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Uptake and diffusion of plasma-generated reactive nitrogen species through keratinized membrane

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Abstract

We propose a mathematical model for uptake and diffusion of air plasma-generated reactive nitrogen species (RNS) in a model keratinized membrane, such as a thin slice of bovine hoof or human nail material. An experimental system consisting of a surface microdischarge (SMD) in air was designed for the purpose of developing and validating a reaction-diffusion model to describe this system. Key variables such as membrane effective diffusivity, surface reaction rate coefficients, and other parameters are determined through comparison between the model predictions and experimental measurements. The model results yield spatial and temporal concentration profiles of RNS inside the keratinized membrane, leading to an improved understanding of transport and reaction of plasma generated RNS through the membrane. This work offers insights into possible mechanisms underlying plasma treatment of toenail fungus.

Keywords: atmospheric pressure plasma, plasma medicine, RONS, plasma–liquid interactions, antifungal, onychomycosis, plasma modeling

(Some figures may appear in colour only in the online journal)

1. Introduction

Cold atmospheric-pressure air plasmas (CAP) have been studied for a wide range of applications in plasma medicine, in part due to the generation of reactive oxygen and nitrogen species (RONS) at ambient or near-ambient conditions [1–6]. It has been demonstrated that atmospheric-pressure, ambient temperature gas plasmas are promising platforms for a wide range of environmental and biomedical applications based on their demonstrated antimicrobial action, treatment of infected tissue, and disinfection of wounds, among many others [1, 7–10]. However, plasma technologies face challenges in many applications because of the intrinsically complex nature of plasma interactions with biomaterials coupled with complex and not fully characterized plasma-biomaterial effects [7]. There is little doubt that the current lack of basic understanding of plasma interactions with biomaterials is a significant challenge that must be overcome in order to more widely enable applications of plasma medicine.

As a part of the ongoing set of efforts to address these emerging issues, this work focuses on modeling diffusive transport of reactive nitrogen species (RNS) with heterogeneous reactions inside a model porous biomaterial. The model is designed to approximate a physical experimental setup detailed previously [11, 12], in which a plasma source was used to inactivate either T. rubrum or Escherichia coli K12 on the far side of a slice of bovine hoof. T. rubrum is a common human toenail fungus responsible for onychomycosis and E. coli serves as a convenient model microbial target. The bovine hoof slice is a keratin membrane that also serves as
a model for human nail [13, 14]. In this work, uptake and delivery of RNS inside the model keratin bio-membrane were analyzed using a transient reaction-diffusion model. In the present model, we postulate that the major active RNS created by the plasma is NO$_2$, and that this NO$_2$ diffuses through the porous keratin to reach microbes residing on the back side of the membrane. When compared with experiments, the reaction-diffusion model shows evidence that RNS cause an antimicrobial effect by penetrating through the porous keratin membrane. Additionally, it is shown that adsorbed H$_2$O on the membrane surface initially causes hydrolysis of plasma-produced NO$_2$, creating HNO$_3$ and preventing NO$_2$ from reaching the far side of the nail. Once the active sites for hydrolysis have been consumed, NO$_2$ can diffuse through the nail and reach the other side. The rate of bacterial inactivation on the back side of the membrane correlated reasonably well with measured rates of NO$_2$ penetration through the nail, lending support to the original hypothesis. These results offer insights into the likely mechanism of plasma treatment of onychomycosis and possibly for other plasma-biomaterial treatments as well.

