

# Quantitative detection of hydrogen peroxide in rain, air, exhaled breath, and biological fluids by NMR spectroscopy

Tayeb Kakeshpour<sup>a</sup>, Belhu Metaferia<sup>a</sup>, Richard N. Zare<sup>b</sup>, and Adriaan Bax<sup>a,1</sup>

<sup>a</sup>Laboratory of Chemical Physics, National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, MD 20892; and <sup>b</sup>Department of Chemistry, Stanford University, Stanford, CA 94305

Edited by Chad Mirkin, Department of Chemistry, Northwestern University, Evanston, IL; received November 27, 2021; accepted January 14, 2022

**Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) plays a key role in environmental chemistry, biology, and medicine. H<sub>2</sub>O<sub>2</sub> concentrations typically are 6 to 10 orders of magnitude lower than that of water, making its quantitative detection challenging. We demonstrate that optimized NMR spectroscopy allows direct, interference-free, quantitative measurements of H<sub>2</sub>O<sub>2</sub> down to submicromolar levels in a wide range of fluids, ranging from exhaled breath and air condensate to rain, blood, urine, and saliva. NMR measurements confirm the previously reported spontaneous generation of H<sub>2</sub>O<sub>2</sub> in microdroplets that form when condensing water vapor on a hydrophobic surface, which can interfere with atmospheric H<sub>2</sub>O<sub>2</sub> measurements. Its antimicrobial activity and strong seasonal variation speculatively could be linked to the seasonality of respiratory viral diseases.**

hydrogen peroxide | microdroplet | exhaled breath condensate | NMR spectroscopy | respiratory droplet

Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is a vital chemical oxidant that plays key roles in many aspects of life, ranging from environmental chemistry to human health. Levels in blood hold diagnostic potential for thyroid disease (1) and have been linked to other maladies, including Alzheimer's disease (2) and cancer (3). Up-regulation of H<sub>2</sub>O<sub>2</sub> production by neutrophils and macrophages in the respiratory epithelium in response to inhaled pathogens mediates inflammation by activating nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a key regulator of the innate immune system (4). Intriguingly, the enzyme myeloperoxidase, whose production is also up-regulated by neutrophils upon infection, converts H<sub>2</sub>O<sub>2</sub> in the presence of chloride into hypochlorite, which may act directly on the pathogen but also has been implicated in the pathogenesis associated with the acute respiratory distress syndrome (5). Elevated levels of H<sub>2</sub>O<sub>2</sub> in exhaled breath condensate (EBC) have been linked to asthma and chronic obstructive pulmonary disease (6). However, detection of H<sub>2</sub>O<sub>2</sub> in breath can be impacted by H<sub>2</sub>O<sub>2</sub>'s variable presence in the atmosphere (7).

Quantitative detection of trace amounts of H<sub>2</sub>O<sub>2</sub> commonly relies on the formation of fluorescent products (8), a process that can suffer from chemical interference, such as quenching, incomplete reactions, or reaction with other reactive oxygen species, which may have contributed to large discrepancies in previously reported values. For example, H<sub>2</sub>O<sub>2</sub> concentrations in blood range anywhere from 0.2 nM to 100 mM (9), and values reported for rain (0.03 μM to 199 μM) and ground-level air (0.02 parts per billion by volume [ppbv] to 180 ppbv) also cover nearly four orders of magnitude (7). The recent discovery of spontaneous generation of levels as high as 100 μM H<sub>2</sub>O<sub>2</sub> upon condensation of water vapor into microdroplets (10) also may interfere with quantitation. Indeed, the very wide range of H<sub>2</sub>O<sub>2</sub> concentrations reported for EBC may also include generation of H<sub>2</sub>O<sub>2</sub> in microdroplets formed in the collection device.

