Nitric Oxide Analysis in Biology and Biomedicine. Modern Trends and Overlook

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Abstract:- Given presentation is a brief review of current state of currently used physico chemical methods of analysis of nitric oxide: optical spectroscopy, fluorescence, chemiluminescence, and electrochemistry. Other less frequently employed approaches include mass spectrometry, X-ray photoelectron spectroscopy, quantum cascade infrared laser spectroscopy, mechanotronics quartz crystal microbalance, and spin trapping also have been mentioned. The attention will be focused on recently proposed methods for real time monitoring nitric oxide dynamics in biological systems based on using supermolecules: fluorophore-nitroxide (FNMA), Fluorescence inductive-resonance method of a (FIRMA), and fluorescence spin exchange (FSEMA) methods of analysis

Key-words:- nitric oxide, optics, fluorescence, chemiluminescence electrochemistry, supermolecules, energy transfer, spin exchang.

1 Introduction

Nitric oxide (NO) is essential in a significant number of vital normal and pathological physiological processes. The scope of importance of nitric oxide spans from normal numerous biological and physiological processes to pathophysiology of the cardiovascular, nervous pulmonary hypertension, chronic obstructive lung diseases, asthma, atherosclerosis, wound inflammation. NO content in human exhalation and liquids can be used for diagnostic purposes. NO is a powerful signaling molecule capable of modulating cytokine production in the immune response and in wound healing. Nowadays a whole arsenal of chemical, physical and biological methods spanning the NO picomolar-to-micromolar concentration range in physiological milieus has been developed and widely used. Nevertheless, real-time monitoring of NO dynamics under physiological conditions of nano and less molar concentrations is still a challenging analytical problem. This has been attributed to the labile nature of NO molecule with half-life of the order of seconds, which both rapidly diffuses through the medium and readily reacts with many scavenger targets. The scope of importance of nitric oxide spans from normal numerous biological and physiological processes to pathophysiology of the cardiovascular, nervous pulmonary, hypertension, chronic obstructive lung diseases, asthma, atherosclerosis, wound inflammation, *etc* [1]. NO content in human exhalation and liquids can be used for diagnostic purposes. NO is a powerful signaling molecule capable of modulating cytokine production in the immune response and in wound healing.

In physiological conditions, scavenger targets for include oxygen, superoxide, amino and mercapto compounds (e.g., cysteine residues, glutathione), hemeproteins, (e.g., hemoglobin, mioglobin), and free radicals (e.g.), and soforth. Avariety of methods for NO detection have been proposed (Fig. 4.1) [2].

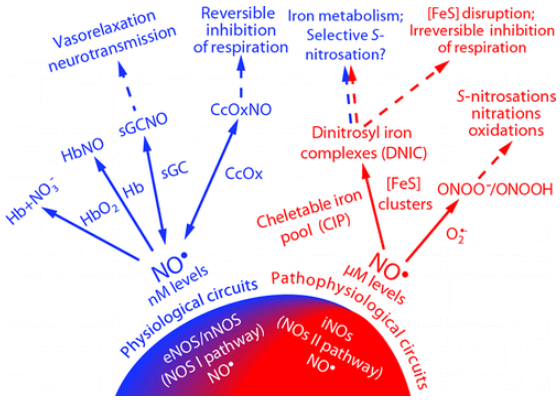

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Figure 1. Methods of nitric oxide analysis and action. [2].

