Electrochemical microwell sensor with Fe–N co-doped carbon catalyst to monitor nitric oxide release from endothelial cell spheroids

Kaoru Hiramoto1 · Kazuyuki Iwase2 · Yoshinobu Utagawa3 · Yuji Nashimoto4 · Itaru Honma2 · Kosuke Ino5 · Hitoshi Shiku5

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Abstract
Endothelial cells have been widely used for vascular biology studies; recent progress in tissue engineering have offered three-dimensional (3D) culture systems for vascular endothelial cells which can be considered as physiologically relevant models. To facilitate the studies, we developed an electrochemical device to detect nitric oxide (NO), a key molecule in the vasculature, for the evaluation of 3D cultured endothelial cells. Using an NO-sensitive catalyst composed of Fe–N co-doped reduced graphene oxide, the real-time monitoring of NO release from the endothelial cell spheroids was demonstrated.

Keywords Nitric Oxide (NO) · Electrocatalysts · Composite carbon materials · Endothelial cells · Spheroids

Introduction
Endothelial cells line the inner surfaces of blood vessels and play crucial roles in vascularized tissues and organs, such as maintaining vascular homeostasis and regulating the transportation of nutrients and signaling molecules. Human umbilical vein endothelial cells (HUVECs) are widely used not only for basic studies of vascular biology, but also for transplantation research [1, 2] and tissue engineering [3, 4]. To date, a wide variety of culture models using HUVECs have been provided, from simple monolayer cultures using plastic plates to more physiologically relevant models using microfluidic devices [5, 6] and/or three-dimensional (3D) culture systems [7, 8]. Augustin et al. reported HUVEC spheroids for an endothelial transplantation assay, demonstrating the successful formation of matured capillary networks from the spheroid and connections to mouse vasculature in vivo [9]. These achievements show that 3D culture models of HUVECs are promising tools for bridging the gap between in vivo and in vitro vascular systems [10]. To facilitate the studies, analytical techniques to evaluate the in vitro vascular systems are highly needed [11].

The production of nitric oxide (NO) is an important biological function of endothelial cells that regulates immune responses, vasodilation, and blood pressure [12]. Thus, the real-time measurement of NO released from endothelial cells is of great interest in physiological and pathological studies. Electrochemical sensors are considered promising for NO detection because of their fast response, high sensitivity, and ease of use. These attributes should overcome the difficulty of measuring NO regarding its short half-life. Since the first electrochemical sensor was reported by Shibuki [13], various electrochemical methods have been developed to measure NO in living systems [14]. In terms of the endothelium, endothelial cells express NO synthase (NOS), which synthesizes NO by converting l.-Arginine...
(L-Arg) to L-citrulline. The NO production occurs not only under chemical but also mechanical stimulations [15, 16]. In literatures, electrodes placed above or under endothelial cells have often been utilized to evaluate the NOS function of the cells. For instance, scanning electrochemical microscopy was utilized to measure NO produced from single HUVECs [17]. Flat electrodes decorated with catalytic materials and/or perm-selective membranes have been used to directly detect NO from endothelial cells adhered onto the electrodes [18, 19]. More recently, a flexible device [20] and a microfluidic system [21] have both been proposed to detect NO production from HUVECs under mechanical stress. These studies provided significant platforms for the in situ detection of NO, although most of these studies have used HUVECs that adhered to the substrates. Considering the growing recognition of its relevance in 3D vascular models, it is important to investigate the cellular functions of 3D constructs of endothelial cells, i.e., spheroids. To date, several electrochemical methods have been developed to measure the cellular activity of spheroids (e.g., oxygen consumption [22–24] and enzymatic activity [25, 26]. Whereas, to the best of our knowledge, NO production of spheroids has never been electrochemically monitored.

Here, we present a microwell-type electrochemical sensor that facilitates the real-time monitoring of NO released from single HUVEC spheroids. Various catalytic materials have been proposed to ensure sufficient sensitivity for the electroanalysis of NO, including metal nanoparticles [27], carbon materials [19, 28], and their composites [29]. Organometallic complexes are promising materials as highly active NO detection catalysts owing to their high design flexibility. Iron (Fe)-macroyclic (Fe-N₄) compounds (e.g., porphyrins or phthalocyanines) have been reported to show increased NO sensing activity among metal-macrocyclic compounds [30, 31]. Previous reports have demonstrated that the NO sensing ability from living cells can be enhanced by modifying electrodes with Fe-N₄ compounds [18, 19]. However, these organometallic complexes might suffer from unstable catalysts because of their solubility or low resistance to the oxidative conditions required for NO detection (~0.9 V vs. Ag/AgCl). Thus, in the present study, we applied Fe-N co-doped carbon material to an NO detection catalyst and fabricated an in-situ detection device for NO produced from HUVECs. We took this approach as doping Fe-N complexes with carbon materials is expected to realize both NO detection activity and stability.

