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Optimized polymeric film-based nitric oxide delivery inhibits bacterial growth in a mouse burn wound model

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Abstract

Nitric oxide (NO) has many biological roles (e.g., antimicrobial agent, promoter of angiogenesis, prevention of platelet activation, etc.) that make NO releasing materials desirable for a variety of biomedical applications. Localized NO release can be achieved from biomedical grade polymers doped with diazeniumdiolated dibutylhexanediamine (DBHD/N₂O₂) and poly(lactic-co-glycolic acid) (PLGA). In this study, the optimization of this chemistry to create film/patches that can be used to decrease microbial infection at wound sites is examined. Two polyurethanes with different water uptakes (Tecoflex SG-80A (6.2 ± 0.7 wt %) and Tecophillic SP-60D-20 (22.5 ± 1.1 wt%)) were doped with 25 wt% DBHD/N₂O₂ and 10 wt% of PLGA with various hydrolysis rates. Films prepared with the polymer that has the higher water uptake (SP-60D-20) were found to have higher NO release and for a longer duration than the polyurethane with lower water uptake (SG-80A). The more hydrophilic polymer enhances the hydrolysis rate of the PLGA additive, thereby providing a more acidic environment that increases the rate of NO release from the NO donor. The optimal NO releasing and control SG-80A patches were then applied to scald burn wounds that were infected with *Acinetobacter baumannii*. The NO released from these patches applied to the wounds is shown to significantly reduce the *A. baumannii* infection after 24 h (~4 log reduction). The NO release patches are also able to reduce the TGF-β levels, in comparison to controls, which can enhance reepithelialization, decrease scarring, and reduce migration of bacteria. The combined DBHD/N₂O₂ and PLGA-doped polymer patches, which could be replaced periodically throughout the wound healing process, demonstrate the potential to reduce risk of bacterial infection and promote the overall wound healing process.

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Keywords

Antimicrobial; Burn Wounds; Diazeniumdiolates; Poly(lactic-co-glycolic acid); Nitric Oxide

1. Introduction

Bacterial infection and biofilm formation is a significant problem with a variety of biomedical devices that can lead to complications, increased medical costs, and increased morbidity [1]. Indwelling medical devices are responsible for more than one million hospital acquired infections, resulting in 99,000 deaths per year in the United States [2, 3]. Another significant area of infection is in wounds. More than one million burn injuries are reported annually in the US [4], resulting in 3,500 deaths per year [5]. Complications of wound infection are also significant and include delayed wound healing, tissue necrosis, spread of infection to the bloodstream and other organs, and transmission of wound-associated bacteria to other patients in hospitals [6]. Treatment of these infections often includes antibiotics and other antimicrobial agents, such as silver [7]. However, these materials often fail to prevent the infection and there is a growing concern for their use due to the emergence of bacterial resistance to antibiotics and antimicrobial agents [7-9]. Recent findings have also suggested that silver delays the wound-healing process and may have serious cytotoxic effects [10]. *Acinetobacter baumannii* is one such bacterial strain that has developed extensive antimicrobial resistance, and it also forms biofilms that are resistant to host defenses and antimicrobial treatment [11]. *A. baumannii* has been named a “new enemy” [12] due to large outbreaks in intensive care units [13-17] and also is a dominant organism isolated from wound infections (e.g., troops injured in Afghanistan and Iraq) [11, 18, 19].

Among its many biological roles, nitric oxide (NO) is known as a potent antimicrobial agent and an accelerant to the wound healing process [1]. Nitric oxide is endogenously synthesized by nitric oxide synthase enzymes (NOS): endothelial (eNOS), neuronal (nNOS), and inducible (iNOS). The iNOS is capable of producing high levels of NO [20] and micromolar concentrations of NO are known to have cytotoxic effects [21-23]. Reactive oxygen species (such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH)) and reactive nitrogen species (such as NO, N_2O_3 , and peroxyxynitrite ($OONO^-$)) are generated by the iNOS and phagocyte oxidase pathways and are responsible for the antimicrobial effects observed due to their interactions with thiols, proteins, DNA, and lipids [20]. The broad-spectrum antibacterial properties of NO against a wide range of microbes have been demonstrated, showing that both gram-positive and gram-negative bacteria can be killed [24]. In addition, bacteria have the ability to form biofilms (communities of bacteria encased in a self-synthesized extracellular matrix), which is one of the mechanisms that bacteria use to survive in adverse environments [25-28]. Indeed, formation of biofilms protects bacteria from antiseptics, antibiotics, and host defenses, making the infections difficult to eradicate [29]. Evidence suggests that biofilms also play a role in wound infections, which may explain the chronic nature of many wounds infections and their resistance to antimicrobial therapy [30]. Low nM concentrations of NO have been shown to be efficient at dispersing biofilms of various bacterial strains [31-34]. Lu et al. also reported

a 5 log reduction in *Pseudomonas aeruginosa* biofilms using diazeniumdiolate-functionalized chitosan oligosaccharides as the NO donor.[35] Therefore, NO releasing materials have great potential in biomedical applications, especially to reduce the risk of infection, promote wound healing, and improve biocompatibility of implantable medical devices [34, 36-38].

