Visualisation of bacterial sequestration and bactericidal activity within hydrating Hydrofiber® wound dressings

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Abstract

The fluid handling and microbiological properties of a non-antimicrobial Hydrofiber® (NAH) wound dressing have been compared with those of a silver salt-containing Hydrofiber® (SCH). Fluorescent dyes (BacLight®TM, Live/Dead®TM Kit) were added to fresh cultures of two wound pathogens (Pseudomonas aeruginosa and Staphylococcus aureus), and used to visualise their viability. Live bacteria stained green and dead/dying bacteria turned red. When inoculated into samples of the NAH and SCH dressings, the viability of the bacteria could be effectively monitored over time using a rapid form of confocal laser scanning microscopy (RCLSM—Leica® UK). When the NAH dressing was hydrated with stained bacterial culture, its fibres swelled quickly, reducing interstitial spaces between the fibres, resulting in the formation of a cohesive gel. Bacteria became immobilised in the gel, forming characteristic clumps, but remained largely green (viable) for more than 20 h with no apparent increase in numbers. The SCH initially behaved in a similar manner, however, using 3-D data from RCLSM time-lapse sequences P. aeruginosa was observed to turn progressively red (i.e. died) within 1.5–3 h and S. aureus similarly turned red within 5–7 h of contact with the SCH dressing. The ability of both Hydrofiber® dressings to sequester and immobilise potentially pathogenic wound micro-organisms has been demonstrated. Additionally the SCH dressing was shown to kill immobilised bacteria, as a consequence of the ionic silver bactericide. These properties of the Hydrofiber® dressings may contribute to providing an environment that is supportive to wound healing.

Keywords: Bacteria; Confocal microscopy; Silver; Hydrofiber® wound dressing; Wound healing

1. Introduction

Many modern wound dressings possess a variety of attributes that are designed to create an environment to encourage conditions that support wound healing. These include the ability to absorb exudate, provide optimum moisture balance at the wound surface and prevent maceration of surrounding tissue. Since bacteria are often present in high numbers in wound fluid, it is also important that dressings with high fluid retention levels are also able to absorb and retain bacteria [1].

Scanning electron microscopy (SEM) has demonstrated the immobilisation of bacteria within a Hydrofiber® [2]. However, the SEM images were generated from critical-point dried, metal-coated samples and were representative only of a single point in time. The visualisation of dynamic events, i.e. swelling of individual fibres and interactions with bacterial populations that occur in ‘real time’ cannot be addressed by this method.

In this study, viable bacteria inoculated into Hydrofiber® dressings were observed directly using a rapid
form of confocal laser scanning microscopy (RCLSM) (Leica\textsuperscript{R} TCS-SP2 RS, Leica\textsuperscript{R} Microsystems AG). As with conventional confocal laser scanning microscopy, RCLSM enables a specimen to be scanned by a beam of light (laser) focused through confocal pinholes, but operates at much faster speeds. Only light from the plane of focus (XY) passes on to the detectors (photomultiplier tubes—PMTs), resulting in a unique image from which all out-of-focus information is eliminated. With little or no disruption to the specimen, stacks of perfectly focused images (or optical sections) can be recorded from different depths (Z planes).

Complex computer software enables the stacks to be reconstructed and analysed in a variety of ways, for example in three-dimensions. The RCLSM employs a powerful resonance scanner to enable much faster scan rates than those available with the standard CLSM. The resulting reduced exposure to laser light and rapid acquisition of images confers many advantages for live cell studies. In the present studies it has allowed the direct visualisation of changes in the three-dimensional structure of Hydrofiber\textsuperscript{R} dressings over time and enabled simultaneous observation of their interactions with viable bacteria.

2. Materials and methods

2.1. Test materials

A non-antimicrobial Hydrofiber\textsuperscript{R} (NAH) dressing (100% sodium carboxymethylcellulose) and a silver-containing form of Hydrofiber\textsuperscript{R} (SCH) dressing (100% sodium carboxymethylcellulose with ionic silver 1.2% w/w) were used.

2.2. Bacterial strains

\textit{Pseudomonas aeruginosa} (NCIMB 8626) and \textit{Staphylococcus aureus} (NCIMB 9518) were cultured in Tryptone Soya Broth (TSB) (Lab M, UK), filtered and re-suspended in TSB.