2- Current Nitric Oxide Assays

Because of its simplicity, availability and the intense color of an adduct, optical spectroscopy for a long time served as main practical tools for nitrite and NO analysis in chemistry and biology The Griess diazotization reaction by Peter Griess), on which the Griess reagent relies was the first assay for nitrite, can be used for an indirect assay of nitric oxide [3]. Nitrite concentration can be measured by a number of additional methods. such as colorimetric measurements [4], chemiluminescence assay [5], fluorescent assay using fluorescence indicator, 2,3-diaminonaphthalene and cadmium reduction of nitrate $NO_3^-$to nitrite NO_2^- [6], the enzymatic conversion of nitrate into nitrite including the reaction modification, Griess reaction coupled to high-performance liquid chromatography (HPLC) including fluorometric method, which involves pre-column derivatization of nitrite with 2,3-diaminonaphthalene [7], gas chromatography-mass spectrometry employing pentafluorobenzyl derivatives [8], and electrochemical detection [9]. Chemical structures of fluorophores routinely employed in biology for analysis of small molecules are shown in Fig. 4.2 [15]. In the last decades the essential progress was achieved in design of novel fluorescent methods of analysis and real-time monitoring nitric oxide [16, 17] and references therein]. A number new non-cytotoxic water soluble and membrane permeable probes including two-photon fluorophores, excitable at low-energy wavelengths were synthesized and investigated employing advanced spectroscopic techniques. One of the general basis of methods for NO analyses is the conversion of a fluorescence silent probe to a compound of high fluorescent quantum yield. This objective can be achieved in various ways. One of the method of such a conversion is to expand aromatic structure of a probe with markedly increase fluorescence intensity of the NO product [20].

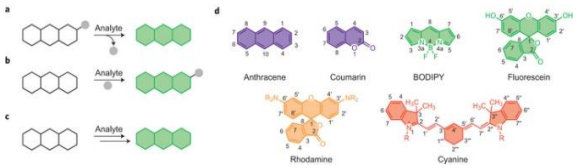

Figure 2 Overview of organic and metal-mediated reaction-based strategies for the chemoselective bioimaging of small-molecule and metal ion analytes in biological systems [15].

Two-photon microscopy (TPM) with two NIR photons offers a number of advantages over one-photon microscopy, including increased depth of penetration (>500 μm),

localized excitation, high spatial resolutions, low photo damage and low cellular auto-fluorescence18]. Utilizing two-photon microscopy made it possible to detect NO in live cells and live tissues at a depth of 90–180 μm.

Due to practically unlimited variety of structures and properties, organometal-based fluorescent probes provide for new possibilities in the field [15, 19] Several mechanisms of metal-transition NO detecting probes are presented in Fig. 3.

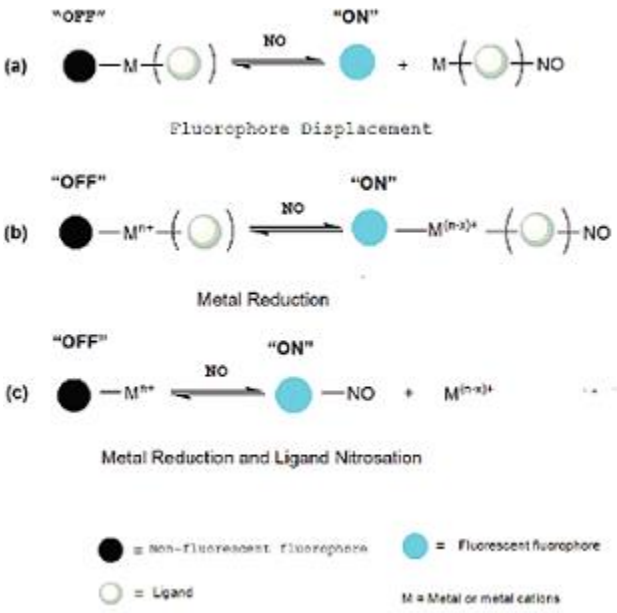

Figure 3. Mechanisms of metal-transition NO detecting probes [20].

Nitric oxide can be detected in the gas phase by the chemiluminescence of its reaction with ozone [21]:

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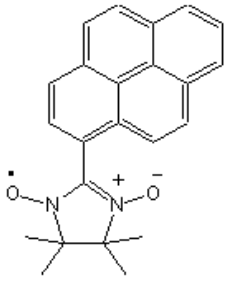
$$NO + O_3 \rightarrow NO_2 + O_2 + h\nu$$

This method is the most sensitive chemical assay currently available for the detection of nitric oxide up to 50 attomole, $5x10^{-17}$ mole. Nevertheless, competition reactions of NO with other species in a system under investigation is expected. As an example, a highly sensitive chemiluminescence approach to direct NO detection in aqueous solutions using a natural nitric oxide target, soluble guanylyl cyclase (sGC), which catalyzes the conversion of guanosine triphosphate to guanosine 3′,5′-cyclic monophosphate and inorganic pyrophosphate was introduced in [22]. Converting inorganic pyrophosphate into ATP catalyzed by ATP sulfurylase is accomplished by light emission from the ATP-dependent luciferin–luciferase reaction.

