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Angeli's Salt ($Na_2N_2O_3$) is a Precursor of HNO and NO: a Voltammetric Study of the Reactive Intermediates Released by Angeli's Salt Decomposition

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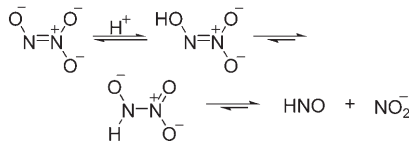
Under physiological conditions, it is usually accepted that the aerobic decomposition of Angeli's salt produces nitrite (NO_2^-) and nitroxyl (HNO), which dimerizes and leads to N_2O. No consensus has yet been established on the formation of nitric oxide (NO) and/or peroxynitrite ($ONOO^-$) by Angeli's salt. Because this salt has recently been shown to have pharmacological properties for the treatment of cardiovascular diseases, identification of its follow-up reactive intermediates is of increasing importance. In this work, we investigated the decomposition mechanism of Angeli's salt by voltammetry performed at platinized carbon fiber microelectrodes. By following the decomposition process of Angeli's salt, we showed that the mechanism depends on the experimental conditions. Under aerobic neutral and slightly alkaline conditions, the formation of HNO, NO_2^-, but also of nitric oxide NO was demonstrated. In strongly alkaline buffer (pH > 10), we observed the formation of peroxynitrite $ONOO^-$ in the presence of oxygen. These electrochemical results are supported by comparison with UV spectrophotometry data.

Introduction

The inorganic salt $Na_2N_2O_3$, named Angeli's salt (AS), is a well-reputed donor of nitroxyl (HNO and NO^-) species[1] and has been used for that reason in many biological and biochemical studies. From a medical point of view, AS also behaves as a nitric oxide donor, presenting both pro-inflammatory properties[2] and cytotoxic effects.[3] However, the cardiovascular properties of AS are discernable from those of NO. For instance there is evidence that AS, at moderate pharmacological doses, leads to vein distension and to the enhancement of myocardial contractility in failing hearts.[4] On the other hand, at high concentrations, Angeli's salt may induce the oxidation of DNA bases.[5] It was proposed that in the presence of oxygen, the nitroxyl anion NO^- may evolve in vivo into peroxynitrite $ONOO^-$, a reactive intermediate which, in addition to the nitration of tyrosine moieties, can also oxidize nucleic bases, phospholipidic membranes, and thiol groups.[4] At physiological pH, HNO is the exclusive form of the nitroxyl species, as its pK_a value was recently estimated at 11.3.[6] Therefore, the potential pharmacological use of Angeli's salt, particularly in the treatment of heart failure, might be considered only after a precise determination of the reactive chemical intermediates released.

Over the past few years, many research groups have studied the decomposition process of AS according to different methods. Hence, it is generally thought that the decomposition mechanism of AS above pH 4 involves protonation of the dianion $N_2O_3^{2-}$ ($pK_a = 9.35$) followed by tautomerization and heterolytic cleavage of the N–N bond to produce HNO and nitrite [Eq. (1)].[7–11]



This mechanism is supported by theoretical calculations,[7] which predict that protonation at the oxygen atom of the nitroso group leads to the most thermodynamically stable monoanionic tautomer. HNO formed is also known to dimerize rapidly ($8 \times 10^6\ M^{-1}\ s^{-1}$) according to reaction (2).[8,9]

$$HNO + HNO \rightarrow H_2N_2O_2 \qquad (2)$$

The product of reaction (2) may then dehydrate into the stable N_2O. Liochev and Fridovich showed that the reverse of reaction (1) is limited by the dimerization of HNO in the absence of O_2 and furthermore by the reaction of HNO with O_2