Due to the potential benefits of NO release, a wide variety of NO releasing polymers have been reported in the literature, and many of these are summarized in a recent review by Carpenter and Schoenfisch [39]. Materials with short durations of NO release may have potential wound healing applications due to the ease of replacing the material periodically throughout the wound healing process. The NO released from these materials may also decrease the risks of infected wounds, thereby reducing the wound healing time and repair chronic wounds [40]. Gaseous nitric oxide treatments and NO releasing materials have been used topically and shown to increase dermal blood flow, increase reepithelialization and angiogenesis, and accelerate wound repair; however, some of these studies have been conducted with uninfected wounds [41-45]. Previous studies have shown NO can be released from polymer films doped with diazeniumdiolate dibutylhexanediamine (DBHD/N₂O₂), which releases NO through proton or thermal driven mechanisms [46-49]. However, the loss of NO from DBHD/N₂O₂ creates free lipophilic amine species within the polymer that react with water, thereby increasing the pH within the polymer phase and effectively turning off the NO release. In a recent report, poly(lactic-co-glycolic) acid was used as an additive to promote and prolong the NO release from poly(vinyl chloride) films doped with DBHD/N₂O₂ [49, 50]. The ester linkages of the PLGA will hydrolyze in the presence of water, producing lactic and glycolic acids that can act as proton sources to promote the NO release from DBHD/N₂O₂-doped polymers. PLGAs can have varying hydrolysis rates, which is primarily determined by the copolymer ratio, the end group chemistry (either a free carboxylic acid or ester end group), and molecular weight. Lactate has been shown to enhance angiogenesis and accelerate wound healing [51], so any lactic acid monomers that leach from the NO releasing patches may also prove beneficial. Previous work with DBHD/N₂O₂-based films have primarily utilized hydrophobic polymers (e.g., PVC) as the base polymer [46, 49]. In this study, we compared the effects of the base polymer, in terms of their water uptake property, on the NO release from polymer films doped with DBHD/N₂O₂ and PLGA. The optimal formulation was then utilized to create NO releasing patches (and corresponding controls) that were applied to partial thickness scald burn wounds in a mouse model that were infected with *A. baumannii* to observe effects of such NO release patches on bacterial growth and TGF- β levels in the wounds after 24 h.

2. Materials and Methods

2.1. Materials

Tecoflex SG-80A and Tecophilic SP-60D-20 were purchased from Lubrizol Advanced Materials Inc. (Cleveland, OH). Anhydrous tetrahydrofuran (THF), anhydrous acetonitrile, sodium chloride, potassium chloride, sodium phosphate dibasic, and potassium phosphate monobasic were products of Sigma-Aldrich Chemical Company (St. Louis, MO). Poly(D,L-

lactide-co-glycolide) 5050DLG1A (1-2 week hydrolysis rate), 5050DLG7E (1-2 month hydrolysis rate), and 6535DLG7E (3-4 month hydrolysis rate) were obtained from SurModics Pharmaceuticals Inc. (Birmingham, AL). N,N'-Dibutyl-1,6-hexanediamine (DBHD) was purchased from Alfa Aesar (Ward Hill, MA). DBHD/N₂O₂ was synthesized by treating DBHD with 80 psi NO gas purchased from Cryogenic Gases (Detroit, MI) at room temperature for 48 h, as previously described [46]. Phosphate buffered saline (PBS), pH 7.4, containing 138 mM NaCl, 2.7 mM KCl, and 10 mM sodium phosphate was used for all *in vitro* experiments.

2.2. Preparation of NO releasing films and patches

The focus of this study was to compare the effects of polymer water uptake on the NO release properties from DBHD/N₂O₂ and PLGA-doped within these base polymers (SG-80A or SP-60D-20). A variety of films (dia. = 2.5 cm) were prepared for the initial NO release profile studies, and ultimately larger patches (5 cm × 6 cm) were prepared for application on the mouse wounds. The PLGA additives used were 5050DLG1A (1-2 week hydrolysis rate), 5050DLG7E (1-2 month hydrolysis rate), and 6535DLG7E (3-4 month hydrolysis rate). The product names identify the copolymer ratio, inherent viscosity (used to determine the molecular weight), and the end group type (acid or ester), which are the main factors that determine the hydrolysis rate of the PLGA. For example, the 5050DLG7E is a PLGA with 50 mol% DL-lactide, 50 mol% glycolide, an inherent viscosity of 0.7 dLg⁻¹, and has an ester end group ('E'). A variety of NO releasing films were prepared via a solvent evaporation method using either SG-80A or SP-60D-20 polyurethanes as the base polymer, while keeping the amount of DBHD/N₂O₂ and PLGA constant at 25 wt% and 10 wt%, respectively. The NO releasing films consisting of 25 wt% DBHD/N₂O₂, 10 wt% PLGA, and 65 wt% polyurethane were prepared by dissolving 80 mg PLGA, 200 mg DBHD/N₂O₂, and 520 mg polyurethane in 5 mL THF. This solution was cast in Teflon rings (dia. = 2.5 cm) and cured under ambient conditions for 2 d. Disks (dia. = 0.9 cm) were cut from the parent films and dip-coated 4 times in a top-coat solution (550 mg of the respective polyurethane in 7.5 mL THF). Four top coat layers of the polymer (without additives) were added to minimize the amounts of DBHD/N₂O₂ and PLGA diffusing to the surface during this process. The polymer top coats were employed for three main reasons: (1) to prevent leaching of DBHD/N₂O₂; (2) to neutralize the surface charge; and (3) to yield a smoother finish to the surface.