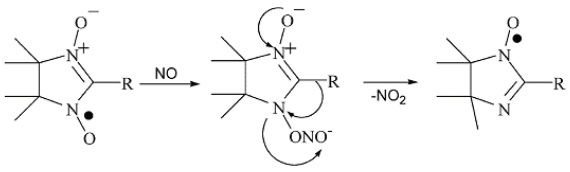
3 Methods Based on Utilization of Super Molecules

3.1 Fluorescence-nitroxide method

An approach to detection of nitric oxide based on the phenomenon of the intramolecular fluorescence quenching of the fluorophore fragment by the nitroxide in a dual fluorescent- nitroxide supermolecula was developed [23, 24]. Specifically, the pyrene-nitronyl (PN)



reacts with NO to yield a pyrene-imino nitroxide radical (PI) (Scheme 1)



Scheme 1. Conversion the pyrene-nitronyl (PN) to imino nitroxide radical (PI) [23].

Conversion of PN to PI resulted in a change of the electron paramagnetic resonance (EPR) spectrum from a five-line pattern (two equivalent N nuclei) into a seven-line pattern (two nonequivalent N nuclei) and accompanied by 80-fold an increase in the fluorescence intensity. The experiments indicate that the fluorescence measurements enable detection of nanomolar concentrations of NO compared to a sensitivity threshold of only several micromolar concentration for the EPR technique (Fig. 4).

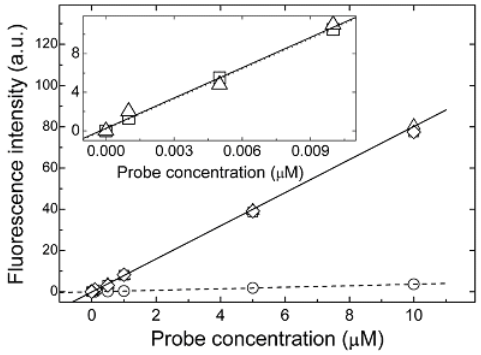
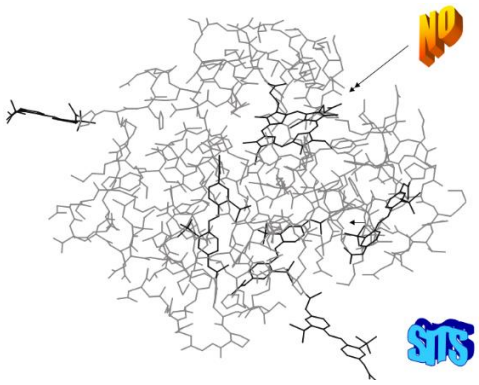


Figure 4. Integrated fluorescence measured before (circles) and after a 5-h incubation of PN with excess N-acetyl-Snitrosopenicillamine (10 μM; squares) and a 10-min incubation with NO (10 μM; diamonds). The fluorescence of the pyrene–imino (triangles) is measured for comparison [23].

3.2 Fluorescence inductive-resonance method of analysis (FIRMA1)

The principle of a fluorescent NO analysis based on the phenomena of inductive-resonance energy transfer from a donor to an acceptor is to construct a donor acceptor pair, in which the donor is a fluorophore (a stilbene probe tethered to myoglobin) in exited singlet state and acceptor is a nitric oxide trap (hemin) (Fig.) [25-27], see also Section 6.2). The proposed methods meet the following strict requirements: 1) The hemin NO trap provides extremely higher selectivity, fast speed formation of the corresponding nitronyl complex and very high stability and 2) Modern fluorescence techniques is able to detect very weak signals, down to single molecule. The obvious consequence of the theory of inductive-resonance energy transfer is that trapping NO by the hemin Fe^{2+} will lead to a change in absorption spectrum and, in consequence of this, to change of the overlap integral and the intensity of fluorescence. The latter makes possible the detection of small quantities of NO by the fluorescence technique of very high sensitivity. This method was tested using myoglobin covalently modified by stilbene label 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS) (Fig. 5) The rapid trapping of NO by myoglobin results in the change of the absorbance of myoglobin, which causes the change of both the absorbance–fluorescence overlap integral value and the fluorescence intensity of myoglobin–STIS.