A schematic illustration of the sensor is shown in Fig. 1. Here, the Fe-N-doped reduced graphene oxide (Fe-N/rGO) catalyst was immobilized in a microwell with an underlying Au electrode to achieve electric conduction. The microwell was fabricated to a suitable size to support HUVEC spheroids, allowing the detection of NO in a confined space between the spheroid and the electrode underneath. Under an injection of L-Arg as a substrate, NO production from HUVEC spheroids was detected amperometrically via an increase in the oxidation current of NO.

**Experimental**

**Synthesis of Fe–N/rGO**

In the present study, Fe–N/rGO was synthesized via the heat treatment of a mixture of a Fe–N complex and graphene oxide (GO) prepared from graphite powder under an N₂ atmosphere [32]. Briefly, 270 mg of FeCl₃·6H₂O (Wako Pure Chemicals Industries Ltd., Japan) and 465 μL of pentaethylenehexamine (TCI, Japan) were dissolved into 10 mL of ethanol (Wako). Then, 200 mg of GO was dispersed in 368 μL of the mixed solution and 5 mL of ethanol using an ultrasonic bath. The mixture was dried at 60ºC. The dried powder was collected and placed in the aluminum crucible, and heated at 900ºC for 1 h under an N₂ atmosphere, and cooled down to room temperature. After cooling, the obtained powder was acid washed by 1 M H₂SO₄ solution for 3 h under 80ºC. Then, the sample was washed with distilled water 3 times and then vacuum filtered. After drying the powder under 60ºC, the catalysts were obtained. N/rGO (without Fe) was synthesized in a similar manner without adding Fe source in the Fe–N complex solution.

**Electrochemical characterization of catalysts toward NO oxidation**

The NO sensing activities of catalysts were measured by cyclic voltammetry (CV) (scan rate: 50 mV s⁻¹) with different NO concentrations prepared by adding NO saturated solution into N₂ saturated phosphate buffer solution (PBS, pH 7.4). The preparation of NO standard solution is described in Supplemental Information. The CV was carried out using HZ-7000 (HOKUTO DENKO Co., Japan) with PDMS Fe-N/rGO-chitosan Au electrode Glass slide L-Arginine NO
a three-electrode setup. For working electrodes, the catalyst ink was prepared by dispersing 3 mg of catalyst (Fe–N/rGO, N/rGO, or GO), 28.5 µL of 5% nafion solution (Sigma) and 300 µL of ethanol (Wako) using an ultrasonic bath for 2 h. Then 5 µL of the catalyst ink was drop-casted onto a glassy carbon electrode (φ3 mm, BAS Co., Ltd, Japan), and the electrode was air-dried. An Ag/AgCl (sat. KCl) and a carbon rod were used as reference and counter electrode, respectively.

**Selectivity test**

The selectivity of Fe–N/rG0 was evaluated by collecting amperometric responses of successive injections of interfering species under a constant potential of 0.85 V. For working electrodes, the catalyst ink was prepared by dispersing 3 mg of Fe–N/rGO in 300 µL of 0.5% chitosan solution (Sigma). Then 5 µL of the catalyst ink was drop-casted onto an Au disk electrode (φ3 mm, BAS Co., Ltd, Japan). The Fe–N/rGO modified working electrode, an Ag/AgCl (sat. KCl) and a Pt wire were set in a beaker with PBS. Under a gentle stirring, interfering species, KCl, H2O2, NaNO2, Na2SO4, glucose (all from Wako), and dopamine hydrochloride (Sigma) were injected by using a pipette at a final concentration of 180 µM, while NO solution was injected at a final concentration of 18 µM.

