Vol. 28 (no. 1, March 2016) pp 1–15

Biokemistri

Nigerian Society for Experimental Biology (http://nisebpublications.org)

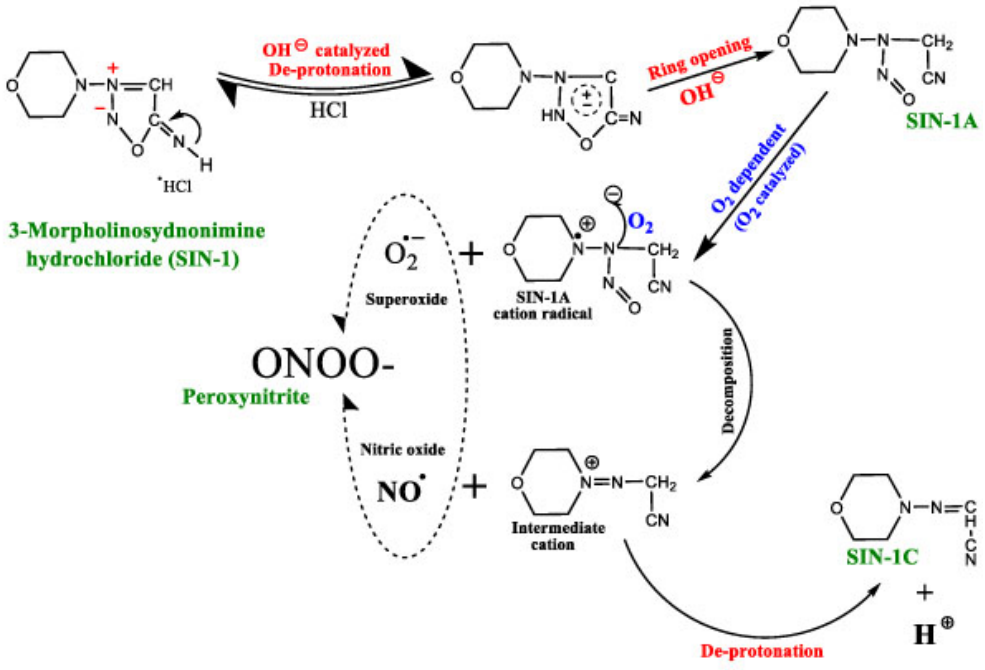
Research Article

In-situ generation of peroxynitrite from 3-morpholinosydnonimine decay in pulmonary cell culture medium

Ejaife Ono Agbani

School of Physiology, Pharmacology & Neuroscience, Faculty of Biomedical Sciences, University of Bristol, University Walk, Bristol, BS8 1TD.

Correspondence: e.agbani@bristol.ac.uk; +44(0)1173311448.



Received: 18 October 2015: Approved: 3 January 2016.

ABSTRACT: Background: Peroxynitrite (ONOO-) is a strong oxidising and nitrating agent generally implicated in oxidative stress. Its cellular action is linked with pulmonary artery cell hyper-proliferation and vascular remodelling seen in pulmonary hypertensive diseases. It is thus vital to elucidate the biological actions of peroxynitrite; however, working with the anion is challenging. Whether supplied commercially or prepared extemporaneously, ONOO- is stabilised and stored under strongly alkaline conditions and the exposure of cells to this form of ONOO- will in tandem increase culture media pH. Accordingly, increasing number of studies are seeking alternative means of generating peroxynitrite and have utilised 3-morpholinosydnonimine (SIN-1) the active metabolite of the vasodilatory drug molisdomine to generate peroxynitrite in-situ. Even so, it is not clear how much authentic ONOO- is generated under these conditions and for how long. Aim: To establish the formation of peroxynitrite and to determine its decay kinetics following SIN-1 decomposition in a medium formulated for the culture of primary human and bovine pulmonary artery cells. Results: The half-life of authentic peroxynitrite was determined to be 1.38s in pulmonary artery cell culture medium. Formation of peroxynitrite during 3-morpholinosydnonimine (SIN-1) decomposition was continuously monitored from the loss in fluorescence associated with the ONOO- oxidation of nicotinamide adenine dinucleotide (NADH) to NAD^+. SIN-1 decayed by 1^{st} order kinetics and 20μM SIN-1 generated ONOO- at the rate of 0.11 μM min^{-1}. SIN-1 decomposition in cell culture medium was associated with the formation of a stable intermediate product SIN-1C with absorbance (λ_{max}) at 279±2nm. The SIN-1 → SIN-1C transformation was oxygen dependent and the result of ^{-}OH catalyzed hydrolytic decomposition. Stopped-flow spectroscopic evidence revealed that SIN-1 can be de-protonated in a pH dependent manner during the phase of the reaction leading up to SIN-1A formation. Conclusion: The formation of ONOO- was demonstrated by the qualitative and quantitative determination of its in-situ generation from the decay of 3-morpholinosydnonimine (SIN-1). This will enable relevant correlations of the life of peroxynitrite in culture conditions to its actions in pulmonary cells.

KEYWORDS: Peroxynitrite, from SIN-1, culture medium

INTRODUCTION

Peroxynitrite (ONOO-) is a potent oxidizing and nitrating radical produced in-vivo from the diffusion controlled reaction of nitric oxide and superoxide; it production has been reported in endothelial cells and activated macrophages [1, 2]. The biochemical effects of ONOO- suggest that the molecule may mediate cell proliferation and account for the cellular hyper-proliferation observed in patients with pulmonary hypertension [3, 4]. In interacting with bio-molecules, peroxynitrite will preferentially nitrite the tyrosine moieties of protein; hence the formation of 3-nitro tyrosine is a widely accepted footprint or marker of the presence or in-situ biosynthesis of peroxynitrite. Accordingly, increasing number of evidence suggest that peroxynitrite is upregulated in the lungs of patients with long standing pulmonary hypertension [5]

Studying the cellular actions of ONOO- is vital to understanding its roles in vascular oxidative stress and diseases; however, working with authentic peroxynitrite (ONOO-) as a drug is challenging. The half-life of ONOO- is 1.9 s at physiological pH [6] and there are but few data on the life and profile of the molecule in cell culture medium. In addition, whether supplied commercially or prepared extemporaneously, ONOO- is stabilised and stored under strongly alkaline conditions [7, 8] and the exposure of cells to this form of ONOO- will in tandem lead to increase in culture media pH [3, 4]. Accordingly, increasing number of studies are seeking alternative means of generating peroxynitrite while retaining physiological pH conditions [9-12]. The molecule 3-morpholinosydnonimine (SIN-1) the active metabolite of the vasodilatory drug molisdomine has been reported to generate both nitric oxide and superoxide anion, thus suggesting that SIN-1 may be a peroxynitrite generator [13-15]. Indeed, SIN-1 decomposes primarily to yield nitric oxide (NO) under physiological conditions or in the presence of electron acceptors which may include those other than oxygen found in biological systems [16].

Consistent with the hallmark reaction of ONOO-, Trackey & co-workers[17] showed that SIN-1 caused a concentration-dependent increase in cortical cell injury associated with a

parallel increase in the release of cellular proteins containing 3-nitro-tyrosine into the culture medium. Furthermore, indirect evidence of the in-situ formation of peroxynitrite from SIN-1 may be deduced from studies using superoxide dismutase (SOD) to scavenge superoxide and thus prevent the formation and action attributed to peroxynitrite during SIN-1 decomposition [7]. Reported peroxynitrite-like effects of SIN-1 include oxidation of low-density lipoproteins [18], degradation of deoxyribose ([19], and inhibition of glyceraldehyde-3-phosphate-dehydrogenase [20]. Indeed, the cytotoxic effects of SIN-1 are comparable with those of authentic peroxynitrite have been reported in various cell types [21-23]. Even so, it is not clear how much authentic ONOO- is generated under these conditions and for how long. This study thus aimed to establish the formation of peroxynitrite and to determine its decay kinetics following SIN-1 decomposition in a medium formulated for the culture of primary human and bovine pulmonary artery cells.

MATERIALS AND METHODS

Materials

3-morpholinosdynonimine (SIN-1) was purchased from Sigma (Poole, UK). Peroxynitrite was supplied (Calbiochem, United Kingdom and Ireland) as a 170 mM solution in 4.7% NaOH. Also, Nicotinamide adenine dinucleotide (β-NADH) and superoxide dismutase from bovine erythrocytes were obtained from Sigma (Poole, UK). Cell culture media included Large vessel endothelial cell basal medium-prf (TCS cell works; Cat # ZHM-2959) and Leibovitz's L-15 medium (Invitrogen® Cat #: 21083-027). Media optimised for the culture of Bovine and human pulmonary artery cells was a 50:50 mix of Ham F-12 Nutrient mixture medium + L-Glutamine (Invitrogen, UK) and Waymouth's MB 752/1 + L-glutamine (Invitrogen, UK) to which 15% foetal bovine serum (FBS) and 5% penicillin (5000 U/ml):streptomycin (5000 mg/ml) (PEN-STREP, BioWhittaker) were added. This is here referred to as BAHPAC medium.