. **Figure 5.** Molecule of myoglobin with attached fluorescence label SITS (Mb(Fe^{2+})-SITS) [25].

The change in emission intensity of the stilbene fragment, versus an increasing concentration of NO precursors, clearly demonstrated the spectral sensitivity required to monitor the formation of a heme–NO complex in a concentration range of 10 nM– 2 μM (Fig. 6).

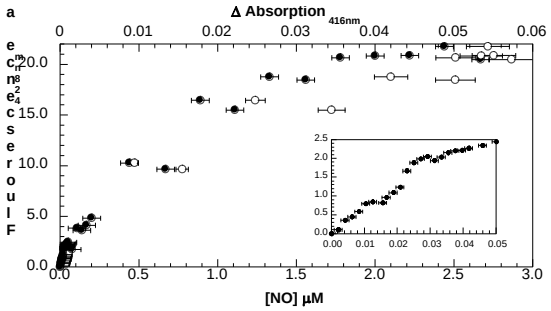


Figure 6. Calibration curve drawn as fluorescent intensity versus absorption (upper x-axis), which is proportional to NO concentration (lower x-axis). The graph inset is an extended version of the low concentration part of the calibration curve [26].

4. Electrochemistry, Cyclic Voltammetry and Amperometry

Electrochemical techniques, cyclic voltammetry and amperometry, together with fluorescence, are the most sensitive and commonly employed analytical method for monitoring nitric oxide. Real-time monitoring, ability to high-spatial resolution, wide variation of electrodes materials and its modification to enhance selectivity and sensitivity provide specific advantages [27-34].

Most electrochemical NO sensors are based on the oxidation of NO to NO_2 though electrocatalytic reduction of nitric oxide also has been reported. Starting from pioneering work of Shibuki [29] in which a Teflon©-coated platinum was used as a working electrode, a number of selective membranes have since been employed to fabricate electrochemical sensors. Among them are cellulose acetate, collodion/polystyrene, polycarbazole, Nafion, polyeugeno, polydimethylsiloxane, phenylenediamine, polysiloxane cross-linked LB films], the xerogel composed of methyltrimethoxysilane and (aminoethylaminomethyl)phenethyltrimethoxysilane, and Nafion hemin, hemin proteins, nickel planar macrocyclic compleX, Co(II) phthalocyanine], films with central ions of Fe, Co, Cu, and Mn , and other (30,31] and references therein.

NO sensors based on electrocatalytic platforms such as ruthenium (colloids, nanoparticles, and nanotubes) and carbon (pastes and nanotubes), acting as catalytic sites for NO oxidation was fabricated and investigated by cyclic voltammetry and amperometry both in solution phase and gas phase [32]. For example, functionalized graphene sheets (FGSs) monolayer electrodes were fabricated employing different heat treatments [33]. Electrocatalytic properties of FGSs in nitric oxide (NO) sensing were determined by cyclic voltammetry which exhibits an NO oxidation peak potential of 794 mV (vs 1 M Ag/AgCl). Porous FGS electrodes indicated a stronger apparent electrocatalytic effect as compared to platinized electrode.

Remarkable advances of electrochemical techniques were in full extend demonstrated in [34] and references therein. A sensor for real time monitoring nitric oxide based on hemin-functionalized graphene field-effect transistors with single atom thickness, and

the highest carrier mobility a sub-nanomolar sensitivity was designed. A graphene–hemin conjugate device designed in [34] is schematically depicted in Fig. 6.

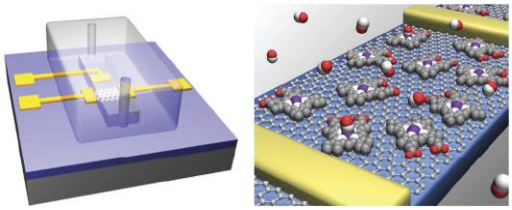


Figure 7 Schematic illustration of a graphene–hemin conjugate device [34]**.**

5. Other NO-detection techniques

The spectroscopic and electrochemical methods described above are the most commonly employed techniques for measuring NO. Other less frequently employed approaches include mass spectrometry [35], X-ray photoelectron spectroscopy [36], quantum cascade infrared laser spectroscopy [37], mechatronics sensoring [37], and quartz crystal microbalance technique [38] Space constraints do not permit us to describe these techniques in detail.

