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Detection of S-nitrosocompounds using mid-IR cavity ringdown spectroscopy

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Abstract

S-nitrosocompounds have received much attention in biological research. In addition to their role as nitric oxide donors, there is a growing evidence that these compounds are involved in signaling processes in biological systems. Determination of S-nitrosylated proteins is of great importance for fundamental biological research and medical applications. The most common method to assay biological S-nitrosocompounds is to chemically or photochemically reduce SNO- functional groups to release nitric oxide that is then entrained in an inert gas stream and detected, usually through chemiluminescence. We report a method of S-nitrosocompounds detection using cavity ring-down measurements of gaseous NO absorbance at 5.2 μ m. The proposed method, in contrast to the chemiluminescence-based approach, can be used to distinguish isotopic forms of NO. We demonstrated sensitivity down to ~2 pmole of S-¹⁴NO groups and ~5 pmole of S-¹⁵NO groups for S-nitrosocompounds in aqueous solutions. The wide dynamic range of cavity ring-down detection allows the measurement of S-nitrosocompounds levels from pico- to nanomole amounts.

Keywords

S-nitrosocompounds; cavity ring-down spectroscopy; nitric oxide

The biological role of nitric oxide (NO) addition to heme groups in soluble guanylyl cyclase (sGC) and in hemoglobin has been well-understood for nearly two decades.^{1,2} Addition of NO to thiols is now appreciated to be an important signaling reaction – termed S-Nitrosylation – that is analogous to phosphorylation, glutathionylation, palmitoylation, acetylation and other physiological protein modifications.¹⁻⁶ There are many examples demonstrating that this type of signaling occurs in a broad range of biological systems.⁷⁻¹⁴ Moreover, disorders of protein S-nitrosylation are relevant to the pathophysiology of many

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Supporting Information Available. Schematics of the ringdown cavity, desorption kinetics of NO from the walls of the isolated cavity, and some characteristics of the spectrometer (dependence of laser frequency on PZT voltage, residence time measurements for constant flow, etc.). This material is available free of charge via the Internet at <http://pubs.acs.org>.

diseases,^{1,3-5,14-22} and S-nitrosylation is emerging as a field relevant to many biological disciplines.²³

In cell and organ systems, S-nitroso proteins level is normally in the range of ~2 pmole/mg protein²⁴, and unfortunately these levels are very close to the limit of sensitivity for most assay systems.²⁵ Moreover, even indirect methods of S-nitroso proteins detection relying on measurements of nitric oxide liberated after chemical or photochemical cleavage of S-NO bond have insufficient sensitivities restricting researcher to work mainly with cell lysates.

The most sensitive assays for S-nitrosylated proteins are based mainly on chemiluminescent detection of NO^{26,27} formed after the reduction of S-nitroso compounds.^{25,28-33} Current state-of-the-art chemiluminescence instruments (for instance, Eco Physics CLD 88) can detect nitric oxide down to a 0.05 ppb level. This technique is based on gas-phase reaction of NO with O₃ giving rise to NO₂* in the excited state, which is responsible for luminescence.^{34,35} However, chemiluminescence intensity is changed in the presence of other gases (for example, water, O₂, CO₂, etc.) that quench luminescence of NO₂*³⁶ and therefore this approach has strict requirements for gas mixture content and purity which may be difficult to satisfy during measurements of biological samples. Chemiluminescence cannot be used to distinguish isotopic forms of NO and thus cannot be used in isotopic labeling experiments. Poor stability of S-nitroso compounds and difficulties with selective reduction of SNO-groups lead to typical assay sensitivity of ~1-5 pmole^{4,25,31,37} while the limit of NO₂⁻ detection was reported to be ~0.12 pmole.³⁸ Similar sensitivity for nitrite detection was demonstrated recently³⁹ using GC/MS method. However, studies of S-nitrosylation signaling in biological systems require better sensitivity; the absence of reliable and sensitive methods of S-nitroso compounds detection in biological samples hampers research progress in this field.²⁵

