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Nitrosopersulfide ($SSNO^-$) decomposes in the presence of sulfide, cyanide or glutathione to give $HSNO/SNO^-$: consequences for the assumed role in cell signalling

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The emergence of hydrogen sulfide (H_2S) as a new signalling molecule able to control vasodilation, neurotransmission and immune response, prompted questions about its possible cross-talk with the other gasontransmitter, nitric oxide (NO). It has been shown that H_2S reacts with NO and its metabolites and several potentially biologically active species have been identified. Thionitrous acid (HSNO) was proposed to be an intermediate product of the reaction of *S*-nitrosothiols with H_2S capable of crossing the membranes and causing further trans-nitrosation of proteins. Alternatively, formation of nitrosopersulfide ($SSNO^-$) has been proposed in this reaction. $SSNO^-$ was claimed to be particularly stable and inert to H_2S, thiols and cyanides. It is suggested that this putative $SSNO^-$ slowly decomposes to give NO, HNO and polysulfides. However, the chemical studies with pure $SSNO^-$ salts showed some conflicting observations. In this study, we work with pure PNP^+SSNO^- to show that contrary to everything that is claimed for the yellow reaction product of GSNO with H_2S, pure $SSNO^-$ decomposes readily in the presence of cyanide, H_2S and glutathione to form SNO^-. Based on literature overview and chemical data about the structures of $HSNO/SNO^-$ and $SSNO^-$ we discuss the biological role these two species could have.

1. Introduction

Hydrogen sulfide (H_2S) emerged as the third gaseous signalling molecule (gasotransmitter), alongside nitric oxide (NO) and carbon monoxide (CO) [1–3]. The similarity of its physiological effects (vasodilation [4,5], cardioprotection [6,7], neurotransmission [8,9], immunomodulation [10], etc.) to those previously reported for NO, prompted questions about possible cross-talk with NO [11].

It is now well established that H_2S strongly interferes with NO signalling, either by reacting directly with NO [12] and its metabolites (*S*-nitrosothiols (RSNO) [13], peroxynitrite [14,15] and nitrite [16–18]) or by modulating NO production [19] and cGMP production [20] and degradation [21,22]. Namely, even the first reports on H_2S-induced vasodilatory effects showed that endogenous H_2S functions as a smooth muscle tone regulating factor in synergy with NO [23]. Inhibition of NO synthase by non-specific inhibitor L-NAME led to inhibition of H_2S-induced vasodilation [24], while the deletion of cystathionine gamma lyase (CSE; an H_2S producing enzyme) prevented vasodilatory effects of acetylcholine and NO [21]. Finally, cardioprotective effects of H_2S are abolished in $eNOS^{-/-}$ mice [25], although mitochondria targeted H_2S donors remain active even in the absence of NO [26,27].

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In their initial studies, Whiteman and co-workers [28,29] observed that different donors of NO or nitrosonium species (NO^+) react with H_2S to give a new *S*-nitrosothiol, which they proposed to be HSNO. Before that, HSNO was reported as a product of *cis*-HNSO photolysis in argon matrices [30,31]. Its isomers (*cis* and *trans*) were studied under these conditions by the means to IR spectroscopy. Seel and co-workers [32] managed to crystalize SNO^- salts with PNP^+ showing charge delocalization over all three atoms. Computational studies suggested that although slightly less stable than RSNOs, HSNO could exist under physiologically relevant conditions [33].

By the means of pulse radiolysis (to generate $NO^\bullet$ and $HS^\bullet$), or by mixing H_2S with either acidified nitrite or GSNO, we also observed that HSNO could be formed under physiologically relevant conditions [13], confirming previous assumptions by Whiteman and co-workers [28]. Furthermore, we showed that HSNO could serve as a shuttle of 'NO^+' from one thiol to another, being thus involved in trans-nitrosation processes [13].

Recently, McCarthy and co-workers gathered highly accurate molecular parameters (rotational, centrifugal distortion and hyperfine constants) of HSNO using Fourier-transform microwave (FTMW) spectroscopy [34]. They confirmed the existence of both *cis* and *trans* isomers and estimated S–N bond length to 1.834 ± 0.002 Å for *cis*-HSNO and 1.852 ± 0.002 Å for *trans*-HSNO, the latter being the longest experimentally measured S–N bond in an *S*-nitrosothiol.

Contrary to that, Feelisch and co-workers failed to identify HSNO when mixing GSNO and H_2S and proposed the formation of another stable *S*-nitroso species, nitrosopersulfide ($SSNO^-$) [35]. In their paper, Cortesse-Krott *et al.* claim that (i) HSNO is too short lived to be identified, (ii) that 'enriched' solutions of $SSNO^-$ could be prepared by having an excess of H_2S over GSNO, (iii) that $SSNO^-$ is stable in the presence of thiols (such as glutathione which is one of the products formed in the reaction of GSNO with H_2S) and (iv) resistant to cyanides [35]. Parallel with the publication of this study, we reported the (bio)chemical characterization of pure crystalline $SSNO^-$ (prepared as PNP^+ $SSNO^-$ salt) [36], following the synthetic protocol reported by Seel *et al.* [32], to show that $SSNO^-$ could not really exist under physiological conditions.

Here, we extend our previous work with pure PNP^+SSNO^- to show that contrary to everything that is claimed for the yellow reaction product of GSNO with H_2S, pure $SSNO^-$ decomposes readily in the presence of cyanide, H_2S and glutathione to form SNO^-, as confirmed by ^{15}N NMR and cryo-spray mass spectrometry. Furthermore, based on, often mis-cited, old literature and actual chemical data about the structures and chemical reactivity of both $HSNO/SNO^-$ and $SSNO^-$, we discuss the biological role these two species could have.