Spin trapping is designed for an investigation of processes with participation of short living particles, reactive free radicals, bearing unpaired electron [39]. The dithiocarbamate-iron spin traps were employed in combination with a liposome-encapsulating technique and ESR spectroscopy [40]. The liposome membrane forms a physical barrier between the spin-trap, catalytic enzymes [i.e., nitricoxidesynthase and nitrate reductase], and most substrates, while permitting the diffusion of NO. It was found that 5-Hydroxy-2,2,6,6-tetramethyl-4-(2-methylprop-1-en-yl)cyclohex-4-ene-1,3-dione behaves as an efficient trap for both NO and NO_2 radicals in the presence of oxygen, yielding EPR observable nitroxide and alk oxynitroxide, respectively [41].

6. Novel High Sensitive and High Selective Fluorescence Methods for Real Time monitoring of Nitric Oxide in Biological Systems in the Picomoleless Range

6.1 Physical background

Although a variety of methods for nitric oxide (NO) detection have been proposed, they are not generally amenable to real-time measurement of endogenous NO in situ. The principle of proposed analysis of nitroxide is design systems of donor acceptor pairs in which the donor is a fluorophore in the excited singlet state and the acceptor of light energy is hemin (System 1), or excited fluorophore is the electron acceptor and hemin (Fe^{2+}) is the electron donor System 2). The proposed approach is based on well-known independent physical phenomena, namely, singlet-singlet inductive-resonance energy transfer from a exited donor to acceptor [42] (in the system 1), or on effect of spin state of acceptor on electron transfer from donor and intersystem crossing (in the system 2). The proposed methods of nitric oxide analysis obey to the following strict requirements: 1) NO trap needs to provide fast formation of the corresponding nitronyl complex, high selectivity and high stability in terms of experience in the presence of oxygen. hemin Fe^{2+} is fully consistent with is this requirement in certain conditions; and 2) For the detection of the formation of this complex, highly sensitive method should be used. Modern fluorescence techniques allow to detect very weak signals, down to single molecule.

Hemin-Fe^{2+} having an intense Soret peak in the blue wavelength region of the visible spectrum ranging around 400 nm can serve as a light energy acceptor or electron donor. As a fluorescent donor it is suggested to utilize fluorophores with corresponding characteristics (stilbene derivatives, for example) and size-tunable CdSe quantum dots [43]. The latter possess unique optical properties. namely, to absorb and to emit light in a wide range of lengths of waves (Fig. 4.36) Abovementioned physical phenomena and optical characteristics of potential donor and acceptor compounds form the basis of two fluorescent methods namely: Fluorescence inductive-resonance method of analysis (FIRMA2) and Fluorescence spin exchange method of analysis (FSEMA)

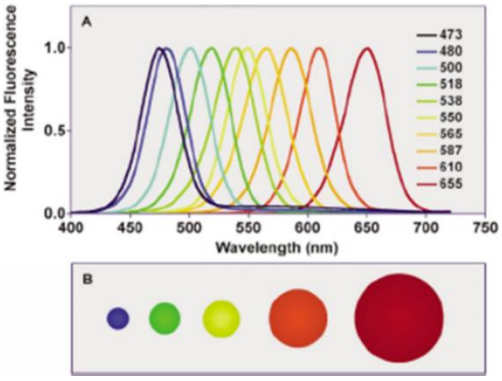


Figure 8. Size-tunable fluorescence spectra of CdSe quantum dots (A), and illustration of the relative particle sizes (B). From left to right, the particle diameters are 2.1 nm, 2.5 nm, 2.9 nm, 4.7 nm, and 7.5 nm [43].

