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Acid-free synthesis of *S*-nitrosothiols at neutral pH by shock-freezing in liquid nitrogen

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Abstract: A laboratory method for the acid-free synthesis of *S*-nitrosothiols (RSNO) from $NaNO_2$ and thiols in aqueous buffer of neutral pH is reported. Equimolar amounts of $NaNO_2$ and thiol are added to a 100-μM Na_4EDTA-containing Na_2HPO_4 buffer (100 mM, pH 7.4). Samples are set into liquid nitrogen for 1 min and then thawed to room temperature or in an ice bath. Dependent upon the RSNO, repetition of the procedure may be required. This method may be of particular importance when the classical acid-based method fails or releases harmful gases such as H_2S.

Key words: LC-MS, nitric oxide, nitrite, *S*-nitrosothiols

1. Introduction

Nitric oxide (NO) is an endogenous, short-lived signalling molecule produced from L-arginine by the catalytic action of NO synthases (NOS).[1] Formally, *S*-nitrosothiols (RSNO) are NO derivatives. *S*-Nitrosothiols are also signalling molecules and possess NO-related biological activities, especially in the cardiovascular system, such as vasodilation and inhibition of platelet aggregation by cyclic guanosine monophosphate (cGMP)-dependent and cGMP-independent mechanisms.[2–4] RSNO can be formed under aerobic conditions from the reaction of higher oxides of NO such as N_2O_3 (R1) with the sulphhydryl group (SH) of thiols (RSH) such as soluble cysteine (Cys) and glutathione (GSH), as well as Cys moieties in proteins including human serum albumin and haemoglobin.[1–4] RSNO can also be formed from the reaction of nitrite (NO_2^-), the autoxidation product of NO in aqueous solution (R2), with RSH in particular organs and acidic media such as in gastric juice (R3).

$$N_2O_3 + 2\,RSH \rightarrow 2\,RSNO + H_2O \quad (R1)$$

$$4\,NO + O_2 + 2\,H_2O \rightarrow 4\,NO_2^- + 4\,H^+ \quad (R2)$$

$$NO_2^- + H^+ + RSH \rightarrow RSNO + H_2O \quad (R3)$$

$$NO_2^- + H^+ \rightarrow HONO \quad (R3a)$$

$$HONO + RSH \rightarrow RSNO + H_2O \quad (R3b)$$

$$R'ONO + RSH \rightarrow R'OH + RSNO \quad (R4)$$

The most convenient laboratory method for preparing low-molecular-mass RSNO is the acid-catalysed *S*-nitrosation of RSH according to reaction (R3).[5] The nitrite:thiol stoichiometry for this reaction is 1:1. RSNO

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formation by reaction (R3) is likely to occur via intermediate nitrous acid (HONO, $pK_a \approx 3.3$) formation from acidified nitrite. RSNO can be prepared at neutral pH by using organic nitrites (R′ONO) such as butyl or amyl nitrite according to reaction (R4). Shortcomings of this method are 1) the need for commercially available R′ONO or their preceding preparation, 2) use of over-stoichiometric amounts of R′ONO, and 3) the presence not only of excess of R′ONO but also of the second reaction alcoholic product R′OH that may potentially interfere in subsequent investigations. Synthesis of low-molecular-mass RSNO based on reaction (R3) is the most feasible method and provides pure RSNO preparations that are stable in their acidic stock solutions and essentially free of reactants provided they are used at equimolar amounts.[5] Interestingly, reaction (R3) allows facile preparation of RSNO labelled with stable isotopes in the *S*-nitroso group, i.e. ^{15}N and/or ^{18}O.[5] This particular feature enables studies on reactions of RSNO and on the metabolic fate of their *S*-nitroso group.[5] In contrast, reaction (R4) requires preceding synthesis of stable-isotope labelled organic nitrites.