2.3. Live/dead\textsuperscript{TM} baclight\textsuperscript{TM} bacterial viability kit

In order to visualise the bacteria and assess their growth characteristics, cell populations were stained using the BacLight\textsuperscript{TM} Bacterial Viability Kit (Molecular Probes\textsuperscript{TM} Inc.). This kit consists of two fluorescent dyes, Syto-9\textsuperscript{TM} (S-9) and propidium iodide (PI), pre-aliquoted in sealed plastic pipettes. Both these dyes have a high affinity for DNA and should be handled with care. All types of bacteria are stained green with the S-9 dye but the red dye (PI) will displace the S-9 dye if the bacterial cell wall is damaged. Consequently, dead or dying bacteria are stained red.

The dry dye reagents were each dissolved separately in 2.5ml of water and used as stock solutions from which the final concentrations were derived. The stock solutions were stored at 4°C in the dark. The dye solutions were mixed together in a 1:1 ratio, to which a fresh culture of a bacterial suspension was added, again in a 1:1 ratio.

2.4. Setting up the RCLSM

The Leica\textsuperscript{R} TCS SP2 RS utilises an inverted microscope so that preparations, such as viable bacteria, when placed on the specimen stage can be viewed through the transparent base of their containers (e.g. Petri dish, coverslip, glass slide, etc.). For low magnification investigations a ×10 objective lens was used with the RCLSM set at a format of 512 × 512 pixels in a 1 mm\textsuperscript{2} scan area. The depth of focus of a ×10 lens is many microns so that usually only one focus or Z-scan position was needed. XYT scanning (2-D imaging over time) enables fast image acquisition for recording rapid, dynamic events. For higher magnification views a ×40/1.3NA objective lens, with a depth of focus of less than 1 μm, was used at a format of 512 × 512 pixels in a 250 μm\textsuperscript{2} scan area. In order to analyse samples many microns thick, it was necessary to take images from a number of focus (Z scan) levels. XYZT (3-D imaging over time) is slower but samples a significant volume of the specimen providing perfectly focused data.

Initially two-channel excitation was set up for detecting green (S-9) and red (PI) fluorescence, simultaneously. Laser power control was set at zero and laser output on all six laser lines is accurately controlled on the RCLSM by acousto-optical tuneable filters (AOTF). Signal strengths were optimised and low line averaging used to remove PMT noise. All settings were saved for use in future experiments. Simultaneous green and red images at low magnification with only one Z position and low line averaging could be rapidly collected (555 ms intervals). At higher magnifications with several Z positions (usually 20–30) and low line averaging, stacks of simultaneous green and red images were collected over slightly longer periods (i.e. 1–1.5 min intervals). Certain samples were viewed in transmission mode using a phase-contrast condenser and a transmitted-light detector.

2.5. Visualisation of Hydrofiber\textsuperscript{R} dressings

In all cases, samples of Hydrofiber\textsuperscript{R} dressings were placed on glass cover slips inserted into modified Petri dishes. Holes (28 mm × 18 mm) were created in the lids of 50 mm Petri dishes with a hot scalpel blade. Any raised edges were carefully removed with abrasive paper. The lids were inverted and a smear of silicon grease was applied to the top surface around the hole. A glass cover slip (32 mm × 22 mm) was then pressed into place over the hole. Petri dishes prepared in this way could be used many times over by replacing the cover slip.

2.5.1. Dry Hydrofiber\textsuperscript{R} dressings

Confocal images of the individual filaments of 3 mm\textsuperscript{2} samples of dry Hydrofiber\textsuperscript{R} dressings were generated from laser light reflected by the fibres (reflection mode at 488 nm).

2.5.2. Hydrated Hydrofiber\textsuperscript{R} dressings

A concentrated dye solution (S-9) was added to dry samples of the Hydrofiber\textsuperscript{R} dressings and, following equilibration for approximately 5 min, confocal images of the partially and fully hydrated fibres were recorded with laser light excitation at
2.5.3. Rapidly hydrating fibres of Hydrofiber® dressings

The RCLSM also has a transmission detector above the specimen so that photons passing through it can be detected as a transmission image. This allowed dynamic observations of the process of hydration within the Hydrofiber® dressings. As fibres absorb water they exhibit a grey/black outline in phase-contrast transmission mode, which makes it an excellent way to show the hydration dynamics of the dressing samples. The 458 nm laser line at 35% AOTF was chosen for transmission. These images were non-confocal.

