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Direct Chemiluminescent Detection of Nitric Oxide in Aqueous Solutions Using the Natural Nitric Oxide Target, Soluble Guanylyl Cyclase

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Abstract

Nitric oxide (NO) is a free radical involved in many physiological processes including regulation of blood pressure, immune response, and neurotransmission. However, the measurement of extremely low, in some cases sub-nanomolar physiological concentrations of nitric oxide presents an analytical challenge. The purpose of this methods article is to introduce a new highly sensitive chemiluminescent approach for 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. The suggested enzymatic assay uses the fact that the rate of the reaction increases about 200 times when NO binds with sGC, and in so doing provides a sensor for nitric oxide. Luminescent detection of the above reaction is accomplished by converting inorganic pyrophosphate into ATP with the help of ATP sulfurylase followed by light emission from ATP-dependent luciferin-luciferase reaction. Detailed protocols for NO quantification in aqueous samples are provided. The examples of applications include measurements of NO generated by nitric oxide donor (PAPA-NONOate), nitric oxide synthase and NO gas dissolved in buffer. The method allows for the measurement of NO concentrations in the nanomolar range and NO generation rates as low as 100 pM/min.

Keywords

Guanylyl cyclase; Nitric Oxide; Nitric oxide Synthase; Chemiluminescence; Luciferase; Pyrophosphate; NO donors

INTRODUCTION

Nitric oxide plays an important role as a signaling molecule in smooth muscle tonus regulation [1], neurotransmission [2] and immune response [3–5]. That is why measurements of nitric oxide production both *in vitro* and *in vivo* are of great interest and are the subject of numerous publications [6–9]. However, the measurement of extremely low, in some cases subnanomolar physiological concentrations of nitric oxide [10–12] presents an analytical challenge. The problem is aggravated by the reactivity of NO towards oxygen and many other substances in the biological milieu.

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The primary methods currently in use for NO detection in biological samples are chemiluminescent reaction with ozone [10–12], electrochemical [9,13], fluorescent [14–16] and EPR approaches [17,18]. All these methods have their own drawbacks that have been thoroughly discussed in the literature. Ozone chemiluminescence is more suited for gaseous samples while electrochemical detection suffers from instability and low sensitivity of electrodes. Fluorescent detection though highly sensitive, requires a high concentration of the probe, yields unstable fluorescent products and can be misinterpreted, as the probe reacts with ascorbic or dehydroascorbic acid yielding the product with a fluorescent spectrum similar to one used for NO detection [19,20]. EPR approaches have certain advantage allowing NO measurements in living tissues but often lack for sensitivity [17,18].

One preferred way to overcome the transient nature of nitric oxide in biological samples is to measure the stable products of its metabolism, nitrate and nitrite. However, this gives only an averaged picture of NO generation, without information on instantaneous NO concentration or rate of NO generation, which can be physiologically important.

The objective of this methods paper is to introduce a new highly sensitive chemiluminescent approach for NO detection in aqueous solutions and to provide detailed experimental protocol of its application.

PRINCIPLES

The concept

To develop the method for detection of ultra-low concentrations of nitric oxide we utilized the following principles: (i) an analytical signal was amplified by using NO as a catalytic molecule rather than a direct participant in the chemical reaction; (ii) the amplified analytical signal was transformed into a chemiluminescent signal, theoretically allowing detection of a single quantum of light. The general outline of the NO detection principle is shown in Scheme 1. The amplification step was accomplished by using soluble guanylyl cyclase (sGC), a natural cellular target of nitric oxide. About a hundred fold increase of activity of sGC over a basal level occurs upon binding of NO. This increase in the rate of sGC-catalyzed conversion of guanosine triphosphate into cGMP and inorganic pyrophosphate (PP_i) provides an amplification of the NO signal. Resulting pyrophosphate reacts with APS in the presence of ATP-sulfurylase, forming ATP and sulfate; ATP then serves as a co-substrate in the luciferase reaction, producing oxoluciferin, AMP, pyrophosphate, CO₂ and a quantum of light with quantum yield about 0.4 [21]. Light output is measured by a luminometer. Essentially, by measuring chemiluminescence, we measure kinetics of pyrophosphate formation [22], the latter being produced by NO-activated guanylyl cyclase. It is important to mention that the nanomolar concentration of ATP generated is far below the luciferase K_m for ATP (160 μM [23]) which results in a linear dependence of luminescence on ATP concentration. The developed approach represents a new highly sensitive tool for detection of NO concentrations in the nanomolar range and NO generation rates in biological samples with sensitivity of 100 pM/min.

Experimental basis

Figure 1 shows linear dependences of the chemiluminescence of luciferase system containing APS and ATP sulfurylase on ATP or pyrophosphate concentration, in agreement with the literature data [22]. This provides an opportunity to monitor NO-dependent pyrophosphate formation by activated guanylyl cyclase. Special precautions described in Caveats section have to be taken against ATP and pyrophosphate impurities in luciferase, APS and GTP samples in order to decrease the background luminescence of the luciferase/sulfurylase system.

