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Article

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Posted Date: May 5th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1591985/v1

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Disposable Electrocatalytic Sensor for Whole Blood NADH Monitoring

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ABSTRACT

In the study, the preparation and electrochemical characterization of screen-printed electrodes (SPE) modified with 4’-mercapto-N-phenylquinone diamine (NPQD) and their behavior as electrocatalysts toward the oxidation of NADH with high stability are described. In particular, NPQD on the SPE substrate was deposited via electro-polymerization. Thus, the modified electrode exhibited a limit of detection (LOD) corresponding to 3.5 µM LOD with a sensitivity corresponding to $0.0076 \pm 0.0006$ µM/µA in the mouse serum. In all cases, NADH oxidation occurred at potentials approximately corresponding to 0.7 V vs Ag/AgCl electrode, and this indicates a decrease in the overpotential. We used the observations as a point of departure and developed an NADH biosensor based on the electrocatalytic oxidation of NADH. The applicability of the NADH sensor was demonstrated for the first time in 1) selectivity/sensitivity test via complex electrolyte as cell culture medium and 2) inflammatory and fibrotic responses by polyhexamethylene guanidine phosphate via NADH sensing in mouse serum.
Introduction

The maintenance of mitochondrial function is critical to preserving homeostasis in the living cell. Mitochondria are the cornerstone of life-supporting metabolic processes such as energy transduction and calcium signaling in biosynthetic pathways. Nicotinamide adenine dinucleotide (NADH/NAD\(^+\)), nicotinamide dinucleotide phosphate (NADP), and flavin adenine dinucleotide (FAD) are the most important coenzymes that exhibit a beneficial effect on the mitochondrial state because ATP production depends on the redox state of these coenzymes. Among them, NADH is the most well-known biomarker of the redox state of a cell. Koidl group reported that NADH deficiency causes an energy production problem and induces Parkinson’s disease due to the lack of ATP. The conventional method to determine NADH corresponds to an optical assay using absorbance or fluorescence and a colorimeter. The aforementioned methods are robust and standardized analytical methods to determine NADH. They also require high maintenance costs and high sample volume, and they exhibit a low limit of detection (LOD). Electrochemical (EC) biosensors for NADH emerged as an alternative analytical tool to conventional methods. The EC biosensor exhibits several advantages including convenience and short analysis time, and they require a small sample volume while maintaining high sensitivity and selectivity. The aforementioned types of sensors use the oxidation/reduction reaction of the NADH/NAD\(^+\) couplet. Specifically, NADH is oxidized to NAD\(^+\) at -0.560 V vs SCE, -0.315 V vs NHE (pH 7.0, 25 °C), and the redox state of NADH/NAD\(^+\) is detected via an electrochemical transducer. However, a few limitations emerged, namely, a large potential is required to oxidize the NADH. This destructs the electrode surface via the fouling effect and decreases efficiency because NAD\(^+\) is easily formed on the electrode surface via a redox reaction at the beginning of development. To solve the problems, several studies were performed using various sensing configurations with a single...
mediator such as 1) K$_3$Fe(CN)$_6$\textsuperscript{0}, 2) a polymer or bulk electrode\textsuperscript{10}, and 3) an enzyme electrocatalytic reaction induced via surface modification issued to lower the redox potential of NADH\textsuperscript{11}. Despite the significant advantages of each method, the development of a rapid, cheap, and high accuracy method that maintains sensitivity is still important in the NADH EC sensor field.