2.6. Visualisation of the bacteria with the BacLight™ dyes

Mixed suspensions of dye and bacteria were kept in foil-covered containers and left to equilibrate for 10 min before RCLSM viewing. The quality of the cultures, e.g. bacterial numbers and the ratio of green (living) to red (dead) bacteria, was assessed using a bright-field fluorescence microscope (Olympus BX51). Only fresh bacterial cultures, stained with recently prepared S-9/PI dyes, in which the vast majority of the bacterial population was viable (i.e. stained green), were used.

The hydration of the dressings with the bacterial suspensions was observed directly on the specimen stage of the RCLSM. A 3 mm² piece of dry wound dressing was positioned above the objective lens on the microscope stage. Dye/bacterial suspension (100 µl) was pipetted onto the dressing whilst under observation. Both the hydration of the Hydrofiber® dressings and the interaction of the bacterial populations with individual fibres were recorded. A humid environment was maintained by the inclusion of a small “doughnut” of filter paper saturated with water and placed into a Petri dish base. This could be inverted over the lid containing the hydrating dressing/bacterial preparation without interfering with the laser beam (particularly transmission imaging).

The S-9 dye was excited with a 488 nm wavelength laser line and detected at a spectrophotometrically optimised wavelength of between 510 and 535 nm. The PI dye was excited with a sub-optimal 543 nm wavelength laser line and detected at a spectrophotometrically optimised wavelength of between 620 and 650 nm. With the 10 magnification lens, AOTF settings of 36% laser output for the excitation of the S-9 dye and 100% for PI dye gave good signal strength, even with the laser power control at zero. Using the 40 magnification lens, only 10% AOTF for the green dye and 52% AOTF for the red dye were required. All parameters were saved on the computer so that experimental conditions could be reproduced for both short and long-term experiments.

2.7. Image acquisition

In order to follow dynamic changes in the early stages of fibre hydration at ×10 magnification, it was necessary to take sequential, single, low magnification images that were rapidly acquired over a 10–15 min period. For long term low magnification experiments, single images were taken every 30 min or 1 h over 20–24 h periods.

With the ×40 magnification lens, 20–30 Z scans through 20–35 µm of dressing/bacterial suspension were taken every 10–20 min over periods of 100–330 min per experiment. The Leica® RCLSM can only collect images from two channels simultaneously, but the two channel limitation of the RCLSM was overcome by collecting the green and red fluorescent images together in one channel, with the transmission image run frame sequentially as a second channel. Acquiring three images in this way took slightly longer (<2 min).

Where necessary, Leica® image-processing software and Bitplane® Imaris®/Surpass® software were used to convert the Z-scan image stacks into maximum projection images (MPI) (extended focus) for each time point of the experiment. Adobe® Photoshop 7 was also used to further process individual images illustrating the interactions between hydrating fibres and viable bacterial populations.

3. Results

The hydration process of the Hydrofiber® dressings and their effects on bacterial population dynamics were studied simultaneously in the RCLSM allowing both short term (i.e. <15 min) and long term (i.e. >20 h) experiments.

Confocal reflection images of the dry Hydrofiber® dressings (Fig. 1a) showed filaments running randomly with a diameter of approximately 10–15 µm. Following hydration with the concentrated green-emitting fluorochrome (S-9), confocal fluorescent images showed that the fibres swelled considerably (Fig. 1b), becoming more vertically aligned as they absorbed fluid, and increased in diameter to 60–100 µm. This figure could be deceptive in that phase-contrast images revealed a variably sized, hydrated “shell” shown as a dark grey ghost around a fluorescent core (not shown). It is likely that only the partially hydrated core of the fibres retains sufficient fluorochrome to generate a signal.