One potential disadvantage of the method based on reaction (R3) is that RSNO stock solutions are acidic and require neutralisation prior to use. Given the particular instability of certain RSNO, notably of *S*-nitrosocysteine,[1] the neutralisation step may be associated with considerable RSNO loss. Our intention was therefore to prepare aqueous buffered solutions of RSNO under very mild, stability-favouring conditions, which can be used directly, without additional, potentially hazardous sample manipulation. Eventually, a much more serious disadvantage of reaction (R3) is the formation of gaseous and toxic volatile thiols, such as hydrogen sulphide (H_2S),[6] during acidification.

Phosphate buffer (pK_{a2}, 7.2) is the most important intracellular buffer system in living organisms and one of the most commonly used buffers in in vitro investigations. Use of aqueous solutions of sodium phosphate (NaP) but not of potassium phosphate (KP) buffers is associated with a pH shift to levels as low as 3.6,[7,8] which is close to the pK_a value (3.34) of the strong nitrosating nitrous acid (ONOH). We[9] and others[10] have shown that freezing of nitrite-containing NaP at –80 °C was associated with formation of RSNO and even partial oxidation of nitrite to nitrate in certain conditions. pH fall in frozen NaP is considered to be due to precipitation of $Na_2HPO_4 \times 12H_2O$.[8] The mechanism by which nitrite oxidises to nitrate in frozen NaP is still elusive. In the presence of aromates such as paracetamol, shock-freezing is a suitable method to synthesise nitro-aromates and polymeric aromates such as nitro-paracetamol and di-paracetamol.[11] These observations prompted us to test the possibility of utilising this particular freeze phenomenon for the synthesis of RSNO in aqueous buffer under exceptionally mild conditions, namely at deep temperatures.

2. Results and discussion

In preliminary experiments, we observed that shock-freezing in liquid nitrogen instead of slow freezing at –20 °C or –80 °C[9] of nitrite- and RSH-containing solutions in sodium phosphate buffer of pH 7.4 (prepared from $Na_2HPO_4 \times 12H_2O$ and $NaH_2PO_4 \times 12H_2O$) was also associated with RSNO formation. The second modification we performed in the present study was the preparation of NaP buffer by using only one salt, i.e. $Na_2HPO_4 \times 12H_2O$, and by adjusting the pH to 7.4 with concentrated H_3PO_4. By including a litmus strip in the thus prepared 100 mM NaP buffer, we noted that pH fell to values between 2 and 1 (red litmus) upon shock-freezing and increased to the original pH value (green litmus) after complete thaw. These conditions seemed to be optimal for the preparation of pH-neutral buffered RSNO solutions. The deeper pH fall seen in the NaP prepared by using only the $Na_2HPO_4 \times 12H_2O$ salt compared to the NaP prepared by using $Na_2HPO_4 \times 12H_2O$ and $NaH_2PO_4 \times 12H_2O$[8] supports the idea that pH fall is caused by $Na_2HPO_4 \times 12H_2O$ precipitation

during shock-freezing. It is worth mentioning that RSNO synthesis from RSH and nitrite at pH values in the pH range 1 to 2 (by using HCl acid) are optimal for instantaneous and quantitative formation of RSNO from RSH and nitrite.[5]

For method optimisation and validation we choose the reduced form of three endogenous cysteinyl thiols, i.e. glutathione (GSH), cysteine (Cys), and *N*-acetylcysteine (NAC), and a synthetic lipophilic thiol, i.e. *N*-acetylcysteine ethyl ester (NACET) (Figure 1). Aqueous solutions of the corresponding RSNO, i.e. *S*-nitrosoglutathione (GSNO), *S*-nitrosocysteine (SNOC), *S*-nitroso-*N*-acetylcysteine (SNAC), and *S*-nitroso-