Stability study of peroxynitrite

Solutions of peroxynitrite at two concentrations (1.7 10^{-3}M and 1.7 10^{-4} M) in NaOH 1M were kept on the shelf at room temperature, at 4° or at -80 °C for 50 days. Absorbance was measured at intervals by placing the peroxynitrite solution in a cuvette into a UV/Visible spectrophotometer (Ultrospec 2000; Pharmacia Biotech), and readings made at selected time points at 302nm to determine ONOO- stability profile. In other experiments, concentration of peroxynitrite solution was determined spectrophotometrically in aliquots thawed just before use based on an extinction coefficient of 1670M-1cm^{-1} at 302 nm in 1 M NaOH.

Decay of Peroxynitrite authentic (ONOO-)

Real time decay of peroxynitrite under conditions of varying pH was evaluated using the Cary50Bio® UV-Visible spectrophotometer (Varian). Using quartz cuvettes, the kinetics of ONOO- were monitored at 302 nm every 10 seconds. Control experiments were also conducted to obtain absorbance spectra of the vehicle (NaOH) and BAHPAC medium.

Decay of 3-morpholinosdynonimine (SIN-1)

Real time decay of SIN-1 under conditions of varying pH was monitored at several wavelengths using quartz cuvettes and Cary50Bio® UV-Visible spectrophotometer (Varian). The spectra and kinetics of SIN-1 were evaluated overnight in BAHPAC and selected mediums. The kinetics of formed authentic peroxynitrite were monitored at 302 nm, the decomposition of SIN-1 and the formation of SIN-1C were monitored at 292 nm at 280 nm nm as anticipated from the literature [16, 24].

The life of authentic and in-situ generated peroxynitrite

These experiments have been performed as previously described [25]. Kinetic data on dynamic processes in chemical and biochemical reactions can be obtained by stopped-flow spectroscopy. Stock solution of peroxynitrite supplied as described above was kept on ice, and diluted into distilled water and immediately used to fill one of the stopped-flow apparatus syringes. In experiments involving SIN-1, one of the stopped flow syringes was filled with double strength of the desired SIN-1 concentration while the other was filled with appropriate buffer of a double strength solution of interest. All reagents were freshly prepared. Solutions were purged with nitrogen in some experiments. Sufficient volume of SIN-1 and NaOH or buffer of interest was pushed through filler ports into the drive syringes or internal tubing; the dead volume is filled and the reaction is initiated in the quartz cell.

Quantification of SIN-1 generated peroxynitrite

Peroxynitrite generated from SIN-1 was quantified by fluorimetry. Fluorescence measurements were carried out with a spectrofluorometer (LS 45 Luminescence Spectrometer, PerkinElmer Instruments). NADH fluorescence was measured with excitation and emission wavelengths of 340 and 460 nm, respectively. The production of peroxynitrite during 3-morpholinosydnonimine (SIN-1) decomposition was continuously monitored in large vessel endothelial cell basal medium-prf as well as BAHPAC medium.

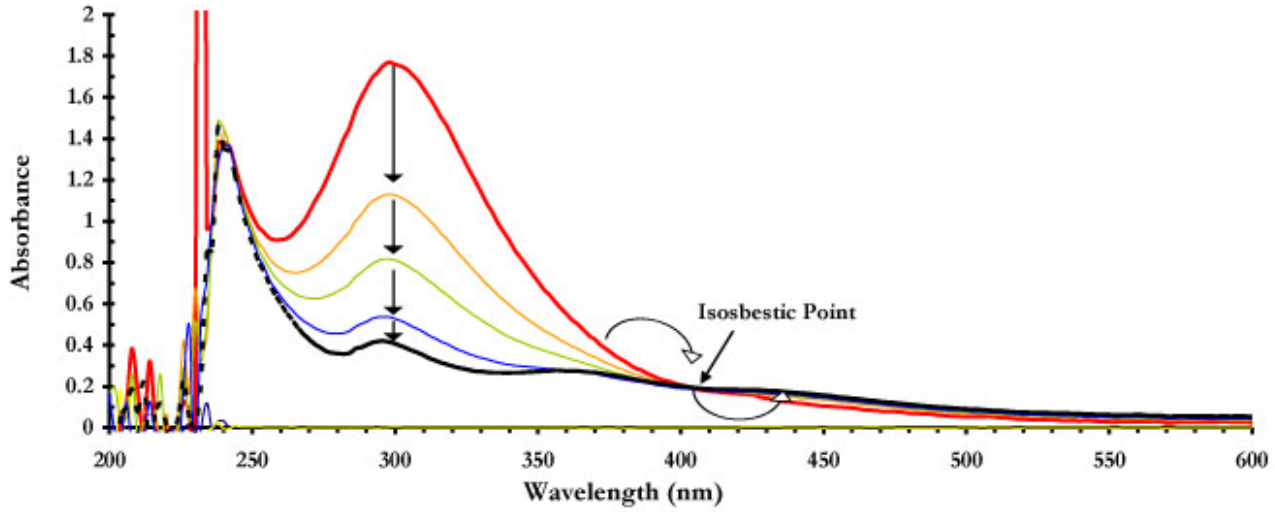


Figure 1: Spectra of the decay of 10^{-3} M peroxynitrite-added, phenol red free, bovine and human pulmonary artery cell culture medium. Peroxynitrite was supplied in 1 M Sodium Hydroxide Solution. Scan was conducted at room temperature every 10 seconds for 15min using the Cary50Bio® UV-Visible spectrophotometer (Varian). First (Red) line represents absorbance measured within first second after injecting peroxynitrite into quartz curvette; subsequent coloured lines are selected decay spectra in the direction of the arrow. The isosbestic point represents the spectroscopic wavelength at which the absorbance of peroxynitrite and a decay product, to which it is being converted, is the same. Experiments were conducted in triplicates.

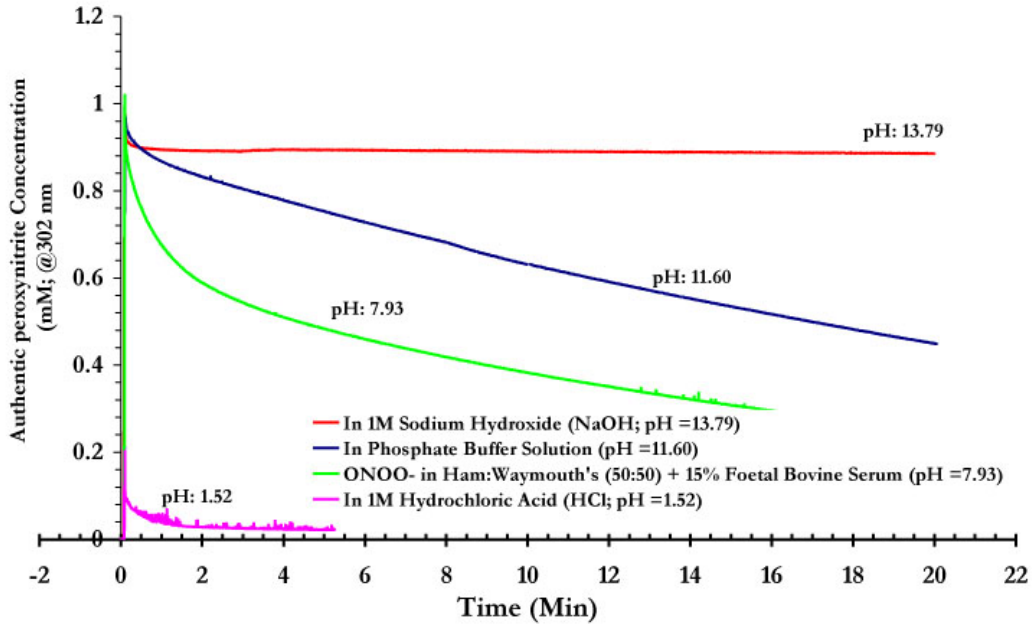


Figure 2: Kinetic profile of 10^{-3} M peroxynitrite decay in various pH conditions. Peroxynitrite was supplied in 1M Sodium Hydroxide Solution. Scan was conducted at room temperature every 0.1 seconds at 302 nm for 15 min using the Cary50Bio® UV-Visible spectrophotometer (Varian). Experiments were conducted in triplicates.

