Binding of two bacterial biofilms to dialkyl carbamoyl chloride (DACC)-coated dressings *in vitro*

• **Objective:** To date only planktonic bacteria have been shown to bind irreversibly to dialkyl carbamoyl chloride (DACC)-coated Cutimed Sorbact dressings. Therefore, this study was designed to determine whether bacterial biofilm bound to the DACC-coated dressing *in vitro*.

• **Method:** Samples of DACC-coated dressings and uncoated control dressings (supplied by BSN medical Ltd, Hull) were placed in contact with plastic coverslips on which biofilms of either *Pseudomonas aeruginosa* or methicillin-resistant *Staphylococcus aureus* (MRSA) had been cultivated for 24 hours. Dressing samples were examined by scanning electron microscopy to detect the presence of biofilm.

• **Results:** *Pseudomonas aeruginosa* biofilm bound avidly to both DACC-coated and uncoated dressing samples. MRSA bound more extensively to DACC-coated dressings than to uncoated samples.

• **Conclusion:** Biofilms of two different test bacteria bound to dressings *in vitro* with the DACC-coating on the dressings enhancing the binding of MRSA biofilm.

• **Declaration of interest:** This study was supported by BSN medical Ltd (Hull). The company had no influence on the experimental design or the interpretation of the results.

DACC; Cutimed Sorbact; biofilm; irreversible binding; MRSA; *Pseudomonas aeruginosa*

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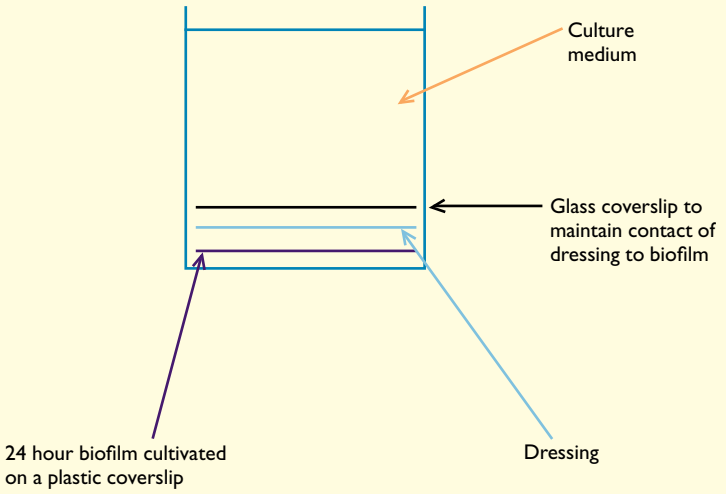
The need to reduce wound bioburden has long been recognised.[1] However, using the ability of microbial species to bind to wound dressings is a relatively recent approach to wound management that provides an antimicrobial effect without the use of an active inhibitory agent or the risk of cytotoxicity to host tissues. Bacteria exist largely in hydrophilic environments where they require water molecules for survival. Their surface layers contain both hydrophilic (water loving) and hydrophobic (water repellent) components which facilitate interaction with either hydrophilic or hydrophobic molecules, respectively. Bacterial cell surfaces contribute to hydrophobic interactions with host cells and inanimate surfaces that are important in the initiation of infections and biofilm formation.[2]

In 2006, the influence of cultural conditions on cell-surface hydrophobicity (CSH) of five planktonic bacteria (*Staphylococcus aureus*, *Staphylococcus haemolyticus*, *Escherichia coli*, *Enterobacter cloacae* and *Pseudomonas aeruginosa*) were investigated, and the binding capacity to a dressing coated with a hydrophobic fatty acid derivative called dialkyl carbamoyl chloride (DACC) was determined using *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans*, *Enterococcus faecalis*, *Bacteroides fragilis* and *Fusobacterium nucleatum*.[3] For *Pseudomonas aeruginosa,* maximum binding was observed at two hours and remained stable for 20 hours, showing that bacteria bound to the dressing did not multiply.[3] A recent investigation into the CSH of *Mycobacterium ulcerans* found it to be higher than that of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*.[4] Additionally, planktonic cultures of *Mycobacterium ulcerans* were found to bind more effectively to DACC-coated dressings than to untreated control dressings *in vitro*, allowing the authors to suggest a possible role for the coated dressing in reducing the bacterial load of Buruli ulcers.[4] Planktonic cultures of two strains of methicillin-sensitive *Staphylococcus aureus* (MSSA) and nine clinical strains of methicillin-resistant *Staphylococcus aureus* MRSA displayed equal binding capacity to DACC-coated dressings.[5] Binding of a range of wound colonising bacterial species to DACC-coated dressing has, therefore, been demonstrated in the laboratory using planktonic cultures.

