Solid Films of Blended Poly(Vinyl Alcohol)/Poly (Vinyl Pyrrolidone) for Topical *S*-Nitrosoglutathione and Nitric Oxide Release

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ABSTRACT: Nitric oxide (NO) is responsible for biological actions in mammals, ranging from the control of arterial pressure to immunological responses. In this study, *S*-nitrosoglutathione (GSNO), a spontaneous NO donor, was incorporated in solid films of blended poly(vinyl alcohol) (PVA) and poly(vinyl pyrrolidone) (PVP) comprising a biomaterial with potential for the local delivery of NO. In dry conditions, the extinction of the absorption bands of GSNO was correlated with the increase of the absorption band of its dimmer, GS–SG, implying NO release through the homolytic cleavage of the S–N bond. Mass spectrometry was used to confirm and to monitor the release of free NO from solid PVA/PVP-GSNO films to the gas phase. Kinetic measurement based on the Griess reaction was used to show that solid PVA/PVP-GSNO films are also capable of releasing both NO and GSNO to aqueous solution trough diffusion. Storage experiments have shown that GSNO is highly stabilized in the dry PVA/PVP matrix. The results indicate that GSNO-containing PVA/PVP films may be used for delivering free NO and/or GSNO topically and controllably. J Pharm Sci 94:994–1003, 2005

Keywords: nitric oxide detection; *S*-nitrosoglutathione; poly(vinyl alcohol); poly(vinyl pyrrolidone); nitric oxide delivery; mass spectrometry; UV/Vis spectroscopy; polymeric drug delivery systems; targeted drug delivery; polymeric biomaterials

INTRODUCTION

The growth interest in the chemistry and biochemistry of nitric oxide (nitrogen monoxide, NO) reflects its current importance as a key molecule that mediates a broad variety of physiologic and pathophysiologic events in cardiovascular, immune and nervous systems in mammals.[1–4] Other recent evidences have shown that NO is also involved in the pathophysiology of cancer[5] and neurodegenerative diseases.[6] Due to the actions of NO in living systems, there is an increasing interest in the development of new biomaterials with potential for controlling the delivery of NO to target areas in tissues where NO could have a biological effect.[7] The incorporation of NO donors into non-toxic polymeric matrices may allow the use of solid materials or gels for topical transdermal applications or subcutaneous implants.[8,9]

Several peptides and proteins containing the sulfhidryl (SH) moiety in cysteine (Cys) residues, like glutathione (GSH), which are found endogenously in the tissues and plasma of mammals, are believed to be involved in the metabolism of NO.[10] GSH is likely to form the *S*-nitrosothiol (RSNO) *S*-nitrosoglutathione (GSNO), which is considered to

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be an NO carrier and donor and may also act as a reservoir of NO *in vivo*.[11] RSNOs are vasodilators and inhibitors of platelet aggregation and have been at the center of several pharmacological studies and strategies concerning the importance of NO in living systems.[9,12,13]

We have shown recently that GSNO can be incorporated into nontoxic solid films of poly(vinyl alcohol) (PVA) and poly(vinyl pyrrolidone) (PVP), two polymers already used in several biomedical applications, and also in blends of PVA/PVP.[14–16] The combination of properties of PVA and PVP in blends leads to a more versatile material, whose physical–chemical properties can be modulated by changing the PVA/PVP ratio. For this reason, a special interest was devoted to this blend.

Free NO released from blended PVA/PVP films containing RSNOs at the film/skin interface is expected to diffuse rapidly through the stratum corneum and into the underling tissue layers. As free NO crosses cell membranes easily, most of its biological effects obtained with the topical application of the film could arise in this case, from free NO, before hydration of the film and diffusion of the water soluble RSNO becomes important.

The characterization of biomaterials such as solid PVA/PVP-GSNO films, which are NO donors, implies the unequivocal detection and quantification of the NO released in different conditions. A range of methods already established can be used for this purpose. In the particular case of RSNOs like GSNO, the NO release reaction can be followed by recording the spectral changes in the UV/VIS region associated with the extinction of the absorption bands at 336 and 545 nm, assigned to the $\pi \rightarrow \pi^*$ and $n_N \rightarrow \pi^*$ electronic transitions, respectively,[17] provided the films are transparent enough for the absorbance measurements. This monitoring gives a true measure of the NO produced according to Equation 1.

$$2\ \mathrm{GSNO} \rightarrow \mathrm{GS\text{-}SG} + 2\ \mathrm{NO} \qquad (1)$$

The fate of NO will depend essentially on the medium in which it is released. In the presence of O_2, NO is readily converted to $^{\bullet}NO_2$ in a termolecular reaction, according to Equation 2:

$$2\ NO^{\bullet}_{(g)} + O_{2(g)} \rightarrow 2\ ^{\bullet}NO_{2(g)} \qquad (2)$$