Nitric oxide has strong ro-vibrational spectrum absorbance lines in the mid-IR range near 5.2 μ m (Fig. 1) and we believe that optical techniques based on absorbance measurements of NO in the gas phase have the potential to achieve improved sensitivity and accuracy in S-nitroso compounds detection. A number of approaches for the optical sensing of trace NO – absorption spectroscopy using multipass cells,⁴⁰ cavity-enhanced methods,^{38,41-43} photoacoustic, Faraday rotation⁴⁴ spectroscopies – have been reviewed recently.⁴⁵

It is important to note that sensitive detection of the absolute amount of NO (but not the NO concentration) is what we are interested in.

Cavity ring-down spectroscopy (CRDS) has been demonstrated to be a powerful method of trace gas detection with ultra high sensitivity and specificity.⁴⁷ The CRDS technique determines the optical absorption of a sample contained inside a low-loss optical cavity from an increase in the decay (“ringdown”) rate of light leaking through the cavity as it travels between highly reflective mirrors (reflectivity $R > 99.9\%$). Use of low loss and thus high finesse cavities allows an increase in the effective pathlength of light in the sample to up to several kilometers. Additionally, intensity fluctuations of the light source do not influence measurement accuracy since changes in the decay lifetime of light in the ring-down cavity (rather than changes in light intensity) are detected. Likewise, analyte absorption outside of

the sample volume does not contribute to the observed concentration. The first CRDS measurements used pulsed light sources with broad spectral width,^{48,49} but another version that utilizes tunable continuous-wave (cw) lasers – cw-CRDS – allows one to realize greater stability in the shot-to-shot fluctuations of the cavity decay rate and this translates into improved sensitivity.^{50,51} In favorable cases, parts per trillion by volume (pptv) level^{52,53} sensitivity has been demonstrated.

Use of cw-CRDS to detect nitric oxide in the mid-IR range were reported previously. Kosterev et al.⁴¹ demonstrated NO detection at parts per billion by volume (ppbv) concentrations with a 0.7-ppbv standard error for 8 s integration time using a cw distributed feedback quantum cascade (QC-DFB) laser. Murtz et al.^{42,54} reported use of a CO sideband laser for ultrasensitive detection of NO isotopologues with sensitivity down to $2 \times 10^{-10} \text{ cm}^{-1} \text{ Hz}^{1/2}$ and demonstrated its application in human breath analysis,⁴³ with a reported noise equivalent detection limit of 7 pptv of ^{15}NO with 70 sec of integration time. In this work, we report similar detection limits but using a laser system that is more robust and portable. The developed method allows the detection of isotopologues of S-nitrosocompounds with sensitivities (3σ) $\sim 1.5 \text{ pmole S}^{14}\text{NO}$ and $\sim 5 \text{ pmole S}^{15}\text{NO}$ groups.

Methods and instrumentation

Figure 2 shows a schematic of our cw-CRD spectrometer that is used to detect trace NO gas released after reduction of SNO- functional groups. A cw, water-cooled, external cavity quantum cascade laser (Daylight Solutions, 21052-MHF) operating at $5.2 \mu\text{m}$ is used as the excitation source. It has a specified mode hop free tuning range of $1880\text{--}1940 \text{ cm}^{-1}$ with output power higher than 30 mW throughout this range ($\sim 60 \text{ mW}$ at 1905 cm^{-1}). The laser can be scanned and coarse-tuned over the entire spectral range using the supplied laser controller, which drives a stepping motor that controls the intracavity grating. Fine-tuning of the laser frequency ($\sim 1 \text{ cm}^{-1}$) was implemented by applying voltage (up to 100 V) to a piezoelectric transducer (PZT) that moves the same grating.

