Combatting Wound Biofilm and Recalcitrance with a Novel Anti-biofilm Hydrofiber Wound Dressing

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Combatting wound biofilm and recalcitrance with a novel anti-biofilm Hydrofiber® wound dressing

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ABSTRACT

Background: Biofilm is an impediment to wound healing as a consequence of its proven ability to impair epithelialization, granulation tissue formation and normal inflammatory processes, as well as protecting wound pathogens from antibiotics and antiseptics. With this in mind, a project was initiated to develop a combined anti-biofilm/antimicrobial technology that could be incorporated into a wound dressing to maximize effectiveness against wound pathogens existing in their predominant biofilm form.
Methods: Initially, a wide range of anti-biofilm agents in combination with ionic silver were screened in a rapid throughput *in vitro* biofilm model. Selected agents were incorporated into a new wound dressing format and subsequently tested *in vitro* against antibiotic-resistant pathogens in their most tolerant biofilm form.
Results: The combination of ionic silver with a metal chelating agent and a surfactant was shown to produce a synergistic effect (referred to as Ag+ Technology) that substantially improved the antimicrobial efficacy of ionic silver against biofilm pathogens in a simulated wound biofilm model.
Conclusion: By combining anti-biofilm and antimicrobial components that work in synergy to disrupt biofilm and expose associated wound pathogens to the antimicrobial action of ionic silver, it is anticipated that this new technology incorporated into an advanced Hydrofiber® wound dressing will contribute significantly to managing biofilm infections and encouraging healing in patients debilitated by recalcitrant wounds.

1. Introduction

Bacteria exist naturally and preferentially in a biofilm mode of life in which they are surface-attached and encased within a matrix of self-produced extracellular polymeric substances (EPS) that provides protection from environmental hostilities such as host defences and biocides. The National Institutes of Health (NIH) has estimated that greater than 80% of human infections involve bacterial biofilm [1].

Pathogenic biofilm can arguably rank alongside antibiotic resistance as being a major concern in healthcare and infectious diseases today. Whereas bacterial resistance is a relatively recent and largely genetically-induced response to external attack from antibiotics, biofilm is a natural, phenotypic state that enables bacteria to tolerate exposure to external threats such as antibiotics and antiseptics [2,3]. Ultimately, the existence of antibiotic-resistant bacteria growing within biofilm presents a real and significant danger to public health. With this in mind, there is a clear need to facilitate the effectiveness of antibiotics and other antimicrobial agents by utilizing anti-biofilm strategies that can disrupt biofilm and expose the bacteria to more effective action of antimicrobial agents.

Recently, biofilm has been recognized as a cause of recalcitrance and infection in chronic wounds, and as an explanation for the frequent failure of antibiotics and antiseptics in these debilitating conditions [4,5]. Ionic silver is an effective and broad-spectrum topical antiseptic agent that is widely used in wound care to manage local infection [6–8], but, as with antibiotics and other antiseptics, its effectiveness against biofilm-protected bacteria is limited [9]. With a view to enhancing the clinical effectiveness of ionic silver in biofilm-impeded wounds, a project was undertaken to identify safe and effective anti-biofilm substances that could be used in combination with ionic silver. The clinical hypothesis was that if wound microflora in recalcitrant wounds could be transformed from a predominantly biofilm (tolerant) population to a

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predominantly planktonic (susceptible) population, then the efficacy of ionic silver is likely to be enhanced considerably. The aim therefore, was to utilize carefully selected anti-biofilm substances to maximize the antimicrobial activity of ionic silver in a commercially-available silver-containing Hydrofiber® dressing, AQUACEL® Ag Extra®.

2. Methods

2.1. Minimum biofilm eradication concentration (MBEC)

This method was used to identify anti-biofilm substances that would work most effectively with ionic silver to enhance the

Table 1
The independent variables studied, their broad classification (bold), type and/or examples of compounds (lists of chemical/biochemical agents screened).