Hydrophobic interaction was the rationale for a clinical study in which DACC-coated dressings were used to investigate reductions in wound bioburden.[6] In this study, quantification of bacterial burden in 20 chronic wounds treated with the DACC-coated dressings showed that of the 15 wounds with a positive clinical outcome, a significant decrease in bacterial load was found in 10 but that it was unchanged in 5. The remaining 5 patients with a negative clinical response showed a non-significant decrease in bacterial load.[6]

Since the demonstration of an association between wound chronicity and the presence of biofilm,[7,8] the need to reduce wound bioburden

Fig 1. Diagrammatic view of a test well within the microtitre plate



including biofilm has been recognised. Binding of biofilm to DACC-coated Cutimed Sorbact dressings has not yet been demonstrated, so this study was designed to investigate whether biofilms of two common wound pathogens bind *in vitro* and if the DACC-coating on these dressings promoted increased binding.

Method

Test organisms and dressings used

Pseudomonas aeruginosa and MRSA were used throughout this study. These had been isolated from different out-patients attending a local wound care clinic and stored at –80°C until required.

BSN medical Ltd provided samples of sterile Cutimed Sorbact dressings with a DACC-coating (72164-01; batch number 807093) and sterile Cutimed Sorbact dressings manufactured without a DACC-coating (72164-01 batch number 72632). Dressing samples were cut under aseptic conditions into circles with a 15mm diameter for testing.

Cultivation of 24 hour established biofilms

A starter culture of each test organism was cultivated in 10ml tryptone soya broth (TSB; Oxoid, Cambridge, UK) overnight at 37°C. Immediately before use, each starter culture was diluted in sterile TSB (1/100 dilution for *Pseudomonas aeruginosa* and 1/500 dilution for MRSA) and 2ml dispensed into wells of a 24-well microtitre plate (Nunc, Roskilde, Denmark) that contained a sterile Thermanox plastic coverslip (Agar Scientific, Stansted, UK). In each plate, three wells contained only 2ml TSB and coverslip to act as a negative control to test for sterility and non-specific binding, and three wells contained only 2ml diluted inoculum as a positive control for untreated biofilm. All plates were incubated at 37°C for 24 hours to allow biofilm to establish on the coverslips.

Fig 2. These images are examples of those the used to assess the extent of biofilm coverage of dressing samples (see Table 1). No biofilm present (a), 1–30% of dressing covered by biofilm (b), 30–60% of dressing covered by biofilm (c), 61–90% dressing covered by biofilm (d), 91–100% dressing covered by biofilm (e)