2. Material and methods

2.1. Chemicals

All chemicals were obtained from Sigma Aldrich if not stated otherwise. All aqueous solutions were prepared using nanopure water. All buffers were supplemented with Chelex-100 resin and kept over the resin to remove traces of transition metal ions. Organic solvents were purchased dry, additionally dried with $MgSO_4$ and stored in argon box.

2.2. Preparation of PNP^+SSNO^-

PNP^+SSNO^- was prepared as previously described [32,36]. Briefly, 0.525 g of $PNP^+NO_2^-$ (900 µmol) and 57 mg of elemental sulfur (1.8 mmol as referred to 'S') were weighed in a vial in the argon box, 15 ml of dry acetone was added. The reaction mixture was stirred overnight, in the argon box, yielding a dark orange-to-red solution. This solution was PTFE-filtered to remove remnants of unreacted sulfur. To the reaction mixture, 10 ml of dried diethylether was added to initiate crystallization. The majority of the crystals were red, with some trace amounts of yellow ones. Crystals were dissolved in acetone and recrystallized by addition of diethylether at least twice.

2.3. Absorption spectroscopy

UV/Vis measurements were performed in anaerobic cuvettes (i.e. cuvettes sealable with a screw cap equipped with a silicon/PTFE septum) using an HP 8452A diode array spectrophotometer connected to a computer equipped with Olis Spectralworks software. Additional measurements were performed on a Specord 200 spectrophotometer by Jena Analytics, connected to a computer equipped with Winaspect software.

2.4. NMR spectroscopy

^{14}N- and ^{15}N-NMR spectra were recorded on a Bruker AVANCE DRX 400WB spectrometer equipped with a Spectrospin superconducting wide-bore magnet operating at a resonance frequency of 28.90 MHz and 40.54 MHz, respectively, at a magnetic induction of 9.4 T. The measurements were performed with a commercial 5 mm Bruker broadband probe thermostated with a Bruker B-VT 3000 variable temperature unit. Chemical shifts given were referenced to nitromethane.

2.5. Electron spray ionization mass spectrometry (ESI-MS)

MS measurements were performed on a UHR-ToF Bruker Daltonik (Bremen, Germany) maXis, which was coupled to a Bruker cryo-spray unit. Detection was in positive and negative ion mode. The flow rates were 200 µl h^{-1}. The dry-gas (N_2) temperature was held at −20°C and the spray-gas temperature was held at −15°C. The instrument was calibrated prior to every experiment via direct infusion of the Agilent ESI-TOF low concentration tuning mixture, which provided an m/z range of singly charged peaks up to 2700 Da in both ion modes.

3. Results and discussion

3.1. UV/Vis spectral properties of $SSNO^-$ in water

A history of the identification of S–S bond-containing compounds in solutions was rich in contradicting conclusions [37], and 'some of those who dared to tackle this challenging task were victims of delusions because such species that were optically (or even by other methods) observed in non-aqueous solutions could not easily be established as defined substances' [38]. This is a translated quotation from Seel & Wagner [38], who were the first to synthesize $SSNO^-$ salt under exclusion of water and dioxygen [32]. Seel and co-workers tackled this challenging chemistry before either NO or H_2S were known as signalling molecules. Besides crystalizing PNP^+SSNO^-, they observed that when NO is

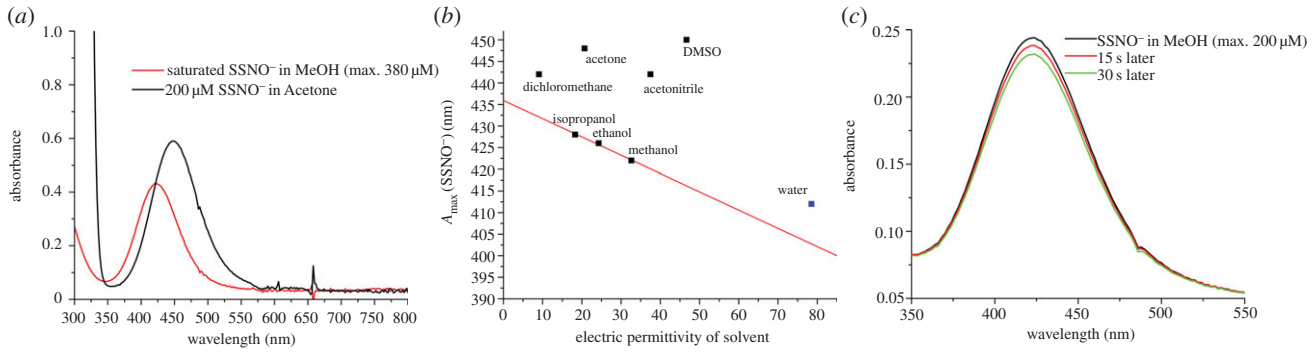

Figure 1. UV/Vis spectral changes of $SSNO^-$ in different solvents. (*a*) Overlaid UV/Vis spectra of PNP^+SSNO^- in acetone (200 μM, black line) and methanol (saturated solution, maximally 380 μM, red line). The absorbance maxima are located at 448 nm and 422 nm, respectively. (*b*) The wavelengths of the absorbance maxima of PNP^+SSNO^- in different organic solvents plotted against the relative permittivity (dielectric constant) of the respective solvent. The putative value of $SSNO^-$ absorbance in water is given as 412 nm according to the proposal of Cortese-Krott *et al*. Contrary to the other tested solvents, the alcohols displayed a pronounced linearity and we used an extrapolated linear fit derived from those three solvents (red line) to estimate the real value for H_2O. The values for relative permittivity of solvents are taken from: Maryott & Smith, 1951 [44]. (*c*) UV/Vis spectra of PNP^+SSNO^- (maximal starting concentration of 200 μM, black) in methanol, recorded 15 s later (red line) and 30 s later (green line). The relatively fast decay of the characteristic $SSNO^-$ peak at 422 nm is clearly visible.