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which is believed to yield ONOOH under aerobic conditions.[12] Conversely, in recent works, Miranda et al. monitored AS decomposition spectrophotometrically and reported that oxygen did not substantially affect the rate at neutral pH.[11] Miranda and co-workers showed a higher influence of O_2 on the decomposition rate of AS in highly alkaline buffer, in correlation with the negative free enthalpy of reaction (3) and the higher redox reactivity of $N_2O_3^{2-}$ towards metal complexes.[13,14]

$$N_2O_3^{2-} + O_2 \xrightarrow{\Delta G = -133\ \text{kcal mol}^{-1}} ONOO^- + NO_2^- \quad (3)$$

These data showed that the hypothesized formation of $ONOO^-$ in aerobic alkaline solution[15–17] may occur via kinetically competitive pathways that involve the reaction of O_2 with $N_2O_3^{2-}$ [Eq. (3)] or with NO^- (following deprotonation of HNO) [Eq. (4)].

$$NO^- + O_2 \rightarrow ONOO^- \quad (4)$$

The effect of oxygen was not so clearly evidenced at neutral and slightly alkaline pH, at which AS is reported to afford HNO. In a study of the nitrosation of tryptophan derivatives by AS, some of us have shown that oxygen is required for the reactivity of Angeli's salt at physiological pH, and we hypothesized the formation of NO, which is enhanced by the addition of melatonin.[18]

All these previous works illustrate that the decomposition of Angeli's salt produces different reactive oxygen and reactive nitrogen species depending on the nature of the environment. However, the determination of the nature of these species is mostly based on indirect clues and is still a matter of controversy. Moreover, previous studies conducted in one of our laboratories showed that some important reactive oxygen species (ROS) and reactive nitrogen species (RNS) including hydrogen peroxide H_2O_2, peroxynitrite $ONOO^-$, nitric oxide NO, and nitrite NO_2^- can be selectively detected by electrochemistry at platinized carbon fiber microelectrodes.[19] Thus, to contribute to the elucidation of the decomposition mechanism of Angeli's salt, we report herein an electrochemical study of Angeli's salt solutions at various pH. The results obtained by using voltammetry in association with UV spectrophotometry, clearly demonstrate the production of nitric oxide over a wide range of pH values including neutral, physiological pH.

Results and Discussion

Oxygen dependence of the decomposition of Angeli's salt

The decomposition of Angeli's salt was first followed by UV spectrophotometry as previously described.[11] The absorbance of AS was recorded in the presence and absence of oxygen in order to assess the effect of oxygen on the decomposition of this salt. Our measurements were made only a few minutes after adding phosphate buffer to the equivalent mass of salt, that is, fresh solutions were prepared for each experiment. This contrasts with previous works, which involved the preparation of a stock solution of AS in alkaline solution and its further dilution into buffer before further measurement.[11,18] Their results hypothesized the formation of nitrite observed at 210 nm and the decrease of a band at 245 nm corresponding to Angeli's salt.

As illustrated in Figure 1, we note on one hand the decrease of the absorbance at 245 nm in both aerobic (Figure 1 A) and anaerobic (Figure 1 B) conditions and the concomitant increase of the absorbance at about 210 nm. This result suggests the decomposition of the anion $HN_2O_3^-$ into HNO and NO_2^-, as described in the literature. Precisely, it was reported that only the protonated form of Angeli's salt, $HN_2O_3^-$, is able to decompose into NO_2^- and HNO under physiological conditions in the presence or in absence of oxygen.[1] The protonation of the basic anion $N_2O_3^{2-}$ as the promoting step of the decomposition of Angeli's salt was revealed simply by the follow-up of the varia-

