Development and evaluation of a hydrogel technology for its potential as a wound dressing

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Technical Report
DRDC Toronto TR 2003-103
August 2003

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Abstract

This report describes the development and *in vivo* testing of a proprietary hydrogel material technology developed under research contract for use as a wound dressing. Application of the liposomal hydrogel for 24 h provided a barrier that effectively prevented contamination of full-thickness wounds and burns in rats. Furthermore, application of liposomal ciprofloxacin-loaded hydrogel (Ciprogel) was also very effective both in limiting the progression of the infection to deeper tissues and, in treating an established wound infection in both rat and pig models of full-thickness wounds. While these studies indicated that Ciprogel was an effective drug delivery system, other experiments suggested that entrapment of ciprofloxacin in liposomes was neither required when large amounts of ciprofloxacin were loaded in the hydrogel nor did it provide a sustained-release bactericidal effect. A number of challenging technical issues for the development of the liposomal antibiotic hydrogel technology as a wound dressing were also identified during the product evaluation. In summary, taking into consideration the results from the assessment of the bactericidal properties of the hydrogel technology as well as the technical issues related to its development, the successful application of the liposomal antibiotic hydrogel material as a wound dressing for front-line military casualties remains to be developed.

Résumé

Ce rapport décrit le développement et la mise à l'essai *in vivo* d'une technologie exclusive d'hydrogel mise au point dans le cadre d'un contrat de recherche pour l'utilisation de ce produit comme pansement. L'application de l'hydrogel liposomal pendant une période de 24 heures constituait une barrière qui prévenait efficacement la contamination des blessures profondes et des brûlures au troisième degré chez des rats. En outre, l'application d'hydrogel liposomal chargé de ciprofloxacine (Ciprogel) s'est également révélée très efficace tant pour ce qui est de limiter la progression de l'infection aux tissus sous-jacents que de traiter une infection établie dans les modèles murin et porcin de blessures entraînant la destruction du derme. Bien que ces études aient indiqué que le Ciprogel était un système efficace d'administration de médicament, d'autres expériences semblent montrer que l'encapsulation de la ciprofloxacine dans des liposomes n'est pas nécessaire lorsque des quantités importantes de ciprofloxacine sont chargées dans l'hydrogel et n'entraînent pas un effet bactéricide prolongé. Au cours de l'évaluation du produit, plusieurs difficultés techniques inhérentes au développement de l'hydrogel liposomal antibiotique devant être utilisé comme pansement ont été mises au jour. En résumé, étant donné les résultats de l'évaluation des propriétés bactéricides de l'hydrogel et les difficultés techniques liées au développement d'un tel produit, l'application efficace de l'hydrogel liposomal antibiotique comme pansement pour les blessures subies par les militaires en première ligne reste à développer.

Executive summary

The present technical report describes the development and *in vivo* testing at DRDC Toronto of a proprietary hydrogel material technology developed under research contract for use as a wound dressing. Prior to testing the prototype hydrogel material *in vivo*, procedures to cast and sterilize the hydrogel sheets were standardized; a packaging system ensuring the long-term hydration status of the hydrogel material was developed; a template to cast homogenous samples of dressing was designed; and, a restraining system for securing the hydrogel material when testing on rat full-thickness wounds was developed. The effect of the sterilization procedures on the integrity of the drug selected (i.e., ciprofloxacin) to achieve a proof-of concept of the antibacterial properties of the liposomal hydrogel wound dressing was also assessed.

Several studies were conducted to assess both *in vitro* and *in vivo* the bactericidal efficacy of liposomal ciprofloxacin-loaded hydrogel (Ciprogel). Application of the liposomal hydrogel material *per se* for 24 h provided a barrier that effectively prevented contamination of full-thickness wounds and burn wounds in rats, regardless of the presence of an antibiotic or not. Furthermore, application of Ciprogel was also very effective both in limiting the progression of the infection to deeper tissues and, in treating an established wound infection in both rat and pig models of full-thickness wounds. While these studies indicated that Ciprogel was an effective drug delivery system, another series of experiments suggested that entrapment of ciprofloxacin in liposomes was not required when large amounts of ciprofloxacin were loaded in the hydrogel. Furthermore, liposomal entrapment of large amounts of ciprofloxacin did not provide a sustained-release bactericidal effect in the porcine model of infected wounds. In fact, the lack of a long-term bactericidal effect of the liposomal hydrogel dressing, taken together with the large amounts of drug remaining in the dressings upon their removal from the infected full-thickness pig wounds, suggested that the liposomal ciprofloxacin remained entrapped in the hydrogel material and was not available to exert a significant bactericidal effect. Considering the present experimental results, and the fact that incorporation of liposomes into a product will introduce additional manufacturing costs, there did not appear to be any commercial or marketing edge to the liposomal hydrogel technology.

A number of technical issues impeding the development of the liposomal antibiotic hydrogel technology as a wound dressing were also identified during the testing and evaluation performed at DRDC. More specifically, challenges in preparing homogenous Ciprogel products and in maintaining an acceptable shelf-life of the liposomal hydrogel under non-refrigeration conditions would need to be addressed and likely result in potentially high costs in packaging the hydrogel products.

In summary, taking into consideration the results from the assessment of the bactericidal properties of the hydrogel technology as well as the technical issues related to its development, further development of the liposomal antibiotic hydrogel material as a wound dressing for front-line military casualties did not appear promising.

Martineau, L., Shek, P.N., 2003. Development and evaluation of a hydrogel technology for its potential as a wound dressing. DRDC Toronto TR 2003-103.

Sommaire

Le présent rapport technique décrit le développement et la mise à l'essai *in vivo* à RDDC Toronto de la technologie exclusive de fabrication d'un hydrogel développé dans le cadre d'un contrat de recherche en vue de son utilisation comme pansement. Avant la mise à l'essai du prototype d'hydrogel *in vivo*, nous avons standardisé les méthodes de moulage et de stérilisation des feuilles d'hydrogel, mis au point un système d'emballage permettant d'assurer l'hydratation à long terme de l'hydrogel, conçu un gabarit pour mouler des échantillons homogènes de pansements et mis au point un système d'immobilisation pour fixer le pansement d'hydrogel lors des essais sur des plaies profondes chez le rat. Nous avons également évalué l'effet des techniques de stérilisation sur l'intégrité du médicament choisi (c.-à-d. la ciprofloxacine) pour valider le principe des propriétés bactéricides du pansement à base d'hydrogel liposomal.

Nous avons réalisé plusieurs études pour évaluer l'efficacité bactéricide tant *in vitro* que *in vivo* d'un hydrogel liposomal chargé de ciprofloxacine (Ciprogel). L'application du produit à base d'hydrogel liposomal seul pendant une période de 24 heures constituait une barrière qui prévenait efficacement la contamination des plaies profondes et des brûlures au troisième degré chez les rats, indépendamment de la présence d'un antibiotique ou non. En outre, l'application de Ciprogel s'est également révélée très efficace tant pour ce qui est de limiter la propagation de l'infection aux tissus plus profonds que de traiter une infection établie d'une plaie dans des modèles porcin et murin de blessures profondes. Bien que ces études aient révélé que le Ciprogel est un système d'administration de médicament efficace, une autre série d'expériences a indiqué que l'encapsulation de la ciprofloxacine dans des liposomes n'est pas nécessaire lorsque de grandes quantités de ciprofloxacine sont chargées dans l'hydrogel. De plus, l'encapsulation liposomale de fortes quantités de ciprofloxacine n'entraînait pas un effet bactéricide prolongé dans le modèle porcin de plaies infectées. Dans les faits, l'absence d'un effet bactéricide à long terme du pansement à base d'hydrogel liposomal ainsi que les grandes quantités de médicament demeurant dans les pansements lors de leur retrait des plaies infectées chez le porc donnaient à entendre que la ciprofloxacine liposomale demeure encapsulée dans l'hydrogel et n'est donc pas disponible pour exercer un effet bactéricide important. À la lumière de ces résultats expérimentaux et vu que l'incorporation de liposome dans un produit entraînera des coûts de fabrication additionnels, on peut conclure qu'il ne semble y avoir aucun avantage à l'hydrogel liposomal que ce soit sur le plan commercial ou de la mise en marché.

Plusieurs difficultés techniques empêchant l'utilisation de l'hydrogel liposomal antibiotique comme pansement ont également été mises en lumière durant la mise à l'essai et l'évaluation réalisées à RDDC. Plus précisément, il faudrait surmonter plusieurs obstacles liés à la préparation de produits homogènes à base de Ciprogel et au maintien d'une durée de vie acceptable de l'hydrogel liposomal en l'absence de réfrigération, et ces problèmes entraîneraient potentiellement des coûts d'emballage élevés des produits à base d'hydrogel.

En résumé, à la lumière des résultats de l'évaluation des propriétés bactéricides de l'hydrogel et des difficultés techniques entourant son développement, la poursuite des travaux de développement d'un produit à base d'hydrogel liposomal antibiotique comme pansement pour les blessures subies par les militaires en première ligne ne semble pas prometteuse.

Martineau, L., and Shek, P.N., 2003. Development and evaluation of a hydrogel technology for its potential as a wound dressing. DRDC Toronto TR 2003-103. Defence R&D Canada – Toronto.

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Acknowledgements

The authors are indebted to Mr. D. Saunders, Miss T. Fitzgibbons, and Mr. L. Chua-Chiaco for expert technical assistance.

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1. Introduction

Wound injuries are a predictable and potentially life-threatening outcome of front-line military operations. Besides the severity of the wounds sustained, and considering the fact that the wounds become readily contaminated, management of military wounds poses challenges that are different from treating comparable civilian wounds. Delayed evacuation of casualties is not uncommon, thus imposing a greater reliance on point-of-wounding care. Therefore, wound care may need to be self-administered or facilitated by untrained personnel in extremely difficult conditions. A military wound dressing must therefore be simple, compact, lightweight, and easy-to-use.

Hydrogels are important advanced second-generation wound care products. In spite of their high water content (up to 96%), hydrogels can further absorb a slight to moderate amount of wound exudate by swelling. They are particularly useful as dressings for many partial-thickness skin defects (e.g., shallow abrasions, superficial wounds), blisters, second-degree burns, and healthy, granulating tissues. However, a distinctive disadvantage of the commercially available hydrogel wound dressings is that they do not provide a barrier against wound infection. It is often recommended clinically that an antimicrobial agent be applied under the hydrogel dressing or blended with the amorphous hydrogel. While this method ensures some control of bacterial growth, it is not always practical as it introduces another step in the wound care management. Furthermore, the therapeutic drugs are not immobilized in the hydrogel material. In the presence of wound exudate, the drug is thus rapidly released at the injured site, so that a long-lasting local therapeutic effect cannot be achieved.

In recent years, liposomes have been increasingly explored as novel drug delivery systems that alleviate this problem. Liposomal drug products provide sustained local concentrations at the site of infection or injury, and are usually deemed more effective and less toxic than conventional drug formulations. Several laboratories have advocated the topical use of liposome-encapsulated drugs for the treatment of wounds [1-10]. Furthermore, Reimer et al. [11] described the use of a topical liposomal Povidone Iodine hydrogel combined with a moisturizer for antiseptic treatment of the wounds. To our knowledge, hydrogel wound dressing sheets that effectively deliver, in a uniform and controlled manner, therapeutic agents entrapped in liposomes have never been described.

Under research contract W7711-6-7360/001, Dr. F. Dicosmo (Dept. of Botany, University of Toronto, Toronto, ON) developed a proprietary method for co-valently attaching liposome-containing hydrogel to a substance such as a catheter (US. Pat. No. 6,475,516). The method exploits the surface area of the device as well as the volume occupied by the hydrogel matrix bonded to the surface. The volume of gel matrix can accommodate large quantities of drug-loaded liposomes and consequently, relatively high doses of a therapeutic drug can be potentially released at specific sites. The hydrogel matrix is biocompatible and biodegradable (i.e. not releasing potentially toxic degradation products). The containment of the liposomes within the gel matrix also creates an opportunity to control drug diffusion rates, thereby affording long-term drug

efflux. The present technical report describes the development and testing of the hydrogel material technology for use as a wound dressing. All experimental results presented in this report were obtained in the laboratories of DRDC Toronto.

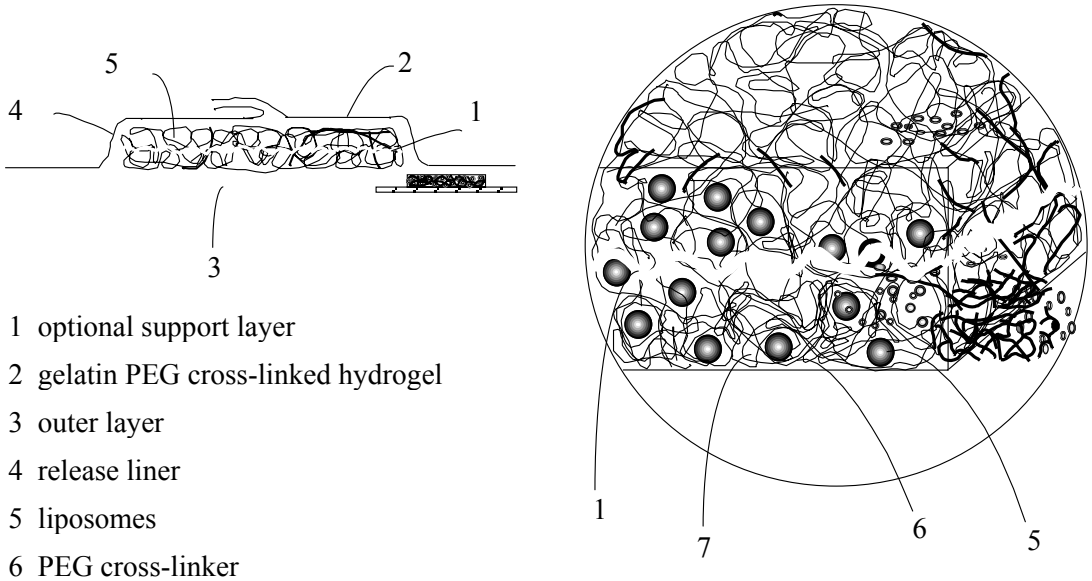
2. Preliminary assessment of a prototype hydrogel material for use as a dressing for non-contaminated full-thickness wounds

2.1 Development of a restraining system for securing the hydrogel material *in vivo*

The hydrogel material was provided as a large sheet (10 cm x 15 cm x 1 mm) wrapped in Saran Wrap®. Prior to testing the hydrogel material on experimental wounds, a system was developed to secure hydrogel material on the dorsum of the animal for at least 7 days. Briefly, three male, Sprague-Dawley rats (325 – 350 g; Charles River, St-Constant, QC) were used for the design of a restraining system that would ensure long-term stabilization of the hydrogel sheet on the wound, as well as maintaining a close contact between the material and the wound surface. All animals were anaesthetized; the dorsum was shaved; and, a piece of hydrogel material (1.5 cm x 1.5 cm x 1 mm) cut out under aseptic conditions (i.e., using sterile instruments in a laminar flow hood) was positioned on the skin. The outer perimeter of the hydrogel sheet was ink-marked, to assess whether the material had shifted from its original location. A folded piece of gauze (2.0 cm x 2.0 cm) was then placed on the hydrogel material, and each animal was fitted with a 5-cm piece of tubular elastic dressing retainer (Surgilast® size 1, Glenwood Inc., Tenafly, NJ) and a rat tether jacket (Ealing Scientific, St-Laurent, QC). The position of the hydrogel sheet on the marked area was checked 24 h later.