NO can further share the unpaired electron with $^{\bullet}NO_2$ yielding dinitrogen trioxide (N_2O_3)[18] according to Equation 3:

$$^{\bullet}NO_{2(g)} + 2\,NO^{\bullet}_{(g)} \rightleftharpoons N_2O_{3(g)} \qquad (3)$$

In aqueous media, N_2O_3 hydrolyses yielding nitrous acid (HONO), which is in equilibrium with its conjugate base (NO_2^-):

$$N_2O_{3(aq)} + H_2O_{(1)} \rightleftharpoons 2\,HNO_{2(aq)} \rightleftharpoons 2\,NO^-_{2(aq)} + 2\,H^+_{(aq)} \qquad (4)$$

Eventually, nitrite (NO_2^-) can be oxidized into nitrate (N_3^-) by dissolved oxygen. The reactions described in Equations 2–4 are among the main reactions responsible for the known short half-life of NO. Other species can also scavenge NO rapidly, such as superoxide ($O_2^{\bullet -}$) or oxyhemoglobin (HBO_2), in addition to those involving the coordination of NO to transition metal ions, (notably iron II). For these reasons, most of the methods designed for identifying and quantifying NO released in solution, are based on the detection of its oxidation products NO_2^- and NO_3^-. Among these, the Griess reaction is one of the most frequently assays used to determine NO_2^- due to its relatively simplicity and inexpensive experimental setup.[19] Although widely used, the Griess reaction has a detection limit in the range 0.1–1.0 μM, what limits its use for measuring NO in biological fluids. A more sensitive method for detecting NO in solution is based on the fluorimetric detection of NO through the nitrosation of 2,3-naphthotriazole (DAN) under acidic conditions, to form the fluorescent product, 2,3-naphthotriazole (NAT).[20] Despite the high sensitivity of this method (detection limit of *ca*. 10 nmol/L), it also applies only if NO is released directly into aqueous solutions. The same limitation applies to the electrochemical detection of NO, which has a linear response[21] to concentrations of up to 300 μmol/L. For detecting and quantifying NO released directly to the gas phase, the current method with best sensitivity is the chemiluminescence method based on the reaction of NO with ozone (O_3). This is a versatile technique and can be used to detect NO released from both liquid or solid samples, once a carrier gas (e.g., Ar) is used to carry NO dissolved in solution or released from a solid sample to the gas phase, until the reaction chamber. Although the chemiluminescent method is highly sensitive, detecting up to 1 pmol/L of NO, it is also relatively complex and expensive.[22]

Mass spectrometry (MS) is a technique with very broad range of applications, and is particularly suitable for the direct detection and characterization of gases and mixtures of gases with high sensitivity and selectivity.[23,24] MS is also one of the few techniques able to detect trace amounts of NO

released directly from solid materials to the gas phase. For example, Avice et al.[25] presented a procedure for NO quantification based on the reaction between nitrite and acid potassium iodide by isotopic ratio MS. The use of MS with a modified dynamic FAB probe technique was also used for detecting NO and other gaseous products released in the photochemical decomposition of sodium nitroprusside (SNP) in aqueous solution.[26] The use of MS to detect NO may represent an important approach being the most sensitive and selective technique to confirm and monitor slow and continuous release of free NO from solid biomaterials to the gas phase.

In this study, MS with 70 eV electron ionization and direct gas was used to sampling and unequivocally detect NO released continuously and thermally from a solid PVA/PVP-GSNO film. The correlation between MS detection of NO and the spectral changes observed in the film has confirmed that the GSNO decomposition pathway in this matrix is the homolytic cleavage of the S−N bond, leading to free NO and oxidized glutathione (GS−SG). In addition, we have shown that solid PVA/PVP-GSNO films immersed in aqueous solution are also able to release both NO and GSNO.

MATERIALS AND METHODS

Materials

Poly(vinyl alcohol) (average M.W. 9000−10,000; 80% hydrolyzed, PVA), poly(vinyl pyrrolidone) (poly[1-(2-oxo-1-pyrrolidinyl)ethylene], average M.W. 1,360,000, PVP), glutathione (γ-Glu-Cys-Glu, GSH), hydrochloric acid, sodium nitrite, acetone and ethyl ether (Aldrich Chemical Co. Inc., St. Louis, MO), 0.01 mol/L phosphate buffered saline (pH 7.4; NaCl 0.138 mol/L; KCl 0.0027 mol/L, PBS) (Sigma, St. Louis, MO), L-cystine (Ecibra, Brazil), sulfanilamide (SULF), and *N*-(1- naphthyl)ethylenediamine dihydrochloride, NEED) (Merck, Berlin, Germany), were used as received. All the experiments were carried out using analytical grade water from a Millipore Milli-Q Gradient filtration system.