RESULTS

Determination of the stability and half-life of peroxynitrite

It was confirmed that peroxynitrite (ONOO-) is relatively stable in strongly alkaline conditions [25]. The absorbance spectrum (λ_{max} 302 nm) of peroxynitrite in aqueous NaOH was similar to what has been reported in the literature [24] Absorbance was linear down to 10^{-4} M ONOO- (Figure 1B). The stability of ONOO- in 1M NaOH was investigated under various storage and intermittent exposure to room temperature conditions. At room temperature ONOO- decay was approximately log-linear with a 10-fold decay after approximately 5 days. At 40 °C, the decay appeared approximately linear with 10-fold decay in approximately 7 days. At -80 °C, decay of ONOO- appeared curvilinear and fell by approximately half in 14 days. This decay rate indicates that at storage condition of -80 °C, higher concentration aliquots of ONOO- can be stored for up to 3 weeks; it will however be necessary to obtain fresh calibration data for each experiment more than a week apart.

Peroxynitrite decay in pulmonary artery cell culture medium

Spectrophotometric detection of peroxynitrite (ONOO-) added to phenol-red free culture medium showed a prominent absorbance maximum at about 302 nm as expected (Figure 1).

This was similar to the spectrum of ONOO- in aqueous alkaline solution although the latter condition was not associated with significant decay (data not shown). Peroxynitrite was seen to decay rapidly in phenol-red free cell culture medium with the appearance of an isosbestic point evident at a wavelength of 410 nm (Figure 1). The isobestic point is suggestive of the formation of new species (i.e. products) which have not been identified and may or may not be involved in the biological effects elicited by ONOO-. Further experimentation is necessary to fully understand the formation and effects of these species in cell culture medium.

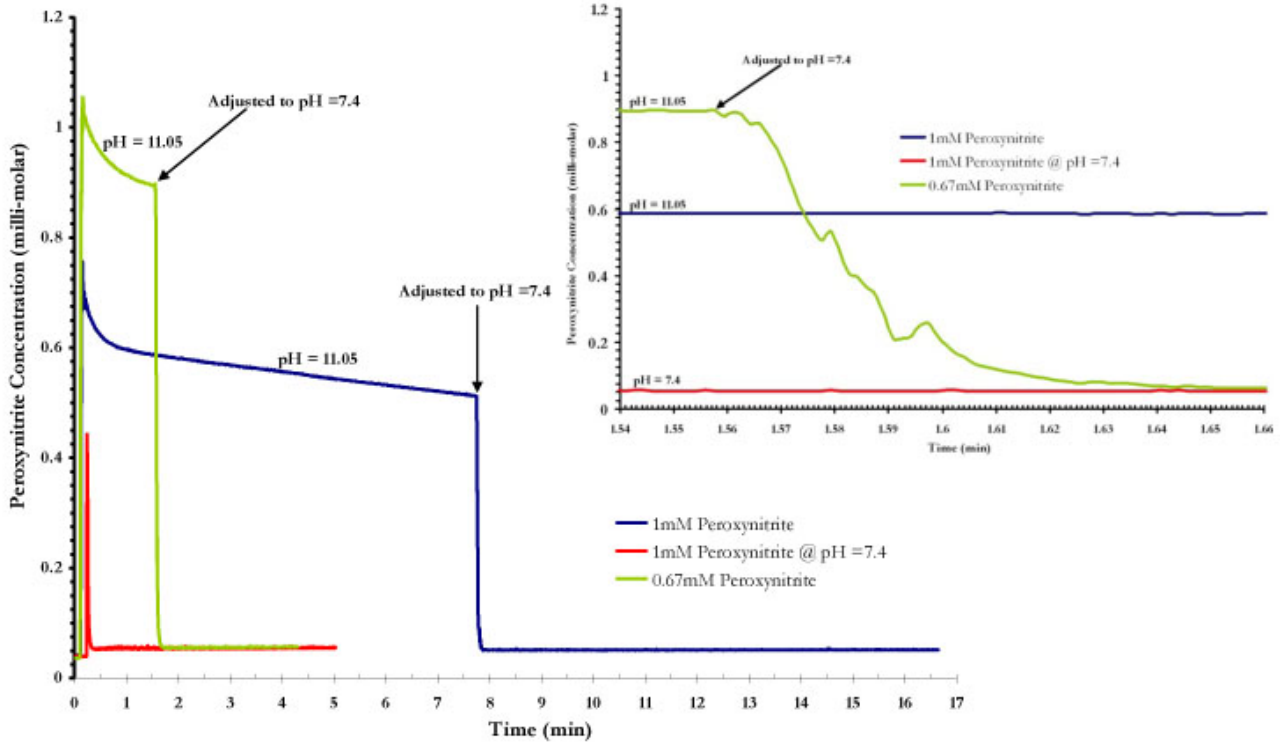


Figure 3: Kinetic profile of 10^{-3} M peroxynitrite decay at pH =7.4 and pH 11.05 adjusted to 7.4 in phosphate buffer solution. Peroxynitrite was supplied in 1 M Sodium Hydroxide Solution. Light absorbance spectrophotometry was conducted at room temperature every. Absorbance at 302 nm was measured every 0.1 interval for 15 min using the Cary50Bio® UV-Visible spectrophotometer (Varian). Experiments were conducted in triplicates.

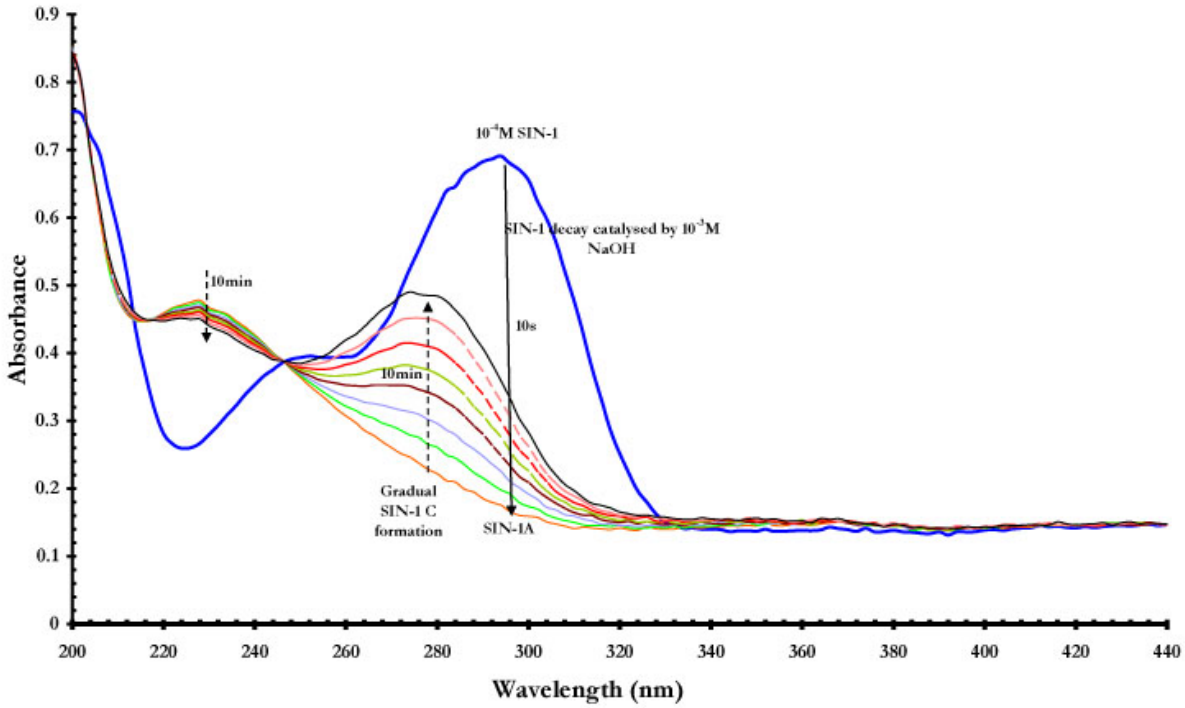


Figure 4. Spectra of 10^{-4}M SIN-1 in Water and Real Time Effect of the Addition of 10^{-3} M NaOH (60 cycles @ 10s/Cycle): Scan was conducted at room temperature using the Cary50Bio® UV-Visible spectrophotometer (Varian). SIN-1 remained stable until the addition of NaOH to the quartz cuvette. The solid arrow indicates rapid decay of SIN-1 while the dashed arrow show the increases in absorbance and the concentration of specie/s formed at 279±2nm immediately after the addition of 10^{-3}M NaOH. Experiments were conducted in triplicates

Control experiments to determine the absorbance spectra of nitrates and nitrites indicate that these breakdown products of peroxynitrite did not show appreciable absorbance at 302 nm. Similarly, the absorbance spectrum of sodium hydroxide (ONOO- solvent) did not show appreciable absorbance at 302 or 320 nm.