The patches for the *in vivo* studies were prepared in a similar manner and consisted of SG-80A doped with 25 wt% DBHD/N₂O₂ and 10 wt% 5050DLG1A. The active layer of the NO releasing patches were prepared by dissolving 1300 mg SG-80A, 200 mg 5050DLG1A, and 500 mg DBHD/N₂O₂ in 20 mL THF. This solution was cast in a Teflon mold (5 cm × 6 cm) and dried under ambient conditions overnight. The control patches were prepared in a similar manner with 5050DLG1A and DBHD amine (non-diazoniumdiolate) in the active layer. The control active layer consisted of 1410 mg SG-80A, 200 mg 5050DLG1A, and 390 mg DBHD amine dissolved in 20 mL THF and then cast in rings as described above for active patches. The patches were dip coated 4 times using the corresponding top-coating solution (750 mg SG-80A in 20 mL THF).

All films and patches were dried under ambient conditions overnight after the top-coating, followed by vacuum drying for 48 h. The final films and patches had a total thickness of ~1000 μm (~600 μm active layer and ~200 μm top-coat), as measured using a Mitutoyo digital micrometer (Metron Precision, Inc.).

2.3 Polymer water uptake

SG-80A and SP-60D-20 polymer films were prepared by the solvent casting method. Polymer solutions consisting of 200 mg polymer dissolved in 5 mL THF were cast in Teflon rings ($d = 2.5$ cm). Disks ($d = 0.9$ cm) were cut from the parent films, weighed, and immersed in PBS buffer for 48 h at 37°C. The wet films were wiped dry and weighed again. The water uptake of the polymer films are reported in weight percent as follows: water uptake (wt%) = $((W_{\text{wet}} - W_{\text{dry}})/W_{\text{dry}}) \times 100$, where W_{wet} and W_{dry} are the weights of the wet and dry films, respectively.

2.4. NO release measurements

Nitric oxide released from the NO release patches was measured using a Sievers chemiluminescence Nitric Oxide Analyzer (NOA) 280 (Boulder, CO). Films were placed in the sample vessel immersed in PBS (pH 7.4). Nitric oxide was continuously purged from the sample vessel and swept from the headspace using a N_2 sweep gas into the chemiluminescence detection chamber. Patches for the *in vivo* studies were wrapped in a moist Kim wipe and Tegaderm dressing (which was replaced daily to mimic the moist environment of the wound) and tested for *in vitro* NO release at 37°C.

2.5. *In vitro* zone inhibition test

Overnight LB (Luria Bertani) broth grown *A. baumannii* ATCC 17978 culture was washed with 1 \times PBS buffer three times by centrifugation, and was then resuspended in 1 \times PBS buffer to make a final cell concentration of approximately 10^5 CFU/mL. For the zone of inhibition, 50 μL of the 10^5 CFU/mL solution was plated onto LB agar plates. NO releasing and control patches (1 cm \times 1 cm square) were placed on the agar plates and incubated at 37°C for 24 h. The zone of inhibition was made by estimating the inhibition zone as circles and measuring the distance from the edge of the sample to the nearest bacterial colony. All the experiments were conducted in triplicate.

2.6. Partial thickness scald burn model in mice

Mouse burn model—The animal handling and surgical procedures used in this study were approved by the University Committee on the Use and Care of Animals (UCUCA) in accordance with university and federal regulations. A total of 9 female pathogen-free C57BL/6 mice (Harlan, Indianapolis, IN), 9-10 weeks old, weighing ~17-23 grams each were used in this study. Mice were housed in standard cages at the University's Unit for Laboratory Animal Medicine Facility and were allowed to acclimate for 7 d after delivery prior to the experiment. The animals were kept on a 12 h light cycle and were provided with rodent chow (LabDiet 5001, PMI Int'l., Richmond, IN) and water *ad libitum* throughout the study. Pentobarbital (Nembutal, Ovation Pharmaceuticals, Inc., Deerfield, IL, manufactured by Hospira, Lake Forest, IL) was administered intraperitoneally (50 mg/kg IP) for

anesthesia. The eyes of the animals were covered with sterile Altalube (Altaire Pharmaceuticals, Aquebogue, NY). During the study, all mice were singly housed and all received 0.1 mg/kg buprenorphine (Buprenex; Reckitt Benckiser Pharmaceuticals Inc., Richmond, VA) subcutaneously (SQ) twice daily for post-burn pain control.