2. Setup

2.1. Experimental

The experimental setup used for comparison to the model is similar to one detailed previously [11]. As shown in inset of figure 1, a surface micro-discharge (SMD) device was used to generate RNS under the higher-power nitrogen oxides-dominated mode [12]. The SMD device configuration, gas-phase spectroscopy, analysis of aqueous chemistry, and antifungal experiments were described in detail in our previous report [12, 15]. Briefly, the SMD, operating in an enclosed acrylic chamber in atmospheric air, was powered with a 2.5 kV sinusoid at a frequency of 25 kHz. As shown in figure 1, the SMD was attached to the top of a chamber set up as a Franz cell, with the top cell and bottom cell separated by a slice of bovine hoof. The seal between the hoof material and the acrylic surfaces of the container was tested to minimize any transport between the membrane and the acrylic. The gas-phase plasma-generated species were quantified above the hoof slice by Fourier transform infrared spectroscopy (FTIR). Penetration of the hoof slice by RNS was measured using Griess reagent (via UV/VIS absorption spectrometry detecting aqueous NO$_2^-$) in phosphate buffered saline (PBS) placed in the bottom cell. For antibacterial experiments, *Escherichia coli* K12 was cultured and prepared as described in previous work [11]. To seed the hoof with bacteria, hoof slices were washed with sterile water and dried until lack of visible hydration before being pelleted with *E. coli* prior to plasma exposure.

Although not shown here, the effective diffusivity ($D_{eff}$) in the hoof disk, in the absence of reaction, was measured directly using an experimental setup similar to the one depicted in figure 1, employing CH$_4$ and CO$_2$. The estimated $D_{eff}$ in this work was $5.0 \times 10^{-5}$ cm$^2$ s$^{-1}$, which is similar to diffusion coefficients of other small molecules diffusing through living tissue [16–19].

2.2. Model

Transport of plasma-produced NO$_2$ through the hoof membrane was modeled by a transient, 1D reaction-diffusion equation within the hoof slice:

$$
\varepsilon \frac{\partial C_{NO_2,\text{hoof}}}{\partial t} = D_{eff} \frac{\partial^2 C_{NO_2,\text{hoof}}}{\partial x^2} - R_{\text{hoof}}
$$

(1)

where $\varepsilon$, $C_{NO_2,\text{hoof}}$, $D_{eff}$, and $R_{\text{hoof}}$ represent the porosity of hoof disk, the concentration of NO$_2$, the effective diffusivity of NO$_2$ within the hoof membrane, and the net rate of chemical reactions with NO$_2$ inside the hoof membrane, respectively. In this work, the porosity of hoof disk was taken as 0.05, based on cumulative mercury intrusion data into bovine hoof reported in the literature [14]. The measured gas phase NO$_2$ concentration, shown in figure 1, was used directly as a time-dependent boundary condition at the interface between the plasma and the top of the hoof membrane.

NO$_2$-consuming reactions inside the porous hoof membrane can in principle occur in both the gas phase and on the surface of the membrane material. While NO$_2$ is fairly stable in the gas phase on the timescales considered here, it is known to undergo reversible dimerization to N$_2$O$_4$, as shown in reaction (R1),

$$
2\text{NO}_2 \xrightleftharpoons[k_{-1}^\text{gas}]{k_1} \text{N}_2\text{O}_4.
$$

(R1)

For the concentrations of NO$_2$ measured in figure 1 and the values of the relevant forward and reverse rate constants ($k_{\text{gas}} = k_1/k_{-1} = 6.86$ atm$^{-1}$) [20], the calculated dimer concentration is approximately two orders of magnitude below that of NO$_2$, as shown in figure 1. Due to the rate constants and the presence of a dense, porous membrane, Knudsen diffusion was assumed within the hoof membrane, allowing for gas-phase dimerization within the hoof volume to be ignored. Thus, only NO$_2$ was considered in equation (1) for diffuse transport and surface reaction.

When considering reactions with the internal surface of the porous hoof membrane, it is important to note that keratin is generally coated with a layer of water adsorbed from the atmosphere, which is likely to dominate NO$_2$-hoof material interactions. Thus, it is important to understand the nature of this adsorbed water. Detailed water uptake isotherms have been reported for keratin tissues such as human nail, horn, hair and wool [21]. It was shown that the water uptake behavior on keratin follows a Langmuir isotherm at lower humidity because water molecules are bonded to hydrophilic surface sites. Therefore, it is clear that the monolayer of adsorbed water is not the equivalent of bulk water. The adsorption of a monolayer of water in a keratin structure has been reported as 0.06 g H$_2$O/g dry keratin [21]; that number is used in this work as the water uptake in the hoof membrane.

