A NOVEL MOIST WOUND HEALING DRESSING THAT PROVIDES SUSTAINED ANTIMICROBIAL ACTIVITY AND PROTEASE INHIBITION TO SPEED WOUND HEALING: WHS Poster #89

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Introduction

The wound dressing described was devised specifically to promote healing of vesicant injuries, and is supported by the US Army Medical Research and Materiel Command under contract W81XWH-06-C-0024. It was recognized that the healing of all injuries relies on fundamentally the same processes, which can be summed neatly as letting the body heal itself with minimum negative influence. The principles of optimal wound healing are summed up in the concepts of wound bed preparation. These concepts borrow from knowledge gained in the treatment of chronic wounds, and apply generally to other wound types including trauma, as well as thermal and vesicant injuries. The critical components of this dressing are the ability to absorb wound exudate while providing a moist wound healing environment, the ability to prevent and combat wound infection through antimicrobial capacity, and the controlled release of a matrix metalloprotease inhibitor (MMPI), to increase the rate of wound healing.

The base material is a rayon substrate processed into a superabsorbent polymer (SAP) by means of a patented NIMBUS™ procedure (yielding NIMBUS-SAP, as shown in **figure 1**). The material has a very high moisture absorption capacity and can provide a moist wound healing interface. Moist wound healing materials are popular with caregivers because they do not adhere to the newly healed tissue, so that dressing changes can be made with minimum pain and destruction of newly healed tissue. Typical commercial moist wound care materials are alginates or carboxymethyl cellulose (CMC), which dissolve in water (or saline, or wound fluid…) in a short time. NIMBUS-SAP maintains its form indefinitely, and has fluid absorption capacities of > 20 times its dry weight for highly ionic fluids (saline), to as much as 200 times for water. This permits the loading of various agents into the material, and subsequent controlled release. The controlled release of doxycycline (an antibiotic of the tetracycline family that is known for it protease inhibiting capability) was demonstrated for a period of over two weeks. The system is also capable of incorporating other agents, such as vitamins and growth factors to provide for improved healing performance.

Discussion

Wound Healing

The fundamental concepts of wound bed preparation include elimination of necrotic tissue and fibrinous exudate, controlling infection, establishing moisture balance, and optimizing the epidermal margin.[1] Control of infection is a critical parameter in this process, and can be achieved with the help of the wide variety of commercially available antimicrobial/antibacterial wound dressings. The diagram in **figure 2** illustrates the cascade of events that occur to impede wound healing. These events are recognized to be closely interrelated, as evidenced by the remarkable success of antimicrobial wound dressings in providing healing to non-responsive chronic wounds. A wound is usually considered to be clinically infected if it harbors a bacterial burden that exceeds 10^5 cfu/g of tissue. Inflammatory responses, however, can be elicited from bacterial burdens that are significantly below the threshold criteria for being clinically infected or 'critically colonized'. Studies have characterized the molecular and cellular environments of chronic skin wounds (shown as a cascade of events in Figure 1).[2]

Wounds of all kinds may contain increased levels of bacteria (that may or may not meet the standard for infection), which (in the sequence illustrated in **figure 2**) cause increased levels of pro-inflammatory cytokines and increased levels of proteases. These factors, in turn, degrade extracellular matrix (ECM) components, growth factors and receptors that are essential for healing.[3] These observations led to the hypothesis that correcting these molecular abnormalities would promote healing of chronic wounds. Studies from Trengove and colleagues[2] support this hypothesis by demonstrating that elevated levels of pro-inflammatory cytokines and proteases decreased in chronic venous stasis ulcers as healing progressed. Additionally, Ladwig and colleagues[4] reported that the elevated ratios of matrix metalloproteinase-9 (MMP-9) to tissue inhibitor of metalloprotease-1 (TIMP-1) in wound fluids from pressure ulcer patients correlated with poor healing. Collectively, these clinical studies suggested that treatments that reduced the levels of bacteria, inflammatory cytokines, and proteases should improve healing of chronic wounds.

The levels of bacteria in wounds can be addressed by use of antimicrobial dressings. These are helpful in part because even initial acute wounds with low levels of bacteria could progress to critically colonized or infected wounds because the bacteria growing in the wound fluid absorbed into common dressings (gauze, foams, alginates etc.) can be shed back into the wound. The bacteria growing in the "reservoir" within these simple dressings re-inoculate the wound and promote progression (**Figures 2 and 3**) to critically colonized levels of bacteria. The integration of protease inhibitors into the dressing is additionally helpful because common wound associated bacteria (*Pseudomonas aeruginosa*) have been implicated in the bacterial production of proteases that degrade healing tissues.