The decay of peroxynitrite in BAHPAC culture medium was compared with the life of the anion under conditions mimicking pH of 1.5, 7.4, 11, 11.6, and 13.8 (Figure 2); buffers were prepared as previously described [26]. The conditions were chosen to represent strongly acidic, strongly basic and intermediate pH states. As shown in Figure 2, ONOO- remained stable in strongly alkaline solution. The rate of decay however increased with decrease in pH with the anion possessing only a fleeting existence at pH 7.4 and 1.5 (Figure 2). If authentic peroxynitrite solution at pH 11.05 (Figure 3) or 14, the pH in which it is supplied commercially) is added to a buffered solution to give an abrupt change to pH 7.4, the peroxynitrite is destroyed rapidly (Figure 3). Half-life of authentic peroxynitrite was calculated to be 1.38 seconds at pH 7.4 (Figure 3), close to the 1.9s value reported by Beckman *et al.*, (1990).

Estimate of the life of authentic ONOO-

In some experiments, bovine endothelial and smooth muscle cells were exposed to authentic peroxynitrite solution in volumes ranging from 0.3-0.6 µl [3]; this resulted in increase in culture medium pH up to 7.93. At pH 7.93 and room temperature, ONOO- decayed to 60 and 50% initial level of activity within 5 and 10 min, respectively (Figure 2). Also, during experimentation with pulmonary cells, it was typical for culture plates to be kept under this condition for period of 5-10 min after ONOO- administration. Culture medium pH reversed to 7.4 when culture plates/flasks were returned to CO_2 incubator (5% CO_2, 37 °C). The return of medium pH to 7.4 will essentially terminate the life of ONOO- (Figure 3). It was therefore expected, under this condition, that the pulmonary artery cells were exposed to substantial levels of ONOO- only within 5-10 min period post ONOO- administration when plates were kept out of the incubator.

SIN-1: stability and decomposition

Decomposition kinetics of SIN-1 was measured by conventional light absorbance spectroscopy at room temperature; SIN-1 showed maximum absorbance (λ_{Max}) at

291±2 nm, close to the reported 290nm value earlier reported [27]. The results showed that at 10^{-4}M SIN-1 was stable when reconstituted in phosphate buffer solution, distilled water and 1M hydrochloric acid, consistent with published works [16, 24]. In addition, SIN-1 did not undergo a spontaneous reaction nor form any product under these conditions described.

Under alkaline condition, SIN-1 dissociated rapidly to form end products (Figure 4). The rapid decomposition of SIN-1 was associated with a shift in absorbance maximum from 291±2 nm to 279±2 nm which is the λ_{max} of SIN-1C [16, 24].

SIN-1 remained stable at λ_{max} 292 nm in distilled water until the addition of 10^{-3} M NaOH which catalyses the decomposition of the molecule (Figure 4). The addition of 10^{-3} M NaOH caused a rapid decline in absorbance at 292 nm reaching a minimum within 10s; this was followed by an increase in absorbance at 279±2 nm. Simultaneously, there was a decrease in absorbance at 230 nm (Figure 4). Previous researchers have shown that these sequences of events represent the formation of SIN-1C [16, 24].

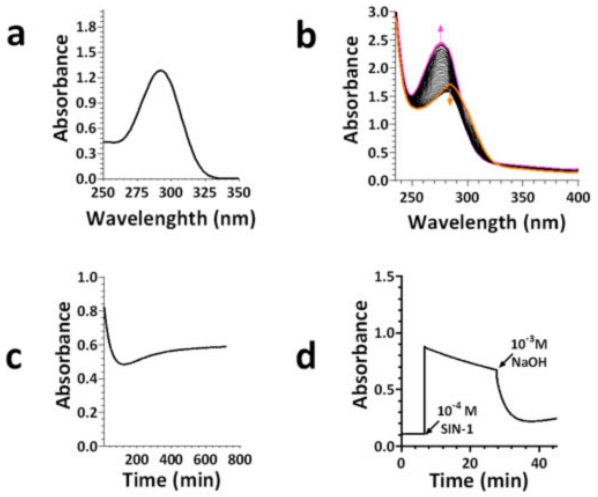


Figure 5: The decomposition spectra and kinetics of 10^{-4} M 3-morpholinosdynonimine (SIN-1) in pulmonary artery cell culture medium. (A) Spectra of 10^{-4} M SIN-1 in culture medium; 60 cycles @ 10 s/cycle was conducted. (B) Spectra of 90 cycles @ 2 min/cycle; the solid arrow indicated the decline in SIN-1 concentration between 0 and 32 min real time period while the broken arrow showed the increases in absorbance and the concentration of a specie formed at 279±2 nm between the 33rd min and 3rd hour of experimentation. (c) Every 2 min scan at 292 nm for 12 h; decomposition kinetics of 10^{-4} M SIN-1 in pulmonary artery cell culture medium showing effect of further increase in pH by 10^{-3} M NaOH. Scan was conducted every 0.1 seconds at 302 nm for 50 min. The slow yet steady decline in SIN-1 absorbance was accelerated by the addition of 10^{-3} M NaOH. Experiments were conducted in triplicates at room temperature using the Cary50Bio® UV-Visible spectrophotometer (Varian).

Spontaneous decomposition of SIN-1 in pulmonary artery cell culture medium

The spectrum of SIN-1 in cell culture medium was similar to that obtained for SIN-1 in water and buffer (Figure 5a). The pH of 10^{-4}M SIN-1 was determined to be 7.53±0.1. SIN-1 decayed slowly in endothelial cell culture medium; based on the kinetics of the decay, the half-life of SIN-1 was calculated to be 80 min (Figure 5b).

However, the decomposition could be made to proceed rapidly with the addition of 10^{-3}M NaOH (pH= 10.04), this indicated that further increase in pH will enhance the decay of the molecule as earlier observed under aqueous conditions (Figure 4). The spectral changes in SIN-1 reported in buffer (Figure 4) were also observed in cell culture media. The slow decay of SIN1 was similarly associated with a shift in absorbance maximum from 291±2nm to 279±2 nm (Figure 5b) which is the λ_{max} of SIN-1C [16, 24]. Without the catalyst effect of 10^{-3}M NaOH, 10^{-4}M SIN-1 decayed in cell culture media until a stable concentration of an intermediate product was reached at 90-100 minutes (Figure 5c, d); following this was a phase of slight increase in absorbance observed for an additional 7 hours (Figure 5c). The kinetics of SIN-1 decay in cell culture medium were essentially similar at 292 and 302 nm (Figure 5). Although SIN-1C has λ_{max} at 279±2 nm; the shoulders of the peak absorbance were detectable also at 290-302 nm. Hence, the slight increase in absorbance at 292nm and 302nm over a 7 hour extended period (Figure 5) was likely indicative of the formation of stable SIN-1C. SIN-1C showed some absorbance and a maximum absorbance (λ_{max}) at 302 and 279±2 nm, respectively. The formation of SIN-1C following the decomposition of SIN-1 has been monitored at wavelengths between 270 and 280 nm in other studies [24, 28].

Therefore, the increases in concentration at these wavelengths, as observed in this study, confirm the formation of this stable end product according (Figure 6). SIN-1C formation was impaired under nitrogen-purged conditions (data not shown). Hence, the formation of SIN-1C under aerobic condition is indirect evidence that superoxide was produced in the process and that SIN-1 isomerised and formed an intermediate product (SIN-1A) and later dissociated to yield nitric oxide as suggested [15, 16, 27]. The study at this stage did not observe several of these steps. The reactions had occurred too quickly to facilitate monitoring by the current approach or other available techniques. The conventional spectroscopy instrument required at least 10 seconds to scan between 200 and 450 nm for activity/absorbance by which time the formation of SIN-1A may have been completed, and gone without detection. Also, it was impossible to assess the activity of SIN-1 within the first 1s, which was crucial to plotting the transition to SIN-1A or SIN-1C.

