Redox Biology 26 (2019) 101281

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Commentary on "Using resonance synchronous spectroscopy to characterize the reactivity and electrophilicity of biologically relevant sulfane sulfur". Evidence that the methodology is inadequate because it only measures unspecific light scattering

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The development of specific and accurate methods for the detection of sulfane sulfur compounds (*i.e.* compounds that have a sulfur atom bound to two or more sulfur atoms or to a sulfur atom and an ionizable hydrogen), such as the biologically-relevant persulfides (RSSH), is thwarted by the instability and complex chemistry shown by most of these compounds. Approaches based on the reaction of the compound of interest with a probe may have kinetic constraints. In addition, they are prone to various interferences and usually unable to distinguish specific molecules among a diversity of compounds. In an effort to bypass these technical inconveniences, Li *et al.* recently published a study in *Redox Biology* on the reactivity of sulfane sulfur-containing molecules based on their quantitative determination by resonance synchronous spectroscopy (RS_2) [1]. This technique is based on using a fluorimeter and scanning the light emitted perpendicularly from a sample while excitation and emission wavelengths are shifted synchronously [2]. RS_2 has been used with samples of nanoparticles [3] or mixtures of fluorophores [4–6], by taking advantage of the ability of these substances to scatter light or, alternatively, to emit Stokes-shifted or on-resonance fluorescence (*i.e.* photon emission wavelength identical to that of the excitation photon). The physical principle lies on the differential scattering, absorption and emission of light along the spectrum according to the characteristic excitation and emission spectra of each analyte.

Li *et al.* [1] claim that RS_2 is a reaction-free method that can specifically determine electrophilic sulfane sulfurs in real time. Based on this method, the paper reports kinetics of sulfane-consuming and sulfane-forming reactions, pK_a values, protein modification, and even sulfane content in whole cells. The sulfane sulfur-containing compounds were initially studied by applying different $\Delta\lambda$ (λ_{em}-λ_{ex}) ranges and the best synchronous scanning spectroscopy signals were obtained when $\Delta\lambda$ was 1 nm. Since excitation was performed with a bandwidth of 5 nm, light detected can only be due to scattering or to on-resonance fluorescence [3]. However, sulfane compounds under scrutiny are neither fluorophores nor optical scatterers, thus, the authors are not measuring sulfanes as they claim. We affirm that the response detected by the fluorimeter is caused by aggregated elemental sulfur (S_n) produced in downstream reactions of sulfane sulfur compounds such as those involving inorganic and organic polysulfides and polysulfanes [7–9].

With the aim of providing support for our assertion, a series of procedures similar to those reported in Ref. [1] were performed in this work. First, a colloidal sulfur suspension was enough to reproduce the signal assigned in Ref. [1] to a broad range of "electrophilic sulfane sulfur" compounds (Fig. 1, red trace). Indeed, the reaction mixtures prepared by the authors produced elemental sulfur as demonstrated by reversed-phase chromatography (shown in Ref. [1], Figure S3). Remarkably, we obtained similar signals to those observed with colloidal sulfur when we used metallic silver nanoparticles or colloidal silica (Ludox AM), that did not contain any sulfur compounds (Fig. 1, black and green traces). In fact, the shape of scattering spectra of different samples resembles the emission spectra of xenon lamps [10], but is affected by the optical components in the fluorimeter, and the size and concentration of the aggregates. Unlike Li et al., we decided not to consider the sample-to-buffer signal intensity ratios (R_2S_2 spectra in Ref. [1]) because, in our case, the signal intensities of the buffer at wavelengths above 600 nm were close to zero, so that the ratios were extremely noisy and provided no information. The results obtained herein with preparations of completely different chemical composition

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https://doi.org/10.1016/j.redox.2019.101281
Received 1 July 2019; Received in revised form 18 July 2019; Accepted 19 July 2019
Available online 20 July 2019

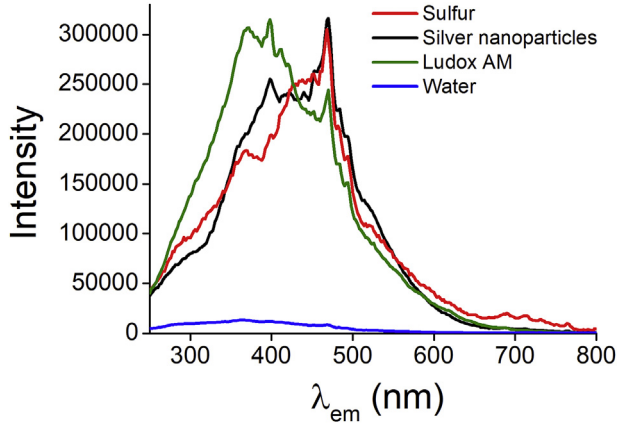

Fig. 1. Synchronous scanning signals obtained from samples of different composition. Spectra obtained from elemental sulfur (red), spherical silver nanoparticles (black) and Ludox AM (green) suspensions in water. Spectra were acquired at an angle of 90° with respect to incident light. The offset between excitation and emission wavelengths was set to 1 nm, slitwidths 8 nm and scan rate 1 nm/s. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

