Review Article

Novel Insights into the Electrochemical Detection of Nitric Oxide in Biological Systems

(nitric oxide / electrochemical detector / biological systems / carbon fibre)

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Abstract. In recent years, microsensor technologies have made a rapid expansion into different fields of physical sciences, engineering, and biomedicine. For analyses of various biomolecules, novel sensors and detection platforms in the electrochemical field have been reported recently. The most important applications based on microelectromechanical systems dramatically reduce the need of manipulation steps with samples, while improving data quality and quantitative capabilities. This is also the case of a special class of electrochemical sensors that allow direct, real-time and non-invasive measurements of nitric oxide, whose determination is crucial for the purposes of basic research, as well as of preclinical and clinical studies. Therefore, this minireview will focus on the description of recent discoveries in the electrochemical determination of nitric oxide, released in different *in vitro* systems.

Nitric oxide as an important regulatory molecule in the organism

Among the molecules that are constantly in the focus of scientific interest is nitric oxide (NO), whose biological and clinical importance lies in its fundamental role in the regulation of vascular and immune functions (Bogdan 2001; Fostermann et al., 2006; Lundberg et al., 2008; Pacher et al., 2007). Importantly, serious human diseases, e.g. systemic and pulmonary hypertension, hypercholesterolaemia, diabetes, and heart failure are accompanied by significant reduction of NO bioavailability in the organism, which probably results from the multifactorial alteration of immune and vascular endothelial functions.

NO plays an important role in many diverse processes, including vasodilatation, immune responses, neurotransmission, and adhesion of platelets and leucocytes to the endothelium (Bogdan 2001; Fostermann et al., 2006). It is synthesized by the enzyme nitric oxide synthase (NOS). There are three different types of NOS: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS). Endothelial and neuronal NOS are constitutively expressed at low levels in a variety of cell types and their activities are dynamically regulated by Ca^{2+} and calmodulin. On the other hand, iNOS is highly inducible in macrophages by bacterial endotoxin and/or Th1 cytokines (e.g. interferon γ), but once expressed it is constitutively active (Mayer and Hemmens, 1997; Bogdan 2001; Krejcova et al., 2009).

As it was mentioned above, down-regulation of NO-dependent processes occurs early in the course of human vascular disease and it is a contributing factor to defective endothelial vasodilator function. Generally, it is accepted that the majority of functional defects of vascular endothelium are associated with impaired eNOS and iNOS activity, which is followed by reduced total NO generation. This reduction of NO formation is associated with increased production of circulating levels of reactive oxygen species (ROS) and impaired endothelial uptake of L-arginine (the only known substrate for NO production) (Mayer and Hemmens, 1997). It is believed that these events could represent the step in the process of endothelial dysfunction in small vessels that evolves through large vessel disease to organ dysfunction and premature cardiovascular morbidity and mortality (Bogdan, 2001; Fostermann et al., 2006; Mayer and Hemmens, 1997; Pacher et al., 2007). Therefore, in order to evaluate the cellular function and bioavailability of NO, direct measurements of its concentration

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Abbreviations: ACh – acetylcholine, CaI – calcium ionophore, eNOS – endothelial NOS, HUVEC – human umbilical vein endothelial cell, iNOS – inducible NOS, LPS – lipopolysaccharide, nNOS – neuronal NOS, NO – nitric oxide, NOS – nitric oxide synthase, ROS – reactive oxygen species.

would be of great value and use in both basic and clinical research.

Detection of NO production in biological systems

NO, which is a free radical, reacts very rapidly and easily with oxygen, peroxides, $O_2^{\cdot-}$ radicals and metal compounds (e.g. metalloproteins). At the same time, the concentration of NO in a free form under physiological conditions has been established to be in nanomolar range (Bogdan 2001; Bryan and Grisham, 2007; Kavdia et al., 2012; Prochazkova et al., 2011). Therefore, there is still a considerable problem when attempting its detection and quantification in biological systems. Most of the methods currently available for detection of NO (e.g. UV-visible spectroscopy, fluorescence, electron paramagnetic resonance spectroscopy) are indirect and some of them are known to be prone to severe interferences and artefacts. They rely on the measurements of secondary species such as nitrite and nitrate or NO-adducts (Bryan and Grisham, 2007). Their disadvantages range from the lack of sensitivity or specificity to interference from the factors commonly present in biological systems. Despite that, several techniques have been used to study the endothelial function or bioavailability of NO within the endothelium. The most commonly used physiology-based method for determination of NO production by endothelial cells is represented by organ chamber experiments evaluating NO-dependent vasodilation. However, there are still many uncertainties remaining in relation to the bioavailability of NO in these systems (Papezikova et al., 2013; Klinke et al., 2014).