The laser beam is sent to an acousto-optic modulator (AOM) (Gooch&Housego, R37040-3-5.4) in a double-pass configuration which is used to shut off the laser light on a trigger pulse generated after sufficient intensity builds up inside the ring-down cavity. The pulse also initiates each measurement of the cavity loss rate. The AOM is driven by a 40 MHz RF oscillator (NeOS / Gooch & Housego) and the first order diffracted beam (diffraction efficiency $d_{\text{eff}} = 0.69$) after two passes through the AOM is sent to the ring-down cavity. We have measured that the first order beam diffracted through the AOM is attenuated with 1/e decay time of $\sim 150 \text{ ns}$ when the control voltage to the RF driver is switched off. One challenge in cw-CRDS with a linear cavity is that light reflected from the front mirror of the cavity (which is usually most of the incident light) can travel back to the laser, leading to frequency instabilities due to optical feedback. The AOM imposes a frequency shift (80 MHz in double pass configuration) on the first order diffracted beam that is doubled for the light that returns from the cavity to the laser, making the returned beam effectively incoherent compared to light in the laser. In addition, the reflected beam intensity was attenuated by a factor of d_{eff}^4 by the AOM on one round trip. Despite this, we observed evidence of the back reflection of the cavity pulling the laser frequency if RF power

corresponding to maximum diffraction efficiency is applied to the AOM, but this can be effectively eliminated by attenuation of RF power going to the AOM by 4-5 dB which decreases d_{eff} . Two uncoated CaF_2 lenses with focal lengths 10 and 7.5 cm were used as a telescope for matching the transverse beam profile of the input laser to the fundamental transverse mode (TEM_{00}) of the ringdown cavity.

A HeNe laser and a pyroelectric beam profiling camera Pyrocam (Spiricon) were used to facilitate aligning of the spectrometer optical components.

The cavity is mounted inside a vacuum-tight cell with CaF_2 windows. The ringdown cell is home-made (Fig. S1) and consists of two stainless steel kinematic mirror mounts attached to flexible hoses (275×275-R-MH4, LDS Vacuum shopper) and reducing cross (275×NW16-RWX, LDS Vacuum shopper) using 2.75" Conflat connections. NW16 flanges of the reducing cross were used as inlet and outlet gas ports of the ringdown cavity. The mirror mounts are mounted on a super-invar plate to reduce thermal drift of the cavity. The high-finesse ($F \sim 10000$) linear optical cavity is formed by a pair of wedged ZnSe supermirrors, one flat and the other concave with a 0.5 m radius of curvature. The mirrors were custom coated by LohnStar with a specified $R = 99.975\%$ at $5.2 \mu\text{m}$ on one side and an AR coating on the backside. Measurements of a ringdown time for an evacuated cavity showed that actual reflectivity of the mirrors at 1900.50 cm^{-1} is equal to 99.972% and mirror transmission $T = 0.020\%$ was also measured. The mirrors are separated by $\sim 35 \text{ cm}$. Scanning the NO absorption spectrum while monitoring the cavity transmission allowed us to determine a cavity free spectral range (FSR) of 427.6 MHz with 0.2% accuracy. The transverse mode spacing was calculated as 135 MHz and the TEM_{00} mode has a calculated beam waist radius of $\omega_0 = 0.69 \text{ mm}$ at 1900 cm^{-1} . The internal diameter of the cell is $\sim 3 \text{ cm}$ and was designed to allow switching from a linear cavity to a 3 mirror "V" shaped cavity or a 4 mirror ring cavity for the purpose of reducing destabilizing feedback to the laser head (Fig. S1). In order to minimize adsorption of nitric oxide onto the cavity cell walls, metal surfaces were coated with an inert layer of SilcoNert 2000 (SilcoTek).