While the restraining system was intact after 24 h, most of the hydrogel sheets recovered were dehydrated to various extents (i.e., 10% to 80% dehydrated) as well as in random patterns (i.e., anterior or posterior periphery, overall, etc.). The same animals were therefore fitted with a new piece of hydrogel material, and a transparent, sterile, semi-permeable adhesive dressing (4 cm x 4 cm; Tegaderm®, 3M, St-Paul, MN) was applied over the folded gauze, to prevent dehydration of the hydrogel while allowing gas exchange. This modification to the securing system did not prove adequate, since the integrity of the hydrogel sheet was maintained for less than 48 h.

After experimenting with several types of polymeric membranes (e.g., silicone, polyvinylchloride (PVC), Teflon®, or polyethylene; n=20 rats), it was determined that a PVC (Elastoplast®, Smith & Nephew, Lachine, QC), non-breathable adhesive backing fulfilled the experimental requirements (i.e., maintenance of hydration of hydrogel material for up to 7 d as well as good contact between the hydrogel material and the surface to which it was applied). A piece of 1-ply gauze was inserted between the PVC tape and the hydrogel material to preserve its integrity and enable its recovery after completion of the experiment. Figure 1 illustrates the concept for a wound dressing based on the hydrogel technology.



1 optional support layer
2 gelatin PEG cross-linked hydrogel
3 outer layer
4 release liner
5 liposomes
6 PEG cross-linker
7 gelatin

Figure 1. *Novel concept for a liposomal drug-loaded hydrogel wound dressing.*

2.2 Rat model of full-thickness wounds

Rats were anaesthetized (2.0% halothane: N_2O: O_2); the dorsum was shaved and sterilized using standard procedures (i.e., alcohol and 10% Povidone Iodine); a custom-made Plexiglas® template with a 1 cm x 1 cm window was positioned over the shaved area; the window was centered between the shoulder blades of the animals; and, the location of the wound was marked by spraying a yellow aerosol powder (Topazone®, Vetoquinol Canada, Joliette, QC) through the window. Povidone Iodine (10% USP) was then applied liberally on the area surrounding the site of incision, to prevent contamination from the normal skin flora, and washed off thoroughly with sterile Phosphate Buffered Saline (PBS) 5 min later. A 1-cm^2 piece of skin was then removed from the back of each animal, exposing the underlying *panniculus carnosus* muscle. All animals received analgesics (buprenorphine, 0.05 mg/kg body weight; s.c.) immediately prior to the surgical procedures, and again 8 h later. Rats were allowed to recover under an infra-red lamp before being housed individually in cages. Usually, no mortality nor untoward bacterial contamination was observed after completing these surgical procedures.

2.3 *In vivo* testing of a prototype hydrogel material

Dr. DiCosmo supplied the hydrogel material was supplied as a large sheet (10 cm x 15 cm x 1 mm) wrapped in Saran Wrap®. Small pieces (1.5 cm x 1.5 cm x 1 mm) were cut out under aseptic conditions. Care was taken to ensure the homogeneity of the hydrogel pieces, discarding any sample showing imperfections or irregularities on its surface. Dr. DiCosmo advised to keep all selected pieces in sterile saline (n=10 per jar) until their use.

Full-thickness wounds were created in five male, Sprague-Dawley rats (325 – 350 g; Charles River, St-Constant, QC), and the hydrogel piece was then secured as previously described (Section 2.1). All animals received analgesics (buprenorphine, 0.05 mg/kg body weight; s.c.) immediately prior to the surgical procedures, and again 8 h later, and were allowed to recover under an infra-red lamp before being housed individually in cages. Each wound dressing was changed 8 h later, and then twice a day (i.e., 08H00 and 16H00) for 2 days (i.e., total of 6 dressings per rat). Each wound was swabbed at each change of hydrogel sheet, to assess for any untoward bacterial contamination. All animals were sacrificed 3 days after inflicting the wound. The *panniculus carnosus* was then removed, and the underlying right *acromiotrapezius* muscle was excised, along with the liver and spleen. These tissues were then weighed; homogenized in sterile PBS; plated onto tryptic soy agar enriched with 5% sheep blood; and, incubated overnight (37°C) to assess the extent of bacterial sequestration. These procedures are referred to as standard microbiological procedures.

Unexpectedly, all animals showed marked bacterial sequestration (mainly *Staphylococcus (Staph.)* sp.) in all tissues and organs sampled. Microbiological assessment of 6 random samples of hydrogel material (taken from an unopened hydrogel material package) revealed marked contamination in all but one of the hydrogel samples tested. Whether or not contamination of the hydrogel sheets occurred during packaging of the hydrogel material or at earlier steps during its preparation could not be determined in the present study.

2.4 Recommendations for further development of the hydrogel material prior to its *in vivo* use

Based on our observations, several recommendations were made to Dr. DiCosmo with regard to the further development of the hydrogel material as a potential wound dressing:

2.4.1 Sterility of hydrogel material

Sterile chemicals should be used when preparing the different hydrogel formulations to be tested. When casting the hydrogel sheets, the liquid hydrogel formulation should be poured under aseptic conditions, preferably under a laminar flow hood. To further ensure maintenance of the sterility of the hydrogel material, the sheets should be pre-cut (1.5 cm x 1.5 cm x 1 mm

pieces) and each piece sterilized using ultraviolet (UV) irradiation. Though not essential for testing of samples at DRDC Toronto, the feasibility of using gamma-irradiation to sterilize the hydrogel material should be considered. Assessment of the sterility of each batch of hydrogel material should be performed at DRDC Toronto prior to the application of the samples on full-thickness wounds, using standard microbiological methods.

2.4.2 Packaging of hydrogel sheets

DRDC Toronto was advised to keep the samples provided in sterile PBS to ensure the maintenance of the hydration status of the hydrogel material. Furthermore, the samples were provided in a rudimentary packaging system (i.e., Saran Wrap®). For obvious reasons, such conditions are not practical in a clinical setting. Therefore, DRDC Toronto recommended the development of a packaging system that would ensure a long-term hydration status of the hydrogel material.

2.4.3 Hydrogel material formulation

The preliminary study has shown that an occlusive backing is essential to prevent dehydration of the hydrogel material. However, it is well established that wound healing is optimized when the dressing allows water and gas exchange [12]. Therefore, DRDC Toronto recommended considering the reformulation of the hydrogel polymer to allow a greater stability of the material, and to minimize its dehydration.

2.4.4 Hydrogel sheet casting procedures

A problem encountered in the preliminary study was the heterogeneity of the hydrogel sheets that were supplied for testing (i.e., presence of air bubbles in hydrogel sheets). DRDC Toronto recommended minimizing this problem by pouring smaller sheets into a ‘mold’, preferably of the required dimensions for the dressings to cover the rat wounds (i.e., 1.5 cm x 1.5 cm x 0.3-0.5 mm).

3. Development of a packaging system for preserving the hydrogel material

The objective of the present series of experiments was to design an experimental packaging system that would preserve the hydration status of the hydrogel material supplied by Dr. DiCosmo for at least 40 days.

All samples tested consisted of 1.5 cm discs prepared by Dr. DiCosmo using a DRDC Toronto-designed template. Briefly, the latter consisted of a 10 cm x 20 cm x 1 cm stainless steel plate in which 60 circular wells (2.0 cm diam.; 1.25 mm deep) had been drilled. The hydrogel formulation was prepared under aseptic conditions, and the liquid gel poured in the pre-autoclaved template within 5 min. Upon gellification, all discs were removed from the template using sterile forceps, UV-irradiated 15 min per side, and stored in sterile PBS (6 discs per vial) until testing was performed.

3.1 Evaluation of different commercial bags

Three different types of bags (10 cm x 15 cm) were tested at DRDC Toronto as potential components of a packaging system. More specifically, they consisted of a polyester bag (Scotchpak®, VWR International, Mississauga, ON); a low-density polyethylene zipper bag (VWR International, Mississauga, ON); and, an aluminum foil pouch (Kapak®, International, Mississauga, ON).

The following procedures were followed for each experiment. The hydrogel disc was weighed, placed in one of the experimental bags, and the weight of the package (i.e., bag and disc) was recorded. The bags were then closed (e.g., polyethylene) or heat-sealed (e.g., Kapak® and polypropylene). The weights of 5 packages per category were recorded at pre-determined time intervals, the bags were then opened, and the hydrogel disc was weighed. These experiments were carried out while applying vacuum or not prior to sealing the packages.

Figure 2 depicts the cumulative changes in weight of the different packages as well as those of the hydrogel discs over 19 days without application of vacuum prior to sealing the bag. While the weights of the packages using either aluminum pouches or polypropylene bags were maintained for the duration of the experiment, those of the polyethylene bags containing the hydrogel discs decreased by approximately 12% over the same period. However, a close examination of the hydrogel discs revealed a marked decrease in their weight after 19 days, ranging from 22.1 ± 0.16% to 58 ± 6% for all bags tested (Figure 2). Application of vacuum prior to sealing the packages seemed to reduce the differences in the extent of dehydration of the hydrogel discs observed between the different experimental groups (Figure 3). However, a 22% reduction in the hydrogel discs was observed after 19 days for all groups. The weight of the hydrogel discs further decreased to 34% of the initial value when they were packaged into an aluminum pouch for 27 days. These data suggest that the level of moisture in the packages containing the hydrogel discs needs to be maintained

artificially close to 100% to prevent dehydration of the gels. Considering the overall better performance of the aluminum pouches in preventing dehydration of the hydrogel discs, together with the fact that the hydrogel discs may be used as a drug delivery system for therapeutic agents that are light-sensitive, DRDC Toronto recommended the use of the opaque aluminum pouches for subsequent experiments.

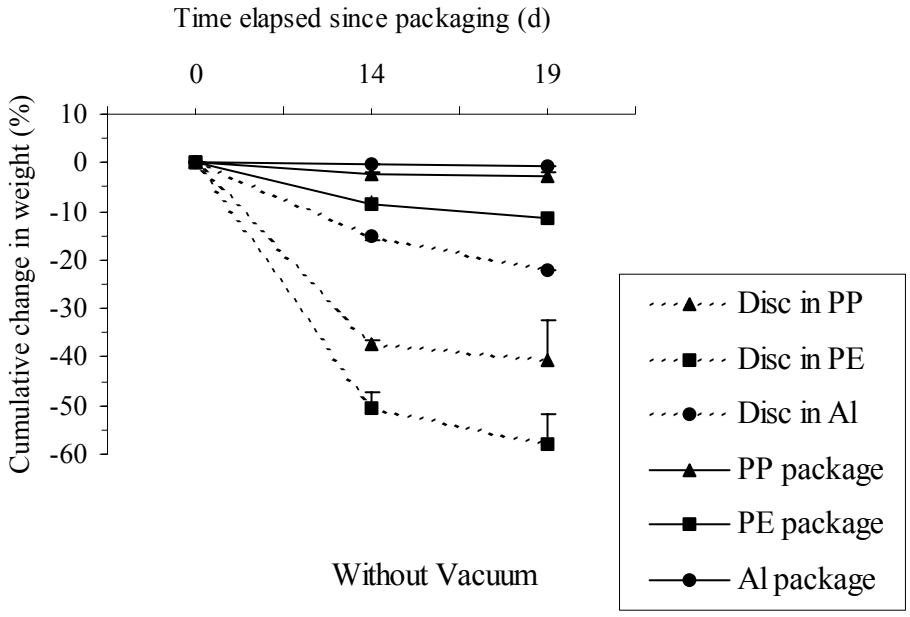


Figure 2. *Cumulative changes in the weights of the hydrogel discs and different packaging systems without application of vacuum prior to closure of the bag. The latter was made of polyethylene (PE), polypropylene (PP) or aluminum (Al). Data are expressed as means ± SEM (n=5 per experimental group).*

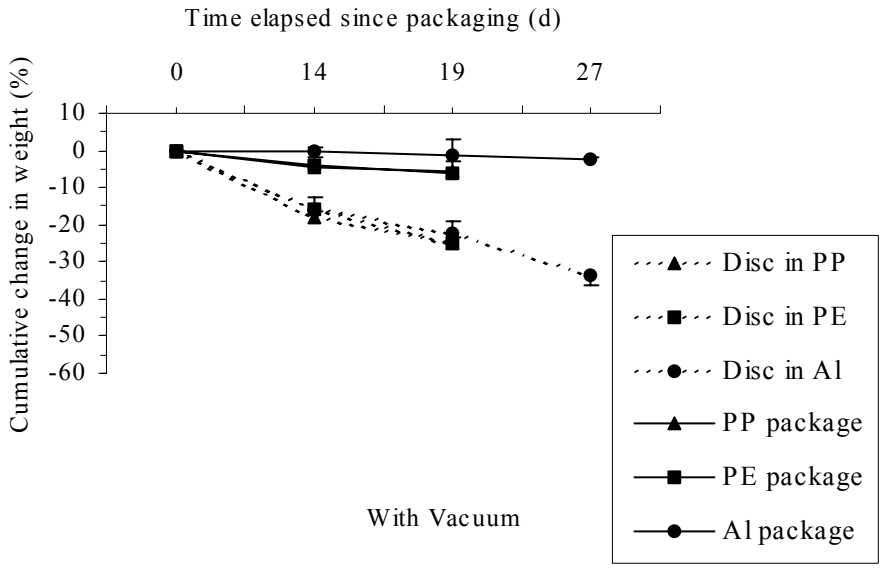


Figure 3. *Cumulative changes in the weights of the hydrogel discs and different packaging systems. Vacuum was applied prior to closure of the bag. The latter was made of polyethylene (PE), polypropylene (PP) or aluminum (Al). Data are expressed as means ± SEM (n=5 per experimental group).*

3.2 Design of a humidifier for incorporation in the packaging system

A humidifier system was designed to nearly saturate the air in the package to prevent dehydration of the hydrogel material. Briefly, one polystyrene weigh boat (4 cm x 4 cm x 0.8 cm; VWR International, Mississauga, ON) was perforated (2 mm holes) in a grid pattern under aseptic conditions using a punch. A 2.5 cm x 2.5 cm polyurethane foam (Allevyn®; Smith & Nephew, Lachine, QC) was cut into small pieces that were then placed into a non-perforated weigh boat. The perforated weigh boat was then inverted over it, and the 2 weigh boats were taped together (PVC Elastoplast®; Smith & Nephew, Lachine, QC). A small volume of sterile PBS was then injected through one of the perforations, and the 'humidifier' was then gently shaken to allow a complete absorption of the PBS by the sponges. The 'humidifier' was then inserted into the packaging system described in section 3.1.

Figure 4 depicts the cumulative changes in the weight of the aluminum foil packages and the hydrogel discs over 7 days when adding either 2 mL or 4 mL of sterile PBS to the humidifier. The data show that the moisture level in the package was better maintained when the humidifier contained 4 mL of sterile PBS.