The viability of freshly cultured bacteria stained with S/9/PI dyes were assessed by fluorescence microscopy (Olympus BX51), and only preparations that showed minimal evidence of red staining were inoculated onto samples of the dry Hydrofiber® dressings. In the RCLSM P. aeruginosa fluoresced brightly at 488 nm excitation (green) and in combined (overlaid) images they were easily visible against the dark grey/black transmission image showing the hydration front spreading through the dressing fibres. Typical short-term effects of the hydrating NAH dressing with a BacLightTM stained culture of P. aeruginosa are shown as an image gallery in Fig. 2. The gallery is of six selected time points that illustrate events over a period of 15 min. The zero time was delayed by a 30–60 s search to obtain a suitable field for investigation. The grey/black, non-confocal, phase-contrast transmission images showed a continual fluid movement (from right to left in Fig. 2) with individual fibres swelling and coalescing. The force
associated with fibre hydration caused green-fluorescent 
clumps of _P. aeruginosa_ cells to move along the direction 
of the fibres until eventually they became immobilised 
within the gelling structure (circled areas, Fig. 2).

These images also illustrated the difficulties associated 
with capturing the continuous changes in shape and 
orientation of the fibres that occur as fluid is absorbed in 
the early stages of hydration. The difficulties associated
with this process were reduced in experiments that were run over much longer periods of time (i.e. >20 h) by waiting for 1 h to establish a more stable preparation. In these studies changes in fibre structure continued over the first 4 h, but the shapes and positions of the main bacterial clusters remained recognisable for at least 21 h (Fig. 3). The bright green fluorescence remained throughout the test period but after 17 h (Fig. 3e) there were slightly more red-stained bacteria than at 1 h and a small population of yellow-stained bacteria was observed. There was no indication of any increase in the green proportion within the field of view.

In experiments with the SCH dressing, similar difficulties associated with the initial movements caused by fibre hydration were encountered. Observations were made over various periods of time (i.e. 2–7 h) and some
Of the two wound pathogens examined, *P. aeruginosa* showed the greater susceptibility to the SCH dressing. Large populations of bacteria could be closely followed because, as previously stated, the images were composed of at least 20 Z scans through approximately 20 μm of the sample. Initially the overwhelming colour of the freshly stained bacterial population was green (Fig. 4a), indicative of viable bacteria, with very few red bacteria visible. However, within 20 min of contact with the SCH dressing (Fig. 4b) bacteria were observed to be dying (i.e. stained red), with virtually all of the bacteria showing red fluorescence within 100 min (Fig. 4f). Evidence that fluid-dependent reactions continued for the whole time period of the experiment was provided by the changes in...

Fig. 4. Time sequence of MIPs from the SCH dressing inoculated with freshly cultured *P. aeruginosa* stained with both S9 and PI dyes, using fluorescence mode only. At *t*₀ (1a) only a scattering of red fluorescent bacteria are present. However, after 20 min (1b) there is a marked increase in dead/dying bacteria (i.e. stained red). The intensity of the red staining increases with time until there are virtually no green, viable bacteria seen (1f).
the contours of the fibres, which in the absence of phase-contrast transmission images are seen as dark spaces between the bacteria, and in the colour of the bacteria from green to red. Generally, the bacteria closest to the SCH hydrating fibres interacted with available silver ions first and the bactericidal effect continued as bacterial suspensions moved along the fibres throughout the extended experimental periods (Fig. 4b–c). From an estimated population of many thousands of bacteria, very few viable bacteria were visible after 100 min (Fig. 4f).

The interaction between the SCH wound-dressing fibres and the *P. aeruginosa* bacteria was more easily observed when the fluorescent images were superimposed on the phase-contrast transmission images (Fig. 5), although often, some of the bacteria were obscured. In the early stages, the initial change of bacterial fluorescence from green to red occurred close to fibres (Fig. 5a–c). The effect then spread more widely until no green fluorescent bacteria could be identified after 3 h (Fig. 5f). At first bacterial cells appeared to be

Fig. 5. Similar to Fig. 4 but with the images of stained *P. aeruginosa* superimposed on non-confocal, grey, phase-contrast transmission images. The hydrating SCH fibres are well shown but can obscure some bacteria. The bacteria turn completely red within 135 min and after 3 h they have a very ragged profile suggesting that they could be disintegrating.
well-formed (Fig. 5a) but after prolonged exposure to the silver ion bactericide they appeared distorted and fragmented (Fig. 5f).

