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Compatibility of Nitric Oxide Release with Implantable Enzymatic Glucose Sensors Based on Osmium (III/II) Mediated Electrochemistry

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Abstract

The compatibility of nitric oxide (NO) release coatings with implantable enzymatic glucose sensors based on osmium (III/II) mediated electrochemical detection is examined for the first time. NO-releasing osmium-mediated glucose sensors are prepared using a *S*-nitrosothiol impregnated outer tubing and are tested *in vitro* in both phosphate buffer (pH 7.4) and within whole porcine blood. It is demonstrated that after 3 days of continuous NO release at or above physiological levels, there are no negative effects on the osmium mediated electrochemical currents. Indeed, such sensors maintain their functionality, sensitivity, and accuracy for detecting glucose levels in blood. The results suggest that improved performance of both intravascular and, potentially, subcutaneous Os(III/II) mediated glucose sensors may be realized by taking advantage of NO's well known anti-clotting, anti-inflammatory and antimicrobial properties.

Graphical abstract

Tight glycemic control is essential to prevent or reduce life-threatening hyper- and hypoglycemic events that can cause serious long-term complications for critically ill

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Notes

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Continuous measurement of glucose in the presence of 300 ppm nitric oxide; *in vitro* continuous measurement of glucose for 3 days in heparinized porcine whole blood plasma (MS word)

patients.¹ Therefore, the development of continuous glucose monitoring systems to quantitate blood glucose levels intravascularly (IV) would greatly enhance glycemic control and, ultimately, improve patient outcomes, especially within intensive care units.^{2–6} FDA approved electrochemical continuous glucose sensors (e.g., commercial Dexcom, Medtronic, and Abbott glucose sensor systems) rely on measurements being made within the subcutaneous interstitial fluid, not within the blood stream. In most cases, frequent calibrations (once or twice per day) are required, by using a blood glucometer test strip and a finger-prick, to update the calibration between blood levels and the sensor's output current. However, it is known that a significant lag time exists between interstitial and blood glucose levels (5–10 min). Further, devices implanted subcutaneously can suffer from a foreign body response (FBR), which can “wall off” the sensor, altering its sensitivity and potentially increasing the lag time to detect changes in blood glucose levels.^{7,8} Therefore, for patients in a hospital setting, implantation of continuous glucose sensing devices within the bloodstream would be convenient and advantageous.

To date, such continuous IV glucose sensors have not been successfully developed due, in part, to activation of platelets that can adhere to and form blood clots on the surface of the sensor. The presence of adhered, metabolically active platelets can change (lower) the local glucose levels at the surface of the device. To overcome this issue, many efforts have been made to reduce cell adhesion on the surface of implanted sensors and other IV devices by using various types of modified polymers,^{9–14} yet none have been successful.

Nitric oxide (NO) is endogenously produced from endothelial cells that line the inner walls of all healthy blood vessels and is known to prevent platelet activation and subsequent thrombosis, as well as serve as a potent antimicrobial/anti-biofilm agent.^{15–18} Therefore, one potential strategy to prevent thrombosis and infection associated with intravascular sensing devices is to utilize polymer coatings that release NO at physiological levels of *ca.* $0.5 \sim 4.0 \times 10^{-10} \text{ mol} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$. Indeed, Yan et al. developed needle-type amperometric glucose sensors based on hydrogen peroxide detection with an NO releasing polymer and demonstrated that such sensors exhibit more accurate glucose measurements, as well as reduced thrombus formation over an 8 h period compared to control sensors when implanted in the veins of rabbits.¹⁹ Soto et al also demonstrated the potential of NO release to enhance the analytical performance of subcutaneous glucose biosensors.²⁰ Further, NO release coatings have also been successfully employed to improve the *in vivo* analytical accuracy of intravascular oxygen sensor.^{21,22} However, in the case of glucose sensors based conventional hydrogen peroxide detection (oxidation), devices with NO-releasing coatings that exhibit higher NO fluxes (desirable for more antithrombotic and antimicrobial activity) exhibit larger background currents in absence of glucose. This is due to the oxidation of NO at +0.65 V on the underlying Pt/Ir working electrode of these sensors. Further, the level of this background current contribution decreases with time, as the NO flux decreases, thereby changing the low-end calibration curve for the sensor. Therefore, using a different glucose sensing method that requires a lower oxidation potential could further improve the compatibility of NO release coatings with implantable enzymatic glucose sensors.