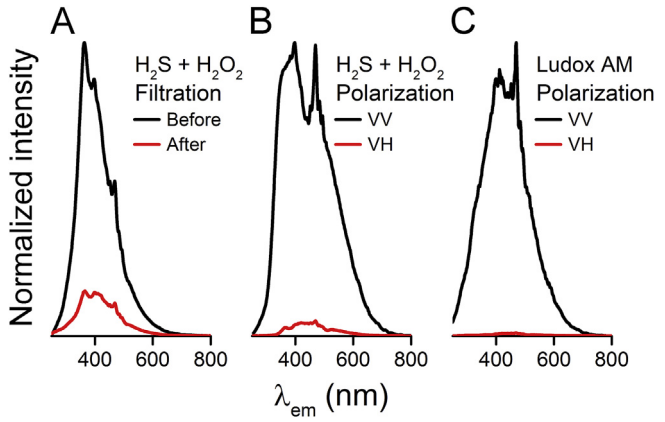

Fig. 2. Signals obtained from mixtures of H_2S and H_2O_2. (A) Synchronous scanning spectra obtained from a mixture of H_2S (1 mM) and H_2O_2 (1 mM) in HEPES buffer (100 mM, pH 7.4) before (black) and after (red) filtration through a 10 kDa MWCO filter. **(B and C)** Polarized synchronous scanning spectra of the mixture of H_2S and H_2O_2, and Ludox AM, respectively, using polarizers in both excitation (vertically polarized) and emission beams (vertically polarized, black, and horizontally polarized, red). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

demonstrate that the synchronous scanning spectroscopy (RS_2 in Ref. [1]) lacks specificity for electrophilic sulfane sulfur compounds, since no sulfur at all is required to obtain the signal.

In our hands, mixtures of H_2S and H_2O_2 also produced the signal reported (Fig. 2A, black trace). However, a simple separation treatment (*i.e.* filtering through a 10 kDa MWCO filter) attenuated the signal (Fig. 2A, red trace), supporting that the optical phenomenon is not produced by soluble compounds but by aggregated elemental sulfur. Furthermore, the use of polarizers in both excitation and emission beams confirms that light scattering is the main contributor to the signal (Fig. 2B). Scattered light is highly anisotropic; polarized incident light is not depolarized because the scattering phenomenon is virtually instantaneous [11]. Thus, when the sample is illuminated with vertically polarized light, the horizontally polarized measured light (VH) is negligible if compared to the vertically polarized (VV), as observed with colloidal silica (Ludox AM, Fig. 2C).

Based on the data shown above and earlier in-depth studies [3], we affirm that synchronous scanning spectroscopy is not a reliable technique to quantify sulfane sulfur compounds. Actually, the term resonance does not apply since the signals observed are simply light scattering. The signals arising from the compounds used in the study by Li *et al.* can be ascribed to the ability to produce elemental sulfur. The signals as well as the kinetics reported were not determined by formation or consumption of electrophilic sulfane sulfurs but by the formation of elemental sulfur, which depends on the conditions used in the experiments, particularly pH, as well as on the rates of the secondary reactions proceeding to elemental sulfur. Last, the signals observed in whole cell experiments (Figure 7 in Ref. [1]) likely arise from light scattering by the cells themselves.

To sum up, since persulfides, polysulfides and other sulfane sulfur compounds are neither fluorophores nor optical scatterers, the resonance synchronous spectroscopy approach proposed by Li *et al.* [1] cannot be used to measure them.

Materials and methods

Spectra were acquired in a ChronosFD spectrofluorometer from ISS (Champaign, IL, USA) equipped with a 300 W high-pressure xenon arc lamp, polarizers and monochromators, with acquisition at 90°. The offset between excitation and emission wavelengths was set to 1 nm, slitwidths 8 nm and scan rate 1 nm/s.

Sulfur powder was suspended in distilled water by vortexing. Silver nanoparticles (100 nm diameter) were from Nanocomposix (San Diego, CA), Ludox AM was from Sigma-Aldrich. $Na_2S{\cdot}9H_2O$ (used as source of H_2S) and H_2O_2 were from Carlo Erba and Sigma, respectively. Vivaspin 500 concentrators (10 kDa MWCO) were from GE.

Acknowledgements

We thank Dr. Eduardo Méndez and Dr. Santiago Botasini (Universidad de la República, Uruguay) for providing the silver nanoparticles.

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