In vitro microbial inhibition and cellular response to novel biodegradable composite wound dressings with controlled release of antibiotics

J.J. Elsner a, I. Berdicevsky b, M. Zilberman a,*

a Department of Biomedical Engineering, Tel-Aviv University, Tel-Aviv 69978, Israel
b Department of Microbiology, Technion – Israel Institute of Technology, Haifa 32000, Israel

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About 70% of all people with severe burns die from related infections, despite advances in treatment regimens and the best efforts of nurses and doctors. Although silver-eluting wound dressings are available for addressing this problem, there is growing evidence of the deleterious effects of such dressings in delaying the healing process owing to cellular toxicity. A new concept of antibiotic-eluting composite wound dressings is described here. These dressings are based on a polyglyconate mesh coated with a porous poly-(α-lactic-co-glycolic acid) matrix loaded with antibiotic drugs. The effect of antibiotic release on bacterial inhibition was studied, and cell cytotoxicity was examined. The dressings resulted in a 99.99% decrease in the viable counts of Pseudomonas aeruginosa and Staphylococcus albus at very high initial inoculations of $10^7–10^8$ CFU ml$^{-1}$ after 1 day, while such a decrease in Staphylococcus aureus was obtained within 3 days. Bacterial inhibition zones around the dressing material were found to persist for 2 weeks, indicating a long-lasting antimicrobial effect. Despite severe toxicity to bacteria, the dressing material was found to have no toxic effect on cultured fibroblasts, indicating that the novel antibiotic-eluting wound dressings represent an effective option for selective treatment of bacterial infections.

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1. Introduction

Burn wound infections are among the most important and potentially serious complications that occur during the acute period following injury. Although burn wound surfaces are sterile immediately following thermal injury, colonization with autogenous micro-organisms (originating from the skin, gastrointestinal and respiratory flora) or through contact with the contaminated environment (water, air and healthcare workers) generally occurs within 48 h [1,2]. The typical burn wound is initially colonized predominantly with Gram-positive organisms, which are replaced by antibiotic-susceptible Gram-negative organisms within ~1 week after the burn injury. If wound closure is delayed and the patient becomes infected, thus requiring treatment with broad-spectrum antibiotics, these flora may be replaced by yeasts, fungi and antibiotic-resistant bacteria [3]. Staphylococcus aureus and Pseudomonas aeruginosa are the most frequently isolated organisms in most burn units [3]. Systemic treatment against infection is limited by inadequate wound perfusion, which restricts migration of host immune cells and the delivery of antimicrobial agents to the wound. In this case, the local concentration of the antibiotics may be insufficient and may lead to bacterial resistance. The widespread application of a topical antimicrobial agent on the open burn wound surface can substantially reduce the microbial load and risk of infection [4]. However, this requires frequent changes of the dressing material, which causes inconvenience to the patient and is a financial burden to the healthcare system. Uncomplicated skin infections account for almost 200 million annual physician-office visits in the US, and treatment of these infections is estimated to cost over US$350 million each year [5].

Improved wound dressings that provide an inherent antimicrobial effect by eluting germicidal compounds have been developed to respond to the problems associated with conventional topical treatments with ointments and creams. Wound dressings that incorporate iodine (Iodosorb® by Smith & Nephew), chlorohexidine (Biopatch® by J&J) or, most frequently, silver ions (e.g., Acticoat® by Smith & Nephew, Actisorb® by J&J and Aquacel® by ConvaTec) as active agents are available on the market. Such dressings are designed to provide controlled release of the active agent through a slow but sustained release mechanism which helps avoid toxicity yet ensures delivery of a therapeutic dose to the wound. Despite frequent use, there is growing evidence that silver is highly toxic to keratinocytes and fibroblasts and may delay burn wound healing if applied indiscriminately to healing tissue areas [6–8]. Furthermore, most of these dressing materials require frequent changes. Bioreversible dressings may successfully address this shortcoming, since they do not need to be removed from the wound surface once they have fulfilled
their role. Biodegradable dressings made of synthetic lactide-caprolactone copolymers [9] and natural polymers such as collagen [10], chitosan/chitin [11] and alginate [12] are already available on the market. Specifically, biological materials such as collagen and chitosan have been reported to perform better than conventional and synthetic dressings in accelerating granulation tissue formation and epithelialization [13,14]. Gentamicin-eluting collagen sponges have been found useful in both partial-thickness and full-thickness burn wounds. Collatamp® (Innocoll GmbH, Germany), Sulmycin®-Implant (Scherer-Plough, USA) and Septocoll® (Biomet Merck, Germany) are examples of such products that have been found to accelerate both granulation tissue formation and epithelialization. Because these products elute gentamicin, they also protect the recovering tissue from potential infection or re-infection [15]. However, the release of antibiotics directly from natural polymers suffers from several disadvantages. First, most natural polymers are hydrophilic and cannot counteract rapid release of the small antibiotic molecules upon water uptake, unless they are highly cross-linked. Second, natural polymers undergo degradation by proteases. The incorporated drug is thus released by a combination of diffusion and natural enzymatic breakdown of the protein, and is dependent on the biochemical wound setting. Consequently, the active agent is rapidly released from these materials [14,16].