We employ NMR spectroscopy to readily quantify H<sub>2</sub>O<sub>2</sub> down to submicromolar concentrations. NMR has been used

previously for H<sub>2</sub>O<sub>2</sub> quantification (11–13), but not for detecting trace amounts. Here, we report its application to samples of interest in medicine and atmospheric chemistry. By taking advantage of fast hydrogen exchange (HX) of H<sub>2</sub>O<sub>2</sub> hydrogens with those of water, the speed of NMR data acquisition can be increased to *ca.* 1,000 scans per minute (14). When combined with improvements in NMR hardware, we achieve a strong enhancement in the sensitivity of H<sub>2</sub>O<sub>2</sub> detection, enabling quantitative measurement in biological fluids.

## Results and Discussion

First, we demonstrate NMR measurement of H<sub>2</sub>O<sub>2</sub> in EBC and how microdroplet formation on the hydrophobic surface of the collection device contributes to the measured concentration. We used a commercial R-tube EBC collection device (<https://respiratoryresearch.com/rtube/>) where water vapor condenses on the inside of a cooled plastic tube. At the start of the condensation process, microdroplets form on the hydrophobic, initially dry inner surface of the R-tube cylinder. Analogous formation of microdroplets on a hydrophobic surface yields H<sub>2</sub>O<sub>2</sub> concentrations in water as high as 100 μM (10), considerably higher than values expected for EBC. However, the initial total volume of microdroplets associated with H<sub>2</sub>O<sub>2</sub> generation, V<sub>1</sub>, is small, and, once droplets become larger, spontaneous H<sub>2</sub>O<sub>2</sub> generation decreases to zero (10). Therefore, subsequent condensation dilutes this initial burst of H<sub>2</sub>O<sub>2</sub> with water vapor that contains true EBC levels of H<sub>2</sub>O<sub>2</sub>.

Fig. 1A displays this behavior: The H<sub>2</sub>O<sub>2</sub> concentration in EBC rapidly decreases to <1 μM with increasing numbers of breaths and total collected condensate. Under these conditions, the averaged concentration, [H<sub>2</sub>O<sub>2</sub>], can be written as

$$[\text{H}_2\text{O}_2] = (C_1V_1 + C_2V_2)/(V_1 + V_2) \approx N_1/V_2 + C_2, \quad [1]$$

in which C and V denote H<sub>2</sub>O<sub>2</sub> concentration (moles per liter) and condensate volume (liter); subscripts 1 and 2 refer to the microdroplets initially formed on the surface of the R tube, and subsequently formed by additional vapor condensation; and N is the amount (moles). Because V<sub>2</sub> is much larger than V<sub>1</sub>, and the minimum amount of liquid that can reproducibly be harvested from the device is *ca.* 25 μL, independent determination of C<sub>1</sub> and V<sub>1</sub> is imprecise. Only the product C<sub>1</sub>V<sub>1</sub> = N<sub>1</sub> and the EBC H<sub>2</sub>O<sub>2</sub> concentration can be reliably extracted from the data, yielding values of N<sub>1</sub> = 104 ± 5 pmol and C<sub>2</sub> = 0.16 ± 0.06 μM when H<sub>2</sub>O<sub>2</sub>-free dry air (relative humidity [RH] < 1%) was

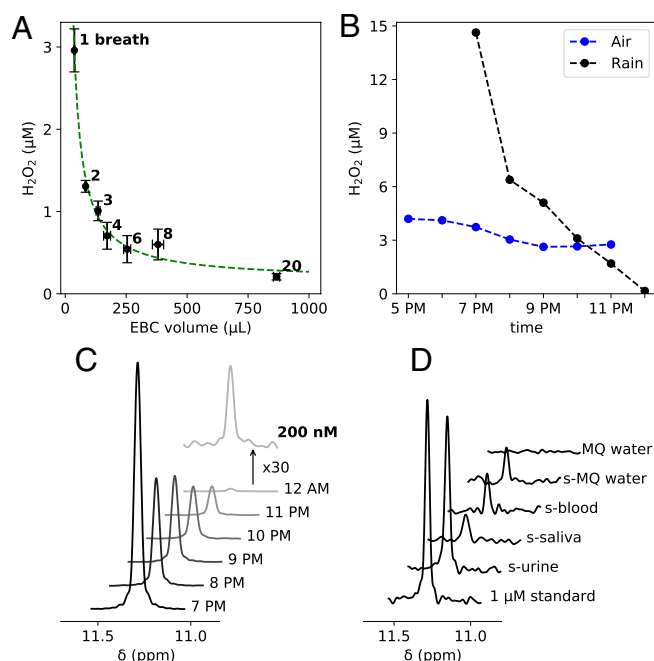
Author contributions: T.K., B.M., and A.B. designed research; T.K. and B.M. performed research; T.K. analyzed data; and R.N.Z. and A.B. wrote the paper.