We demonstrate a screen-printed electrode (SPE)-based biosensor with an electrocatalyst immobilized on the SPE by a surface modification to decrease the redox potential of the NADH/NAD$^+$ couplet. The SPE is a promising analytical tool as an NADH biosensor platform with cost effectiveness\textsuperscript{12}. We systematically investigated electrocatalytic NADH oxidation, and the results indicated that the electrode exhibits a limit of detection (LOD) corresponding to 3.5 µM and sensitivity corresponding to 0.0076 ± 0.0006 µM/µA in a mouse serum. Additionally, we investigated the electrocatalytic reaction in a complex medium to prove that the proposed sensor maintained its sensitivity by lowering the interference signal. Finally, we challenged the NADH electrochemical sensor with diluted mouse serum and observed that it performed well in further \textit{ex vivo} experiments. An animal model of a PHMG-p induced lung injury was used, and the NADH was quantified and compared via a control model. To the best of the authors’ knowledge, the study is the first attempt to quantify NADH electrochemically \textit{ex vivo}. This suggests that the sensor can be a promising tool for NADH monitoring of mitochondrial function and offers exciting opportunities to treat a diverse range of diseases.
3. Results and Discussion

3.1. Surface modification of SPE
Figure 1. (a) Schematic of NPQD modification. (b–c) CV data obtained during the electrochemical functionalization of the 4-ATP Au electrode in (b) 100 mM PBS (pH 7.4) and (c) 10 mM PBS (pH 7.4) after (b). the scan rate is 100 mV/s. (d–e) Single-step and double-step scanning electron micrograph image of the SPE surface after NADH measurement (5 times) by changing surface modification method. The reference and working electrodes are denoted by RE and WE. (f) Contact angle measurement of each electrode. (g) Nyquist plot for each electrode.

**Figure 1a** shows a schematic illustration of the formation of the NPQD layer for the electrocatalytic reaction of NADH. The substance 4’-mercapto-N-phenylquinone diamine (NPQD) was selected as the electrocatalyst and immobilized via electrochemical functionalization due to its good redox behavior and rigid structure. Additionally, it lowers the oxidation potential and enhances the current because diamines are electroactive, easily oxidized, and can transfer two electrons via NADH oxidation to NAD$^+$ (one-electron transfer). This NPQD layer can be constructed via the functionalization of 4-aminothiophenol (4-ATP), which was previously immobilized on the Au layer by covalent bonding based on Au-thiol bonding as initially developed by the Takeo group. Although NPQD functionalization is similar, we selected a different electrochemical functionalization method and used a different substrate as the SPE. Figure 1b shows repeated cyclic voltammogram (CV) data as obtained during the functionalization of 4-ATP in 100-mM PBS buffer (pH 7.2). The large anodic & cathodic peaks nearby 0.7 V and small cathodic peak at 50 mV smoothly decreased by a CV cycle. This result is similar but different from previous reports because cathodic current at 0.55 V and anodic current at -0.2 V decreased and the reversible redox peak at 0.23 V newly emerged via a repeating CV cycle. However, the redox peak at 0.23 V was not observed, and we speculated that this was due to differences in our proposed
system. The SPE consists of Au working, Au counter, and Ag reference electrodes, and 4-ATP was immobilized on the working electrode and the counter electrode. This caused electrochemical motion in a manner different from that in previous reports because only the Au working electrode and not the counter electrode was functionalized. Given that the repeated CV signal in 100-mM PBS buffer is unstable, we performed a “double-step” electrochemical functionalization using a low concentration of PBS buffer (10 mM con, pH 7.4) after a high concentration PBS buffer for stabilization while maintaining other CV parameters. Although the current approximately corresponding to -0.4 V did not exhibit significant fluctuation, changes observed in the high concentration were not observed in the 10-mM PBS buffer (Figure 1b-c).

The NADH sensing performance of the single-step electrochemical functionalization using 100-mM PBS buffer and double-step electrochemical functionalization sequentially using 100-mM and 10-mM PBS buffer are shown in SEM images (Figures 1d and e). A stability problem arose when repeated measurements were performed with a single step. Following five measurements as shown by SEM, the reference region of the single-step modified electrode was burned and the grain boundary dimmed. Given the reference electrode problem, the working region also exhibited damage because it was unable to maintain a constant potential. However, concerning repeated measurements, the stability of the double-step modified electrode significantly improved when compared with that of the single step. Grain boundaries of Ag and Au were clearly observed.