Addition of guanylyl cyclase to the luciferase-sulfurylase reaction system results in a linear increase in luminescence for at least 10 minutes (Fig.2, inset). This increase is not observed in the absence of either GTP or sGC and represents basal (not NO-stimulated) sGC activity. Addition of NO donor (PAPA NONOate, $t_{1/2}=77$ min at 22°C, pH 7.4 [24]) or bolus addition of NO dissolved in buffer to the reaction mixture dramatically increased the rate of luminescence change (Fig. 2 and 3, respectively). We observed that the kinetics of the NO-induced luminescence was strongly affected by superoxide dismutase (SOD) (Fig. 3). Addition of SOD significantly improved sensitivity of the approach, particularly at low NO concentrations. For this reason, SOD addition to detection system is strongly recommended for quantitative NO measurements and has been used in all further applications described here. Further studies are required to identify the possible source of the superoxide production in the detection system. The possible source is the reaction of luciferin with molecular oxygen producing dehydroluciferin and hydrogen peroxide, or, possibly, superoxide as a minor product.

MATERIALS

Note: Not all the reagents that follow are needed for each specific application of the chemiluminescent assay. Before starting, select the appropriate protocol to determine which reagents will be needed for a particular application. We recommend that GTP and APS of highest purity grade were used and when necessary additionally purified as described below.

Enzymatic NO detection system

Chemicals—ATP (10127523001) (Roche Diagnostics, Indianapolis, IN), sodium pyrophosphate (205975000), diethylenetriamine-pentaacetic acid (114322500, DTPA) (Acros Organics), D-luciferin (L9504), GTP (G8877), adenosine-5'-phosphosulfate sodium salt (A5508, APS), Trizma base (T1503), dithiothreitol (D0632, DTT) (Sigma-Aldrich).

Enzymes—Guanylyl cyclase (ALX-202-039, soluble, bovine lung, 10 μ mol cGMP/min per mg protein, Alexis Biochemicals, San Diego, CA), ATP sulfurylase (M0394S, recombinant, from *S. cerevisiae*, 300 units/mL, New England Biolabs, Ipswich, MA), inorganic pyrophosphatase (10108987001, 200 U/mg), hexokinase (11426362001, 450 U/mg) (Roche Diagnostics, Indianapolis, IN), firefly luciferase (L9009, lyophilized powder, $\sim 10^7$ light units/mg protein), superoxide dismutase (S5395, from bovine erythrocytes, 5000 U/mg) (Sigma-Aldrich).

Composition of the reaction mixture—Unless otherwise stated, the reaction mixture for luminescence measurements (300 μ L) contained: 1 mM $MgCl_2$, 1 mM DTE, 0.1 mM DTPA, 0.1 mg/mL BSA, 0.014 mM D-luciferin, 0.2 μ g luciferase, 0.002 U PPase, guanylyl cyclase (25–50 ng total), 0.01 U sulfurylase, 50 U of superoxide dismutase, 0.01 mM APS and 0.1 mM GTP (both APS and GTP were treated as described below) in 0.1 M Tris-HCl, pH 7.5.

Purification of GTP and APS—To remove ATP contamination, GTP was treated as follows: reaction mixture containing 40 μ L of 0.1 M GTP, 4.0 μ L 1 M $MgCl_2$, 4 μ L 1 M glucose, 1 μ L hexokinase (1.5 u/ μ L), 351 μ L 0.1 M Tris-HCl, pH 7.5, was incubated at room temperature for 20 min, then filtered through Microcon Ultracel centrifuge filter YM-3 (Millipore, Bedford, MA) for 20 min at 14000g, 4° C for removal of hexokinase. APS was treated the same way, with reaction mixture containing 20 μ L of 20 mM APS, 1 μ L 1 M $MgCl_2$, 1 μ L 1 M glucose, 0.5 μ L hexokinase (1.5 u/ μ L), 78 μ L 0.1 M Tris-HCl, pH 7.5. GTP and APS concentrations in filtrate were determined spectrophotometrically using $\epsilon = 1.37 \cdot 10^4$ M⁻¹cm⁻¹ at 253 nm (GTP) and $\epsilon = 1.25 \cdot 10^4$ M⁻¹cm⁻¹ at 260 nm (APS). Treated solutions of GTP and APS were aliquoted (we used 30 μ L aliquots) and kept at -80 °C.

