Electrochemical detection of nitric oxide and S-nitrosothiols in biological systems: past, present & future

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Manuscript Details

Manuscript number	COELEC_2018_39
Title	Electrochemical detection of nitric oxide and S-nitrosothiols in biological systems: past, present & future
Short title	Electrochemical detection of nitric oxide and S-nitrosothiols
Article type	Review article

Abstract

Electrochemical detection of nitric oxide using different electrode materials and strategies exploded after the discovery of nitric oxide as important biological messenger. S-nitrosothiols (RSNOs), which result from interaction of NO with peptides and proteins, were shown to be important pools of NO that interfere in different physiological and pathological conditions. This lead to development of several decomposition methods to detect RSNOs electrochemically. This mini-review summarizes the beginning and the current investigations in electrochemical methods to detect NO and RSNOs. Indeed, it describes the latest trends to detect NO and RSNO using microfluidic technologies coupled to electrochemistry and discuss the future of NO and RSNOs detection.

Keywords	S-nitrosothiols; nitric oxide; electrochemical detection; ultramicroelectrodes; microfluidics; microchip capillary electrophoresis; nanoparticles; nanomaterials

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Suggested reviewers	Frederic Lemaître, Tebello Nyokong, Stéphane ARBAULT

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highlights:

- Electrochemical detection of nitric oxide using ultramicroelectrodes and the methods envisaged to enhance its selectivity and sensitivity
- RSNO decomposition methods in order to indirectly quantify RSNOs by electrochemical methods in biological fluids are summarized
- Detection of NO and of RSNO inside microfluidic devices is the new trend

Electrochemical detection of nitric oxide and S-nitrosothiols in biological systems: past, present & future

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Research highlights:

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Abstract :

Electrochemical detection of nitric oxide using different electrode materials and strategies exploded after the discovery of nitric oxide as important biological messenger. S-nitrosothiols (RSNOs), which result from interaction of NO with peptides and proteins, were shown to be important pools of NO that interfere in different physiological and pathological conditions. This lead to development of several decomposition methods to detect RSNOs electrochemically. This mini-review summarizes the beginning and the current investigations in electrochemical methods to detect NO and RSNOs. Indeed, it describes the latest trends to detect NO and RSNO using microfluidic technologies coupled to electrochemistry and discuss the future of NO and RSNOs detection.

Electrochemical detection of NO

Nitric oxide, NO was first characterized in 1987 as endothelial-derived relaxing factor and it was reported that it is produced by endothelial cells in blood vessels and diffuses to the adjacent smooth muscles to cause vasodilatation** [1]. Since this initial report that resulted in *R. F. Furchgott* , *L. J. Ignarro* and *F. Murad* receiving the Nobel Prize in Physiology and Medicine in 1998, there has been an explosion of research activity showing that NO release occurs not only from endothelial cells but also from neuronal [2], tumoral [3] and immune system cells [4] etc. The huge and intense research activities in these fields have resulted in more than 268 000 papers being published in the literature during the last 20 years. NO is a free radical that reacts very fast with oxygen, peroxides, O_2-radicals (superoxide $O_2^{\bullet-}$), metallic ions and metalloproteins [5]. This explains its fleeting existence and extremely low concentrations in biological systems. Its total free concentration in physiological conditions has been established recently in a range to be 0.1 µM down to 5 nM, which is orders of magnitude lower than once though a decade ago [6].

Several strategies have been proposed for NO detection. The only ones that allow direct, real time, label free and *in vivo* detection of NO are those based on the electrochemical detection of NO using ultramicroelectrodes (UMEs) , electrodes having one dimension less than 25 µm **[7-44]. Electrochemical NO-sensors based on UMEs offer: *(i)* good selectivity factors > 100;

(ii) good sensitivity (down to the nanomolar range); *(iii)* fast response (within the millisecond scale time); *(iv)* long-term stability (over 1-2 hours); *(v)* non-destructive technique in close proximity to the site of release (single cells, organelles). Several groups have worked on the detection of NO itself which can be oxidized or reduced depending on the potential utilized. It can undergo mild cathodic reduction by gaining one electron at potentials from -0.5 to -1.4 V *vs* Ag / AgCl. However, the detection of NO through its reduction suffers from severe limitations due to the presence of O_2 which is a major interfering because it is more easily reduced than NO. Direct electro-oxidation of NO is the most used approach for its detection. NO oxidizes at potentials higher than 0.8 V *vs* Ag / AgCl. This leads to selectivity problems because a lot of biologically relevant molecules such as nitrite, dopamine, and urea are oxidized at such high potentials.