In the 1990's, Heller and coworkers introduced the concept of a wired-enzyme electrode that is now utilized by Abbott Laboratory in their subcutaneous, continuous glucose-monitoring

system.²³ Such an enzyme electrode is based on “wiring” glucose oxidase (GOX) to an electron-transfer osmium ion mediator, Os(bpy)₂Cl^{+/2+} (osmium(II) complex with bipyridine), further complexed to poly(1-vinylimidazol) (PVI-Os). The potential required for oxidation of Os(II) to Os(III) is very low, < +400 mV vs. SCE, a potential that should avoid the additional background current from the oxidation of nitric oxide (to nitrate) at the inner working electrode, when NO release polymers are used to coat/contain the devices.

Herein, we demonstrate the compatibility of nitric oxide with implantable enzymatic glucose sensors based on the osmium (III/II) chemistry by fabricating NO-releasing osmium-mediated glucose sensors and testing them in both phosphate buffer (pH 7.4) and *in vitro* within whole porcine blood at 37 °C. It will be shown that amperometric glucose response is preserved in the presence of continuous NO release from the surrounding NO-releasing polymer. This suggests that NO does not interact in any way with the Os(III/II) redox center of Pt/Ir working electrode, and thus does not alter the amperometric signals of such glucose sensing devices.

Two fabrication methods were employed to prepare the two types of needle-type glucose sensors employed in this work. One (Fig. 1a) is based on the configuration reported by Bindra et al.,²⁴ Gifford et al.²⁵ and Yan et al.¹⁹ One μ L of 4.5 mg/mL PVI-Os (III) in methanol, 1 μ L of 0.9 mg/mL glucose oxidase (Type VII, from aspergillus niger) in a mix of methanol, and HEPES (1:1 wt%) and 1 μ L of 0.3 mg/mL poly(ethylene glycol) diglycidyl ether (PEGDE) were dropped sequentially into a 1 mm cavity created on the side of an insulated platinum-iridium wire (see Figure 1a). The sensor was then rinsed with water and allowed to dry for 1 h. Then, the sensor was dip-coated with a thin layer of Nafion to enhance selectivity over potential anionic interferences such as ascorbic and uric acid, and allowed to dry overnight in a 37 °C oven. The sensor was then loop-coated with ca. 1% (wt/vol) PurSil polymer solution in THF and dried for 8 h to remove any THF.

Figure 2 shows that the basic osmium-mediated glucose sensors (Fig. 1a) without any NO-releasing outer tubing exhibit stable amperometric responses to glucose in the range of 0–20 mM using an applied potential of only 200 mV between the Pt/Ir electrode and the wrapped Ag/AgCl reference electrode. As also shown, this response is quite stable for at least 5 days, when the sensor is stored and tested in phosphate buffer solution (PBS, pH 7.4) at 37 °C, with excellent linearity ($R^2 = 0.9963$) and reasonably fast response times (3–5 min). The sensitivity of glucose sensor slowly changed during storage in PBS at 37 °C over 5 consecutive days, which is typical for devices prepared with a PurSil (PU-based) outer coating as it swells by taking up water during the initial hydration period.^{24,26} The normalized sensitivity of the basic osmium-mediated glucose sensors (n=3) on day 1, 3 and 7 day are as follows: 117.4 and 118.4 % on Day 3 and 7, respectively. These values were calculated by dividing the sensitivity on each day by that on day 1.