Measurements of NOS-mediated NO generation

Chemicals—HEPES (H9897), imidazole (I5513), δ -aminolevulinic acid (A7793, δ -ALA), chloramphenicol (C0857), (Sigma-Aldrich), terrific broth (22711-022), carbenicillin (10177-012), isopropyl- β -D-thiogalactoside (15529-019, IPTG) (Invitrogen), NADPH (N4505), arginine (A8094) (Sigma-Aldrich), N^G -monomethyl- L- arginine (80200, NMMA), tetrahydrobiopterin (81880, BH₄) (Cayman Chemical, Ann Arbor, MI), proteinase inhibitor cocktail tablets (11697498001) (Roche Diagnostics, Indianapolis, IN).

Preparation of iNOS—Overexpression of active inducible nitric oxide synthase (iNOS) in *Escherichia coli* was enhanced by coexpression with calmodulin (pCaM). Plasmids containing iNOS and CaM/pACYC were transformed into $\Delta 65$ protease-deficient *E. coli* BL21(DE3). The iNOS/CaM-expressed BL21 cells were cultured on LB agar plate containing carbenicillin (125 g/mL) and chloramphenicol (35 μ g/mL). One liter cultures of terrific broth containing 125 μ g/mL carbenicillin, 35 μ g/mL chloramphenicol, and 8 mL of glycerol were inoculated with 100 mL of overnight bacterial culture and shaken 200 rpm at 37°C. Expression of protein was induced by adding δ -aminolevulinic acid to final concentration of 500 μ M and IPTG to final concentration of 1 mM to the culture when it reached an optical density of 0.8 at 600 nm. Cells were harvested by centrifugation 20 h after induction. The cells from 4 L of culture were resuspended in minimum volume of lysis buffer A, containing 40 mM HEPES, 150 mM NaCl, 20 mM imidazole, 10% glycerol, 3 mM DTT and protease inhibitor cocktail tablets at pH 7.4. Cells were lysed by two passes through an Emulsiflex C3 at 12–15 kpsi. The lysate was centrifuged at 48,000g for 60 min. The supernatant was loaded onto a 5 mL HisTrap column (GE Biosciences) and equilibrated with buffer A. Column was extensively washed with buffer B: 40 mM HEPES, 450 mM NaCl, 10 % glycerol, 40 mM imidazole, 3 mM DTT, pH 7.4. Bound protein was eluted with buffer C, containing 40 mM HEPES, 450 mM NaCl, 10 % glycerol, 250 mM imidazole, 3 mM DTT, pH 7.4. Fractions containing iNOS were pooled and concentrated using an Amicon Ultra 100,000 MW cut off concentrator (Millipore). The concentrated proteins were applied to a Superdex 200 Hiload size exclusion column (GE Biosciences) and eluted with 40 mM HEPES, 150 mM NaCl, 10 % glycerol pH 7.4. The iNOS fractions were concentrated, divided into aliquots, quickly frozen in liquid nitrogen and stored at -80°C . The iNOS concentration was determined using the Bradford assay (Bio-Rad) with bovine serum albumin (BSA) as the standard. The purity of iNOS was above 90% as determined by SDS-PAGE with Coomassie Blue staining. The activity of iNOS was determined using the oxyhemoglobin capture assay [25], the typical activity being above 800 nmol $\text{mg}^{-1} \text{min}^{-1}$.

Measurements of NO release from NO donors

Note: PAPA-NONONOate has been used in the exemplified application given in this paper. Similar assay can be used to study NO release by other NO donors. Stock solution of PAPA NONONOate (82140, Cayman Chemical, Ann Arbor, MI) was prepared in 0.01 M NaOH, and its concentration was determined using $\epsilon = 8050 \text{ M}^{-1}\text{cm}^{-1}$ at 250 nm. Solution is stable for 24 hrs.

INSTRUMENTATION

Most of commercially available luminometers can be used for the luminescence measurements described. In this work all the measurements were conducted at 27°C using LB9505 luminometer (Berthold Analytical Instruments, Nashua, NH).

PROTOCOL

Measurement of NO concentrations in aqueous solutions

A. Preparation of Solutions

1. Five mL of the main buffer contain 4.84 mL Tris-HCl (0.1 M, pH 7.5), 50 μ L of DTPA (10mM), 50 μ L of BSA (10mg/mL), 5 μ L of $MgCl_2$ (1 M), 25 μ L of DTE (0.2M), 14 μ L of luciferin (5 mM), 17 μ L of SOD (10 mg/mL) and 1.7 μ L of luciferase (2 mg/mL). Keep on ice.
2. One hundred μ L of APS, GTP and pyrophosphatase mixture (AGP mix) contains 52 μ L of GTP (10 mM, treated as described), 13 μ L of APS (4 mM, treated as described) and 35 μ L of pyrophosphatase (1 U/mL). Keep this solution on ice for no more than 3 hrs due to instability of APS.
3. One hundred μ L of NO synthase activity mixture (iNOS activity mix) contained 63 μ L of arginine (10 mM), 6.3 μ L of NADPH (100 mM) and 31 μ L of BH_4 (10 mM). Keep on ice for no more than 3 hrs due to oxidation of BH_4 .