There has, as yet, been no report in the literature regarding the detailed mechanism of NO$_2$ uptake by keratin-based material. However, it has been reported that the heterogeneous hydrolysis reaction of NO$_2$ on carbonaceous particles and protein dust is the main mechanism to explain NO$_2$ uptake in the presence of humidity. Furthermore, there appears to be
relatively low direct reactivity of NO2 with protein-containing materials in the absence of humidity [22–27]. Because of high water uptake of keratin-based materials, it is reasonable to assume that the hydrolysis reaction of NO2 with absorbed water on the surface of membrane fibers would lead to NO2 uptake on the hoof disk interior surfaces. As described in a later section, it should be noted that our experimental evidence of NO2 penetration supports the plausibility of this assumption.

In part, the proposed uptake model is based on similar research from the [28–31]. NO2 uptake in the presence of water on mineral dust or soot particles suspended in the atmosphere is an important process in models of atmospheric chemistry. The uptake model we chose is based on kinetic theory, assuming an ideal gas and an Eley–Rideal mechanism, in which the rate-limiting step is assumed to be the adsorption of NO2 as shown in reaction (R2) [28, 32, 33]:

\[
2\text{NO}_2(g) + \text{H}_2\text{O} (s) \rightarrow \text{N}_2\text{O}_4 \cdot \text{H}_2\text{O} (s) \quad (\text{R2})
\]

\[
\text{N}_2\text{O}_4 \cdot \text{H}_2\text{O} (s) \rightarrow \text{HN}_2\text{O}_2 (g, s) + \text{HNO}_3 (s). \quad (\text{R3})
\]

Since reaction (R2) is a surface reaction, a surface site balance equation is written for adsorbed water reacting with gas phase NO2, in the following form:

\[
\frac{d\theta_{\text{H}_2\text{O}}}{dt} = -\gamma_{\text{NO}_2}\theta_{\text{H}_2\text{O}}J_{\text{NO}_2}\sigma_{\text{H}_2\text{O}}. \quad (2)
\]

Here, \(\theta_{\text{H}_2\text{O}}\) denotes the fractional surface coverage of H2O that has not yet reacted with NO2; thus, as more H2O reacts with NO2, the coverage of H2O still available for NO2 uptake reactions decreases. In the present model, we assume that NO2, after adsorbing on the surface water layer, is inactive and the resulting water-NO2 surface compound will not adsorb any additional water. The uptake coefficient of NO2 on water absorbed in keratin material, \(\gamma_{\text{NO}_2}\), has been intensively researched to develop realistic atmospheric chemistry models, but values reported in the literature range from ~10\(^{-4}\) to ~10\(^{-6}\) based on whether the coefficient was measured for droplets, aerosols, or thin films [28, 34, 35]. The thin film uptake coefficient of 10\(^{-6}\) will be used in this paper as it seems to be most appropriate for porous keratin. The effective molecular cross section of water on the surface, \(\sigma_{\text{H}_2\text{O}}\), is assumed to be 0.124 nm\(^2\). The surface collision frequency per unit area, \(J_{\text{NO}_2}\), can be expressed as the average gas kinetic flux in equation (3),

\[
J_{\text{NO}_2} = \frac{C_{\text{NO}_2, \text{hoof}}\omega_{\text{NO}_2}}{4} \quad (3)
\]

where \(\omega_{\text{NO}_2}\) is thermal velocity of NO2, given by equation (4):

\[
\omega_{\text{NO}_2} = \sqrt{\frac{8RT}{\pi m_{\text{NO}_2}}} \quad (4)
\]

Here, \(m_{\text{NO}_2}\) is the molar mass of NO2, \(R\) is the gas constant, and \(T\) is the absolute temperature.