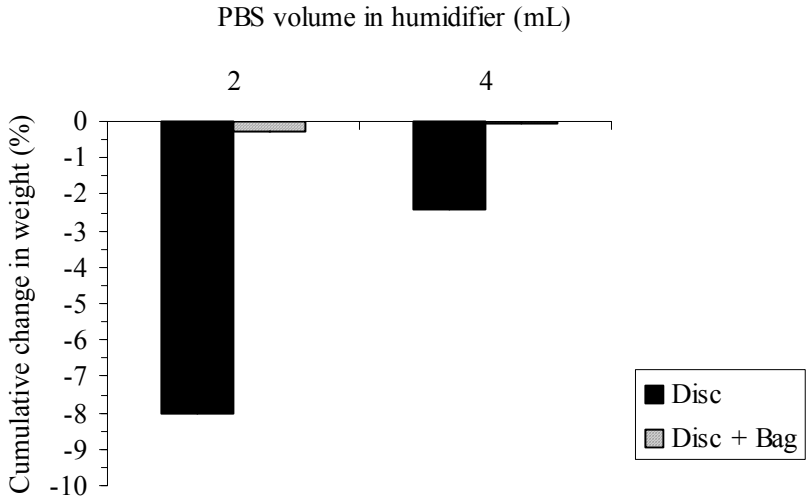


Figure 4. *Cumulative changes over 7 days in the weights of the hydrogel discs and the aluminum foil packages containing a custom-designed humidifier injected with either 2 mL or 4 mL of PBS. Data are expressed as means ± SEM (n=3 per experimental group).*

While the hydrogel discs lost less than 3% of their weight over 7 days (Figure 4), one disc was 10 % dehydrated after 14 days (personal observation). Another packaging system was therefore designed to further minimize the dehydration of the hydrogel discs over longer periods of time. More specifically, the hydrogel discs were packaged in a sterile polyethylene zipper bag, along with a humidifier containing 4 mL of sterile PBS. The polyethylene zipper bag was closed and inserted in an aluminum foil pouch that was then heat-sealed.

Figure 5 depicts the cumulative changes in the weight of the double-bag packages and the hydrogel discs over 42 days. The data show that integrity of the hydrogel discs was well maintained over the experimental period. The designed packaging system was therefore used for all subsequent experimental studies. However, DRDC Toronto advised Dr. DiCosmo to consider alternate strategies to circumvent the marked

dehydration of the hydrogel when marketing his product, as this packaging is not 'clinically-friendly'.

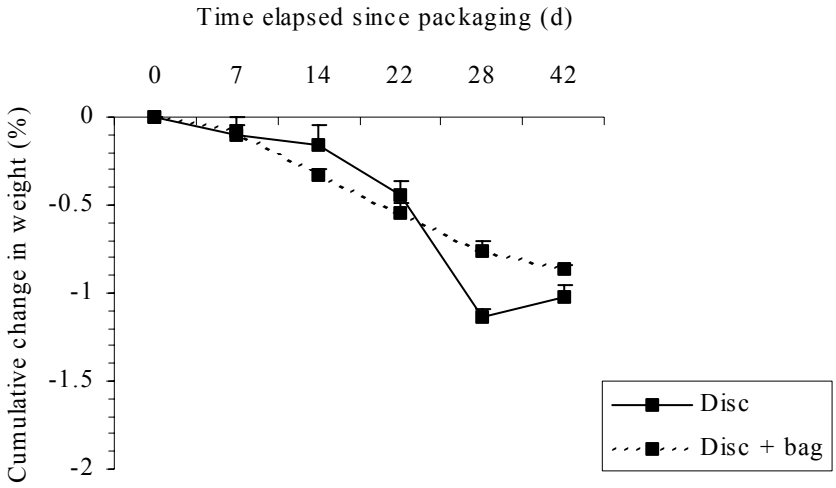


Figure 5. *Cumulative changes over 42 days in the weights of the hydrogel discs and the polyethylene-aluminum packages containing a custom-designed humidifier injected with 4 mL of sterile PBS. Data are expressed as means ± SEM (n=5 per experimental group).*

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4. Standardization of UV sterilization procedures of hydrogel materials

4.1 Determination of optimal sterilization parameters

DRDC Toronto's previous finding of the contamination of the hydrogel samples supplied (reportedly prepared under aseptic conditions and sterilized by UV irradiation) highlighted the need for standardization of the sterilization procedures prior to completion of any further *in vivo* studies. A series of *in vitro* experiments were therefore performed to define the appropriate irradiation parameters (e.g., duration of UV exposure, distance between sample and UV lamp) required to achieve complete sterilization of the hydrogel material.

In a first study, one mL of a *Pseudomonas* (*Ps*.) *aeruginosa* bacterial suspension (American Type Culture Collection (ATCC) 27317; approx 10^9 colony forming units (CFU)/mL) was streaked on the surface of a hydrogel sheet (10 cm x 10 cm) that was then inserted into a polyethylene zipper bag (VWR International, Mississauga, ON). The height of the UV lamp (Sterilaire®, UVP, Upland, CA) was adjusted to 12.5 cm, 25.4 cm or 50.8 cm. For each height setting, 3 bags were UV-irradiated for 1, 2, 5, 7 and 10 min per side. After completion of the irradiation procedures, the inoculum was removed from the bag, plated on tryptic soy agar enriched with 5% sheep blood, and incubated overnight (37°C). Table 1 shows that surface-sterilization of the hydrogel sheets occurred when the material was UV-irradiated for at least 2 min per side, regardless of the distance between the sample and the UV source.

Table 1. *Effect of varying various UV irradiation parameters on the decontamination of superficially contaminated hydrogel sheets*

EXPOSURE TIME (MIN PER SIDE)	**DISTANCE BETWEEN SAMPLE AND UV SOURCE (CM)**		
	12.7	***25.4***	***50.8***
1	+	+	+
2	-	-	-
5	-	-	-
7	-	-	-
10	-	-	-

$^{+}$ Bacterial growth. $^{-}$ No bacterial growth

While the previous study simulated a superficially contaminated material, it was clear that bacteria could also be introduced at various critical steps in the preparation of the hydrogel material. Another study was therefore performed to define the optimal sterilization parameters for deeply contaminated hydrogel sheets. Briefly, 4 pieces of hydrogel (2.5 cm x 2.5 cm; 2.0 mm thick) were soaked up in a *Ps. aeruginosa* bacterial suspension (ATCC 27317; approx 10^5 CFU/mL) for 5.5 h. The hydrogel sheets were then inserted into a polyethylene zipper bag (VWR International, Mississauga, ON), and irradiated as previously described. After completion of the irradiation procedures, the dressings were removed from their bag, incubated overnight in 10 mL of tryptic soy broth (37°C). The broth was then plated in triplicate on tryptic soy agar enriched with 5% sheep blood, and incubated overnight (37°C) to assess bacterial growth. Table 2 shows that the exposure time required to completely sterilize the samples increased markedly when the samples were deeply contaminated. Interestingly, UV-irradiation of deeply contaminated samples twice the thickness of those in the present study failed to be sterilized after 10 min of UV irradiation (data not shown).

Table 2. *Effect of varying various UV irradiation parameters on the decontamination of deeply contaminated hydrogel sheets*

EXPOSURE TIME (MIN PER SIDE)	**DISTANCE BETWEEN SAMPLE AND UV SOURCE (CM)**		
	12.7	***25.4***	***50.8***
1	+	+	+
2	+	+	+
5	+	+	+
7	-	+	+
10	-	-	-

[+]Bacterial growth. [-]No bacterial growth

These data suggest that complete sterilization of the hydrogel material depends on the distance between the sheets and the UV lamp, the thickness of the hydrogel, as well as the initial level of bacterial load in the sample. These findings highlighted the need for performing all steps involved in the preparation of the hydrogel sheets under strict aseptic conditions.

4.2 Effect of sterilization process on the integrity of ciprofloxacin

Dr. DiCosmo advised DRDC Toronto that he had selected ciprofloxacin hydrochloride (Cipro®, Bayer Pharmaceutical Group, Toronto, ON) to achieve a proof-of-concept of the antibacterial properties of the liposomal hydrogel wound dressing. While the previous series of experiments determined the optimal parameters for complete sterilization of drug-free hydrogel samples, experiments were conducted to determine whether these sterilization procedures would affect the integrity of ciprofloxacin.

Thus, 5 aliquots of various concentrations of ciprofloxacin solutions were prepared, spread into polyethylene zipper bags, and UV-irradiated as described in the previous section. Ciprofloxacin levels in UV-irradiated and non-irradiated samples were then assessed using High Performance Liquid Chromatography (HPLC). Figure 6 shows that UV irradiation altered the integrity of ciprofloxacin, the amount of intact ciprofloxacin remaining in the irradiated sample being dependent on the initial concentration of the drug prior to exposure to UV light. In fact, a significant peak of a chemical eluted prior to ciprofloxacin appeared in most of the UV-irradiated samples, while the non-irradiated samples showed only the ciprofloxacin peak (data not shown), suggesting significant breakdown of the drug. It can be assumed that the bactericidal activity of the irradiated samples would be considerably affected by the irradiation procedures.

To ensure the integrity of the drug incorporated into the hydrogel wound dressing, DRDC Toronto recommended that ciprofloxacin be loaded after completion of the sterilization procedures. More specifically, hydrogel wound dressings should be placed directly under the UV lamp (i.e., not inserted in the polyethylene zipper bag) for sterilization (7 min per side, 25 cm below UV lamp). The dressings should then be transferred using aseptic technique into the appropriate ciprofloxacin bathing solution, and immersed for one hour. Each dressing should then be inserted into a pre-sterilized polyethylene bag using aseptic technique. All procedures (i.e., sterilization, drug-loading and packaging) should be performed in a biological safety cabinet.

These new procedures should ensure the integrity of the ciprofloxacin. However, DRDC Toronto recommended that consideration be given to finding an alternate method of sterilization, possibly gamma–irradiation, and assessing its effect on the polymerization and physico-chemical characteristics of the hydrogel and the loaded drug.

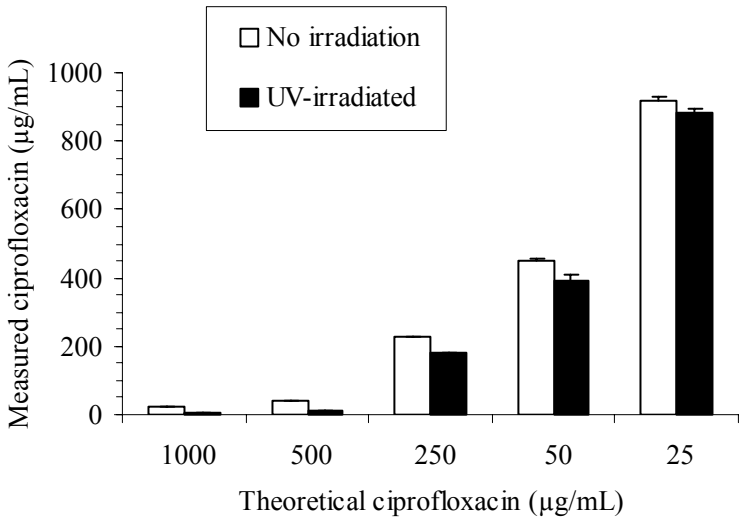

Figure 6. *Effect of UV irradiation on the integrity of ciprofloxacin, measured using HPLC. Data are expressed as means ± SEM (n=5 per experimental group).*

5. *In vivo* assessment of the bactericidal activity of liposomal ciprofloxacin-loaded hydrogel discs

5.1 Hydrogel preparation for rat wound model studies

All hydrogel material tested by DRDC Toronto was prepared in Dr. DiCosmo's laboratory. Liposomes were composed of dipalmitoylphosphatidylcholine (DPPC)/cholesterol (1:1). The lipids were dissolved in a small volume of chloroform and the solvent was removed in vacuo for 2 h. The resulting lipid film was hydrated with 300 mM ammonium sulfate at 45°C. Liposomes were then frozen in liquid nitrogen and thawed in a 45°C water bath (3X), followed by high-pressure extrusion through two 100 nm-pore membranes (5X) giving large unilamellar vesicles. Liposomes suspensions were dialysed overnight against 1000 volumes of PBS.

Polyethylene glycol (PEG) cross-linked hydrogels consisted of gelatin and p-nitrophenylphosphorylcholine-PEG (100 mg/mL and 60 mg/mL, respectively) and were prepared by dissolving the components in PBS, with or without liposomes, at 45°C for 30 min with occasional shaking. The fluid material (400 µL/mold) was transferred to 2 cm diameter molds (1.25 mm deep). The mixture was allowed to set for 5 min, before incubation at 4°C for 10 min. Hydrogel discs were cross-linked by immersion in 200 mM borate buffer, pH 9.0, for one hour. Cross-linking by-products were removed from the gels by continuous washing with PBS over 16 h.

For ciprofloxacin loading, liposomal gels were placed in ciprofloxacin-saline solutions, such that the final drug concentration was equivalent to 2.7 mg drug per disc, followed by heating at 50°C for 60 min. All discs were sterilized under UV light for 7 min per side before being placed in the double-bag packaging system previously described in Section 3 (6 dressings per package).

Sterility of the hydrogel discs of each batch was assessed 2 days prior to their application on the wounds. Briefly, one dressing from each package was removed under aseptic conditions (i.e., under flow hood, using sterilized forceps), and the package re-sealed. The sample disc was incubated 24 h at 37°C in tryptic soy broth. An aliquot of broth was then plated onto tryptic soy agar and incubated for 24 h. All hydrogel dressings tested were sterile.

The hydrogel discs tested in the following experiments were very flexible, thus ensuring a close contact of the disc with the wound (yet without adhering to it), as well as delivery of the antibiotic to the site of infection. However, these discs were very fragile, and extreme care was required in handling them to prevent their splitting. It is also noteworthy that the hydrogel discs were homogenous (i.e., no air bubbles were observed).

5.2 Bacterial challenge

A bacterial suspension of *Ps. aeruginosa* (ATCC 27317) was used to infect the wounds, created as described in Section 1.1. The bacterial strain was grown at 37°C in tryptic soy broth for 18 h in a shaking water bath to obtain a log-phase growth culture. The suspension was washed twice in sterile PBS, resuspended in sterile PBS, and diluted to approximately 10^8 CFU per mL. Bacterial concentrations in the inoculum were assessed by plating serial dilutions on tryptic soy agar enriched with 5% sheep blood.

5.3 Prevention of contamination of wounds

This study was designed to assess the bactericidal efficacy of liposomal ciprofloxacin-loaded hydrogel in preventing contamination of wounds.

On the day of the experiment, rats were anesthetized and a wound was made, as previously described. A 1.5-cm hydrogel disc containing either plain liposomes (Lipogels; n=6) or liposomal ciprofloxacin (Ciprogels; 2.7 mg drug per disc; n=6) was immediately applied to cover entirely the wound. Approximately 3 x 10^9 CFU (in 50 µL) of *Ps. aeruginosa* was placed directly on the hydrogel disc. Each dressing was then covered as described in Section 2.1. All animals received analgesics (buprenorphine, 0.05 mg/kg body weight; s.c.) immediately prior to the surgical procedures, and again 8 h later. Rats were sacrificed 24 h after application of the hydrogel disc, and their muscle tissues (i.e., *panniculus carnosus* and *acromiotrapezius*) were excised under aseptic conditions; homogenized in sterile, ice-cold PBS; and, serially diluted. Each dilution was plated on tryptic soy agar enriched with 5% sheep blood, and incubated overnight at 37°C.

Swabs taken from the wounds revealed the presence of 1-5 CFU per wound. This minor contamination may have occurred during the removal of the disc, as suggested by the fact that no bacterial contamination was detected in the *panniculus carnosus* and *acromiotrapezius* muscles of the rats treated either with Lipogels and Ciprogels. These data suggest that application of the hydrogel material per se for a short period (e.g., 24 h) provides a barrier that effectively prevents contamination of the wound, regardless of the presence of an antibiotic or not. However, it is unlikely that the Lipogel discs would have remained an effective barrier over a longer period. Indeed, while the Ciprogel remained intact after 24 h, the Lipogel discs were partly disintegrated.