bubbled into alkaline solutions of sulfide (pH > 10) a yellow species is formed with an absorbance maximum at 412 nm [39]. Based on their observation that solutions of crystalline $SSNO^-$ absorbs at 448 nm in acetone, they assumed that this was a solvent-induced shift and that the 412 nm product, observed in the reaction of NO with alkaline sulfide solutions, might also be $SSNO^-$. They estimated that the yield of this putative $SSNO^-$ is not more than 10%, but the authors clearly stated that the proposed chemical nature of this product was questionable, because $SSNO^-$ could not be confirmed by ^{15}N NMR [39].

When studying the reaction of S-nitrosoglutathione with H_2S, at pH 7.4 we observed the formation of yellow product with absorbance at 412 nm but because the time-resolved ^{15}N NMR studies and time-resolved IR studies confirmed that the ^{15}N NMR signal and N = O vibrational band, respectively, were only present for short amount of time, while the 'yellow' product remained stable for couple of hours (slowly decaying with sulfur precipitation), we proposed that this is a mixture of polysulfides/sulfur sols [13,36]. Working with pure $SSNO^-$ we could not test whether there was a water-induced hypsochromic shift, as PNP^+SSNO^-, as in the Seel's report [32], decomposed when 10% of water was added into the acetone solution of the salt [36]. The matured mixture of GSNO and H_2S, called '$SSNO^-$ mix', has been referred to as enriched $SSNO^-$ in several papers [35,40–42], solely based on 412 nm absorbance, although no real chemical evidence was provided to prove that this 'yellow' mixture consists of $SSNO^-$. Recently, Olabe and co-workers performed a computational study to predict the UV/Vis spectrum of $SSNO^-$ in water and they also suggested that $SSNO^-$ should indeed absorb at 412 nm when dissolved in water [43].

We wanted to address this experimentally, by recording absorbance maximum shift in different solvents and then plotting the data over the dielectric constant (ε) of the particular solvent. First obvious observation is that it is impossible to establish any correlation for most of the tested solvents (figure 1). For example, the absorbance maximum of $SSNO^-$ in acetonitrile and dichloromethane is quite similar (442 nm) while the difference in their ε values is quite large (ε = 8.93 for dichloromethane and ε = 37.5 for acetonitrile) (figure 1). Therefore, the absorbance at 448 nm that others and we reported for the acetone solution can hardly be used as a reference point for predicting the UV/Vis features of $SSNO^-$ in other solvents, particularly in water. Several of the tested alcohols however showed nice linear dependence (figure 1*b*). Assuming that water will behave in a similar way, we used the obtained correlation between absorbance maximum and dielectric constant of alcohols to estimate the former value for water, being 402 ± 2 nm. It should be stressed that if we consider a linear correlation observed only for small water-soluble alcohols, the widely used 412 ± 2 nm value is quite off the observed linear correlation (figure 1*b*). By way of comparison, the estimated absorbance maximum is even lower than what we calculated and experimentally measured for $HSNO_2$ (408 nm; [14,15]). It is worth mentioning that $SSNO^-$ solutions in methanol and ethanol were not as stable as they were in acetone with 250 μM $SSNO^-$ disappearing from the ethanol solution in 30 min (not shown). This contradicts the statement that $SSNO^-$ is stable for hours in aqueous solutions at pH 7.4, especially in high concentrations in '$SSNO^-$-enriched mixtures' ('$SSNO^-$ mix') [35].

3.2. Biologically relevant reactivity of $SSNO^-$

In their paper Cortese-Krott and co-workers claim (i) that 'enriched' solutions of $SSNO^-$ could be prepared by having an excess of H_2S over GSNO, (ii) that $SSNO^-$ is stable in the presence of thiols (such as glutathione which is one of the products formed in the reaction of GSNO with H_2S) and (iii) resistant to cyanides [35]. This is surprising, considering that X-ray structural analysis of $SSNO^-$ suggests a structure that could best be described as cage structure of [S_2——NO^-] [36], therefore one of the two sulfur atoms should be available for nucleophilic attack by either HS^-, GS^- or CN^-.

We previously reported that $SSNO^-$ reacts rapidly with sulfide [36]. Now, we combined ^{15}N NMR with ESI-TOF-MS data to observe the reaction product of $SSNO^-$ with sulfide. When acetone solutions of $PNP^+SS^{15}NO^-$ were mixed with Na_2S and the reaction monitored by ^{15}N NMR, a clear shift in ^{15}N NMR from 354 ppm to 314 ppm is noticeable. The NMR (314 ppm) signal was also observed previously in the