To determine whether nitric oxide can cause additional background current to glucose sensors based on hydrogen peroxide detection (+0.65 V), but not to the sensors based on osmium-mediated chemistry (+0.2 V), both types of glucose sensors, were prepared without any NO-releasing outer tubing, and were placed in a bulk PBS solution (pH 7.4) with 0 mM glucose in the absence (purged with N₂) and the presence of 300 ppm NO. Notably, the

concentration of a saturated NO solution is reported to be 1.74 ± 0.03 mM (equivalent to 52.2 ppm).²⁷ As shown in Figure 3, when 300 ppm NO was introduced at the 10 min mark, the osmium-mediated glucose sensor exhibits no additional background current while the sensor based on hydrogen peroxide detection exhibits a significant increase in current (~ 5 nA) (equivalent to 4.1 mM glucose based on prior calibration curve for that sensor) due to electrochemical oxidation of NO. The result confirms that the application of lower redox potential (+0.2 V) can clearly help avoid the unnecessary additional background current from oxidation of NO at the surface of sensing area.

To determine whether nitric oxide has any adverse effects on the electron-transfer process during glucose measurements, the osmium-mediated glucose sensors prepared without any NO release outer tubing (Fig. 1a) were used to measure 14 mM glucose in bulk PBS solution (pH 7.4) while simultaneously purging the solution continuously with 300 ppm NO gas over a 25 h period. The osmium-mediated glucose sensor exhibits very stable and continuous measurement of 0 mM glucose (background current) between 0–3 h and 14 mM glucose between 3–25 h in both the absence (0–1 h) and presence (1–25 h) of 300 ppm NO (see Fig. S-1 in Supporting Information). Furthermore, not only did NO not provide any additional background current at the applied potential of 200 mV (as shown in Fig. S-1 between 1–3 h), but also the sensor exhibited relatively stable and continuous measurement of the 14 mM glucose in the presence of 300 ppm NO, more than the concentration of saturated NO in solution (52.2 ppm),²⁷ for 22 h ($< 4\%$ change in current over this period). These measurements match the calibration curve (N_2 -purged solution) obtained prior to the experiment (inset in Figure S-1). As a result, the osmium-mediated glucose sensor exhibits continuous glucose sensing capability in the presence of highly concentrated levels of NO within the test solution.

To fabricate the NO-releasing osmium-mediated glucose sensor (Fig. 1b), a Pt-Ir wire insulated with a Teflon coating was inserted into a commercial silicone tubing (Artec) that was impregnated with the NO donor, *S*-nitroso-*N*-acetylpenicillamine (SNAP), via a solvent swelling method using 125 mg/ml SNAP in THF.²⁸ Before insertion, the Teflon coating was coated with a layer of copper nanoparticles (Cu-NPs, 5 wt % and 40–60 nm) in Carbosil (2080A, DSM). Then, at the distal tip of the device, sequential layers of glucose oxidase, PVI-Os (III) and PEGDE, then Nafion and PurSil were applied with drying in between each layer (see Fig 1b). The analogous control sensors were prepared by only inserting the osmium-mediated glucose sensor (with the sensing area at the distal tip) into silicone tubing, but without the SNAP and Cu-NP layers.

As shown in Figure 4, the additional layer of Cu-NPs in Carbosil between Teflon coating on the Pt working electrode and SNAP-impregnated silicone tubing increased NO release flux. The Cu^+ ions, produced from oxidation of Cu^0 nanoparticles in the presence of moisture, catalyze the generation of NO from RSNOs such as SNAP.^{29–31} The SNAP-impregnated silicone tubing without the Cu-NP coatings exhibited lower NO fluxes compared to the Cu-NP based devices. The NO fluxes from SNAP with Cu-NPs correspond to the physiological levels ($0.5 - 4 \times 10^{-10} \text{ mol} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$) of NO released by endothelial cells that line the walls of all blood vessels.³² Nitric oxide released from the osmium-mediated glucose sensor

was measured in real-time via chemiluminescence using a Sievers Nitric Oxide Analyzer (NOA) 280.

As shown in Figure 5, although the NO-releasing glucose sensor exhibits slightly different glucose sensitivity than the control, the difference is not due to interference from NO, but is due to the fabrication process, as the amount of reagent (GOX, PVI-Os, and outer PU coating) deposited on sensing area dictates the current output for glucose measurements. Most importantly, continuous NO release from SNAP did not exhibit any negative effect on the osmium-mediated chemistry for glucose measurement over a 3 day period. The normalized sensitivity of NO-releasing and controls sensors (n=3) over 3 days are as follows: 109.1 and 86.4 % on Day 2 and 3 for NO-releasing sensors, respectively; 87.7 and 97.7 % on Day 2 and 3 for control sensors, respectively. The percentage was calculated from dividing the sensitivity on each day by that on day 1.