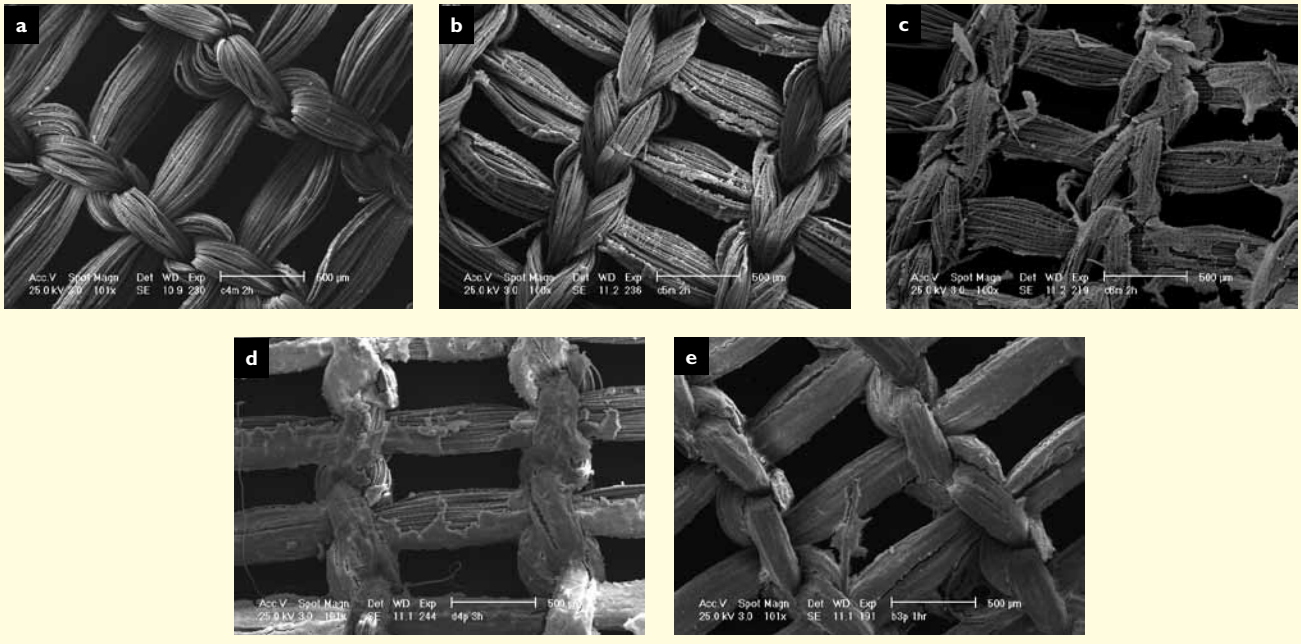


Table 1. Scoring system used by trained volunteers to evaluate the extent of coverage of biofilm associated with dressing samples

Score	Biofilm covering of the wound dressing	Example image
0	No biofilm visible	Fig 2a
1	Limited coverage and most of the dressing fibres are still visible (1–30% of the dressing covered)	Fig 2b
2	Moderate coverage (31–60% of the dressing covered)	Fig 2c
3	Marked coverage but some parts of the dressing still visible (61–90% of the dressing covered)	Fig 2d
4	Extensive coverage with hardly any parts of the dressing visible (91–100% of the dressing covered)	Fig 2e

Binding of 24 hours established biofilm to dressing samples

A circular dressing sample was aseptically introduced in selected wells, followed immediately by a sterile glass coverslip to ensure contact between dressing and biofilm and to prevent the dressing floating away from the biofilm layer (Fig 1). DACC-coated and uncoated dressings were tested in duplicate in the same microtitre plate; positive controls (no dressings) and negative controls (no bacteria) were included in all plates. Plates were incubated at 37°C and at known time intervals (normally up to 3 hours) and wells were sampled to retrieve the dressing, making sure that the orientation of the sample was known (i.e. surface in contact with the biofilm). Biofilm on the surface of the dressing was visualised by scanning electron microscopy (SEM).

Scanning electron microscopy of dressing samples

Dressing samples were transferred to wells in fresh microtitre plates containing 200µl 2.5% glutaraldehyde for 5 minutes to fix the attached biofilm. After gentle washing in phosphate buffered saline (PBS; Oxoid, Cambridge, UK) and storage overnight at 4°C, fixed samples were treated with 1% osmium tetroxide for 45 minutes, dehydrated in each of 50, 70 and 90% ethanol, followed by three changes of absolute alcohol for 10 minutes. Fixed dressings were then mounted onto pins dried in a critical dryer, coated by gold spluttering and examined in a 5200LV Jeol scanning electron microscope (Jeol Ltd, Hertfordshire, UK). For each sample in every experiment at least four representative images were captured, usually three at low magnification (typically 100X) and at least one at a higher magnification size (between 200 and 10,000X)

Images of the dressing samples were evaluated for the extent of biofilm coverage by six volunteers. These were postgraduate biomedical science students and research technicians who had undergone a training programme using suitable sample images and a scoring system (Table 1 and Fig 2). For each time point and each test organism, three images coded to ensure anonymity were scored between 0 (no binding) and 4 (extensive binding of biofilm to dressing) by each volunteer, who worked independ-

Fig 3. Biofilm of *Pseudomonas aeruginosa* bound to dressing samples after one hour contact. The dressing surface in direct contact with the biofilm established on the plastic coverslip in test well (a). The surface not in contact with biofilm in the test well (b)

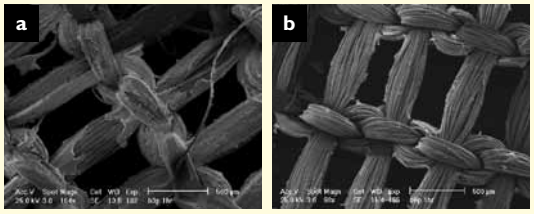


Fig 4. Binding of *Pseudomonas aeruginosa* biofilm to dressing samples. Uncoated after 1 hour contact (a), uncoated after 3 hour contact (b), DACC-coated after 1 hour contact (c), DACC-coated after 3 hour contact (d)