The depression of protease levels is also pursued by the use of such commercial products as Promogran™, which is a collagen matrix wound dressing that acts as a protease sink. The only commercial product that we are aware of that combines antimicrobial and protease inhibiting features is a silver based antimicrobial form of Promogran, marketed under the name Prisma™ at a retail price of ~ $17 for an approximately 4" dressing.

Benefits of Antimicrobial wound dressings:

- Promise to lower the bacterial challenge to the wound:
  - By providing a bacterial barrier to help prevent nosocomial infection.
  - By preventing the scenario of **figure 3**, where the dressing acts as an incubator for bacteria that can be shed back into the wound.
  - By leaching antimicrobial agents into the wound itself to directly control the bacterial population
- Can help prevent the spread of infectious organisms
  - By using a microbicidal dressing, organisms from one patient cannot exit the wound and be carried over to caregiver and/or other patients. This is particularly important for preventing the spread of resistant organisms like MRSA or VRE, against which patient may have little defense.

Controlled release of doxycycline from the matrix.

The dressing described forms a polyelectrolyte network of anionic base material (rayon), to which a cationic quaternary polymer has been graft copolymerized. Doxycycline is incorporated and retained by the polyelectrolyte network because the network has pockets of charge inhomogeneity (with a net positive balance due to the high charge density of the cationic polymer) that slow release of the doxycycline. The doxycycline becomes increasingly negatively charged as pH in the environment increases through the pK_a range (7.7 pH). Normal physiological pH is 7.4. Infection and attendant inflammation depress the pH of the wound environment, and will drive the doxycycline molecule to a more positive state, which will in turn speed release from the matrix of net positive character.

Figure 1 (below). NIMBUS-SAP material in dry state (left) and fully hydrated with DI water (right). Both pieces were 29 mg dry weight. The left (dry) piece measures 33 mm.

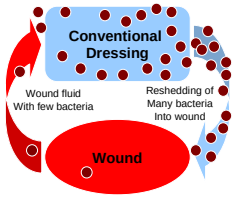


Figure 3. Reshedding of bacteria into wound. Wound fluid absorbed by a conventional (non–antimicrobial) dressing serves as nutrient to grow bacteria shed by the wound. The bacteria grown in the dressing can shed back into the wound to provide reinoculation.

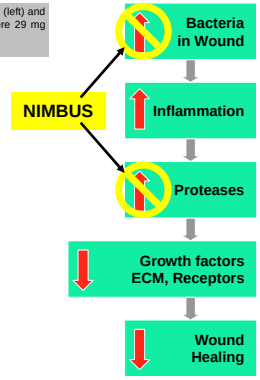
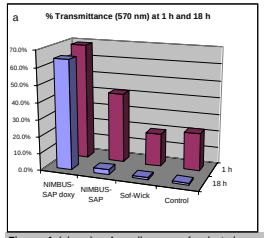


Figure 2. Cascade of events initiated by the colonization of a wound by bacteria. The colonizing bacteria induce inflammation in the wound that, through the sequence of events depicted, results in decreased rate of healing for the wound.

Protease Inhibition in the NIMBUS-SAP doxycycline releasing dressing.

The dressing material was compared to both NIMBUS-SAP without doxycycline, and to unprocessed rayon substrate (J&J SofWick®). The results in **Figure 4** show that the NIMBUS-SAP material has significantly enhanced protease inhibition over control material and no material, while the NIMBUS-SAP with doxycycline has greatly enhanced protease inhibition. Calibration data not shown demonstrates that the levels of protease activity seen with the NIMBUS-SAP doxycycline dressing correspond to better than a 90 % suppression of protease activity. The clinical efficacy of utilizing topical doxycycline to promote wound healing in chronic diabetic foot ulcers (wounds that were not clinically infected, but were not healing) is charted in the two panels of **figure 5**. In a pilot prospective, randomized, double blind clinical trial of the topical treatment of chronic diabetic foot ulcers with 1 % doxycycline in CMC gel, doxycycline treatment was found to stimulate healing better than the controls (p=0.05, Chi-squared test for healing).