To ensure laser light would couple into the TEM_{00} cavity mode, the position of the plane mirror and therefore cavity length were changed by applying a sine-wave voltage in parallel to three PZTs (AE0505D08F, Thorlabs) attached to the mirror mount using function generator (HP3312A, Agilent) and piezo driver (P-863, Physik Instrumente). The amplitude of the mirror motion was selected to scan slightly over FSR of the ringdown cavity, ensuring that at least one TEM_{00} mode will pass through resonance with the laser on each half cycle of the modulation. The frequency of the cavity length modulation was in the range 20-40 Hz, well below mechanical resonance conditions seen above 80 Hz. This is also sufficiently slow to ensure the cavity excitation reaches steady-state.

The IR radiation transmitted through the cavity was focused, using an off axis parabolic mirror, and detected with a fast (rise time $< 100 \text{ ns}$) liquid nitrogen-cooled InSb detector (KISDP-0.5-FJ/GE, Kolmar) with photosensitive area $S = 0.5 \text{ mm}^2$. The detector signal was amplified by a matched DC to 20 MHz transimpedance preamplifier (Kolmar).

When the detector signal exceeds a specific threshold level (typically +1.5 V) a trigger pulse is generated by a pulse generator (DG535, Stanford Research Systems) and this is used to switch-off the AOM for 40 μ s (approx. 10 times the ringdown time) and to initiate acquisition of the ringdown signal by a 100MHz 14-bit USB oscilloscope (HS5-530XM, Tiepie, Netherlands). A 12-bit digitizer board (PCI-DAS4020/12, Measurement Computing) was used to monitor pressure and record signals from etalon and reference cell detectors. Software for instrument control, data processing and analysis is developed using Delphi 7.0 programming language. Levenberg-Marquardt algorithm^{55,56} was used for non-linear least squares fitting of experimental data to exponential model.^{57,58}

It was found that the fine-tuning system of the laser frequency using the PZT attached to external cavity grating has a non-linear dependence of detuning vs. applied voltage (Fig. S2, see Support Information) and absolute value calibration of wavenumber scale is needed.

A scanning internally-coupled Fabry-Perot etalon with finesse $F \sim 21$, similar to one described earlier,⁵⁹ was developed for this purpose using 2 spherical gold mirrors (radius of curvature 50 mm) in a confocal configuration for ease of alignment and mode spacing. One of its mirrors was attached to PZT and ramped by a Burleigh PZT driver (model #RC-43) at 50 Hz. A special circuit box (Wavelength Monitor, designed by W.S. Woodward for the group of R.E. Miller) was used to track the PZT voltage where the maximum etalon transmission signal occurred which was used to monitor the laser frequency position within one etalon FSR at millisecond timescale. Non-linearity of the mirror displacement on PZT voltage of the etalon was corrected by calibration with a HeNe laser. The etalon FSR of 749.3 MHz was measured using the frequencies of ¹⁴NO Q-branch lines from HITRAN database.^{46,60}

In addition, a reference gas cell filled with ~ 200 mTorr ¹⁴NO (or ¹⁵NO) was used for calibration of the spectrometer frequency scale and for locking of laser frequency to an absorption line of nitric oxide. To increase accuracy of gas cell transmission measurements, the light intensity was modulated by a chopper with $f = 200$ Hz and a lock-in amplifier (SR-510, Stanford Research Systems) was used to demodulate the signal.

Locking of the laser frequency to the ¹⁴NO R_{3/2}(6.5) transition line at 1900.5 cm⁻¹ and the ¹⁵NO R_{3/2}(18.5) line at 1900.4 cm⁻¹ was achieved using a 1f modulation technique. Laser frequency was modulated by 14 MHz peak-to-peak by applying a 100 Hz sinusoidal voltage to the laser PZT. The lock-in output signal was read by a 12-bit digitizer and used for stabilization of the laser frequency on the absorbance line by changing the DC voltage offset on the laser PZT. We found that laser frequency was reliably locked to NO line transition within ± 2 MHz at millisecond time scale.