The electrode materials usually employed are platinum** [7], platinum alloy (90% Pt, 10% Ir) [9], gold** [10], glassy carbon [18] and carbon fibers [12]. The electrode material and surface characteristics affect the potential at which NO is oxidized, the selectivity, the sensitivity, the signal stability and the quality of the ensuing analytical measurements [16,**19,30,38]. For example, the platinization of the platinum electrode or carbon nanoelectrode surface gives faster electron transfer and lowers NO reduction [31,32] or oxidation [33] potentials, respectively. Recently, modification of glassy-carbon electrodes with graphene-gold nanocomposite [34] or elaborating a 3D nanoporous gold microelectrode [35] improved the performances of the sensors. Govindhan et al. [36] summarized the recent advances in platinum-based nanomaterials for amelioration of sensitivity and selectivity of NO detection by changing the electronic exchange surface properties. To overcome the problems of selectivity two main approaches have been developed through the use of membranes selective to NO and / or the use of electrocatalytic moieties that decrease the potential of NO oxidation.

The first “NO sensor” in brain tissue was Shibuki’s electrode** [7] (**Figure 1a**). The author succeeded in detecting NO by modifying of the Clark’s electrode that was developed for O_2. The gas permeable membrane used was wax printed and sealed twicely with chloroperene rubber. This approach improved the selectivity against nitrite but lacked reproducibility. Although this sensor allows the very first studies of *in vivo* NO, its response time (*ca.* 1 s) and the fluctuations of the background signals make it useless for most biomedical applications.

The second approach of elaborating NO-sensor iss based on the immobilization of NO-permeable membranes directly on the metal without the use of internal solution (**Figure 1b**). Such an electrode is amenable to miniaturization and uses a multilayer membranes that permits selectivity against several possible biological interferences [*16,**19,30,38]. Indeed, several kinds of membranes have been deposited on electrodes. Anionic and cationic membranes act by electrostatic repulsion improving selectivity against interferences. Bedioui et al.** [10] used for the first time microelectrodes of gold coated by Nafion for detection of NO. The linearity of the calibration curve was between 10-100 µM. Other membranes were evaluated, including polycarbazole, polydimethylsiloxane, polystyrene (PS), fluorinated xerogel, polytetrafluoroethylene (PTFE) and o-phenylenediamine (o-PD) [*16,**19,30,31,38]. Multilayered polymers have also be used to ensure the impermeability to the largest amount possible of analytes except NO. A compromise should be found between selectivity and sensitivity since thicker membranes lead to lower sensitivity. Among the various methods of depositing membranes on the surface of the electrodes, electropolymerisation represents an elegant method since it permits controlling the thickness of film and covering small irregular spaces* [16]. The monomers usually used are eugenol, phenol, aniline, o-PD.

The third approach involves the use of an electrocatalyst (metallo-porphyrins or metallo-phtalocyanines) to improve the electron transfer inducing a negative shift of the oxidation potential by 0.15 V and an increase in sensitivity by *ca* 1.5-3 times [**19,**39-41] (**Figure 1c**). The electrocatalyst layer is deposited directly on the metal and other layers (size or charge excrusion) can be added to provide the sensor with more selectivity. A fourth rarely used approach consists in the use of a composite material made from catalyst and permselective membrane [38].

In all cases the performances of the prepared sensors depend on several parameters such as *(i)* the electrode material and the conditions of polymerization that affect the properties of a electrochemically prepared polymer modified electrode* [17], *(ii)* the potential necessary for NO detection which depends on the nature of the substrate, the electrocatalytic properties of the membrane and on the surface roughness [30] and *(iii)* the electrochemical techniques which are usually simple amperometry, pulsed chronamperometry, differential normal pulse amperometry and differential normal pulse voltamperometry* [16].