Electrochemical sensors as a promising methodological approach to direct measurement of NO

The only known strategy that allows direct detection of NO is based on the use of electrochemical sensors, which build a bridge between the powerful analytical methods and the recognition process of the biological specificity. Through the use of the electrochemical sensors, both the kinetics of NO formation and its corresponding concentration range can be quantified, allowing us to obtain very fruitful information about its bioavailability. Indeed, the electrode design and fabrication are now reaching a very high level of sophistication, which actively contributes to promoting the use of electrochemical techniques for *in vivo*, *ex vivo*, as well as *in vitro* determination of NO (Tanaka and Sackmann, 2005; Borgmann et al., 2006; Barbosa et al., 2008; Pekarova et al., 2009a; Crespi et al., 2010). They offer the following characteristics: good selectivity toward NO and efficient discrimination against other species, good sensitivity, fast response, long-term stability, small size to offer non-invasive and non-destructive close proximity to the site of NO release (single cells, organelles) and ease of handling (Oni et al., 2004; Castillo et al., 2005, Chang et al., 2005; Tanaka and Sackmann, 2005; Borgmann et al., 2006; Hrbac et al., 2007; Pekarova et al., 2009a).

Electrochemical sensors can be classified into amperometric, potentiometric or conductometric sensors based on whether the current, potential or resistance is being measured. In the case of amperometric sensors, which are the most relevant devices for the task of NO quantification, the current is measured during an electrochemical reaction (oxidation or reduction) between the analyte of interest and the electrode surface subjected to appropriate potential (de Vooys et al., 2004; Lang et al., 2013).

Basically, there are two types of amperometric electrochemical sensors, widely used for determination of NO in biological systems. The first type of electrochemical NO detectors are membrane electrodes, which are analogous to the Clark O_2 electrode system, where NO diffuses through the selective membrane into the internal compartment of the sensor, where it undergoes the electron reaction (Millar et al., 2003; de Vooys et al., 2004). Their main adverse problems are insufficient long-term stability and reliability associated with incorporation of liquid electrolytes, lifetime and cycle-time issues due to the small amount of consumable materials (e.g. electrode components such as Ag/AgCl). Secondly, non-specific reactions taking place between the electroactive impurities on the surface and the sample also limit the sensitivity of these sensors (Millar et al., 2003; de Vooys et al., 2004).

The second type of electrochemical NO detectors is represented by a very promising group of carbon fibre microelectrodes, which are used to be coated with ultrathin NO-selective layers that can solve the problem of their lower selectivity against NO (de Vooys et al., 2004; Castillo et al., 2005; Chang et al., 2005; Hrbac et al., 2007; Pekarova et al., 2009a). Importantly, they enable real-time measurements of NO production with a time-response in milliseconds and very high sensitivity (< 1 nmol.dm^{-3} NO) in both *in vitro* and *in vivo* systems (Zhang et al., 2002; Hurst and Clark, 2003; Hrbac et al., 2007; Barbosa et al., 2008; Pekarova et al., 2009a; Crespi et al., 2010). However, there are possible complications that could cause problems during the *in vitro* measurements: (a) the precise positioning of the microelectrodes and their fragility, (b) the sensitivity of microelectrodes to changes in pH, temperature, as well as cultivation media interference levels (Zhang et al., 2002; Hurst and Clark, 2003; Barbosa et al., 2008; Pekarova et al., 2009a). Therefore, it is important to critically control these influences during the measurements. On the other hand, the changes in pH and the presence of different bioactive molecules, which could affect detection of NO under *in vivo* or *ex vivo* conditions, are most likely associated with development of pathophysiological states described in the first chapter (Bogdan, 2001; Fostermann et al., 2006; Pacher et al., 2007; Barbosa et

al., 2008; Lundberg et al., 2008; Crespi et al., 2010; Rudolph et al., 2012).

Modified carbon-based electrodes for detection of NO in *in vitro* systems

As it has been mentioned above, direct measurement of NO production using electrochemical sensors offers a valuable tool for evaluation of NO production and bioavailability during different pathophysiological conditions. Moreover, recent findings show that the use of human isolated cells has great potential for NO production screening for the purposes of basic research (Borgmann et al., 2006; Diring et al., 2013). Prospective research topics include the changes in the L-arginine availability for the NOS enzyme, the NOS expression/structure, NO signalling, and destruction of NO by other ROS. In our group, this methodological approach was effectively used and improved in several *in vitro* and *ex vivo* models within the last years (Hrbac et al., 2007; Kralova et al., 2008, 2009; Papezikova et al., 2008, 2009, 2013; Pekarova et al., 2009a, b, 2011; Ambrozova et al., 2011; Jakubec et al., 2012). We focused on electrochemical detection of NO production using the carbon fibre microelectrodes coated with the Nafion layer (Hrbac et al., 2007; Papezikova et al., 2008, 2009; Pekarova et al., 2009a, 2013a, b). The basic performance of the NO sensors was reported elsewhere (Hrbac et al., 2007).