By way of comparison with the results shown in Fig. 4 for SCH, control experiments were run at ×40 magnification over a similar time period (i.e. 80 min) on NAH in which the bacterial population was aligned along the fibres but, in the absence of silver ions, remained green (Fig. 6).

Interactions between the SCH dressing and *S. aureus* were very similar to those seen with *P. aeruginosa* but slower. As a result, experiments examining the bactericidal effects of the ionic silver on *S. aureus* were carried out over much longer time periods than those on *P. aeruginosa*. Initially, there often appeared to be only a slight diminution of green fluorescence (Fig. 6a and b) but after 60 min the change in colour of the bacteria to red became much more apparent. However, although the spread of red fluorescence again followed the orientation of the fibres (seen in Fig. 7 as dark spaces between bacteria) some bacteria at a distance from the main fibres were still fluorescing green after 5.5 h (Fig. 7f). Generally it could take up to 7 h for the *S. aureus* population to be killed.

4. Discussion

A key challenge for maximising the healing response in wounds has been the advent of modern fibrous dressings to absorb large volumes of exudate, whilst still providing moisture balance in the wound environment [3]. Equally important, however, is the ability of these dressings to immobilise wound exudate, which may contain pathogenic bacteria (e.g. *P. aeruginosa*). Bowler et al., [4] have suggested that as Hydrofiber® dressings absorb wound exudate, this in turn reduces the interstitial spaces between individual fibres within the dressing as they coalesce resulting in bacterial immobilisation. Subsequent studies using SEM have corroborated these findings [2].

The visualisation of what appears to be randomly orientated fibres present in the dry Hydrofiber® dressings are in good agreement with previous observations [5]. Individual dry fibres were shown to be approximately 10–12 µm in diameter, and following the application of an aqueous solution the fibres started to swell rapidly, and partially hydrated fibres were seen to achieve a diameter of at least 60–100 µm. As more fluid was absorbed the fibres continued to swell, resulting in
the elimination of inter-fibre space and the formation of a cohesively gelled dressing. Concentrated solutions of the green fluorescent dye (S-9) were required to highlight fibre structure and even then it was often only the centres of individual fibres that had retained sufficient fluorescence to produce an image. A completely hydrated fibre could, therefore, be much bigger in diameter than its fluorescent image.

When used at much lower concentrations, the green fluorescent dye (S-9) only stained the freshly cultured bacterial cells and these were easily resolved by the RCLSM. When applied together (i.e. green dye:red dye, 1:1) to the NAH dressing the bacteria predominately fluoresced green, indicating their viability. The progress of the viable bacterial populations along the fibres of the hydrating dressing could be followed in transmission mode, even at low magnification, accompanying the moving fluid fronts. In the short-term experiments (i.e. <1 h) as the fibres absorbed fluid and began to swell and coalesce, immobilisation of the bacteria was observed.

Fig. 7. Time sequence of MIPs from SCH dressing inoculated with *S. aureus*. At *t*₀ (4a) almost all the bacteria are green fluorescent but a progressive increase in red fluorescence spreads through the bacterial population. The change to red fluorescence is slower than with *P. aeruginosa* indicating a greater resistance to the silver ion bactericide.
Within a short period of time (5–10 min) clusters of bacteria showed no visible movement, little change in shape and no change in colour from green, suggesting that, although immobilised by the dressing they were still viable.

In longer-term experiments (i.e. in excess of 20 h), similar observations were noted, in that after approximately 4 h there was minimal fluid movement or change to the population density of the bacteria, confirming their immobilisation within the dressing. There was, however, a slight increase in the fluorescent red-stained bacterial population (indicative of dead bacteria) and a visible minority of yellow bacteria, possibly stained with equal amounts of both S-9 and PI dyes. This is not an unexpected finding knowing that a cohesive gel forms when Hydrofiber® dressings are hydrated leading to fluid immobilisation and reduced water activity. As bacteria are immobilised in the newly forming gel, natural cell death would be expected, particularly over prolonged time periods, and this was made visible by use of the BacLight® bacterial viability test kit.