The skin over the lumbrosacral and back region of the mice was clipped using a 35-W model 5-55E electrical clipper (Oyster-Golden A-S, Head no.80, blade size 50). To create the burn, anesthetized mice were placed in an insulated, custom-made mold that exposes only the lumbrosacral and back region that is approximately 30% of the total body area (calculated using Meeh's formula [52]). Partial thickness burns were achieved by exposure of the skin to 60 °C water for 18 s. The burn was then wiped with sterile gauze. The burn sites were immediately inoculated with *A. baumannii* bacteria (200 µL of 10⁶ CFU/mL) and covered with Tegaderm dressing (3M, Minneapolis, MN). Because the wounds were approximately 10 cm², 200 µL was an appropriate volume of bacteria solution to completely cover the wound site. The mice were returned to cages in a 37°C incubator until fully ambulatory. Each mouse was given a 1 mL injection of 5% Dextrose and Lactated Ringer's Injection (Baxter) IP and another 500 µL injection SQ on the back of its hind leg.

Application of NO release and control patches—The *A. baumannii* infection was allowed to grow in the wounds for a 24 h period prior to the application of the patches. The mice were divided into 3 groups receiving the following treatments: control (DBHD + PLGA patch), NO releasing patch, and control (no patch). NO release and control patches were soaked in sterile saline for approximately 15 min before attachment. Soaking the patches in saline solution helps initiate the PLGA hydrolysis and NO release reactions. The Tegaderm dressing was removed, patches were applied to the wounds, and fresh Tegaderm was used to cover and hold the patch in place on the wound. The Tegaderm was removed and replaced for mice that did not receive the patch treatment.

Tissue collection—At the time-point for tissue harvest (24 h after NO release and control patch application) the mice were given IP injections of pentobarbital (100 mg/kg) and exsanguinated followed by a bilateral pneumothorax. Skin samples were collected for bacterial counts, slides/staining, and mRNA isolation for TGF-β PCR. For the bacterial counts, skin tissue samples were excised and weighed, homogenized in 2 mL of PBS for 30 s, and cultured to determine the number of living *A. baumannii* microorganisms. Plate counting was conducted with LB agar plates.

The relative TGF-β mRNA levels were determined using TGF-β1 and GAPDH primers previously reported [53, 54]. The skin samples were immediately frozen in liquid nitrogen and stored overnight at -80°C. Samples were thawed briefly and homogenized in TRizol (Invitrogen, Carlsbad, CA) and RNA was extracted according to the manufacturer's instructions. RNA (2 µg) was utilized to make cDNA using the ABI High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Two hundred ng of cDNA was used to perform PCR with iQ SYBRGreen Supermix (Bio-Rad, Hercules, CA) on an Eppendorf Mastercycler eppgradient S realplex 4 thermocycler (Eppendorf North America, Hauppauge, NY). After an initial denaturation for 2 min at 95 °C, samples were

subjected to 50 cycles of 95 °C for 20 s, annealing at 60°C for 30 s, and extension at 72 °C for 20 s.

2.7. Statistical analysis

Data are expressed as mean \pm SEM (standard error of the mean). Comparison of results between the control and NO releasing patches were analyzed by a comparison of means using Student's t-test. Values of $p < 0.05$ were considered statistically significant for all tests.

3. Results and Discussion

3.1 *In vitro* NO release measurements from films and patches

Diazoniumdiolates are a group of widely studied NO donor molecules that release NO through proton or thermal driven mechanisms [47-49]. Previous studies have shown NO can be released from polymer films doped with DBHD/N₂O₂ [46, 49]. Poly(lactic-co-glycolic) acid additives have been shown recently help promote and prolong the NO release from poly(vinyl chloride) films doped with DBHD/N₂O₂ [49]. The ester linkages of the PLGA will hydrolyze in the presence of water, producing lactic and glycolic acids that can act as a proton source to sustain the NO release from DBHD/N₂O₂-doped polymers. Previous work with DBHD/N₂O₂ films have primarily utilized hydrophobic polymers (e.g., PVC) as the base polymer [46, 49]; however, in this study we compared the effects of the base polymer, in terms of their water uptake property, on the NO release from combined DBHD/N₂O₂ and PLGA-doped films. In order to measure the water uptake of the two base polymers used in this study, films of the polymers were cast without any additives. The water uptake was then determined by the weight difference of the polymer film before and after soaking in PBS at 37°C for 48 h. As shown in Table 1, the Tecoflex SG-80A polymer is more hydrophobic and has a significantly lower water uptake than the Tecophillic SP-60D-20.