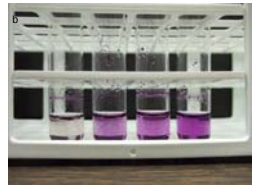


Figure 4 (above). Azocoll assay of selected samples. In the azocoll assay, a protease (10^{-5} g/ml concentration collagenase) solution is exposed to the sample for 30 min, then centrifuged off and assayed by adding 200 µl to 1800 µl to 0.5 % AzoColl solution. Dye is released from the matrix as the protease dissolves it – higher absorbance indicates lower protease inhibition activity by the sample. Figure 4a shows the absorbance readings after 1 h and after 18 h of incubation. Figure 4b shows the AzoColl solutions after 1 h incubation with the test collagenase solutions. Samples on both images are in the order depicted for 4a.

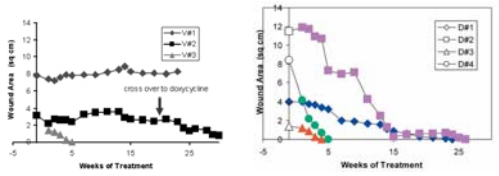


Figure 5. The results of a clinical trial (at the University of Florida) applying 1 % doxycycline gel topically onto chronic diabetic foot ulcers is shown in the two panels at left. The top panel shows three patients randomized to treatment with CMC gel. Of the two non-healing patients, one (V#2) crossed over to doxycycline after the 20 week masked treatment phase, and healed within 10 weeks. The panel below shows that all 4 patients randomized to the doxycycline treatment arm healed within 10 weeks of treatment.[5]

Microbial control in the NIMBUS-SAP doxycycline releasing dressing.

The NIMBUS-SAP dressing treated with doxycycline has redundant microbial control capacity, as well as providing protease inhibition.

Microbial control is provided by two means. The MMPI doxycycline is a potent antibiotic (it is recommended against anthrax), which is shown to be released in a controlled manner for long periods of time in **figure 6**. This image shows a NIMBUS-SAP doxycycline sample, as used in the protease inhibition experiments detailed in **figure 4a and 4b**. After 5 extraction cycles, the sample still demonstrated a zone of inhibition consistent with a released concentration of doxycycline of approximately 0.1 %. The intrinsic microbicidal property of the NIMBUS-SAP remains active as well, as is further detailed in **table 2**.

Tables 1 and 2 detail some of the pathogens against which NIMBUS treated substrates have been tested, using an AATCC method 100 test protocol, as well as time kill data, persistence of activity and activity against resistant organisms, the latter 3 directly on the NIMBUS-SAP material. The NIMBUS process renders the substrate microbicidal by binding an intrinsically microbicidal cationic quaternary ammonia polymer to that substrate. This is particularly useful for the containment of the much-dreaded resistant strains, known by such daunting terms as 'superbugs' through media. Because the polymeric quaternary microbicide acts on the cell wall of the bacteria instead of by interrupting metabolic pathways as antibiotics do, it does not provide the opportunity for resistance generation and transmission between bacteria. Even in the worst-case scenario of a doxycycline resistant bacteria populating the wound, the dressing matrix would continue to provide its barrier function, and prevent reinoculation of the wound from the dressing itself.

Bacteriocidal Efficacy Testing

Table 1 Bacteriocidal test results from NIMBUS treated materials, as per AATCC method 100-1999

Organism	% wound infection**	% killed	*ATCC#*
The most common wound-associated bacteria			
Staphylococcus aureus	20%	>99.9999%	12600, 6538
Staphylococcus epidermidis	14%	>99.9999%	12228
Enterococci spp	12%	>99.9999%	19433
Escherichia coli	8%	>99.9999%	15597, 8739
Pseudomonas aeruginosa	8%	>99.9999%	51447, 15442, 9027
Enterobacter spp	7%	>99.9999%	13048
Proteus spp	3%	>99.9999%	13115
Klebsiella pneumoniae	3%	>99.9999%	13833
Streptococci	3%	>99.9999%	10096
Candida albicans	3%	>99.9995%	
**CDC, 1996, common bacterial species associated with wound infections			
Additional organisms associated with:			
Corynebacterium xerosis	body odor	>99.9999%	7711
Corynebacterium diptheriae	body odor	>99.9999%	43145
Micrococcus luteus	body odor	>99.9999%	21102
Proteus vulgaris	body odor	>99.9999%	13115, 29905
Listeria monocytogenes	food contamination	>99.9999%	13932
Salmonella choleraesuis	food contamination	>99.9999%	10708
Serratia marcescens	burn wounds	>99.9999%	13880
Resistant organisms			
MRSA: methycilin resistant staphylococcus aureus		>99.9999% (full kill)	BAA-44
VRE: Vancomycin resistant enterococci		>99.9996% (full kill)	700221

