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Scientific Research

A Carbon Monoxide Sensing Film Based on Hemoglobin Allostery

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Abstract

Carbon monoxide (CO) is a gaseous mediator, which is generated via anenzymatic reaction of heme oxygenase, and it plays physiological roles in regulating cellular respiration and blood flow in the liver. The concentration and distribution of CO molecules in the living body is unknown owing to a lack of a suitable technique for measuring them *in vivo*. A needle-type CO sensor has been used for bioinstrumentation, but it is inappropriate for implantation *in vivo* and long-term monitoring. We developed a CO sensor sheet based on hemoglobin (Hb) allostery, as Hb undergoes a conformational change on CO binding. Hb was extracted from mice blood and mixed with agarose gel with a reducer to stabilize deoxy-Hb in the gel. CO-releasing molecules (CORM) were used to mimic CO-generating tissue, and the sensitivity of the Hb gel could be regulated by Hb concentration. We defined the CO-Hb index, an absorbance ratio at 539 and 557 nm, to estimate the accumulation of captured CO in the gel. It correlatively increased with CORM dose, indicating that gel-embedded Hb underwent a conformational change on CO binding, thereby acting as a CO sensor. We subsequently used the Hb-sensor sheet for two-dimensional imaging of CO distribution. CORM-containing gels with different sizes and doses were layered on this sheet. Size- and dose-dependent CO distribution was visualized by scanning the CO-Hb index in the sheet. Our Hb-based CO sensor sheet is composed of biocompatible materials and can be applied to detect low-level CO sources in the living body.

Keywords

Carbon Monoxide; Gas Sensor; Hemeoxygenase; Tumor Hypoxia

1. Introduction

Carbon monoxide (CO) is generated via anenzyme reaction in which heme oxygenase (HO) catalyzes the oxida-

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tive conversion of heme to biliverdin IXα and iron *in vivo* [1] [2]. Because CO is a gaseous molecule, it freely diffuses in the living body and is used for the regulation of physiological functions. CO regulates vessel diameter and blood flow in the liver [3] and exerts protective effects on cells [4]. In particular, in tumor environments, heterogeneously distributed hypoxic areas are caused by an imbalance between oxygen supply and consumption in tumor tissues because of immature neovessels and blood flow disturbance [5]. Hypoxia inducible factor-1 (HIF-1) is a transcription factor that transactivates genes encoding proteins contributing to homeostatic responses to hypoxia [6]. HO-1 is located downstream to HIF-1; thus, CO gases can be generated in hypoxic tumor tissues [7]. It is proposed that CO molecules are generated in a tumor bind to hemoglobin (Hb) in erythrocytes and diffused in blood. However, the details of the pathological roles of CO in cancer remain unknown [8]. Extraneous CO gas, e.g., which is generated upon incomplete combustion, is taken up via pulmonary respiration and binds to Hb. Because the affinity of CO for Hb is 240 times stronger than that for oxygen [9], excess binding of CO to Hb impairs oxygen transport to tissues, leading to CO poisoning.

Commercially developed CO sensors are mainly of the needle type. When they are inserted in the living body, invasion is inevitable; further, this sensor type is unsuitable for long-term monitoring. Because the amount of CO produced *in vivo* is infinitesimal, the sensor has higher sensitivity and biocompatibility. We applied the conformational change of Hb by CO binding as a CO gas sensor. It is well known that the difference in Hb spectra between oxy-Hb and deoxy-Hb is used in pulse oximetry because the spectra differ substantially. The spectrum change depends on allosteric change of Hb conformation: relaxed (R) state in oxy-Hb and tense (T) state in deoxy-Hb. CO-Hb, similar to oxy-Hb, assumes the R state, and therefore, the absorption spectrum of CO-Hb is similar to that of oxy-Hb. Reports have described hypoxia sensing with Hb *in vivo* [10] [11]. However, Hb has not been used as a CO sensor because distinction between the spectra of CO-Hb and oxy-Hb is technically difficult.

In this study, we developed a sensor sheet made with biocompatible materials for detecting CO based on Hb allostery. All Hb molecules in the sensor sheet are stabilized to the T state by inclusion of a reducing agent, and only when CO binds to Hb, the conformation changes to the R state (**Figure 1**). CO-releasing molecules (CORM) were used as a CO source to mimic living tissues, such as tumor tissues. We determined the sensitivity of the sensor sheet, and CO distribution was two-dimensionally (2D) imaged by scanning the absorption spectra.