Finally, it is necessary to write an equation for the total uptake of NO2 by water on the surface of the hoof membrane fibers, which will correspond to \(R_{\text{hoof}}\), the total loss of NO2 from reactions inside the hoof volume in equation (1). This uptake will be proportional to the total number of available water sites per unit volume of hoof material, the flux of NO2, and the NO2 uptake coefficient. The total number of available sites for NO2 reaction can be written as:

\[
\text{#available sites} = \frac{\rho_{\text{hoof}}}{M_w} v_{\text{reactive}}\sigma_{\text{H}_2\text{O}}\theta_{\text{H}_2\text{O}} \quad (5)
\]

where \(\rho_{\text{hoof}}\) is the mass density of the hoof membrane, \(M_w\) is the molar mass of H2O, and \(v_{\text{reactive}}\) is the amount of water that actually takes part in hydrolysis of NO2. The dry density of bovine hoof is regarded as 1.3 g cm\(^{-3}\) of human nail with the similar keratin structure due to absence of proper experimental

Figure 1. NO2 gas concentration as a function of time measured by gas-phase FTIR absorption spectroscopy. The data points are numerically fitted to provide an input of NO2 for the reaction-diffusion model boundary condition at the top of the hoof disk. The gas phase concentration of N2O4, calculated based on equilibrium data, is insignificantly small under these conditions. The inset represents a schematic diagram of the experiment.
data [36]. Not all adsorbed water takes part in hydrolysis, and $v_{\text{measured}}$ is calculated as

$$v_{\text{measured}} = \phi v_m$$  \hspace{1cm} (6)

where $\phi$ is an adjustable constant determined by comparison to experiments. The determination of this constant will be discussed in the next section. Multiplying equation (5) by the flux of NO$_2$ and the NO$_2$ uptake coefficient yields equation (7) for $R_{\text{hoof}}$:

$$R_{\text{hoof}} = \frac{\gamma_{\text{NO}_2} h_{\text{H}_2\text{O}} I_{\text{NO}_2} \rho_{\text{hoof}} v_{\text{in,reactive}} \sigma_{\text{H}_2\text{O}}}{M_w}.$$  \hspace{1cm} (7)

Thus, if equation (7) is inserted into equations (1) and (3) is plugged into equation (2), equations (1) and (2) become a coupled set of differential equations that can be solved for the concentration of NO$_2$ and the fractional coverage of available surface sites.

Once NO$_2$ penetrates through the entire hoof membrane, it then diffuses through a small air gap of 5 mm and into a reservoir of PBS solution containing Griess reagent (see figure 1). In modeling this transport and water solvation, we followed the approach reported by Deen’s group. In this approach, the differential equations and computational complexity were simplified by defining a ‘total reactive nitrogen’ concentration [20];

$$C_N = C_{\text{NO}_2} + 2C_{\text{N}_2\text{O}_4}.$$  \hspace{1cm} (8)

According to this approach, it is assumed that NO$_2$ and N$_2$O$_4$ are near equilibrium in the liquid or gas. Thus, solving coupled diffusion-reaction equations for each species is unnecessary, and based on reaction (R1), the concentrations of N$_2$O$_4$ in air and water can be written as

$$C_{\text{N}_2\text{O}_4,\text{air}} = K_{1,\text{air}}RTC_{\text{NO}_2,\text{air}}^2$$  \hspace{1cm} (9)

$$C_{\text{N}_2\text{O}_4,\text{water}} = K_{1,\text{water}}C_{\text{NO}_2,\text{water}}^2$$  \hspace{1cm} (10)

where $K_{1,\text{water}}$ represents the dimerization equilibrium constant of NO$_2$ in the liquid phase and is assumed to be 6.54 × 10$^3$ M$^{-1}$ [20, 33]. Assuming that the gas-phase and liquid-phase diffusivities ($D_{\text{air}}$ and $D_{\text{water}}$) of NO$_2$ and N$_2$O$_4$ are equal, the transient diffusion-reaction equation for each phase can be written as:

$$\frac{\partial C_{N,1}}{\partial t} = D_{N,1} \frac{\partial^2 C_{N,1}}{\partial x^2} + R_1$$  \hspace{1cm} (11)

where $I$ denotes either air or water. Here, $D_{\text{N,air}}$ and $D_{\text{N,liquid}}$ are taken as 1.7 × 10$^{-5}$ and 1.85 × 10$^{-9}$ m$^2$ s$^{-1}$, respectively [35]. At the interface between air and water, the liquid-phase concentration is governed by Henry’s Law:

$$C_{\text{N,water,interfacial}} = RT \left( h_{\text{N}_2\text{O}_4} C_{\text{NO}_2,\text{gas,interfacial}} + h_{\text{N}_2\text{O}_4} C_{\text{N}_2\text{O}_4,\text{gas,interfacial}} \right).$$  \hspace{1cm} (12)

Henry’s law coefficients $h_{\text{NO}_2}$ and $h_{\text{N}_2\text{O}_4}$ are 9 × 10$^{-3}$ and 0.77 M atm$^{-1}$, respectively [20, 37].

Due to the ‘total reactive nitrogen’ assumption in equations (8)–(10), the loss due to reactions in the gas phase in equation (11) is set to 0. In buffered liquid solutions containing Griess reagent, we considered the following hydrolysis reaction of N$_2$O$_4$ which is in equilibrium with NO$_2$, as justified elsewhere [20].

$$\text{N}_2\text{O}_4 + \text{H}_2\text{O} \xleftarrow{\delta} \text{HNO}_2 + \text{HNO}_3.$$  \hspace{1cm} (R4)

The uptake and hydrolysis of gas phase NO$_2$ in liquid water play important roles in various fundamental atmospheric and environmental chemistries [20, 22, 30]. Due to the apparent absence of real-time monitoring of these species under hydrolysis conditions, most analyses rely on theoretical estimates in order to determine chemical mechanisms and rates [22, 38–40]. Recently, Deen’s group reported that the accumulation rates of NO$_2$ and NO$_3^−$ in buffered aqueous solutions with exposure to NO$_2$ gas follow the stoichiometry of reaction (R4) [20]. Their reported experimental measurements were consistent with these assumptions. We have therefore followed their approach in the present work.

The loss due to reactions in the liquid phase is given by equation (13):

$$R_{\text{liquid}} = -k_3 C_{\text{N}_2\text{O}_4}$$  \hspace{1cm} (13)

where $k_3$ is the reaction rate coefficient in reaction (R4) (assumed to be the same as in reaction (R3)), given as 10$^3$ s$^{-1}$ [20]. The detailed boundary conditions expressions using total reactive nitrogen concentration are found in [20]. Note that the model prediction of formation of HNO$_3$ in (R4) is assumed to lead to the formation of nitrate (NO$_3^−$), the compound detected experimentally using Griess reagent.

The 1D transient reaction-diffusion equation for transport and reaction in the porous membrane (equation (1)) and for subsequent diffusion and water dissolution (equation (11)) are coupled with equation (2) for water surface coverage at each point within the porous membrane as a function of time. There, equations were numerically solved using Mathematica.

3. Results and discussion

In this model, all variables except the proportionality constant ($\phi$) can be independently estimated using published data. Adjusting $\phi$ effectively alters the extent to which NO$_2$ uptake occurs within the membrane. Setting this parameter to zero eliminates uptake, resulting in pure diffusion of NO$_2$ through the membrane with no loss at the porous nail surface. Physically, this key parameter corresponds to the fraction of water that participates in uptake. Water uptake inside the keratin fibers can be reduced during NO$_2$ uptake because the surface reactions of (R2) and (R3) in the liquid phase are exothermic reactions with reaction enthalpy of −63.38 KJ mol$^{-1}$. In addition to that, it is desirable that we also consider the existence of both ‘bound’ and ‘free’ water on the keratin fibers, where ‘bound’ water is not available to take part in reactions. To the best of the authors’ knowledge, the details regarding the above phenomena have not been reported in the literature so far, and rates for these processes are difficult to theoretically estimate. Therefore, the inclusion of this parameter in this work is to simplify the complexities of NO$_2$ uptake.
It was inferred in this work by comparison between model and measurements.

