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REAL TIME AND *IN VIVO* MONITORING OF NITRIC OXIDE BY ELECTROCEHMICAL SENSORS- FROM DREAM TO REALITY

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1. ABSTRACT

Nitric oxide is a key intercellular messenger in the human and animal bodies. The identification of nitric oxide (NO) as the endothelium-derived relaxing factor (EDRF) has driven an enormous effort to further elucidate the chemistry, biology and therapeutic actions of this important molecule. It has found that nitric oxide is involved in many disease states such as such as chronic heart failure, stroke, impotent (erectile dysfunction). The bioactivity of nitric oxide intrinsically linked to its diffusion from its site production to the sites of action. Accurate reliable in real time detection of NO in various biological systems is therefore crucial to understanding its biological role. However, the instability of NO in aqueous solution and its high reactivity with other molecules can cause difficulties for its measurement depending on the detection method employed. Although a variety of methods have been described to measure NO in aqueous environments, it is now generally accepted that electrochemical (amperometric) detection using NO-specific electrodes is the most reliable and sensitive technique available for real-time *in situ* detection of NO. In 1992 the first commercial NO electrode-based amperometric detection system was developed by WPI. The system has been used successfully for a number of years in a wide range of research applications, both *in vitro* and *in vivo*. Recently, many new electrochemical nitric sensors have been invented and commercialized. Here we describe some of the background principles in NO sensors design, methodology and their applications.

2. INTRODUCTION

Nitric oxide (NO), is thought to play a key role in numerous physiological, pathological and pharmacological processes (1), identified as being responsible for the physiological actions of endothelium-derived relaxing factor EDRF (2), and numerous other medically important processes. NO was eventually declared "Molecule of the Year" in 1992 in the *Science* magazine. Subsequently NO has been shown to be involved in many more biomedical processes including; vasodilatation and as a molecular messenger (3); penile erection (4); neurotransmission (5-6); as a mediator in a wide range of both anti-tumor and anti-microbial activities (7, 8) and, inhibition of platelet aggregation (9). In recognition of their contribution to the field of NO research, three American scientists; Robert F. Furchgott, Louis J. Ignarro and Ferid Murad were jointly awarded the 1998 Nobel Prize for physiology and medicine. However, despite the many thousands of scientific publications over the last decade dedicated to the field of NO research, less than 10% actually involve direct measurement of NO.

In the majority of NO studies successful detection of NO requires the ability to detect NO in the presence of other potentially interfering species such as; nitrite, nitrate, dopamine, ascorbate, and L-arginine. It is also often necessary to be able to make such measurements, *in situ* and in real-time. Several techniques have been developed for the measurement of NO. These have been reviewed extensively elsewhere (10-14).

The most commonly used NO analysis methods include techniques such as chemiluminescence (15,16);

Griess method (17); paramagnetic resonance spectrometry (18); paramagnetic resonance imaging; spectrophotometry (19); and bioassay (20). However each of these methods has significant limitations when it comes to monitoring NO continuously and in real time. Other drawbacks include; poor sensitivity and the need for complex and often expensive experimental apparatus. The inability to measure NO *in situ* and in real time is probably the most significant shortfall. Consequently, none of these methods are able to provide the necessary quantitative data required in many NO research applications. Electrochemical (amperometric) detection of NO is currently the only sensitive technique available that is able to satisfy the real-time and *in vivo* measurement requirement of many NO related studies.

The first amperometric NO electrode used for direct measurement was described in 1990 (21). In 1992 WPI developed the first commercial NO sensor system called, the ISO-NO. Over subsequent years a range of highly specialized and sensitive NO electrodes have been developed offering detection limits for NO ranging from below 1 nM up to 100 µM (13). Most recently, a unique range of high sensitivity NO sensors based on a membrane coated activated carbon microelectrode have been developed by WPI. These electrodes exhibit superior performance during NO measurement and feature a detection limit of less than 0.5 nM NO.

3. ELECTROCHEMICAL DETECTION OF NO

Several electrochemical techniques can be employed to measure NO. One such technique is the amperometric method. This involves monitoring the very low redox current (e.g. pA's) produced by the oxidation of NO over time at a fixed (poise) voltage potential. The response time of this method is typically less than a few seconds, and coupled with its high sensitivity, it provides fast, quantitative measurement of very small changes of NO concentration. Alternative electrochemical techniques can also be employed to detect NO including; differential pulse voltammetry (DPV), differential normal pulse voltammetry (DNPV), linear scanning voltammetry (LSV), square wave voltammetry (SWV) and fast scan voltammetry (FSV). Typically these methods employ a classical 3-electrode configuration consisting of a working electrode, reference electrode, and a counter electrode. Scanning techniques, with the exception of fast scanning voltammetry, require approximately ten seconds for the voltammogram to be recorded and this therefore precludes their use in most NO research applications. Moreover, since scanning voltammetry-based. NO apparatus is not commercially available, investigators on nitric oxide typically rely on the use of a 2-electrode amperometric method to measure NO (as described below).