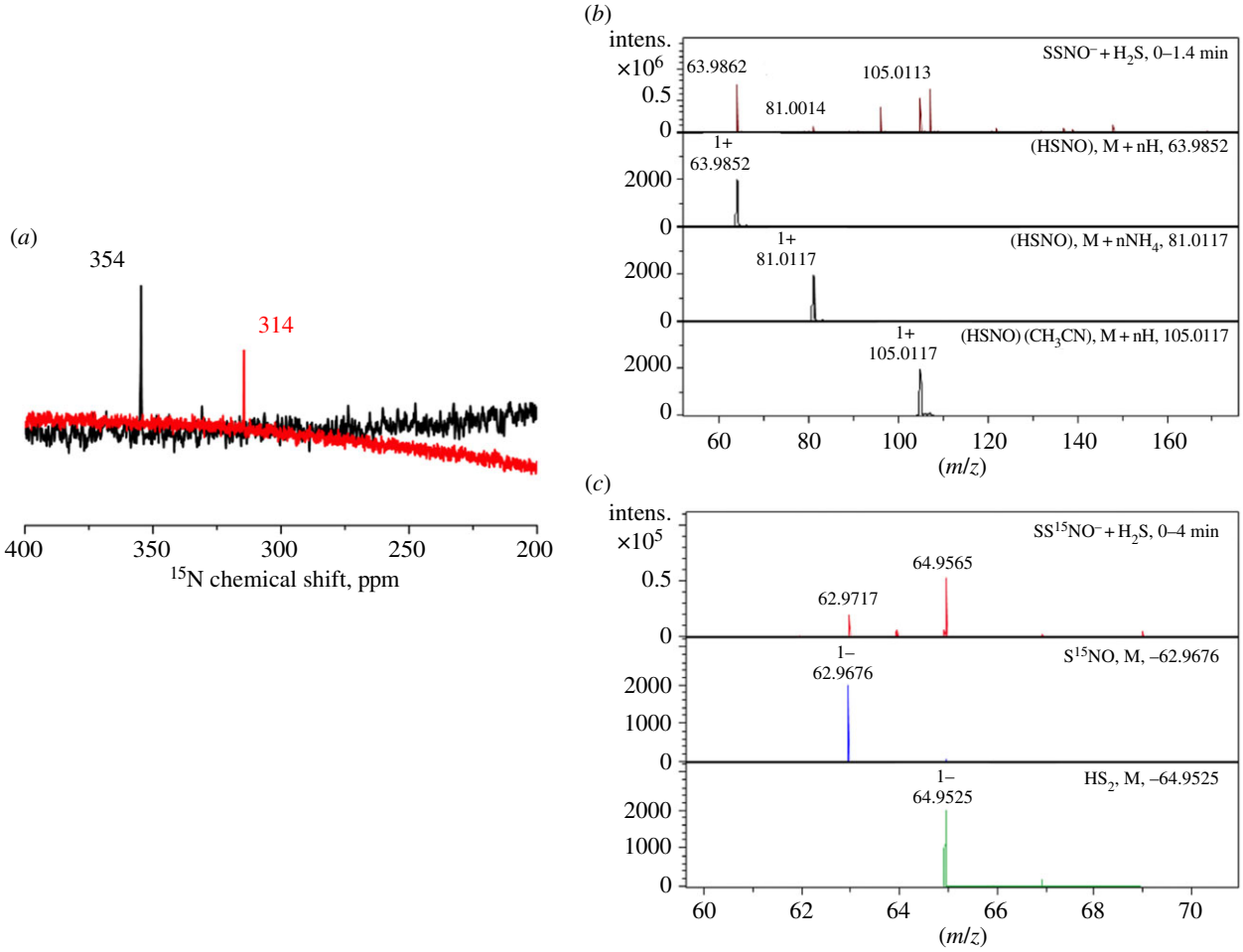

Figure 2. SNO^- is the reaction product of HS^- with nitrosopersulfide. (*a*) Overlayed ^{15}N NMR spectra of $PNP^+SS^{15}NO^-$ (100 mM in acetone, black line) as well as of a mixture of $PNP^+SS^{15}NO^-$ (30 mM) and H_2S (50 mM) in a solution of acetone : water 9 : 1, recorded 1 h after mixing (red line). While we ascribe the signal at 354 ppm to $SS^{15}NO^-$, the newly appearing signal at 314 ppm in the nitrosopersulfide/hydrogen sulfide mixture is assigned to $S^{15}NO^-$ (figure 4*b*). (*b*) Identification of HSNO as a reaction product of PNP^+SSNO^- with H_2S. 250 μM PNP^+SSNO^- was mixed with an equimolar amount of H_2S (solution in THF) and reaction monitored in positive mode. Simulated mass spectra for some of the species identified during the measurement are shown in black. (*c*) Negative ion mode ionization of a reaction mixture containing 250 μM $PNP^+SS^{15}NO^-$ and H_2S. Recorded spectrum is shown in red, and simulated mass spectra of $S^{15}NO^-$ and HS_2^- are shown in blue and green, respectively.

reaction of $GS^{15}NO$ and H_2S and we assigned it to the ^{15}N NMR of $HSNO/SNO^-$ (figure 2*a*). To confirm this we performed cryo-electron spray ionization mass spectrometry, spraying the acetonitrile mixture of PNP^+SSNO^- (figure 2*b*) or $PNP^+SS^{15}NO^-$ (figure 2*c*) and H_2S at −20°C. Recording spectra in positive mode we could clearly demonstrate the formation of HSNO, either as $[HSNO + H]^+$ or $[HSNO + CH_3CN + H]^+$. The observed and predicted masses matched nicely (figure 2*b*). When we sprayed the reaction mixture in the negative ion mode, we could see the formation of SNO^- with both PNP^+SSNO^- (not shown) and $PNP^+SS^{15}NO^-$ (figure 2*c*). These data clearly confirm that SNO^- is the product of the reaction (equation (3.1); figure 2*b*). The use of cryo-ESI-TOF-MS once again proved to be a useful tool for facile detection of $HSNO/SNO^-$.