B. Sample preparation and luminescence recording—Put 293 μ L of main buffer into a luminometer sample tube and leave for temperature equilibration in the luminometer cell compartment for 7 minutes. Add AGP mixture (5.8 μ L) and incubate for 2 minutes to allow pyrophosphatase to hydrolyse pyrophosphate impurity present in APS and GTP. Then, add 1 μ L of sulfurylase (0.01 U). At this point luminescence recording starts. After recording baseline luminescence for 1–2 minutes, add guanylyl cyclase (2 μ L, 25 ng), continue registration of luminescence. The observed gradual increase of luminescence represents unstimulated guanylyl cyclase reaction. Add an aliquot of NO donor or NO gas solution (0.5–5 μ L) and record NO-stimulated luminescence kinetics. For the registration of NO synthase activity, the sample preparation is the same except 10 μ L of iNOS activity mix is added the same time as AGP mix and reaction is started by addition of iNOS.

Note 1: concentrations of GTP and APS stock solutions can vary from what is stated in Section A2; adjust the amount in AGP mix and amount added to the sample accordingly to have the final concentrations of 10 μ M (APS) and 100 μ M (GTP) in the sample.

Note 2: addition of calmodulin in the reaction mixture does not change iNOS activity and addition of calcium inhibits guanylyl cyclase [26].

Note 3: Inducible NOS has been used in this application. Similar assay can be used to study NO generation by other NOS isoforms.

Calculations and Results

Measurement of NO concentrations in aqueous solutions—The proposed approach provides the data in the form of the kinetics of the luminescence change. It is expected that constant NO concentration in solution should result in a linear increase of the luminescence, as pyrophosphate generation by sGC proceeds at a constant rate. Therefore, the rate of the luminescence change calculated as a slope of the kinetics is expected to be proportional to NO concentration and can be used for NO quantification in the sample.

Typical kinetics of the luminescence increase observed after addition of nanomolar concentrations of anaerobic NO solution to the NO-detection system are shown in Fig. 4. The dependence of the initial rate of luminescence change on NO concentration is shown in Figure 5. As expected it was found to be proportional to NO concentration with the sensitivity limit about 1 nM. However, the slope of the kinetic curve decreased with time for the kinetics

initiated by dissolved NO gas (Figure 3 and Figure 4) probably due to NO depletion in solution. The direct uncatalyzed reaction with molecular oxygen cannot explain this decrease, as the NO first half-life at 100 nM would be about 1.5 hrs and even longer for lower concentrations [27,28].

Measurement of the rates of NO generation: It is expected that the rate of the initial luminescence change after initiation of NO generation will be indistinguishable from the background level due to insufficient NO accumulation at zero time point. On the other hand, NO generation will result in accumulation of NO and “acceleration” of the luminescence change which should be proportional to NO generation rate. Therefore, luminescence “acceleration” calculated as second derivative of the luminescence curve, is expected to be proportional to NO generation rate and can be used for its quantitation.

Typical luminescence kinetics observed after addition of NO donor, PAPA NONOate, are shown in Fig. 6. The initial “acceleration” of the luminescence (second derivative of the luminescence curve at zero time point) was found to be proportional to the concentration of NO donor (Fig. 7), as expected. This agrees with “acceleration” being proportional to NO generation rate. It was also observed that the maximal rate of luminescence change is proportional to the concentration of NO donor (Fig.8). This can be explained by the fact that steady state level of NO accumulated in solution is proportional to the rate of NO generation, i.e. to the concentration of the NO donor. The maximal rate of luminescence change observed in the presence of 3.3 nM of PAPA NONOate (corresponds to NO release rate of about 100 pM/min) exceeded the basal rate of luminescence change about 3 times.

The extraordinary high sensitivity of the approach was further confirmed by measurements of NO generation by purified inducible nitric oxide synthase (Fig. 9). Luminescence intensity increase here depended on the presence of NADPH and arginine and was inhibited by specific NOS inhibitor, NMMA (data not shown). The rate of luminescence change was proportional to iNOS concentration allowing for detection of NO generated by only 20 pg of purified protein. Similar shapes of the luminescence kinetics were observed upon NO release by NO donor (Fig. 6) and NO generation by iNOS (Fig.9). The dependence of the initial “acceleration” of the luminescence (second derivative of the luminescence curve at zero time point) on iNOS concentration shown in Figure 10 exhibits the same proportionality as in the case of NO release by NO donor (cf. Figure 10 and Figure 7). Taking into account that NO generation rate by PAPA NONOate is known, the observed dependence of initial luminescence “acceleration” on the concentration of NO donor (Fig.7) can be used as a calibration curve for the calculation of the rate of NO production by iNOS (Fig. 10, left axis). The dependence of NO generation rate versus iNOS amount shown in Fig.10 yields the activity of iNOS equal to 1100 nmol NO·min⁻¹·mg⁻¹. This value is in good agreement with the activity obtained by oxyhemoglobin assay, 800 nmol NO·min⁻¹·mg⁻¹.