NPQD functionalization was also demonstrated by the contact angle measurement of a water droplet since the -NH functional group in NPQD is polarized and hydrophilic. As shown in Figure 1f, the contact angle of the bare electrode is 108.4°, and for the 4-ATP modified electrode, it is 54.4°, a value not much different from that of the bare electrode. However, the NPQD-modified electrode showed a much lower value of 33.9° due to the amine group of NPQD. In addition to
surface angle measurement, the generation of NPQD as a redox-active monolayer was also observed by electrical impedance spectroscopy (EIS). Figure 1g is the Nyquist plot of the impedance for the formation of NPQD monolayers. EIS was used to investigate unmodified, 4-ATP- and NPQD-modified Au surfaces at a constant concentration of redox species of Fe(CN)₆³⁻/⁴⁻, and we found a charge transfer resistance (R_{ct}) that was expressed by the diameter of a semicircle in a Nyquist plot of the NPQD-modified surface, which had a maximum value (3133 Ω) compared with the unmodified and ATP modified electrodes (433 Ω and 819 Ω, respectively). Additionally, a 45° line in the Nyquist plot indicates a Warburg region of semi-infinite diffusion of a species in the modified electrode and the NPQD-modified electrode clearly shows a diffusion process governed by the mass transport of the redox molecules from the solution to the electrode [14]. Table 1 shows the specific EIS parameters of each electrode.

| Parameter | Bare           | 4-ATP          | NPQD          |
|-----------|----------------|----------------|---------------|
| R_{sol} (ohm) | 11.68          | 11.59          | 12.02         |
| R_{ct} (ohm)  | 433            | 819            | 3133          |
| W           | 5.86e-4        | 196.5          | 25.44         |
| Q (0.785)    | 1.67e-5        | 9.33e-4        | 9.75e-4       |
| Q (0.8195)   | 1.17e-5        | 4.64e-5        |               |
| Q (5.64e-5)  | 0.7221         |                |               |
| Q (2.602e-4)|                |                |               |

Table 1. electrical circuit simulation by a Nyquist plot.
3.2. NADH sensor sensitivity and selectivity

We already show the electrocatalytic activity of the NPQD-Au electrode for NADH oxidation in the cell culture medium\textsuperscript{15}. The electrocatalytic sensor was used to detect NADH in different electrolytes using a cell culture medium because it is important to monitor the NADH concentration in the cell to directly investigate the mitochondrial dysfunction status\textsuperscript{17-19}. The cyclic voltammogram signal in the mouse serum is similar to the signal in the medium, and it proves that the proposed sensor also works in the mouse serum (\textbf{Figure 2a-b}). The current was caused by NADH oxidation to NAD\textsuperscript{+} that regenerates the diamine as previously shown in the schematic (\textbf{Figure 1a}). To oxidize NADH to NAD\textsuperscript{+}, a potential is required as the driving force.

Chronoamperometry was used to make a calibration plot of NADH. Furthermore, a short and fixed potential analysis time (10 s) per sample was required when compared with cyclic voltammetry\textsuperscript{16}.

Finally, the NADH concentration in the mouse serum followed the adjusted equation I (nA) = {(-7.55)[NADH], µM -1.89} (R$^2$=0.981) with a range of linearity approximately between 16 – 1000 µM and a LOD corresponding to 3.5 µM (\textbf{Figure 2c–d}).
Figure 2. a-d) Electrochemical results for NADH in the mouse serum, a) Cyclic voltammogram, b) cyclic voltammogram from the circled part of the cyclic voltammogram of a), c) Chronoamperometry, d) Specific range in the mouse serum (0–150 µM) (n=5).