$$SS^{15}NO^- + HS^- \rightarrow S^{15}NO^- + HS_2^-. \qquad (3.1)$$

Sulfur precipitated from this reaction, most probably from HS_2^-. We previously reported that synthetic H_2S_2 explodes when exposed to the air humidity giving H_2S and elemental sulfur [36]. But after filtration of this mixture of decomposition products, the yellow species remained and it absorbs exactly at 412 nm, just like putative '$SSNO^-$ mix'. This yellow species was resistant to reducing agents such as DTT (reacting with it rather slowly) [36], just like what Cortese-Krott *et al*. reported for their '$SSNO^-$ mix' [35]. Based on ^{15}N NMR and cryo-ESI-TOF-MS data that show only SNO^- as the nitrogen containing species, and knowing that SNO^- absorbs at 340 nm, this chemical evidence suggests that 412 nm absorbance may only be attributed to the presence of the mixture of polysulfides/sulfur sols. Importantly, contrary to previous claims [35,40], these results demonstrate that real $SSNO^-$ cannot be stabilized in solutions containing excess of H_2S because it reacts with it.

Comparably, thiols should also be capable of nucleophilic attack. In fact, in our previous work we estimated that the redox potential of $SSNO^-$ is −0.2 V (versus NHE) [36], a value that is easily reached in the biological environment by simple thiol buffers such as the glutathione buffer. We showed previously that $SSNO^-$ decomposes readily in the presence of DTT and we wanted now to address its ability to react with GSH. Owing to the limited solubility of GSH in other solvents, the reaction had to be carried out in DMSO. Addition of 4 mM GSH into 400 μM $SSNO^-$ resulted in a steady decay of $SSNO^-$ with a half-life of 10 min at 21°C (figure 3). Taking into account that GSH is not deprotonated under these conditions, its nucleophilicity is lower than what

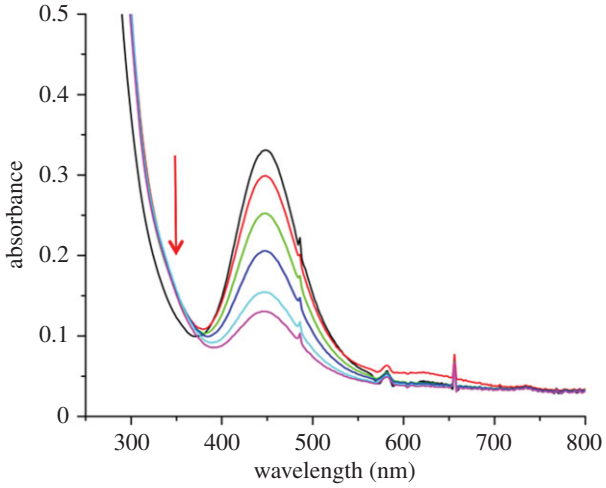

Figure 3. Glutathione causes decomposition of $SSNO^-$. Time-resolved UV/Vis spectra of the reaction mixture containing $SSNO^-$ (400 µM PNP^+SSNO^-) with glutathione (4 mM) in DMSO over 30 min. The colours of the lines correspond to the respective observation times as follows: immediately after mixing (black line), 1 min later (red line), 5 min later (green line), 10 min later (deep blue line), 20 min later (light blue line), 30 min later (purple line).

it would be under physiological conditions, where the reaction would probably proceed even faster. But even with such a reaction rate and by assuming that the physiological concentration of GSH is 5 mM, and that there is only a five-fold increase in the reaction rate at 37°C when compared with 21°C, the half-life of $SSNO^-$ becomes less than 100 s. The isosbestic point and the shoulder at approximately 340 nm is indicative of formation of another species, most likely $HSNO/SNO^-$ (equation (3.2)) [13].

$$GSH + SSNO^- \rightarrow GSSH + SNO^- \qquad (3.2)$$

and

$$GSH + SSNO^- \rightarrow GSNO + HS_2^-. \qquad (3.3)$$

A trans-nitrosation reaction to give GSNO and HS_2^- (equation (3.3) is a possibility but considering that there were no characteristic UV/Vis features of polysulfides/sulfur sols formed as decomposition products of HS_2^- (as in the case of the reaction with HS^-) we concluded that reaction described by equation (3.2) is the most likely one. In addition, when we studied the reaction of H_2S_2 with NO^+BF_4, we observed that although the product is $SSNO^-$ it is not nitrosopersulfide but its isomer, dithionitrate, with distinct UV/Vis, ^{15}N NMR and IR characteristics and different chemical reactivity [36].

Finally, cyanides are known to react with sulfane sulfur [45]; however, the putative '$SSNO^-$ mix' was resistant to the cyanide treatment [35]. In order to experimentally address this contradiction, we firstly followed the decay of PNP^+SSNO^- upon addition of potassium cyanide into the acetone solution. $SSNO^-$ started decaying rather fast with the reaction being over in approximately 200 s (figure 4*a*,*b*). If CN^- indeed reacted with sulfane sulfur then the reaction products should be SCN^- and SNO^- (equation (3.4)). To resolve the product identity, we mixed $SS^{15}NO^-$ with $C^{14}N^-$ and recorded both ^{14}N and ^{15}N NMR (figure 4; equation (3.4)).

$$SS^{15}NO^- + C^{14}N^- \rightarrow SC^{14}N^- + S^{15}NO^- \qquad (3.4)$$

In the ^{15}N NMR, we again observed the 314 ppm signal which we also saw when sulfide reacted with $SS^{15}NO^-$ and which corresponds to $S^{15}NO^-$ (figure 4*c*). In ^{14}N NMR, we could see two signals, one corresponding to the unreacted CN^- and the other to newly formed SCN^- as confirmed by comparing the ^{14}N NMR chemical shifts of separately recorded cyanide and thiocyanate salts (figure 4*d*). These results unambiguously showed that $SSNO^-$ does react readily with CN^- forming more stable SNO^-, contrary to the chemical behaviour of putative '$SSNO^-$ mix' described in literature [35].