CAVEATS

Background luminescence

The reaction mixture without sGC demonstrates background luminescence originating from ATP and pyrophosphate impurities in luciferase, APS and GTP. Out of the aforementioned, the predominant problem is ATP and pyrophosphate contamination in GTP, as it is present in the highest concentration (0.1 mM) in the reaction mixture. GTP (and possibly APS) is also a substrate for luciferase, as has been shown previously [29]. To find out how much this side reaction of luciferase can add to the background luminescence, we added hexokinase and glucose to the luciferase reaction system containing either ATP or GTP (Fig.11). ATP-depleting hexokinase activity reduces luminescence to the background level in the reaction mixture containing ATP. However, luminescence drops substantially but not to the background

in the presence of GTP. As GTP is not a substrate for hexokinase, drop of luminescence for GTP-containing sample after addition of hexokinase shows that contaminating ATP contributes mainly to GTP-originating fluorescence. Still, some luminescence is produced by GTP serving as an alternative substrate for luciferase (residual hexokinase-resistant luminescence in GTP-containing sample). Taking into account higher GTP concentration, we concluded that GTP is about 14000 times less effective in activation of luciferase luminescence than ATP. In further experiments GTP and APS were treated with hexokinase as described in *Materials*.

Commercial GTP preparations also contain substantial amounts of pyrophosphate. This has been found by observing a sharp increase in the luminescence of the luciferase reaction system containing GTP upon addition of APS and ATP sulfurylase. In order to remove initially present pyrophosphate, small amount of pyrophosphatase was added to the reaction mixture *before* addition of sulfurylase, as described in *Sample Preparation*. The added amount was adjusted to be sufficient to hydrolyze practically all contaminating pyrophosphate in about two minutes before sulfurylase addition. Note that in the presence of sulfurylase most of pyrophosphate produced in guanylyl cyclase reaction is converted to ATP rather than to phosphate due to predominant activity of sulfurylase over pyrophosphatase. Separate experiments proved that the amount of pyrophosphatase included in the reaction mixture does not significantly affect the luminescence kinetics (data not shown).

Concluding Remarks

Here we presented a new method for the measurement of nitric oxide concentrations and rates of NO generation by using a natural target of the nitric oxide, soluble guanylyl cyclase. Method allows for monitoring of the guanylyl cyclase reaction, both basal and NO-stimulated, using a highly sensitive chemiluminescent detection technique. High degree of stimulation of the sGC reaction by NO provides the way for detection of nanomolar concentrations of nitric oxide and the rates of NO generation as low as 0.1 nM/min. The stimulation ratio in our experiments was in the range of 140 to 160 times, which is comparable to values (130 – 670) reported in the literature [30–33]. It should be noted, that basal guanylyl cyclase activity observed in our experiments was significantly lower than one reported in a recent publication [34], namely 10–40 nmol PP_i/(mg GC·min), depending on the batch of the enzyme, and did not vary substantially from day to day. This could reflect, according to the authors of mentioned publication, the quality of air (NO level) in Columbus, OH vs. London, UK.

The proposed chemiluminescent approach can be useful for mechanistic studies of the guanylyl cyclase reaction. To our best knowledge, this is the first method describing *continuous* registration of guanylyl cyclase kinetics. As can be seen from Fig. 5, the rate of the guanylyl cyclase reaction detected by chemiluminescent method linearly depends on NO concentration in nanomolar range. It means that the apparent dissociation constant for NO cannot be lower than 10 nM, which is in agreement with some literature data [35–37] and contradicts other [34]. Luminescence kinetics under conditions of maximal stimulation by NO (3–8 μM of PAPA NONOate) allowed for calculation of the specific activity of guanylyl cyclase in the reaction mixture, which was found to be $1.8 \cdot 10^3$ nmole PP_i · min⁻¹ · mg⁻¹ at 27° C. The manufacturer reports about 10^4 nmole cGMP · min⁻¹ · mg⁻¹ at 37°C. Taking into account a 10°C temperature difference and possible non-optimal conditions for enzyme activity (buffer composition, GTP concentration, etc.) the agreement appears to be reasonable. It should be noted that the assay described here can be also used to study NO-independent regulation mechanisms of sGC (e.g. by CO and YC-1, a synthetic benzyldiazole derivative [38]) or particulate GC [39].