The main problems with the NADH sensor are stability due to fouling that occurred on the electrode surface when NADH is oxidized to NAD$^+$, and the selectivity due to NADH oxidation is largely influenced by the oxidation of AA (ascorbic acid)$^{18}$. A selectivity test was performed by adding the same concentrations of NADH and AA (1000 µM), and no significant difference was found when 1000 µM AA was added (the black dotted line) compared with a medium that contains no AA and NADH (the black solid line). The detection of NADH with our sensor was not affected
by AA, as evidenced by an NADH signal (red solid line) at 600 mV similar to the signal of an NADH + AA complex (red dotted line) (Figure 3a). Not only AA, to demonstrate the selectivity of the measurement, but NADH was also measured in a cell culture medium, and different concentrations of other biomarkers were added to the electrode surface to capture NADH, which is a biomarker for lung disease. Because cell culture medium contains many other impurities, such as albumin, glucose, and other inorganic salts, that may interfere with obtaining an accurate signal for NADH. Typically, glucose and urea exist at high concentrations in biological samples, so it is adequate to observe selectivity using this marker. The concentration of glucose and urea is fixed at 5 mM, 7 mM and that is the normal level. As shown in Figure 3b-d, the current was not stabilized at 500, 800 mV potential. We observed CV graph was not stabilized at 500 mV, and 800 mV potential. However, the signal was stabilized at 600 mV, and the signal is similar even in the presence of glucose and urea. This means that only NADH was oxidized at 600 mV potential, demonstrating the good selectivity of this modified electrode.
Figure 3. a) selectivity test with NADH and AA (ascorbic acid). b-d) signal difference for NADH with adding other impurities at b) 500 mV, c) 600 mV, d) 800 mV. e) stability test of NADH with repeated measurement (500 μM NADH solution was used).
To demonstrate stability, repeated measurements were performed in the presence of a high concentration of NADH (500 µM). Surprisingly, the signal for the 5th run had a similar value to the signal for the 1st run (0.17 % difference), which demonstrates that our NADH sensor maintained its electrocatalytic ability after several measurements were made even under harsh conditions such as in the presence of a higher concentration of NADH (Figure 3e). The achieved sensitivity and sensing range were applied to cell studies and non-clinical animal experiments (e.g., mice) because the known concentrations in animal cells approximately corresponded to 0.3 mM^{19-23}.

3.3. Electrochemical monitoring of mitochondrial NADH from an ex vivo system

To apply the proposed technique in an ex vivo system, we investigated changes in the NADH concentrations with time in whole blood during the initiation and development of PHMG-p-induced lung inflammation and fibrosis. Specifically, NADH is an important component of redox systems that plays a key role in various human diseases including cancer and lung disorders. The NADH concentration can change during many pathophysiological processes. We hypothesized that NADH can correspond to a potential biomarker that reflects the aforementioned pathophysiological processes and that a change in the NADH level can precede the onset of a metabolic disorder as evidenced by the previous result that indicated a relationship between cell viability and magnitude of the change in the amount of NADH. To test the hypothesis, we performed NADH sensing using mouse whole blood to determine whether its performance was comparable in terms of maintaining sensitivity and selectivity. First, we optimized the variables involved in the preparation of the NADH electrocatalytic sensor in the blood. We individually
tested each variable for each sample via a single NPQD-modified SPE, which included a) optimization of the concentration of EDTA used as an anticoagulant, b) blood dilution factor, and c) applied potential. The details of the optimization studies are summarized in Table 2.

| Variable                  | Tested                  | Selected |
|---------------------------|-------------------------|----------|
| Blood dilution factor     | 1:10, 1:20, 1:40, 1:80, 1:160 | 1:40     |
| EDTA concentration, mM    | 0, 0.05, 0.5, 5, 50, 500 | 5        |
| Applied potential, mV     | 500, 600, 700, 800      | 700      |

Table 2. Optimization of the experimental variables affecting the performance of NADH electrocatalytic sensor using blood.

\[
\text{% Matrix effect} = \left( \frac{\text{Response (in spiked sample)}}{\text{Response (in standard solution)}} - 1 \right) \times 100
\]

Equation 1. Matrix effect coefficient.