3.3. HSNO versus $SSNO^-$: relevance for cell signalling

In our previous attempt to prove that HSNO could be formed under physiologically relevant conditions, (water, pH 7.4) we used three approaches to generate HSNO: pulse radiolysis (to monitor the reaction of $HS^\bullet$ with $NO^\bullet$), reaction of H_2S with acidified nitrite, and trans-nitrosation reaction between *S*-nitrosoglutathione and H_2S [13]. Using pulse radiolysis to generate $HS^\bullet$ and $NO^\bullet$, we observed transient formation of a new species with spectral characteristics of an *S*-nitrosothiol, i.e. absorbance maximum at approximately 340 nm.

Nitrite and H_2S do not react at neutral pH [16], but acidified nitrite is used to induce S-nitrosothiol formation through the formation of $[H_2NO_2]^+$ species.

$$HNO_2 + H^+ \rightarrow [H_2NO_2]^+ \rightarrow [H_2O + NO^+]. \qquad (3.5)$$

By adding H_2S to acidified nitrite, we could detect transient red-brown coloration and identify HSNO by ESI-TOF-MS. Finally, using ESI-TOF-MS, $^{14}N/^{15}N$ IR and ^{15}N NMR we also studied the trans-nitrosation reaction between S-nitrosoglutathione and H_2S. HSNO, more precisely *cis*-HSNO has been shown to be formed. HSNO was not stable and decayed in time, particularly depending on the GSNO/H_2S ratio [13]. In addition, coordinated HSNO has been observed in nitroprusside [46], water-soluble haem centres [17] and Ru(III)EDTA [47].

The most surprising was the ability of HSNO to act as a carrier of 'NO^+' from one protein to another, even through the cell membrane. Namely, when *S*-nitrosated bovine serum albumin (BSA-SNO) was placed in a dialysis bag, which was immersed into the solution of BSA, addition of H_2S led to nitrosation of the outer solution containing BSA. A similar observation was made with haemoglobin and red blood cells (RBC): RBC incubated with BSA-SNO in the presence of H_2S showed increased haemoglobin *S*-nitrosation [13].

Protein *S*-nitrosation is considered to be an important post-translational modification. The role of *S*-nitrosation in controlling the protein function has been extensively covered in the past decade [48–54]. *S*-nitrosation has been implicated in the regulation of proteins involved in muscle contractility, neuronal transmission, host defence, cell trafficking, apoptosis, cardioprotection, etc. [48–54]. One of the unsolved questions, however, is the means by which trans-nitrosation (formal transfer of 'NO^+' moiety) proceeds from one *S*-nitrosothiol to another [54–56]. Most *S*-nitrosothiols do not freely diffuse into the cells. A protein transporter for *S*-nitrosocysteine has been reported but it cannot explain [54], for example, almost immediate *S*-nitrosation of complex I in mitochondria achieved