To adapt the technique for *in vivo* and *in situ* applications, a few problems must be resolved. First, as the method is based on the detection of ATP and PP_i, molecules that are ubiquitous in biological milieu, the whole detection system should be encapsulated, e.g. in liposomes

[40]. As the liposomal membrane is permeable for nitric oxide, but not for ATP and PP_i, detection is possible in this configuration. Second, *in vivo* applications can be hampered by strong absorption of 560 nm light, emitted by firefly luciferase, in biological tissues. Recently constructed luciferase mutants [23,41] emit at 615 nm, fitting perfectly into the spectral window (*ca.* 600–710 nm) where such emissions can be measured in living tissues.

Abbreviations

APS, adenosine-5'-phosphosulfate
 BH₄, tetrahydrobiopterin
 BSA, bovine serum albumin
 cGMP, guanosine 3',5'-cyclic monophosphate
 cpm, counts per minute
 DTE, 1,4-dithioerythritol
 DTPA, diethylenetriamine-pentaacetic acid
 DTT, dithiothreitol
 iNOS, inducible nitric oxide synthase
 IPTG, isopropyl-β-D-thiogalactoside
 NMMA, N^G-monomethyl-L- arginine
 PAPA NONOate, propylamine propylamine NONOate
 PP_i, inorganic pyrophosphate
 PPase, inorganic pyrophosphatase
 sGC, soluble guanylyl cyclase
 SOD, superoxide dismutase
 AGP mix, the mixture of APS, GTP and pyrophosphatase.

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REFERENCES

1. Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. U. S. A* 1987;84:9265–9269. [PubMed: 2827174]
2. Garthwaite J, Charles SL, Chess-Williams R. Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature* 1988;336:385–388. [PubMed: 2904125]
3. Tripathi P. Nitric oxide and immune response. *Indian J. Biochem. Biophys* 2007;44:310–319. [PubMed: 18341206]
4. MacMicking J, Xie QW, Nathan C. Nitric oxide and macrophage function. *Annu. Rev. Immunol* 1997;15:323–350. [PubMed: 9143691]
5. Moilanen E, Vapaatalo H. Nitric oxide in inflammation and immune response. *Ann. Med* 1995;27:359–367. [PubMed: 7546626]
6. Nagano T. Practical methods for detection of nitric oxide. *Luminescence* 1999;14:283–290. [PubMed: 10602296]
7. Laver JR, Stevanin TM, Read RC. Chemiluminescence quantification of NO and its derivatives in liquid samples. *Methods Enzymol* 2008;436:113–127. [PubMed: 18237630]
8. Bryan NS, Grisham MB. Methods to detect nitric oxide and its metabolites in biological samples. *Free Radic. Biol. Med* 2007;43:645–657. [PubMed: 17664129]
9. Zhang X. Real time and in vivo monitoring of nitric oxide by electrochemical sensors--from dream to reality. *Front. Biosci* 2004;9:3434–3446. [PubMed: 15353368]