\[
\text{% Matrix effect (serum)} = \left[ \frac{0.00958}{0.01012} - 1 \right] \times 100 = 2.34 \%
\]

Equation 2. Matrix coefficient of serum.

\[
\text{% Matrix effect (blood)} = \left[ \frac{0.00865}{0.01012} - 1 \right] \times 100 = 4.53 \%
\]

Equation 3. Matrix coefficient of blood.
As shown in **Figure 4a** a significant effect on the NADH electrocatalytic sensor response was not evident with the presence of any substances in the serum or blood, thereby demonstrating that excellent selectivity and sensitivity were maintained. Finally, the matrix coefficient obtained by calculation using **Equation 1-3** corresponds to 2.34 % for serum and 4.53 % for blood. Following the optimization, the effect of potential interfering compounds that can be present in conjunction with the NADH in various biological samples on the electrochemical responses obtained with the NPQD-modified SPE was examined. Matrix effect experiments were performed to determine problems with the sensor by using spiked samples, and a known amount of NADH was added to PBS buffer, mouse serum, or mouse blood.

**Figure 4.** a) Calibration plot for NADH obtained with pure PBS buffer, mouse serum, and blood (n=5). B) NADH profiles of the control and PHMG exposure groups as obtained via an electrocatalytic assay (n=5).

After optimization and the construction of a calibration plot, we performed time-course analyses of NADH in the blood obtained from PHMG-p-induced mice via the proposed electrocatalytic
sensor. Although PHMG-p exhibits biocidal properties relative to various bacteria, yeast, and molds, epidemiological studies revealed that inhalation of PHMG induces lung and physiologic toxicity in Korea\textsuperscript{24,25}. Previously, we already established an animal model of PHMG-p-induced lung inflammatory and fibrotic responses and reported that PHMG-p induces polymorphonuclear cells (PMN) and macrophage-dominant lung inflammation during week 1 and marked lung fibrosis from weeks 2 to 10 via histologic analyses of H&E and Masson's trichrome stained preparations\textsuperscript{26}.

The NADH concentration peaked on day 7 in the blood of PHMG-induced mice when compared to control mice and then continuously decreased up to day 44 (Figure 4b). The concentration of NADH in the control at day 44 corresponded to 72.55 ± 7.99 µM and that in the PHMG-p group on day 44 corresponded to 54.24 ± 5.77 µM. Our results indicated that changes in the NADH concentration were related to lung inflammatory and fibrotic responses caused by PHMG-p. Individual data are shown in Figures 5-6. The results also suggested that our surface-modified SPE-based electrocatalytic system can experimentally monitor the \textit{ex vivo} release of NADH.
Figure 5. NADH profiles of the control groups as obtained via an electrocatalytic assay (n=5).
The sensitivity of the developed sensor was improved by double-step electro functionalization when compared with that by the single-step functionalization that was performed by Takeo’s group (LOD: 3.5 µM vs 10 µM). Additionally, the stability was also improved even for repeated measurements. We can assume that the difference is due to NPQD generation efficiency that is
related to the length of the electric double layer given the occurrence of NPQD functionalization in the electric double layer. The Debye length (i.e., the thickness of the double layer that forms at the charged surface in 100 mM PBS buffer) corresponds to 0.34 nm although it is approximately 0.7 nm in the 10-mM PBS buffer. A thicker double layer leads to deeper penetration of the electric potential of the target NPQD and exceeds the Debye length in 100 mM PBS buffer. However, the penetration rate is lower in the 10 mM PBS buffer, which displays a greater Debye length. The NPQD generation rate increases because the penetration rate of the electric potential on the electrode decreases. This contributes to sensitivity because NADH is easily oxidized via transferring electrons from NPQD.