Variable 1 (Biofilm-Disrupting Agent)			Variable 2 (Surfactant)	Variable 3 (pH)
Enzymatic			**Nonionic (no charge)**	
α amylase			Fatty alcohols	
α dornase			Fatty alcohol ethoxylates	
uronase			Alkyl phenol ethoxylates	
alginate lyase			Polyethylene glycol (and esters)	
non-specific proteainase			Polyoxyethylene alkyl ethers	**Acidic (<5)**
Dispersin B			Sorbitan esters	
			Ethoxylated Sorbitan esters	
			Alkyl polyglycosides	
			Sucrose esters	
			Alkanolamides	
			Polymers of alkylene oxides	
Metal Chelation			**Anionic (-ve charge)**	
Acetylacetone	Dimercaprol	o-Phenylenediamine	Alkyl sulphate	
BAPTA	2,3-Dimercapto-1-	Phytochelatin	Alkyl ether sulphate	
Bipyridine (bipy)	propanesulfonic acid	Porphin	Sulphated ethoxylate	**Mildly acidic (~6)**
2,2'-Bipyridine	Dimercaptosuccinic acid	Porphyrins	Sulphated alkanolamides	
Chlorophyll	Dimethylglyoxime	Pyrocatechol	Alkyl sulphosuccinates	
Citric acid	Dithiolene	Scorpionate ligand	Alkyl carboxylate (soap)	
Corrole	1,2-Ethanedithiol	Diethyldithiocarbamate	Alkyl ethoxycarboxylate	
Crown ether	Ethylenediamine	Poly(aspartate)	Acyl Sarcosinates	
18-Crown-6	1,4,7-Trithiacyclononane	Orthophosphate		
Cryptand	Gluconic acid	Pyrophosphate		**Neutral (7)**
Cyclen	Hemoglobin	Polyphosphate		
Deferasirox	Hexafluoroacetylacetone	Terpyridine		
Deferiprone	Histidine	Tetramethylethylenediamine		
Deferoxamine	Indo-1	Trans-1,2-		
Dexrazoxane	Maleic acid	Diaminocyclohexane		
Diethylenetriamine	Nitrilotriacetic acid (NTA)	1,4,7-Triazacyclononane		
Diglyme	Penicillamine	Triethylenetetramine		
EDTA	Aminoethylethanolamine	Bis(dimethylarsino)benzene		
EGTA	Sodium Tartrate	Bis(diphenylphosphino)ethane		
Fura-2	Pentetic acid	Phenanthroline		
Ion Exchange Polymers			**Cationic (+ve charge)**	
Bentonite		Gelatin	Arylalkyl quaternary ammonium	
Carboxymethylcellulose (CMC)		Pectin	Alkyl quaternary ammonium	**Mildly alkaline (~8)**
Colloidal silicate		Chitosan	Imidazolium	
Magnesium aluminium silicate		Starch	Ethoxylated quaternary ammonium	
Calcium silicate		Alginate	Amine oxide	
Agar			Alkyl amine	
EPS Softening / Solubilisation			**Amphoteric (+ve and -ve charges)**	
Propylene glycol		Xylitol	Alkyl glycinates	
Polyethylene glycol		Glycerol	Alkylaminopropionates	
Triethanolamine		Sorbitol	Alkyl betaines	
Cyclomethicone		Maltitol	Sulphobetaines	
Ammonium lactate		Mannitol		
Glycerol esters		Maltodextrin		**Alkaline (>9)**
Monosaccharides (glucose)		Polydextrose		
Disaccharides (sucrose)		Carrageenan gum		
Trisaccharides (trehalose)		Locust bean gum		
Polydextrose		Guar gum		
Sorbitol		Tragacanth gum		
Xanthan gum				

activity of the commercially-available silver Hydrofiber® dressing, AQUACEL® Ag (SH).

First published in 1999, the MBEC method was designed as a high-throughput *in vitro* method to measure antibiotic susceptibility in the presence of bacterial biofilm [10]. It has been adapted here to investigate the efficacy of compounds in conjunction with ionic silver.