Solvent Cast Films Blended PVA/PVP Containing GSNO

The synthesis of GSNO and the preparation of solids films of PVA/PVP-GSNO were already described elsewhere.[14] In brief, aqueous GSNO solution (186 mmol/L) was added to a previously prepared aqueous PVA/PVP solution. A volume of 300 μL of the final solution was introduced into the cavity of a quartz cell with a detachable window (Hellma 106-QS optical path 0.1 mm) and allowed to dry during 4 h at room temperature in the dark to cast films. After water evaporation, the films were further dried in a desiccator with activated silica for 4 h in the dark at 25°C, prior to the analysis. This procedure led to the preparation of PVA/PVP blends with a PVA:PVP ratio of 7.7:2.3 (wt/wt), containing 20.4 wt% of GSNO.

In order to measure the residual water content in the solid films prepared as described above, a known mass of PVA/PVP-GSNO film was further dried in a freeze-drier (Terroni-Fauve, LT 1000/8, São Paulo, Brazil) for 24 h and the water content was determined gravimetrically and expressed as wt% of water.

Kinetics of GSNO Decomposition with NO Release

Spectral changes of solid PVA/PVP-GSNO films were recorded in the range 220−1100 nm in the dark at 37°C, referenced against air, using an HP 8453 Diode Array Spectrophotometer (Hewlett-Packard, model 8453, Palo Alto, CA) with a temperature-controlled cuvet holder. Kinetic curves of GSNO decomposition in the dry films were obtained from the absorption changes at 336 nm in time intervals of 10 min, at 37°C. The amount of NO released over time was calculated directly from the amount of GSNO decomposed. This calculation was based on the fact that the decay of the absorption band of GSNO at 336 nm can be associated solely to the homolytic cleavage of the S−N bond with NO release, according to Equation 1. Thus, the increase in the concentration of NO released over time ($[NO]_t$), was calculated from the changes in GSNO concentration ($[GSNO]_o - [GSNO]_t$), according to Beer's law:[14]

$$\begin{aligned}[NO]_t &= [GSNO] - [GSNO]_t \\ &= (A_o b/\varepsilon_{GSNO}) - (A_t b/\varepsilon_{GSNO})\end{aligned} \quad (5)$$

where: A_o and A_t are the GSNO absorbances at 336 nm at the beginning of the monitoring and at time t, respectively, $[GSNO]_o$ and $[GSNO]_t$ are the concentrations of GSNO at the beginning of the reaction and at time t, respectively, ε_{GSNO} is the molar absorption coefficient of GSNO at 336 nm in the PVA/PVP film ($\varepsilon_{GSNO} = 233$ mol/L/cm)[14] and b is the optical path of the film, which corresponds to the optical path of the detachable quartz cell cuvette (0.1 mm). The molar amount of NO released from solid PVA/PVP-GSNO films

was calculated directly from the molar GSNO concentration decomposed in a volume of 300 μL. This volume corresponds to the volume of the polymeric film, which fills the cavity of the detachable quartz cuvette employed.

Corresponding kinetic curves of GS−SG formation were obtained from the absorption changes at 274 nm. Corrected absorption values were obtained through the deconvolution of the overlapped UV and visible bands, using the software Origin™. Each point in the kinetic curves represents the average of three experiments, with the error bars expressed by their standard error of the mean (SEM). The absorption spectrum of L-cystine was obtained for comparison using an aqueous L-cystine solution (0.43 mmol/L) in acidic medium (HCl 1 mol/L). Initial rates (I_R) of thermal GSNO decomposition with NO release and GSSG formation were obtained by linear regression from the slopes of the initial sections of the kinetic curves, according to:

$$I_R = \Delta[A]/\Delta t \quad (6)$$

where, $\Delta[A]$ and Δt are the changes in the absorbance of GSNO (or GS−SG) and the corresponding time intervals, respectively. The kinetic curves of NO released versus time were fit to a first order exponential growth, according to the equation

$$[NO]_t = [NO]_f - [NO]_{fe}^{-kt} \quad (7)$$

where, $[NO]_t$ is the NO concentration at each time t, $[NO]_f$ is the final NO concentration, and k is the first order rate constant.

Diffusion of GSNO from PVA/PVP-GSNO Film Immersed in Aqueous Solution

Solid dry PVA/PVP films containing 20.4 wt% of GSNO, cast into the cavity of the detachable quartz cell, were immersed in 15 mL of PBS (pH = 7.4) in a quartz cuvette (2.5 × 2.5 × 3.0 cm) thermostatized at 37°C. The increase in the absorption band at 336 nm in the PBS solution, due to the diffusion of GSNO from the film, was monitored under stirring with a magnetic bar. The amount of GSNO, which diffuses from PVA/PVP films to PBS solution over time, was calculated from the intensity of the absorbance band at 336 nm, which is assigned to the S−N bond of GSNO according to:

$$[GSNO]_t = (A_t b/\varepsilon_{GSNO})b \quad (8)$$

where ε_{GSNO} is the molar absorption coefficient of GSNO at 336 nm in aqueous solution (800 mol/L/cm) [17] and b is 3 cm.