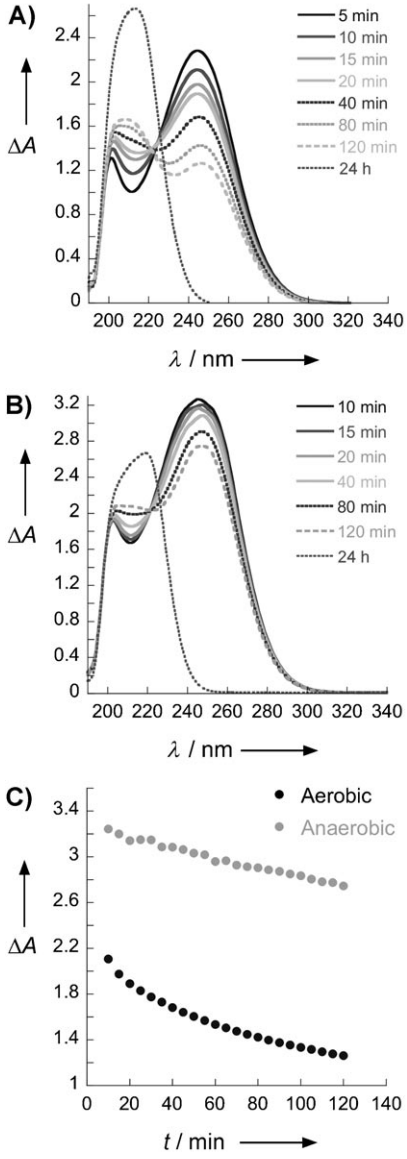

Figure 1. Spectrophotometric monitoring of the decomposition ($T = 22\,^\circ C$) of Angeli's salt in A) aerobic and B) anaerobic phosphate-buffered saline (PBS). Spectra were recorded every 5 min. C) Kinetic evolution of the absorbance at 245 nm of Angeli's salt in aerobic and anaerobic PBS. In both cases, the initial concentration (at $t = 0$) of Angeli's salt was 5 mM.

tion of the solution pH in aerobic media. We observed that the addition of the basic form of Angeli's salt $N_2O_3^{2-}$ at 5 mM in 10 mM PBS modifies the pH. A rapid increase of the pH occurs in less than 2 min, from 7.4 to 9.5, or 6 to 6.8, respectively, and then remains quite constant along the whole decomposition process.

On the other hand, one observes in Figure 1B that the absorbance at 245 nm in the anaerobic buffer is larger by one unit than that measured at the same initial AS concentration (5 mM) and at the same times under aerobic conditions. This observation proves that oxygen substantially affects the initial evolution of Angeli's salt at physiological pH. This is evidenced in Figure 1C, which compares the kinetic evolution of the absorbance at 245 nm under both conditions (same initial concentration of AS solutions). In contrast with previous reports,[1,11] the presence of oxygen accelerates the decomposition of Angeli's salt, particularly during the first 20 min of its decay. Furthermore, experiments with oxygen-saturated solutions of AS showed a faster decrease of the absorbance at 245 nm and supported this assumption. In addition, the variations in the rate of AS disappearance as function of oxygen were correlated with the quantity of nitrite (and possibly nitrate) accumulating in the solution, as measured by the absorbance at 210 nm. The concentration of nitrite increased more rapidly when the uptake of oxygen was higher (compare Figure 1A and B). Though the initial decomposition of Angeli's salt appeared sensitive to oxygen, the kinetic analysis of its band amplitude establishes that its global decay under aerobic conditions follows an exponential variation, as shown previously.[11] The corresponding rate constant k is $3\times10^{-4}\ s^{-1}$ at pH 9.5 and $11\times10^{-4}\ s^{-1}$ at pH 6.8, both measured at 22 °C and in agreement with the values previously reported ($k = 1.2–6.8\times10^{-4}\ s^{-1}$ at 25 °C)[1,20,21] at pH 7.4.