In vitro continuous glucose measurement in heparinized whole porcine blood (female, 120 kg, provided by Extracorporeal Life Support Laboratory at University of Michigan) was conducted with NO-releasing and control osmium-mediated glucose sensors for 3 consecutive days. On each day, glucose values were back-calculated using the calibration obtained prior to the *in vitro* blood testing. To confirm the accuracy of the glucose sensors, a commercial blood glucometer (Accu-Chek® Nano, Roche Diabetes Care) was used every 10 min to measure (in triplicate) the whole blood glucose level. This value was compared to the value obtained from the sensors using the prior calibration data. NO release was monitored for the three days via collecting NOA data for one hour each day, prior to glucose calibration (0–20 mM) in PBS (pH 7.4, 37 °C) and the *in vitro* continuous glucose measurements in porcine blood. The sensors were stored in PBS (pH 7.4) at 37 °C in the dark between measurements to ensure continuous NO release from SNAP.

As shown in Figure S-2 (Supplemental file), the intermittent glucometer glucose measurement values in whole blood are very comparable to the continuous glucose measurements obtained by the osmium-mediated glucose sensors with the pre-calibration made in PBS on each day. The discrepancies in glucose value between NO-releasing glucose sensor and glucometer observed at the 2 min mark may be due to the glucometer test strips having the standard accuracy of $\pm 15\%$ for measured glucose concentration. Nonetheless, it is clear that during 3 days of continuous NO release from the sensors, at or above physiological levels, there is no negative effects on the osmium-mediated wired enzyme electrode chemistry.

In summary, it has been shown that *in vivo* type amperometric glucose sensors prepared with the Os(III/II)-bipyridine-PVI complex do maintain continuous analytical functionality and measurement accuracy in the presence of nitric oxide. Further, there is no evidence that NO can be oxidized at the underlying Pt/Ir working electrode at the applied potential of 200 mV used to record the current response from these devices. In addition, there does not appear to be any direct interaction between the NO and the Os(III/II) redox center that inhibits electron transfer from the glucose oxidase enzyme and this redox site. While the ultimate focus of this effort is to eventually prepare useful IV glucose sensors, the use of NO release also has the potential to improve the performance (reduce drift, require less frequent

calibrations) of such wired enzyme electrodes that are employed for subcutaneous glucose sensing. This is because, as mentioned previously, it is well known that NO release can also greatly reduce the foreign body inflammatory response that occurs for devices placed in the subcutaneous space.^{33,34}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Designs of implantable (a) osmium-mediated glucose sensor and (b) NO-releasing osmium-mediated glucose sensor.

Figure 2. Amperometric response to glucose (0–20 mM) obtained with non-NO release Os(III/II)-mediated implantable glucose sensor (Fig. 1a) with applied potential of 200 mV on Days 1 – 5.

Figure 3.

A comparison of continuous current measurements for 0 mM glucose using a glucose sensor based on hydrogen peroxide detection (+0.65 V) and osmium-mediated chemistry (+0.2 V) in the absence and presence of 300 ppm NO purged into the solution at the 10 min mark. Current was normalized by subtracting the background current at 0 mM glucose for both sensors.

Figure 4. NO flux analysis of SNAP-based NO release glucose sensors prepared with and without Cu-NPs for 3 consecutive days (note: 1 flux unit = $1 \times 10^{-10} \text{ mol min}^{-1} \text{cm}^{-2}$). The error bars represent standard deviation for $n=3$.

Figure 5. Glucose calibrations (0–20 mM) with applied potential of 200 mV in PBS (pH 7.4, 37 °C) for 3 consecutive days. (a) NO-releasing glucose sensor; (b) control glucose sensor. (note 1 flux unit = $1 \times 10^{-10} \text{ mol} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$).