The authors declare no competing interest.

This open access article is distributed under [Creative Commons Attribution License 4.0 \(CC BY\)](https://creativecommons.org/licenses/by/4.0/).

<sup>1</sup>To whom correspondence may be addressed. Email: bax@nih.gov.

Published February 14, 2022.



**Fig. 1.** NMR detection of H<sub>2</sub>O<sub>2</sub> in various fluids. (A) Dependence of H<sub>2</sub>O<sub>2</sub> concentration in EBC on the collected volume. The green dashed line represents the best fit to Eq. 1. (B) H<sub>2</sub>O<sub>2</sub> levels in rainwater and samples condensed from ambient air on April 24, 2021. (C) NMR spectra of the rainwater samples. (D) NMR spectra of a 1-μM standard sample, sublimed (s-)urine, s-saliva, s-blood, s-MQ water, and MQ water.

inhaled. The surface area of the inside of the R tube is 123 cm<sup>2</sup>, and a rough estimate for the aqueous volume of the microdroplets, V<sub>1</sub>, formed on the inside of the R tube considers their total volume equivalent to that of a homogeneous water layer of 1-μm thickness, yielding C<sub>1</sub> = 104 pmol/12.3 μL = 8.5 μM. This value is in fair agreement with exhaling a single shallow breath into a falcon tube (polypropylene) followed by centrifuging the condensation fog to the bottom (repeated 20 times), which yielded 12.7 μM H<sub>2</sub>O<sub>2</sub>. The H<sub>2</sub>O<sub>2</sub> level of EBC may also depend on its concentration in the inhaled air source (15). The concentration of H<sub>2</sub>O<sub>2</sub> in the condensate from 20 breaths using H<sub>2</sub>O<sub>2</sub>-free dry air was 0.21 ± 0.04 μM, slightly smaller than the 0.27 ± 0.05 μM value obtained using ambient air (RH 25%) that contained 4 μM H<sub>2</sub>O<sub>2</sub> in its condensate.

The same R-tube device was used for quantifying H<sub>2</sub>O<sub>2</sub> in ambient air. We slightly modified the commercial R-tube device (details in *Materials and Methods*), which allowed us to pass a constant airflow through it while the aluminum cooling sleeve was in contact with dry ice. On April 21, 2021, we started collecting indoor air samples about 2 h before the forecasted rain. From 5 PM to 6 PM (Fig. 1B), the air H<sub>2</sub>O<sub>2</sub> level was constant at around 4.2 μM. However, as soon as rain started around 6 PM, it decreased to 2.6 μM over the course of 2 h. We were able to collect the first rain sample at 7 PM briefly after the rain started. This sample contained 14.6 μM of H<sub>2</sub>O<sub>2</sub>, but it decreased to as low as 0.2 μM at its endpoint (Fig. 1B and C). Such high levels of H<sub>2</sub>O<sub>2</sub> in the initial stages of rain and its subsequent decline agree with prior observations (16) and mirror a rain sample collected at the start of Hurricane Ida (September 1, 2021; Bethesda, MD) that contained 56.5 μM H<sub>2</sub>O<sub>2</sub>.