A new concept in wound dressing design which combines the advantages of occlusive dressings with biodegradability and effective intrinsic topical antibiotic treatment has recently been developed and studied. The new composite dressing material, which is based on a polyglyconate mesh coated with a porous poly(DL-lactide-co-glycolic acid) matrix, is designed to protect the wound until it is no longer needed, after which it dissolves into non-toxic end products by chemical (hydrolytic) degradation. Taken together, both mesh and matrix materials synergistically produce properties unavailable in the individual constituent materials, allowing the designer to choose an optimum combination. The reinforcing polyglyconate mesh affords the necessary mechanical strength to the dressing, whereas the porous PDLGA binding matrix is aimed to provide adequate moisture control and release of antibiotics between several days and 3 weeks, to protect the wound bed from infection and promote healing. Being biodegradable and drug-eluting, this material can prevent the need for constant wound cleaning and redressing and should enable the body to cope better with healing and reduce patient pain and suffering. This dressing concept also solves current mechanical and physical limitations in wound dressing techniques and may afford physicians a new and more effective platform for treating wounds. Such biodegradable drug-eluting wound dressings, which present an alternative to silver-eluting dressings, are currently not available on the market. The present paper focuses on the antibiotic release profile from this bioresorbable material and its effect on bacterial inhibition. Cytotoxicity results are also described.

2. Materials and methods

2.1. Materials

Maxon™ polyglyconate monofilament sutures with a diameter of 0.20–0.25 mm (United States Surgical Inc.) were surface-treated in order to dispose of the original fiber’s coating and enhance adhesion between the fiber and the coating. The Maxon fibers were dipped in 1,1,3,3-tetrafluoro-2-propanol (Spectrum Chemical Mfg. Corp. H1008) for 40 s and then washed with 70% ethanol and dried before being woven to produce the mesh foundation for the wound dressing. For the mesh coating, 50/50 poly-(DL-lactic-co-glycolic acid) (PDLGA), MW ~100 KDa (Absorbable Polymer Technologies, Inc., USA) was used. The antibiotics used were ceftazidime hydrate (Sigma C-3809) and gentamicin sulfate (Sigma G-1264). The surface active agents were bovine serum albumin (BSA), molecular weight, 66,000 Da, Sigma (A-4503) and sorbitan mono-oleate (Span 80, Sigma 85548).

2.2. Emulsion preparation

The inverted emulsions used in this study were prepared by the homogenization of two immiscible phases: an organic solution containing 20% w/v PDLGA in chloroform, and an aqueous phase containing 5%, 10% or 15% w/w antibiotics (either ceftazidime or gentamicin) in double-distilled water. BSA or Span was included at 1% w/v in the aqueous or organic phase, respectively, as a surfactant. Homogenization of the two phases, at an organic to aqueous ratio (O:A) of 6:1 (BSA) or 12:1 (Span) was performed for the duration of 90 s at 16,000 rpm using a Kinematica PT-3100 Polytron homogenizer. These combinations of phase ratios and surfactants were chosen based on their ability to induce medium-high (BSA) and low (Span) burst release rates followed by continuous antibiotic release spanning several days to 2 weeks [17].

2.3. Preparation of the composite dressing

Composite wound dressings were created using the freeze-drying of an inverted emulsion technique. In brief, the woven fibers were dip-coated in freshly prepared inverted (water in oil) emulsions containing the drug and polymer and then immediately frozen in a liquid nitrogen bath. The samples were then placed in a pre-cooled (~105 °C) freeze-dryer (VirTis 101) and freeze-dried overnight in order to preserve the microstructure of the emulsion-based structures.

2.4. Morphological characterization

The morphology of the wound dressing’s structures was observed by scanning electron microscopy (SEM) using a Jeol JSM-6300 instrument at an accelerating voltage of 5 kV. Cryogenically fractured surfaces were Pd-sputtered prior to observation.

2.5. In vitro drug release studies

The composite wound dressings were immersed in phosphate buffered saline (PBS) at 37 °C for 28 days in order to determine the various drug release kinetics from these structures. The release studies were conducted in closed glass vessels containing 1.5 ml PBS. The medium was removed (completely) periodically at each sampling time (6, 12 h, 1, 2, 3, 5, 7, 14, 21 days), and fresh medium was introduced.

2.5.1. Ceftazidime assay

The medium’s ceftazidime content was determined using a Jasco HPLC with a UV 2075 plus detector and a reverse phase column (Interstil® ODS-3V 5 mm; inner diameter, 4.6 mm; length, 250 mm) kept at 25 °C. The mobile phase consisted of a mixture of PBS and acetonitrile (95/5, v/v) at a flow rate of 1 ml min⁻¹ with a quaternary gradient pump (PU 2089 plus) without gradient. Twenty-microliter samples were injected with an autosampler (AS 2057 Plus). The column effluent was eluted for 22 min and detected at 254 nm. The area of each eluted peak was integrated using EZstart software version 3.1.7. A calibration curve was prepared for concentrations ranging from 1.0 to 200.0 µg ml⁻¹ (correlation coefficient >0.999; slope, 0.0000318).