10. Michelakis ED, Archer SL. The measurement of NO in biological systems using chemiluminescence. *Methods Mol. Biol* 1998;100:111–127. [PubMed: 10906999]
11. Dunham AJ, Barkley RM, Sievers RE. Aqueous nitrite ion determination by selective reduction and gas phase nitric oxide chemiluminescence. *Anal. Chem* 1995;67:220–224. [PubMed: 11536661]
12. Fareed D, Iqbal O, Tobu M, Hoppensteadt DA, Fareed J. Blood levels of nitric oxide, C-reactive protein, and tumor necrosis factor-alpha are upregulated in patients with malignancy-associated hypercoagulable state: pathophysiologic implications. *Clin Appl Thromb Hemost* 2004;10:357–364. [PubMed: 15497022]
13. Barbosa RM, Lourenco CF, Santos RM, Pomerleau F, Huettl P, Gerhardt GA, Laranjinha J. Chapter 20 in vivo real-time measurement of nitric oxide in anesthetized rat brain. *Methods Enzymol* 2008;441:351–367. [PubMed: 18554545]
14. Zhang, G.; Shu, FP.; Robinson, CJ. Design and characterization of a nano-encapsulated self-referenced fluorescent nitric oxide sensor for wide-field optical imaging; *Conf Proc IEEE Eng Med Biol Soc*; 2007. p. 103-106.
15. Wardman P. Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: progress, pitfalls, and prospects. *Free Radic. Biol. Med* 2007;43:995–1022. [PubMed: 17761297]
16. Gomes A, Fernandes E, Lima JL. Use of fluorescence probes for detection of reactive nitrogen species: a review. *J Fluoresc* 2006;16:119–139. [PubMed: 16477509]
17. Mordvintcev P, Mulsch A, Busse R, Vanin A. On-line detection of nitric oxide formation in liquid aqueous phase by electron paramagnetic resonance spectroscopy. *Anal Biochem* 1991;199:142–146. [PubMed: 1666941]
18. Samouilov A, Zweier JL. Analytical implications of iron dithiocarbamates for measurement of nitric oxide. *Methods Enzymol* 2002;352:506–522. [PubMed: 12125375]
19. Rodriguez JSV, Maloney R, Jourdeuil D, Feelisch M. Performance of diamino fluorophores for the localization of sources and targets of nitric oxide. *Free Radic Biol Med* 2005;38:356–368. [PubMed: 15629864]
20. Zhang X, Kim W-S, Hatcher N, Potgieter K, Moroz LL, Gillette R, Sweedler JV. Interfering with Nitric Oxide Measurements. 4,5-Diaminofluorescein Reacts with Dehydroascorbic Acid and Ascorbic Acid. *J. Biol. Chem* 2002;277:48472–48478. [PubMed: 12370177]
21. Ando Y, Niwa K, Yamada N, Enomoto T, Irie T, Kubota H, Ohmiya Y, Akiyama H. Firefly bioluminescence quantum yield and colour change by pH-sensitive green emission. *Nat Photon* 2008;2:44–47.
22. Nyren P, Lundin A. Enzymatic method for continuous monitoring of inorganic pyrophosphate synthesis. *Anal. Biochem* 1985;151:504–509. [PubMed: 3006540]
23. Branchini BR, Southworth TL, Khattak NF, Michelini E, Roda A. Red- and green-emitting firefly luciferase mutants for bioluminescent reporter applications. *Anal. Biochem* 2005;345:140–148. [PubMed: 16125663]
24. Keefer LK, Nims RW, Davies KM, Wink DA. "NONOates" (1-substituted diazen-1-ium-1,2-diols) as nitric oxide donors: convenient nitric oxide dosage forms. *Methods Enzymol* 1996;268:281–293. [PubMed: 8782594]
25. Gross SS. Microtiter plate assay for determining kinetics of nitric oxide synthesis. *Methods Enzymol* 1996;268:159–168. [PubMed: 8782582]
26. Serfass L, Carr HS, Aschenbrenner LM, Burstyn JN. Calcium ion downregulates soluble guanylyl cyclase activity: evidence for a two-metal ion catalytic mechanism. *Arch. Biochem. Biophys* 2001;387:47–56. [PubMed: 11368183]
27. Kharitonov VG, Sundquist AR, Sharma VS. Kinetics of nitric oxide autoxidation in aqueous solution. *J. Biol. Chem* 1994;269:5881–5883. [PubMed: 8119931]
28. Lewis RS, Deen WM. Kinetics of the reaction of nitric oxide with oxygen in aqueous solutions. *Chem. Res. Toxicol* 1994;7:568–574. [PubMed: 7981422]
29. Moyer JD, Henderson JF. Nucleoside triphosphate specificity of firefly luciferase. *Anal. Biochem* 1983;131:187–189. [PubMed: 6614450]

30. Brandish PE, Buechler W, Marletta MA. Regeneration of the ferrous heme of soluble guanylate cyclase from the nitric oxide complex: acceleration by thiols and oxyhemoglobin. *Biochemistry* 1998;37:16898–16907. [PubMed: 9836582]
31. Stone JR, Marletta MA. Soluble guanylate cyclase from bovine lung: activation with nitric oxide and carbon monoxide and spectral characterization of the ferrous and ferric states. *Biochemistry* 1994;33:5636–5640. [PubMed: 7910035]
32. Stone JR, Marletta MA. Heme stoichiometry of heterodimeric soluble guanylate cyclase. *Biochemistry* 1995;34:14668–14674. [PubMed: 7578074]
33. Stone JR, Marletta MA. Spectral and kinetic studies on the activation of soluble guanylate cyclase by nitric oxide. *Biochemistry* 1996;35:1093–1099. [PubMed: 8573563]
34. Roy B, Halvey EJ, Garthwaite J. An enzyme-linked receptor mechanism for nitric oxide-activated guanylyl cyclase. *J. Biol. Chem* 2008;283:18841–18851. [PubMed: 18463095]
35. Stone JR, Sands RH, Dunham WR, Marletta MA. Spectral and ligand-binding properties of an unusual hemoprotein, the ferric form of soluble guanylate cyclase. *Biochemistry* 1996;35:3258–3262. [PubMed: 8605161]
36. Bellamy TC, Wood J, Garthwaite J. On the activation of soluble guanylyl cyclase by nitric oxide. *Proc. Natl. Acad. Sci. U. S. A* 2002;99:507–510. [PubMed: 11752394]
37. Condorelli P, George SC. In vivo control of soluble guanylate cyclase activation by nitric oxide: a kinetic analysis. *Biophys. J* 2001;80:2110–2119. [PubMed: 11325714]
38. Denninger JW, Schelvis JP, Brandish PE, Zhao Y, Babcock GT, Marletta MA. Interaction of soluble guanylate cyclase with YC-1: kinetic and resonance Raman studies. *Biochemistry* 2000;39:4191–4198. [PubMed: 10747811]
39. Kobińska M, Gorczyca WA. Particulate guanylyl cyclases: multiple mechanisms of activation. *Acta Biochim. Polonica* 2000;47:517–528.
40. Woldman YY, Semenov SV, Bobko AA, Kirilyuk IA, Polienko JF, Voinov MA, Bagryanskaya EG, Khramtsov VV. Design of liposome-based pH sensitive nanoSPIN probes: nano-sized particles with incorporated nitroxides. *Analyst* 2009;134:904–910. [PubMed: 19381383]
41. Branchini BR, Ablamsky DM, Murtiashaw MH, Uzasci L, Fraga H, Southworth TL. Thermostable red and green light-producing firefly luciferase mutants for bioluminescent reporter applications. *Anal. Biochem* 2007;361:253–262. [PubMed: 17181991]