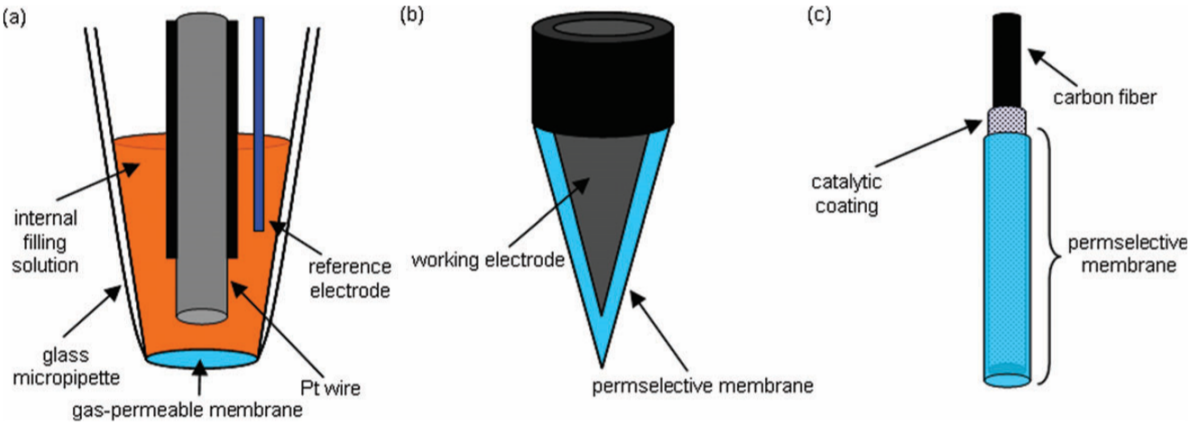

Figure 1: General types of electrochemical NO sensors (a) Clark's type electrode, (b) whole solid type electrode and (c) composite electrode. Adapted from [30]

Electrochemical detection of S-nitrosothiols

In order to be transported and stored in biological fluids, NO binds to the sulfhydryl groups of peptides and proteins forming S-nitrosothiols that play important roles in several physiological functions **[45-52] and physiopathological events [53-58]. They exist in biological media at concentrations that vary between tenth of nanomolar to less than ten micromolar **[59-61] and there is no gold standard method to determine their biological concentrations. They can be directly detected if the RS-NO bond remains intact or otherwise indirectly determined [62,63]. Indirect detection methods are based on a two-step protocol: decomposition of the RS-NO bond followed by the detection of the decomposition products (NO, nitrite or thionyl moiety) using electrochemical, spectrophotometric, or fluorescent methods, biotin switch methods or chemiluminiscence assays [31,**59,64-67] **(figure 2)**. Electrochemical methods represent direct, real time, and label-free detection approach that can be used for *in vivo* applications [31,*60]. RSNOs can be decomposed through different pathways [68] such as metal cation catalysis *[69], ascorbic acid [70], heat [69,71], infrared, ultraviolet [72-74] or visible light [46,72,75,76]. However, they mainly lead to partial and non-reproducible decomposition that can be detrimental for accurate detection. Furthermore the decomposition processes can be multiple: homolytic cleavage giving rise to the formation of unstable $NO^{\bullet}$ and $RS^{\bullet}$ that can lead to nitrite and other end-products, or heterolytic cleavage leading to RS^{-} and NO^{+} which rapidly forms nitrite (**figure 2**).