2.5.2. Gentamicin assay

Determination of the medium’s gentamicin content was carried out using an Abbott Therapeutic Drug Monitoring System (TDX, Abbott Laboratories) according to the manufacturer’s instructions.
This machine enables the gentamicin concentration to be determined based on a polarization fluoroimmunoassay, using fluorescein as a tracer. Briefly, the latter is excited by polarized light. Polarization of the emitted light is dependent on molecule size. The free and labeled drug competes for binding sites. The drug concentration in the sample is proportional to the scatter of polarized light caused by the free labeled drug. The measurable concentration range without dilution is 0.0–100 μg ml⁻¹. Higher drug concentrations were measured after carrying out manual dilution.

2.6. Antimicrobial activity

_Staphylococcus aureus_ (S. aureus), _Staphylococcus albus_ (S. Albus) and _Pseudomonas aeruginosa_ (P. aeruginosa) were used in this study. All three strains were clinically isolated at the Microbiological Laboratory of Rambam Medical Center (Haifa, Israel), and their minimal inhibitory concentration (MIC) values were evaluated (Table 1). The strains were grown overnight on Müller–Hinton (Difco) agar plates at 37 °C prior to use. The bacterial cells were collected and re-suspended in saline, and adjusted to 10⁷ CFU ml⁻¹ (colony forming units) by visual comparison with a 0.5 McFarland standard. Drug release-induced bacterial inhibition was evaluated using the following two methods.

2.6.1. Corrected zone of inhibition

The corrected zone of inhibition test (CZOI) was used to determine the time-dependence of the antimicrobial activity of the wound dressing. In this modified version of the Kirby–Bauer disc diffusion test, which is typically used to determine bacterial susceptibility to antibiotics, round pieces of dressing materials (D = 10 mm) were placed on a bacterial lawn (100 μl of inoculum (~10⁷ CFU ml⁻¹), seeded on Müller–Hinton agar plates), incubated overnight at 37 °C and then photographed. The inhibition zone area around the dressings (A) was measured from the images by means of digital image processing software (SigmaScan Pro) by placing a circular mark to cover the circumference of the round inhibition zone (ignoring unclear interlapping between adjacent samples, as demonstrated in Fig. 4b). An effective diameter (D) of the circle was calculated according to Eq. (1):

\[
D = \sqrt{\frac{4 \cdot A}{\pi}}
\]

The CZOI was then calculated by subtracting the dressing diameter from D. This procedure was repeated on dressing materials which were incubated in PBS, with daily replacement of the medium for 3, 5, 7 and 14 days prior to testing. The test was performed in triplicate for each type of dressing and for each of the three micro-organisms: _S. aureus_, _S. albus_ and _P. aeruginosa_.

2.6.2. Viable counts

A release study from selected wound dressings in the presence of bacteria was performed in order to study the effect of drug release on the kinetics of residual bacteria. At the beginning of the release study, 1 ml of the bacterial strains at a concentration of 1 × 10⁷ CFU ml⁻¹ was added, and the effect of the antibiotics released from the dressings on residual bacteria was tested. Microorganisms in the presence of PBS only served as controls. Samples of 10 or 100 μl were collected at time 0 and after 6 h, 24 h, 3 d, 7 d and 14 d, and spread on agar plates containing Müller–Hinton. CFU ml⁻¹ were counted after 24 h incubation at 37 °C.

2.7. Cytotoxicity

2.7.1. Cell culture

Primary cultures of human fibroblasts were obtained from neonatal foreskins. The isolated cells were plated in 25 cm² T-flasks with modified Eagle's medium supplemented with heat-inactivated fetal bovine serum (10% v/v), l-glutamine (2 mM), penicillin (100 U ml⁻¹) and streptomycin (100 mg ml⁻¹). The cells were kept in culture at 37 °C in a humidified atmosphere with 5% CO₂. After reaching 70–80% confluence, the cells were sub-cultivated by 1-min incubation in 0.18% trypsin and 5 mM EDTA. The free cells were added to three times the volume of the original culture medium and re-suspended equally between three flasks. Once an adequate number of cells had been attained, the cells (at passages 9–12) were seeded in 6-well plates using the same medium without penicillin and streptomycin (1.5 ml/well). The medium was replaced with fresh medium 24 and 72 h after seeding. After reaching 70–80% confluence, round discs of the tested dressing material (D = 10 mm) were introduced into the medium.

2.7.2. Alamar Blue assay of cell viability

The Alamar Blue (AB) assay was used to assess the effect of the dressing material, and specifically the active agents incorporated in it, on cell viability [18–20]. This method involves the addition of a non-toxic fluorogenic redox indicator to the culture medium. The oxidized form of AB has a dark blue color and little intrinsic fluorescence. When taken up by cells, the dye becomes reduced and turns red. This reduced form of AB is highly fluorescent. The extent of the AB conversion, which is a reflection of cell viability, can be quantified spectrophotometrically at wavelengths of 570 and 600 nm.