Amperometric detection of NO involves the oxidation of NO on the surface of an electrode and the measurement of the subsequent (redox) current generated. In principle the technique works as follows. When an electrochemical NO sensor (consisting of a working and reference electrode pair) is immersed in a solution containing NO and a positive potential of approximately 860 mV (vs. a Ag/AgCl reference electrode) is applied, NO will be oxidized at the working electrode surface producing a redox current. This oxidation proceeds via an electrochemical reaction followed by a chemical reaction. The electrochemical reaction is a one electron transfer from the NO molecule to the electrode, resulting in the formation of a cation, the nitrosonium ion:

$$NO - e \rightarrow NO^+$$

NO^+ is a relatively strong Lewis acid and in the presence of OH^-, it is converted into nitrite (NO_2^-):

Hence; $$NO^+ + OH^- \rightarrow HNO_2 \rightarrow H^+ + NO_2^-$$

Nitrite can then be further oxidized into nitrate.

The amount of NO oxidized is thus proportional to the current flow between the working and reference electrodes, which is measured by an NO meter. Typically the working electrode is covered with a gas-permeable NO-selective membrane, which also encloses an internal electrolyte. NO gas then diffuses through the membrane and internal electrolyte, and is oxidized at the surface of the working electrode generating the current.

The amount of redox current typically generated by the oxidation of NO in the biological system is typically extremely small (e.g. 1 pA to 10 pA). The design of an amperometric-based electrode NO detection system therefore requires extremely sensitive electronics and ultra low noise amplification circuitry. These measurement limitations were overcome by WPI with the development of the ISO-NO and ISO-NO Mark II NO meters. The ISO-NO Mark II employed a unique electrically isolated low noise circuit, this permitted measurement of redox currents as small as 0.1 pA. The design of the instrument also allowed measurement of NO to be performed without the need for special electrical screening, such as a Faraday cage. Recently, the world 's only fully integrated multiple channels electrochemical nitric oxide /free radical detection system, Apollo-4000 was developed. The detection system is an optically isolated multiple-channel nitric oxide/free radical analyzer designed specially for the detection nitric oxide and other free radicals such as oxygen, hydrogen peroxide, superoxide *et al.* (21)

4. ELECTROCHEMICAL NO SENSORS

Several different types of electrochemical NO sensors have been described over the years for use in a variety of measurement applications. The following section summarizes the main types.

4.1. Clark type NO sensors

The first described electrochemical NO sensor was based on a classical Clark electrode design, where NO was directly oxidized on the working surface of the electrode (22). The NO sensor comprised of a fine platinum wire and a separate silver wire, which were then inserted into a glass micropipette. The micropipette was then filled with 30 mM NaCl and 0.3 mM HCl and sealed at the tip with

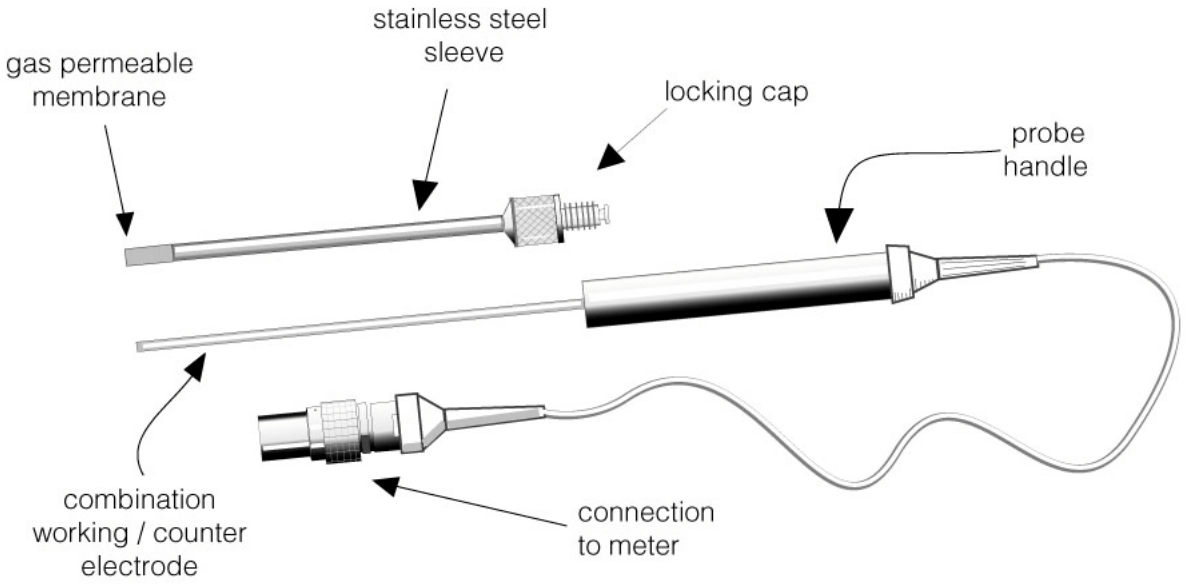

Figure 1. Illustration of WPI's ISO-NOP NO sensor.

a chloroprene rubber membrane. The platinum (working) electrode was positioned close to the surface of the membrane. The silver wire was then used as the reference-counter electrode. Although such electrodes could be used to measure NO, their inherent low sensitivity, narrow linear concentration measurement range and fragility rendered them unsuitable for most research applications. In 1992, utilizing the Clark type design WPI produced the first commercial electrochemical NO sensor (ISO-NOP) for use with their NO detection meter (ISO-NO). The ISO-NOP sensor consisted of a platinum wire disk working electrode and an Ag/AgCl reference electrode. Both electrodes were encased within a protective Faraday-shielded stainless steel sleeve. The tip of sleeve was covered with a NO-selective membrane and the sleeve itself contained an electrolyte. The rugged design of this sensor made it extremely convenient in many research applications and the sensor became widely used and established in numerous NO measurement research applications. The basic design of this type of NO sensor is illustrated in Figure 1.