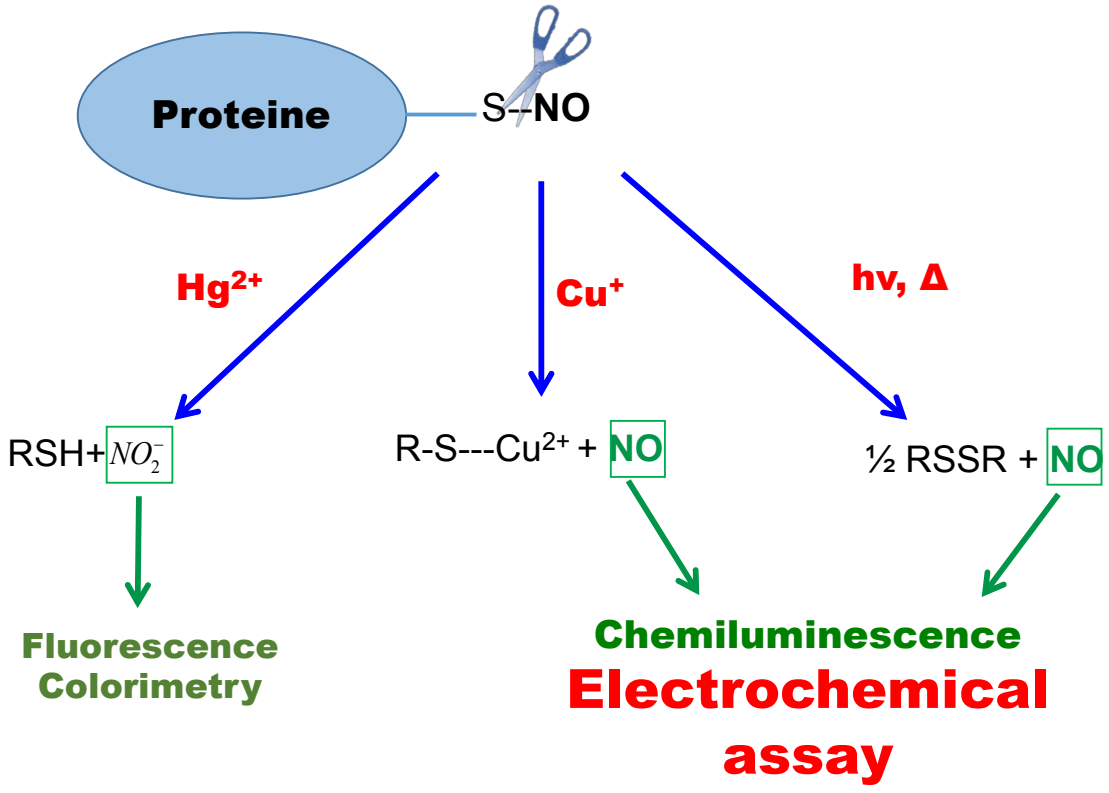

Figure 2: General scheme for S-nitrosothiols (RSNOs) indirect quantitation. Decomposition is done using mercuric (II) ion (Hg^{2+}), cuprous ions (Cu^{+}), heat (Δ) or light (hv). This decomposition is followed, depending on the nascent product, by adequate detection method (fluorescence, colorimetry, chemiluminescence, or electrochemistry)..

Electrochemistry can be implemented for RSNOs detection through the determination of NO released upon their decomposition. Only one study reported on the direct RSNOs detection thanks to their reduction at gold and glassy carbon electrodes in acidic medium at pH 4 [77]. Very little application of RSNOs electrochemical reduction (between -0.6 and -0.9 V *vs* Ag / AgCl) is found in literature, probably due to the possible interferences of O_2 and other molecules and to the low sensitivity of the method. Thus, the most used strategy consists in chemical decomposition of RSNOs into NO or nitrite and then the detection of the resulting molecules by electrochemistry. The decomposition is accomplished mostly by Cu^{+} catalyst. Several methods have been used (**Table 1**) *(i)* Cu^{+} can be added directly using CuCl , *(ii)* Cu^{2+} can be added with a reducing agent such as thiol or ascorbic acid to give Cu^{+} *[78] or *(iii)* Cu metal can be oxidized to produce Cu^{+}. For example, Meyerhoff et al. [79-**82] have used a strategy where the catalyst is immobilized in a polymeric membrane near to the NO sensor. The catalysts used were copper, organoselenium and organotelluride nanoparticles. Recently, a new

method was developed by Baldim et al. to analyze RSNO electrochemically after decomposition by gold nanoparticles *[83]. Light decomposition and electrochemical detection of NO have also been used to analyze RSNOs [76,**84].

Table 1: Different methods of decomposition by copper and other organometals to detect RSNOs. Adapted from [*60,76,*78,*83]

RSNOs decomposition	RSNOs sensor configuration	E (V/ref)	LOD	RSNO tested	Sensor lifetime	Interfering compounds tested	Biological samples
Catalyst: $Cu(NO_3)_2$ + thiol (L-cysteine or glutathione)	ISO-NO WPI	0.8 V	≈ 50 nM	GSNO, SNAP, S-nitrosated bovine serum albumin	n.d.	Nitrosomorpholine (no response up to 1 nM), N-nitroso-N-methylurea (no response up to 1 mM), Isoamylnitrite (no response up to 0.7 mM), Nitroglycerol (no response up to 4 mM), NO_2^- (no response up to 50 μM)	–
Catalyst: CuCl	ISO-NO Mark II WPI: carbon fiber (=100 nm diameter) / Nafion / WPI membrane	0.865 V	≈ 10 nM	GSNO, SNAP, AlbSNO	n.d.	NO_2^- (no response at 50 μM), AA (no response at 50 μM), L-Arg (no response up to 100 μM), DA (no response at 10 μM), $NH_{3(g)}$ (no response at 1 μM), $CO_{2(g)}$ (no response at 1 μM), $CO_{(g)}$ (no response at 1 μM)	–
Catalyst: CuCl	Pt disk (200 μM diameter) / poly-Cu(II)TAPc / Nafion	DPV	≈ 4 nM	GSNO	n.d.	NO_2^- (no response at 10 μM), UA (no response at 100 μM), AA (no response at 100 μM), 5-hydroxyindole-3-acetic acid (no response at 10 μM), 3,4-dihydroxyphenylacetic acid (no response at 10 μM), DA (peak at 0.3 V above 1 μM), Epinephrine (above 1 μM, peak observed at 0.3 V), 5-hydroxytyptamine (above 1 μM, peak observed at 0.3 V)	Whole blood
Catalyst: Cu(II)-ligand complex or Cu(II)phosphate or Cu(0) particles as the copper source + ascorbate	Platinized Pt disk (250 μMdiameter) / PTFE / Copper-based catalytic membrane	0.75 V	n.d.	SNAP, SNAC, CysNO, GSNO, AlbSNO	10 days	NO_2^- (no response from 0.1 to 100 μM)	Whole blood
Catalyst: $CuSO_4$ + GSH	UME Platinum (25 µm)	0.8 V	100 nM	GSNO	1 day	nitrite	–
Catalyst: organoselenium	Platinized Pt disk (250 μMdiameter) / PTFE / organoselenium catalytic hydrogel	0.75 V	<0.1 μM	SNAC, SNAP, SPA, CysNO, GSNO, AlbSNO	10 days	n.d.	Whole blood
Catalyst: organotelluride	Platinized Pt disk (250 μMdiameter) / PTFE / organotelluride based hydrogel	0.75 V	<0.1 μM	SNAP, GSNO, CySNO, AlbSNO	>1 month	n.d.	Whole blood
Catalyst: organoselenium	Platinized Pt disk (250 μMdiameter) / PTFE / organoselenium	0.75 V	<20 μM	GSNO, CySNO, AlbSNO	10 days	NO_2^-: sensitivity ratio $S(NO_2^-)/S(NO) = 10^{-6}$, AA: sensitivity ratio $S(NO)/S(AA) = 10^{-6}$, N-nitroso-1-proline sensitivity ratio: S(N-	Whole blood