| Electrode          | LOD    | Linear Range | Sample   | Ref |
|--------------------|--------|--------------|----------|-----|
| AuNPs/PB/GCE       | 0.21 μmol/L | 0.5 – 1000 μmol/L | PBS      | 29  |
| PEDOT CM/GCE       | 5.3 μM    | 20 – 240 μM | PBS      | 30  |
| MoSe₂/HEG          | 1 μM      | 1 – 2380 μM | PBS      | 31  |
| POA-Ag/GCE         | 0.05 μM   | 5 – 270 μM | Urine, PBS | 32  |
| NPQD-Au/SPE        | 3.5 μM    | 16–1000 μM | PBS, medium, whole blood | This work |

**Table 3.** Comparison of NADH detection based on an electrochemical reaction. Note: LOD: limit of detection, PEDOT: Poly(ethylene dioxythiophene), CM: Colloidal microparticles, GCE: glassy carbon electrode, HEG: Hydrogen exfoliated graphene, ADH: Alcohol dehydrogenase, POA: poly(o-anisidine), NPQD: 4’-mercapto-N-phenylquinone diamine, SPE: Screen printed electrode.
Continuous monitoring of NADH in *ex vivo* studies is an application of the sensor. The results suggest that the technique enables monitoring of mitochondrial energetics via measuring the change in the NADH concentration during the initiation and development of lung inflammation and fibrosis (or lung injury) that are induced by PHMG-p. To the best of the authors’ knowledge, only a few electrochemical sensors are employed for NADH measurement directly in whole blood (*Table 3*). As previously mentioned, NADH is an important marker of the cellular redox state and mitochondrial function and plays a crucial role in the maintenance of cellular homeostasis via regulating cellular metabolism and energy production\(^{33}\). Nevertheless, NADH accumulates when the cellular redox state is out of balance due to various physiological stimuli (e.g., exercise, diet, or hormones) and stressors, and this leads to various human diseases, including lung diseases caused by mitochondrial dysfunction. Our results indicated that in the PHMG-p group, NADH rapidly increased during initial lung injury and then significantly decreased during a prolonged lung injury with fibrotic features. The results were consistent with the NADH pattern displayed due to a rapid imbalance between NADH generation and oxidation in an ischemia-reperfusion animal model\(^{34}\). Although it is essential to further examine the relationship between NADH and various diseases including lung diseases, the results indicate that the sensor can be applied to an *ex vivo* system.
Conclusion

We demonstrated the development and application of double-step functionalization by lowering the electrolyte concentration from 100 mM to 10 mM in PBS buffer to prepare an electrocatalytic modified electrode for NADH oxidation and biosensing. NADH sensor exhibits good analytical performance to detect NADH with a linear response corresponding to 16 and 1000 µM, and a LOD corresponding to 3.5 µM in the mouse serum. Additionally, the technique permits the sensing of NADH redox signaling without interference by impurities while maintaining high sensitivity and selectivity, and thus it is an appropriate platform for continuous monitoring of NADH. The *ex vivo* PHMG-induced mice blood analysis substantiated the performance of the NADH sensor. Future efforts aim at establishing an NADH mechanism and its effect on lung disease that is induced by PHMG-p, and the aforementioned developments should lead to a better understanding of PHMG-p cytotoxicity.
Materials and Methods

Reagents and Chemicals

Polyhexamethyleneguanidine phosphate (PHMG-p) was generously donated by SK chemicals (Seongnam, South Korea). Potassium ferricyanide \([\text{K}_3\text{Fe(CN)}_6]\), 4-aminothiophenol (4-ATP), 100 mM and 10 mM of Dulbecco’s phosphate-buffered saline (DPBS), Tween 20, and absolute ethanol were purchased from Sigma-Aldrich (St. Louis, USA) and used without further purification. All reagents used in the investigation were of analytical grade.