5.4 Prevention of the progression of infection in contaminated wounds

This study was designed to simulate a battlefield scenario where a 'fresh' full-thickness wound was contaminated with Gram-negative bacteria. However, due to operational requirements, cleaning or debriding of the wound could not be performed immediately. Under those circumstances, first aid treatment consisted of applying an

antibiotic-loaded dressing to attempt to limit the progression of the superficial infection to deeper tissues.

Twelve male, Sprague-Dawley rats (325 – 350 g; Charles River, St-Constant, QC) were anesthetized, and a wound was made as previously described. A small piece of gauze was placed on the wound, and wetted with approximately 2 x 10^9 CFU (in 500 µL) of *Ps. aeruginosa* (ATCC 27317). The abdomen of the animal was then wrapped in a sterile plastic membrane (Saran Wrap®) for 15 min, after which time the gauze was discarded. A sterile hydrogel disc containing either plain liposomes (n=6; Lipogel) or liposomal ciprofloxacin (n=6; 2.7 mg ciprofloxacin per disc; Ciprogel) was applied to cover the entire wound. Each hydrogel disc was then covered using the restraining system described previously. All animals received analgesics (buprenorphine, 0.05 mg/kg body weight; s.c.) immediately prior to the surgical procedures, and again 8 h later. All animals were sacrificed 24 h after application of the hydrogel discs. The *panniculus carnosus* as well as the underlying *acromiotrapezius* muscles were removed under aseptic conditions; homogenized in sterile, ice-cold PBS; and, serially diluted. Each dilution was plated on tryptic soy agar enriched with 5% sheep blood, and incubated overnight at 37°C.

All the hydrogel discs recovered were intact (i.e., no apparent dehydration). The *panniculus carnosus* appeared marginally inflamed 24 h after application of the Ciprogel dressings. In contrast, the *panniculus carnosus* covered by the Lipogels for 24 h were swollen (1-3 mm) and opaque over the entire surface of the wound. The appearance of the *acromiotrapezius* muscle was similar in both experimental groups.

Assessment of bacterial counts in both the superficial and deep muscles revealed approximately 5-log and 3-log reductions, respectively, in the number of bacteria recovered in these tissues after application of the Ciprogels for 24 h compared to that of Lipogels (Figure 7). It is noteworthy that the levels of contamination in both tissues were maintained below the accepted clinical threshold of contamination of 10^5 CFU/g tissue. These data suggest that incorporation of liposomal ciprofloxacin in the hydrogel material is very effective in limiting the progression of the infection to deeper tissues in this rat model of infected wounds. However, there is no indication from this series of experiments whether the encapsulation of ciprofloxacin in liposomes is required to exert this significant bactericidal effect.

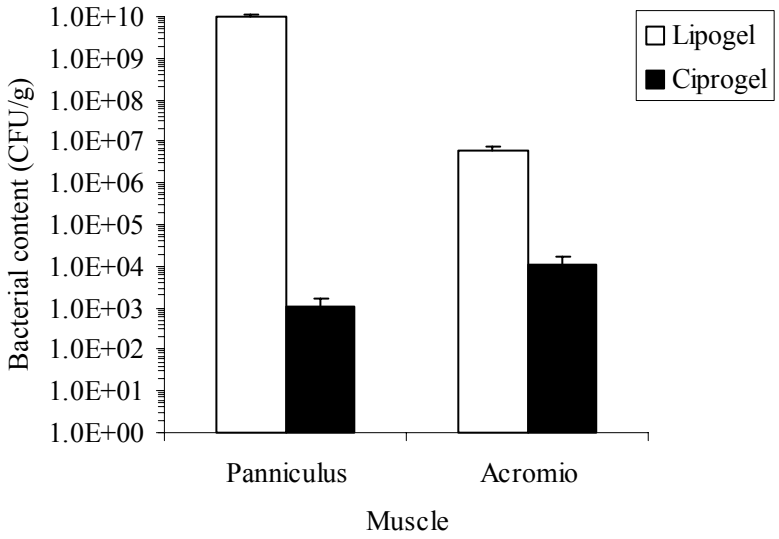

Figure 7. *Bactericidal efficacy of liposomal ciprofloxacin-loaded hydrogel (Ciprogel) or plain liposomal gel (Lipogel) in preventing the progression of infection in the panniculus carnosus (Panniculus) or acromiotrapezius (Acromio) muscles. Data are expressed as means ± SEM.*

5.5 Treatment of established wound infections

The objective of the present study was to determine the effectiveness of the liposomal ciprofloxacin-loaded hydrogel in treating established wound infections using a rat model of full-thickness wounds.

Sterility of the liposomal ciprofloxacin-loaded hydrogel discs (i.e., Ciprogels) or plain liposome-loaded hydrogel discs (i.e., Lipogels) was assessed 2 days prior to performing the surgical procedures. On the experimental day, a full-thickness wound was made in twelve male, Sprague-Dawley rats (325 – 350 g; Charles River, St-Constant, QC). Approximately 5 x 10^8 CFU (in 500 µL) of *Ps. aeruginosa* (ATCC 27317) was injected under the fascia of the *panniculus carnosus* muscle in each rat. DRDC Toronto had shown in preliminary experiments that this procedure induced a local infection, which was maintained for at least 7 days (unpublished data); thus, we refer to this protocol as that of an 'established' wound infection. A Lipogel (n=5) or Ciprogel (n=7; 2.7 mg ciprofloxacin per disc) was immediately applied to cover entirely the wound. Each hydrogel disc was then covered using the restraining system described in Section 2.1. All animals received analgesics (buprenorphine, 0.05 mg/kg body weight; s.c.) immediately prior to the surgical procedures, and again 8 h later.

All animals were sacrificed 24 h after application of the dressing. The *panniculus carnosus* and the underlying *acromiotrapezius* muscle were removed under aseptic conditions. All tissues were homogenized in sterile, ice-cold PBS, and serially diluted. Each dilution was plated on tryptic soy agar enriched with 5% sheep blood, and incubated overnight at 37°C.

The *panniculus carnosus* muscle was very inflamed 24 h after completion of the infection procedures. Furthermore, the *acromiotrapezius* muscle was often discolored (greenish hue), suggesting that the bacteria had migrated to the muscle. A 5-log reduction in bacterial counts measured in the superficial muscle tissue was observed after application of the Ciprogel discs for 24 h compared to that of Lipogel discs (Figure 8). In contrast, there was a significantly lower reduction (half-log) in bacterial counts measured in the *acromiotrapezius* muscle (i.e., the established site of infection) after application of the Ciprogel discs for 24 h compared to Lipogel discs.

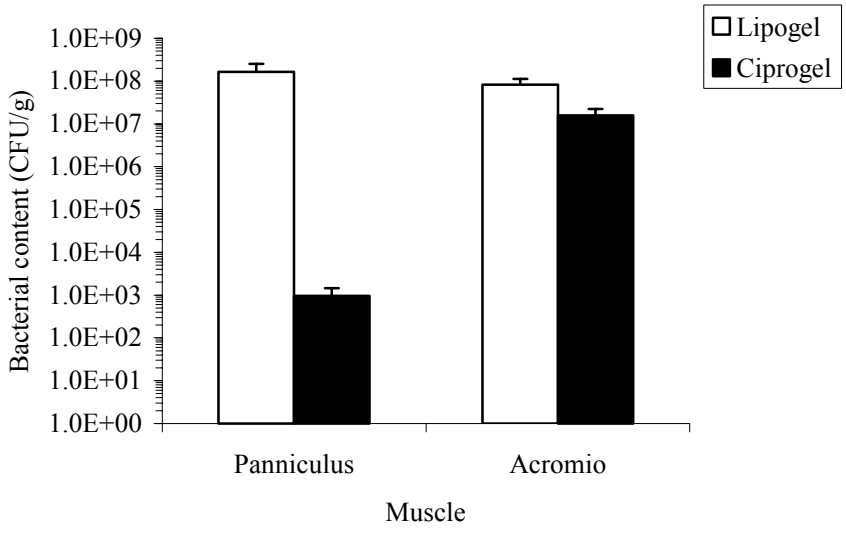

Figure 8. *Effectiveness of liposomal ciprofloxacin-loaded hydrogel (Ciprogel) and plain liposomal gel (Lipogel) in reducing the bacterial load in the panniculus carnosus (Panniculus) or acromiotrapezius (Acromio) muscles. Data are expressed as means ± SEM.*

These data suggest that application of a liposomal ciprofloxacin hydrogel disc for 24 h is an effective method to treat an established wound infection in this rat model of full-thickness wounds. However, this bactericidal effect is markedly reduced in deeper tissues.

5.6 Evaluation of long-term bactericidal efficacy of the liposomal ciprofloxacin-loaded hydrogel

While data from previous experiments suggested that Ciprogel was an effective drug delivery system, there was no indication whether the encapsulation of ciprofloxacin in liposomes was required to exert the significant bactericidal effects observed. Furthermore, there was also no indication that liposomal entrapment of ciprofloxacin provided a sustained-release bactericidal effect. The present study was thus designed to compare the efficacy of liposomal and free ciprofloxacin-loaded hydrogel in reducing the bacterial load of full-thickness contaminated wounds after a single application of the experimental dressing for up to 7 days.

Sterility of the liposomal ciprofloxacin-loaded hydrogel discs (i.e., Ciprogel) or ciprofloxacin-loaded hydrogel discs (i.e., Freegel) was assessed 2 days prior to performing the surgical procedures. On the experimental day, a full-thickness wound was created in 36 male, Sprague-Dawley rats (325 – 350 g; Charles River, St-Constant, QC). Approximately 5 x 10^8 CFU (in 500 μL) of *Ps. aeruginosa* (ATCC 27317) was injected under the fascia of the *panniculus carnosus* muscle in each rat. A hydrogel disc containing either free ciprofloxacin (n=18; 0.8 mg drug per disc) or liposomal ciprofloxacin (n=18; 3.1 mg drug per disc) was immediately applied to cover entirely the wound. Each hydrogel disc was then covered using the restraining system previously described. All animals received analgesics (buprenorphine, 0.05 mg/kg body weight; s.c.) immediately prior to the surgical procedures, and again 8 h later.

Animals were sacrificed 1, 3 or 7 days after application of the dressing (n=6 per experimental group). The *panniculus carnosus* and the underlying *acromiotrapezius* muscle were removed under aseptic conditions. All tissues were homogenized in sterile, ice-cold PBS, and serially diluted. Each dilution was plated on tryptic soy agar enriched with 5% sheep blood, and incubated overnight at 37°C. The ciprofloxacin content of all dressings applied to the wounds as well as that of unused dressings (n=6) was assessed using fluorimetry.

Figure 9 shows the changes in bacterial content of a superficial muscle (*panniculus carnosus*) and a deep muscle (*acromiotrapezius*) at different times following application of the antibacterial dressings. Application of a Ciprogel for 24 h did not alter the level of contamination of the deep muscle, regardless of whether the drug was entrapped in the liposomes or not. However, unlike our previous finding of a marked bactericidal effect in the *panniculus carnosus* when applying Ciprogel for 24 h (Figure 8), no significant reduction in bacterial load was observed in the present study after 24 h (Figure 9). However, the amounts of ciprofloxacin released from all drug-loaded dressings (Figure 10) exceeded 10 times the Minimum Inhibitory Concentration (MIC) required to exert a bactericidal effect against this strain of *Ps. aeruginosa* (i.e., 30 ug; unpublished data). Whether or not this discrepancy between the two studies was due to an inactivation of the ciprofloxacin due to the UV procedures (see section 4.2 for a full discussion), and therefore a reduction in the bactericidal efficacy of the drug, could not be ascertained from the present study.

Nevertheless, application of a Ciprogel for up to 7 days exerted a greater bactericidal effect than that of a Freegel, this difference in the efficacy of the two hydrogel preparations being more apparent in the *panniculus carnosus* muscle than in the *acromiotrapezius* muscle (Figure 9). However, this finding does indicate that the liposomal entrapment of the drug is an essential component of the hydrogel technology, as the levels of ciprofloxacin released from the Ciprogel were 5 times greater than those released to wounds covered with the Freegel (Figure 10). Furthermore, the application of a Freegel allowed the maintenance of the contamination in the tissues below the acceptable clinical threshold of contamination of 10^5 CFU per g tissue (Figure 9).

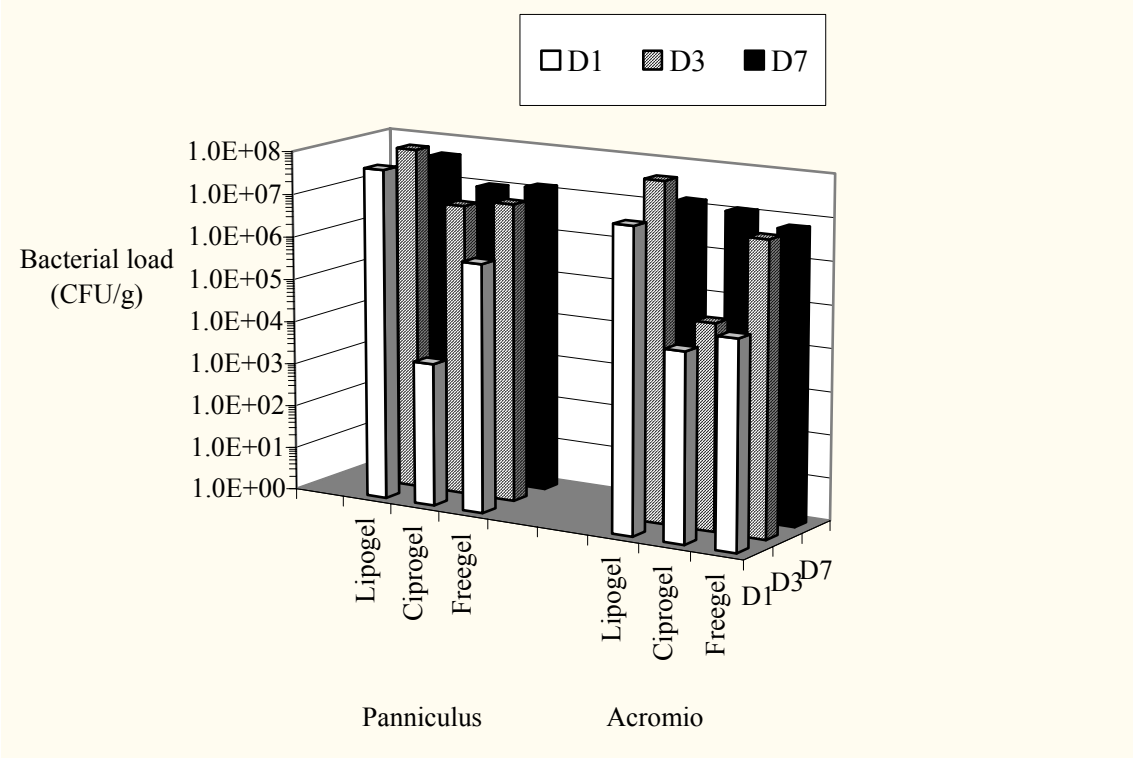

Figure 9. *Ps. aeruginosa load in the panniculus carnosus (Panniculus) and the acromiotrapezius (Acromio) muscles underlying wounds covered with a hydrogel disc containing plain liposomes (Lipogel), liposomal ciprofloxacin (Ciprogel) or free ciprofloxacin (Freegel) for up to 7 days. Data are expressed as means ± SEM (n=6 per group).*

These data suggest that the entrapment of ciprofloxacin in liposomes is not required when large amounts of ciprofloxacin are loaded in the hydrogel. However, considering that the clinical use of large amounts of antibiotics is associated with the emergence of strains of drug-resistant bacteria [13], DRDC Toronto recommended that

the further development of the hydrogel technology includes the incorporation of ciprofloxacin in concentrations corresponding to a maximum of 2-3 times the MIC. Furthermore, consideration should be given to determining a procedure for loading similar amounts of the drug in the hydrogel material to allow a definitive assessment of whether or not the liposomal entrapment of the drug is essential for exerting a long-term bactericidal effect.