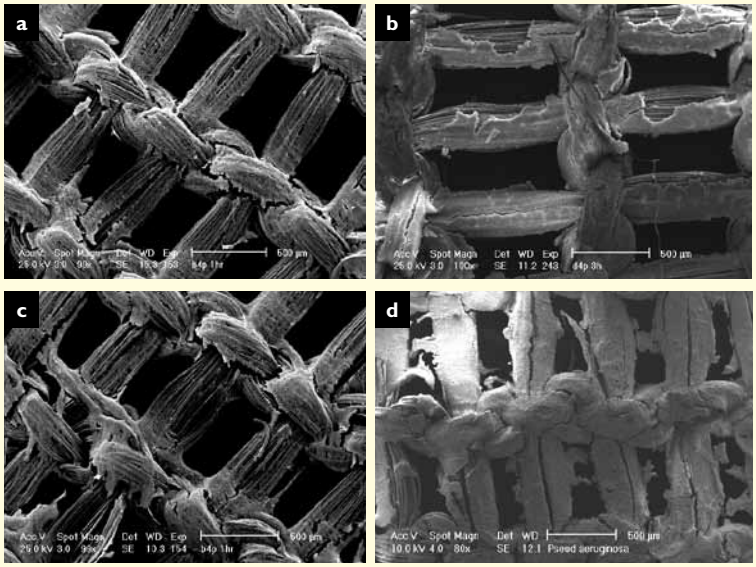


Fig 5. Binding of MRSA biofilm to dressing samples. Uncoated after 1 hour contact (a), uncoated after 3 hour contact (b), DACC-coated after 1 hour contact (c), DACC-coated after 3 hours contact (d)

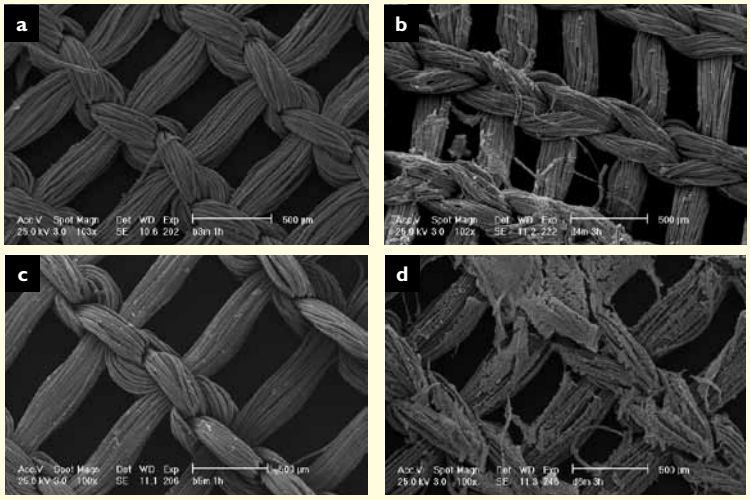
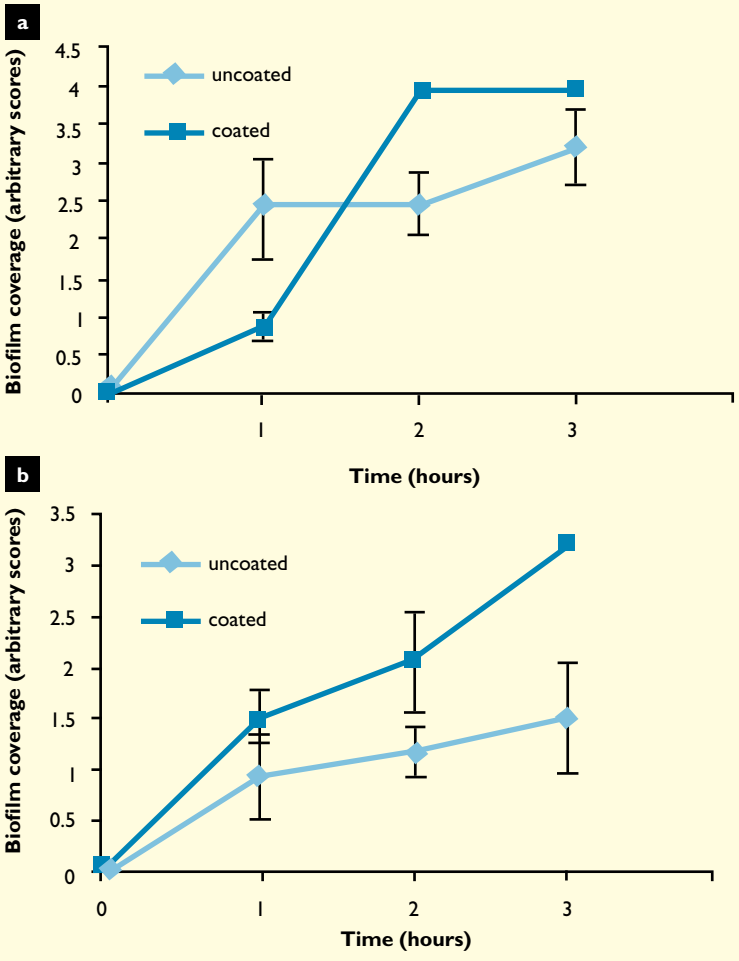