Kinetics of NO Release from Hydrated PVA/PVP/GSNO Films

The release do NO from solid films in hydrating conditions, was measured with the films immersed in PBS solution at 25°C, using the Griess method.[27] A small piece (c. 14.0 mg) of freshly prepared PVA/PVP-GSNO film, dried as described above (not freeze dried), containing 20.4 wt% (53 μmol/L) of GSNO, was immersed in a quartz cuvette containing Griess solution (SULF 0.2 mol/L; NEED 0.2 mol/L; HCl 4.0 mol/L). The absorbance at 550 nm, which provides a quantitative measure of NO released to the solution was kinetically monitored at 25°C for more than 20 h in triplicate. A calibration curve based on the absorption at 550 nm was obtained with acidic sodium nitrite solutions in the range 3–100 μmol/L (data not shown).

Mass Spectrometry Measurements

Figure 1 shows a diagram of the MS system used. Dried PVA/PVP films (70 mg) containing 20.4 wt% of GSNO were removed from the quartz cell and cut into small pieces, and then placed into a small ampoule (A), which was connected to the ion

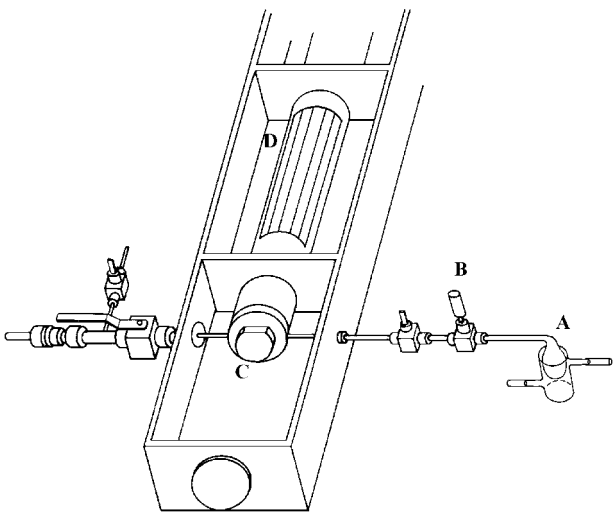

Figure 1. Schedule of the mass spectrometry (MS) system used for detecting free nitric oxide (NO) in the nearly air-free headspace of a solid PVA/PVP-GSNO film in dry conditions. (A) Thermostatizated ampoule containing pieces of the film; (B) needle valve; (C) EI ion source of the mass spectrometer; (D) high-transmission $\frac{3}{4}$ inches. mass analyzer quadrupole.

source of the mass spectrometer (C) via a needle valve and a 1/4 inches stain-steel line (B). The ampoule (A) was placed inside a double walled water-circulating flask connected to a thermostatic bath, and the sample flask was kept at constant temperatures of 37°C and 45°C. An AAB Extrel (Pittsburgh, PA) quadrupole mass spectrometer described in details elsewhere[28] fitted with a high-transmission 3/4 inches. mass analyzer quadrupole (D) was used. MS detection was performed using 70 eV electron ionization (EI). Mass spectra were first acquired in the range of m/z 25–200 to screen for any gaseous compound, and then from m/z 25 to 35 to selectively monitor the continuous release of NO (detected in its ionized form NO^+ of m/z 30) from the PVA/PVP-GSNO solid films at 37 and 45°C.

RESULTS AND DISCUSSION

Kinetics of NO Release from PVA/PVP-GSNO Films

GSNO decomposition with NO release leads to the formation of the dimmer GSSG (oxidized glutathione), according to Equation 1. Thus, the extinction of the band at 336 nm is expected to lead to the simultaneous appearance of a new band, with maximum around 274 nm, assigned to the formation of GSSG.[29] Figure 2A shows the spectral changes in the UV/VIS region, observed during the spontaneous thermal decomposition of GSNO at 37°C in the PVA/PVP film, in dry conditions. There is a clear increase in the intensity of absorption in the range 250–300 nm in the film, whilst the absorption band at 336 nm decreases. The association between these two spectral changes is confirmed by the detection of a well-defined isosbestic point at 312 nm. This result can be taken as an evidence that NO is released thermally through the homolytic S–N bond cleavage of the GSNO molecule, followed by dimerization through the formation of a sulfur bridge between two glutathionil ($GS^\bullet$) radicals (Eqs. 9 and 10).

$$GSNO \rightarrow GS^\bullet + NO^\bullet \quad (9)$$

$$GS^\bullet + GSNO \rightarrow GSSG + NO^\bullet \quad (10)$$

Owing to its bulkiness (molar mass 612.6 g/mol), GSSG is expected to display low diffusion coefficient in the highly viscous solid PVA/PVP film but the small NO molecules are expected to diffuse rapidly through the matrix and so to escape to the gas phase.