The AB assay was performed on the cell cultures before introducing the dressing materials and then every 24 h for 3 days. The evaluation process included replacing the original medium with fresh medium containing AB (10% v/v), incubating the cells for 4 h, and taking spectrophotometric readings at 570 and 600 nm. Thereafter, the medium was again replaced with fresh medium, and the cultures were returned for incubation until the next reading point. Relative changes in the readings, which are indicative of changes in cell proliferation, were compared with the initial reading (t₀). Calculation of the percentage change in AB reduction over time (%AB reduction) per culture plate was performed according to the manufacturer’s protocol:

\[
\% \text{AB reduction} = \frac{(A_{570}^t - A_{570}^0) - (A_{600}^t - A_{600}^0)}{(A_{570}^0 - A_{600}^0)}
\]

where A₅₇₀ and A₆₀₀ represent the molar extinction coefficients of oxidized AB at 570 and 600 nm, respectively. A₅₇₀ and A₆₀₀ represent the absorbance of test and control cells (media with AB and no cells), respectively, at 570 and 600 nm. Comparisons were then made between the dressing materials and two controls: cultures grown without dressing material and cultures exposed to a dressing devoid of any drug. The concentrations of cefazidime/gentamicin in the removed growth medium were determined as described above at each time point.

2.8. Statistical analysis

Data points are expressed as means ± standard deviations. Differences between means were analyzed for statistical significance.
using one-way ANOVA with the Tukey–Kramer multiple comparisons post-test (SPSS version 17.0). \( p \) values \( \leq 0.05 \) were considered significant.

3. Results

3.1. Dressing structure

A composite wound dressing composed of a plain-woven polyglyconate mesh bound by a continuous poly-(DL-lactic-co-glycolic acid) (PDLGA) porous matrix loaded with antibiotics was developed and studied (Fig. 1a). The PDLGA matrix adhered well to the fibers, forming a skin layer with a thickness of \( \sim 60 \mu m \) (Fig. 1b). The technique of freeze-drying of inverted emulsions which was used to create the PDLGA binding matrix enabled a highly porous microstructure to be achieved (shown in Fig. 1c and d), which also acted as a reservoir for the antibiotic incorporated within it.

3.2. In vitro drug release studies

The cumulative release of antibiotics from dressings based on emulsion formulations containing 5%, 10% and 15% (w/w) of either gentamicin or ceftazidime stabilized with BSA or Span are presented in Figs. 2 and 3, respectively. Release profiles from dressings stabilized with BSA typically demonstrated a high burst release of antibiotics, followed by a gradual release at a decreasing rate over time (Fig. 2). The relative portion of drug released in the burst effect decreased when a higher drug loading was used, and the lowest burst release ratios for gentamicin and ceftazidime (59% and 42%, respectively) were obtained with a 15% drug loading. Release profiles from dressings stabilized with Span (Fig. 3) differed substantially from those obtained with BSA-stabilized emulsions. First, the burst release of both gentamicin and ceftazidime was reduced considerably to only 4% and 6%, respectively. Second, the overall release rate of both drugs was reduced. Dressings containing gentamicin displayed a dual-phase release profile, consisting of a slow release over 5 days followed by an increased release over the next 2 weeks. Dressings containing ceftazidime demonstrated a constant release over the first 3–5 days, followed by release at a decreasing rate.

3.3. CZOI studies

Representative photographs of inhibition zones created around dressing materials loaded with 10% gentamicin or ceftazidime and stabilized with BSA are presented in Fig. 4a–f. All three bacterial strains used in the study (\( P. aeruginosa \), \( S. aureus \), \( S. albus \)) demonstrated susceptibility to gentamicin and ceftazidime at the initial time point. Similar wound dressings which were incubated in PBS for 3, 5, 7 and 14 days prior to the bacterial challenge demonstrated various levels of bacterial inhibition, indicating that the dressing material retains its antimicrobial properties over time. Gentamicin is a more potent antimicrobial drug (Table 1) and was generally released in larger quantities (Fig. 2). It displayed better results than ceftazidime in terms of inhibition zone diameter and lasting antibacterial effect. The presence of bacterial inhibition in an area that exceeds the dressing material (CZOI > 0) can be considered beneficial. CZOI results for dressings derived from emulsions containing 5%, 10% and 15% antibiotics and stabilized with BSA are presented in Fig. 5. Dressing materials containing gentamicin maintained their antibacterial potency against all the micro-organisms over 2 weeks. However, they were most effective in killing \( S. aureus \) (Fig. 5b) and least effective in killing \( P. aeruginosa \) (Fig. 5a). Dressings containing ceftazidime were most effective in killing \( P. aeruginosa \) (Fig. 5d), and their inhibition zone was dose- and time-dependent across all specimens. Only samples loaded with 10% and 15% ceftazidime retained some degree of inhibition after 1 week.

The CZOI was also evaluated for dressing materials with a reduced burst release, stabilized by Span, focusing on the mid-range 10% antibiotic loading ratio. Samples containing gentamicin exhibited inhibition zones similar to those shown when BSA was used as surfactant. In contradistinction, both the magnitude and the dura-

Fig. 1. The structure of the biodegradable composite wound dressing composed of polyglyconate fibers surrounded by a continuous PDLGA porous matrix. (a) Photograph of the wound dressing; (b) cross-sectional cryo-fractured SEM image demonstrating the plain-weave basic unit structure; (c and d) the microstructure of the porous matrix.
tion of the inhibition effect of ceftazidime-loaded materials were reduced (Fig. 6).