2. Materials and Methods

2.1. Hb Preparation and CORM-Containing Gel as a CO Source

All experimental protocols were approved by the Animal Care Committee of Keio University School of Medicine. Fresh heparinized venous blood was obtained from male BALB/c mice. Erythrocytes were separated by centrifugation at 2500 rpm for 5 min. Plasma and buffy coat were removed, and erythrocytes were washed thrice using phosphate-buffered saline (PBS). The packed erythrocytes were diluted using 10 times their volume of distilled water in order to lyse them. After centrifugation at 10,000 g for 30 min, the supernatant, without membrane component, was sampled. Agarose powder (500 mg) was suspended in 500 ml of PBS and dissolved by

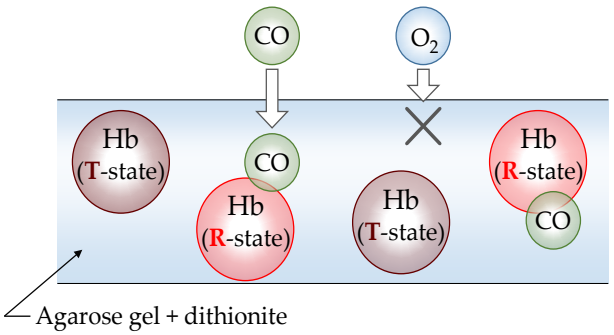

Figure 1. Schematic illustration of Hb-based CO sensor in gel. Conformation of Hb is stabilized by dithionite to the Tense (T) state and changed to the Relaxed (R) state only when CO binds to Hb.

heating in a microwave oven. When the temperature decreased to 50˚C, Hb solution and dithionite [12]-[14] were added to the agarose solution, and the mixture was stored at 4˚C until use. By mixing dithionite in the agarose gel, Hb conformation was stabilized to the T state until CO gas bound to the heme. For 2D imaging of CO-Hb, the Hb gel was made in the form of a sheet (thickness, 2.5 mm; diameter, 35 mm). For measurement of the absorption spectra, the Hb gel was placed in an optical cell. CORM was used as a low-level CO generation source. CORM was dissolved in agarose at various concentrations and placed on the Hb gel for spectrum analysis and CO imaging under a microscope.

2.2. Maintaining Hb Absorption Spectra and Defining CO-Hb Index

The absorption spectra of the Hb gel were measured using a spectrophotometer (Smart Spec Plus, Biorad). **Figure 2** shows the spectra of air-saturated oxy-Hb, dithionite-treated deoxy-Hb, and CORM-layered CO-Hb. As noted above, dithionite changed all oxy-Hb (R state) to deoxy-Hb (T state) so that only deoxy-Hb or CO-Hb could be present in the gel. The proportion of CO-Hb present in the gel was quantified by theratio of absorbance at 539 nm, the absorbance peak of CO-Hb, to that at 557 nm, the absorbance peak of deoxy-Hb, according to the following Formula (1).

$$\text{CO-Hb index} \equiv \frac{OD_{539}}{OD_{557}} \tag{1}$$

The Hb gels in optical cuvettes for spectral measurement are shown in **Figure 3**. Oxy-Hb and CO-Hb were bright red, indicating the R state, whereas deoxy-Hb was dark purple, indicating that Hb conformation

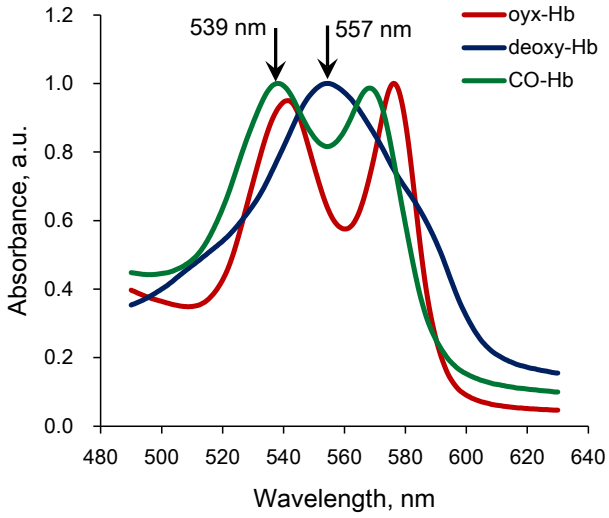

Figure 2. Absorption spectra of air-saturated oxy-Hb, deoxy-Hb, and CO-Hb extracted from mice erythrocytes. Absorption ratio at 539 and 557 nm was used for CO-Hb index.

Figure 3. Images of Hb gel in glass cells for spectrum analysis. From the left, air-saturated oxy-Hb, deoxy-Hb deoxygenated with dithionite, and CO-Hb covered with a layer of CORM-containing gel.

changed due to deoxyge nation and CO binding to Hb molecules.