Arguably, blood represents the most important but also the most challenging fluid for quantitative measurement of [H<sub>2</sub>O<sub>2</sub>]. Its relatively high ionic strength, including HX-catalyzing phosphate ions that hinder NMR observation, and chemical substances, including the enzymes catalase and glutathione peroxidase that regulate [H<sub>2</sub>O<sub>2</sub>], can interfere with the mea-

surement. In addition, conversion of superoxide into H<sub>2</sub>O<sub>2</sub> by superoxide dismutase can increase [H<sub>2</sub>O<sub>2</sub>]. To avoid disturbing the equilibrium [H<sub>2</sub>O<sub>2</sub>] in blood, we fast froze a freshly collected (<3 min) human blood sample into liquid isopentane at ca. −140 °C. The water and H<sub>2</sub>O<sub>2</sub> were then sublimed from the blood while keeping the blood frozen, thereby also removing molecules with functional groups that promote HX (*Materials and Methods*). Human whole blood contains 77 to 82% water by weight, and we sublimed 78% of the whole blood mass. The deposited water on the cold finger contained 0.19 μM H<sub>2</sub>O<sub>2</sub> (Fig. 1D), considerably lower than most literature values (9). However, sublimation of the same volume of milli-Q (MQ) water under identical conditions yields a very similar H<sub>2</sub>O<sub>2</sub> concentration (Fig. 1D and Table 1), indicating that, analogous to vapor condensation (10), sublimation also generates H<sub>2</sub>O<sub>2</sub>. Therefore, the true blood H<sub>2</sub>O<sub>2</sub> level must have been considerably below its measured value. Similarly, a sublimed sample of saliva contained about the same amount of H<sub>2</sub>O<sub>2</sub>, suggesting an upper limit of ≤ 100 nM. Sublimation of a urine sample resulted in 0.77 μM H<sub>2</sub>O<sub>2</sub>, suggesting at least 0.58 μM H<sub>2</sub>O<sub>2</sub> in urine itself (Fig. 1D and Table 1).

Our measurements demonstrate that NMR spectroscopy is a quite sensitive method for quantitative detection of [H<sub>2</sub>O<sub>2</sub>] in fluids. Because the method directly observes the H<sub>2</sub>O<sub>2</sub> hydrogen signal that falls in a remote spectral region, it does not suffer from chemical interference or resonance overlap. We anticipate that quantitation of H<sub>2</sub>O<sub>2</sub> by NMR spectroscopy will prove valuable not only for direct measurement but also as a “gold standard” to calibrate other methods that have resulted in highly divergent sets of literature [H<sub>2</sub>O<sub>2</sub>] values.

Prior to their desiccation, atmospheric H<sub>2</sub>O<sub>2</sub> is in rapid exchange with the aqueous fraction of airborne droplets. Respiratory fluids contain myeloperoxidase, which converts H<sub>2</sub>O<sub>2</sub> into the much more powerful hypochlorite oxidant, as well as catalase, which breaks down H<sub>2</sub>O<sub>2</sub>. Continuous influx of atmospheric H<sub>2</sub>O<sub>2</sub> possibly could result in antimicrobial levels of hypochlorite, but only at RH levels that prevent evaporation for a sufficient time for reaction to cause pathogen inactivation (17) in droplets. Although elevated levels of H<sub>2</sub>O<sub>2</sub> are known to be an airway irritant, the naturally occurring summertime H<sub>2</sub>O<sub>2</sub> levels may play a role in limiting the duration airborne virus remains viable when humidity is sufficiently high to prevent rapid desiccation of respiratory droplets.

## Materials and Methods

**NMR Spectroscopy.** NMR samples contained 1 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer and 2% vol/vol D<sub>2</sub>O, pH 6.00 ± 0.05 (14). A low concentration of MES buffer was used to reduce catalysis of HX. Spectra were recorded at 2 °C to further minimize HX and retain a fairly narrow H<sub>2</sub>O<sub>2</sub> resonance (line width ca. 15 Hz to 20 Hz). Selective excitation of the downfield spectral region, using a 3-ms Gaussian pulse,

**Table 1.** Observed H<sub>2</sub>O<sub>2</sub> concentrations in various fluids

Substance	[H <sub>2</sub> O <sub>2</sub> ] (μM)
EBC (20 breaths, dry air)*	0.21 ± 0.04
EBC (20 breaths, ambient air)*,†	0.27 ± 0.05
Rain	0.2–56.5
Ambient air	2.6–4.2
MQ water	≤ 0.02
Sublimed MQ water‡	0.19 ± 0.02
Sublimed saliva	≤ 0.2
Sublimed blood	≤ 0.2
Sublimed urine§	0.38–0.77

\*Uncertainties obtained from triplicate measurements.