Voltammetric study of Angeli's salt decomposition at physiological pH

To further probe the decomposition mechanism of Angeli's salt, we examined the nature of the electroactive species released under aerobic or anaerobic conditions using voltammetry at platinized carbon fiber microelectrodes. We have shown previously that this type of modified microelectrode provides a direct and selective measurement by oxidation of several major ROS and RNS including NO, $O_2^{\bullet-}$, H_2O_2, $ONOO^-$, and NO_2^-.[19] Thus, the oxidative response of AS solutions (at millimolar concentration) was studied by voltammetry, and the evolution of this response was followed with time. Rapidly following preparation of the solution, voltammograms exhibited three irreversible and reproducible waves, I, II, and III (Figure 2). Their apparent half-wave potentials were determined to be 200, 580, and 730 mV (±5 mV) versus SSCE, respectively. It must be emphasized that all the following studies and comparisons were conducted at 100 mV s^{-1}, because this rate provided the most well-defined electrochemical waves, that is, it afforded the better compromise between the rate of analysis, the rate of AS decay, and the rates of electronic transfer for the oxidation of compounds under study. The three

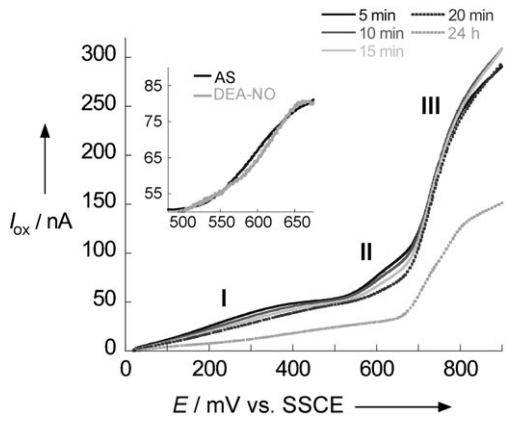

Figure 2. Voltammograms generated by detection with a platinized carbon fiber microelectrode (radius = 5 μm) in an Angeli's salt solution (5 mM) in PBS ($T = 22$ °C) as a function of time; scan rate: $v = 100$ mV s^{-1}. The inset compares the oxidation wave for nitric oxide detected in a DEA-NONOate solution (DEA-NO, 5 mM) and wave II (AS) as monitored under identical conditions.

electrochemical waves were then assigned based on their comparison with known responses of several ROS and RNS on the platinized microelectrodes.

On this basis, wave I could not be simply attributed, as it did not correspond to the oxidation of one of the species previously characterized with microelectrodes in terms of potential and electron transfer rate (slope; see above). The specific investigation of wave I necessitated further studies with another nitroxyl donor, and are detailed below. Wave II could be attributed to the oxidation of nitric oxide. As shown in Figure 2 (inset), this wave matches that recorded in solutions of DEA-NONOate (a chemical donor of NO in the presence of oxygen) under the same experimental conditions very satisfactorily. This observation was confirmed by the decrease of the current corresponding to the transient NO formed during AS decomposition. Moreover, for measurements performed in the deaerated buffer (data not shown), wave II was absent, and the kinetics of decomposition slowed down, as mentioned above (see Figure 1C). This observation indicates that the decomposition of Angeli's salt strongly depends on oxygen and that the latter is necessary for the formation of nitric oxide. Moreover, our results establish that unless drastic anaerobic storage conditions apply, stock solutions of Angeli's salt may react with oxygen to yield NO. This conclusion is in agreement with previous works, which suggest the occurrence of a reaction between the nitroxyl dianion $N_2O_3^{2-}$ and oxygen to form an oxidant, or possibly nitric oxide, at neutral pH.[11,22,23]

Wave III, detected between +650 and +850 mV versus SSCE, may be ascribed to the oxidation of nitrite.[19,24] However, its amplitude current at the very beginning of the decomposition appears too large, being equivalent to what would be expected for a concentration equal to that of Angeli's salt initially. Furthermore, its current plateau does not change drastically with time which is not compatible with the supposed accumulation of nitrite in the solution with time. Owing to the similarity of the developed formula of Angeli's salt and nitrite, we attributed this wave to the simultaneous oxidation of Angeli's

salt, particularly $N_2O_3^{2-}$, and nitrite issued from the decomposition of the salt. This explains in particular the near invariance of this wave with the reaction time. Furthermore, an oxidation of Angeli's salt at a half-wave potential of 730 mV versus SSCE is in agreement with quantum calculations reported in the literature,[14] as a value of the oxidation potential of the $N_2O_3^{-}$/$N_2O_3^{2-}$ couple at ~0.5 V versus NHE at pH 7 was predicted. Considering the sluggishness of the wave due to slow charge-transfer kinetics, such a shift in potential is perfectly acceptable.[25]