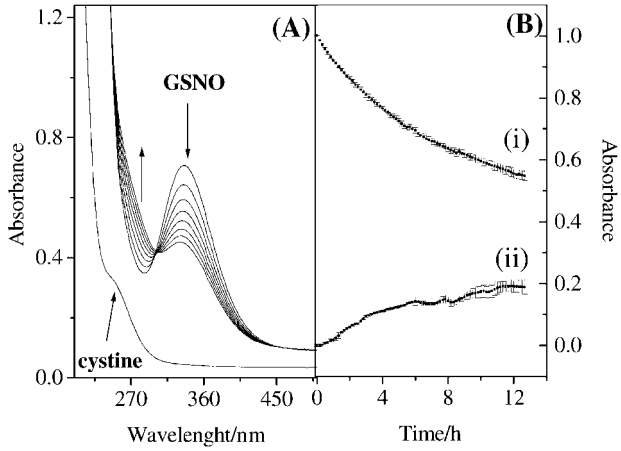

Figure 2. (A) Spectral changes in the UV/VIS region due to the decomposition of GSNO incorporated in solid PVA/PVP films at 37°C and absorption spectra of L-cystine (0.43 mmol/L) in aqueous HCl 1 mol/L solution. (B) Kinetic curves of GSNO decomposition monitored at (i) 336 and (ii) 274 nm.

Figure 2A also shows the absorption spectra of L-cystine in aqueous solution. As for GSSG, L-cystine (the dimmer of cysteine) also displays a sulfur bridge responsible for the absorption band around 270 nm. A comparison between this spectrum and the increase in the absorption band at *ca.* 274 nm in the decomposition of GSNO is further evidence that the release of NO occurs through a homolytic pathway. Figure 2B shows the corresponding decay and growth kinetic curves monitored at (i) 336 nm and (ii) 274 nm, respectively. The initial rates (I_R) calculated from these curves were 38 ± 1.6 for the decay curve and 29 ± 2.3 mmol/L/h for the growth curve. The I_R 1.3-fold times higher, obtained for the decay process is in accordance with the fact that the primary decomposition of GSNO follows a first order process, while the formation of the dimmer is a second order reaction, subjected to diffusion constrains imposed by the solid viscous matrix on the bulk $GS^\bullet$ radicals.

In dilute conditions, the decomposition of GSNO could be considered a first order reaction. However, at high concentrations, an autocatalytic process becomes operative.[17] In this process, $GS^\bullet$ radicals formed in the primary S–N bond cleavage reaction can react with native GSNO molecules in a second order reaction, leading to the primary formation of GS–SG and further free NO (Eqs. 9 and 10). GS–SG can also form in the collision between two diffusing $GS^\cdot$ radicals, although in the beginning of the reaction this must be a less

probable process. In addition, recombination between $GS^{\bullet}$ and $NO^{\bullet}$ also takes place and is favored by the viscosity of the solvent. In the present case, the high viscosity of the solid matrix imposes an important cage recombination effect, which stabilizes GSNO, compared to its decomposition in water, for example.[14] These simultaneous reactions make any attempt of a kinetic treatment of the data very difficult. A further complication arises from the fact that the absorption bands of GSNO and GS–SG in the UV region are highly overlapped, although deconvolution of the spectra could be partially applied. The dimmer GS–SG cannot be reconverted to GSNO by free NO, therefore, every GS–SG formed implies two NO molecules released. Free NO is expected to have a low solubility in the polar PVA/PVP matrix and thus most NO formed must escape to the gas phase.

Figure 3A shows the kinetic curve of NO release from GSNO 20.43 wt% incorporated in solid blended PVA/PVP films in dry conditions at 37°C. This curve corresponds to the spontaneous decomposition of GSNO according to Equation 1, implying the release of NO. The magnitude of the first order rate constants (k) of NO release, calculated from this curve was found to be $12 \pm 0.2 \times 10^{-2}$/h.