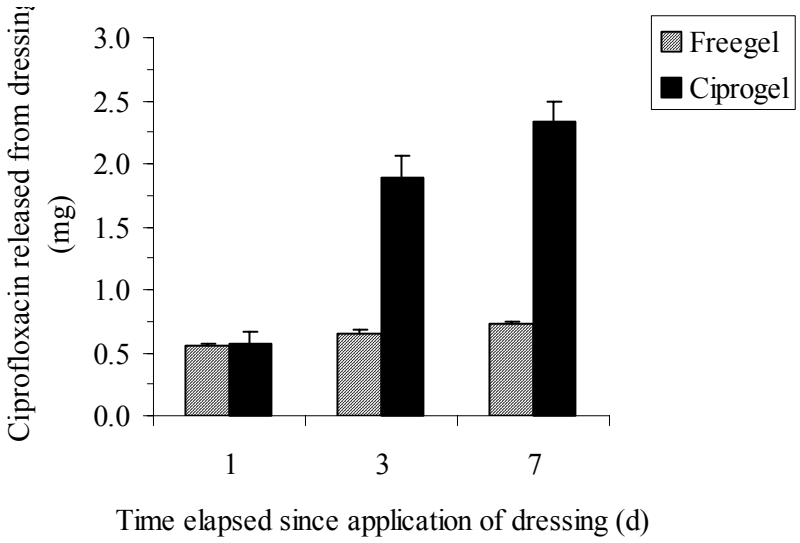

Figure 10. *Amount of ciprofloxacin released to wounds covered for up to 7 days with a hydrogel disc containing liposomal (Ciprogel) or free ciprofloxacin (Freegel). Data are expressed as means ± SEM (n=6 per group).*

6. Evaluation of the commercialization potential of liposomal ciprofloxacin-loaded hydrogel technology

Considering the results obtained in the *in vivo* experiments performed at DRDC Toronto, a decision was made to approach a small Canadian biomedical company, Westaim Biomedical Corp. (Fort Saskatchewan, AB), to seek their interest in the commercialization of the liposomal antibiotic hydrogel technology. Westaim Biomedical Corp. had developed a proprietary antimicrobial coating technology that functioned through the dissolution of metal films, especially silver-based, at controlled specific rates. These films could be tailored to provide tangible antimicrobial benefits to a variety of medical devices, including wound dressings. The Acticoat™ Antimicrobial Barrier Dressing is such an example of a silver-coated dressing. Following presentation of the experimental results, Westaim Biomedical Corp. requested that specific questions be addressed in order to make a go-no-go decision regarding future financial support for the hydrogel technology, namely:

1. What is the release kinetics of liposomal ciprofloxacin-PEG-gelatin hydrogel material when evaluated over time in calf serum?

2. What is the antimicrobial potential of liposomal ciprofloxacin-PEG-gelatin hydrogel material when evaluated *in vitro*?

3. Is the liposomal ciprofloxacin-PEG-gelatin hydrogel material effective in reducing the bacterial count and facilitating wound healing in a chronic wound infection model?

DRDC Toronto assisted in providing answers to the last two questions. All experiments were commissioned by Westaim Biomedical Corp., and performed in the laboratories of the Biofilm Research Group (Dr. Merle Olson, University of Calgary, AB).

6.1 Hydrogel preparation for porcine wound model study

All hydrogel material were prepared in the laboratories of Dr. DiCosmo. Liposomes were composed of DPPC/cholesterol (1:1). The lipids were dissolved in a small volume of chloroform and the solvent was removed *in vacuo* for 6 h. The resulting lipid film was hydrated with 300 mM ammonium sulfate at 45°C. Liposomes were then frozen in liquid nitrogen and thawed in a 45°C water bath (3X), followed by high-pressure extrusion through two 100 nm-pore membranes (5X) giving large unilamellar vesicles. Liposomes suspensions were dialysed over 36 h against 100 volumes of PBS (3 changes).

PEG cross-linked hydrogels consisted of gelatin and p-nitrophenylphosphorylcholine-PEG (100 mg/mL and 60 mg/mL, respectively) and were prepared by dissolving the

components in PBS, with or without liposomes, at 45°C for 30 min with occasional shaking. The fluid material was transferred to 10 cm x 10 cm molds (13 mL/mold) each containing a 10 cm x 10 cm piece of gauze. The mixture was allowed to set (5 min), before incubation at 4°C for 10 min. Hydrogel sheets were cross-linked by immersion in 200 mM borate buffer, pH 9.0, for one hour. Cross-linking by-products were removed from the gels by continuous washing with PBS over 16h.

For ciprofloxacin loading, liposomal gels were placed in ciprofloxacin-saline solutions, such that the final drug concentration was equivalent to 100 mg drug per gel sheet, followed by heating at 50°C for 60 min. All gel sheets were placed in a plastic bag and sterilized under UV light (254 nm) for 10 min per side before being placed in a pre-sterilized aluminum foil pouch that was heat-sealed.

6.2 *In vitro* assessment of Ciprogel as a barrier against bacterial contamination

The aim of this study was to assess the *in vitro* efficacy of liposomal ciprofloxacin-loaded hydrogel (Ciprogel) and Acticoat™ wound dressings (Westaim Biomedicals Corp., Fort Saskatchewan, AB) as a barrier against contamination with Gram-positive (*Staph. aureus*) and Gram-negative (*Ps. aeruginosa*) bacteria.

Twelve control dressings (6 Sontara gauze, i.e. the base component of Acticoat™; 6 Lipogel, i.e. plain liposomal hydrogel) and twelve experimental dressings (i.e., 6 Ciprogel containing 2.7 mg ciprofloxacin per g hydrogel; 6 Acticoat™) were placed on different blood agar plates. The hydrogel discs were then covered with a small sterile gauze, to ensure that the bacterial inoculum would remain in contact with the hydrogel discs and not slide off its surface. A bacterial inoculum containing either *Ps. aeruginosa* (ATCC 27317; approximately 10^7 CFU in 200 µl) or *Staph. aureus* (approximately 10^6 CFU in 200 µl) was then applied to each of the experimental dressings. After incubation at 37°C for 2 h, bacterial counts in each dressing were assessed using standard microbiological procedures.

Figures 11 and 12 show the bacterial counts measured in various dressings that were contaminated with a bacterial inoculum containing either Gram-positive or Gram-negative bacteria, respectively. The reduction in *Ps. aeruginosa* counts after 2 h of contact with the Acticoat™ wound dressings was 26% greater than that observed for the Ciprogel discs (Figure 11). The efficacy of the Acticoat™ wound dressings to act as a barrier against bacterial contamination was also 26% greater against *Ps. aeruginosa* than *Staph. aureus*. While a 3.1 log reduction in *Ps. aeruginosa* counts was observed after applying the Ciprogel discs for 2 h (Figure 11), the levels of *Staph. aureus* contamination in the Ciprogel group remained comparable to those of the Lipogel group (Figure 12). This result was expected as ciprofloxacin bactericidal activity against Gram-positive bacteria is typically minimal.

These data suggest that while Acticoat™ may provide a better barrier against bacterial contamination than the liposomal ciprofloxacin hydrogel technology, application of

the Ciprogels was nevertheless an effective antibacterial method, reducing the levels of contamination below the accepted clinical threshold of wound contamination (i.e., 10^5 CFU/g tissue). However, DRDC Toronto recommended that while ciprofloxacin may be an acceptable bactericidal agent to achieve a proof-of-concept, broad-spectrum anti-infective agents other than antibiotics should be incorporated in the product to be commercialized by Dr. DiCosmo, to minimize the problems associated with the emergence of strains of antibiotic-resistant bacteria.

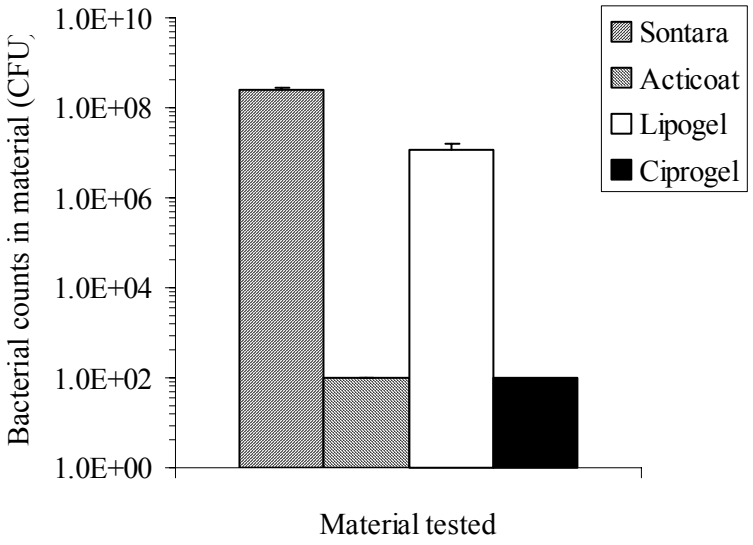

Figure 11. *In vitro comparison of the bactericidal activity of liposomal ciprofloxacin-loaded hydrogel (Ciprogel) and Acticoat™ against Ps. aeruginosa. Data are expressed as means ± SEM (n=4 per experimental group).*

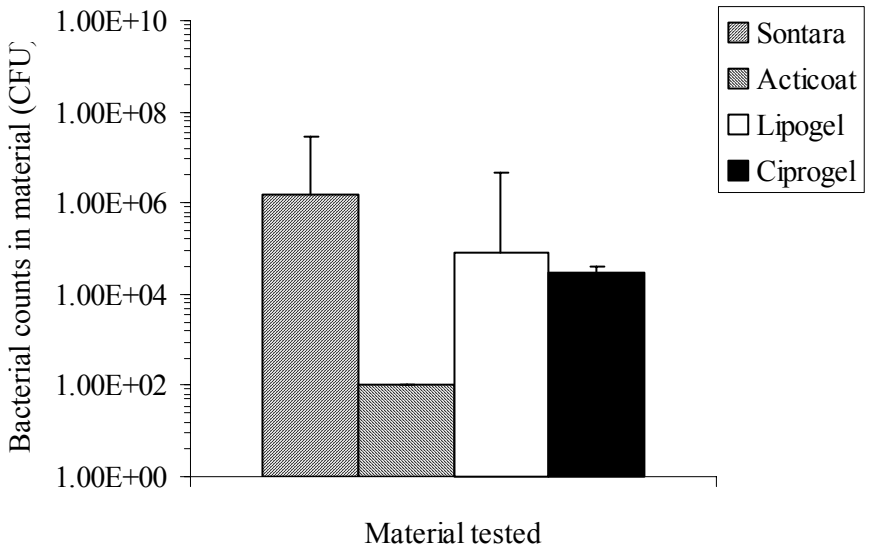

Figure 12. *In vitro comparison of the bactericidal activity of liposomal ciprofloxacin-loaded hydrogel (Ciprogel) and Acticoat™ against Staph. aureus. Data are expressed as means ± SEM (n=4 per experimental group).*

6.3 Assessment of the efficacy of Ciprogel in preventing wound infection in a rat model of infected burns

A pilot experiment was performed to compare the bactericidal efficacy of the liposomal ciprofloxacin-loaded hydrogel discs (Ciprogel; 2.7 mg liposomal ciprofloxacin per disc) to that of a 0.5% silver nitrate solution (Sigma, St-Louis, MI), a 1% silver sulfadiazine (Silvadene™, King Pharmaceuticals, Bristol, TN), and the Acticoat™ Antimicrobial Barrier Dressing (Westaim Biomedical Corp., Fort Saskatchewan, AB) in preventing wound infection using a modified Walker-Mason burn wound model (Dr. Merle Olson, Biofilm Research Group; unpublished data). Briefly, eight male, Sprague-Dawley rats (300-325 g; University of Calgary inbred colony) were anesthetized using ketamine (i.m.; 10 mg/kg body weight) and xylazine (i.m.; 1 mg/kg body weight). The dorsum was shaved and cleansed using standard procedures. Three 2 cm x 2 cm burn wounds were made on each side of the animal by applying a heated (100°C) brass rod for 40 s on the skin. Previous studies have shown that these procedures induce a full-thickness, non-lethal burn (Olson, personal communication). Three 2.5 cm x 2.5 cm pieces of sterile Sontara gauze and three 2.5 cm x 2.5 cm Acticoat™ dressings were applied to cover the wounds in two rats; two other rats received three 2.5 cm x 2.5 cm Lipogel sheets and three Ciprogel sheets.

The hydrogel discs were then covered with sterile gauze, to ensure that the bacterial inoculum would remain in contact with the hydrogel dressings and not slide off its surface. Silvadene™ cream (200 µL) or a placebo cream was applied liberally over the wounds in two rats. The cream was then covered with sterile gauze to provide a surface for inoculation of the bacteria. Finally, wounds in the last 2 rats were covered with a piece of sterile gauze that was wetted with either 200 µL of PBS or 0.5% silver nitrate. A mixture of *Ps. aeruginosa* Utah strain (approximately 10^7 CFU in 200 µl) was then applied to each of the dressings. The animals were sacrificed either 30 min (n=2) or 120 min (n=2) after inoculation; the burn wounds were then excised under aseptic conditions. The wounds (n=3 per experimental group) were sonicated separately and bacterial counts were assessed using standard microbiological procedures.

Figure 13 shows that application of silver nitrate to burn wounds could not prevent their infection. While application of sulfadiazine cream was ineffective in preventing the contamination of the burns in the first 30 min, a one-log reduction in bacterial load was observed in the wounds excised 2 h after treatment. In contrast, both Ciprogel and Acticoat™ provided an immediate barrier against infection, the reductions in bacterial counts in the wounds 2 h after application of the Ciprogel discs and Acticoat™ wound dressings being comparable (i.e., 2-log reduction). While these data suggest that Acticoat™ and Ciprogel have comparable short-term bactericidal efficacies in burn wounds, DRDC recommended that future *in vivo* studies further assess the long-term bactericidal efficacies of the two dressings.

It is noteworthy that the *Ps. aeruginosa* strain used in the present study was able to migrate through the hydrogel sheet and infect the underlying tissues. This is in contrast to the results presented in section 4.3 using a rat model of infected full-thickness wounds. This may be partly due to the use a more virulent strain of *Ps. aeruginosa* in the present series of experiments. Indeed, all rats used for the infected burn wound model would have died in 48 h-60 h (Dr. Olson, personal communication). No mortality has ever been observed following infection of full-thickness wounds using the *Ps. aeruginosa* ATCC 27317 strain used for all studies at DRDC Toronto. These data suggest that the long-term bactericidal efficacy of the Ciprogel may be reduced if the environment is harsh enough to compromise the integrity of the hydrogel material. Frequent changes of the wound dressing would therefore be recommended to alleviate this problem.