4.2. Metal porphyrin modified NO sensors

Surface modified NO sensors incorporate an electrode surface that has been modified or treated in some way so as to increase the selectivity of the sensor for NO and promote catalytic oxidation of NO. Early designs of this type of NO sensor were based on carbon fiber electrodes coated with metal porphyrins (23-26). Nafion was then coated over the porphyrin layer. Although these electrodes were used to some extent successfully in several applications (27-29); subsequent studies have shown that carbon fibers modified using porphyrins lacking a coordinated metal can also detect NO with significant sensitivity, as can a bare carbon fiber without any porphyrin coating (30,31). The sensitivity and selectivity of the porphyrinic NO sensors varied significantly from electrode to electrode and depended not only on the potential at which NO oxidizes, but also on the surface effects axial ligation to the central metal in the porphyrin, modification/treatment procedure and other experimental variations. Furthermore, because the surface of the electrode remained in direct contact with the measurement medium a variety of biological species were shown to interfere (i.e. give false responses) during NO measurement. Other practical problems with these electrodes have limited their usefulness in most applications (32,33). Other metal porphyrins such as manganese porphyrin (34), ion porphyrin (35), cobalt porphyrin (36) were also employed as catalysts for construction of NO sensors.

4.3. Combination NO Sensors

During the mid to late 1990's a new range of combination NO sensors with tip diameters between from 7μm to 200 μm were developed by WPI (37,38). These sensors combined a carbon fiber working electrode with a separate integrated Ag/AgCl reference electrode. The resulting combination sensor was then coated with a proprietary gas permeable selective membrane mixture. Susceptibility to environmental noise was minimized by the use of a high performance Faraday-shielded layer applied to the sensor outer. The combination electrode design facilitated measurement, particularly in small volumes and confined spaces. Using proprietary diffusion membranes and a novel design, these sensors discriminated efficiently against a wide range of species known to cause interference during NO measurement (e.g. ascorbic acid, nitrite and dopamine). Later during 2000 a unique "microchip" combination sensor was developed by WPI (39). This sensor was based on a design that utilized laser lithographic to create a microelectrode array on a single silicon chip substrate. The resulting sensor (ISO-NOP-MC) exhibited an extremely low detection limit for NO (i.e. below 300 pM) and superior recording performance. Recently, WPI developed a novel combination NO 'nanosensor', which had a tip diameter of just 100 nm (40). More recently, a flexible non breakable NO microsensor was reported by Zhang's group (41). This sensor can be used for *in vivo* measurement.

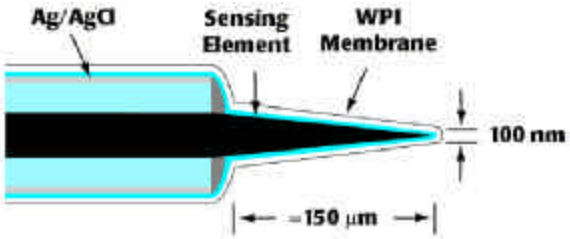

Figure 2. Illustration of WPI's ISO-NOPNM 100 nm combination NO nanosensor.

4.4. Other Sensor Types

Various other types of carbon fiber NO sensors that utilize a variety of different coatings have been described. Coatings used in these sensors include; conducting and non-conducting polymers (42-44); multiple membranes (45,46); ruthenium (47); iridium and palladium (48); heated-denatured Cytochrome C (49); nafion-CoII-1, 10-phenanthroline (50); ferrioxamine (51); microcoaxial microelectrode was reported for *in vivo* nitric oxide measurement (52); siloxane polymer (53); nafion and cellulose (54); Hb/phosphatidylcholin films (55); hemoglobin-DNA film (56,57), and ionic polymers and a-cyclodextrin (58). Meyerhoff'group descripted an improved plannar amperometric NO sensor based on platinized anode (59, 60) and its application for measurement of NO release from NO donors. Scheler *et al.* explored using myoglobin-clay modified electrode for NO detection (61). Kamei *et al.* fabricated NO sensing device for drug sceening (62). Indium hexacyanoferrate film-modified electrodes was used for NO detection by Casero *et al.* (63). Schuhmann' group (64) developed a device for *in situ* formation and scanning electrochemical microscopy assisted positioning of NO-sensors above human umbilical vein endothelial cells for the detection of nitric oxide release.

Unfortunately, despite the various novel approaches incorporated into these designs, none of the sensors have stood the test of time, due mostly to various practical difficulties and/or poor sensitivity/selectivity. Furthermore, the lack of any published data describing the use of these sensors in any biological research applications limits any conclusion that can be made on their individual performance.

5. CALIBRATION OF NO SENSORS

Routine calibration of an NO sensor is essential in order to ensure accurate experimental measurements. Three calibration techniques will be described in the following section, these have already been the subject of several reviews (10-13) and will therefore only be described in summary here. NO sensors are typically sensitive to temperature; accordingly calibration is usually best performed at the experimental temperature at which the measurements will be made.