The NO releasing films and patches used in this study were prepared using a two layer configuration, top-coat and active coat, as shown in Fig. 1. The active coat was doped with 25 wt% DBHD/N₂O₂ and 10 wt% PLGA (either 5050DLG1A, 5050DLG7E, or 6535DLG7E). The NO release from these films was measured using a chemiluminescence NO analyzer at 37°C while immersed in PBS buffer. The films doped with 5050DLG1A have a significant burst of NO during the first day of soaking (see Fig. 2). This high initial burst can be attributed to the higher residual acid content (from the carboxylic end groups and residual monomers). The NO release rapidly decreases by day 3 for the SG-80A films, due to the low water uptake of this polymer, which slows the PLGA hydrolysis rate. The films made with SP-60D-20 continue to release higher levels of NO for up to 16 d. SP-60D-20 has a higher water uptake, which continues to allow water to diffuse into the film and promote PLGA hydrolysis and NO release. In contrast, the films doped with the ester-capped PLGAs (either 5050DLG7E or 6535DLG7E) do not exhibit an initial burst during the first day (Figs. 3 and 4). The SP-60D-20 based films have higher NO release that has a longer duration than the corresponding SG-80A based films. This trend is most noticeable in the films doped with PLGAs that have lower hydrolysis rates (5050DLG1A

and 5050DLG7E). The higher water uptake of the SP-60D-20 polymer facilitates the PLGA hydrolysis, which then continues to promote NO release.

Although these NO releasing polymers have a wide variety of potential biomedical applications requiring various levels and duration of NO release [39], in this study we examined their potential use to reduce infection in burn wounds. For wound healing applications, the NO release from patches can have short durations because new patches could be applied daily. The polyurethanes used in this study are not typically used for wound healing applications, but we used these polyurethanes as an example of how to deliver NO to the wound site. The NO donor can be incorporated into other polymers as well for wound healing applications, but the polymers should not have too high a water uptake, otherwise there is risk of losing the free DBHD amine formed into the wound site. To choose the appropriate polymer composition for the *in vivo* studies, patches were prepared with Tecoflex SG-80A as the base polymer (due to the lower initial burst observed on the first day of soaking) and doped with 25 wt% DBHD/N₂O₂ and 10 wt% PLGA (either 5050DLG1A or 5050DLG7E). In order to mimic the moist environment of the wounds, NO releasing patches were wrapped with a moist Kim wipe and Tegaderm dressing, and tested at 37°C for their NO release. The patches doped with 5050DLG1A maintained a NO flux in the range of $12.8\text{--}14.5 \times 10^{-10} \text{ mol cm}^{-2} \text{ min}^{-1}$ for the first 24 h under physiological conditions (Fig. 5). In contrast, the patches doped with the 5050DLG7E exhibit a significantly lower flux of $\sim 3 \times 10^{-10} \text{ mol cm}^{-2} \text{ min}^{-1}$ under the same moist conditions and this flux is also sustained for the first 24 h. The NO release from both patches begins to diminish on the third day due to the accumulation DBHD amine and the concomitant slower hydrolysis of the PLGA additive (from the reduced amount of water that diffuses into the polymer). For the *in vivo* studies, the SG-80A patch type doped with 25 wt% DBHD/N₂O₂ and 10 wt% 5050DLG1A was selected due to its higher NO release.

3.2. Effects of NO and control patches on zone inhibition and bacteria count in mouse burn model

The NO releasing (SG-80A doped with 25 wt% DBHD/N₂O₂ and 10 wt% 5050DLG1A) and control (SG-80A doped with 20 wt% DBHD amine and 10 wt% 5050DLG1A) used for the *in vitro* zone inhibition test and *in vivo* studies were prepared as described in Section 2.2. The control patches were prepared with equal moles of the DBHD amine, where the additional weight from the mass of the diaziniumdiolate NONO group was compensated by additional SG-80A, in order to observe the effects of the NO release vs. control. Prior to the *in vivo* experiments, the NO releasing and control patches were tested *in vitro* for their zone of inhibition. *A. baumannii* was spread on agar plates and the patches (1 cm × 1 cm square) were placed in the center of the plate. After a 24 h incubation at 37 °C, the NO release patch created a zone of inhibition that had a diameter of ca. 4.3 ± 0.9 cm. The control patch showed no zone of inhibition, where only the bacteria underneath the patch were slightly suppressed. This zone of inhibition test mimics the nutrient environment of the wound and demonstrates that the NO released from patches has the potential to diffuse in and around the wound site, and ultimately reduce the bacteria and infection.