3.4. Viable counts

The purpose of these experiments was to monitor the effectiveness of cumulative antibiotic release from the wound dressings in terms of the residual bacteria compared with initial bacterial inoculations of $10^7$–$10^8$ CFU ml$^{-1}$, which correspond to severe infection. This investigation focused on samples based on BSA-stabilized emulsions containing 10% antibiotics. Bacteria present in PBS only served as the control. The results are presented in Fig. 7. Relatively high numbers of bacteria, above $10^5$ CFU ml$^{-1}$, survived in $P.\ aeruginosa$ and $S.\ albus$ controls over 1 week, whereas $S.\ aureus$ displayed lower survival in the presence of PBS. Total eradication of $P.\ aeruginosa$ and $S.\ albus$ due to gentamicin release from dressing samples occurred within 3 days (Fig. 7a and b). At this time point, the amount of $S.\ aureus$ was reduced considerably, by four orders of magnitude, to $<10^5$ CFU ml$^{-1}$. However, in this case total eradication was reached only after 1 week (Fig. 7c). Samples containing ceftazidime exhibited a milder effect of reducing bacterial growth. Total eradication of $S.\ albus$ was reached after 1 week, whereas eradication of $P.\ aeruginosa$ and $S.\ aureus$ was obtained within 2 weeks.

3.5. Cell cytotoxicity tests

The cultured fibroblasts responded well to the different dressing materials. The basic dressing material, consisting of the fully processed freeze-dried polyglyconate/PDLGA composite without additional antibiotics, was not found to affect cell viability compared with control cell cultures (Fig. 8). Similarly, fibroblast viability was maintained in the presence of dressing materials loaded with 5% gentamicin (Fig. 8a). Some decrease in viability occurred in the presence of a gentamicin loading of 15%. However, this decrease was small and was not statistically different from the control. Dressing material loaded with the antibiotic drug ceftazidime (at 5% and 15%, stabilized with BSA and Span) also maintained cell viability at levels similar to the control or higher (Fig. 8b).

Additional cytotoxicity tests were conducted to determine the threshold for an acute toxic reaction to the antibiotics. Fibroblast cell cultures exposed to a medium containing known antibiotic concentrations higher than those measured in the presence of the dressing materials (Table 2) were monitored after 24 h. Inspection of the cultures by inverted light microscopy demonstrated that cells exposed to gentamicin concentrations as high as 1500 lgm l$^{-1}$ remained confluent and with a gross appearance similar to the control (Fig. 9a and e). In cultures exposed to ceftazidime, sporadic cell detachment from the culture plate occurred already at 500 lgm l$^{-1}$ (Fig. 9b) and became widespread at 1000 lgm l$^{-1}$ and higher, where the majority of the fibroblasts became apoptotic and exhibited a typical spherical shape (Fig. 9c). Alamar Blue reduction assays confirmed that gentamicin has a relatively mild effect on cell viability, with a 20% reduction in fibroblast viability subsequent to a 24 h exposure to 1500 lgm l$^{-1}$. Ceftazidime was found to be more cytotoxic, since cell viability was reduced by 25% at 500 lgm l$^{-1}$ and by ~50% and 70% at 750 and 1500 lgm l$^{-1}$, respectively, after a 24 h exposure.
4. Discussion

In this study, a biodegradable composite wound dressing based on a fibrous polyglyconate mesh and a porous antibiotic-loaded PDLGA binding matrix was developed and studied. It was hypothesized that incorporation of broad-spectrum antibiotics such as gentamicin or ceftazidime in wound dressings would help to reduce the bio-burden in the wound bed and thus prevent infection and accelerate wound healing. The findings show that the in vitro controlled release of these antibiotics from the wound dressing can eradicate large numbers of bacteria for up to 2 weeks, without adversely affecting the viability of fibroblast cells, which play an active role in the wound healing process.

Silver ions, which are the most commonly used topical antimicrobial agent in burn wound care products, do not discriminate between cells involved in the healing process and pathogenic bacteria. Several recent tissue culture studies have shown that silver ions can cause lethal damage to both keratinocytes and fibroblasts [6,7,21,22]. Such tests are probably a severe estimate, since rapid inactivation by chloride and protein occur in the wound in the clinical environment [6]. However, the inactivation of silver ions concomitantly reduces their antibacterial potency. Since the bacterial and cellular toxic silver dose is within a similar range (7–55 mg ml⁻¹) [6], it has been suggested that silver-based products should be used with caution in situations where rapidly proliferating cells may be harmed, such as in superficial burns and the application of cultured cells [6,22].

Despite growing evidence of bacterial resistance, antibiotics still represent an effective and selective treatment option against bacterial infections. Aminoglycosides such as gentamicin are often used as prophylaxis when skin excisions and transplantation are undertaken. However, aminoglycosides have a narrow therapeutic index and are known for their potential nephro- and ototoxicity when used systemically, such that frequent drug and renal function monitoring are mandatory [23]. Ceftazidime is a third generation cephalosporin antibiotic which is very active against Gram-negative bacteria, including P. aeruginosa, and is a suitable antibiotic for the prophylaxis and therapy of bacterial infections in patients with severe burns [23,24]. The release of these antibiotics directly to the wound, and in a controlled manner, should enable a high local concentration to be reached, while avoiding systemic toxicity.