To address this issue, we used Griess reagent measurements to determine the accumulation of aqueous nitrite formed by the plasma-generated RNS inside the water reservoir after penetration of the hoof, as shown in figure 1. The hoof membrane thickness was varied, and multiple experiments were conducted with different membrane thicknesses. As shown in figure 2, the measured nitrite concentration in the water after 20 min exposure strongly depends on the thickness of the bovine hoof. This indicates clearly that the plasma-generated NO$_2$ can be transported by diffusion through the hoof membrane. However, if $\varphi$ is set to 0 (corresponding to no surface loss of NO$_2$) and only diffusion through the porous nail is considered, the model results do not agree well with the experimental results. Thus, it is likely that diffusive transport through the membrane is hindered by uptake with surface-bound, reactive water. By including uptake in the model with a proportionality constant of $\varphi = 10^{-3}$, agreement between experiment and model is significantly improved. This result suggests that a small portion of adsorbed water takes part in NO$_2$ uptake by hydrolysis, inhibiting transport through the hoof membrane.

Figure 2. Penetration of the hoof membrane by NO$_2$ as a function of hoof thickness, as measured by nitrite ion concentration accumulated in the water reservoir beneath the hoof membrane after 20 min exposure. If only diffusion is considered in the model, far more penetration of NO$_2$ is predicted than what is experimentally observed. If NO$_2$ uptake by surface hydrolysis in adsorbed water is included with a proportionality constant of $\varphi = 10^{-3}$, reasonable agreement is found between the model and experiment (see text). This suggests that approximately 0.1% of the adsorbed water takes part in NO$_2$ uptake by hydrolysis, inhibiting transport through the hoof membrane.

Figure 3. Modeling results of the time- and space-profiles of nitrite ion concentration diffusing into the water surface, shown in the lower region of the container in figure 1. Each profile represents a 4 min increment, starting at 0 min. It should be noted that, for minutes 8–20, the concentration of nitrite at the water surface (0 mm) rises by the same amount every increment; this implies a linear rise of nitrite at the water surface as a function of time.
Having estimated the proportionality factor for free water in this manner, the parameter was held constant for the remainder of the simulations. The model was used to explore physical processes occurring in the penetration of NO$_2$ through the hoof membrane as well as accumulation of nitrite both within the nail material as well as in the reservoir of water (see figure 1) below the nail. Figure 3 shows predicted nitrite concentration spatial profiles near the surface of the pool of water (on the back side of the membrane) for a 250 µm-thick hoof (approximately the same thickness as a human nail) at time increments of 4 min.

As shown in figure 3, the concentration of nitrite at the surface of the water initially rises slowly. However, after the first 8 min, this concentration appears to rise linearly in time, increasing by approximately 9 mM every 4 min. This effect is further explored in figure 4. If the nitrite concentration profile is spatially averaged over the pool of water (to simulate mixing), the average nitrite concentration is shown to follow this type of behavior for a variety of hoof thicknesses.

Figure 4 shows nitrite accumulation profiles in the pool of water beneath the hoof (following hoof membrane penetration) as a function of time for various hoof thicknesses. This corresponds to penetration of the hoof membrane by NO$_2$. As expected, penetration decreases with increasing hoof thickness. Additionally, all hoof membranes appear to display an ‘induction time’ in which the nitrite concentration remains near 0, followed by a period in which the nitrite concentration increases linearly (corresponding to constant penetration). The induction time corresponds to NO$_2$ uptake by adsorbed water on the keratin fibers; only after uptake is complete can NO$_2$ penetrate the hoof membrane.