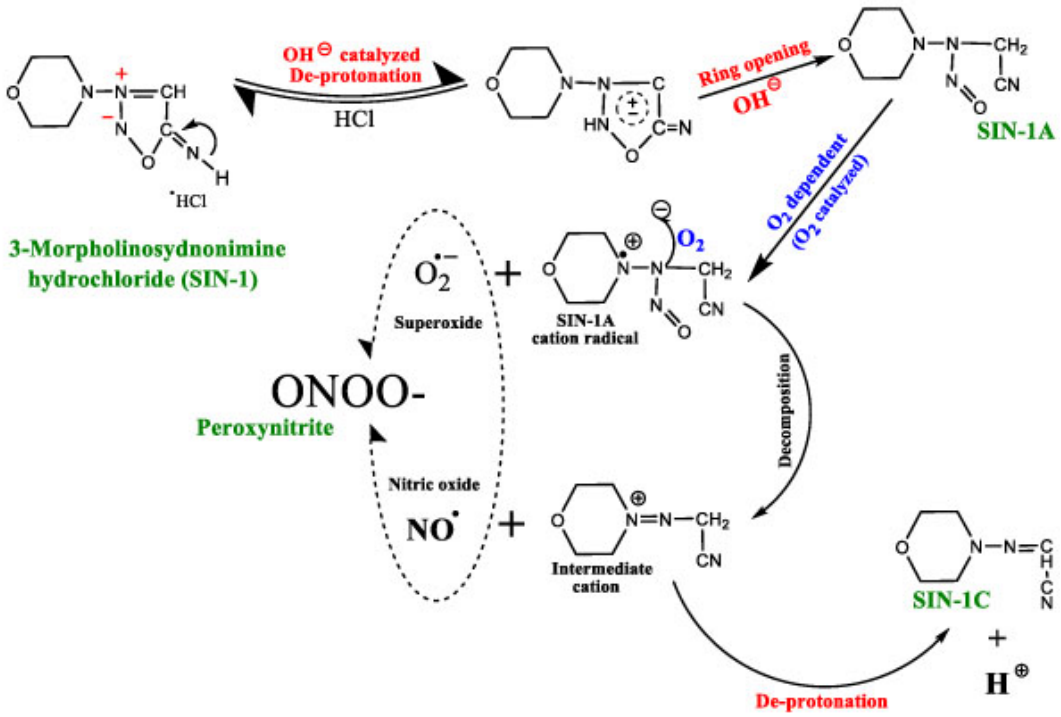


Figure 6: A schematic representation of the generation of peroxynitrite from 3-morpholinosdynonimine (SIN-1): SIN-1 isomerises and undergoes ring opening to form SIN-1A. Under aerobic condition, dioxygen undergoes one electron reduction to form superoxide. This is may have been initiated by the one electron oxidation of SIN-1A to form an intermediate radical cation of SIN-1A. This decomposed to release $NO^{\bullet}$ and form stable SIN-1C. This study has demonstrated that (1) the rate of SIN-1 decomposition is pH dependent (2) the rate and amount of SIN-1C formation is oxygen dependent. The timing of the appearance and increases of the absorbance peak at 279±2 nm in aqueous confirmed the formation of SIN-1C and provide indirect evidence for the release of $NO^{\bullet}$. (3) For the first time, this study provided stopped-flow spectroscopic evidence that SIN-1 is de-protonated in the initial phase of the reaction leading up to SIN-1A formation in a pH dependent manner. This reaction may be the initiator of SIN-1 decay.

This study therefore utilised stopped-flow spectroscopy to further study the formation of ONOO- from SIN-1. Stopped-flow system can monitor changes in absorbance between 200-450 nm in increments of 0.6s.

Generation of peroxynitrite by SIN-1: the formation of SIN-1A can be ⌈OH] catalysed

SIN-1 decomposed in a pH dependent manner to form the stable active metabolite- SIN-1C (Figure7 a-f) as medium pH increases. Stopped-flow spectroscopy results summarized in Figure 2.8 showed that an aqueous solution of SIN-1 (pH 5.1±0.04) decays only slowly throughout the period of experimentation; there were no changes in absorbance maximum absorbance a 120 s period (Figure 2.8A). However, as medium pH increased from 6.2 to 11.9, the time taken for the formation of the intermediate product (SIN-1A) decreased from 7.5 min to 0.6 s, respectively (Figure 2.8). This was further illustrated in Figure 2.9A. At pH 6.7, the decay of SIN-1 was slow and the formation of SIN-1C was detectable after 5 h. There were decreases in absorbance at 240nm (Figure 2.8b); this coincided with the decay of SIN-1 monitored at 292 nm thus suggesting that at slightly acidic pH the decay of SIN-1 may be monitored at 240 or 292 nm (Figure 2.8b). A hint at the complex nature of SIN-1 decomposition was seen as medium pH was increased from 6.7 to 8.6 (Figure 2.8c). For the first time, the study observed clear evidence of the broadening of SIN-1 peak from 325nm (Figure 2.8a) to 380nm on the right hand shoulder of the SIN-1 spectrum (Figure 2.8c). In addition, 2 absorbance peaks were visible, namely at 290 and 330 nm recorded at pH 8.6 (Figure 2.8c).