Diffusion of GSNO from PVA/PVP-GSNO Films

Figure 3B shows the kinetic curve of GSNO diffusion from solid PVA/PVP-GSNO, cast in the cavity of a quartz cell, to PBS solution and 37°C after the immersion of the dry film in PBS solution. It can be seen that GSNO diffuses very rapidly from the film to the aqueous solution, with

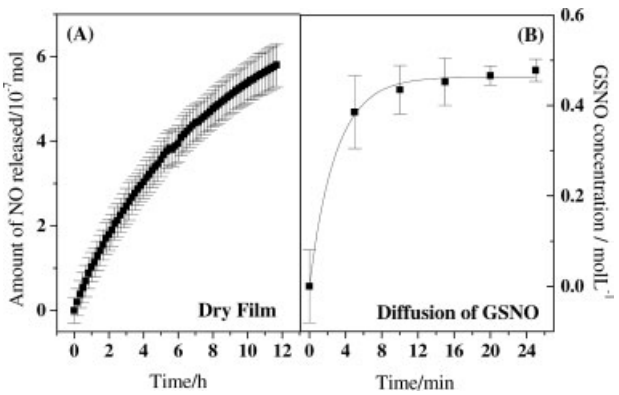

Figure 3. (A) Kinetic curve monitored at 336 nm for the NO release from GSNO incorporated in blended PVA/PVP films at 20.4 wt% in dry conditions at 37°C; (B) Kinetic curve monitored at 336 nm due to GSNO diffusion from solid PVA/PVP-GSNO to PBS solution.

a concentration plateau being reached in *ca.* 25 min. As hydrophilic materials, dehydrated PVA/PVP films will absorb water from the skin or from other tissues. However, in such conditions hydration will be much slower than with the films immersed in PBS solution, as shown here. Consequently, the diffusion of GSNO will also be slower and could allow for a prolonged biological effect in topical applications. It must be pointed out that in this case, the biological effects achieved could arise from both the diffusion of GSNO to the tissue and from the diffusion of free NO resulting from GSNO decomposition.

NO Release from Hydrated PVA/PVP-GSNO Films Immersed in Aqueous Medium

Figure 4 shows the kinetic curves monitored at $\lambda = 550$ nm, associated with the release of free NO from solid PVA/PVP-GSNO immersed in aerated acidic aqueous solution, over a period of 23 h. In this condition, NO undergoes a very fast reaction with dissolved O_2, forming NO_2. Hydrolyses of NO_2 in acidic conditions leads to the formation of nitrous acid, according to Equations 1–4. Nitrous acid, in turn, nitrosates sulphanilic acid of the Griess solution, forming a diazonium ion which couples to *N*-(1-napthyl)ethylenamidiamine, leading to the formation of a chromophoric azo-derivative detected through its absorbance band at 550 nm. The molar amount the azo-derivative formed is equal to the molar amount of NO released from the films to the solution. The Griess method is widely used to quantitatively measure nitrite (or NO after its conversion to nitrite).[32] In the present case, the Griess method was used to continually follow the kinetics of NO release from the films in hydrating conditions. The kinetic curve of Figure 4 reaches an apparent plateau after *ca.* 2.5 h of immersion with a $t_{1/2}$ of 35.5 min. The amount of NO released at this time leads to an NO concentration in the solution of *ca.*50 μmol/L. This concentration corresponds to a decomposition of *ca.* 74% of the initial molar GSNO stock in the film. It must be considered that GSNO not only decomposes inside the film, releasing free NO to the solution, but also diffuses intact to the solution as shown in Figure 3B. Thus, the total amount of NO detected in the solution is sum of the NO released inside the film, plus the NO released by native GSNO, which has previously diffused to the solution and decomposed afterwards. Regarding the diffusion phenomenon, it must be noted in Figure 3B that

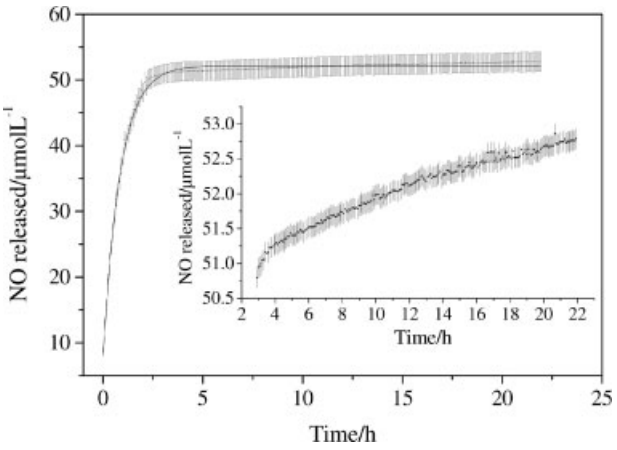

Figure 4. Kinetic curve showing the increase in NO concentration in a Griess solution due to the release of NO from a PVA/PVP film containing 20.4 wt% of GSNO immersed in the solution at 25°C. Formation of the chromophoric azo-derivative in the solution was monitored at $\lambda = 550$ nm. Inset: Detail of the section of the kinetic curve after 3 h, showing the slow increase in NO concentration.