2.3. Fabrication of the Hb Gel Sheet for CO-Hb Imaging

The Hb gel sheet was transformed into a disk (thickness, 2.5 mm; diameter, 35 mm)in a cell culture dish. CORM gel was set into circular sheets with a diameter of 3.5, 5.2, and 7.5 mm at a fixed CORM concentration of 10 mM. In a similar manner, gels were set using CORM concentrations of 5, 10, and 20 mM at a fixed diameter of 9 mm. These CO-releasing gel sheets were layered on the Hb gel sheet for 2D imaging of CO-Hb.

2.4. Optical Setup for CO Imaging on Sensor Film

The optical setup for imaging CO-Hb in the gel sheet is shown in **Figure 4**. The Hb gel was placed under an objective lens (20×) and irradiated with white light transmitted from an LED under the Hb gel. The light transmitted through the Hb sheet passed through the objective, and absorption spectra were obtained with a multi-channel CCD via a polychro meter (C5094, Hamamatsu Photonics). The CO-Hb index was calculated from the absorption spectra. The Hb gel sheet was scanned in 20 mm square with a spatial resolution of 0.5 mm using a X-Y stage installed in the microscope. The CORM-containing gel, as a source of CO gas, was placed on a Hb gel sheet for 15 min until scanning. Two sets of CO-releasing gel sheets were prepared as follows: samples with varying CORM concentrations of 5, 10, and 20 mM at a constant size of 9 mm, and samples with varying gel sizes of 3.5, 5.2, and 7.5 mm at a constant CORM concentration of 10 mM. The scanned CO-Hb index was imaged in pseudo color.

3. Results

3.1. Time Course Change in CO-Hb Index Depending on CORM Concentration

Figure 5 shows the time course change in CO-Hb index in an optical cell with a Hb concentration of 7.5%. CORM gels with varying concentrations were placed on the Hb gel in the optical cell at time 0. CO-Hb index increased in a short time with an increase in the concentration of CORM, but no change was observed in the absence of CORM. At a CORM concentration of 1.0 mM, CO-Hb index continued to increase over 3 h and stabilized after 10 h. However, at a low concentration of 0.3 mM, CO-Hb index did not reach CO-Hb index 1.2, owing to the complete release of CO molecules from CORM after 3 h. This experiment showed that the T state molecules bound minute amounts of CO gas and changed to the Rstate.CO-Hb index represents the total amount of CO molecules gradually released from CORM molecules, indicating that this Hb-gel sensor can sense a CO source that continuously and infinitesimally generates CO, such astumor tissues, in the living body.

Figure 6 shows the time course change in CO-Hb index with Hb concentration in the gel sheet at a fixed CORM concentration of 0.5 mM. The lower the Hb concentration in the gel, the more rapidly CO-Hb index responded and became saturated. At 7.5% Hb, the index continued to increase after 3 h. The Hb concentration corresponds to the number of acceptors for CO molecules; a higher concentration of Hb in the gel leads to

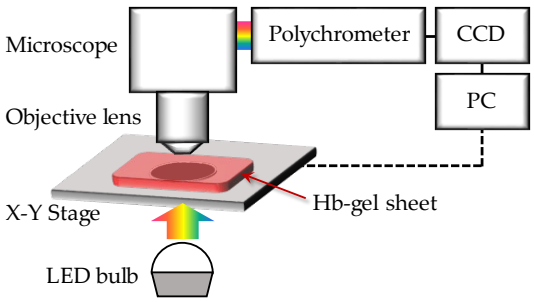

Figure 4. Microscope setup for 2D imaging of CO-Hb in the gel sheet. White light transmitted from an LED passed through the Hb gel sheet and was corrected with an objective lens. The spectrum was obtained using a polychrometer. X-Y stage was controlled to visualize 2D image of CO–Hb index.

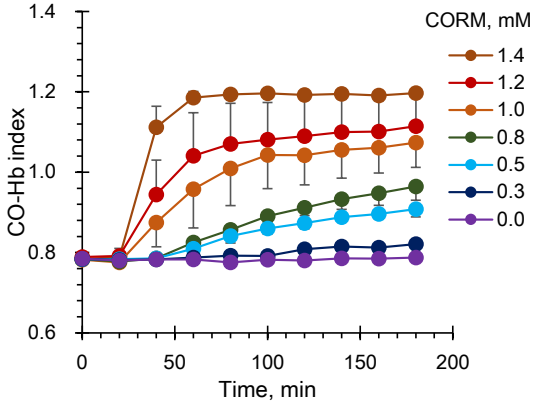

Figure 5. Time course change in CO-Hb index with CORM concentration in the layered gel on the Hb gel.