Voltammetric study of Angeli's salt decomposition at alkaline pH

To confirm that Angeli's salt leads itself to an oxidation wave, we conducted its voltammetric analysis in an alkaline buffer, because Angeli's salt is known to be much more stable under such conditions.[26] As illustrated in Figure 3, the corresponding

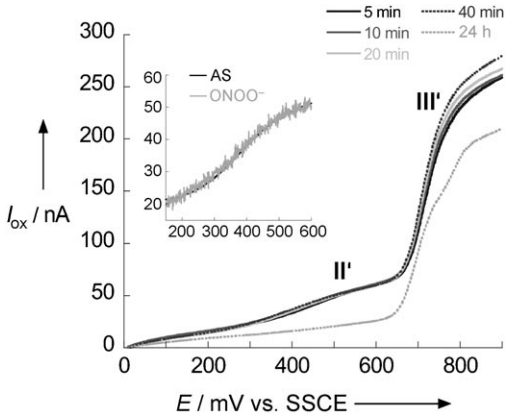

Figure 3. Voltammograms generated by detection with a platinized carbon fiber microelectrode (radius = 5 μm) in an Angeli's salt solution (5 mM) in alkaline PBS (pH 11) as a function of time; scan rate: $v = 100\ mV\,s^{-1}$. The inset compares wave II′ with the oxidation wave detected in an alkaline peroxynitrite solution ($ONOO^-$, 0.4 mM).

voltammogram under aerobic conditions exhibits two well-defined irreversible waves (termed II′ and III′ in comparison with the curves in Figure 2). The first (wave II′), detected between 300 and 500 mV and defined by a half-wave potential of 430 mV versus SSCE, is very similar to that recorded for solutions of the peroxynitrite anion ($ONOO^-$) under the same conditions (see inset in Figure 3). Conversely, only wave III′ could be detected in an anaerobic buffer. These observations establish that wave III, identical to wave III′, represents the oxidation of Angeli's salt as well as that of its decomposition product, NO_2^-. Furthermore, the presence of wave II′ suggests that in aerobic alkaline buffer, Angeli's salt reacts with oxygen to form, at the same rate, nitric oxide and superoxide anion, which recombine extremely fast ($k = 4–19 \times 10^9\ M^{-1}\,s^{-1}$)[27] to yield the peroxynitrite anion [Eq. (5)]:

$$N_2O_3^{2-} + O_2 \rightarrow NO + O_2^- + NO_2^- \rightarrow ONOO^- + NO_2^- \qquad (5)$$

Then, peroxynitrite is sufficiently stable in alkaline buffer at pH 11 to accumulate with time in the solution and to reach a concentration (typically in the millimolar range) detectable by voltammetry.[28] Conversely, the instability of peroxynitrite (peroxynitrous acid) at physiological pH prevents its detection and characterization in stationary conditions. Moreover, the formation of peroxynitrite from Angeli's salt is in agreement with both quantum mechanical predictions and spectrophotometric observations reported by Miranda et al.[11] However, we could not confirm their results during our spectrophotometric study (data not shown), as the variation of absorbance at a wavelength of 302 nm corresponding to peroxynitrite was negligible.