5.1 Calibration based on chemical generation of NO

This method of calibration generates known concentrations of NO based on the reaction of nitrite with iodide in acid in accordance to the following equation:

$$2\ KNO_2 + 2\ KI + 2\ H_2SO_4 \rightarrow 2\ NO + I_2 + 2\ H_2O + 2\ K_2SO_4$$

The NO generated from the reaction is then used to calibrate the sensor. Since the conversion of NO_2^- to NO is stoichometric (and KI and H_2SO_4 are present in excess) the final concentration of NO generated is equal to the concentration of KNO_2 in the solution. Hence the concentration of NO can be easily calculated by simple dilution factors. Experiments have demonstrated that NO generated from this reaction will persist sufficiently long enough to calibrate an NO sensor. However, since the technique involves the use of a strong acid, which can damage the delicate selective membrane of most NO microsensors, it is only suitable for use with Clark type stainless steel encased NO sensors (e.g. ISO-NOP). Figure 3 illustrates the amperometric response of a 2.0 mm ISO-NOP sensor following exposure to increasing concentrations of NO. The sensor responds rapidly to NO and reaches steady state current within a few seconds. Data generated from Figure 3 is then used to construct a final calibration curve (Figure 3 inset). The calibration curve illustrates the good linearity that exists between NO concentration and the current produced by its oxidation.

5.2. Calibration using NO standard solution

This technique involves the production of an NO stock solution using a supply of compressed NO gas. One advantage of this method is that it allows an NO sensor to be calibrated in a similar environment in which the experimental measurements are to be made. However, the major drawback is that it requires a source of compressed NO gas and since NO gas is toxic the whole procedure must be performed in a fume hood.

The method can be summarized as follows. A vacutainer is first filled with 10ml deionized water and agitated ultrasonically for 10 minutes. Purified argon is then be passed through an alkaline pyrogallol (5% w/v) solution to scavenge any traces of oxygen before being purged through the deionized water solution for 30 min. NO stock solution is prepared by bubbling compressed NO gas through the argon-treated water for 30 min (66). The NO gas is first purified by passing it through 5% pyrogallol solution in saturated potassium hydroxide (to remove oxygen) and 10%(w/v) potassium hydroxide (to remove all other nitrogen oxides). The resultant concentration of saturated NO in the water is 2 mM at 22 ^{0}C (67). This can be confirmed further by a photometric method based on the conversion of oxyhemoglobin to methemoglobin in the presence of NO (68). NO standard solutions can then be freshly prepared by serial dilution of saturated NO solution with oxygen-free deionized water prior to each experiment.

5.3. Calibration based on decomposition of SNAP

In this method S-nitroso-N- acetyl-D, L-penicillamine (SNAP) is decomposed to NO in solution in the presence of a catalyst, Cu(I). The resultant NO generated can then be used to calibrate the sensor. The reaction proceeds in accordance to the following reaction:

$$2RSNO \rightarrow 2\ NO + RS\text{-}SR$$

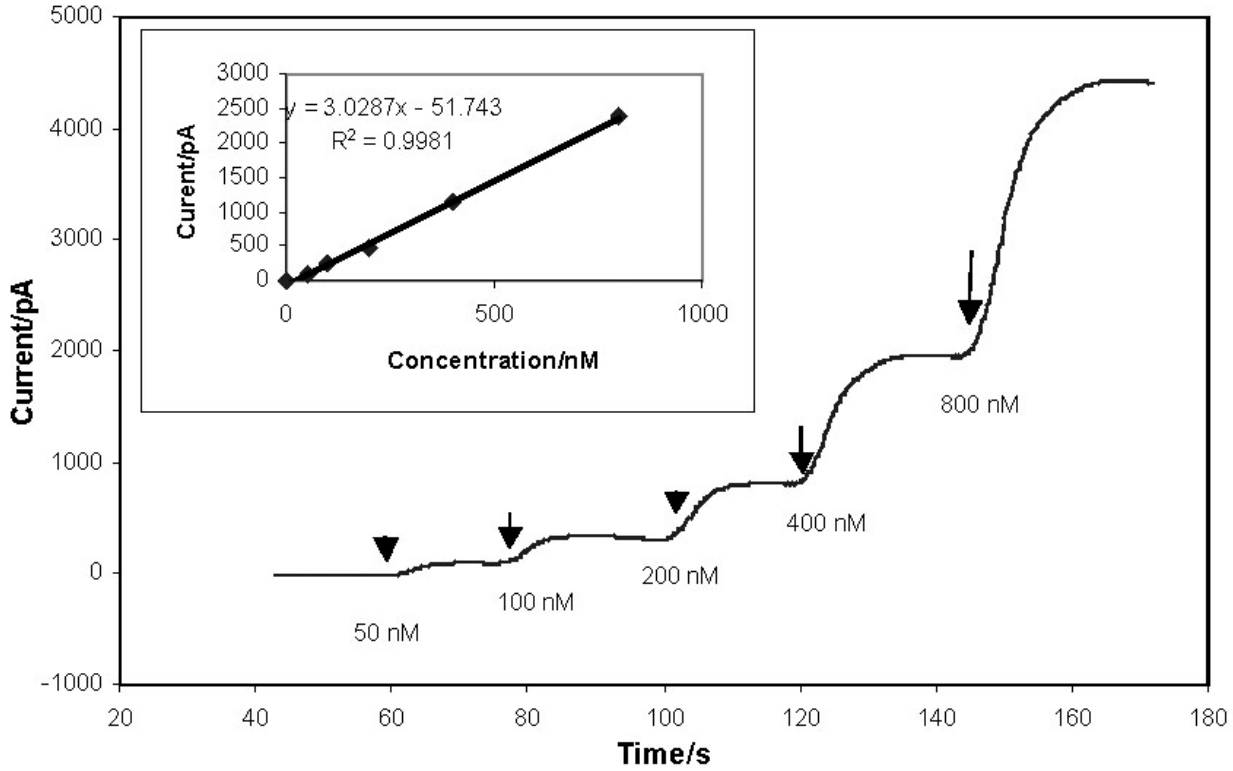

Figure 3. Response of WPI's Clark type NO sensor to increasing concentration of NO, inset shows the resulting calibration.

The stoichiometry of the reaction dictates that the final concentration of generated NO will be equal to the concentration of SNAP in the solution. The method can be summarized as follows. Saturated cuprous chloride solution is first prepared by adding 150 mg CuCl to 500 ml distilled deoxygenated water. The distilled water is then deoxygenated, by purging with pure nitrogen or argon gas for 15 min. The final saturated CuCl solution will have a concentration of approximately 2.4 mM at room temperature. The solution is light sensitive and must therefore be kept in the dark prior to use.

The SNAP solution is then prepared separately as follows. EDTA (5 mg) is dissolved in 250 ml of HPLC pure water (HPLC grade, Sigma) and then adjusted to pH 9.0 using 0.1 M NaOH. The solution is then deoxygenated using the method described above. SNAP (5.6 mg) is then added to the solution and molarity of the SNAP solution (SNAP f.w. = 220.3) can then be calculated. SNAP solution is also extremely sensitive to light and temperature and must therefore be stored in the dark and in a refrigerator until required. Under these conditions and in the presence of the cheating reagent (EDTA), the decomposition of SNAP occurs extremely slowly. This allows the solution to be used to calibrate NO probes throughout the day. In practice actual calibration is performed by placing an NO sensor into a vial containing a measured amount of the CuCl solution. Known volumes of the SNAP stock solution are then injected into the vial and the final concentration of NO can be calculated using dilution factors.

The concentration of SNAP in the stock solution is calculated as follows:

$$[C] = [A*W/(M*V)]\ 1000$$

Where C = concentration of SNAP in micromoles (μM); A = purity of SNAP; W = weight of SNAP in milligrams (mg); and V = volume of the solution in liters (l).

If SNAP purity, for example, is 98.5% then the concentration of SNAP is calculated as:

$$[C] = [98.5\% \text{ X } 5.6/ (220.3\text{X}0.25)] \text{ x}1000 = 100.1\ \mu M$$

Figure 4 shows a typical calibration curve generated using an NO micro sensor and the SNAP method described.

6. CHARACTERIZATION OF NO SENSORS

NO sensors can be characterized in terms of sensitivity, detection limit, selectivity, stability, linear range, and response time. Sensitivity as discussed here, refers to the gradient/slope of response of the sensor observed when plotting the redox current against NO concentration. In most applications detection limit and selectivity are usually the most important requirements.