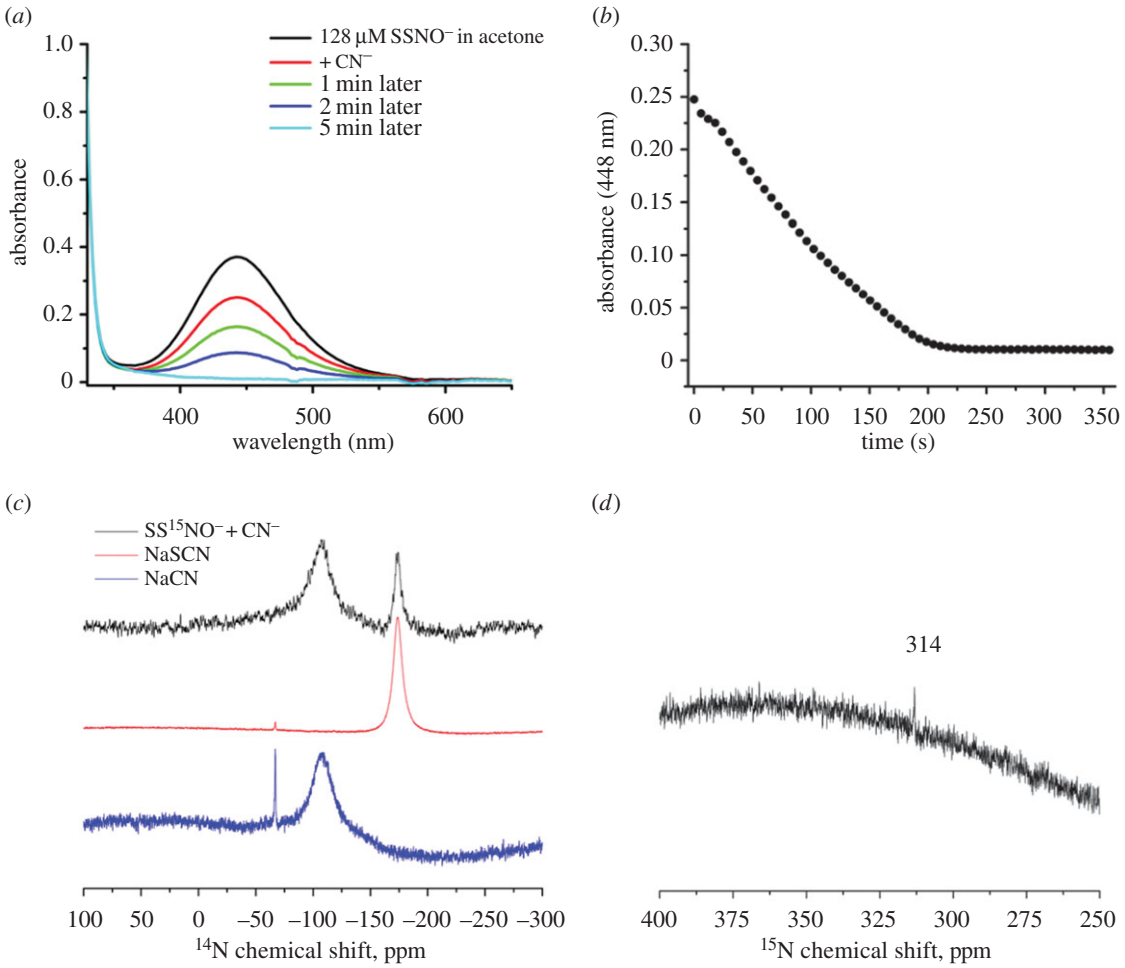

Figure 4. $SSNO^-$ reacts with cyanide to give SNO^- and SCN^-. (*a*) Time-resolved spectra of $SSNO^-$ decomposition upon addition of KCN. (*b*) Kinetics of $SSNO^-$ decomposition in acetone upon addition of KCN. (*c*) ^{14}N NMR spectrum of a mixture of 32 mM $PNP^+SS^{15}NO^-$ and 200 mM NaCN in 5 : 2 acetone : methanol (black line) solution, recorded 4 h after mixing. The two broad signals at about −173 ppm and at about −107 ppm are ascribed to SCN^- and unreacted CN^-, respectively, by measuring ^{14}N NMR spectra of solutions of NaSCN (about 1 M, red line) and NaCN (about 200 mM, blue line) in the same solvent mixture. (*d*) ^{15}N NMR spectrum of a mixture of 32 mM $PNP^+SS^{15}NO^-$ and 200 mM NaCN in 5 : 2 acetone : methanol solution (the same sample as shown in the figure 4*c*). The signal at 314 ppm is ascribed to $S^{15}NO^-$ (figure 2).

by treating the cells with *S*-nitrosothiols [57,58]. By reacting with H_2S, low-molecular weight (LMW) and protein *S*-nitrosothiols could generate HSNO and we showed previously that HSNO could fulfil the criteria of acting as a shuttle of 'NO^+', which classifies it as a new signalling species [13]. Indeed, inhibition of H_2S production led to lower intracellular *S*-nitrosation, while the combination of H_2S with RSNO increased the total *S*-nitrosothiol content when compared with a treatment with RSNO alone [59].

Recently, HSNO has been made at room temperature by diluting NO and H_2S gases with Ne [34]. The reaction took place due to the fact that under this experimental condition there is a surface-catalysed NO disproportionation and formation of N_2O_3 which then reacted with H_2S to give HSNO. McCarthy and co-workers used FTMW spectroscopy to prove facile HSNO formation from N_2O_3 and H_2S [34]. Maximal HSNO concentration was reached when all N_2O_3 was consumed. Further addition of H_2S led to disappearance of HSNO and formation of N_2O. Using ^{18}O labelled N_2O_3 (equation (3.6); * represents the ^{18}O) the authors detected $HSN^{18}O$ and then $N_2^{18}O$, which suggests that HNO was generated in between (equation (3.7)), confirming our previous findings from the buffered solutions [13].

$$N_2O_3^* + H_2S \rightarrow HSNO^* + HNO_2^*, \tag{3.6}$$

$$HSNO^* + H_2S \rightarrow HNO^* + HSSH \tag{3.7}$$

and

$$2HNO^* \rightarrow N_2O^* + H_2O^* \tag{3.8}$$

Although reaction between HSNO and H_2S to give HNO (equation (3.7)) is thermodynamically unfavourable ($\Delta_{rxn}G^{0\prime} = +32$ kJ mol^{-1}) [60], if the products get removed/decomposed (e.g. considering the dimerization of HNO to N_2O (equation (3.8)), the reaction of HNO with RSH or H_2S_2 decomposition) reaction could be physiologically relevant. Interesting computational studies by Timerghazin and co-workers proposed that HSNO could isomerize to form Y-isomer SN(H)O, a process that is additionally favoured by water [61]. This Y-isomer could then react with H_2S to give HNO. The lowering of the barrier to isomerization by water and formation of the Y-isomer, as well as other isomers such as HONS, could explain the peculiar observation that (i) HSNO ionizes as $[HSNO + H]^+$ in the MS experiments even at pH 7.4 ([13], this study), (ii) that it remains protonated when coordinated [17,47] and (iii) that it can cross the membrane freely [13,59]. Namely, chemical logic implies that the pK_a of HSNO should be lower than that of HNO_2 (4.1), therefore the predominant species in the solution should be SNO^-. However, other isomers would have a higher pK_a.

Facile formation of HSNO from N_2O_3 and H_2S could be of importance for intracellular RSNO generation. Although intracellular N_2O_3 formation is kinetically/statistically improbable, N_2O_3 can easily be formed in lipid bilayers where both NO and O_2 accumulate [62]. The H_2S concentration should also be higher in lipid bilayers than in the cytosol [63] creating ideal conditions for HSNO formation that can act as further trans-nitrosating agent.