Fig 6. Biofilm coverage of dressing samples *Pseudomonas aeruginosa* (a) and MRSA (b)



ently. Mean scores and standard deviations were calculated and plotted versus time. Experiments were performed on two occasions.

Results

The presence of biofilm on dressing samples was determined using the surface that had been in contact with the biofilm established on the plastic coverslip, rather than the distal surface that had been in contact with the glass coverslip (Fig 1). It was seen that biofilm transferred from the plastic coverslips directly to dressing samples (Fig 3a) and did not migrate extensively through the dressing sample to the distal surface during the contact times tested here (up to 3 hours) (Fig 3b). Dressing samples exposed to *Pseudomonas aeruginosa* biofilm indicated rapid and extensive acquisition of biofilm (Fig 4); the extent of biofilm associated with uncoated (Fig 4a and 4b) and DACC-coated dressings (Fig 4c and d) showed no marked differences. Binding of MRSA biofilm to dressings was initially (Fig 5a and 5c) at a slower rate compared to *Pseudomonas aeruginosa* (Fig 4a and 4c). After a 3-hour contact period the coverage of uncoated dressing samples by MRSA biofilm (Fig 5b) was not as extensive as that of DACC-coated samples (Fig 5d), suggesting that the presence of the hydrophobic fatty acid derivative on the dressing surface did enhance biofilm binding. These observations were supported by the dressing coverage evaluations performed by the volunteer group (Fig 6).

In order to determine whether the bacterial cells attached to DACC-coated dressings were present as planktonic cells or as biofilms, some images at higher magnification were collected from samples tested with each of *Pseudomonas aeruginosa* and MRSA, biofilm structures were evident (Fig 7 and Fig 8, respectively).

Discussion

Using SEM to observe the extent of binding of established biofilm it was found that DACC enhanced the binding of MRSA biofilm compared with uncoated dressing samples (Fig 5d and 6a); this is in line with previous work on the binding of planktonic staphylococci to DACC-coated dressings.[5] *Pseudomonas aeruginosa*, however, bound similarly to coated and uncoated dressings. These differences probably reflect the distinct adhesins present on the surface of each species and the sticky nature of the extracellular polymeric material produced by *Pseudomonas*. Binding of established biofilms to dressing samples started within an hour of contact time for both test organisms. This concurs with the observation made by Ljungh et al., using planktonic *Pseudomonas aeruginosa,* that maximum binding to the DACC-coated dressing occurred at 120 minutes *in vitro*.[3]