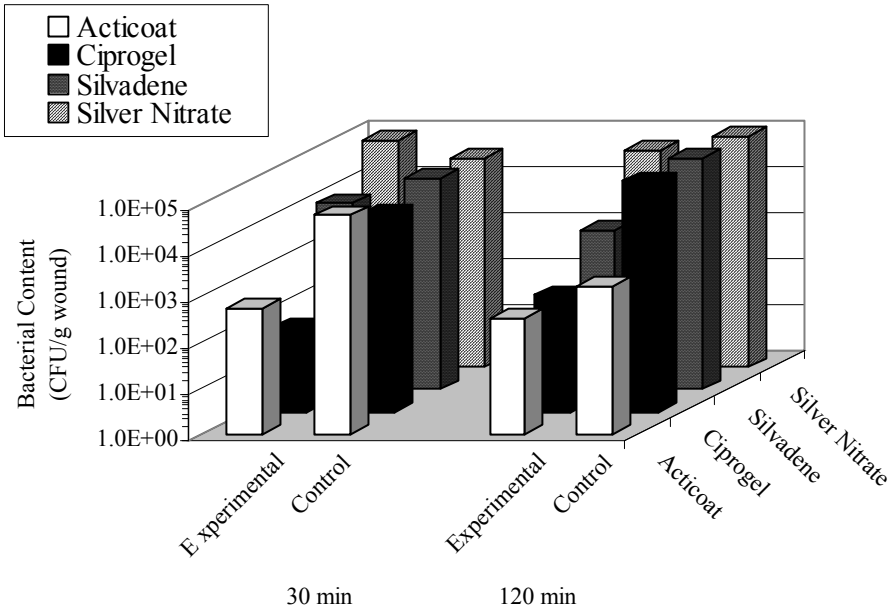

Figure 13. *Comparison of bactericidal activities of various silver-based dressings with liposomal ciprofloxacin-loaded hydrogel (Ciprogel) in a burn model of infected wounds. Data are expressed as means ± SEM (n=3 per group).*

6.4 Preliminary assessment of the effect of Ciprogel on the bacterial burden of full-thickness, contaminated porcine wounds

The purpose of this experiment was to conduct a preliminary investigation into the effectiveness of the liposomal ciprofloxacin hydrogel using a porcine model of contaminated wounds established in the laboratories of the University of Calgary Biofilm Group. This porcine model of contaminated wounds is currently the most clinically-relevant model currently available in Canada to test wound dressings. The effectiveness of the dressing at reducing the bacterial load of full-thickness, contaminated wounds as well as its wound healing properties were evaluated over a 7-d study period.

6.4.1 Experimental procedures

Bacterial cultures containing *Ps. aeruginosa*, *Staph. epidermidis*, and a clinical isolate of *Fusobacterium* sp. were prepared using standard microbiological procedures. On the experimental day, the three bacterial

cultures were mixed together in a ratio approximating 1:1:0.5 (*Pseudomonas: Staphylococcus: Fusobacterium* sp.; 10^7 CFU/mL per culture). One pig was then anesthetized, clipped and the skin prepared for wounding by washing with an antibiotic-free soap. Twenty full-thickness wounds (ten on each side of the pig's dorsum) were created using a 2-cm diameter trephine. A saline/epinephrine solution was applied until complete haemostasis had occurred. The wounds were then covered with gauze sponges, and inoculated with the bacterial suspension. The wounds were then covered for 15 min with an occlusive film to prevent drying.

At the end of the infection period, the size of the wound was measured in two directions. Four adjacent wounds were dressed with a 10 cm x 10 cm hydrogel sheet containing plain liposomes (Lipogel), free ciprofloxacin (Freegel) or liposomal ciprofloxacin (Ciprogel). Though DRDC Toronto had previously recommended against the use of large sheets for experimental protocol due to the technical difficulties encountered in fabricating homogenous sheets, this format was nevertheless selected for this pilot experiment because of Westaim Biomedical Corp.'s requirement to assess the wound healing properties of the hydrogel technology. To minimize the incorporation of air bubbles in the large sheets as previously observed (see Section 2.3), Dr. DiCosmo prepared the sheets by slowly pouring the liquid hydrogel solution on a slanted glass surface.

The wounds were dressed as described in Table 3.

Table 3. *Position of the various dressings on the pig's dorsum*

COL A	COL B	MIDLINE	COL C	COL D
Lipogel	Lipogel	Row 1	Freegel	Freegel
Lipogel	Lipogel	Row 2	Freegel	Freegel
Lipogel	Lipogel	Row 3	Freegel	Freegel
Ciprogel	Ciprogel	Row 4	Ciprogel	Ciprogel
Ciprogel	Ciprogel	Row 5	Ciprogel	Ciprogel

^{Ciprogel} Liposomal ciprofloxacin-loaded hydrogel ^{Lipogel} Plain liposomal hydrogel
^{Freegel} Free ciprofloxacin-loaded hydrogel

Each experimental dressing was then covered with a piece of sterile gauze (Surgipad Combine™ dressing, Johnson & Johnson, New Brunswick, NJ). Layers of adhesive PVC tape (Elastoplast™, Smith & Nephew, Lachine, QC) were then applied to hold down firmly the gauze dressings. The entire trunk of the pig was then wrapped with a layer of elastic self-adhesive bandage

(Elastoplast™, Smith & Nephew, Lachine, QC). A dose of narcotic (butorphenol, 0.6 mg/kg body weight sc.) was administered prior to returning the animal to its pen.

The dressings were replaced on days 1 and 4, as described in Table 3. Thus, the first batch of hydrogel dressings remained on the wounds for 24 h, while the wounds were subsequently covered with two other dressings, each of them remaining on the wounds for 3 days. At each dressing change, the size of the wound was measured in two directions. A 2-mm biopsy was taken at the centre of 2 wounds in each experimental group on days 0, 1, 4, and 7. Tissues were homogenized in cold PBS containing 5 mM $FeCl_3$ (to neutralize ciprofloxacin), and plated to determine the microbiological counts on Minimal *Staphylococcus* agar, Brain Heart Infusion agar, and *Pseudomonas* Isolation agar. The sampling schedule is described in Table 4.

Samples of the hydrogel dressings were recovered at each dressing change. From each dressing, three 6-mm samples were taken from an area over the wound. Furthermore, 3 samples were taken prior to applying a new dressing. Each sample was placed in an empty Eppendorf tube for subsequent determination of the ciprofloxacin content by fluorimetry.

It is noteworthy that concurrently to the pilot experiment, Westaim Biomedical Corp. ran another pig study to assess the bactericidal efficacy of Acticoat™ under the same experimental conditions.

Table 4. *Experimental wound sampling schedule*

SAMPLE DAY	TREATMENT	WOUND POSITION
Day 0	Lipogel	A1 B1
Day 0	Ciprogel	A5 B5
Day 0	Freegel	C1 D1
Day 1	Lipogel	A1 B1
Day 1	Ciprogel	A5 B5
Day 1	Freegel	C1 D1
Day 4	Lipogel	A2 B2
Day 4	Ciprogel	A4 B4
Day 4	Freegel	C2 D2
Day 7	Lipogel	A3 B3
Day 7	Ciprogel	C4 D4 C5 D5
Day 7	Freegel	C3 D3

Ciprogel Liposomal ciprofloxacin-loaded hydrogel Lipogel Plain liposomal hydrogel Freegel Free ciprofloxacin-loaded hydrogel

6.4.2 Results and discussion

Despite the precautions used in preparing the large hydrogel sheets, the Ciprogel were 'wavy' and upon close examination, contained very small air bubbles. Dr. DiCosmo explained that there had been a rapid polymerization of the liquid hydrogel solution into the flask during its pouring onto the slanted template used to prepare the sheets. In contrast, the Freegel showed no irregularities.

While *Ps. aeruginosa* levels in control wounds increased progressively for 4 days after completion of the infection procedures, levels of *Staph. epidermidis* reached a plateau after 24 h (Figure 14). Application for 24 h of

a hydrogel wound dressing containing either free (Freegel) or liposomal ciprofloxacin (Ciprogel) decreased *Ps. aeruginosa* levels below the detection level. This bactericidal effect was maintained for the next 3 days in the free ciprofloxacin-treated group following application of a new Freegel. In contrast, levels of *Ps. aeruginosa* in the Ciprogel-treated wounds increased to approximately 10^5 CFU/g, and remained elevated for the remainder of the study, albeit to levels 2.5 log lower than those of control wounds (Figure 14). Despite application of a third Freegel on day 4, *Ps. aeruginosa* levels had increased by day 7 to values comparable to those of Ciprogel-treated wounds (i.e., 10^5 CFU/g).

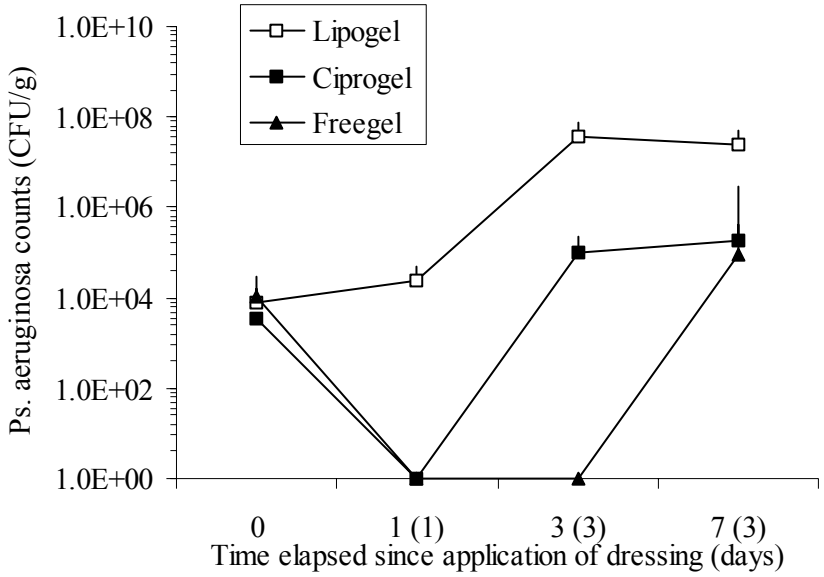

Figure 14. *Ps. aeruginosa load of contaminated wounds treated with hydrogel sheets containing plain liposomes (Lipogel), liposomal ciprofloxacin (Ciprogel) or free ciprofloxacin (Freegel). Number in parenthesis represents the number of days of application of a given dressing on the wound. Data are expressed as means ± SEM (n=2 per group except Ciprogel n=4).*

Application of a Ciprogel wound dressing for 24 h decreased *Staph. epidermidis* levels by approximately 3-log compared to control wounds. However, subsequent applications of Ciprogel dressings had no further bactericidal effect (Figure 15). In contrast, application of a hydrogel wound

dressing containing free ciprofloxacin for 24 h decreased *Staph. epidermidis* levels below detection level. However, levels of *Staph. epidermidis* in Freegel-treated wounds increased subsequently to levels comparable to those of Ciprogel-treated wounds despite application of two 'fresh' Freegel wound dressings. It is noteworthy that the bacterial load in wounds of the pig treated with the Acticoat™ barrier dressings remained below 10^2 CFU/g at all time intervals sampled for the entire duration of the study (personal communication, Dr. Olson). Furthermore, no significant change in the size of the wounds was observed over the 7-d study period for either Freegel-treated (1.9 ± 0.1 cm) or Ciprogel-treated wounds (1.8 ± 0.1 cm).

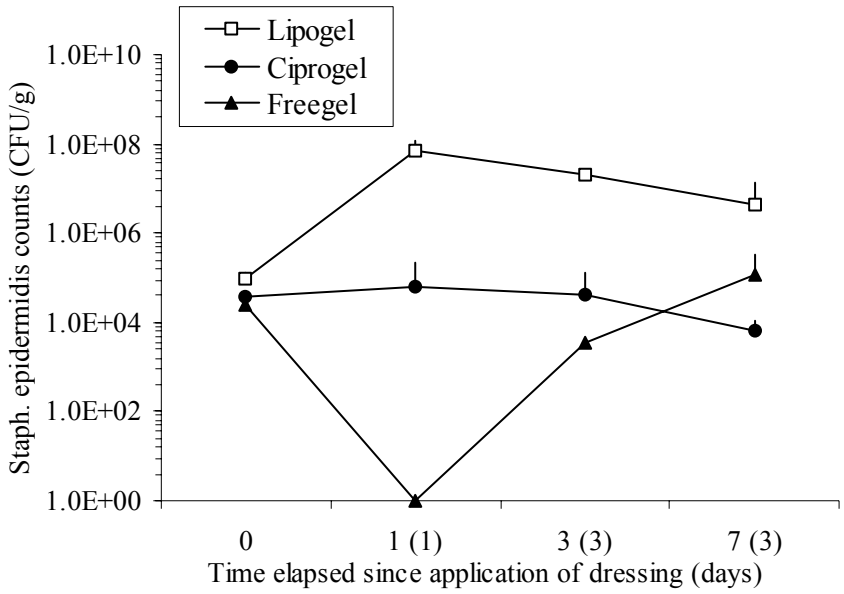

Figure 15. *Staph. epidermidis load of contaminated wounds treated with hydrogel sheets containing plain liposomes (Lipogel), liposomal ciprofloxacin (Ciprogel) or free ciprofloxacin (Freegel). Number in parenthesis represents the number of days of application of a given dressing on the wound. Data are expressed as means ± SEM (n=2 per group except Ciprogel n=4).*

The hydrogel wound dressings loaded with free ciprofloxacin contained 253 ± 3 ug ciprofloxacin/cm^2 prior to their application to the wounds. The drug was evenly distributed in these wound dressings, as suggested by an overall coefficient of variation averaging 5.8%. In contrast, there was a large

variability in the ciprofloxacin levels of the different Ciprogel wound dressings applied over the 7-d study period, as suggested by the large coefficient of variation in drug content (28%). Moreover, there was 3.4 times more drug entrapped in the liposomal ciprofloxacin-loaded wound dressings (859 ± 56 ug/cm^2) than in the Freegel.

Similar concentrations of ciprofloxacin were released from the Ciprogel and Freegel 24 h after their application over the wounds (Figure 16). These values represented 14% and 54% of the initial ciprofloxacin concentration in the Ciprogel and Freegel wound dressings, respectively. Furthermore, the cumulative dose of ciprofloxacin released from the Ciprogel wound dressings over the 7-d study period was 1.4 ± 0.3 mg/cm^2 (i.e., 54% of total dose in the 3 dressings applied over a same wound) compared to 0.50 ± 0.03 mg/cm^2 (i.e., 71% of total dose) for the Freegel dressings.