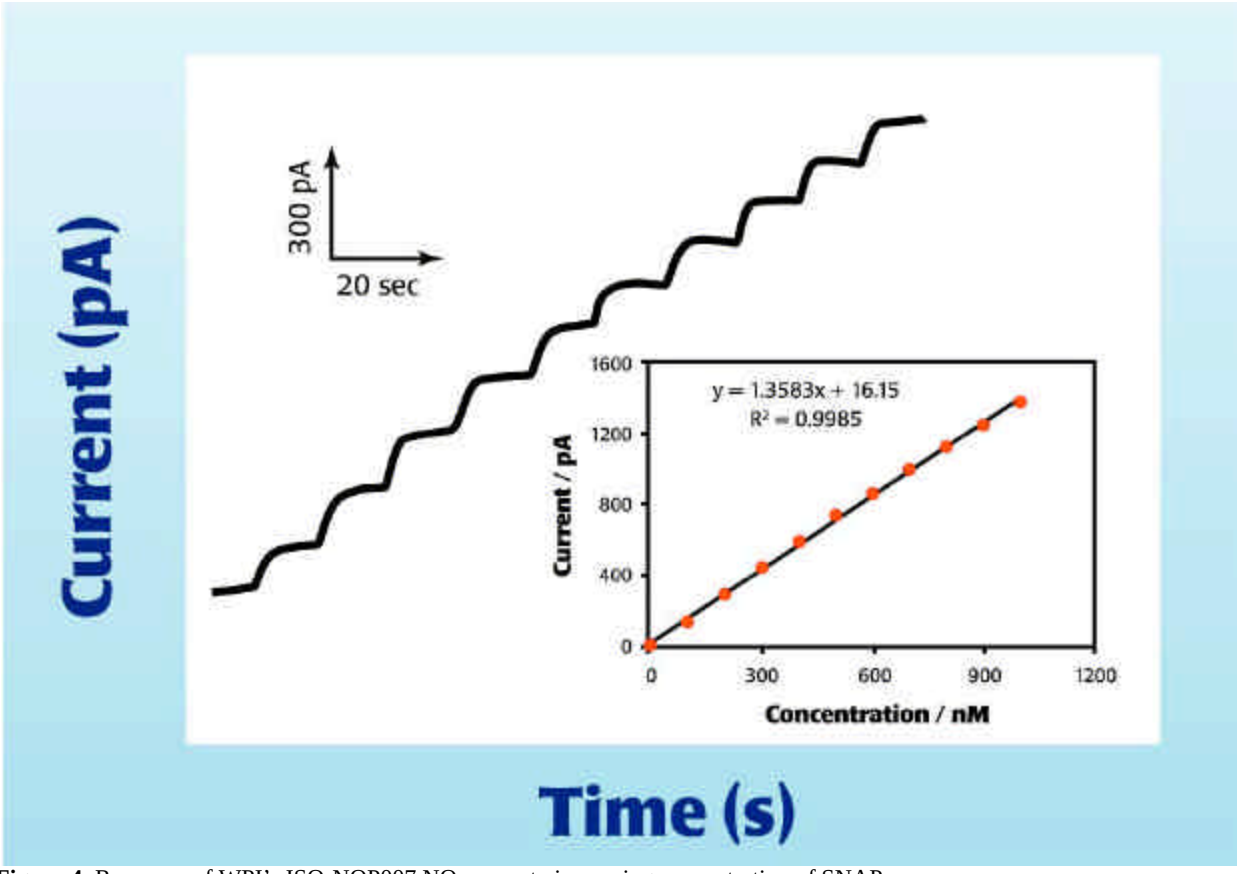

Figure 4. Response of WPI's ISO-NOP007 NO sensor to increasing concentration of SNAP.

6.1. Sensitivity and detection limit

The sensitivity of an NO sensor depends largely on the reactive surface area of the sensor and the electrode materials used in the design. An electrode with a small surface area will generally have a lower sensitivity compared to one with a larger surface area. Although sensitivity of the sensor is clearly important, end detection limit is often more important to the investigator. High sensitivity of a sensor does not necessarily equate to a low detection limit. For example, a highly sensitive NO sensor may have a high background noise level, which at a high NO concentration may not be a problem. However at lower NO concentrations measurement can be hindered by excessive noise. Accordingly, in evaluating the performance of an NO sensor, the ultimate detection limit is usually more critical than the sensitivity. Fortunately, most commercial NO sensors can detect NO at levels of 1 nM or less and are therefore well suited for the majority of research applications.

6.2. Selectivity

An NO sensor is practically useless unless it is immune to interference from other species likely to be present in the measurement environment. Selectivity is usually controlled by both the (redox) poise voltage (i.e. the voltage between the working and reference electrode) and the selective membrane used to coat the sensor. Many species present in a biological matrix are easily oxidized at the poise voltage employed to detect NO (i.e. +860 mV vs Ag/AgCl). For example, monoamines such as dopamine (DA), 5-hydroxytryptamine (5-HT), and norepinephrine (NE), as well as their primary metabolites, can be oxidized at 0.3V (and higher) vs Ag/AgCl. . Ascorbic acid can be oxidized at 0.4 V (and higher). A Clark type NO sensor (e.g. ISO-NOP) is covered with a gas permeable membrane, hence the selectivity of such sensors in biological samples is extremely good. With other types of NO sensors selectivity is usually achieved by coating the sensor surface with Nafion and other gas permeable membranes. Nafion is widely used to eliminate interference caused by anions (e.g. ascorbic acid, nitrite etc.) during measurement of catecholamine species. When used for NO detection the negatively charged Nafion layer can stabilize NO^+ formed upon the oxidation of NO and prevent a complicated pattern of reactions that could lead to the formation of nitrite and nitrate. However, the main drawback with Nafion is that it does not eliminate interference from cationic molecules such as dopamine, serotonin, epinephrine and other catecholamines. Consequently, selectivity of the conventional Nafion coated NO sensors is very poor. Nafion coated NO sensors also exhibit other undesirable characteristics including; unstable background current, continuous drift in the base line, and extended polarization requirements. These problems significantly limit the use of Nafion coated carbon fiber electrodes for measurement of NO. During the late 1990's WPI developed

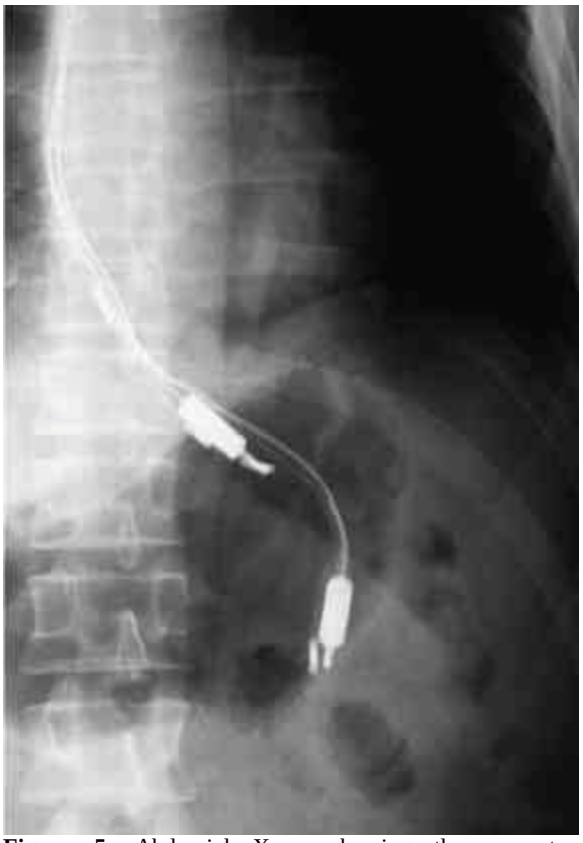

Figure 5. Abdomial X-ray showing the apparatus consisting 2 nitric oxide sensor, 4-channel pH catheter, and Teflon nasogastric tube.

of unique multi-layered proprietary membrane configuration. NO sensors coated with this membrane exhibited increased selectivity and sensitivity for NO, and moreover were shown to be immune from interference caused from a wide range of potentially interfering species (38).