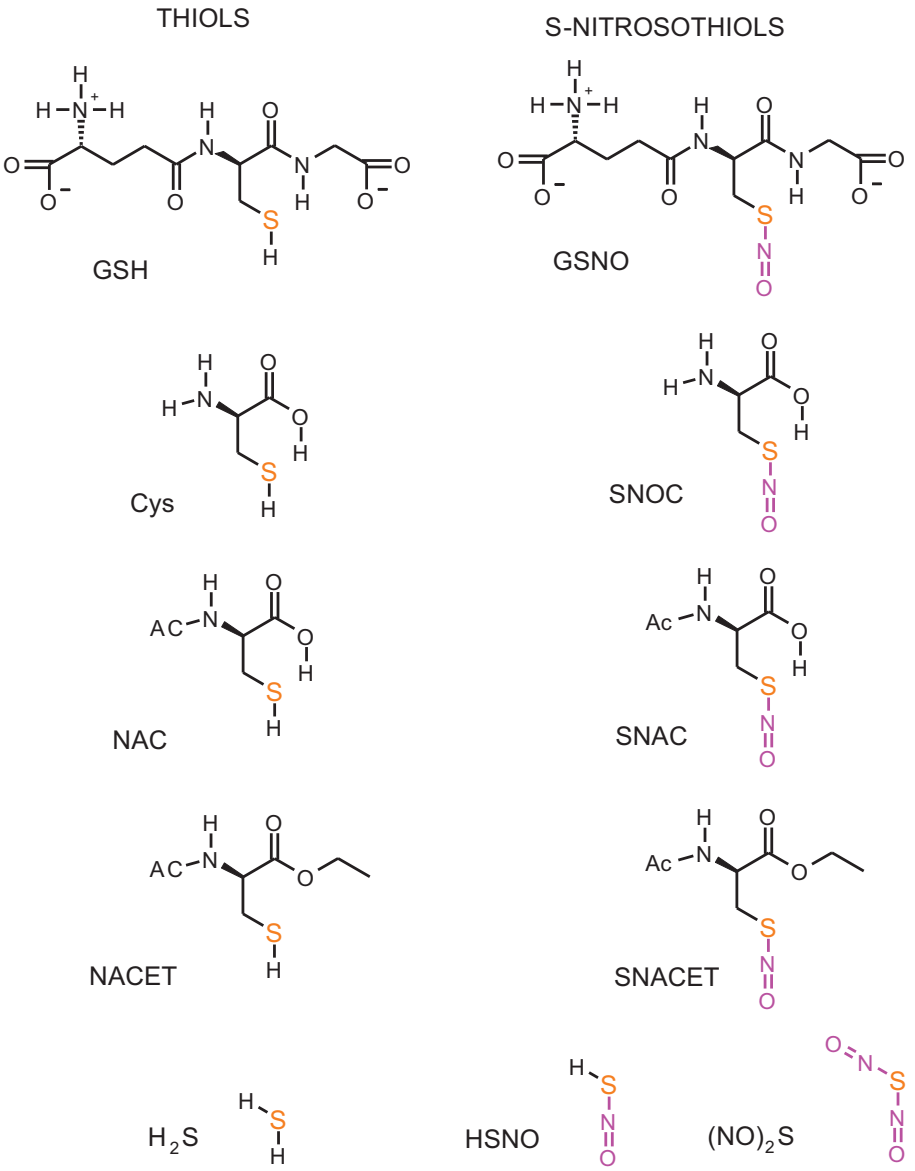

Figure 1. Chemical structures of the thiols (RSH) and the corresponding *S*-nitrosothiols (RSNO) investigated in the present work. Glutathione (GSH), cysteine (CysSH), *N*-acetylcysteine (NAC), *N*-acetylcysteine ethyl ester (NACET), hydrogen sulphide (H_2S), *S*-nitrosoglutathione (GSNO), *S*-nitrosocysteine (SNOC), *S*-nitroso-*N*-acetylcysteine (SNAC), *S*-nitroso-*N*-acetylcysteine ethyl ester (SNACET), *S*-nitroso-sulphide (HSNO), *S*-dinitroso-sulphide ($(NO)_2S$).

N-acetylcysteine ethyl ester (SNACET) (Figure 1), are purple at 10-mM concentrations and absorb light in the visible range around 334 nm, with a molar absorptivity of about 0.7 $mM^{-1} \times cm^{-1}$.[5] This physicochemical property was utilised for the qualitative and quantitative evaluation of the present shock-freezing procedure. LC-MS and LC-MS/MS approaches were used to identify reaction products and possible side-products.