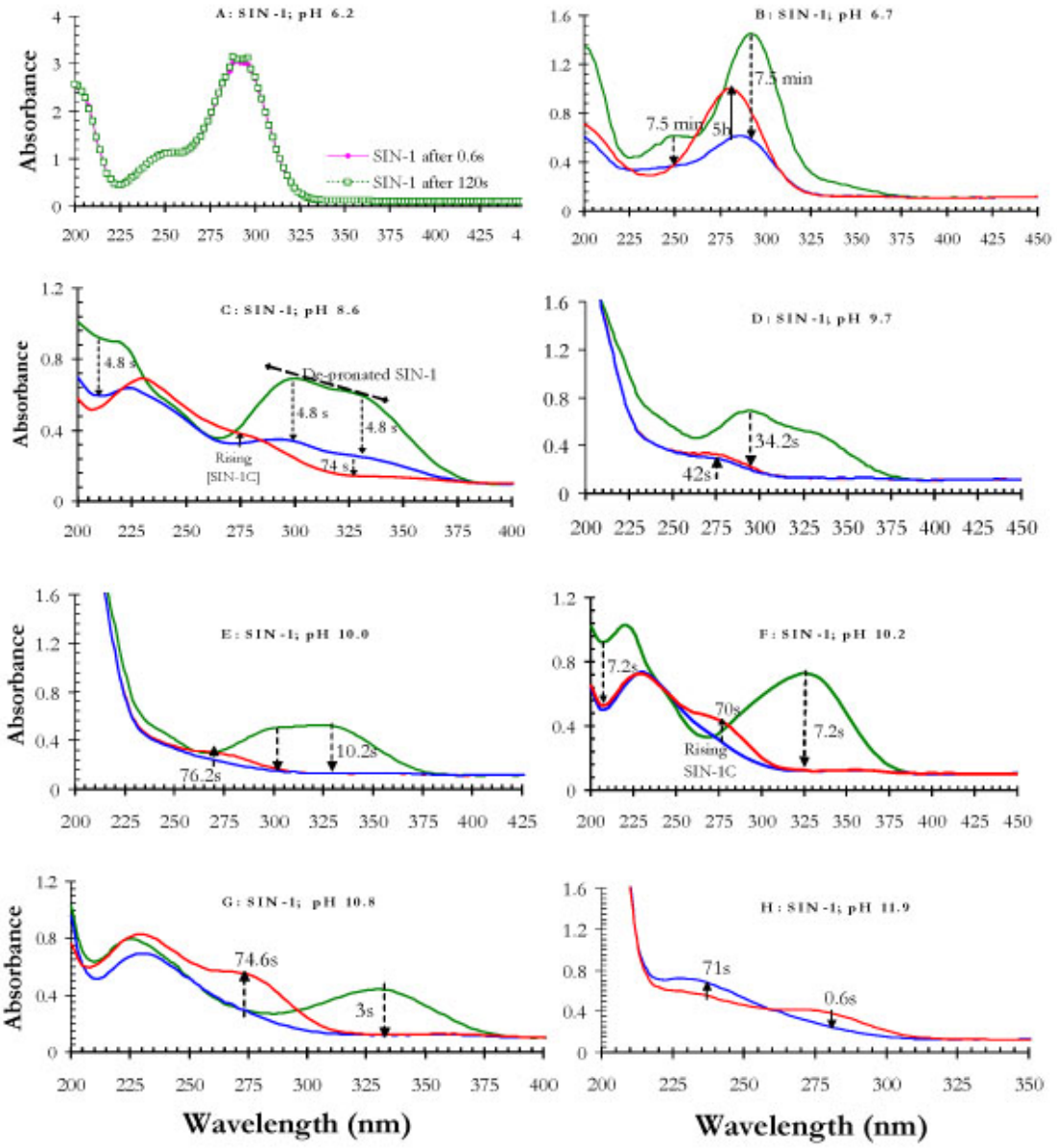


Figure 7: Significant time Points selected from the real time stopped-flow spectra of aqueous 10^{-4} M SIN-1 solution alone or mixed with equal volume buffer or NaOH. The selected time points highlight the decay of SIN-1 (Green line) to an intermediate product (Blue lines) followed by the gradual formation of stable SIN-1C (red line) (A): $2x10^{-4}$ M SIN-1 mixed with equal volume $2x10^{-4}$ M SIN-1 solution (pH 6.2) Scan setting: 200 cycles @ 0.6s/Cycle. (B): $2x10^{-4}$ M SIN-1 solution mixed with equal volume $2x10^{-5}$ M NaOH (pH = 6.5; setting: 128 cycles @ 2.5 min/cycle). (C): $2x10^{-4}$ M SIN-1 solution mixed with equal volume $5x10^{-5}$ M NaOH; pH = 8.6). (D): $2x10^{-4}$ M SIN-1 solution at pH 9.7 (Carbonates/NaOH Buffer); pH = 9.7) (E): 10^{-4}M SIN-1 solution at pH 10 (Carbonates/NaOH Buffer; actual pH 10.0) (F): Mixture of aqueous $2x10^{-4}$ M SIN-1 solution + $1x10^{-4}$ M NaOH (pH 10.2). (G): Mixture of aqueous $2x10^{-4}$ M SIN-1 solution + $2x10^{-4}$ M NaOH (pH 10.8) (H): $2x10^{-4}$ M SIN-1 solution mixed with $2x10^{-3}$ M NaOH. (pH= 11.9) Experiments were conducted in triplicates at room temperature using the Cary50Bio® UV-Visible stopped-flow spectrophotometer (Varian). Scan setting for C-H: 128 cycles @ 0.6s/Cycle. The gradual decay of SIN-1 to SIN-1A and the subsequent increase in SIN-1C (280 nm) absorbance were monitored in real time. Experiments were conducted in triplicates.

Decline in absorbance at these points was 2-phased. Firstly, within 4.8s SIN-1 absorbance declined to form an intermediate (SIN-1A) with trough absorbance at 270nm and an isobestic point at 285 nm (Figure 7c). This was immediately followed by 2nd decay phase characterised by further decline in absorbance at 292 and 330 nm but with simultaneous increase in absorbance at 270 nm with the isobestic point remaining at 285 nm (Figure 7c), thus indicating the formation of SIN-1C. At pH 9.7, much of this insight into the multi-phased decay of SIN-1 was not apparent. Under the same scan settings as in Figure 7c, the formation of SIN-1A appeared to have been completed within 35 s. The two absorbance peaks of the broadened SIN-1 spectrum noticed at pH 8.6 (Figure 7c) were also observed at 290 and 330nm in addition to the early stages of SIN-1C formation (Figure 7d). The spectral changes at pH 10.0, 10.2, 10.8 and 11.9 were similar to that reported at pH 9.7; however, SIN-1A formation appeared to have occurred within 11, 8, 3 and 0.6s respectively (Figure 7 e-h). In all 5 pH conditions, the formation of SIN-1C was evident at 280nm (Figure 7 d-h).

These results suggest that the formation of SIN-1A can be modified by pH changes; with the hydroxyl ion (^{-}OH) serving as a catalyst for this phase of SIN-1 decay (Figure 6, 7). The results also demonstrate that formation of SIN-1A and the generation of stable SIN-1C are mutually exclusive events in which the latter proceeds only after the completion of the former (Figure 2.7). In summary, the gradual decay of SIN-1 to SIN-1A has occurred in pH dependent time frames; at all pH levels investigated, this was accompanied for the most part with decline in absorbance at 291±2 nm and with increases in absorbance at 279±2 nm which indicated the formation of stable SIN-1C (Figure 7).

SIN-1 decay: the de-protonation sub-phase of SIN-1A formation

Beginning at pH 8.6, the sharp SIN-1 peak seen normally at 291±2 nm was broadened to have a right shoulder extending from 325 nm (Figure 7a) to 375nm (Figure 7a-e). The presence of an isobetic point at approximately 315nm represented a pKa transition (Figure 7; pH 6.7-9); thus suggesting the existence of 2 directly related species.

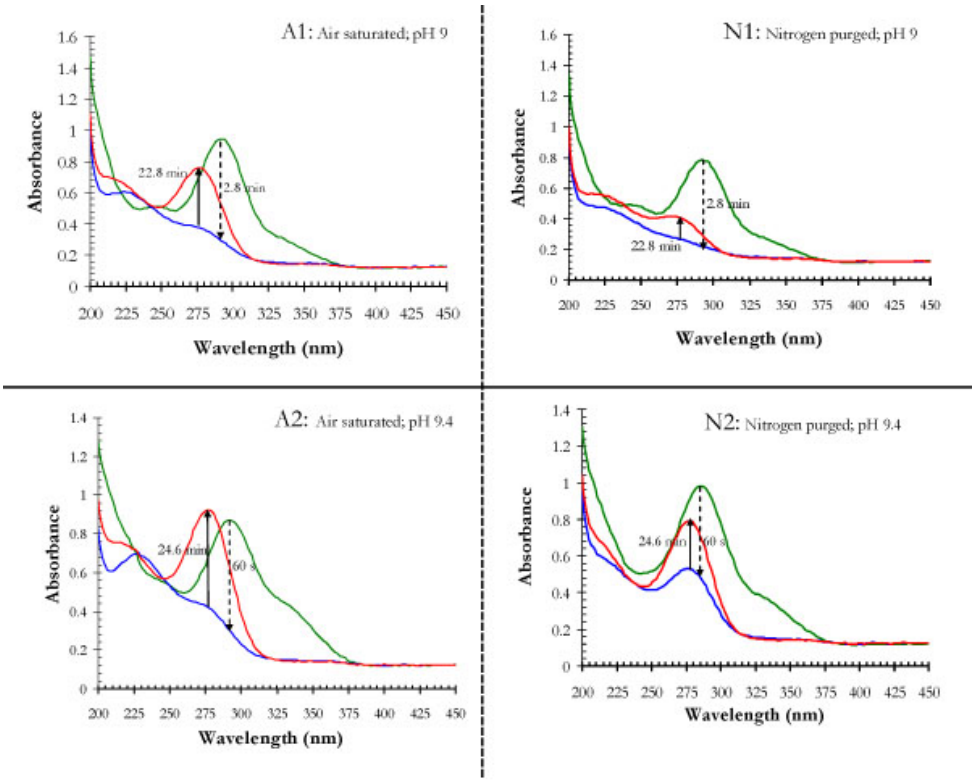


Figure 8: Significant time points selected from real time stopped-flow spectra of aqueous air saturated or nitrogen purged 10^{-4} M SIN-1 solution at pH 9.0 (Borax/HCl Buffer) and 9.4 (Borax/NaOH Buffer). Experiments were conducted in triplicate (128 cycles @ 12s/Cycle) with air saturated (Figures A1 & A2) and nitrogen purged (Figures N1 & N2) SIN-1 samples at pH 9 and 9.4, respectively. Scan was conducted in triplicates at room temperature using the Cary50Bio® UV-Visible stopped-flow spectrophotometer (Varian).

A possible explanation is the formation of a de-protonated intermediate (Figure 6) before the formation of SIN-1A (Figure 2.9B). This phenomenon appeared to have occurred so fast that it is hardly seen even at an acquisition rate of 0.6 s of SIN-1 in a pH 11.9 medium (compare Figure 7a-e with Figure 7f).

The formation of SIN-1C is oxygen dependent

The formation of SIN-1C was significantly impaired under nitrogen purged conditions (Figure 2.10; $p < 0.05$; t-test). At pH 9.0 and using nitrogen purged SIN-1/buffer mixtures, SIN-1 decayed to intermediate SIN-1A within 2.8min; this was monitrored at 292nm (Figure 8, N1). This was immediately followed by a steady increase in SIN-1C concentration at 280 nm (Figure 8, N1). Repeating the experiments with air saturated SIN-1/buffer mixtures showed that the final concentration of SIN-1C increased from 54.7±3.3% under nitrogen purged conditions to 71.4 ±4.6% initial SIN-1 concentration under air saturated conditions over the same time period (Figure 8; A1, N1).