Apparatus and electrode

Commercial screen-printed electrodes (SPE, Model no: DRP 220 AT, \(\Phi= 4 \text{ mm}\)) were purchased from the DropSens Co. Chronoamperometry (CA) and cyclic voltammetry (CV). Furthermore, electrochemical impedance spectroscopy (EIS) was performed with a multi-channel potentiostat obtained from CH Instruments (Texas, USA, Model no: CH 1030C) and the Wizmac Co. Ltd. (Daejeon, South Korea, Model no: WIZECM-1200Premium). All electrochemical (EC) measurements were performed at room temperature in a Faraday cage to ensure electromagnetic shielding.

Surface modification of the SPE

Before assembly, the SPE was pretreated by placing a 50-\(\mu\)L drop of a 10 mM H\(_2\)SO\(_4\) solution on it, and cyclic voltammetry was performed from 0 V to 1.8 V at a scan rate of 100 mV/s to remove dust. Subsequently, the SPE was washed with DI water and dried with nitrogen. After it was dried
in a stream of N₂, the 4-ATP self-assembled monolayer (SAM) was prepared on the SPE by incubating the electrode in 10 mM 4-ATP dissolved in absolute ethanol for 2 h at room temperature. Subsequently, the SPE was washed with absolute ethanol for 1 min and then washed again with 0.05 % Tween 20 in 10 mM DPBS (pH 7.2) to remove the remaining chemicals. A 4’-mercapto-N-phenylquinone diamine (NPQD) layer was generated on the Au electrode via a two-step electrochemical surface modification. After drying in a stream of N₂, 1) 50-µL of 100 mM DPBS buffer (pH 7.2) was dropped on the SPE, and CV was performed by applying a potential ranging between 0.8 V and -0.4 V 30 times. The electrode was washed by using 10-mM DPBS buffer (pH 7.2), and 2) a CV step identical to that in 1) was performed again by changing the 100 mM DPBS buffer to 10 mM DPBS buffer. Following surface modification, the electrode was washed with 0.05% Tween 20 in 10 mM and kept in a 10 mM DPBS buffer.

**Characterization of the surface-modified electrode**

**Scanning Electron Microscopy**

SEM micrographs were recorded with an EVO MA 10 (Carl Zeiss Ag, Germany). The samples were sputtered with Au before microscopic analyses.

**Contact Angle Measurements**

The hydrophilic properties of the surface-modified layer were characterized via contact angle measurement. The contact angle data of each layer was obtained by dropping 10-µL of DI water on the modified surface of the Au working electrode, and the image was analyzed via the ImageJ contact angle program. All measurements were performed on the air-facing surfaces of the films.
Five different measurements were performed on each modified electrode, and the standard error was determined.

**Electrochemical impedance spectroscopy measurement**

The impedance spectra were recorded from 1 MHz to 0.1 Hz at an AC signal amplitude corresponding to 50 mV. A CV with a different scan rate and impedance was recorded in 5 mM K$_3$Fe(CN)$_6$ + 0.1 M KCl in pH 7.4 PBS buffer as the electrolyte solution at room temperature.

**Calibration of NADH in DPBS buffer and cell culture medium**

Similar experimental procedures were followed for DPBS and culture medium. To construct a calibration curve for NADH, first, various concentrations of NADH (0–1000 µM) were separately prepared in the DPBS and culture medium. Subsequently, CV at a scan rate corresponding to 100 mV/s was used to electrochemically characterize the electron transfer between NPQD-modified surface and NADH under both conditions after dropping 50-µL of an NADH solution on the electrode.

Current versus time curves were recorded via applying a fixed potential determined by CV. A computer-controlled EC analyzer was used for signal readout at a fixed potential of 0.7 V (culture medium) with a pulse width corresponding to 10 s and a sample interval corresponding to 0.05 s. By plotting the current intensities when a steady state was reached, a standard curve for NADH was obtained. Several calibration curves were obtained (n=5) with each concentration under the same conditions to calculate the average and standard deviation. The limit of detection (LOD) was determined via gathering the background signal and 3 standard deviations (3σ).
Ex vivo evaluation

Ethics statement

All the experiments were approved by the Institutional Animal Care and Use Committee of the Korea Institute of Toxicology (KIT) and conducted based on the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International (B218004). In addition, the RRIVE guidelines were followed to carry out the present study.