4.1. Effect of the loaded drugs and their content on the release profile and the bacterial inhibition

In the current study, a variety of antibiotic release profiles were obtained by modification of the composition and structure of the binding matrix (drug reservoir). Specifically, the release of antibiotic contents at high (>90%), intermediate (40–60%) and low (<5%) burst release rates and over release spans ranging from several days to 3 weeks was demonstrated. The versatility of the drug release profiles from porous freeze-dried PDLGA matrices has already been demonstrated in a previous work, which explains the

Fig. 3. Cumulative release of antibiotics from wound dressings derived from emulsions containing 5% (w/w) ( ), 10% (w/w) ( ) and 15% (v/w) ( ) antibiotics and stabilized with 1% (w/v) Span. (a) Absolute quantity of released gentamicin (per D = 10 mm disc); (b) % release of total encapsulated gentamicin; (c) absolute quantity of released ceftazidime (per D = 10 mm disc); (d) % release of total encapsulated ceftazidime.
effects of the inverted emulsion’s formulation parameters on the porous structure and on the resulting antibiotic release profile in detail [25]. In particular, lower burst release rates and longer elution durations can be achieved through structuring towards a reduced pore size, pore connectivity and total porosity. For example, increasing the polymer content in the emulsion and using a higher polymer molar weight were found to delay drug release through structuring. Another interesting finding was that surfactants included as emulsion stabilizers may also serve to control the drug release profile through specific binding to the drug [25].

The strategy of drug release to a wound depends on the condition of the wound. After the onset of an infection, it is crucial to respond immediately to the presence of large numbers of bacteria (>10^5 CFU ml^−1) which may already be present in the biofilm [26], and which may require antibiotic doses of up to 1000 times those needed in suspension [27,28]. Following the initial release, sustained release at an effective level over a period of time can prevent the occurrence of latent infection. It was shown that the proposed system can comply with these requirements. Both antibiotics demonstrated a combination of a medium to high burst release from BSA-stabilized dressings followed by release at a decreasing rate over 1–2 weeks (Fig. 2). Generally, increasing drug loads from 5% to 10% and 15% resulted in a decrease in the fraction of burst release and extended the duration of release. Even though comparable formulations and drug contents were used for both antibiotics, gentamicin was released overall in larger quantities than ceftazidime, reflecting higher encapsulation efficiency. A possible explanation for this finding may be gentamicin’s greater water-solubility compared with ceftazidime (100 and 5 mg ml^−1, respectively).

The time-dependent antimicrobial efficacy of these wound dressing formulations was tested in vitro by two complementary methods. The disc diffusion test is a good representation of the clinical situation, where the dressing material is applied to the wound surface, allowing the drug to diffuse to the wound bed. The results from this method are dependent on the rate of diffusion of the active agent from the dressing, set against the growth rate of the bacterial species growing on the lawn, and are highly dependent on the physicochemical environment. Viable counts provide valuable information on the kill rate, which is a key comparator for different formulations and physicochemical conditions. Wound dressings containing gentamicin demonstrated excellent antimicrobial properties over 2 weeks, with bacterial inhibition zones extending well beyond the dressing margin at most times. Interestingly, inhibition zones around dressing materials containing gentamicin remained close to constant over time and for the different drug loads. The largest CZOI were measured for the Gram-positive bacteria (S. aureus and S. albus) and especially for S. albus. Despite having the lowest MIC (Table 1), the Gram-negative P. aeruginosa was least inhibited, and exhibited the smallest CZOI (Fig. 4). This was not the case for ceftazidime-loaded materials, for which CZOI were found to decrease over time, and with lower drug loads. In contradistinction to gentamicin-loaded materials, ceftazidime was found to be most effective against P. aeruginosa and less effective against S. albus and S. aureus, and in good correlation with their MIC (Table 1). Curves describing the decrease in the number of bacteria as a result of antibiotic release also demonstrate the superiority of gentamicin-loaded dressing materials over ceftazidime (Fig. 7). High bacterial inoculations of 10^7–10^8 CFU ml^−1 were decreased by 99.99% after 1 (P. aeruginosa and S. albus) to 3 days (S. aureus) in the presence of the gentamicin-loaded wound dressing material. Under similar conditions, the ceftazidime-loaded dressing material demonstrated a 99% decrease in P. aeruginosa and S. albus only after 3 days, and its effect on S. aureus was even lower. Even though gentamicin is more

Fig. 4. The inhibition of P. aeruginosa, S. albus and S. aureus growth around wound dressings based on the emulsion formulations containing 10% gentamicin or 10% ceftazidime and stabilized with 1% BSA, PBS incubation times prior to the test are indicated next to each sample.
potent than ceftazidime (Table 1) and is therefore expected to perform better, the authors believe that the better overall performance of dressing materials containing gentamicin is associated with the better encapsulation of this drug compared with ceftazidime, portrayed in the release of larger quantities over time (Figs. 2 and 3).