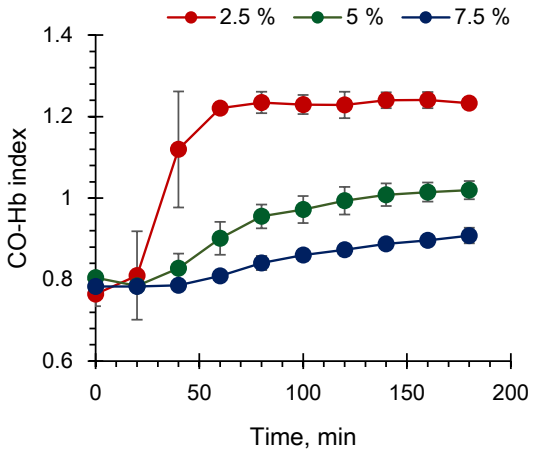

Figure 6. Time course change in CO-Hb index with Hb concentration in the Hb gel.

agradual increase in CO-Hb index with a constant CO binding rate. Therefore, the Hb concentration in the gel is a determinant of sensitivity of the gel sheet.

3.2. CO-Dependent Change in CO-Hb Index at Fixed Time Interval

Figure 7 shows the change in CO-Hb index with a CORM concentration at 5% on the Hb gel. CO-Hb index did not change in the absence of CORM, as shown in **Figure 5**. At 180 min, the index increased dose-dependently at a low CORM concentration, indicating that Hb in the gel sheet continually bound to the released CO. These results indicate that the Hb gel sheet can be applied to detect low levels of CO on long-term accumulation. Because Hb concentration determines the sensor sensitivity as shown in **Figure 6**, it is important to establish the Hb concentration required for measurement by considering the amounts of CO released from the target. CO-Hb index should be below 1.2 at the end point of measurement for accurate measurement.

3.3. Spatial Imaging of CO Concentration on Sensor Film

The 2D change in CO-Hb index was imaged by scanning the Hb gel sheet. A double-layered CORM-containing gel on the Hb gel was removed after 15 min and absorption spectra were obtained with the microscope system illustrated in **Figure 4**. **Figure 8(a)** shows a pseudo color image of CO-Hb index in the Hb gel sheet. CORM concentrations in the upper gels varied between 5, 10, and 20 mM with the same diameter (9 mm). Circular changes in the Hb index depending on CORM concentration were observed. Higher CORM doses resulted in an increase in the size, owing to the diffusion of CO gas in the Hb gel sheet. **Figure 8(b)** illustrates the size-de-

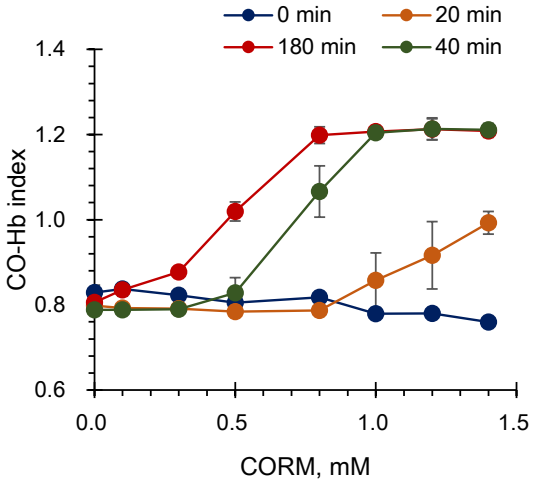

Figure 7. Relationship between CORM concentration and CORM index at the contact time of the Hb gel with CORM-containing gel.

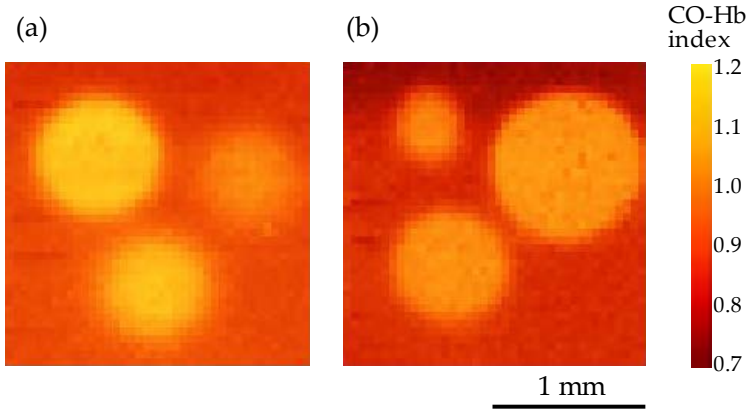

Figure 8. Visualization of CO-Hb index in the Hb gel sheet. Circular gels with a diameter of 9 mm at CORM concentration of 5, 10, and 20 mM were layered on the Hb gel sheet (a); Likewise, gels of diameter 3.5, 5.2 and 7.5 mm at a fixed concentration of CORM at 10 mM were imaged (b).