3.3 Effects of NO and control patches on bacteria counts and TGF- β mRNA in mouse burn model

A mouse burn model was used to observe the effects of the NO releasing patch on an infected wound. Partial thickness scald burn wounds on mice were inoculated with *A. baumannii* and covered with Tegaderm dressing. The bacteria were allowed to grow in the wound for 24 h prior to the application of NO release patches, control patches, or control (no patch). After 24 h of patch or control treatment, skin tissue was harvested, homogenized in PBS, and grown on agar plates to assess the effects of NO on bacterial growth in the wounds. As shown in Fig. 6, the NO releasing patches significantly reduced the amount of *A. baumannii* bacteria present in the wounds after 24 h application (~ 4 log reduction) in comparison to the control patches (which did not have NO release). The wounds that receive the control treatment of no patch (only Tegaderm dressing covering the wound) had similar bacteria counts as the other control patch group (data not shown). Part of the reduced bacteria counts can be attributed to neutrophil infiltration, which happens in the case of all treatment groups in this study. However, the additional NO that is supplied by the NO releasing patches has the potential to improve the overall healing of the wound by reducing the infection and bacterial growth.

Burn wounds have been shown to have increased TGF- β mRNA levels, which contributes to immunosuppression [55], impairs humoral immunity (antibody generation) [56], and contributes to scar formation [57]. In this study, the harvested skin tissue was also assessed for the expression of TGF- β mRNA levels using RT-PCR. The wounds with the NO releasing patches created a significant reduction in TGF- β levels in comparison to the control patches (Fig. 7). This reduction may be due to the inhibition of T cell proliferation by NO [58]. Reduction of TGF- β has been shown to enhance reepithelialization, decrease post-burn scarring, and reduce trans-epithelial migration of bacteria [59, 60]. In addition to the promising bacteria and TGF- β results, the UM pathology report indicated that the NO releasing patches did not worsen the injury and indicated that there was less overall damage in the wounds in comparison to the controls. Nitric oxide has many biological roles, including reducing bacterial infection and decreasing TGF- β mRNA levels as addressed here, and both processes can be quite beneficial to the wound healing process [39]. The nitric oxide releasing patches used in this study could be replaced daily, in order to maintain consistent NO delivery to the wound site.

4. Conclusions

In summary, this study demonstrates that the water uptake properties of the base polymer can be used to further control the NO release rates from polymeric films/patches doped with DBHD/N₂O₂ and PLGA. Films prepared with a more hydrophobic polyurethane (SG-80A) exhibit NO release that is lower and shorter in duration than the polyurethane with 20% water uptake (SP-60D-20). The more hydrophilic base polymer increases the rate of hydrolysis of the PLGA additive, supplying more protons locally within the polymeric phase that thereby increase the NO release rate. These new DBHD/N₂O₂ and PLGA-doped SG-80A patches demonstrate the potential to improve the healing of burn wounds by reducing the bacterial infection. Indeed, the NO released from the patches is clearly shown

to be able to significantly reduce the *A. baumannii* infection after 24 h application to scald burn wounds. The NO release patches are also shown to be able to reduce the TGF- β levels, in comparison to controls, and this species has been reported to enhance reepithelialization, decrease scarring, and reduce migration of bacteria. The novel NO releasing patches developed here could be replaced frequently throughout the duration of the wound healing process in order to further promote and expedite the healthy wound healing process by maintaining exposure of the wound to a higher flux rate of NO. This study demonstrated that NO releasing polymers can be used to kill antibiotic resistant bacteria (*A. baumannii*) in burn wounds. A more elaborate study comparing the overall healing process of these new NO releasing polymers to appropriate controls, including silver-based materials, is a future direction and currently under investigation.

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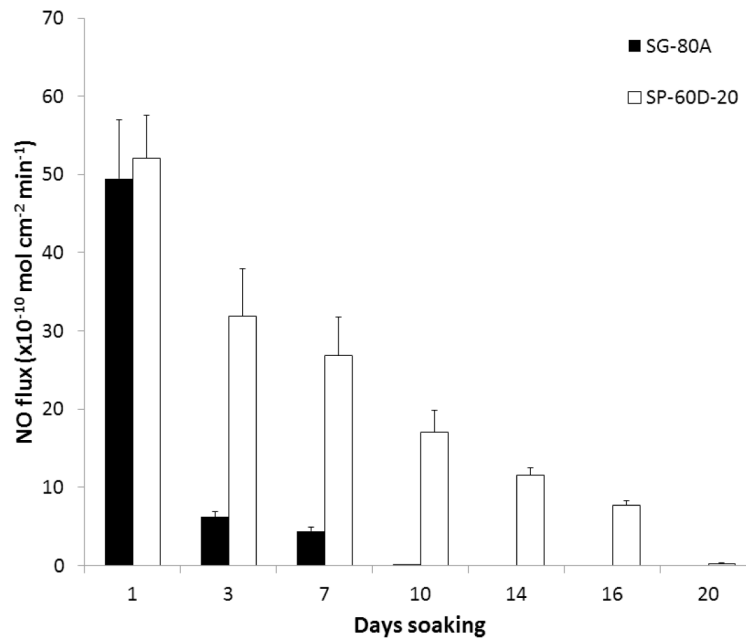


Fig. 2. NO surface flux from SG-80A and SP-60D-20 films doped with 25 wt% DBHD/N₂O₂ and 10 wt% 5050DLG1A (1-2 week hydrolysis rate) PLGA additives. Films were incubated in PBS buffer at 37°C during the testing period. The data are means ± SEM (n=3).