6. 2 Fluorescence inductive-resonance method of analysis (FIRMA2)

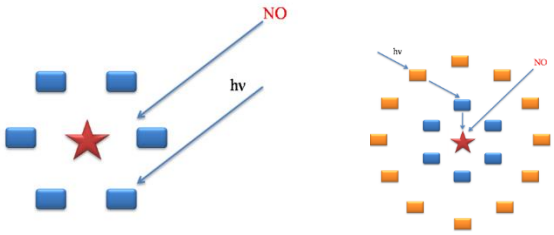
FIRMA 2 is a development of the approach of analysis of nitric oxide (FIRMA1) described in Section 3.2. Suggested method also based on phenomena of the singlet-singlet energy transfer by inductive resonance mechanism, due to the dipole-dipole interaction between excited fluorescent donor (D*) and nonexcited acceptor (A) According to the Forster theory, rate constant of the energy transfer depends on the donor acceptor distance and overlap between spectra of donor fluorescence and acceptor absorption [43]. The proposed method is based on the obvious consequence of the theory, that trapping NO by the hemin Fe^{2+} will lead to a change in absorption spectrum and, in consequence of this, change of the overlap integral $\int$ and, therefore, the intensity of fluorescence. The latter makes possible the detection of small quantities of NO by the fluorescence technique of very high sensitivity. A pair of hemin (absorption in the Soret region) and a corresponding CdSe quantum dot (Fig. 8) obeys the requirement of efficient overlap of the donor emission spectrum and the acceptor absorption spectrum.

Figs. 9 I, II illustrates schematically two donor -acceptor (D-A) models, having stilbene derivative as a donor and hemin as an acceptor, with direct D-A energy transfer and transfer via a light harvesting antenna, correspondingly. The latter is an analog of the photosynthetic light harvesting antenna. The harvesting light system is expected to increase sensitivity of detection of nitric oxide trapped by the hemin acceptor. In the frame of this conception, supermolecules composed with acceptor (an excited fluorophore) and donor (hemin Fe2+) segments tethered via a bridge can be designed (Fig. 9 III)) (Models 3).

6.3 Fluorescence Spin Exchange Method of Analysis (FSEMA)

- The method is based on measuring the difference in efficiency of spin exchange

(electron transfer, intersystem crossing) in supermolecules, consisting of NO trap (hemin Fe^{2+}) and a fluorophore, by detecting fluorescence in absent and present NO (Fig. 9 IV). Binding of NO by the trap fragment should influence on efficiency of the spin exchange between excited fluorophore and the nitryl complex and, as consequence, on the fluorescence intensity. The ET rate can be exponentially dependent on the distance between donor and acceptor [44, 45]. At a distance of about 12-14 Å the ET rate is comparable with the rate of spontaneous emission of fluorophore acceptor (typically, $1/\tau_D = 10^8 - 10^9$ s^{-1}). Thus, this distance would be optimum for detection effect of change of fluorescence after the NO trapping by hemin $Fe2^+$. Intersystem crossing (ISC) is a process in which a singlet state nonradiatively transforms into a triplet state. According to theory [24] the intersystem crossing efficiancy also dependent on distance between the donor and acceptor. In the framework of FSEMA, to prevent a competing effect of the inductive resonance process, which is very strong at distances of 12 -14 Å, is indispensably to choose a fluorophore with a minimum overlap of its emission spectrum and the acceptor absorption spectrum. The menu of the Size-tunable fluorescence spectra of CdSe quantum dots (Fig. 8) offers the way for the selection of a corresponding pair in a supermolecule indicated in Fig. 8.



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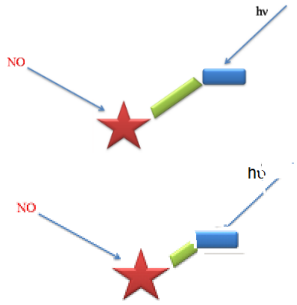

III **IV**

Figure 9. Schematic illustration of NO trapping hemin (star) and systems of fluorophores: I- Direct energy transfer from a fluorescent donor (rectangle) to the hemin acceptor (star) (Model 1); II - Energy transfer via inductive resonance exciton cascade (light harvesting antenna), rectangles are fluorescent donor (Model 2); III - Donor (excited CdSe quantum dots, rectangle) acceptor) pair connected with a bridge (trapezium) (Model 3); IV - Electron donor (hemin $Fe2^+$, star) – electron acceptor (an exited fluorophore (rectangle) connected via a bridge optimum for exchange interaction (Model 4).

Suggested methods can be employed for research of processes of NO flux and adsorption in cells, tissues, organs, animals, biological liquids and the human body, in the future. The methods can make basis for design of portable simple and cheap biosensor for NO for wide use in research laboratories and medical practice.

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