4.2. Effect of surfactant type on the drug release profile and the bacterial inhibition

The replacement of BSA with the surfactant Span enabled a stable emulsion to be obtained at a more extreme 12:1 O:A phase ratio, which had a profound effect on the release rate of both antibiotics from the dressing (Fig. 3). First, it drastically reduced the burst release. For instance, dressings containing 5% ceftazidime only had a 6% burst release at 6 h compared with an 88% burst release when stabilized with BSA. Similarly, for gentamicin, the burst release was reduced from 85% to 4%. While most of the ceftazidime was released at a near-constant rate over 3 days, followed by a decreasing release rate, gentamicin exhibited a double-phase release pattern: a continuous release at a decreasing rate until release of 15–30% of the encapsulated drug (5 days) followed by a second phase of release of a similar nature, reaching 99% release after 3 weeks (Fig. 3). Employing a high aqueous-to-organic volume ratio, such as 12:1, makes emulsion formation difficult. Employing Span to enable a better stabilization effect than that obtained with BSA allowed this ratio to be reached and a stable emulsion to be maintained. Following freeze-drying, a lower aqueous volume fraction is portrayed in lower porosity and less pore connectivity, which in effect reduce the release rate of the hydrophilic low MW antibiotic molecules [25]. The incorporation of Span as an oil-soluble surface active agent may also act to restrict the diffusion of the antibiotics by increasing the diffusion coefficient through the polymer walls or by promoting hydrophobic interactions with the drug molecules. The second release phase demonstrated for gentamicin is probably governed by degradation of the host polymer, which enables trapped drug molecules to diffuse out through newly formed elution paths. Ceftazidime release was governed primarily by diffusion, since almost the entire amount

Fig. 5. Histograms showing the effect of drug release on CZOI around (1% w/v) BSA loaded wound dressings (n = 3) containing 5% (w/w) (■), 10% (w/w) (□) and 15% (w/w) (■) drug, as a function of pre-incubation time in PBS: (a–c) gentamicin-loaded wound dressings; (d–f) ceftazidime-loaded dressings. The bacterial strain (P. aeruginosa, S. albus and S. aureus) is indicated.
of drug was released before polymer degradation would have been able to contribute to its release.

The decrease in the release rate of gentamicin only slightly reduced the CZOI at the start time. For ceftazidime, the decrease in the burst release had a significant negative effect on the initial response of the dressing material, especially against staphylococci (Fig. 6). The antibacterial response for this dressing material was found to be greatest at 3 days rather than at the start point, which correlates with a maximal release rate between days 1 and 3 (Fig. 3).

The burst release is an important factor in the immediate performance of the dressing. A high burst release followed by a decreased release over several days, as achieved for BSA-stabilized dressings, can provide a robust response and eliminate large numbers of bacteria, and can therefore be considered suitable for cases of developed infection. Alternately, when the release of antibiotics is intended for prophylaxis, a low burst release and constant re-

Fig. 6. CZOI around Span-loaded (1% w/v) wound dressings (n = 3) containing 10% (w/w) gentamicin [■] and ceftazidime [■] as a function of pre-incubation time in PBS. CZOI was evaluated on cultures of (a) P. aeruginosa, (b) S. albus and (c) S. aureus.

Fig. 7. Number of CFU vs time when initial concentrations of $10^7$–$10^8$ CFU ml$^{-1}$ were used: (a) P. aeruginosa; (b) S. albus; (c) S. aureus. The releasing wound dressing discs ($D = 10$ mm) were derived from 1% (w/v) BSA-stabilized emulsions containing 10% (w/w) gentamicin [■] or 10% (w/w) ceftazidime [■]. Bacteria in the presence of PBS only served as controls [■].
lease rate would probably be more suitable. Nevertheless, in this case it must be verified that the antibiotics are released at effective levels in order to prevent the appearance of antibiotic-resistant strains. In this case, a clear advantage to using gentamicin over ceftazidime is seen.

4.3. Cell cytotoxicity

In order to complete the results of bacterial inhibition, it is also necessary to ensure that the dressing material developed is not toxic to the cells that participate in the healing process. Previous studies have shown that dressing materials may impose a toxic effect on cells, caused by the dressing material itself, its processing or due to the incorporation of antimicrobials [8,22]. Cell viability was assessed by observations of cell morphology and use of the AB assay, which is comparable with the MTT assay in measuring changes in cellular metabolic activity [19]. The AB assay is advantageous in that it does not necessitate killing the cells (as in the MTT assay), thus enabling day by day monitoring of the cell cultures. No difference in the appearance of the cell cultures was seen over the 3 days during which they were exposed to the dressing material devoid of antibiotics. The AB assay also shows a stable preservation of cellular viability. Thus, the authors are assured that the dressing material itself and its processing by freeze-drying of inverted emulsions do not inflict a toxic effect. Similar results were obtained for all the dressing materials containing antibiotics. No more than a 10% reduction in the metabolic activity of cell cultures was measured and, in most cases, metabolic activity even increased as the cells became more confluent (Fig. 9). These results are promising compared with studies reporting similar testing of commonly used silver-based dressing materials. Burd et al. [7] and Paddle-Ledinek et al. [22] reported that such dressings induce a mild to severe cytotoxic effect on keratinocytes and fibroblasts grown in culture, which correlated with the silver released to the culture medium. Specifically, it was shown that commercial dressings such as Acticoat™, Aquacel® Ag and Contreet® Ag reduce fibroblast viability in culture by 70% or more [7,22]. All silver dressings were shown to delay wound re-epithelialization in an explant culture model, and Aquacel® Ag and Contreet® Ag were found to significantly delay re-epithelialization in a mouse excisional wound model [7].
These findings emphasize the superiority of the proposed new antibiotic-eluting wound dressings over dressings loaded with silver ions. It is important to note that, while providing excellent antibacterial protection, none of the dressing materials developed in this study exerted a cytotoxic effect on cells. Antibiotics seem to be tolerated better by the host tissue, and tissue concentrations of gentamicin as high as 2000 \(\mu g\) ml\(^{-1}\) by local release from collagen sponges have been reported to exist and result in good outcomes [29]. The proposed system provides better control over the release rate of antibiotics, and therefore such high fluxes of antibiotics were not noted even when the highest drug load of 15% w/w was used. Nevertheless, the effect of higher concentrations of gentamicin (more than two orders of magnitude higher than the bacterial MIC) in the cell culture model were investigated in order to elucidate the safety margin for use of these antibiotics. It was found that fibroblasts are hardly affected by gentamicin up to 1000 \(\mu g\) ml\(^{-1}\) and maintain 80% of their viability even at 1500 \(\mu g\) ml\(^{-1}\). Cells were found to be more susceptible to ceftazidime, which reduced cell viability by \(\sim 40\%\) when exposed to