Table 2. Repeated inoculation and time kill data of NIMBUS-SAP materials with *P. aeruginosa (PA)* per AATCC method 100-1999

2a Time Kill of NIMBUS-SAP w doxycycline on PA

Time	Average log reduction	% kill
1 min	3.88	99.987 %
10 min	5.78 (full kill)	99.9998 %
60 min	5.78 (full kill)	99.9998 %
120 min	5.78 (full kill)	99.9998 %

2b

	Doxycycline SAP	NIMBUS A SAP
Day 1	7.48*	5.65
Day 2	7.64*	7.64*
Day 3	7.90*	6.23
Day 4	8.82*	8.82*
Day 5	9.03*	6.93
Day 6	8.99*	8.99*
Day 7	9.01*	4.31

* Denotes full kill. Numbers variation due to individual controls used.

Figure 6 (right). Photograph of NIMBUS-SAP doxycycline sample that has been subjected to 5 extraction cycles. The first cycle added 500 µl buffer to the sample, incubated for 30 min, then centrifuged at 10,000 rpm for 4 min. Subsequent cycles added only 200 µl. The dry weight of material was 20 mg. All plates are lawn-spreads of *Pseudomonas aeruginosa*. The two top plates had 20 µl of 0.1 % and 0.01 % freshly prepared doxycycline solution pipetted onto the center, as indicated. The NIMBUS sample had just enough fluid added to permit slight leaching. The zone of inhibition around the sample demonstrates that the extracted doxycycline concentration is very close to 0.1 %.

Safety Testing Data

*Testing performed on NIMBUS™ treated cotton gauze, by Toxikon Laboratories, Bedford, MA

NIMBUS materials have passed all standard toxicology tests for prolonged use materials (1-30 days) in direct contact with breached or compromised skin. The FDA is currently reviewing a 510(k) submission on NIMBUS treated cotton materials.

Safety tests performed and passed, as per ISO 10993

- Agar diffusion test
- Intracutaneous Injection test
- Kligman Maximization test (sensitization test)
- Systemic Injection test

NIMBUS™ Materials and Methods

The NIMBUS™ family of processes has applications developed that are suitable for a wide variety of substrates, to render the material of choice antimicrobial. The NIMBUS™ process entails the permanent binding of a polymeric form of quaternary ammonium based antimicrobial onto a surface. The details of the binding are specific to the substrate and application. The cationic polymer enables the binding of a second species for release if this is desired.

For medical grade applications the quality of binding is assessed by performing an extraction assay – where the substrate is incubated in saline at 70° C for 24 h or at 50° C for 72 h, and the extract is tested for antimicrobial activity against *Staphylococcus aureus*. Zone of inhibition (ZOI) experiments have also been conducted to demonstrate that no leachable agents are responsible for microbicidal activity.

Microbicidal assays on dressing material samples were performed using the AATCC method 100-1999 testing protocol, modified to more closely approximate anticipated use conditions (including the use of 10 % serum as medium). Briefly, swatches of material were inoculated with appropriate titers of bacteria (typically 10^6 cfu/ml, incubated overnight, extracted and grown on nutrient plates to enable comparison to control samples.) This general protocol was followed for all testing involving swatches of substrate (such as woven cottons, dressing materials, etc.), with time points indicated where relevant, such as for re-inoculation testing, time to kill, or persistence of activity. While all results presented are not for identical substrates, the controls for each experiment were always untreated substrates of the same composition.

Azocoll assays were used to measure levels of MMP activity. The assay utilizes insoluble bovine hide that is covalently derivatized with a dye molecule. This assay has been used extensively to measure matrix metalloprotease (MMP) levels in various human wound fluids. When the collagen molecules are proteolytically cut by MMPs, small soluble fragments of collagen containing the dye are released and after centrifugation to pellet the remaining insoluble hide particles, the absorbance of the supernatant solution is measured with a 96-well plate reader, or an absorbance spectrometer.

References

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