diffusion of GSNO to the solution reaches a plateau after *ca*. 12 min. Thus, the plateau reached in Figure 4, after 2.5 h, cannot be correlated to the fast diffusion step of Figure 3B followed by decomposition, and reflects a slower diffusion of free NO from the film. Moreover, a close inspection of the plateau of Figure 4 shows that the conversion of the Griess reactants is still in progress until 23 h after the immersion of the film. This result can be clearly seen in the expanded scale of the inset of Figure 4. Although the amount of NO released in this step is very small leading to an increase of NO concentration in the solution of *ca*. 2 μmol/L, it shows that a slow release process is taking place, after the initial burst of NO in the first 2 h. The estimated rate of increase of NO concentration in the solution is this step is *ca*. 84 nmol/h.

Any analysis here, must firstly take into account that the film was immersed in aqueous solution, what represents an extreme situation regarding the rate of NO (or GSNO) release. In practical applications, where the film will be in contact with tissues (specially onto the skin) the rates of such diffusion processes are expected to be attenuated, leading to much longer releasing times.

Stability Under Storage

We have already shown that during the casting of PVA/PVP-GSNO films the extent of GSNO decomposition is *ca*. 3.3% of the initial GSNO molar concentration.[14] In a practical situation, this percentage will be lower because this estimate was carried out with the polymer solution kept in a closed cuvette. In an open system, viscosity of the solution will increase continuously, with the loss of water, reducing GSNO decomposition. This stabilization effect promoted by viscous matrices on *S*-nitrosothiols was already described in a previous study.[29] Films dried only in the desiccator were found to be stable with non-detectable decomposition after 30 days of storage in a normal refrigerator (5°C). In the freezer (−20°C), the films showed negligible decomposition after more than 3 months of storage. At 25°C, in the dark, 33% of the initial molar GSNO amount was lost after 30 days. These data show that the films can be stored under normal refrigeration conditions for at least 2 months. However, it must be noted that this film was not completely dried. The residual water content in the film was estimated gravimetrically as 4.8 wt%. The stability of GSNO in freeze-dried films is expected to increase, once water acts as a plasticizer of hydrophilic polymers, increasing the mobility of the polymeric matrix. Removal of water decreases the matrix mobility and the lower the mobility of the matrix, the higher the stability of GSNO, or other *S*-nitrosothiols.[29]

Detection of Free NO by MS

Figure 5 shows the evolution of the 70 eV EI mass spectra in the mass range from *m*/*z* 25 to 35 obtained for the continuous MS monitoring of the "headspace" gas composition above solid dry films of either PVA/PVP or PVA/PVP-GSNO at 37 (A) and 45°C (B). The "blank mass spectrum" for the headspace gas from the reference PVA/PVP film displays mainly two ions of *m*/*z* 28 and 32 in an abundance ratio of near 3:1, owing therefore to residual air, that is $^{14}N_2^{+\bullet}$ of *m*/*z* 28 and $^{16}O_2^{+\bullet}$ of *m*/*z* 32. Despite the high vacuum (10^{-7} torr) cleanup of the headspace region, air is still present, although in very low concentrations, and is therefore its main components are clearly detected by MS. After 25 min at 37°C the mass spectrum for the PVA/PVP-GSNO film clearly displays, however, an additional and relatively very abundant ion of *m*/*z* 30, which can be unequivocally assigned to the detection of free NO ($^{14}N^{16}O^{+}$) (spectrum b). The intensity of the ion of *m*/*z* 30 was found to increase up to 35 min (spectrum c) and then to decrease after 45 min (spectrum d) at 37°C. The evolution of the mass

spectra of a second sample of PVA/PVP-GSNO film kept at 45°C is shown in Figure 5B. The signal corresponding to the *m/z* 30 ion was detected initially after 60 min (spectrum f). It can be seen that the intensity of this signal increases continually in the spectra collected after 85 (spectrum g), 90 (spectrum h), and 100 min (spectrum i). The corresponding intensities of the *m/z* 30 signal in Figure 5A,B are plotted against time in Figure 6 and shows that although the release of NO from the film starts to decrease after 45 min at 37°C, at 45°C the film continues to release increasing amounts of NO for at least 100 min. This result shows that the rate of NO release from the film can be modulated by temperature and is in accordance with a mechanism based on the thermal homolytic cleavage of the S–N bond of GSNO.