By using equimolar concentrations (10 mM) of RSH and nitrite in NaP, we noticed that colour did not develop during shock-freezing but mainly during sample thawing to room temperature. Moreover, colour developed differently for the individual RSNO. LC-MS and LC-MS/MS analyses confirmed the formation of SNACET, SNAC, SNOC, and GSNO (Figure 2A and Figure 2B, respectively). Major ions in the mass spectra were ions due to $[M+H]^+$ and $[M - NO + H]^{\cdot +}$. Collision-induced dissociation of $[M + H]^+$ yielded the major product ion $[M - NO + H]^{\cdot +}$. The $[M + H]^+$ and $[M - NO + H]^{\cdot +}$ ions observed from all synthesised RSNO unequivocally identify the formation of the respective RSNO with an intact *S*-nitroso group.[12,13] LC-MS analysis of the thus synthesised RSNO did not reveal the formation of other theoretically possible reaction products such as *N*-nitrosothiols, *S*,*N*-dinitrosothiols, phosphorylated derivatives, or thiol disulphides (data not shown).

UV/vis spectroscopy analysis indicated absorbance maxima at 336 nm and 546 nm for all investigated RSNO, which are characteristic for the *S*-nitroso group (S-NO) of cysteinyl RSNO.[5] Repetition of the shock-freezing/thaw procedure increased colour intensity and absorbance at 334 nm for all RSNO only when the chelator Na_4EDTA (100 μM) was present (Figure 3A). In the absence of Na_4EDTA, performance of the shock-freezing/thaw procedure three times led to decomposition of SNOC (Figure 3A). It is worth mentioning that SNOC is one of the most short-lived and strongest NO donors in aqueous solution of neutral pH.[5] Incorporation of Na_4EDTA or sodium but not potassium salts of other chelators in the NaP buffer is advantageous for SNOC. Figure 3A indicates that different conditions may be required for the synthesis of RSNO by the shock-freeze procedure.

The absorbance value at 336 nm was comparable for all *S*-nitrosocysteinyl thiol preparations and of the same order, at a molar basis, of reported values for these and other RSNO.[5] Under practically identical conditions, preparation of RSNO by the present method was reproducible. For instance, the intra-run reproducibility for the synthesis of SNOC, the most labile *S*-nitrosothiol, varied by 3% to 5% as determined by spectrophotometry (Figure 3B). By using RSNO prepared by the "classical" method based on reaction (R3), we found that the present method produced RSNO with a yield ranging between 80% and 100% using two freeze/thaw steps. We found that the freeze protocol also applies to the preparation of RSNO of highly volatile thiols such as H_2S, for instance by using buffered solutions of Na_2S. However, the recovery rate is much lower compared to RSH (Figure 3C), and furthermore water-insoluble sulphur-containing species precipitate. In addition, it is unclear whether under these or other conditions Na_2S (and/or NaHS) reacts to produce HSNO and/or $S(NO)_2$. In any case, HSNO and $S(NO)_2$ are far less stable than *S*-nitrosothiols (RSNO) from cysteinyl thiols (RSH).

3. Experimental

Stock solutions of $NaNO_2$ (1 M; Riedel-de-Haën, Seelze, Germany), thiols (0.1 M), and Na_4EDTA (10 mM; Merck, Munich, Germany) were freshly prepared in distilled water and stored in an ice bath in the dark (aluminium foil) during the procedure. NaP was prepared by diluting $Na_2HPO_4 \times 12H_2O$ (50 mmol; Merck, Munich, Germany) in 0.5 L of distilled water. The pH was adjusted to 7.4 by using concentrated *ortho*-phosphoric acid (Merck, Munich, Germany) and a glass electrode, and the buffer was stored in a 0.5-L glass flask