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Drug load: (w/w)</th>
<th>Ceftazidime ((\mu g) ml(^{-1}))</th>
<th>Gentamicin ((\mu g) ml(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5% BSA</td>
<td>5% Span</td>
<td>15% BSA</td>
</tr>
<tr>
<td>24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>47.9 ± 1.3</td>
<td>18.4 ± 5.3</td>
<td>125.0 ± 14.7</td>
</tr>
<tr>
<td>48</td>
<td>16.1 ± 1.9</td>
<td>24.2 ± 1.8</td>
<td>106.2 ± 27.2</td>
</tr>
<tr>
<td>72</td>
<td>7.2 ± 0.8</td>
<td>11.0 ± 1.1</td>
<td>20.9 ± 3.2</td>
</tr>
</tbody>
</table>

Fig. 9. Photographs demonstrating the appearance of fibroblasts after 24 h incubation in a known concentration of antibiotics: (a) control (no antibiotics); (b) 500 \(\mu g\) ml\(^{-1}\) ceftazidime; (c) 1500 \(\mu g\) ml\(^{-1}\) ceftazidime; (d) 500 \(\mu g\) ml\(^{-1}\) gentamicin; (e) 1500 \(\mu g\) ml\(^{-1}\) gentamicin.
500 µg ml⁻¹ and progressively more as the concentration was increased to 1500 µg ml⁻¹. Based on these results, it is recommended that care be taken to avoid a local concentration that exceeds 500 µg ml⁻¹ ceftazidime when selecting a release profile for this drug.

5. Summary and conclusions

Novel antibiotic-eluting biodegradable wound dressings based on a polylactone mesh and a porous PDLGA binding matrix were developed and studied. These composite dressings were prepared by dip-coating woven meshes in inverted emulsions, followed by freeze-drying. Their investigation focused on the release profiles of gentamicin and ceftazidime from the dressing and on their effect on bacterial inhibition. Cell cytotoxicity was studied using human fibroblasts.

Release profiles from dressings derived from emulsions stabilized with BSA typically demonstrated medium–high burst release of the antibiotics, followed by gradual release at a decreasing rate over time. In contradistinction, release profiles from dressings derived from emulsions stabilized with Span demonstrated a low burst release followed by a lower rate of release.

Both types of microbiological studies showed that the investigated antibiotic-eluting wound dressings are highly effective against the three relevant bacterial strains. The viability results indicated that gentamicin-eluting dressings preserved their antibacterial potency over 2 weeks. The viable count results showed that ceftazidime-eluting dressings preserved their antibacterial potency over 2 weeks. The viable count results indicated that free gentamicin from emulsions stabilized with BSA typically demonstrated medium–high burst release followed by a lower rate of release.

Based on these results, it is recommended to gentamicin and ceftazidime in the wound environment due to gentamicin burst release followed by a lower rate of release. Both types of microbiological studies showed that the investigated antibiotic-eluting wound dressings are highly effective against the three relevant bacterial strains. The CZOI results showed that gentamicin-eluting dressings preserved their antibacterial potency over 2 weeks. The viable count results indicated that ceftazidime-eluting dressings preserved their antibacterial potency over 2 weeks.

References


[14] Elsner JJ, Shefy-Peleg A, Zilberman M. Novel biodegradable composite wound dressings with controlled release of the antibiotic drugs ceftazidime and gentamicin from the binding matrix, and are therefore potentially very useful as burn dressings. Changing the emulsion formulation enables the desired properties to be adapted to the wound characteristics, and can thus enhance wound healing. Despite severe toxicity to bacteria, the dressing material was not found to have a toxic effect on cultured fibroblasts, indicating that the new antibiotic-eluting wound dressings represent an effective and selective treatment option against bacterial infection.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figures 1–8, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.actbio.2010.07.013.