**Device fabrication**

The fabrication process of the Fe–N/rGO modified microwell sensor is detailed in Supplemental Information and Fig. S4. Briefly, an Au sputtered slide glass (S1126, Matsunami, Japan) was used for an underlaid electrode for electric conduction. To construct a microwell, two sheets of polydimethylsiloxane (PDMS, SYLGARD™ 184 Silicone Elastomer, TORAY, Japan, 0.1 mm thick, each) with a hole of φ0.5 mm was overlaid on the Au substrate. The catalyst ink of 1 w/v% of Fe–N/rGO in 0.5% chitosan solution was casted onto the Au electrode with the PDMS sheets and left at room temperature for drying. Approximately after 30 min, 1 µL of 0.05% chitosan solution was overlaid on the catalyst and dried again to make sure the catalyst was immobilized on the substrate. After that, the top layer of the PDMS sheets was peeled off from the substrate so that overflowed catalyst was removed. Finally, cured PDMS (0.3 cm thick) with a hole of φ8 mm was bonded on the substrate as a reservoir.

**Preparation of HUVEC spheroids**

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Switzerland) and cultured in Endothelial Cell Growth Medium 2 (ECGM2, PromoCell, Germany) at 37°C under 5% CO2. The HUVECs at passage 5 or 6 were used for subsequent fabrication of HUVEC spheroids. HUVEC spheroids were prepared according to the methods described in a previous report [9, 33], with slight modifications. The stock solution of Methocel was prepared in advance. Briefly, 600 mg of methyl cellulose (Methocel® A4M, Sigma) was diluted in 50 mL of ECGM2 under stirring for 24 h, and stored at 4 °C. For the formation of the spheroids, a 96-well plate with a U-shaped bottom well was purchased from Sumitomo Bakelite (Japan). HUVECs were seeded at 1 × 10⁴ cells per well with 200 µL of ECGM2 containing 0.2% Methocel. The HUVECs spontaneously formed spheroids within 24 h. For fixation of cells, HUVEC spheroids were immersed in 4% paraformaldehyde (Wako) for 2 h at room temperature. The fixed samples were washed twice with PBS and stored at 4 °C.

**Electrochemical measurement of NO from HUVEC spheroids**

Before the experiment, the microwell sensor with Fe–N/rGO was treated with a plasma ashing device for 30 s. Then, 100 µL of PBS (Code No. 14249, Nacalai tesque) was introduced to the device. A HUVEC spheroid was gently washed with PBS and transferred to the microwell using a micropipette. An Ag/AgCl electrode and a Pt wire as reference and counter electrodes, respectively, were inserted into the device. Amperometry was performed using HA1010mM4 (HOKUTO DENKO Co.) under a constant potential of 0.85 V. For induction of NO production of HUVEC spheroids, 10 mM L-Arg (Nacalai tesque) was diluted in PBS and 5 µL of the solution was injected into the device. The final concentration of L-Arg was calculated to be 0.5 mM in the microwell sensor containing PBS. Whereas, to test the suppression of NO production, 10 mM Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME, Nacalai tesque) in PBS was injected for 5 µL.

For quantification of NO released from a HUVEC spheroid, the amperometry of the sensor upon successive injection of NO solutions (NO concentrations of 0–900 nM) was used. The increase in the current after the injection of NO was plotted against the NO concentration and a calibration curve of $y = 0.143 + 0.0193x$ (nM) was derived (Fig. 4e insert). From this equation, NO production of a HUVEC spheroid was estimated.

**Results and discussions**

**Surface characterization of Fe–N/rGO**

Scanning electron microscopy (SEM) images (Fig. S1) revealed that the particle size of Fe–N/rGO was ~1–5 µm. Transmission electron microscopy (TEM) images of Fe–N/
rGO (Fig. S2) showed that aggregated species with ~20 nm in size were present on the carbon layer, suggesting that some of the Fe species existed as Fe-based agglomerates, such as Fe oxides. Fe and N doping were verified using X-ray photoelectron spectroscopy (XPS, Fig. S3). In Fe–N/rGO, the peak position of Fe-2p at 710.5 eV in Fe–N/rGO can be assigned to Fe$^{3+}$ [34, 35]. The slight shift of the peak position of Fe$^{3+}$ can be attributed to the formation of aggregated Fe species. The semi-quantitative analyses conducted using XPS are summarized in Table S1.