Figure 3 shows a schematic of the gas sampling system for S-nitrosocompound detection. S-nitrosocompounds solution is injected through the septum port into the sample chamber and UV irradiation or chemical reaction with Cu(I)/Cys are used to cleave S-NO bond and release nitric oxide gas, whose concentration is measured by the CRDS-instrument. Carrier gas with a constant flow controlled by a mass-flow controller (5850E, MKS) is bubbled through the sample solution and facilitated NO diffusion to the ringdown cavity.

Temperature of the glass cold trap was maintained at ~195K (with a cryogenic bath of dry ice and ethanol) to remove water vapor. The ringdown cell pressure was regulated by a needle valve between the cell and pump and was monitored using a 100 torr capacitance pressure gauge (MKS). The total volume of the sample chamber, connecting tubings, cold trap, and the cavity was determined to be ~730 ml. Residency time for a molecule is proportional to pressure inside the cavity. For a 116 sccm (standard cm³ per minute) He flow and 20 torr cavity pressure, a residence time $\tau_{\text{res}} \sim 9.2$ s was determined. Higher flow rates of the carrier gas did not provide good mixing with NO and uniform distribution of gas mixture in the cavity.

Samples preparation

Glutathione (GSH), L-cysteine, copper (I) chloride, NaNO₂, Na¹⁵NO₂, N-(1-naphthyl)ethylenediamine dihydrochloride (NED), sulfanilamide, HgCl₂, KI, bovine serum albumin (BSA) were purchased from Sigma-Aldrich. Sulfuric acid was from Mallinckrodt Chemicals (USA). Nitric oxide (>99.5%) was purchased from GTS-Welco (USA). Aqueous solutions were freshly prepared using milliQ water.

¹⁴N, ¹⁵N- labeled S-nitrosoglutatione (GSNO) were prepared by acid-catalyzed nitrosation of GSH according to the method described previously.⁶¹ GSNO concentration were measured spectrophotometrically using molar extinction coefficient 922 M⁻¹cm⁻¹ at 335 nm.^{61,62}

S-nitrosoalbumin was synthesized according to the method reported earlier⁶³ without preliminary reduction of disulfide bonds. SNO-groups content in the protein was determined using the Saville assay.⁶⁴ The copper/cysteine solution for SNO-groups reduction was prepared as described earlier.⁶⁵

¹⁵NO was synthesized by reduction of Na¹⁵NO₂ in acidic conditions. Briefly, a 120 ml screw-cap septum bottle (23230-U, Supelco) was connected to two cold traps in series maintained at 196 K and 77 K respectively and evacuated using a mechanical pump. 0.4 ml 0.2 M Na¹⁵NO₂, 1 ml 0.2 M KI, and 1 ml of concentrated H₂SO₄ were injected through the septum to the glass bottle under evacuation. Water vapor and residual NO₂ were intercepted by the first cold trap, while ¹⁵NO was condensed in the trap maintained at 77K.

Results and discussion

We examined measurement statistics of the CRDS-spectrometer and estimated experimentally achievable limit of detection for NO concentration based on noise level and temporal stability of ringdown times for the empty cavity. Allan deviation (a criterion first developed to quantify stability of clocks and oscillators⁶⁶) of absorption coefficient (σ_a) was used to identify the optimal measurement time that can be utilized for data averaging, i.e. integration over this period leads to increase of signal-to-noise ratio. Figure 4a shows that for an optimum averaging time of 200 s the minimum detectable absorption coefficient (1σ) was 3.7×10^{-10} cm⁻¹. This corresponds to a minimal detectable concentration 5×10^7 cm⁻³ of ¹⁴NO molecules in helium at 20 torr (77 pptv) or ~0.06 pmole ¹⁴NO inside the cavity

with 730 ml volume. Ringdown time $t_0 \sim 4.2 \mu\text{s}$ (Fig.4b) in absence of NO indicates that the effective pathlength of light travelling between the cavity mirrors is $\sim 1.2 \text{ km}$.