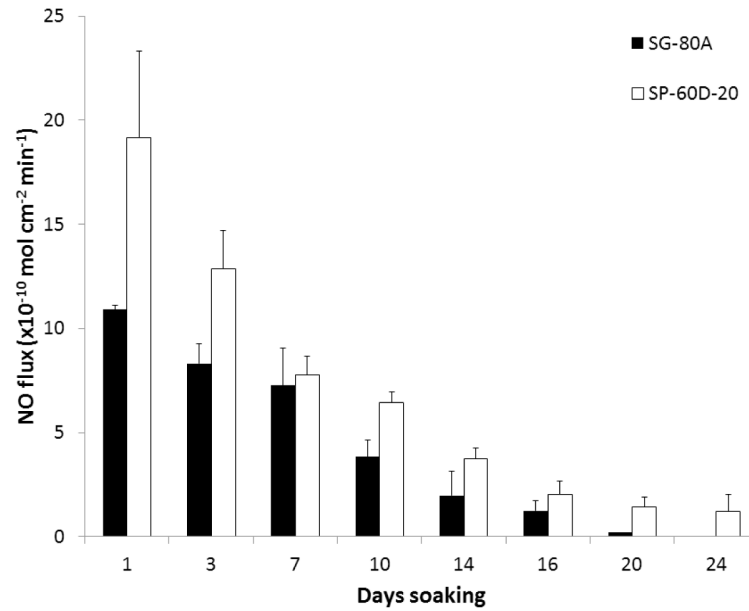


Fig. 3. NO surface flux from SG-80A and SP-60D-20 films doped with 25 wt% DBHD/N₂O₂ and 10 wt% 5050DLG7E (1-2 month hydrolysis rate) PLGA additives. Films were incubated in PBS buffer at 37°C during the testing period. The data are means ± SEM (n=3).

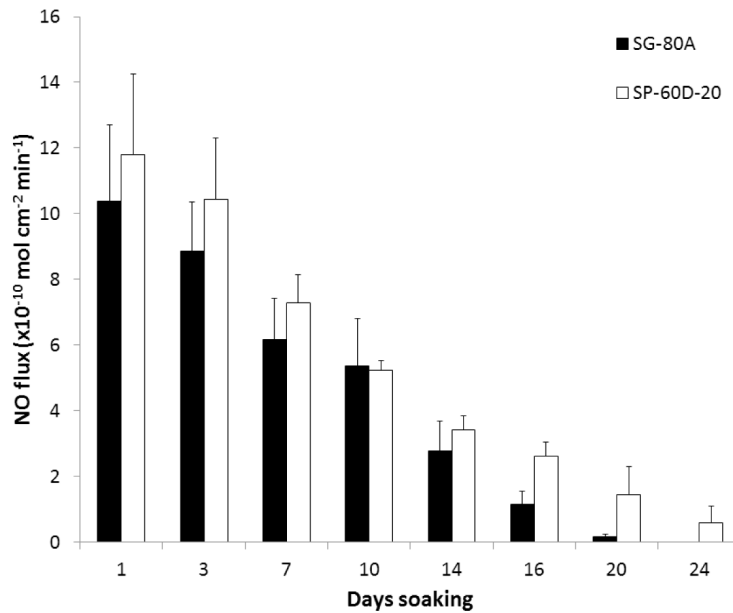


Fig. 4. NO surface flux from SG-80A and SP-60D-20 films doped with 25 wt% DBHD/N₂O₂ and 10 wt% 6535DLG7E (3-4 month hydrolysis rate) PLGA additives. Films were incubated in PBS buffer at 37°C during the testing period. The data are means ± SEM (n=3).