pendent change in CO-Hb index for 3.5, 5.2, and 7.5-mm gels at a constant CORM concentration. These results indicate that the Hb gel sheet can detect and image CO-releasing substrates with different sizes and concentrations.

4. Discussion

Here we developed a CO sensor based on Hb allostery. CO-Hb index derived from the ratio of Hb absorptions at 539 and 557 nm was effective for estimating CO concentrations. Although several types of gas sensors have been developed [15]-[17], in general, CO gas sensors have been limited to the needle type for bioinstrumentation. Because our sensor was basically composed of materials with a high biocompatibility, it can be implanted for long-term monitoring of CO generation *in vivo*. We used CORM as a small generator of CO for potential biometric applications. However, our Hb gel sheet can be applied for industrialor general use by increasing the Hb content in the gel.

The addition of a reducer to the gel removed oxygen molecules and stabilized Hb in the T state. An increase in the R state, equivalent to an increase in CO-Hb index, corresponds to acon formational change of Hb by binding CO, and not oxygen. Although the absorption spectra of oxy-Hb and CO-Hb are similar, the absorption peaks of CO-Hb shift toward shorter wavelengths compared with oxy-Hb [18]. The quantification of CO-Hb

using this shift has been described [19]. However, acquiring Hb spectra is required for estimating the amount of the shift, consuming time for measurement, and data processing. In particular, when CO-Hb is scanned for 2D imaging, absorption data should be processed at each point (pixel) in the scan area. In our method, because oxy-Hb was not present in the gel, only absorption at two wavelengths, those characterizing the R and T states, need to be measured. We used a polychrometer to obtain Hb spectra for CO imaging. However, CO-Hb index could be directly calculated using two photo detectors with filters for 539 and 557 nm, allowing simultaneous measurement. This method should be appropriate for fast scanning of CO-Hb in the gel sheet.

To remove oxygen and to change oxy-Hb to deoxy-Hb in the gel, sodium dithionite was used as a reducing agent [12]-[14], and all Hb was stabilized to the T state. However, dithionite does not durably remove oxygen and the effect fades with time. In addition, escape of dithionite from the gel could reduce the T state stabilization effect. We applied glucose oxidase and catalase system [20] to remove oxygen molecules, but glucose oxidaseis yellow, which affects absorption measurements (data not shown). Another method should be contrived to maintain the T state of Hb without absorption at 539 and 557 nm.

The sensitivity can be adjusted by changing Hb concentration in the gel sheet, as shown in **Figure 6**. If Hb concentration is lowered, CO-Hb index will increase at low CO levels, corresponding to enhanced sensitivity. However, in general, lower Hb concentrations lead to a reduced signal-to-noise ratio owing to low absorbance. Some type of noise reduction is required, e.g., by averaging signals. Our sensor sheet detects cumulative amounts of CO-Hb and not momentary absolute values. It should thus be suitable for CO detection in tissues that continuously generate minute amounts of CO over long periods, such astumor tissues.

Here we used Hb extracted from mouse blood by centrifugation and membrane removal. However, the extract contained not only Hb but also several ions and proteins, such as glycolytic enzymes, in erythrocytes. We supposed that molecules other than Hb were not in sufficient abundance to affect the absorption used for determining CO-Hb index. However, the dissociation curve of Hb shifted by binding 2,3-bisphosphoglycerate, an allosteric effector, so that purification could change the property of R–T transition by CO binding. In addition, the increase of content of met-Hb with repeated purification decreases sensitivity, indicating that the relationship between purification and sensitivity should be determined for future practical application. Finally, the durability of the sensor should be tested for long-term use because the Hb protein can denature or degrade with time.

5. Conclusion

We developed a CO sensor based on Hb allostery and successfully imaged CO-generating gels, mimicking CO sources such as tumor tissues in the living body. Further studies of Hb purification, quantification of CO, and durability of the gel sheet are needed for practical application. Because the sensor has a high biocompatibility, it may be expected to be useful for long-term monitoring *in vivo* as well as for other general uses.

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