Animals

Six-week-old male C57BL/6 mice were purchased from Orient Bio, Inc. (Seongnam, Korea). The mice were housed in an environmentally controlled animal room that was maintained at a temperature of 22 ± 3°C, relative humidity of 50 ± 20%, and air ventilation rate of 10–20 changes/h with a 12-h light/dark cycle. Sterile pelleted food for experimental animals (PM Nutrition International, Richmond, USA) and UV-irradiated (Steritron SX-1; Daeyoung, Inc., Korea) and filtered (1 μm) tap water was provided. The mice were acclimatized for 6 d. All the experiments were approved by the Institutional Animal Care and Use Committee of the Korea Institute of Toxicology (KIT) and conducted based on the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International (B218004).

Treatments and experimental groups

Mice (n=10) were randomly assigned to one of the following three weight-matched experimental groups via the Pristina System (Version 7.3; Xybion Medical system Co., USA), (six mice per group): mice treated with Saline for group 1 (control), mice treated with PHMG for group 2. The mice in group 1 were instilled with 50 μL of saline solution using an automatic video instiller.
Mice in the PHMG (25 %; SK Chemicals, Seoul, Korea) groups received a single intratracheal instillation (ITI) of 1.1 mg/kg PHMG in 50-μL saline solutions via the same route.

**Intratracheal instillation**

A mouse was anesthetized with isoflurane and placed on its back in a head-up position on a flat board with an inclined angle. Concerning the ITI, the upper incisors of the mouse were held by a rubber band that was fixed on the board. The tongue was gently moved to one side of the oral cavity, and the mouse was then instilled with 50-μL of each sample by using an automated syringe pump and video instiller.

**Blood collection**

Before commencing the collection, we diluted a 5-mM EDTA solution with PBS. A mouse was gently placed in a restrainer, and its tail was washed and cleaned using a wipe with 70% alcohol. After grasping the distal end of the tail vein line, we inserted a 1-mL needle with the bevel side up and parallel to the skin. The needle was placed correctly in the vein, and it was then slowly removed. Blood was collected from the vein until at least 10 μL of blood was obtained. A 10-μL aliquot of blood was directly mixed with 90-μL EDTA solution in a tube and stored at -80 °C.

**Quantification of NADH in mouse blood**

To quantify NADH in mouse blood, 50-μL of diluted blood extracted as detailed in the previous section was dropped on the NPQD-modified electrode. The NADH quantification was performed within 10 min after extraction to prevent contamination. Five replicates were used for each sample, and non-treated PHMG-p mice were used as the positive control group. A potential corresponding to 0.7 V was applied, and the current was read at a stabilization state after 20 s.
Statistical Analysis

All values were expressed as the mean±standard deviation. Statistical analyses were performed using a two-tailed t-test. Statistical significance was defined as \( p<0.05 \). All assays were run five times and mean and standard deviation were calculated at each concentration to generate the calibration curve. Each replicate was measured with a new screen-printed electrode. The analyte NADH was newly made at each time measurement to maintain fresh conditions. Nonlinear curve fitting was performed with the Origin 8.0 program.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.
Supporting Information

Acknowledgments
This research was supported by the Kumoh National Institute of Technology (2019-104-044).

Author Contributions
JuKyung Lee wrote the main manuscript and performed overall experiments. Han Nah Suh and Hyebin Park, Sae Young Ahn performed an ex vivo experiment. Hyung Jin Kim and Sang Hee Kim directed the paper. All authors reviewed the manuscript.

Conflicts of Interest
The authors declare no competing financial interest.

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Research Highlight

**Figure.** Electrocatalytic NADH monitoring using mouse whole blood. In this research, inflammatory and fibrotic responses by polyhexamethylene guanidine phosphate via NADH sensing in mouse whole blood was used as a model analyte.