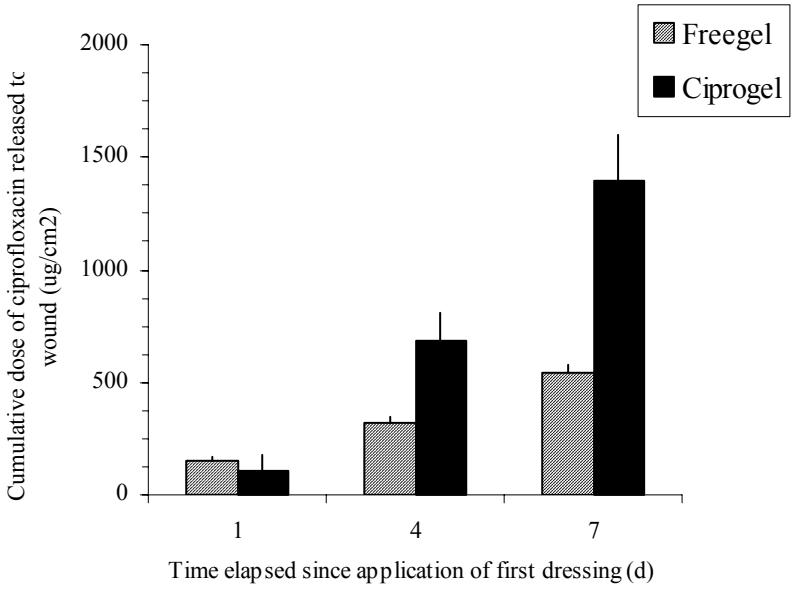

Figure 16. *Cumulative dose of ciprofloxacin released to the wound following application of free ciprofloxacin-loaded (Freegel) or a liposomal ciprofloxacin-loaded wound dressings (Ciprogel). Data are expressed as means ± SEM (n=6 per group).*

These data suggest that application of a given liposomal ciprofloxacin-loaded hydrogel material for up to 3 days was effective in maintaining the bacterial contamination in the wounds below the accepted clinical threshold of contamination (i.e., 10^5 CFU/g). However, despite significantly higher drug levels in the Ciprogel than in the Freegel, the bactericidal activity of the two hydrogels against *Ps. aeruginosa* was comparable. These data, taken together with the large amounts of drug remaining in the free ciprofloxacin-loaded hydrogel dressings, would suggest that the liposomal ciprofloxacin remained entrapped in the hydrogel material, and was not available to exert further bactericidal effect. Thus, liposomal entrapment of the antibiotic was not necessary for the drug to exert its bactericidal effect, nor did it provide a sustained drug release to the wound site

6.4.3 Recommendations

The results from the pilot pig study suggest that the hydrogel technology may be a useful drug delivery system. However, a few technical problems were encountered in performing the study. DRDC Toronto made a series of recommendations to Dr. DiCosmo for improving the dressing to ensure that an adequate product evaluation is carried out in their planned definitive pig study.

6.4.3.1 *Measurement of ciprofloxacin*

Ciprofloxacin levels were determined by fluorimetry in the pilot pig study. In light of the potential alterations in the structure and/or biological activity of ciprofloxacin (e.g., due to over-exposure to UV light; see section 4.2 for details), DRDC Toronto recommended using HPLC to routinely determine the integrity and drug levels of the ciprofloxacin in the dressings. The specificity of this method will ensure that only ciprofloxacin unaffected by the UV irradiation procedures would be measured. To illustrate the potential importance of this issue, ciprofloxacin concentrations in small (n=10; 5.5 mm diameter) and large samples (n=10; 15.8 mm diameter) of the Ciprogel used in the pilot experiment were measured using HPLC. Concentration values of 588 ± 32 ug/cm^2 and 680 ± 34 ug/cm^2 were obtained for the small and large samples, respectively. These values are much lower than that assessed using fluorimetry (859 ± 56 ug/cm^2), suggesting the latter method also measured therapeutically inactive dimers of ciprofloxacin. The large difference observed in the ciprofloxacin content when altering the size of the sample analyzed highlighted the importance of sampling most of the hydrogel dressing covering the wound, rather than 3 small 5-mm samples. Finally, since the hydrogel material was prone to dehydration, the ciprofloxacin concentration should be expressed

per unit weight (i.e., g or mg) of hydrogel dressing rather than per cm^2.

6.4.3.2 *Drug loading procedures*

There were marked differences in the initial doses of free and liposomal ciprofloxacin loaded in the hydrogel dressings. For obvious reasons, such discrepancies in initial drug levels made it difficult to assess conclusively whether liposomes were an essential component in determining the bactericidal efficacy of the hydrogel wound dressing. Typically, Dr. DiCosmo prepared the hydrogel wound dressings with or without liposomes, and then placed the dressings in a bathing solution containing similar ciprofloxacin concentrations. Considering that incorporation of liposomes into a 'vehicle' typically enhances its drug loading capacity, the drug-loading procedures used for the pilot experiment could therefore explain the marked differences in drug levels. Simple modifications of these procedures should normalize the levels of ciprofloxacin in the dressings.

Loading procedures of the different hydrogel dressings (i.e., Freegel and Ciprogel) should be performed using various concentrations of ciprofloxacin in the bathing solution. The final concentrations of free and liposomal drug in the hydrogel wound dressings should then be measured in random samples using HPLC. These calibration curves should enable the selection of the appropriate concentration of the bathing solutions for the Ciprogel and the Freegel dressings that would load comparable initial drug concentrations. These levels should optimally be within 5-10% for different lots of ciprofloxacin-loaded hydrogel wound dressings as well as within a given lot of Ciprogel and Free Cipro dressings.

A preliminary study was performed to validate these drug-loading procedures. Briefly, two batches of hydrogel wound dressings (2.5-cm discs, 2.5 mm thick) were prepared with or without liposomes, using the procedures previously described. Loading of the ciprofloxacin into triplicate hydrogel wound dressings was performed using various concentrations of drug in the bathing solution. The final concentrations of free and liposomal drug in the hydrogel wound dressings (as well as that in the different bathing solutions) were measured in triplicate in each sample using HPLC.

Figure 17 depicts the amount of ciprofloxacin loaded into clear hydrogels (Freegel) and liposomal hydrogels (Ciprogel) when using various bathing solutions. The calibration curves obtained were different for each batch of gels loaded, the differences being

more important for the Ciprogel than the Freegel as well as greater at high concentrations of ciprofloxacin. Based on these findings, DRDC Toronto therefore recommended that new calibration curves be generated for each new lot of hydrogel dressings to ensure that the clear and liposomal hydrogels contain similar amounts of drug. Furthermore, it was strongly suggested to determine the reproducibility of these calibration curves for a given lot of gels. This could be achieved by generating calibration curves on different days using the same lot of clear or liposomal hydrogels.

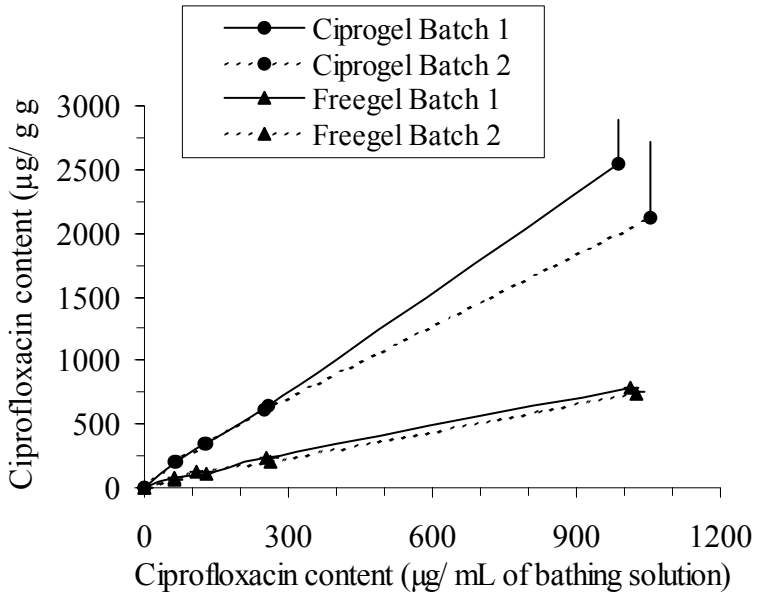

Figure 17. *Effect of varying the concentration of ciprofloxacin in the bathing solution on the drug loading in different batches of clear hydrogels (Freegel) and liposomal gels (Ciprogel). Data are expressed as means ± SEM (n=3 per group).*

Determination of the amount of ciprofloxacin (in triplicate) in the different bathing solutions or in the clear hydrogels yielded relatively small coefficients of variation (<5-8%). In contrast, there was a large variability in the drug content of the liposomal hydrogels, with coefficients of variation ranging from 5-60%. This heterogeneity of the liposomal hydrogels may be due to a large inter-dressing variability in the number of liposomes. Typically, liposomal hydrogels were prepared by pipetting a given

volume of liquid gel solution while gently 'swirling' the beaker. DRDC Toronto recommended that continuous mechanical stirring of the liposomal hydrogel preparation be performed during the preparation of the dressings, to ensure homogeneity of the hydrogel solution.

6.4.3.3 *Optimization of wound contact to dressings*

Incorporation of liposomes in the hydrogel material reduced their flexibility compared to that of the free ciprofloxacin hydrogels. This raised questions whether the method used to secure the hydrogel wound dressings ensured a good contact of the dressings with the pig's dorsum. Though darker 'imprints' of the wounds were observed on all the drug-loaded dressings after 3 days, this phenomenon may have been due to an alteration of the hydrogel material due to evaporation from the wound bed and/or fluids exudating from the wounds rather than caused by a direct contact of the dressing with the wound. Therefore, a study was designed to assess whether the method used to secure the hydrogel dressings ensured a good contact of the dressing with the wound bed.

Briefly, a thermoplastic material (3 mm thick) was shaped and perforated to mimic the first two rows of wounds on the pig's dorsum (Figure 18). A piece of Medipore® adhesive gauze (3M, St-Paul, MN) was applied under the template to close each hole; this created a 3-mm deep "wound". Each pair of "wounds" was covered with a 5 cm x 10 cm piece of hydrogel previously dipped into 10% Povidone Iodine. A Surgipad Combine® dressing (Johnson & Johnson, New Brunswick, NJ) was then applied on top of each 2 pairs of hydrogel dressings, and secured in place with a Transpore® adhesive tape (3M, St-Paul, MN). The entire "wound" area was finally covered with Elastoplast® PVC tape (Smith & Nephew, Lachine, QC). Two types of gauze bolsters were then taped over each "wound area". One strip of tape was then applied across the middle of the template, and several strips were applied from left to right, each almost reaching the edge of the template. The imprint left on the "wound bed" was measured to assess the depth of intrusion of the hydrogel into the simulated wound.

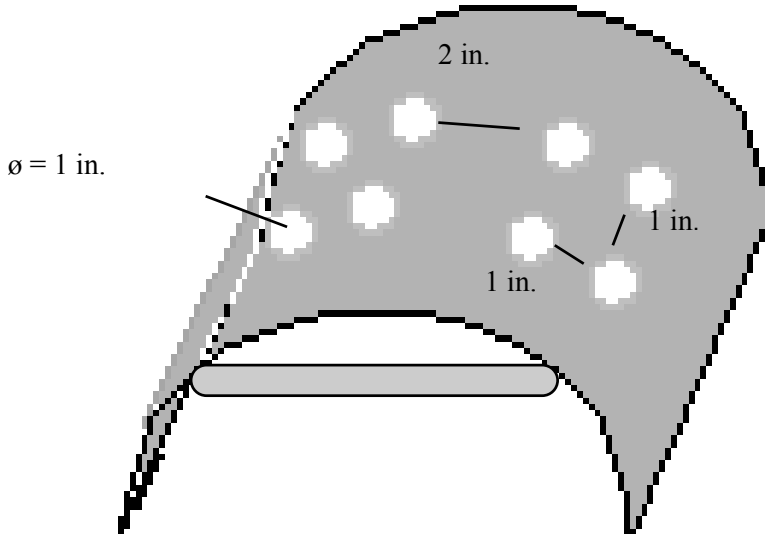

Figure 18. *Diagram of the template simulating the pig's dorsum.*

There were no Povidone Iodine imprints left on the "wound bed" when gauze bolsters were not used under the Elastoplast® PVC tape. This result contrasts with the finding of a bactericidal effect in the pig wounds after a 24 h application of the dressing using similar taping procedures. The lack of "wound contact" in the first test may be due to the fact that the thermoplastic material used to make the template is not compressible. Therefore, the following results obtained using various types of gauze bolsters likely represent a worst-case scenario.

In a second test, gauze bolsters were made by folding a Combine Surgipad® dressing into a 2.5 cm x 2.5 cm square with a thickness of 2.5 cm. The bolsters were then centered over the underlying wound area, using the markings on the template as a guide (e.g., A1, B4, etc.). We expected that the gauze bolsters would press the hydrogel dressing firmly against the "wound bed" when the Elastoplast® PVC tape was applied. Indeed, the imprint observed represented 41 ± 1 % of the surface of the wound (n=24). Furthermore, there was a large variability (CV=15%) in the area

of the "wounds" covered by the dressings, the imprints in the middle columns of "wounds" (i.e., B and C) seeming smaller than those measured in the outer columns of "wounds" (i.e., A and D).

In a third test, we therefore prepared gauze bolsters by enclosing a Combine Surgipad® dressing (2 cm x 2 cm) into the cap of a 25-mL centrifuge tube (2.8 cm diameter). We expected that the pressure applied on the hydrogel dressings would be similar across all 4 wounds of a given row (i.e., row 1 or 2), and would be greater than that exerted when using the previous type of (compressible) gauze bolster. The imprint observed represented 57 ± 1 % of the surface of the wound (n=24), the variability in the area of the "wounds" covered by the dressings being reduced to 11%. However, the imprints left in the middle columns of "wounds" (i.e., B and C) remained smaller (52± 1 %) than those measured in the outer columns (63± 1 %) of "wounds" (i.e., A and D).

These preliminary tests highlighted two important technical considerations for future experiments using the pig model. While the hydrogel wound dressing is relatively thin (2.5 mm) and flexible, it is unlikely to cover entirely the wound bed, regardless of the method used to apply downward pressure on the dressing. As shown in Figure 19, the periphery of the wound bed will never be in contact with the dressing, unless the hydrogel sheets are much thinner than those currently used, and thus become more flexible and compressible. Less ciprofloxacin would obviously be entrapped in thinner hydrogel wound sheets. However, a reduction in the drug concentration may not necessarily be critical (down to a minimal thickness of the hydrogel sheet), since maximal amounts of ciprofloxacin were entrapped in the dressings so far, these amounts corresponding to several MIC.

Another potential concern relates to whether or not the differential effective contact area between the wound and the dressing depending on its location on the pig's dorsum (i.e., 52 % vs. 63%) will also be observed *in vivo*. As mentioned above, the current *in vitro* experimental set-up likely represented a worst-case scenario, since the pig's skin is somewhat compressible. Nevertheless, options are available to minimize these potential problems related to the extent of contact between the wound bed and the hydrogel sheet.