Test variables, which included biofilm disrupting substances, surfactants and pH (Table 1), were investigated individually or in combination against a doubling-dilution gradient of ionic silver (128 μg/ml reducing to 1 μg/ml). Briefly, the method used 96-well microtiter plates (wells) with lids with corresponding protruding spikes (pegs) [Nunc, Denmark] (Fig. 2). *Pseudomonas aeruginosa* (NCIMB 8626) in planktonic form (suspension) was inoculated into each well (1×10^5 in 150 μl) and the peg lid was fitted. Biofilm formation was encouraged by incubation with gentle agitation (24 h/35 °C/gyration). Pegs were then washed (×2) to remove planktonic bacteria and inserted into fresh wells containing the test substance(s) (30 min, 35 °C). Treated pegs were neutralized by washing with isotonic saline (×5), then placed into further wells containing a growth medium (Mueller Hinton Broth, MHB) and sonicated (5 min) to destroy residual biofilm EPS, thus releasing planktonic bacteria. The peg lid was then discarded and replaced by a plain lid. Wells were incubated (24 h/35 °C) and optical density at 595 nm was then determined using a microtiter plate spectrophotometer [Powerwave XS, BioTek Instruments Inc.]. Anti-biofilm activity was determined semi-quantitatively by the degree of opacity being proportional to biofilm survival.

2.2. Gauze biofilm model [11]—simulated use testing of the formulated product

Having identified the anti-biofilm substances that worked most effectively with ionic silver to provide a combined anti-biofilm/antimicrobial effect, the anti-biofilm silver-containing Hydrofiber® dressing subsequently formulated (AQUACEL® Ag+ Extra [ABSH]) was compared with three other, commercially-available silver-containing wound dressings (see Table 2) in a stringent *in vitro* gauze biofilm model. All three of the comparator silver-containing dressings are widely used in wound care of which one was the same silver-containing Hydrofiber® dressing without the addition of anti-biofilm substances.

This method was developed from the observation that implanted gauze dressing in experimental wounds promoted and prolonged infection [12] which was likely induced by biofilm formation on the gauze implant. All three dressings tested in this model are indicated for use on wounds that are locally infected or at risk of infection.

Mature *P. aeruginosa* (NCTC 13437) or community-associated Methicillin-resistant *Staphylococcus aureus* (CA-MRSA, USA-300) biofilm was grown by inoculating 1×10^6 CFU/ml in a nutrient-rich medium onto pieces of sterile gauze [N-A knitted viscose primary dressing (Systagenix)] and incubating (48 h/35 °C/gyration) (Fig. 1A). Biofilm-gauze substrates (BGSs) were rinsed (×2; Fig. 1B) and the presence of biofilm was confirmed by environmental scanning electron microscopy (ESEM, Quanta200, FEI) (Fig. 1C) and confocal laser-scanning microscopy (CLSM, TCS SP2, Leica) (Fig. 1D). BGSs were cut to 35 mm diameter circles and transferred onto Tryptone Soy Agar contact plates (the simulated wound bed) which were fitted into a Perspex block covered with dried leather (simulating peri-wound skin) (Fig. 2A). Test dressings were placed over the BGS, hydrated with a simulated wound fluid [6] and covered with an adhesive secondary cover dressing [AQUACEL™ Foam Dressing, ConvaTec] (Fig. 2B and C). Test models were then incubated for up to nine days, and after 4–5 days, BGSs were re-inoculated with 1×10^5 CFU bacteria to challenge the dressing's ability to prevent biofilm re-formation (Fig. 2E) over a further 3–4 days. After 4 h and daily time points biofilm-gauze substrates (5 per treatment time per test dressing) were removed (Fig. 2D), placed in neutralising broth and stomached (4 min, high setting) to detach and disperse residual biofilm. Quantitative microbiological methods were then used to determine the bioburden in the resultant suspension and hence calculate the survival of bacteria in the BGSs.

3. Results

3.1. MBEC screening study

Three variables were studied: biofilm disrupting substances, surfactants and pH (Table 1). Approximately 250,000 potential combinations were identified but this was reduced to ~60,000 tests by eliminating potentially unsafe or chemically incompatible options.

Of the biofilm-disrupting substances, metal chelation and ion exchange polymers performed most effectively in the MBEC model. Enzymes that were considered were generally inhibited by ionic silver; they interfered with antimicrobial activity, and were strongly pH-dependent and were hence excluded from further consideration. The biofilm softening/solubilizing candidates were ineffective and in many cases encouraged biofilm proliferation. For the surfactants, the majority of anionic surfactants were precipitated by ionic silver and quaternary cationic surfactants were most effective.