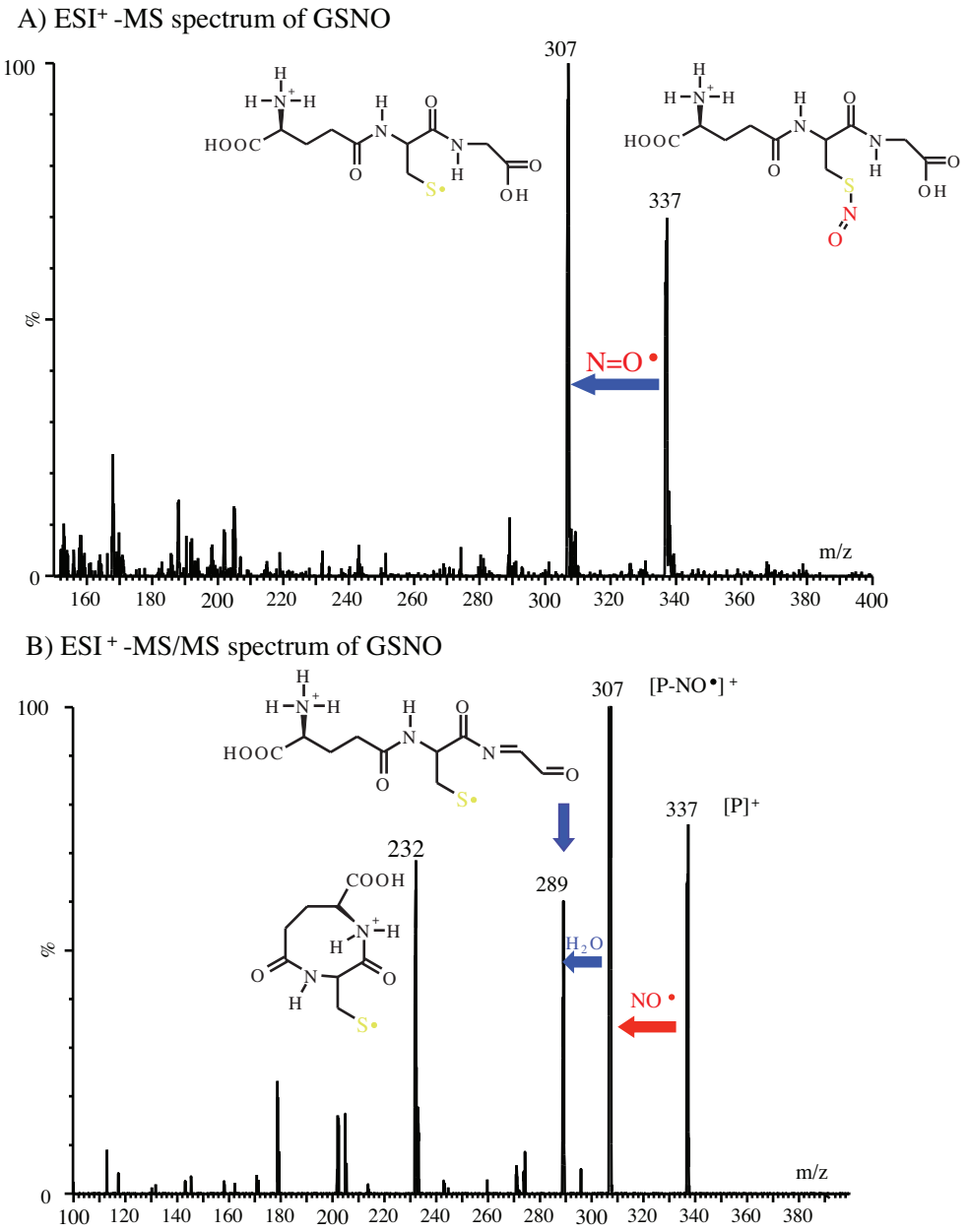

Figure 2. ESI^+ LC-MS (A) and LC-MS/MS (B) spectra of *S*-nitrosoglutathione (GSNO). GSNO was prepared by a two-cycle freezing/thawing (–196 °C/room temperature) procedure using a solution of $NaNO_2$ (10 mM) and GSH (10 mM) in 100 mM sodium phosphate buffer, pH 7.4. In LC-MS/MS, the precursor ion (P) $[M+H]^+$ was subjected to collision-induced dissociation. Scanning rate and collision energy were 1 s and 22 eV, respectively. Insets show the proposed structures of detected ions.