Similarly, bacterial immobilisation of both P. aeruginosa and S. aureus has been shown in an in vivo infected skin ulcer model in the rat [6]. In these studies the Hydrofiber® dressing was shown to immobilise a greater bacterial population in the dressing when compared to alginate dressings [6]. Clinically this could be an important consideration as the ability of a dressing to sequester and immobilise potentially pathogenic wound micro-organisms in the dressing may contribute to providing an environment that is supportive to wound healing.

RCLSM was able to demonstrate both the sequestration and the progressive killing of the bacteria associated with exposure to ionic silver in the SCH dressing. The use of RCLSM resolved some of the problems that would have made recording dynamic changes in the topography of the hydrating SCH dressing and its effects on bacterial populations almost impossible by other means. For example, conventional fluorescence light microscopy has only a limited depth of focus (>1 µm at ×40 magnification) and field of view, which can lead to confused and fuzzy images. However, with rapid optical sectioning, confocal microscopy has the advantage of being able to analyse samples of the dressing in depth (e.g. 20–35 µm) and even at ×40 magnification, a relatively large area of the sample (0.25 mm²) could be scanned, allowing a much greater number of bacteria to be imaged in sharp focus than would be possible with bright-field methods. The images, presented here in the form of time-lapse sequences, have a two-dimensional appearance. However, they are MPIs each composed of all the optical sections collected at an individual experimental time-point, and shown simultaneously. They therefore include a significant amount of three-dimensional data.

The bright-green fluorescent intensity of the freshly cultured, viable bacterial cells when added to the SCH dressing quickly changed to a spreading red fluorescence indicating their death. Cultures of P. aeruginosa were particularly vulnerable and were usually dead in less than 2 h whilst those of S. aureus took longer to kill (approximately 7 h). One possible explanation for this could be related to the presence of a thinner cell wall in Gram-negative bacteria like P. aeruginosa compared with the much thicker, and more complex, cell wall present in Gram-positive bacteria like S. aureus. Feng et al., [7] have suggested that the presence of a much thicker peptidoglycan layer that is present in Gram-positive cell walls “is of immense practical importance in protecting the cell from penetration of silver ions into the cytoplasm”. Studies are under way to examine the effects of ionic silver on the bacterial cell wall.

The time taken for bacteria to succumb to the antimicrobial effects of ionic silver in the SCH dressing may relate only not to the structure of their cell walls but also to the production of bio-film by bacteria cultured for longer periods. In cultures left overnight or longer, bacteria multiply in number and secrete extracellular polymeric substances [8]. Some initial experimentation in this area suggests that bacteria from these overnight cultures are more resistant to the bactericide and take longer to kill than freshly cultured bacteria (data not shown) [8]. Further investigations in this area continue.

The continuous changes in contour and colour seen during the course of numerous experiments showed that water dependant activities, although reduced by the formation of a cohesive gel, continued throughout the experimental time periods and consequently this excluded the possibility that, for example, drying-out of the samples may have influenced the results. In addition, the recording of events at specific time intervals showed that the destruction of the bacteria was progressive with, in some cases, green-fluorescent bacteria still visible after several hours indicating that the loss of green fluorescence cannot simply be explained by laser bleaching. Further, the continuing death of bacteria over hours of exposure to silver ions in the SCH dressing was indicative of a sustainable bactericidal effect by the dressing, which could be clinically relevant in minimising the opportunity for wound infection.

5. Conclusion

Modern wound dressings can help facilitate wound healing, particularly those that are able to effectively manage exudates, maintain a moist wound environment and control bacterial populations. In these studies a RSLCM technique has demonstrated the ability of a Hydrofiber® dressing to immobilise bacteria and delay their growth characteristics within the dressing over a 21 h period. Additionally, the same Hydrofiber®
dressing containing ionic silver has been shown to kill immobilised bacteria within the dressing.

The fluid handling and microbiological characteristics of the Hydrofiber® dressing are likely to be beneficial in the management of wounds that are in bacterial balance. In wounds that are infected or at risk of infection the Hydrofiber® dressing containing ionic silver may be more appropriate to correct bacterial imbalance and create an environment supportive of wound healing.

References