In our studies we used two different types of cells, known to be in the focus of scientific interest when considering the NO production: macrophages and endothelial cells. First, we detected NO production in murine macrophages induced by bacterial lipopolysaccharide (LPS) (Pekarova et al., 2009a). A constant temperature (37 °C) was kept during the time of the experiments by using a measurement chamber (Fig. 1). The NO production was recorded for 45 h, which confirmed the stability of carbon fibre microelectrodes during a long period. These results proved that modified carbon-based microsensors are able to measure the dynamic changes in NO production in activated immune cells (Pekarova et al., 2009a).

The most important experiments were performed with human endothelial cells, which are known to release low but biologically relevant levels of NO (Forstermann et al., 2006). We found that electrochemical microsensors (Hrbac et al., 2007; Pekarova et al., 2009a) were able to detect the changes in NO production from human umbilical vein endothelial cells (HUVECs) stimulated with calcium ionophore (CaI) and acetylcholine (ACh) (Fig. 1). After the signal stabilization, the current response proportional to the instantaneous level of ACh, CaI-derived NO release was recorded for the period of 15–20 min (Papezikova et al., 2009, 2013). The NO production was inhibited by a specific inhibitor, L-NG-nitroarginine methyl ester. It turned out that the modified carbon fibre microelectrodes are crucial for monitoring NO production from endothelial cells, because other methods failed to record the dynamic changes in its production. Moreover, in most of the indirect methods (based on Griess reaction or fluorochromes) the signal from NO was below their detection limits (Pekarova et al., 2009a; Papezikova et al., 2013).

Conclusions

Based on the results described above, we conclude that the direct electrochemical method employing modified carbon fibres is very useful for measurement of NO production in different *in vitro* models. Importantly, this methodological approach allows obtaining unique results. This approach might have a great potential in the field of biology, biochemistry, as well as medicine for identification and characterization of the significant effects of biologically relevant drugs on the NO production (prediction of their effects in humans), as well as drug discovery and validation of compounds and their targets, when testing the efficiency of specific modulators of NO production in different *in vitro* models.

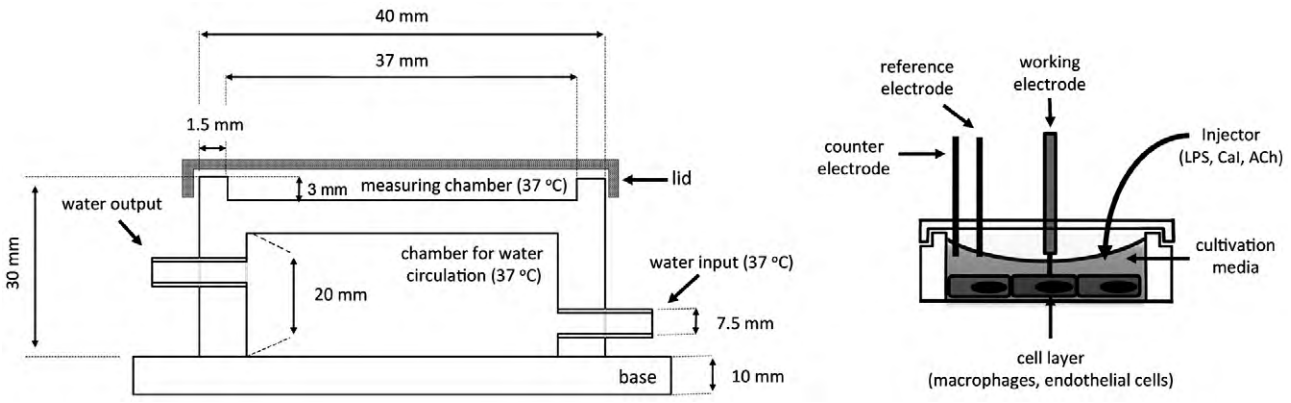

Fig. 1. Scheme and design of the measurement chamber for detection of NO production in *in vitro* models. The NO production is detected under stable temperature (37 °C) in a specific measuring chamber. The electrochemical cell consists of a three-electrode system: working electrode (carbon fibre), counter electrode (platinum), reference electrode (silver), and injector for application of LPS, CaI, ACh. Cells (as the source for NO production) are cultivated at the bottom of the electrochemical cell in the presence of cultivation media.

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