**Electrochemical characterization of Fe–N/rGO**

The NO oxidation activities of Fe–N/rGO were examined using cyclic voltammetry (CV) under different NO concentrations. The preparation of standard NO solutions is detailed in Supporting Information. The representative CVs are summarized in Fig. 2a. The oxidation current clearly increased with an increase in NO concentration, revealing that Fe–N/rGO exhibited a NO detection ability. The NO oxidation currents at 1.1 V for Fe–N/rGO, N/rGO (without Fe doping), and GO are summarized in Fig. 2b. Among them, Fe–N/rGO showed the highest NO sensitivity, and its oxidation current increased linearly with NO concentration. Considering that Fe metallophthalocyanines with Fe–N structure can efficiently catalyze NO oxidation [19], the current synthesis successfully introduced Fe–N structure in a carbon layer to form a porphyrin-like structure that accelerate the adsorption of NO and electron transfer to oxidize NO [36]. This suggests that Fe–N/rGO is suitable for quantitative NO detection. The selectivity of Fe–N/rGO was also studied by investigating the amperometric responses of potential biological interfering species (Fig. 3a). Amperometric responses were collected while stirring phosphate-buffered saline (PBS) under a constant potential of 0.85 V. Interfering species, KCl (Cl$^-$), H$_2$O$_2$, Na$_2$SO$_4$ (SO$_4^{2-}$), NaNO$_2$ (NO$_2^-$), glucose (Glu), and dopamine (DA) were injected into the PBS at a final concentration of 180 µM, whereas
NO solution was injected at a concentration of 18 µM. We subtracted the current before the introduction of interfering species from that after the introduction. As the background current gradually decreased during the measurement, the responses of Cl⁻ and Glu were minus, indicating that these species were very inert to the Fe–N/rGO electrode. Moreover, although H₂O₂, NO₂⁻, and DA led to an increase in current, the sensor showed a remarkably larger response to NO even at one-tenth of the concentration of those interfering species (Fig. 3). This result demonstrates the good affinity of NO to Fe and N sites, catalytic activity of Fe–N/rGO to NO oxidation.

Device characterization

To monitor the NO release from HUVEC spheroids in real-time, a Fe–N/rGO modified sensor well was developed. Figure 4a (i) displays the whole image of the device. The device was composed of four individual wells made with polydimethylsiloxane (PDMS), and an underlying Au electrode for electrical conduction. Each well contains a microwell (diameter: 0.5 mm, height: 0.1 mm) with Fe–N/rGO modified at the bottom, to serve as an electrochemical sensor for monitoring NO release from a single HUVEC spheroid. To modify the electrode with Fe–N/rGO, a chitosan solution was chosen as the sticking agent. Owing to its high mechanical strength and good biocompatibility, chitosan has been used as a binder of electroactive materials to electrochemical NO sensors and successfully applied for biological and pharmaceutical measurements [37, 38]. In the current case, Fe–N/rGO was homogeneously dispersed in chitosan solution which was favorable for the drop-casting process. The fabrication process is detailed in Supporting Information.
NO, CV was performed in homogeneously stirred PBS with different concentrations of NO. As shown in Fig. 4b, NO oxidation peak was observed at 0.85 V, whereas the reduction peak observed at 0.5 V may be attributed to the reduction of oxide film on partially exposed Au surfaces. The peak current at 0.85 V directly corresponded to NO concentrations, showing the ideal electrocatalytic effect of Fe–N/rGO on the microwell (Fig. 4c). To further investigate the amperometric response of the sensor toward NO, we monitored the current response under a constant applied potential of 0.85 V (Fig. 4d). To mimic the experimental conditions of the spheroid measurement, this experiment was conducted under a static condition with close injections of 10 µL of NO solutions into the microwell sensor using a micropipette. A rapid increase in the current was observed when the NO solution was injected; the sharp peak-like responses may be attributed to the close injection of NO solution to the modified electrode surface. In Fig. 4e, the difference between the current values before ($I_{BG}$) and after ($I$) each injection was plotted against the NO concentrations. The current values corresponded directly to NO concentrations, although the $I - I_{BG}$ value at 0 µM NO was negative due to the gradual decrease of the current during the measurement. Nevertheless, the microwell sensor could detect several nano-molar of NO solution, which is comparable to previous works [19, 39].