The optimum combination with ionic silver was the metal chelating substance ethylenediaminetetra-acetic acid (EDTA), the quaternary cationic surfactant benzethonium chloride and a pH of approximately 5.5. None of the individual components showed activity in isolation, but were strongly synergistic when brought together.

Table 2
Test dressings (wound dressing details).

Dressing Reference	Dressing	Description (*United Kingdom IFU description*)	Silver Content (by assay)
SH	AQUACEl®Ag Extra™ (ConvaTec Inc.)	*Two layers of 1.2% ionic silver impregnated sodium carboxymethylcellulose stitched together with strengthening fibers.*	0.16 mg/cm^2 (1.2% w/w dry)
ABSH	AQUACEL®Ag+ Extra™ (ConvaTec Inc.)	*Two layers of sodium carboxymethylcellulose impregnated with 1.2% ionic silver (an antimicrobial agent), enhanced by ethylenediaminetetra-acetic acid di-sodium salt (EDTA) and benzethonium chloride, and strengthened by regenerated cellulose fibre.*	0.16 mg/cm^2 (1.2% w/w dry)
SNA	Silvercel®NA (Systagenix)	*Non-woven pad composed of high G (guluronic acid) alginate, carboxymethyl cellulose (CMC) and elemental silver-coated nylon fibers, laminated to a perforated, non-adherent ethylene methyl acrylate (EMA) wound contact layer.*	0.74 mg/cm^2 (2.2% w/w)
AC7	Acticoat ™ 7 (Smith & Nephew Medical Limited)	A layered dressing consisting of *two layers of absorbent rayon/polyester leaved between three layers of elemental nanocrystalline silver-coated, low adherent polyethylene net.*	1.8 mg/cm^2 (11.2% w/w)

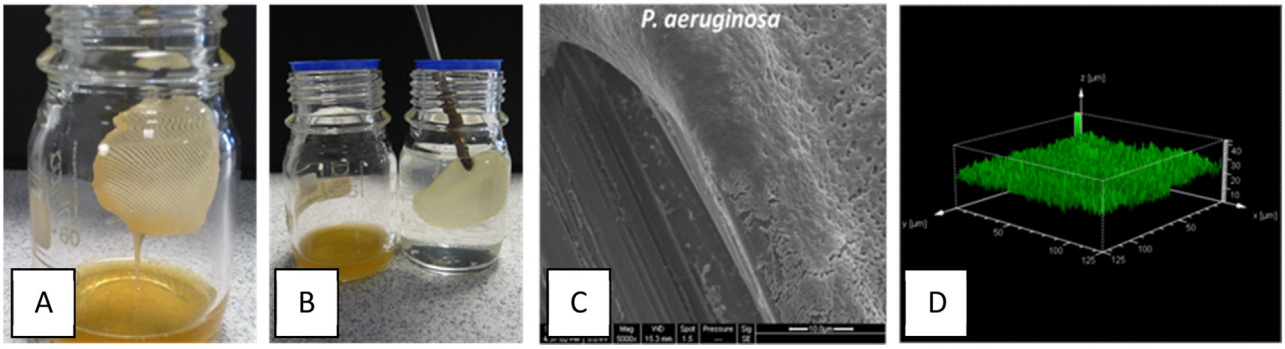

Fig 1. *P. aeruginosa* biofilm on gauze; rinsing of biofilm gauze to remove planktonic bacteria; confirmation of biofilm on gauze using ESEM; confocal microscopy.

3.2. Gauze biofilm model

Four silver-containing antimicrobial wound dressings were tested (Table 2). The anti-biofilm silver-containing Hydrofiber® dressing (ABSH) was the only antimicrobial dressing tested in this *in vitro* model that was capable of killing a highly antibiotic-resistant strain of *S. aureus* (CA-MRSA) in biofilm form (Fig. 3). The same silver-containing dressing without anti-biofilm enhancement (Ag+ Technology) (SH) was not able to eliminate CA-MRSA biofilm. Other high silver-content dressings (AC7 and SNA) were ineffective against CA-MRSA biofilm in this model.