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Figure 4. The average simulated nitrite concentration in the reservoir of water beneath the hoof is shown as a function of time for varying hoof thicknesses. This corresponds to penetration of the hoof membrane by NO$_2$. As expected, penetration decreases with increasing hoof thickness. Additionally, all hoof membranes appear to display an ‘induction time’ in which the nitrite concentration remains near 0, followed by a period in which the nitrite concentration increases linearly (corresponding to constant penetration). The induction time corresponds to NO$_2$ uptake by adsorbed water on the keratin fibers; only after uptake is complete can NO$_2$ penetrate the hoof membrane.

This interpretation can be directly verified by exploring phenomena within the hoof membrane. Figure 5 shows fractional coverage of the keratin fibers by available hydrolysis surface sites inside the 250 µm-thick at 2 min intervals. As time increases, NO$_2$ first depletes the available surface sites near the top of the hoof membrane, sending $\theta_{H_2O}$ to 0 at that location rather quickly. As uptake is completed near the top of the hoof, NO$_2$ progresses deeper into the hoof, depleting surface sites and causing $\theta_{H_2O}$ to drop to 0 farther below the surface of the hoof. After 8 min, $\theta_{H_2O}$ does not reach 100% at any location inside the membrane; by 12 min, it does not rise above 20% even at the far end of the hoof. This corresponds to the time in figure 4 at which NO$_2$ begins to significantly penetrate the hoof and accumulate as nitrite in the underlying water. Figure 6 displays the same phenomenon in terms of NO$_2$ gas phase concentration inside the hoof membrane. The propagation of NO$_2$ species inside the hoof is characterized by relatively slow diffusion through the pores of the membrane as well as by the uptake of NO$_2$ by water on the surface of the keratin. When compared to figure 5, it is clear that NO$_2$ penetration drops to 0 at the point where active surface sites have not yet been depleted. As NO$_2$ depletes active surface sites, it is able to penetrate deeper into the nail, eventually reaching the far side. The time- and position-dependent profile of NO$_2$ concentration within the membrane behaves as a propagating wave.
Finally, to understand the effect of these modeled mechanisms on through-nail plasma treatment, antibacterial activity was investigated experimentally and compared to simulations. Antibacterial activity was measured by means of log reductions after plasma treatments of varying time, in which the log reduction is given by

$$\log \text{ reduction} = \log \frac{N_0}{N}$$

(14)

where $N_0$ is the number of viable cells present in an untreated sample and $N$ is the number of cells that remained viable treatment after plasma exposures. Detailed experimental procedures for the bacterial experiments were reported previously, as noted above [11, 12]. Experiments were undertaken with *E. coli* on both the top of the hoof membrane and the bottom of the hoof membrane with membrane thickness of 250 µm. Although the primary interest is in measuring the bacterial kill on the backside of the membrane, the top side measurements help establish the concentration-time relationship between NO$_2$ and bacterial inactivation rates and can be compared to previous results to assure consistency. Figure 7 shows a comparison of the measured averaged NO$_2$ gas concentration and bacteria log reduction as a function of time at the top of the hoof.

As shown in figure 7, bacterial inactivation at the top of the hoof initially correlates reasonably well with NO$_2$...
concentration and is consistent with previously reported data for similar experiments in killing *E. coli* using SMD air plasma [11, 12]. The bacterial log reduction appears to saturate after 10 min exposure, since the maximum log reduction possible, given the initial bacterial load, was about 8.

Figure 8 shows results for treatment of bacteria on the far side of the hoof membrane, which is more applicable to scenarios such as through-nail treatment of onychomycosis. As shown in figure 8, the log reduction of bacteria on the bottom side of the hoof appears to correlate with predicted NO\(_2\) concentration at this location. Since NO\(_2\) concentrations are smaller than on the top of the hoof, the log reduction is not saturated even after 20 min exposure. Note that the 20 min NO\(_2\) concentration at the bottom of the hoof is about the same as the 2 min NO\(_2\) concentration at the top of the hoof, shown in figure 7. Both cases display approximately 2 log reductions corresponding to about the same NO\(_2\) concentration (~2–3 µM), supporting the hypothesis that bacterial inactivation is strongly dependent on NO\(_2\) exposure time and concentration. Furthermore, the bacterial inactivation at the bottom of the hoof displays approximately the same 10 min ‘induction time’ as the model NO\(_2\) concentration. This correlation supports the hypothesis that antibacterial effects are due to NO\(_2\) diffusing through the nail membrane.