**Real-time detection of NO release from HUVECs**

For practical applications, NO released from HUVEC spheroids was monitored using amperometric detection. The configuration of the device and a spheroid is illustrated in Fig. S5. The diameter of the well (φ0.5 mm) was almost the same as the diameter of the HUVEC spheroid so that the spheroid was likely to attach the surface of the electrode. We used the spheroids formed on day 1 for the measurement because older spheroids were easy to fall apart when they were transferred to the device. We suppose aggregation of HUVECs are fundamentally weak, and we need to optimize the concentration of Methocell or consider other sticking agent to form stiffer constructs. Nevertheless, the literature utilized endothelial cell spheroids of day 1 for biological study such as angiogenesis and transplanting, therefore, we used the spheroids of the same culture period. Figure 5a shows the amperometric current when a constant potential of 0.85 V was applied to the electrode. After l-Arg stimulation at 400 s, the current gradually increased and reached a maximum at around 800 s, indicating that NO was produced from the spheroid. The amount of NO was estimated to be 8.0 nM when the current increase (9.6 nA in Fig. 5a) was considered to be generated from NO. In this study, HUVEC spheroids fixed with 4% paraformaldehyde were also measured. As cellular activity is terminated in those cells, no change in current was observed under the same conditions. Although we did not investigate the reusability of the sensor in detail, we could use one sensor for 3 or 4 times after washing the sensor with MilliQ. Therefore, the same sensor was used in Fig. 5a for measuring the NO production ability of living and fixed spheroids. Figure 5b shows the current values at 800 s after l-Arg stimulation to the living and fixed spheroids. As it can be seen, the current response of the living spheroids was much larger than that of the fixed spheroids ($p < 0.05$). The variations of the current in the living spheroids may attributed to the cellular activity of the spheroids, which possibly became weak during transferring and/or measurement. On the other hand, some current noises were also observed in the fixed spheroids after l-Arg was introduced (Fig. S6). We could not determine the factor of the noise, but it may be attributed to the fixation process. For a detailed examination of eNOS activity in HUVEC spheroids, one can conduct genetic modification, although it is out of scope in this study. Instead, we injected l-NAME, a competitive eNOS inhibitor, into living spheroids after the l-Arg stimulation. The current increased with the l-Arg stimulation and apparently

**Fig. 5** Amperometry with HUVEC spheroids. a Amperometric responses of the sensor with living and fixed spheroids, indicated as red and black line, respectively. A drop of 5 µL of 10 mM l-Arg was injected into the well at 400 s. b Box-and-whisker plot of average current between 770 and 800 s obtained from the amperometry with living and fixed spheroids under l-Arg stimulation. $N = 15$ and 10 for living and fixed spheroids, respectively.
decreased with the succeeded injection of l-NAME. On the other hand, no changes in the current occurred following the injection of PBS as a control (Fig. S7). These results demonstrated that the active response of eNOS was successfully monitored by the sensor. Indeed, the histochemical staining of HUVEC spheroids revealed the expression of eNOS in most of the cells (Fig. S8). Overall, there were some differences between the present study and previous reports in which HUVECs were attached to electrodes to form a monolayer; the increase of the current after l-Arg stimulation was relatively slow (~100 s) in HUVEC spheroids compared to the attached cells (~10 s), and the amount of released NO was also much less than that of the attached cells. Although further investigations are needed to discuss this matter, we think this is attributed to the limited exposed area of cell membranes of the spheroids for the first contact of l-Arg. In addition, the consumption of l-Arg and the product NO inside the spheroid may have been rate-limiting which may have delayed the HUVECs to release extracellular NO.

**Conclusion**

In summary, we present the synthesis and fabrication of an Fe–N/rGO-immobilized microwell sensor for monitoring NO release from endothelial cell spheroids. A facile introduction of Fe and N composites to reduced graphene oxide structure exhibited favorable sensitivity and selectivity for electrochemical detection of NO. Although we could not conduct a further experiment, sensitivity to NO may be improved by altering the composition rate of Fe and N. By modifying Fe–N/rGO on the bottom of the microwell, NO released from an endothelial cell spheroid settled in the microwell could be monitored in real time. As the studies of cellular activity of endothelial cell spheroids are relatively scarce, the present paper provides some insights into both in vitro vascular study and analytical techniques for tissue engineering. In these days, in vitro 3D vascular models are becoming increasingly important, thus the present sensor provides a potential platform for monitoring the cellular functions of vascular tissue models.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s44211-022-00160-0.

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**Data availability** Authors can confirm that all relevant data are included in the article and/or its supporting information files.

**Declarations**

**Conflict of interest** There are no conflicts to declare.

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