						nitrosoproline)/S(NO)= 10^{-4}, NH_3/NH_4^+	
Catalyst: electrochemically oxidized Cu(0) in the presence of ascorbate	Micrometric ring-disc: central disc (50 μM diameter) = Cu(0), ring = NO sensor (gold / poly-eugenol / polyphenol)	0.70 V	n.d.	GSNO	n.d.	NO_2^- (sensitivity ratio: 9x10-4), H_2O_2 (sensitivity ratio: $3.9x10^{-2}$), AA (sensitivity ratio: $1.8x10^{-2}$)	GSNO in serum
Catalyst: gold nanoparticles	UME Platinum (25 µm)	0.8 V	100 nM	GSNO and total RSNOs in plasma	1 day	nitrite	Total RSNOs in human plasma
Catalyst: visible light	Xerogel-modified platinum disk (2 mm) electrode	0.8 V	CySNO (40 nM) GSNO (30 nM) AlbSNo (0.42 µM)	CySNO, GSNO, AlbSNO		nitrite	Low molecular weight RSNOs in Porcine plasma

Microfluidic devices with electrochemical detection for analysis of NO and RSNOs : current prospects and their future development

Microfluidic devices permit the use of significantly reduced sample volumes, improving amenability to clinical analysis [*85,86]. Bedioui et al. **[87] was the first to elaborate an amperometric fluidic microchip array for the detection of NO. Later on several researchers have detected NO in microfluidic devices using amperometry [88-90]. Most of these NO detections were done for NO produced from cells cultured inside the microfluidic device. Only Hunter et al [88] have detected NO in a spiked wound fluid and in whole blood. Gunasekara et al. *[91] used microchip electrophoresis with amperometric detection for the study of the generation of NO by NO-donor molecules such as NONOate salts. They separated NO from nitrite and NONOate in less than 1 min. To the best of our knowledge, only one study analyzed total RSNOs in microfluidic devices with electrochemical detection **[84]. No separation step was performed and the decomposition duration was quite long (100 s). The use of a microfluidic device permitted more complete sample irradiation and thus higher conversion of RSNOs to NO after a specific time. Detection methods, other than electrochemistry, were applied to detect RSNOs after their separation [92-94]. Introducing electrochemical detection instead of fluorescence detection after RSNOs separation sill offer more simple, specific, and portable devices.

Concluding remarks

Evolution of NO detection in biological media using UME is still under investigation mainly in the variation of electrodes surface composition (nanoparticles and nanocomposites) and in

membrane's permselective and catalytic properties. In case of NO bound to peptides and proteins in form of RSNO analysis, advance has been made mainly in decomposition pathways such as copper catalysis and gold nanoparticles techniques. Microfluidics is the new trend in biomedical applications. Few studies were done about the detection of RSNO and NO in microfluidic devices despite its importance in lowering sample volume and improving sensitivity due to confined diffusion volumes. Elaborating a point of care devices as diagnostic tool to separate then detect electrochemically a mixture of RSNO's and NO is still a challenge to be resolved in the coming years

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