7. APPLICATION OF NO SENSORS

Several hundreds of research papers have been published over the last 10 years in which amperometric NO sensors have been used to detect NO in a variety of biological research applications both *in vitro* and *in vivo* (69,70). Such applications include NO measurements in; guinea pig isolated and intact hearts (71-73); human monocyte cells (74); human endothelial cells (75); granulocytes, invertebrate ganglia and immunocytes (76,77); choroidal endothelial cells (78); peripheral blood (79); Levine, *et al,* first reported real-time profiling of kidney tubular fluid nitric oxide concentration *in vivo* (80,81); There are many reports of other application of NO sensors to detect NO in rat kidney (82); brain (83), and mitochondria (84,85). In a recent example to illustrate a typical use for an NO electrode, Hurst & Clark (86) describe how to monitor NO produced from human vascular endothelial cells (HUVEC). More recently investigators using a specially customized ISO-NOP have described a technique for real-time monitoring of NO production in the stomach and esophagus of human patients (87). In this method the patient first swallowed two NO electrodes (see Figure 5), which were then withdrawn slowly at 1 cm increments every 2 minutes. The investigators were then able to establish a profile of NO concentration in the upper gastrointestinal tract.

In addition to their various uses in animal tissues, NO sensors have also been used to monitor NO produced by plants (88-90). Simonsen *et al.* successfully simultaneously measured relaxation and nitric oxide concentration in rat superior artery using a NO microsensor (91). This elegant work, in Figure 6, shows simultaneous measurement of force(upper traces) and NO concentration(lower traces) in an endothelium intact(+E) segment of rat superior mesenteric artery and same segment after mechanical endothelial cell removal(-E). Based on this work, the same group reported measurement of NO and hyporeactivity in rat superior mesenteric artery (92), and measurement of NO released in artery from hypertensive rat (93) and isolated human small arteries (94).

Kellogg *et al.* reported measurement of NO under human skin using a flexible 200 um NO micreoelectrode (95). It demonstrated that NO concentration increases in the cutaneous interstitial space during heat stress in humans. Figure 7 illustrates protocol and results for laser-Doppler flowmetry and amperometric NO electrode measurement under human skin. Same group also explored detection of NO during reactive hyperemia in human skin (96).

Recently, Millar studied real time monitoring of NO in bovine perfused eye trabecular network using a NO microelectrode (97). Nuttal *et al.* pioneered in research on nitric oxide in ear employing NO microelectrode (98, 99). Abu-Soud *et al.* (100) studied Myeloperoxidase up-regulates the catalytic activity of inducible nitric oxide synthase by preventing nitric oxide feedback inhibition. Gladwin's group used microchip NO sensor to reveal cell free hemoglobin limits nitric oxide bioavailability in sickle-cell disease (101). Interestingly, some NO sensors not only can be used to detect dissolved NO in solution, but also can be employed to detect NO in gas phase (102). Different applications need different NO sensors. Table 1 lists some commercial available NO sensors from WPI and their specifications and theirs applications.

8. CONCLUSION

The use of the electrochemical NO sensor provides an elegant and convenient way to detect NO in real time in biological samples. Currently they provide the only means by which to measure NO continuously, accurately and directly within tissues. The increasing acceptance of such sensors and their diversity of use in many NO research applications will help to further current understanding of the various clinical roles of this interesting and ubiquitous molecule. Continual improvements being made to NO sensor design and technology will facilitate these studies for the foreseeable future.