The results showed similar trend at pH 9.4 (Figure 8; A2, N2). In this study, it was not possible to create oxygen free medium or saturate SIN-1/buffer mixtures with pure oxygen, however the amount of SIN-1C formed during these period increased significantly under air saturated conditions (Figure 8). This supposes an important role for oxygen in this phase of SIN-1 decay.

In these experiments, NaOH was utilized to provide the ^{-}OH ion needed to catalyse the decay of SIN-1. The stopped-flow spectroscopy data confirmed that the decay of SIN-1 to SIN-1A (Phase 1) can be accelerated by hydroxyl ion, while the subsequent formation of SIN-1C from SIN-1A can be catalysed by oxygen. This shows that the SIN-1 to SIN-1C transformation was the result of the ^{-}OH catalyzed hydrolytic decomposition (Figure 7) which occurred under cell culture medium condition. The broadened (pH 8.6, 9.7. 10, 10.2, 10.8; Figure 7c-g) and characteristic double peak (pH 8.6, 9.7. 10; Figure 7d-e) seen during the decay of SIN-1 to SIN-1A was an indication that SIN-1 formed a putative de-protonation species during the process. Based on these, this study provides a scheme for the decomposition pathway of SIN-1 in cell culture medium (Figure 6).

Peroxynitrite formation by SIN-1 as quantified by NADH fluorescence quenching

NADH was found to fluoresce between emission wavelengths of 420 and 520 nm with a peak at 460nm. Phenol red free culture media showed insignificant background fluorescence and a satisfactory 5-fold increase (measured at 460 nm) on addition of NADH 30 µM (data not shown). NADH fluorescence at 460 nm was approximately linear with NADH concentration up to 35 µM in both cell culture medium and de-ionised water (result not shown). Thereafter the relationship was curvilinear, possibly suggesting some self-quenching of emission (result not shown). Experimentations in cell culture medium therefore involved the use of 30 µM NADH.

Bolus addition of authentic peroxynitrite caused a concentration dependent rapid quenching of NADH fluorescence without significant distortion of the fluorescence emission spectra (result not shown). This though was associated with an increase in fluorescence emission at approximately 410 nm due likely to a fluorescing adduct (result not shown). Loss of NADH fluorescence was due likely to the conversion of NADH to NAD^{+} [29]. Also, it was confirmed that NADH underwent slight air oxidation giving a gradual decline in fluorescence at 460 nm, as previously found [29]. Over a period of 120 minutes, fluorescence diminished from 22.11 to 20.15, a decline of 9% due to air oxidation (Figure 9a).

SIN-1 20 µM caused a gradual decline in NADH fluorescence over a period of approximately 2 hours (Figure 9a). Increasing SIN-1 concentration 5 fold caused a more rapid decline in NADH fluorescence; this was similar to background medium fluorescence within 90 minutes. These effects were blocked by the co-addition of superoxide dismutase, a superoxide scavenger (Figure 9b), consistent with the reaction mechanism that showed the release of a superoxide intermediate from SIN-1 (Figure 6). With the addition of 1mM SIN-1, the fluorescence emission of 30µM NADH diminished to the level of the culture medium basal fluorescence in approximately 30min. Thus it appears that 1 mM SIN-1 generated more ONOO- than was required to react with 30 µM NADH (Figure 9b). Subtraction of the decline due to air oxidation of NADH from the results in Figure 9b generated the true rate of ONOO- formation shown in Figure 9c. This showed close to linear formation of ONOO-. At 20 µM, SIN-1 caused a 2.20 unit decline in fluorescence emission over a period of 88.85 min (Figure 9). The rate of NAD+ formation by 20 µM SIN-1, which also corresponded to the rate of ONOO- formation, was therefore calculated to be 0.025 fluorescence unit/min (Figure 9c). Under the same conditions, the effect of authentic peroxynitrite on NADH fluorescence was further investigated by step-wise titration experiments involving the addition of pre-synthesised ONOO- to cuvette containing 30 µM NADH culture medium.

The real time data showed that quenching of NADH fluorescence due to ONOO- was rapid and cumulative (Figure 9d). The fluorescence decline at each step in Figure 9c was measured, and plotted against cumulative ONOO- added to give Fig 10. (Figure 10a shows the data without correction for air oxidation, and Figure 10b includes correction for air oxidation).

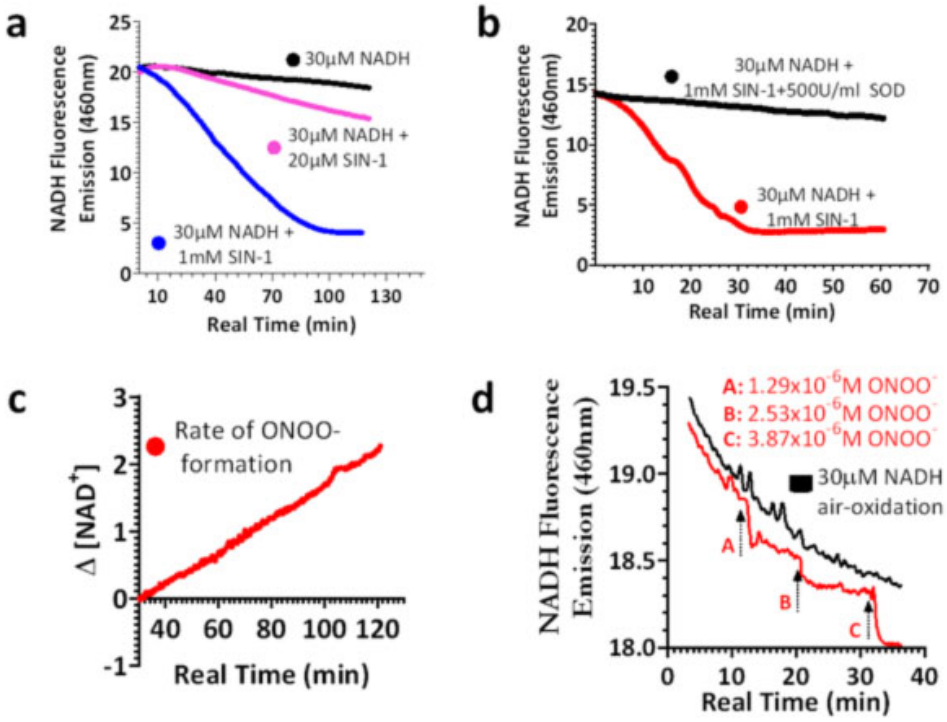


Figure: 9: Quantification of peroxynitrite generation from SIN-1. (a) Kinetics of SIN-1 induced quenching of 30 µM NADH fluorescence. NADH fluorescence was investigated in cell culture medium to which SIN-1 was added (pH = 7.41±0.23 at 25 ^{0}C) and measured with excitation and emission wavelengths at 340 and 460 nm, respectively. Separate experiments investigated the quenching of 30 µM NADH fluorescence in the presence of 20 µM & 1 mM SIN-1 in a cuvette. **(b) Attenuation of SIN-1 induced oxidation of 30 µM NADH fluorescence by superoxide dismutase (SOD)**. NADH fluorescence was investigated in cell culture medium to which SIN-1 and SOD were added (pH = 7.41±0.23 at 25 ^{0}C) and measured with excitation and emission wavelengths at 340 and 460 nm, respectively. **(c) The production of peroxynitrite during SIN-1 decomposition. Loss is NADH fluorescence correlates with increased NAD^{+} formation.** The figure shows increase in NAD^{+} formation in the presence of 20 µM SIN-1 in pulmonary cell culture medium (pH = 7.41±0.23 at 25 ^{0}C) and measured with excitation and emission wavelengths at 340 and 460 nm, respectively. Oxidation of NADH to NAD^{+} was due to peroxynitrite (ONOO-) generated from SIN-1. The data was corrected for NADH air-oxidation. **(d) Stepwise titration of 30µM NADH fluorescence with authentic peroxynitrite.** The tracing shows the quenching of the fluorescence of 30 µM NADH by the sequential addition of 1 µL volume pulses of authentic peroxynitrite from 3.87x10^{-3} M stock solutions to 3 ml NADH/Medium in a cuvette. Cumulative authentic peroxynitrite concentrations are indicated by the arrow at each point of addition. NADH fluorescence was investigated in pulmonary cell culture medium to which authentic ONOO- was added (pH = 7.47±0.2 at 25 ^{0}C) and measured with excitation and emission wavelengths at 340 and 460 nm, respectively. Experiments were conducted in triplicates. Measurements in (a) and (b) were taken following a rapid mixing period of approximately 10 s. Experiments were conducted in triplicates.