in a refrigerator at 8 °C. NaP aliquots (1 mL) were transferred into 1.3-mL polypropylene tubes. Subsequently, aliquots of the Na_4EDTA (where required) and nitrite and thiols solutions were added and mixed by vortexing to reach final concentrations of 100 μM, 10 mM, and 10 mM, respectively. Under these conditions (about 22 °C) no colour develops. By means of long tweezers, the closed polypropylene tubes were set in series into liquid nitrogen (Linde, Hannover, Germany) placed in a small-volume (e.g., 0.5 L) Dewar vessel and held therein

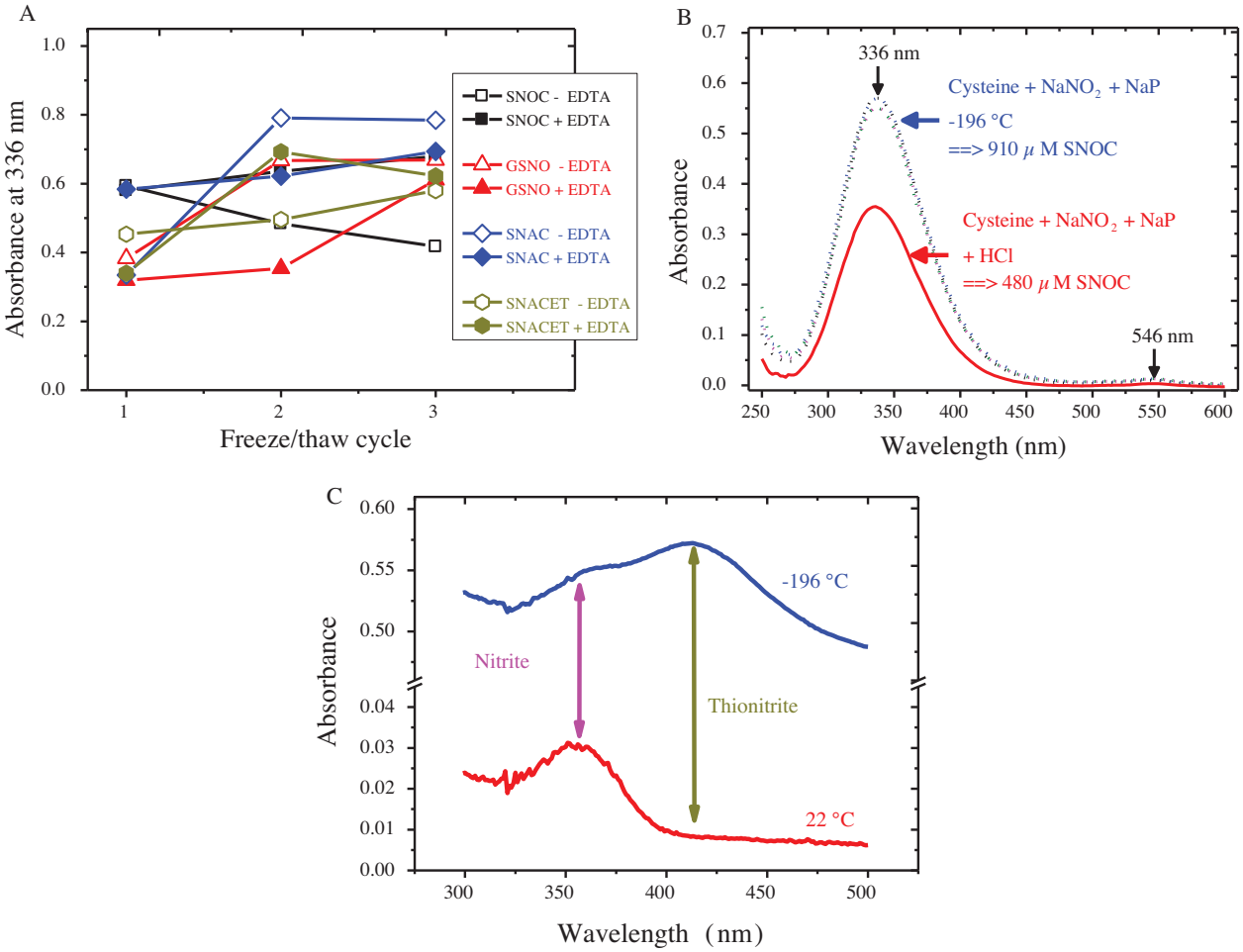

Figure 3. (A) UV absorbance at 336 nm of individual solutions of L-Cys, GSH, *N*-acetylcysteine, and *N*-acetyl cysteine ethyl ester (each 10 mM) and $NaNO_2$ (each 10 mM) in 100 mM sodium phosphate buffer, pH 7.4, in the absence or in the presence of Na_4EDTA (100 μM), measured after the 1st, 2nd, and 3rd freezing and thawing to room temperature (22 °C). The corresponding RSNO are SNOC, GSNO, SNAC, and SNACET. (B) UV/vis spectra of SNOC prepared by a two-step freezing/thawing (–196 °C/22 °C) procedure of four separate solutions of $NaNO_2$ (10 mM) and L-Cys (10 mM) in 100 mM sodium phosphate buffer, pH 7.4. A 4.8-mM solution of SNOC was also prepared by the classic method using HCl acidification.[5] (C) UV/vis spectra of a mixture of Na_2S (5 mM) and $NaNO_2$ (10 mM) in 100 mM sodium phosphate buffer, pH 7.4, before freezing and after a single freezing at –196 °C and thawing in an ice bath. In all cases, original solutions were diluted with the same buffer (1:10, v/v) immediately prior to UV/vis spectroscopy analysis, which was performed on the spectrophotometer (Analytik Jena, Jena, Germany).

for 1 min. Then the samples were allowed to thaw to room temperature or in an ice bath. Subsequently, the procedure was repeated where required (see below). Aliquots of the samples were taken and analyzed by UV/vis spectroscopy and LC-MS.