†[H<sub>2</sub>O<sub>2</sub>] remained ca. 4 μM during EBC collection.

‡Uncertainty is calculated from signal to noise.

§Values are not corrected for H<sub>2</sub>O<sub>2</sub> generation during sublimation.

prevented excitation of the H<sub>2</sub>O resonance. Even under conditions that minimize HX of H<sub>2</sub>O<sub>2</sub> with water, HX remains sufficiently fast to allow rapid signal averaging without saturating the H<sub>2</sub>O<sub>2</sub> resonance (14). Spectra were recorded with 30,720 scans in ca. 30 min each, using a 700-MHz Bruker Avance-III NMR spectrometer equipped with a cryogenic probehead.

**Air Condensate and Rainwater Collection.** A commercial R tube was modified slightly for air sample collection. The mouthpiece was replaced by a septum connecting a hose to the input of the R tube to enable controlling the ambient airflow rate through the R tube using a gauge. Another septum was used at the exit of the R tube, allowing pulling of air through the device using a house vacuum line. An airflow of 10 L/min was passed through the R tube for 30 min while inserted into the aluminum sleeve in contact with dry ice. For rainwater collection, the narrow end of a plastic funnel was fitted into a 15-mL falcon tube.

**EBC Collection.** A K-type thermocouple was attached to the outside of the cooling aluminum sleeve. The sleeve was first cooled to  $-5^{\circ}\text{C}$  in a freezer, followed by slowly warming to  $0^{\circ}\text{C}$ . During this time, eight deep inhalations of dry ( $<1\%$  RH), high-efficiency-particulate-air (HEPA)-filtered air were made, followed by nose exhalation. As soon as the cooling sleeve reached  $0^{\circ}\text{C}$ , the mouthpiece, which was slightly warmed by a heat gun to prevent condensation, was connected to the R tube, the R tube was inserted into the sleeve, and breathing started with an inhale through the mouthpiece of the device which is equipped with its own unidirectionally valved HEPA filter. During sample collection, each deep inhalation (ca. 4 L) was timed to take 5 s, followed by 3 s of breath holding, and exhaling at an approximately constant flow for 12 s. The total time of each breath cycle was 20 s. For quantifying H<sub>2</sub>O<sub>2</sub> formation on a polypropylene surface, single exhales were performed into four 50-mL falcon tubes at room temperature, followed by centrifugation ( $20,000 \times g$ ,  $20^{\circ}\text{C}$ ). This procedure was repeated five times to yield a total of 110  $\mu\text{L}$  of EBC (i.e., 5.5  $\mu\text{L}$  per exhale). EBC samples smaller than 500  $\mu\text{L}$  were supplemented with MQ water to permit measurement in standard NMR sample tubes.

**Blood, Urine, and Saliva Sample Preparation.** Within 3 min of drawing, fresh human blood (ca. 1 mL) was added dropwise to magnetically stirred isopentane (20 mL) cooled to ca.  $-140^{\circ}\text{C}$  in a 100-mL flask. The addition was performed under an inert atmosphere using an N<sub>2</sub> balloon. The flask was then stored at dry ice temperature before it was opened in a N<sub>2</sub>-filled glove bag for removing isopentane from the solid blood pellets, using a pipette. Next, a sublimation cold finger equipped with a vacuum valve was inserted into the flask (Sigma-Aldrich; product numbers Z129607 and Z515574), and the remaining isopentane was removed using a lyophilizer while keeping the bottom of the flask in contact with dry ice. Subsequently, the vacuum valve was closed, and the sample was sublimed by warming the frozen blood to about  $-15^{\circ}\text{C}$  to  $-10^{\circ}\text{C}$  and running liquid N<sub>2</sub> through the cold finger for 4 h. Then, the sublimation device was filled with N<sub>2</sub> gas and warmed to ambient temperature. During this time, the deposited ice on the cold finger thawed and was collected in a cup at the tip of the cold finger (875 mg). The fraction of the blood that did not sublime (245 mg) remained fully dehydrated after warming to ambient temperature, indicating completeness of the sublimation process. Urine and saliva samples were processed analogously to blood, and liquid fractions of 95 and 98 wt/wt% were collected on the cold finger after sublimation, consistent with their respective known water contents. All samples were deidentified.