We considered two approaches to quantify the amount of NO released from S-nitrosothiols. Our initial approach was to evacuate the ring-down cavity and sample chamber, isolate them from pump, and initiate NO release from the sample by chemical or photochemical reaction and to fill the ring-down cavity. To ensure uniform distribution of NO in the cavity, cold trap, and sample chamber a short pulse of He carrier gas was injected which facilitated transport of NO and increased total pressure (normally up to 2-5 torr). Then measurements of NO in gas phase were carried out using the cavity ring-down technique. This approach potentially allows simultaneous measurement of absolute concentration of ^{14}NO and ^{15}NO from the same sample and, from the known volume of gas sampling system (730 ml), one can determine the absolute amount of reduced SNO-groups. It also would minimize the dilution of the generated NO in the carrier gas and also allow measurements to be averaged to achieve whatever signal to noise ratio is required, down to a minimum determined by long term drift in the instrument baseline.

Unfortunately, we have found that this technique does not provide reproducible results of S-nitrosothiols concentration due to significant (~ 3 times) and non-controlled losses of NO (Fig.5). We believe that the losses are mainly related to sticking of NO to the surfaces of the cavity and gas sampling system. Extensive literature on nitric oxide absorption on metal surfaces can be found elsewhere ⁶⁷⁻⁶⁹. Slow desorption of NO isotopologues adsorbed onto the cavity walls were observed in the isolated and preliminary evacuated ringdown cavity (Fig. S3). Passivation of the cavity metal parts with a chemically inert SilcoNert coating did not eliminate the problem with the losses.

More encouragingly, we found that we can reproducibly measure NO concentration when a constant flow of calibrated gas mixture (0.725 ppm ^{14}NO in He) was established through the cavity (Fig.6). The limit of detection obtained by monitoring the ^{14}NO $R_{1/2}(6.5)$ transition at 1900.5 cm^{-1} was determined to be $4 \times 10^9 \text{ cm}^{-3}$ (at $\sim 0.2 \text{ s}$ integration time), that corresponded to $\sim 5 \text{ pmoles}$ of nitric oxide in 0.73 L volume (Fig.6). The negative value of linear fit intercept shifted from zero by more than 3σ may indicate manifestation of saturation effects of optical transition at low pressures.

Therefore, we found that only measurements in flow mode could provide reliable quantitative information of NO concentration and this approach was implemented for S-nitrosothiols detection.

Detection of NO and S-nitrosothiols in flow mode

For these measurements, a constant flow of carrier gas was established through the sample chamber and the optical cavity; steady-state pressure inside the cavity was adjusted using the needle valve (Fig. 3). Laser frequency was locked to the maximum of NO absorbance line. For ^{14}N -labeled NO and SNO-group containing compounds detection the $R_{3/2}(6.5)$ transition of ^{14}NO located at 1900.5 cm^{-1} was used. Changes of the cavity ring-down time were monitored in time after sample injection through the septum into the sample chamber.

Change in ringdown decay rate $k=1/\tau$ is proportional to concentration $N(t)$ of NO molecules in the cavity

$$k(t) = 1/\tau(t) = cN(t)\sigma_p + k_0, \quad (1)$$

where c - speed of light, σ_p - absorption cross-section of NO in helium at center of the transition at pressure p , and k_0 - the empty cavity loss rate which is determined before NO measurement. The pressure broadening coefficient of NO line by helium is needed for σ_p calculation when pressure broadening makes a significant contribution to the lineshape. The measured pressure broadening coefficient of lorentzian half-width at half-medium for ^{14}NO $R_{3/2}(6.5)$ transition $b=6.9\times 10^{-5} \text{ cm}^{-1}\text{torr}^{-1}$ was in good agreement with the data reported earlier.⁷⁰

One can estimate optimal pressure of carrier gas to maximize sensitivity of CRDS measurements in the flow mode. Signal-to-noise ratio is proportional to $\sigma_p \sqrt{N_m}$, where N_m - number of measurements to be averaged. N_m is proportional to the residence time τ_{res} of a molecule inside the ringdown cavity, which for constant gas flow has a linear dependence on pressure (Fig. S4). Therefore, the signal-to-noise ratio will have the following dependence

$$S/N \sim \sigma_p \cdot \sqrt{p} \quad (2)$$

Taking into account that the pressure broadening coefficient for ^{14}NO $R_{3/2}(6.5)$ transition one can find that optimal pressure for the flow mode measurements will be ~ 20 torr (Fig. S5).

