get a stable background signal for 1 min, (b) then it was stepped to reduce NB to its colorless state and held there for 2 min, and (c) finally the potential was stepped back to its original value for another 2 min to regenerate the parent, absorbing NB. The step potentials were chosen to be the same as the switching potentials for the cyclic voltammetry experiments. Prior to these measurements, a potential (Ea) of 0.5 V was applied for 15 min to accumulate the labeled ligand into the film. Thus, the total analysis time, which includes the incubation step with protein and accumulation of the labeled ligand into the film, is ~50 min. This is almost the same as the analysis time of the electrochemical

binding assay using an electroactive labeled ligand that we reported previously.\textsuperscript{37}

The plots of changes in percent transmittance vs time for 10^{-5} M LB and several concentrations of avidin measured by the double-potential step are shown in Figure 8A. Before the solution was loaded into the spectroelectrochemical cell, LB and avidin were mixed in Tris-HCl buffer (pH 7.5) for 30 min at room temperature to reach the binding equilibrium. The amplitude of the optical signal change decreased significantly in the presence of avidin. Figure 8B shows the binding curve of LB to avidin obtained from the optical signal for the reduction of LB plotted as a function of increasing avidin concentration (curve a). The change in the signal of 10^{-5} M LB obtained by the spectroelectrochemical measurements decreases with increasing concentration of avidin and was close to zero at 10^{-5} M avidin. The same measurements were made using NB instead of LB (curve b). In this case, the amplitude of the optical signal changes for 10^{-5} M NB remained at the original value, at all concentrations of avidin used to generate curve a. Therefore, the decrease in the spectroelectrochemical signal of LB is not due to nonspecific binding between the NB portion in LB and avidin.
The results obtained from the spectroelectrochemical binding assay for the E2–anti-E2 Ab system are shown in Figure 9. Similar to the avidin–biotin system, the spectroelectrochemical signal of $10^{-5}$ M LE decreases with increasing concentration of anti-E2 Ab (curve a). These results indicate that the binding of a small molecule such as LB and LE with a large molecule like a protein prevents accumulation into the film. Therefore, this principle for the spectroelectrochemical binding assay is based on “sequestration electrochemistry”. It is possible to detect E2 by the competitive reaction between E2 and LE for the limited number of antibody binding sites. Figure 9 (curve b) shows the competitive binding curve obtained from E2. The measurements were made from $10^{-7}$ to $10^{-4}$ M E2 in the buffer, including $10^{-5}$ M LE and 1 $\mu$g/L anti-E2 Ab. In the absence of E2, the spectroelectrochemical signal of LE decreases as a result of the specific binding with the antibody. However, the presence of a large amount of E2 in the solution does not allow LE to bind to the antibody because E2 occupies the limited binding sites on the antibody. Consequently, the spectroelectrochemical signal of LE increases.

**CONCLUSIONS**

Proof of concept for a novel binding assay strategy has been demonstrated with the detection of a protein. Most importantly, this is a homogeneous assay that does not need the rinsing/separation step characteristic of heterogeneous assays. Homogeneous assays are desirable because a procedure without a rinsing step is simpler and easier to automate. However, homogeneous assays are difficult to develop because of the need to distinguish between bound label and unbound label without a rinsing step to remove one of the forms. The few commercially available homogeneous assays accomplish this by distinguishing between bound and unbound label based on differences in fluorescence polarization or enzyme activity. A key feature of the spectroelectrochemical assay is the film covering the OTE, which allows the Ab-bound label and unbound label to be distinguished between. The Nafion sol-gel film accomplishes this by excluding the Ab-bound label on the basis of its larger size compared to the free label. Thus, the spectroelectrochemical assay technique adds to the relatively few existing strategies for homogeneous assays.

In heterogeneous assays the detection method needs little selectivity for the label since the sample components are all rinsed away except the captured analyte. This means that label is detected by the instrument in a relatively pure solution without interferences from the sample. However, selectivity for label detection is critically important in homogeneous assays where the sample components are not rinsed away in a separation step and may contribute to the detection signal. This spectroelectrochemical assay takes advantage of the improved selectivity provided by combining the three elements of partitioning into a semiselective film, electrochemical modulation in a restricted potential range, and choice of wavelength for the optical detection. The effectiveness of this combination has been clearly demonstrated by the determination of ferrocyanide in nuclear waste, which is an extraordinarily challenging sample with respect to selectivity.

Label requirements are more stringent for the spectroelectrochemical assay than is typical for assays, which ordinarily need only one measurable property such as absorbance, fluorescence, or radioactivity. Here the label must pass three requirements: electroactivity for potential modulation, optical signal that changes with electrochemistry for detection, and properties consistent with preconcentration into a film.

The limit of detection reported here is about $10^{-7}$ M with absorbance as the mode of detection. However, this can be improved. Strategies to be explored for improving assay sensitivity include increasing the absorbance signal by (a) enhancing preconcentration into the film through modification of appropriate properties of the label and the film to increase the partitioning coefficient and (b) increasing $\Delta \varepsilon$ for the two forms of the label involved in electrochemical modulation. Also, the considerably lower limit of detection for spectroelectrochemical sensing demonstrated for fluorescence compared to absorbance can be achieved in assays by using a fluorescent label.

This new spectroelectrochemical assay should be applicable to any binding assay in which properties of the free and ligand-bound species are sufficiently different to be distinguished on the basis of partitioning into the film on the OTE, making the assay widely applicable.

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