Voltammetric study of the decomposition of methylsulfonylhydroxylamine

Because Angeli's salt is a reputed HNO donor, we performed a series of additional electrochemical experiments to check for the formation of this species and therefore to attribute unequivocally wave I of Figure 2 to the oxidation of HNO. With that purpose, we followed the decomposition of methylsulfonylhydroxylamine CH_3SO_2NHOH (MSHA), a known chemical donor of HNO, by voltammetry. The ensuing curves (Figure 4)

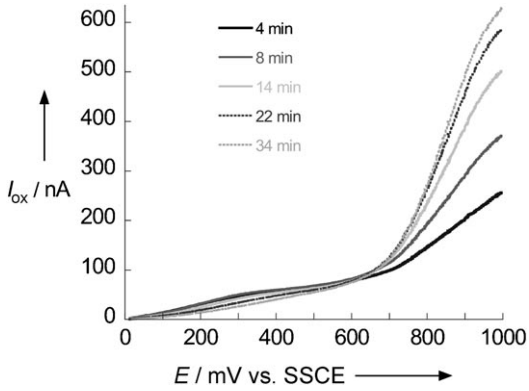

Figure 4. Voltammograms generated by detection with a platinized carbon fiber microelectrode (radius = 5 μm) in a solution of methylsulfonylhydroxylamine (MSHA, 10 mM) in alkaline PBS (pH 11) investigated as a function of time; scan rate: $v = 100\ mV\,s^{-1}$.

show two well-defined irreversible waves. The less anodic wave, with a half-wave potential of $E_{1/2} = 200 \pm 5$ mV versus SSCE matches wave I ($E_{1/2} = 200$ mV versus SSCE) detected in AS solutions (Figure 2) very well. An additional experiment based on the recording of the voltammogram corresponding to sodium methane sulfate (CH_3SO_2Na) demonstrated that the second wave detected between 650 and 900 mV, defined by a half-wave potential of about 820 mV versus SSCE, can be attributed to the oxidation of sodium methane sulfate, a by-product of MSHA decomposition. Therefore, this wave is irrelevant to our study, although it establishes the effectiveness of HNO production by MSHA decomposition. Due to the fact that HNO and sodium methane sulfate were the only electroactive products produced by the decomposition of methylsulfonylhy-

droxylamine, we attribute with high confidence the first wave, and consequently wave I, in AS solutions to the oxidation of HNO.

Moreover, when the same experiment was performed in the presence of ferric ions, we observed the disappearance of wave I ascribed to HNO and the concomitant increase of another wave with a plateau potential close to 650 mV versus SSCE, corresponding to the oxidation of nitric oxide. This result is further evidence that HNO is formed and may react with Fe^{3+} to yield NO according to the known reaction in equation (6):

$$HNO + Fe^{3+} \rightarrow Fe^{2+} + NO + H^{+} \quad (6)$$

Conclusion

The combination of voltammetry at microelectrodes with UV spectrophotometry data allowed us to determine the nature of major species actually released by Angeli's salt during its decomposition in aqueous solution. Under physiological conditions, HNO as well as NO were unequivocally detected, whereas $ONOO^{-}$ could only be observed at alkaline pH. Thus, the present results should certainly narrow the spectrum of possible mechanisms that explain the pharmacological properties of Angeli's salt.