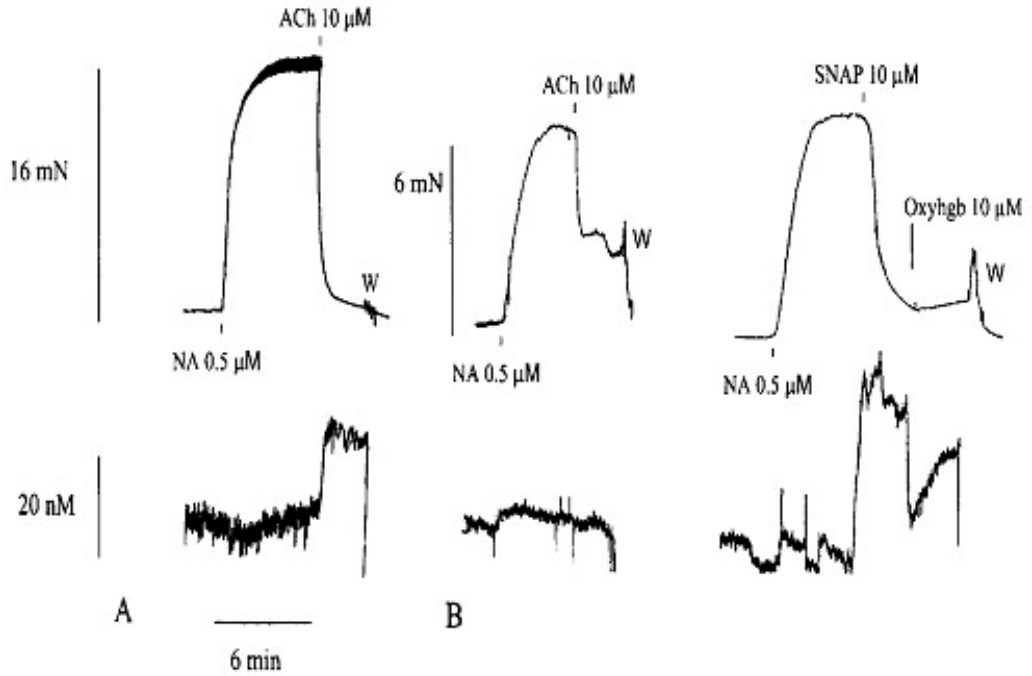

Figure 6. The effect of mechanical endothelial cell removal on simultaneously obtained relaxation and increase in NO concentration Simultaneous measurements of force(upper traces) and NO concentration(lower traces) in an endothelium intact(+E) segment of rat superior mesenteric artery contracted with 0.5 µM noradrenaline(NA) and relaxed with either 10 µM acetylcholine(Ach) (A), or 10 µM SNAP (B), and the lack of relaxation to Ach, but relaxation and increases in NO induced by SNAP in the same segment after mechanical endothelial cell removal(-E) (C). the traces are representive of 4 experiments. W, whashout.

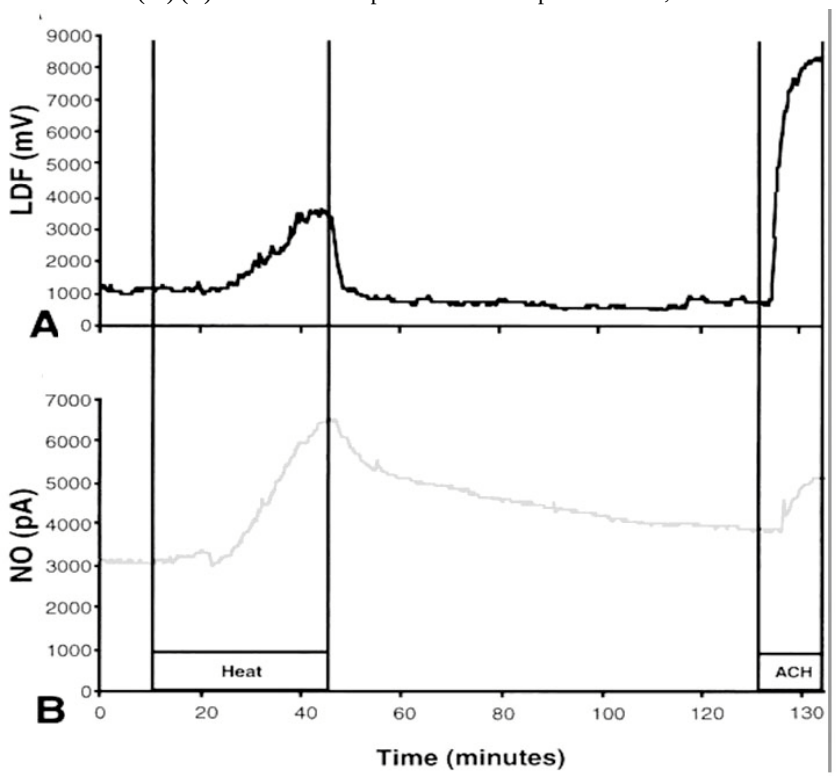

Figure 7. Illustration of protocol and results for laser-doppler flowmetry(LDF) and amperometric nitric oxide (NO) electrode measurement from 1 subject. The protocol began with a normothermic control period, which was followed by whole body heating to induce heat stress and thus activate the cutaneous vasodilators system. After heat stress, subjects were cooled and ACh was administered by intradermal microdialysis to confirm the ability of the amperometric electrode to measure NO concentrations.

Table 1. Nitric oxide sensors application guide

Sensor Name	**ISO-NOPMC**	**ISO-NOP**	**ISO-NOP30**	**ISO-NOP007**	**ISO-NOPNM**	**ISO-NOPF100/200**
Sensor Diameter	4mm	2mm	30μm	7μm	100nm	100μm /200μm
Detection Limit	300pM	1nM	1nM	3nM	5nM	100pM
Drift	None	None	None	None	None	None
Physiological Interference	None	None	None	None	None	None
Meter Resolution	0.1pA	0.1pA	0.1pA	0.1pA	0.1pA	0.1pA
Sensitivity	15-20pA/nM	1-3pA/nM	2-5pA/nM	0.5-1.5pA/nM	0.1pA/nM	20-30pA/nM
Applications	Cell culture	Cell culture	*In vivo*	*In vivo*	Single cell	*In vivo* tissue
		NO-2, NO-3	Microvessels	Microtubular		

9. ACKNOWLEDGMENT

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