Contrary to all these claims where the existence of HSNO was proved by all available analytical methods [13,17,29,31,32,34], Cortese-Krott *et al*. proposed that $SSNO^-$ has to be one of three key bioactive reaction products of the reaction of H_2S with *S*-nitrosothiols [35,40]. Based on these authors' claims, $SSNO^-$ can be obtained in high yields in aqueous solutions at pH 7.4 (even in the presence of oxygen) and it is stable for hours. $HSNO/SNO^-$, however, was marked as unstable to be detected even at low temperatures as it reacts fast to eventually form a stable $SSNO^-$ [35]. This is in contradiction with the previous study by the same authors where HSNO formation was observed at room temperature from a 1 : 1 mixture of RSNO and sulfide in water (pH 7.4) at even higher yield than the putative '$SSNO^-$ mix' [40].

It is claimed that HSSNO is more stable than HSNO, because HSSNO supposedly has increased electron density on the proximal sulfur (although no experimental support for such claim was provided) and therefore does not easily react with HS^- and positive metal centres [35]. This putative $SSNO^-$ was reported to be relatively stable in general, and particularly stable in H_2S, so the synthetic procedure for preparation of its enriched solutions from RSNO and H_2S was proposed [35,40]. Furthermore, this putative $SSNO^-$ showed no reactivity with biological thiols or with cyanide. The facts are quite different: (i) the proximal-S has a +0.24 charge, (ii) the S–N bonds in HSSNO and $SSNO^-$ (calculated BDE 16.0 and 22.1 kcal mol^{-1}, respectively; B3LYP/aug-cc-pv5z, in the presence of solvent/water) are weaker than those in HSNO and SNO^- (BDE 27.74 and 36.21 kcal mol^{-1}, respectively), which makes (H)SSNO more prone to homolysis than (H)SNO [36]. We also demonstrate here quite the opposite reactivity, when using the crystalline $SSNO^-$ (characterized previously by ^{15}N NMR, IR, EPR, MS, X-ray analysis and electrochemical methods). Namely, real $SSNO^-$ reacts with all three, HS^-, CN^- and GSH to actually produce SNO^-, which appears to be more stable species than $SSNO^-$.

$SSNO^-$ formation from RSNO and H_2S is proposed to happen through following reaction steps:

$$\mathrm{RSNO} + \mathrm{HS}^- \rightarrow \mathrm{SNO}^- + \mathrm{RSH}, \quad (3.9)$$

$$\mathrm{SNO}^- + \mathrm{HS}^- \rightarrow \mathrm{HS}_2^- + \mathrm{HNO} \quad (3.10)$$

$$\text{and } \mathrm{HS}_2^- + \mathrm{RSNO\,(or\ HSNO)} \rightarrow \mathrm{SSNO}^- + \mathrm{RSH\,(or H_2S)}. \quad (3.11)$$

The first two reactions (equation (3.9) and (3.10)) have been characterized in our previous studies and confirmed recently by McCarthy and co-workers [34]. The reaction shown in equation (3.11) is in equilibrium and under physiological conditions or the experimental conditions where thiols/H_2S are in excess it will proceed in an opposite direction, as demonstrated in the figures 2 and 3 and equations (3.1)–(3.2). Both thiols and H_2S react with $SSNO^-$ to give $HSNO/SNO^-$ and HS_2^- as confirmed by ^{15}N NMR and ESI-TOF-MS (figures 2 and 3).

The '$SSNO^-$ mix' has been used to treat animals and cells, and is proposed to represent storage of NO and to be an activator of Keap-1/Nrf-2 signalling [35,40,41,64]. Reanalysing the original data published by Cortese-Krott *et al*. [35], Koppenol & Bounds discussed recently the probability for $SSNO^-$ formation under physiological conditions following the reaction steps shown in equations (3.9)–(3.11) [60]. The kinetics of $SSNO^-$ formation, based on the reported experimental data [35], was estimated to be 10^{-14} M^{-1} s^{-1} [60]. In other words, even if we assume that $SSNO^-$ remains stable in the cell, it would take more than 1 day to generate 1 nM concentration from 1 μM H_2S, which is, for most of the tissues, an unreachable concentration under physiological conditions. Instability aside, given the starting concentrations of NO and H_2S inside a cell and the likelihood of them interacting at a chemical level in the presence of outcompeting substrates, it is more likely that it would take even more than 1 day to form 1 nM $SSNO^-$. Therefore, it becomes unclear what would be the physiological (and even pharmacological) relevance of treating the cells and animals with 2 mM combination of NO and H_2S [35] or 40 μM of '$SSNO^-$ mix' [41], for example. If 1 nM $SSNO^-$ cannot be formed in the cells what would be the physiological relevance of 40 μM?

In conclusion, it is of utmost importance that eventual studies with $SSNO^-$ are performed using clean and defined substance, which is simple to prepare, than to use reaction mixtures of unidentified composition, as the latter might lead to erroneous conclusions.

Furthermore, it is very important to separate the physiological relevance of all these proposed reactions from those that are just possible *in vitro*. Considering the very low HS^- concentration [65,66] and very high thiol concentrations, the reaction shown in equation (3.10) is less likely to occur (although the rate constant for that reaction is approximately 10^7 M^{-1} s^{-1} [60]), let alone form HS_2^-, which is intrinsically unstable [67,68] and could easily be reduced by the intracellular thiol pool, and wait to meet another *S*-nitrosothiol (or, even less probable, another HSNO) to form $SSNO^-$. $HSNO/SNO^-$ remains therefore the most probable signalling molecule able to react with the abundant thiol pool to either cause further trans-nitrosation or HNO and RSS^- formation, in specific protein environments [69].

Competing interests. We declare we have no competing interests.

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