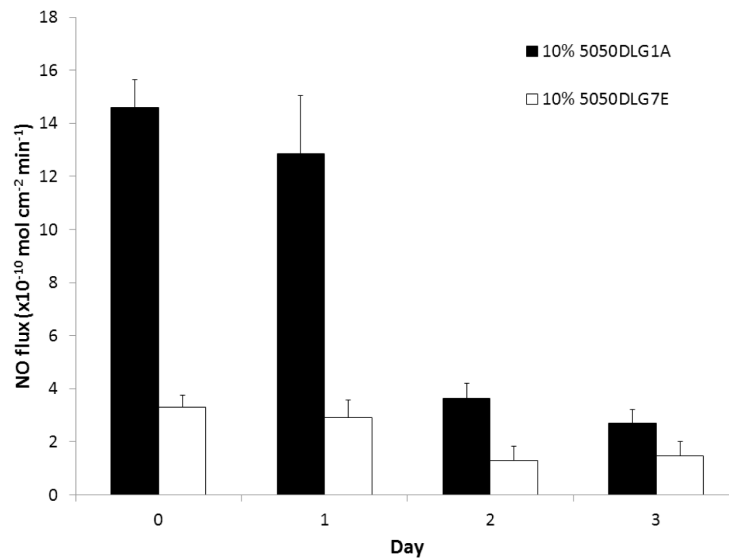


Fig. 5. NO release from SG-80A patched doped with 25 wt% DBHD/N₂O₂ and 10 wt% of either 5050DLG1A (1-2 week hydrolysis rate) or 5050DLG7E (1-2 month hydrolysis rate) PLGA additives. Films were wrapped in moist Kim wipes and Tegederm dressing at 37°C during the testing period. The data are means ± SEM (n=3).

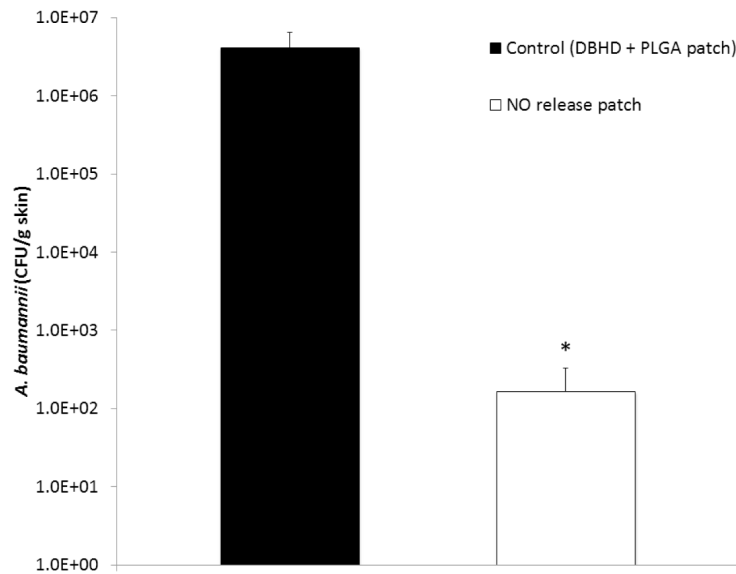


Fig. 6. Plate counting of *A. baumannii* cells on the wounded skin of mice after 24 h application of SG-80A based NO releasing and control patches. NO releasing and control patches were applied to wounds 24 h after inoculation with *A. baumannii*. After 24 h, skin tissue was harvested, homogenized, serially diluted, and grown on agar plates. The data are means \pm SEM (n=3). * = p <

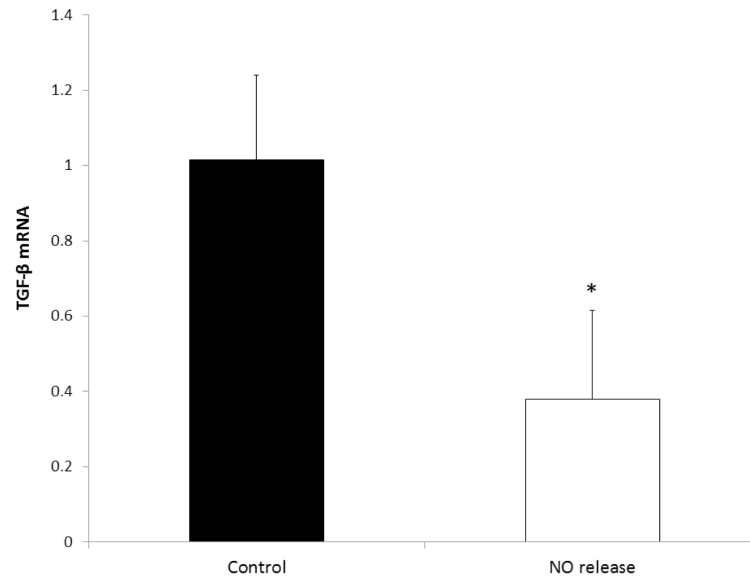


Fig. 7.

Expression of TGF- β mRNA after application of the SG-80A based NO releasing and control patches. RNA was extracted from the homogenized skin tissue and the expression of TGF- β was determined using RT-PCR and expressed as the ratio to that of untreated mice. The data are means \pm SEM (n=3). * = $p < 0.05$.

Table 1

The water uptake of SG-80A and SP-60D-20 polyurethanes. Polymer films were weighed prior to immersing in PBS for 48 h at 37°C. The wet films were wiped dry and weighed again. The water uptake of the polymer films are reported in weight percent as follows: water uptake (wt%) = $(W_{\text{wet}} - W_{\text{dry}})/W_{\text{dry}} \times 100$, where W_{wet} and W_{dry} are the weights of the wet and dry films, respectively.

Polymer	Water Uptake (wt%)
SG80A	6.2 ± 0.7
SP-60D-20	22.5 ± 1.1