Reproducibility of repeated injections (100 μl) of ^{14}NO using a gas-tight syringe is shown in Fig.7. Peaks were fitted using Exponentially Modified Gaussians (convolution of Gaussian with exponential)

$$EMG(t) = \frac{Area}{\sigma \sqrt{2\pi}} \exp\left(-\frac{(t-t_0)^2}{2\sigma^2}\right) \otimes \tau^2 \exp(-t/\tau^0), \quad (3)$$

where τ^0 corresponded to the residence time of NO molecules inside the cavity (Fig. S6). We found that for the constant flow of 116 sccm through the gas sampling system of 0.73 L at 20 torr pressure the residence time was ~ 9.2 s.

A relative error of 2% in the determination of the peak area was observed and indicated that measurement-related uncertainty was comparable to precision of sample volume injection using the syringe (specified as $\sim 1\%$).

Measurements of S-nitrosothiols were carried out in a similar way but with the SNO-group reducing solution of Cu(I)/Cys (~ 5 ml) placed in the sample chamber before the injections. After injecting small amounts of GSNO, where the timescale of the NO release reaction was less than ~ 1 s, the shape of the peaks observed could be fairly described using EMG function (3) with residence time ~ 9 -10 s. For higher amounts of injected S-

nitrosocompounds the shape was distorted due to finite time of SNO-groups reduction and it was fitted to a linear combination of EMG functions.

Calibration curves for ^{14}N -labeled S-nitrosocompounds detection using Cu(I)/Cysteine assay are shown in Fig.8. We estimated an uncertainty in the concentration of prepared standards to be $\varepsilon \approx 5\%$ and corresponding weight factors were assigned to data points. Figure 8a shows good linearity for a wide dynamic range (more than 2 orders of magnitude) of GS ^{14}NO amounts with the limit of detection (3σ) ~ 1.5 pmoles. A similar limit of detection ~ 2 pmole was found for BSA-S ^{14}NO (Fig. 8b). The presence of protein introduced some problems during the measurements due to foaming of the solution in the sample chamber as carrier gas bubbled through it. However addition of antifoam compound to the solution helped to minimize this effect.

Analogous dependence for GS ^{15}NO (Fig.9) shows a slightly worse sensitivity of ~ 5.4 pmole which is explained by ~ 4 times difference in line strengths of ^{14}NO and ^{15}NO transitions in this range.

We demonstrated that cavity ring-down spectroscopy method of S-nitrosocompounds detection in aqueous solutions has similar sensitivity as state-of-the-art reduction-chemiluminescence technique and, additionally, allows to distinguish $^{14}\text{N}/^{15}\text{N}$ -labeled S-nitrosocompounds. It is noteworthy that utmost sensitivity of the proposed method is determined by quality of the supermirrors of the ringdown cavity. Because of the importance of this emerging field to medicine and experimental biology, assays with improved sensitivity can be predicted to be important for diagnosis and monitoring of a wide range of biological fluids, including blood, urine, cerebrospinal fluid, bronchoalveolar lavage fluid.

In distinction to chemiluminescent method the developed technique relies on absorbance measurements of nitric oxide within its rovibrational spectrum. In order to ensure specificity of NO detection the absorbance line used for laser frequency locking and detection should not overlap with absorption lines of any volatile impurities that may be present in biological mixtures (mostly water and CO_2). This condition can be easily implemented because of narrow widths of molecular absorption lines in gas phase at low pressures, and the ^{14}NO $R_{3/2}(6.5)$ transition line at 1900.5 cm^{-1} and the ^{15}NO $R_{3/2}(18.5)$ line at 1900.4 cm^{-1} have been selected for detection that are located far from water or CO_2 absorbance lines.