This showed an approximately linear relationship between ONOO- formation and fluorescence quenching. From Figure 10, it can be seen that decline by 1 fluorescence unit occurred with addition of 4.6µM of ONOO-. Since decline in NADH fluorescence with 20 µM SIN-1 was 0.025 fluorescence unit per min, 20 µM SIN-1 was thus generating 1.9 nM ONOO- per second or 0.11 µM min^{-1}. Introducing this figure into the rate of fluorescence decline with 20 µM SIN-1 (Figure 9a) gives an estimate for ONOO- generation of 10 µM over approximately 90min period. Comparing SIN-1 induced NADH fluorescence quenching (Figure 9a-c) and the **s**tepwise titration of NADH fluorescence with authentic peroxynitrite (Figure 9c, 10) showed that 20 µM SIN-1 generated ONOO- at 0.11 µM min^{-1} over ~ 90 min period.

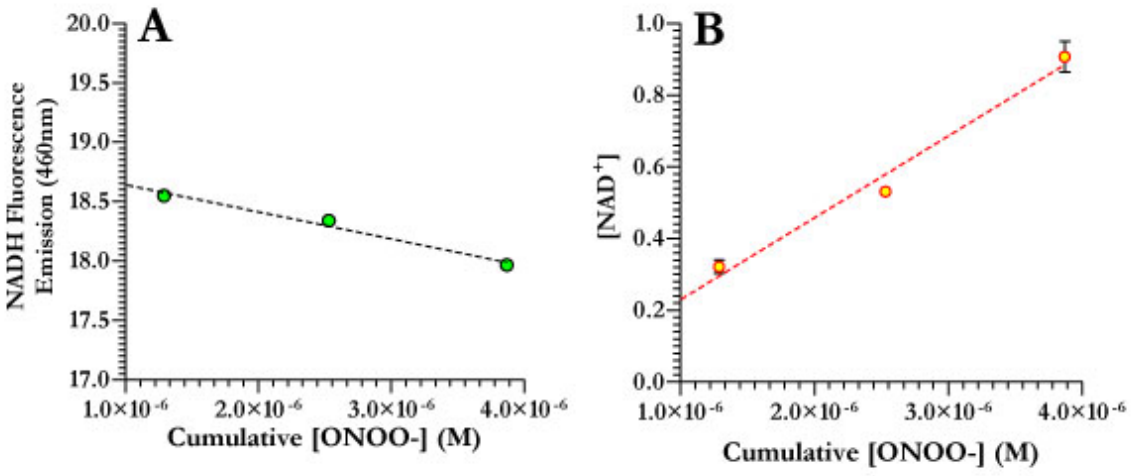


Figure 10: Analysis of stepwise titration of 30 µM NADH fluorescence with authentic peroxynitrite. Loss is NADH fluorescence correlates with increased NAD^+ formation. NADH fluorescence was investigated in pulmonary artery cell culture medium to which authentic ONOO- was added (pH = 7.47±0.21 at 25 ^{0}C) and measured with excitation and emission wavelengths at 340 and 460 nm, respectively. Panel A shows the cumulative quenching of the fluorescence of 30 µM NADH achieved by cumulative authentic peroxynitrite concentration. Panel B shows the increase in NAD^+ with cumulative [ONOO-]

DISCUSSION

Although peroxynitrite is stable in strongly alkaline solution, working with the molecule at physiological pH poses a major challenge because of its unstable nature and fleeting existence at physiological pH. This study confirmed earlier findings that $ONOO^-$ is short-lived at physiological pH; ONOO- half-life was calculated to be 1.38s at pH 7.4 [6]. Also, studies involving bolus and direct addition of ONOO- to cells engender significant loss of ONOO- before reaching the cells. It has therefore been argued that this method of adding ONOO- simulate the physiologically relevant steady state ONOO- concentration *in vivo* [30, 31]. In addition, this study showed that the slight increase in pH of buffered cell culture medium to 7.93 following the addition of strongly alkaline solution ONOO- enabled the activity of the anion for a much longer period.

This study confirm published evidence for the accepted mechanism for the generation of ONOO- from SIN-1 [15] and provides additional evidence that further the understanding of the decay process. The present work demonstrates that (1) the rate of SIN-1 decomposition can be catalysed by -OH ions, and (2) the rate and amount of SIN-1C formation during this process is oxygen dependent (Figure 6). In addition, this study provide for the first time, stopped-flow spectroscopic evidence that SIN-1 is de-protonated in a pH dependent manner during the phase of the reaction leading up to SIN-1A formation. It is possible that the depronation of SIN-1 under physiological pH initiated its decay. Studies on the mechanism of nitric oxide release from SIN-1 showed a positive correlation between O_2 consumption and superoxide formation [32]. As here confirmed, the formation SIN-1C during SIN-1 decay was reported to be oxygen dependent [15]. Under aerobic condition, oxygen initiated SIN-1A decay. This was thought to have occurred via the one electron abstraction from the nitrogen connected to the N-NO group as shown in Figure 6 [15]; oxygen is thereafter reduced to superoxide and the SIN-1A is oxidized to an unstable radical cation that further decomposes to release $NO^\bullet$ [16]. In this study, the timing of the appearance and increases of the absorbance peak at 279±2nm in aerobic condition (Figure 7) confirmed the formation of SIN-1C and provide indirect evidence for the release of $NO^\bullet$ (Figure 6). It has been proven under this condition that $NO^\bullet$ and superoxide combine to form peroxynitrite [14, 15]. Previous work has showed that decay of SIN-1 and formation of ONOO- can be sustained at physiological or pH 7.4 under aerobic condition [29]. In this study, this was confirmed by experiments showing the decay of SIN-1 under *in-vitro* pulmonary artery cell culture conditions (BAHPAC medium; Figure 5). The formation of peroxynitrite under this condition may have in the present study occurred over a period in excess of 75 min (Figure 5).

Absorbance spectroscopy is useful in elucidating the decay mechanism for the release of ONOO- from SIN-1. However,

the technique was unsuitable for the determination of the amount of ONOO- released per unit time or molecule from SIN-1. A key reason for this is that SIN-1 and ONOO- shows considerable overlap in absorbance at both 292 and 302 nm (Figure 1, 4). The use of another method was thus indicated.

The work of Martin-Romero et al [29] showed that the production of peroxynitrite during 3-morpholinosydnonimine (SIN-1) decomposition can be continuously monitored from the loss in fluorescence associated with the ONOO- oxidation of nicotinamide adenine dinucleotide (NADH) to NAD^+. This reaction was claimed to be specific for peroxynitrite and provided a cumulative record of peroxynitrite formation. Unlike the Dihydrorhodamine 123 method [33], this approach was more specific for ONOO- and was not affected by the influence of hydrogen peroxide or other oxidants. The kinetics of NADH fluorescence quenching by SIN-1 was shown to overlap with the kinetics of NADH oxidation to NAD^+, monitored by the decay of absorbance at 340 nm [29]. Thus this study employed this method to demonstrate the release and to quantify the yield of ONOO- from SIN-1 in pulmonary artery cell culture medium.

The decay rate of 20 µM and 1 mM SIN-1 (Figure 9) suggests that the process was governed by 1st order kinetics. This implied that the half-life of the anion= 0.693/k, where k is the decay constant of ONOO-. The half-life of ONOO- was determined to be 1.38s in pulmonary artery cell culture medium (Figure 3); hence $k=0.5s^{-1}$. In addition, this study estimated that under this condition, 20 µM SIN-1 generated ONOO- at the rate of 0.11µM min^{-1}. Dividing this production rate by the decay constant amounted to a steady state ONOO- concentration of 0.95nM over 90min period (Figure 9, 10). This takes into cognisance that ONOO- is concurrently being generated and destroyed. The sensitivity of the NADH fluorescence quenching method did not allow for reproducible estimation of ONOO- formation by lower SIN-1 concentration. Peroxynitrite formation by 100 µM SIN-1 was calculated to be 3.2 µM ± 0.2 min^{-1} in medium formulated for the growth of pulmonary artery cells. Overall, the rate ONOO- generation from SIN-1 depended on SIN-1 concentration.

Conclusion

The formation of ONOO- was demonstrated by the qualitative and quantitative determination of its in-situ generation from the decay of 3-morpholinosydnonimine (SIN-1). This will enable relevant correlations of the life of peroxynitrite in culture conditions to its cellular actions in pulmonary cells.

Acknowledgment

E.O Agbani was supported by the University of Strathclyde John Anderson Scholarship and The United Kingdom Overseas Research Students Award Scheme. This work was done under the supervision of late Professor Roger Wadsworth of the University of Strathclyde, Glasgow, Scotland.

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