Figure 1.

Luminescence of luciferase-sulfurylase reaction system upon addition of (A) ATP or (B) pyrophosphate; each addition is 1 nM. Spikes are from removing and replacing the sample and should be disregarded. (C) Dependence of luminescence on concentration of ATP or pyrophosphate (nM range). The samples contained 1 mM MgCl₂, 1 mM DTE, 0.1 mM DTPA, 0.1 mg/mL BSA, 0.014 mM D-luciferin, 0.01 mM APS, 0.1 mM GTP (APS and GTP were treated as described in *Materials*), 0.2 μ g luciferase, 0.01 U sulfurylase in 0.1 M Tris-HCl, pH 7.5.

Figure 2.

Luminescence of the luciferase-sulfurylase reaction system (the same as in Fig. 1, except GTP) upon addition of 0.1 mM GTP treated as described in *Materials*, guanylyl cyclase (50 ng) and NO donor (PAPA NONOate, 67 μ M). *Inset*: 10x expansion of vertical scale.

Figure 3.

Influence of SOD on the luminescence kinetics of the NO-detection system. The samples contain all the components of luciferase-sulfonylase reaction mixture described in the legend to Figure 1 with addition of pyrophosphatase (2 mU) and sGC (50 ng). The kinetics were measured in the presence of SOD (50 U, solid lines) or its absence (dashed lines) and initiated by addition of the aliquots of anaerobically prepared solution of NO gas in buffer (final NO concentrations shown).

Figure 4.

Typical luminescence kinetics of the NO-detection system initiated by addition of NO gas in buffer (final NO concentrations shown). The NO-detection system contains all the components of luciferase-sulphydrylase reaction mixture described in the legend to Figure 1 with addition of pyrophosphatase (2 mU), sGC (50 ng) and SOD (50 U). Zero time points of kinetics after addition of NO are indicated by arrows.

Figure 5.

The dependence of the initial rate of the luminescence increase of NO-detection system on concentration of NO. The rates were calculated by fitting the first minute of kinetics with quadratic function and calculating the derivative at zero time point (see Figure 4 for the typical kinetics).

Figure 6. Luminescence kinetics of the NO-detection system in the presence of different concentrations of PAPA NONOate. The NO-detection system composition was the same as described in the legend to Fig. 4. Reaction was started by addition of PAPA NONOate.

Figure 7. Dependence of the initial acceleration of the luminescence of NO-detection system on the concentration of PAPA NONOate. The accelerations were calculated as a second derivative of the kinetic curve at zero time point (see Figure 6 for the typical kinetics).

Figure 8.
Dependence of maximal rate of luminescence increase of NO-detection system on PAPA NONOate concentration calculated from the kinetic curves (see Figure 6 for the typical kinetics).

Figure 9.

Luminescence kinetics of the NO-detection system in the presence of various amounts of iNOS. The NO-detection system composition was the same as described in the legend to Fig. 4 with addition of the components required for iNOS activity (arginine, 0.2 mM; NADPH, 0.2 mM; BH₄, 0.1 mM). Zero time points of kinetics after addition of iNOS are indicated by arrows.

Figure 10.

Dependence of the initial acceleration of the luminescence of NO-detection system (right axis) and rate of NO generation by iNOS (left axis) on the amount of iNOS present. Initial acceleration was calculated as a second derivative of the kinetic curve at zero time point (see Figure 9). The rate of NO production by iNOS was calculated based on the known rate of NO production by PAPA NONOate (see text for details).

Figure 11.

Luminescence of the luciferase reaction system in the presence of ATP (solid line) or GTP (dashed line). Indicated by arrows are time points of additions of (a) ATP (40 nM) or GTP (80 μM); (b) glucose (3mM) and (c) hexokinase (0.15 U). Reaction mixtures before additions contained 1 mM MgCl_2 , 1 mM DTE, 0.1 mM DTPA, 0.1 mg/mL BSA, 0.014 mM D-luciferin, 0.2 μg luciferase in 0.1 M Tris-HCl, pH 7.5.

Scheme 1.
General outline of the chemiluminescent detection of nitric oxide.