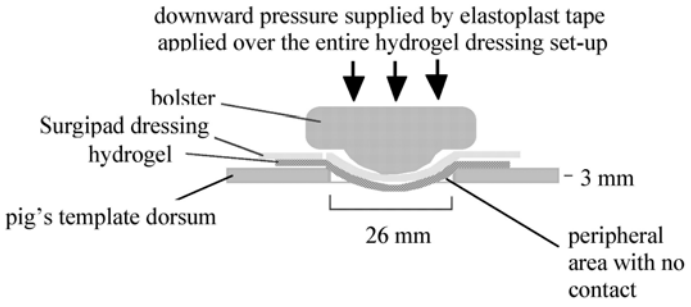

Figure 19. *Diagram describing the experimental set-up for determining the wound surface area in contact with the experimental dressing.*

For the next experiment using the pig model of contaminated wounds, DRDC Toronto recommended modifying the product and the method for applying it as follows. Briefly, hydrogel discs should be cut out from the 5 cm x 10 cm sheet (2.0-2.3 mm thick), using the trephine used for creating the pig's wounds. Each disc would then be snugly inserted into the wound, applied onto the wound bed, and covered with a piece of Allevyn® trilaminate polyurethane dressing (Smith & Nephew, Lachine, QC). The Allevyn® dressing will be applied as recommended by the manufacturer for the first 24 h (i.e., waterproof and bacteria-proof layer facing up). This will allow any significant amount of exudate from the wound to be absorbed through the wound contact layer into the absorbent central foam core. However, upon application of the second hydrogel wound dressing (which will remain in place for 3 or 7 days), the waterproof and bacteria-proof layer of the Allevyn® dressing will be applied face down, to prevent any dehydration of the hydrogel over the duration of the experiment. Similar procedures have been followed successfully using DRDC Toronto's rat model of infected wounds. The pig's dorsum will then be covered with strips of Elastoplast® PVC tape and fabric adhesive tape as previously described. After 3 or 7

days, the hydrogel discs will be collected, and ciprofloxacin content analyzed by HPLC.

We believe that the protocol described above is adequate to provide a proof-of-concept of the bactericidal effect of the hydrogel wound dressings using the pig model. However, for obvious reasons, inserting the non-bioresorbable drug-loaded hydrogel dressing into the wound would not be appropriate for future experiments assessing both the bactericidal effect and the wound healing properties of the product. In such experiments, DRDC Toronto recommended using the same protocol as that used in the pilot pig study for applying the dressing, with the exception of applying downward pressure on the dressing with gauze bolsters to ensure an optimal contact with the wound bed.

6.4.3.4 *Establishment of Standard Operating Procedures*

The technical problems encountered in attempting to achieve a proof-of-concept of the liposomal hydrogel wound dressing using a porcine model of contaminated wounds indicate a need for establishing strict quality control procedures, prior to testing future lots of the product *in vivo*. More specifically, these procedures should include:

1. Preparation of the hydrogel wound dressings under strict aseptic conditions. This will involve using sterile chemicals, autoclaving all laboratory glassware, and performing all critical procedures under a laminar flow hood.

2. Preparation of small volumes of hydrogel solution at any given time, to prevent unforeseen polymerization in the last step of the procedure.

3. Determination of the thickness of the drug-loaded wound dressings in 5 randomly selected samples per batch produced. The lot will be 'red-flagged' if the variability in thickness between Freegel and Ciprogel dressings exceeds 10%.

4. Sterilization of the pre-packaged product using standardized procedures (7 min per side; 250 cm under UV lamp; 8 units per session).

5. Assessment of the integrity of ciprofloxacin by HPLC in 18 samples taken randomly from 3 hydrogel wound dressings in each of the experimental groups (i.e., Ciprogel or Freegel). The lot will be discarded if the structure of ciprofloxacin has been affected during the preparation of the dressings.

6. If the lot fulfills the Quality Control/Quality Assessment (QC/QA) requirements, the ciprofloxacin levels will be measured by HPLC, and expressed in mg per unit wet weight of dressing. The variation in ciprofloxacin levels between the Ciprogel and Freegel dressings should not exceed 5% to proceed to the next step.

7. Upon successful completion of the previous QC/QA steps, the lot of hydrogel wound dressings would be deemed suitable for testing using an *in vivo* animal model of contaminated wounds.

8. Determination of the minimum dose of ciprofloxacin in a hydrogel wound dressing exerting a significant long-term bactericidal effect.

Maximum doses of ciprofloxacin were loaded into the hydrogel wound dressings supplied for testing at DRDC Toronto. While this strategy ensures that all wounds (including those with heavy bacterial loads) will be decontaminated, it can deter from finding out whether liposomes are an essential component in improving the bactericidal efficacy of the hydrogel wound dressings. Thus, DRDC Toronto recommended that the minimum dose of ciprofloxacin, in a liposomal hydrogel wound dressing required to exert a maximum bactericidal effect for up to 7 days, be determined using DRDC's *in vitro* system. Hydrogel wound dressings containing various concentrations of free or liposomal ciprofloxacin will be prepared, and applied on the polyurethane sponges for 1, 3 or 7 days. The appropriate control liposomal hydrogel wound dressing will also be included. The minimum concentration of ciprofloxacin selected will be used in the product to be tested in the *in vivo* animal models.

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7. Conclusions

The present report highlighted a number of technical issues impeding the development of the liposomal antibiotic hydrogel technology as a wound dressing. For example, preparation of homogenous ciprogel products remained challenging, despite introduction by Dr. DiCosmo of several steps to enhance the mixing of the hydrogel formulation and/or slow down the gellification process of the hydrogel mixture. The present results also demonstrated that the current hydrogel formulation was fairly unstable, being more prone to dehydration than some of the commercially available hydrogel products tested at DRDC, such as Vigilon™ (Bard), Elasto-gel™ (Southwest Technologies), CarraDres™ (Carrington Laboratories Inc.), 2nd Skin™ (Spenco Medical Ltd), FLEXDERM™ (Dow Hickam Pharmaceuticals Inc.), ClearSite™ (Conmed Corporation), and NU-GEL™ (Johnson & Johnson). This technical issue could be solved either through further altering the formulation of the hydrogel or marketing the product using a sophisticated packaging system such as that designed by DRDC. However, the latter solution would likely result in potentially high costs in packaging the hydrogel products.

Our observations also pointed to a challenge in maintaining an acceptable shelf-life of the liposomal hydrogel products under non-refrigeration conditions. That concern, taken together with the relatively low absorbency and extreme fragility of the liposomal hydrogel material tested, would indicate that the hydrogel material is not the dressing of choice for front-line casualties, who are typically exposed to adverse environmental conditions. Indeed, one can foresee that the current hydrogel product would easily freeze in cold environments and, conversely, dehydrate under hot conditions, therefore becoming in both cases stiff and unmanageable. It is also noteworthy that the integrity of the hydrogel material may also be compromised due to excessive hydrolysis of the hydrogel material from the bacteria present in the wound environment.

The potential susceptibility of many antimicrobial drugs to standard sterilization procedures, with detrimental side effects on their therapeutic activity levels, remained also a significant challenge in further developing the product that would need to be addressed seriously prior to marketing it.

Regardless of the technical issues raised during the evaluation and testing of the liposomal hydrogel technology, the data obtained both *in vitro* and *in vivo* suggest that this technology could be successfully used as a drug delivery system. Indeed, incorporation of liposomal ciprofloxacin in the hydrogel material was very effective in limiting the progression of infection to deeper tissues in the DRDC rat model of infected full-thickness wounds as well as in infected full-thickness burns. Furthermore, application of liposomal ciprofloxacin hydrogel to full-thickness wounds was also effective in treating an established infection in both rat and pig models.

However, there was no therapeutic advantage of entrapping an antibiotic (e.g. ciprofloxacin) into liposomes, as suggested by comparable bactericidal activities

against Gram-negative bacteria of hydrogels loaded with either free or liposomal ciprofloxacin. Our observation that application of a free ciprofloxacin-loaded dressing maintained the bacterial load in infected wounds below the accepted clinical threshold of infection further pointed to a minimal benefit of the liposomal entrapment. Furthermore, liposomal entrapment of large amounts of ciprofloxacin did not provide a sustained-release bactericidal effect in the porcine model of infected wounds. In fact, the lack of a long-term bactericidal effect of the liposomal hydrogel dressing, taken together with the large amounts of drug remaining in the dressings upon their removal from the infected full-thickness pig wounds, suggested that the liposomal ciprofloxacin remained entrapped in the hydrogel material and was not available to exert a significant bactericidal effect. Considering the present experimental results, and the fact that incorporation of liposomes into a product will introduce additional manufacturing costs, there did not appear to be any commercial or marketing edge to the liposomal hydrogel technology.

In summary, taking into consideration the results from the assessment of the bactericidal properties of the hydrogel technology as well as the technical issues related to its development, further development of the liposomal antibiotic hydrogel material as a wound dressing for front-line military casualties did not appear promising. Therefore, we advised Dr. DiCosmo that no further R&D efforts would be undertaken by DRDC.

8. References

1. Gilenko, I.O., Shevchenko, V.S., Dikii, I.L., Strel'nikov, L.S., and Bashtan, V.P.. (1996). Use of liposomes and biologically active soluble antibiotics in the prevention and treatment of post-operative infectious-suppurative complications. *Klin. Khir.*, 10: 53-54.

2. Grayson, L.S., Hansbrough, J.F., Zapata-Sirvent, R., Roehrborn, A.J., Kim, T., and Kim, S. (1995). Soft tissue infection prophylaxis with gentamicin encapsulated multivesicular liposomes: results from a prospective, randomized trial.. *Crit. Care Med.* 23(1): 84-91.

3. Lichtenstein, A., and Margalit, R. (1995). Liposome-encapsulated silver sulfadiazine (SSD) for the topical treatment of infected burns: thermodynamics of drug encapsulation and kinetics of drug release. *J. Inorg. Biochem.* 60(3): 187-198.

4. Margalit, R. (1995). Liposome-mediated drug targeting in topical and regional therapies. *Crit. Rev. Therap. Drug Carrier Syst.* 12(2-3): 233-261.

5. Pallua, N., and Schneider, W. (1992). Candida infection in the severely burned patient – a successful treatment concept with liposomal amphotericin B. *Unfallchirurg.* 95(12): 641-643.

6. Price, C.I, Horton, J.W., and Baxter, C.R. (1990). Topical liposomal delivery of antibiotics in soft tissue infection. *J. Surg. Res.* 49(2): 174-178.

7. Price, C.I, Horton, J.W., and Baxter, C.R. (1992). Liposomal delivery of aminoglycosides in burn wounds. *Surg. Gynecol. Obstret.* 174(5): 414-418.

8. Price, C.I, Horton, J.W., and Baxter, C.R. (1994). Liposome encapsulation: a method for enhancing the effectiveness of local antibiotics. *Surgery* 115(4): 480-487

9. Shraer, T.I., Kreines, V.M., Golubchikova, N.A., Buinyi, V.V., and, Vamburg, I.M. (1988). Use of liposome suspensions in experimental local suppurative processes. *Khirurgiia*, 4:30-44.

10. Shraer, T.I., Shaposhnikov, I.U., and Kreines, V.M. (1994). Useof liposomes in early treatment of experimental wounds. *Khirurgiia.* 12: 35-38.

11. Reimer, K., Fleischer, W., Brogmann, B., Schreier, H., Burkhard, P., Lazendorfer, A., Gumbel, H., Hoekstra, H., and Behrens-Baumann, W. (1997). Povidone-iodine liposomes – an overview. *Dermatology*. 195(Spuppl 2): 93-99.

12. Eaglstein, W.H. (2001). Moist wound healing with occlusive dressings: a clinical focus. *Dermatol. Surg.* 27(2): 175-181.

13. dig out reference on antibiotic resistance.

List of symbols/abbreviations/acronyms/initialisms

ATCC	American Type Culture Collection
CFU	Colony Forming Unit
Ciprogel	Liposomal ciprofloxacin-loaded hydrogel
DND	Department of National Defence
DPPC	dipalmitoylphosphatidylcholine
Freegel	Free ciprofloxacin
HPLC	High Performance Liquid Chromatography
Lipogel	Plain liposomal hydrogel
MIC	Minimum Inhibitory Concentration
PBS	Phosphate Buffered Saline
PEG	Polyethylene glycol
PVC	Polyvinylchloride
Ps.	*Pseudomonas*
QC/QA	Quality Control/Quality Assessment
Staph.	*Staphyloccoccus*
UV	Ultraviolet

DOCUMENT CONTROL DATA SHEET

1a. PERFORMING AGENCY
DRDC Toronto

1b. PUBLISHING AGENCY
DRDC Toronto

2. SECURITY CLASSIFICATION

UNCLASSIFIED
Unlimited distribution -

3. TITLE

(U) Development and evaluation of a hydrogel technology for its potential as a wound dressing

4. AUTHORS

Martineau, L., Shek, P.N.

5. DATE OF PUBLICATION

August 10 , 2003

6. NO. OF PAGES

51

7. DESCRIPTIVE NOTES

8. SPONSORING/MONITORING/CONTRACTING/TASKING AGENCY
Sponsoring Agency:
Monitoring Agency:
Contracting Agency :
Tasking Agency:

9. ORIGINATORS DOCUMENT NO.

Technical Report TR 2003-103

10. CONTRACT GRANT AND/OR PROJECT NO.

11. OTHER DOCUMENT NOS.

12. DOCUMENT RELEASABILITY

Unlimited distribution

13. DOCUMENT ANNOUNCEMENT

Unlimited announcement

14. ABSTRACT

(U) This report describes the development and in vivo testing of a proprietary hydrogel material technology developed under research contract for use as a wound dressing. Application of the liposomal hydrogel for 24 h provided a barrier that effectively prevented contamination of full-thickness wounds and burns in rats. Furthermore, application of liposomal ciprofloxacin-loaded hydrogel (Ciprogel) was also very effective both in limiting the progression of the infection to deeper tissues and, in treating an established wound infection in both rat and pig models of full-thickness wounds. While these studies indicated that Ciprogel was an effective drug delivery system, other experiments suggested that entrapment of ciprofloxacin in liposomes was neither required when large amounts of ciprofloxacin were loaded in the hydrogel nor did it provide a sustained-release bactericidal effect. A number of challenging technical issues for the development of the liposomal antibiotic hydrogel technology as a wound dressing were also identified during the product evaluation. In summary, taking into consideration the results from the assessment of the bactericidal properties of the hydrogel technology as well as the technical issues related to its development, the successful application of the liposomal antibiotic hydrogel material as a wound dressing for front-line military casualties remains to be developed.

(U) Ce rapport décrit le développement et la mise à l'essai in vivo d'une technologie exclusive d'hydrogel mise au point dans le cadre d'un contrat de recherche pour l'utilisation de ce produit comme pansement. L'application de l'hydrogel liposomal pendant une période de 24 heures constituait une barrière qui prévenait efficacement la contamination des blessures profondes et des brûlures au troisième degré chez des rats. En outre, l'application d'hydrogel liposomal chargé de ciprofloxacine (Ciprogel) s'est également révélée très efficace tant pour ce qui est de limiter la progression de l'infection aux tissus sous?jacents que de traiter une infection établie dans les modèles murin et porcin de blessures entraînant la destruction du derme. Bien que ces études aient indiqué que le Ciprogel était un système efficace d'administration de médicament, d'autres expériences semblent montrer que l'encapsulation de la ciprofloxacine dans des liposomes n'est pas nécessaire lorsque des quantités importantes de ciprofloxacine sont chargées dans l'hydrogel et n'entraînent pas un effet bactéricide prolongé. Au cours de l'évaluation du produit, plusieurs difficultés techniques inhérentes au développement de l'hydrogel liposomal antibiotique devant être utilisé comme pansement ont été mises au jour. En résumé, étant donné les résultats de l'évaluation des propriétés bactéricides de l'hydrogel et les difficultés techniques liées au développement d'un tel produit, l'application efficace de l'hydrogel liposomal antibiotique comme pansement pour les blessures subies par les militaires en première ligne reste à développer.

15. KEYWORDS, DESCRIPTORS or IDENTIFIERS

(U) hydrogel; wound dressing; bactericidal