**Data Availability.** All study data are included in the main text.

**ACKNOWLEDGMENTS.** This work was supported by the Intramural Research Program of the National Institute of Diabetes and Digestive and Kidney Diseases. We thank Jinfa Ying, James Baber, and Blake Wilson for valuable assistance and Nancy Alexander for assistance with obtaining an exemption from the NIH institutional review board. R.N.Z. gratefully acknowledges support from the Multidisciplinary University Research Initiative program (Air Force Office of Scientific Research Grant FA9550-21-1-0170).

1. H. Ohye, M. Sugawara, Dual oxidase, hydrogen peroxide and thyroid diseases. *Exp. Biol. Med. (Maywood)* **235**, 424–433 (2010).
2. C. Behl, J. B. Davis, R. Lesley, D. Schubert, Hydrogen peroxide mediates amyloid beta protein toxicity. *Cell* **77**, 817–827 (1994).
3. M. P. Lisanti *et al.*, Hydrogen peroxide fuels aging, inflammation, cancer metabolism and metastasis: The seed and soil also needs “fertilizer.” *Cell Cycle* **10**, 2440–2449 (2011).
4. C. Johansson, F. C. M. Kirsebom, Neutrophils in respiratory viral infections. *Mucosal Immunol.* **14**, 815–827 (2021).
5. R. Sugamata *et al.*, Contribution of neutrophil-derived myeloperoxidase in the early phase of fulminant acute respiratory distress syndrome induced by influenza virus infection. *Microbiol. Immunol.* **56**, 171–182 (2012).
6. Y. Teng *et al.*, Hydrogen peroxide in exhaled breath condensate in patients with asthma: A promising biomarker? *Chest* **140**, 108–116 (2011).
7. H. Sakugawa, I. R. Kaplan, W. Tsai, Y. Cohen, Atmospheric hydrogen peroxide. *Environ. Sci. Technol.* **24**, 1452–1462 (1990).
8. F. Rezende, R. P. Brandes, K. Schröder, Detection of hydrogen peroxide with fluorescent dyes. *Antioxid. Redox Signal.* **29**, 585–602 (2018).
9. H. J. Forman, A. Bernardo, K. J. Davies, What is the concentration of hydrogen peroxide in blood and plasma? *Arch. Biochem. Biophys.* **603**, 48–53 (2016).
10. J. K. Lee *et al.*, Condensing water vapor to droplets generates hydrogen peroxide. *Proc. Natl. Acad. Sci. U.S.A.* **117**, 30934–30941 (2020).
11. N. A. Stephenson, A. T. Bell, Quantitative analysis of hydrogen peroxide by <sup>1</sup>H NMR spectroscopy. *Anal. Bioanal. Chem.* **381**, 1289–1293 (2005).
12. C. G. Tsiafoulis, I. P. Gerothanassis, A novel NMR method for the determination and monitoring of evolution of hydrogen peroxide in aqueous solutions. *Anal. Bioanal. Chem.* **406**, 3371–3375 (2014).
13. D. Ryoo *et al.*, Detection and quantification of hydrogen peroxide in aqueous solutions using chemical exchange saturation transfer. *Anal. Chem.* **89**, 7758–7764 (2017).
14. T. Kakeshpour, A. Bax, NMR characterization of H<sub>2</sub>O<sub>2</sub> hydrogen exchange. *J. Magn. Reson.* **333**, 107092 (2021).
15. S. Peters *et al.*, Hydrogen peroxide in exhaled air: A source of error, a paradox and its resolution. *ERJ Open Res.* **2**, 00052-2015 (2016).
16. G. L. Kok, Measurements of hydrogen peroxide in rainwater. *Atmos. Environ. (1967)* **14**, 653–656 (1980).
17. M. T. Dulay *et al.*, Spraying small water droplets acts as a bactericide. *QRB Discov.* **1**, e3 (2020).