Experimental Section

Materials and solutions: All chemicals used throughout these studies were purchased from Sigma (St. Louis, MO, USA) except Angeli's salt (sodium α-oxyhyponitrite, $Na_2N_2O_3$) and DEA-NONOate [diethylammonium (*Z*)-1-(*N*,*N*-diethylamino)diazen-1-ium-1,2-diolate] that were provided by Cayman Chemical (Ann Arbor, MI, USA). Stock solutions of peroxynitrite [oxoperoxonitrate^{1-}, $ONOO^{-}$] were synthesized by the method of Uppu and Pryor,[29] and were stored at $-20\,^{\circ}C$. Their current stock concentration was determined by measuring the absorbance at 302 nm ($\varepsilon = 1670\ M^{-1}cm^{-1}$) in NaOH (0.02 M). MSHA [*N*-(methanesulfonyl)hydroxylamine, CH_3SO_2NHOH] was synthesized following the description by King and Nagasawa.[30] Sodium methanesulfonate [*N*-(methanesulfonyl)-sodium, CH_3SO_2Na] was stored at $-20\,^{\circ}C$ until use. Phosphate-buffered saline (PBS: 137 mM NaCl, 10 mM Na_2HPO_4, and 3 mM KCl at pH 7.4) was used as buffer throughout the experiments. PBS was prepared from tablets dissolved in pure water (resistivity = 18 $M\Omega\,cm^{-1}$; Milli-Q system, Millipore, Billerica, MA, USA). The aqueous solutions of AS, DEA-NONOate and MSHA were prepared freshly for each experiment by dissolving the compound into PBS and using it immediately. Studies on Angeli's salt solutions were made in the presence of DTPA (diethylenetriaminepentaacetic acid, $C_{14}H_{23}N_3O_{10}$, 50 μM) for the complexation of metallic ions. The complete decay profiles of Angeli's salt concentration in either aerated or deaerated PBS at various pH values were monitored by UV spectrophotometry ($\varepsilon = 8000\ M^{-1}cm^{-1}$, UV Safas spectrophotometer, MONACO) at controlled temperature (22 °C). The sample was not exposed to the instrument light source during time intervals. Deaerated PBS was prepared by purging with argon in bulk (> 20 min) and then again (5 min) following Hamilton syringe transfer (3 mL) to an argon-flushed container. Similarly, oxygen-saturated solutions were prepared by bubbling PBS with synthetic pure oxygen (99.9%) and following the same procedure.

Electrochemical measurements: A detailed procedure for the fabrication of carbon fiber microelectrodes has been described elsewhere.[31] The main steps are as follows: individual carbon fibers (diameter = 10 μm; Cytec Engineered Materials, West Paterson, NJ, USA) were aspirated into 1.2-mm diameter glass capillary tubing (GC120F-10, Clark Electromedical Instruments, Harvard Apparatus, Edenbridge, UK); each capillary was then pulled with a microelectrode puller (Model PB-7, Narishige, Tokyo, Japan), and the carbon fiber protruding from the glass tip was insulated by the electrochemical deposition of polyoxyphenylene according to a method previously described.[32] The polymerization solution contained allylamine (0.4 M), 2-allylphenol (0.23 M), and 2-butoxyethanol (0.23 M) in water/methanol (1:1, *v*/*v*). Deposition was accomplished by applying a potential of +4 V for 3 min with a platinum wire as the counterelectrode. The efficiency of the polymer deposition was verified under a microscope. Subsequently, the microelectrodes were washed in distilled water, and the polymer was cured (3 h, 150 °C) to reticulate and form an insulating shielding on the carbon fiber surface. The tip of the microelectrode was polished on a diamond particle whetstone microgrinder (Model EG-4, Narishige, Tokyo, Japan) at an angle of 45° for 3 min to make a clean and regular surface of freshly exposed carbon. In order to increase the microelectrode sensitivity and selectivity versus the electroactive reactive oxygen and nitrogen species, the polished carbon surface was platinized by reducing hydrogen hexachloroplatinate in the presence of lead acetate at −60 mV versus SSCE.[31,33] The linear increase of the reductive deposition current was followed on a computer, and the process was interrupted when the electrical charge of the signal reached the desired value of 30–40 μC, which corresponds to the optimal activity of the electrode surface for the present experiments.

Electrochemical experiments were performed in a Faraday cage at a controlled room temperature (22 °C). Linear scan voltammetry (Ensman EI 400, Ensman Instruments, Bloomington, IN, USA) was used to record the oxidative response of Angeli's salt solutions and their evolution with time. The voltammetric curves were monitored and stored on a PC computer (Dell, Austin, TX, USA) via a D/A converter (Powerlab 4SP, ADInstruments, Colorado Springs, CO, USA) and its software interface (Chart version 5.0).

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Keywords: Angeli's salt · electrochemistry · microelectrodes · nitric oxide · nitroxyl · peroxynitrite

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