It should be noted that the apparent ‘premature’ inactivation at 5 and 10 min is within experimental error of 0. It is also
possible that a small amount of ‘premature’ inactivation could actually occur due to the fact that, as seen in reaction (R3), uptake and hydrolysis of NO₂ produces both HNO₂ and HNO₃. While HNO₃ does not leave the surface of the keratin fibers, HNO₂ can desorb into the gas phase given its known vapor pressure. Thus, one version of the model was expanded to include HNO₂ desorption from the surface following NO₂ adsorption and hydrolysis followed by gas phase diffusion. This analysis assumed HNO₂ reaching the top of the hoof diffused away and HNO₂ reaching the bottom of the hoof was confined by layers of E. coli. As shown in figure 9, HNO₂ is predicted to reach concentrations higher than 20 µM at the far side of the hoof over 10 min given these assumptions. This effect may be responsible for the relatively weak antibacterial effect seen before full penetration of NO₂. However, further studies should be done to better understand this potential mechanism.

4. Conclusions

Many applications of plasma medicine, such as through-nail plasma treatment are dependent on transport of plasma-produced species through a membrane of biological tissue. In this work, we proposed a transient reaction-diffusion model for transport of plasma-generated RNS species through a bovine hoof slice, which serves as a porous biological membrane and a model for a human nail. In the present model, we took NO₂ as the model RNS responsible for most of the observed antimicrobial effects, although it is likely that other species are involved as well. It was shown that physical diffusion alone could not account for the observed time dependence of hoof penetration by NO₂. Rather, transport through the hoof membrane was inhibited by uptake and hydrolysis of NO₂ by water adsorbed on the surface of keratin fibers; only after active surface sites of water were depleted could NO₂ penetrate the hoof membrane. When the fraction of adsorbed water available for NO₂ uptake was set to 10⁻³, experimental penetration of the hoof membrane agreed well with theoretical results; this suggests that, while NO₂ uptake into the nail is significant, most adsorbed water is strongly bound and cannot take part in uptake.

Comparison to experiments also shows that the uptake-dependent transport of NO₂ through the hoof membrane corresponds to inactivation of E. coli on the far side of the hoof. Thus, this model potentially captures the mechanisms responsible for through-nail plasma treatment, observed in a previous report [11]. After an ‘induction time’ corresponding to the uptake of NO₂ and deactivation of surface sites, NO₂ is predicted to freely diffuse through the hoof membrane and take part in antibacterial (or, depending on the application, antifungal) activity. The thickness of the nail determines not only the steady-state NO₂ penetration rate but also the time needed for diffusing NO₂ to penetrate the membrane. Only minimal bacterial inactivation was observed during the apparent induction time. The observed nonzero inactivation during the induction period is provisionally attributed to the diffusion of HNO₂ formed by hydrolysis of NO₂ within the nail. This effect is relatively weak in comparison to the inactivation that takes place after the induction time is over and NO₂ fully penetrates the nail.

This work provides a theoretical and quantitative model of the mechanisms of through-nail plasma treatment, which is relevant to applications such as plasma treatment of onychomycosis. The importance of NO₂ uptake by adsorbed water is shown, and a correlation is observed between bacterial inactivation and the reaction-diffusion mechanisms presented.
in this paper. While this study used a biological membrane, the findings are relevant in a broader context as well, since adsorbed water will commonly be present on membranes in atmospheric air. Thus, the findings of this paper potentially provide basic insights into other plasma treatments through porous membranes at atmospheric pressure.

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