LC-MS and LC-MS/MS analyses were performed using a Waters ACQUITY UPLC–MS/MS system consisting of a solvent delivery device, an autosampler, a column thermostat, and the tandem quadrupole mass spectrometer XEVO TQ MS (Waters, Milford, MA, USA). The mobile phase consisted of a mixture (1:1, v/v) of daily prepared deionised water (Milli-Q Synthesis A10 System; Millipore, Billerica, MA, USA) and LC-MS

grade acetonitrile (Mallinckrodt Baker, Deventer, Netherlands) and contained 25 mM ammonium formate and 0.1 vol% HCOOH. The mobile phase pH was adjusted to 7 by means of aqueous ammonia. The flow rate was kept constant at 0.2 mL/min. The autosampler was equipped with a 10-μL sample loop, and 10-μL aliquots of 1:1 (v/v) dilutions of samples in the mobile phase were injected. Separation of the analytes was carried out on a Merck SeQuant ZIC HILIC column (100 $\times$ 2.1 mm internal diameter, 3.5 μm particle size) at 35 °C. Electrospray ionisation in the positive mode (ESI^+) was used with nitrogen (600 °C, flow rate of 1000 L/h) as the desolvation gas. The capillary voltage was set to 0.8 kV and the ion source was kept at 150 °C. Argon served as the collision gas (0.13 mL/min, 1.8 $\times$ 10^{-3} mbar). System operation, data acquisition, and data processing were accomplished with the software MassLynx V4.1 from Waters.

4. Conclusions

In summary, aqueous solutions of RSNO are best prepared by acidifying equimolar amounts of nitrite and thiols. Here an alternative laboratory method is described for the acid-free preparation of RSNO in sodium phosphate buffer of neutral pH by shock-freezing in liquid nitrogen. The method provides pH-neutral buffered solutions of RSNO ready for use in basic chemical and biochemical mechanistic studies on topics related to NO. The present method is particularly useful to prepare RSNO when the "classical" acid-based method fails or is associated with a risk of formation of harmful gases such as H_2S.

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