The ability of some wound dressings to sequester

and immobilise microbial cells from simulated wound fluid *in vitro* has been described and benefits to infection control recognised.[9] Although the findings of this small laboratory study suggest a potential for DACC-coated dressings to lower the surface bioburden of wounds by binding biofilms as well as planktonic bacteria, it can only be confirmed *in vivo*. Recently, two pertinent studies have demonstrated a reduction in wound bioburden levels following the use of a DACC-coated dressings, although the presence of biofilm in neither study was tested. In one, using traditional culturing techniques of wound swabs to monitor the bioburden of aerobic bacteria in hard-to-heal leg ulcers, application of either Aquacel Ag or Cutimed Sorbact changed daily for a total observation period of four days, showed significant reductions in bioburden.[10] In the other, a molecular approach using punch biopsies collected weekly from chronic leg ulcers treated twice a week with DACC-coated dressing over a four-week period showed a significant decrease in the bacterial load of 10 out of 15 healing wounds, but no change in 5 out of 5 non-healing wounds. Clinical observations indicated that DACC-coated dressing resulted in completely successful therapy of 7 out of 20 patients and an improvement for 9 further patients. However, an analysis of information on bacterial load obtained from wound swabs taken from the same patients did not correlate with clinical outcome.[11] This raises the importance of considering the differential effects of topical interventions on bacterial species unequally distributed throughout the wound environment.[12] Biofilm is not universally located at the surface of the wound,[13] and it may be embedded within deeper tissue where it may not be affected by a therapy confined to the wound bed. The capacity of topical antimicrobial interventions to control biofilm in deep tissue must, therefore, always be evaluated clinically.

The fact that biofilms are especially tolerant to antibiotics[14] explains why some wounds fail to respond to antimicrobial interventions. Until effective antibiofilm agents are developed, the ability of a dressing to bind biofilms provides a non-invasive means to remove biofilm from the surface layer of a wound without sharp debridement or potentially cytotoxic chemical interventions. Another advantage of this approach is the diminished risk of the emergence of dressings-resistant species.

Limitations

An important limitation of this study is that it contains *in vitro* data, which is not necessarily transferable to the clinical situation. We used pure cultures of two representative bacteria that had been isolated from out-patients with chronic wounds attending a local hospital and cultivated them in microtitre plates for 24 hours before contact with dressing samples. Under these 'artificial conditions', the biofilms generated would not have been mature and would have behaved differently if they had been established for longer periods. There are many laboratory models for the study of biofilms, but none can accurately reproduce the complex conditions within a wound. Many experiments have used *Pseudomonas aeruginosa* cultivated in flow chambers where it grows to produce mushroom-like structures, but the relevance of these systems to human chronic infections has been questioned,[15] since such structures have not yet been observed in wounds.[16] Most chronic wounds are characterised by polymicrobial communities of microbial species *in vivo*[17–19] and mixed cultures are used in some experimental models.[20–22] Animal models can also provide more realistic conditions, even if confined to pure cultures.[23]

Laboratory investigations may help to elucidate mechanisms of action, but standardised methods for evaluating anti-biofilm agents are yet to be devised. However, it is clear that only clinical observations can establish the efficacy of antimicrobial interventions.

Fig 7. *Pseudomonas aeruginosa* biofilm attached to DACC-coated dressings at 1000x magnification (a) and 10000x magnification (b)

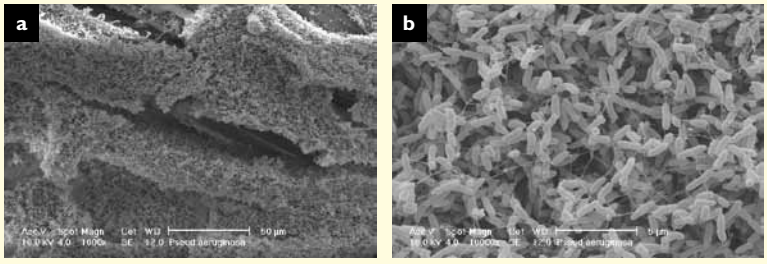
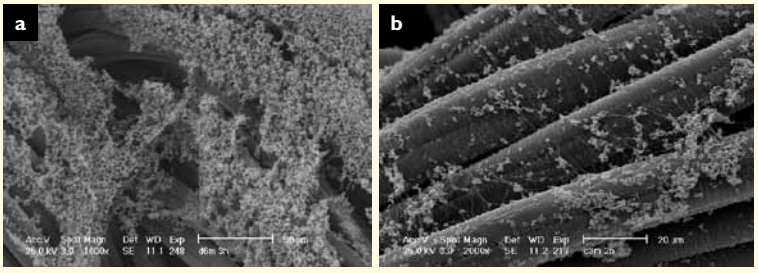


Fig 8. MRSA biofilm attached to DACC-coated dressings at 1000x magnification (a) and 2000x magnification (b)



Conclusion

This is the first demonstration that DACC-coated dressings bind MRSA and *Pseudomonas aeruginosa* biofilms *in vitro*. Whether this occurs widely *in vivo* has yet to be demonstrated, but this will only be known after the development of a routine biofilm diagnostic test that can be used before and after the clinical use of these dressings. ■

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