The unequivocal MS detection of free NO released from the PVA/PVP-GSNO film (Figures 5 and 6), along with the spectral changes associated with the extinction of the GSNO band and the formation of the dimmer band in Figure 2A, confirms that NO is released through the homolytic bong cleavage of the S–N bond of GSNO, according to Equation 1. In hydrating conditions, GSNO was shown to diffuse to the aqueous environment before undergoing decomposition with NO release (Figure 3B). Thus, in topical applications of this material on the skin, NO action can involve either the previous homolitic S–N bond cleavage with the release of free NO or a direct transnitrosation reaction between diffusing GSNO molecules and NO receptors like free thiols according to:

$$\mathrm{GSNO} + \mathrm{RSH} \rightarrow \mathrm{GSH} + \mathrm{RSNO} \qquad (11)$$

Where R can be a transmembrane protein with a free thiol, for example.

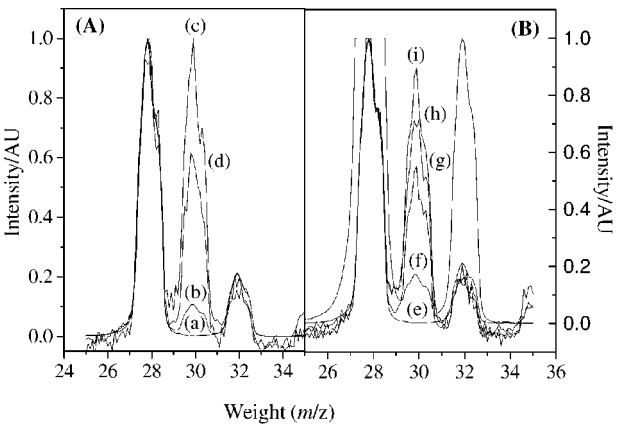

Figure 5. 70 eV EI mass spectra of the headspace gas for (a) solid PVA/PVP film (blank) and for the PVA/PVP-GSNO film after (b) 25 min, (c) 35 min, and (d) 45 min at 37°C (A) and for (e) solid PVA/PVP film (blank) and PVA/PVP-GSNO film after (f) 60 min, (g) 85 min, (h) 90 min and (i) 100 min at 45°C (B).

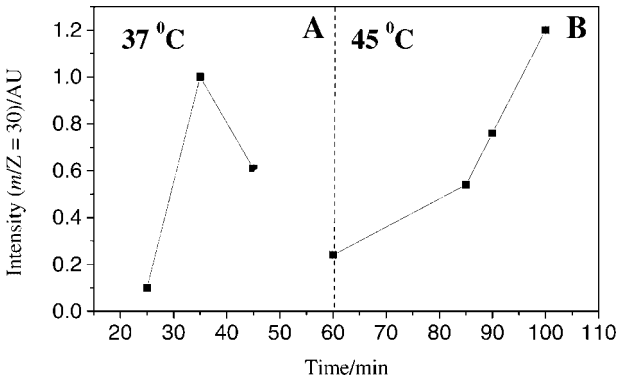

Figure 6. Mass spectrometry kinetic monitoring of NO released to the gas phase by solid films of PVA/PVP-GSNO kept at 37°C for 45 min (A) and at 45°C for 100 min (B). The normalized intensities of the *m/z* 30 ($^{14}N^{16}O^{+}$) signals correspond to intensities of these signals in the spectra of Figure 5.

Potential Uses of PVA/PVP-GSNO for Topical Applications

The incorporation of the NO donor GSNO in PVA/PVP films may represent a new strategy for the topical release of either NO or GSNO. Thus, the biological effects of these two species could be used in biomedical applications by promoting either a local vasodilation or a cytotoxic effect. We have recently reported the local increase of blood flow with the topical application of GSNO-containing hydrogels of poly(ethylene glycol)-poly (propylene glycol)-poly(ethylene glycol) (PEO-PPO-PEO) in the forearm of humans subjects.[9] Similarly, the topical application of PVA/PVP-GSNO could be used for enhancing wound healing in diabetes, for example, once this pathology is accompanied by a severe vasoconstriction.[33] Another potential topical application is for killing cutaneous leishmania once other works have already shown that NO in toxic against leishmania parasites.[34]

GSNO is completely dissolved in the polymeric PVA/PVP matrix and a phase separation will only happen after saturating the matrix with GSNO, (beyond 50 wt% of GSNO). Thus, the amount of NO released from PVA/PVP films can be adjusted in the solubility range, depending on the application.

For example, NO released from PVA/PVP-GSNO in the mmol/L range is high enough to provide a transdermal delivery of NO in a topical application.[9,30] The concentration 10 nmol/L is the minimal NO concentration required in order to cause vascular smooth muscle via cyclic GMP.[31] Exposure to NO released by the PVA/PVP-GSNO in the mmol/L range is expected to produce biological effects or to have cytotoxic action in topical applications, while exposure to NO in the nmol/L range is expect to lead to a vasodilatation effect. Such different effects can be achieved by controlling the amount of GSNO incorporated in PVA/PVP films.

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