Other possible sources of artefacts that may affect reliability and performance of CRDS technique are: 1) pressure instability in the ringdown cavity during the sample injection (however, these cases can be easily identified by monitoring pressure during the measurements) and 2) presence of volatile compounds (for example ethanol) in high amounts (more than 1 mL) in the sample chamber. In the latter case the volatile compound vapor entrained by carrier gas may not be completely intercepted by the cold trap at -80°C and can condense and produce aerosole in the cavity resulting in broad-band optical losses due to scattering. Effect of broad-band optical losses can be mitigated by periodic measurements of ringdown time “on” and “off” the absorbance peak at some cost in sensitivity, however occurrence of this situation is very unlikely since sample injection volume normally does not exceed 100 μL .

Conclusions

Here we report a cw-CRD spectrometer for S-nitroso compounds detection with limits of detection ~2 pmole for S-¹⁴NO and ~5 pmole for S-¹⁵NO groups of proteins. We have found that quantitative measurements are possible only in a flow mode. The dynamic range of S-nitrosothiols measurements reaches 3 orders of magnitude and extends from pico- to nanomoles. The advantages of the proposed method to assay S-nitrosylated proteins are: 1) high sensitivity and large dynamic range; 2) ability to detect and metabolically track ¹⁵N-labeled S-nitrosothiols in cells and organisms.

We believe that making use of long integration time available for averaging and increasing data acquisition rate will result in sensitivity improvement of the CRDS-spectrometer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Simulation of absorption spectra for NO isotopologues and H_2O in helium (100 torr) using the data from the HITRAN-database.⁴⁶ Different wavenumber ranges are shown

Figure 2.

Principal scheme of cw-CRDS instrument for ultrasensitive detection of S-nitrosocompounds. EC-QCL – external cavity quantum cascade laser, PZT- piezoelectric transducer, AOM- acousto-optical modulator

Figure 3.
Schematic of gas sampling system. MFC – mass flow controller

Figure 4.

A) Allan deviation plot of absorbance coefficient at 1900.5 cm^{-1} vs time in absence of NO. Flow of He (116 sccm) was established through the ringdown cavity at 20.3 torr pressure. Minimum detectable absorption coefficient of $3.7 \times 10^{-10} \text{ cm}^{-1}$ can be reached in $\sim 200 \text{ s}$ integration time. B) Distribution of the ringdown times is shown.

Figure 5. Detected amount of ^{15}NO (A) and ^{14}NO (B) released from corresponding N-labeled GSNO in reaction with chemical reducer (Cu(I)/Cys assay). Dotted line shows predicted amount of NO isotopologues based on GSNO concentration.

Figure 6.

Plot of experimental concentration of ^{14}NO for different pressures of 0.72 ppm NO in He measured in a flow mode. Constant flow of 17 sccm was established through the cavity. Limit of detection is $4 \times 10^9 \text{ cm}^{-3}$ or $\sim 5 \text{ pmole}$ of ^{14}NO for 0.73 L volume.

Figure 7.

Reproducibility of 100 μl injections of the same ^{14}NO amount measured using the flow mode. Variability in areas of the peaks does not exceed 2 %. Carrier gas flow 116 sccm, pressure 30 torr. SD- standard deviation.

Figure 8.
Limit of GS¹⁴NO (A) and BSA-S¹⁴NO (B) detection using CRDS-technique. Carrier gas flow 116 sccm, pressure – 20 torr.

Figure 9.

Limit of GS¹⁵NO detection using CRDS technique. Carrier gas flow 116 sccm, pressure – 20 torr. Laser was locked to the ¹⁵NO transition at 1899.4 cm⁻¹.