The ABSH dressing was superior to SH against a multidrug resistant *P. aeruginosa* biofilm; AC7 and SNA dressings had no antimicrobial/anti-biofilm effect (Fig. 4). The ABSH dressing was the most effective in preventing biofilm re-formation following re-inoculation of the gauze substrate during the test period.

4. Discussion

An expert panel representing the European Society for Clinical Microbiology and Infectious Diseases (ESCMID) recently published a guideline for the diagnosis and treatment of biofilm infections, in which new combinations of antibiotics with biofilm-dissolving agents were reported to be urgently needed [13]. Management of biofilm infections is considered to require multi-disciplinary intervention, involving removal of infected tissues or related devices, and use of well-penetrating antimicrobial agents in combination with biofilm dispersal agents [13]. In biofilm-

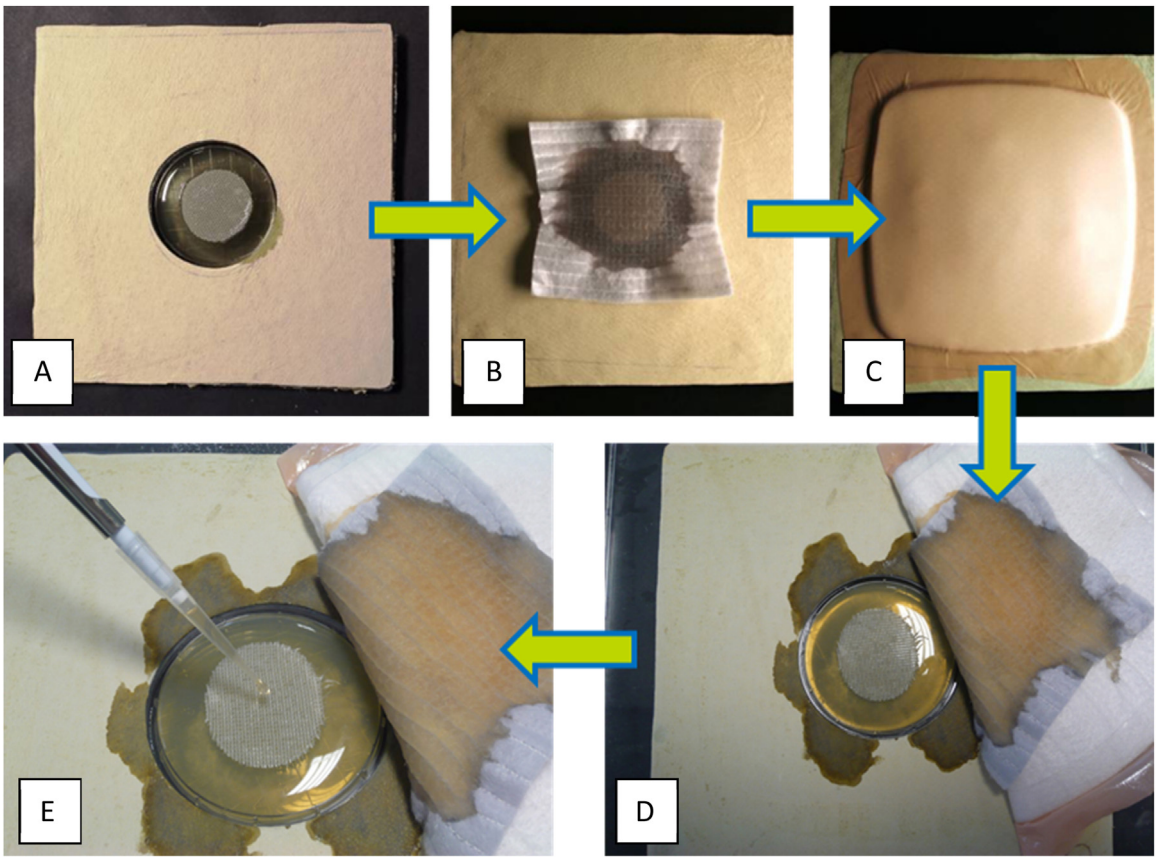

Fig. 2. Gauze biofilm model. Agar contact plate inserted into center of the model surrounded by simulated skin and biofilm-colonized gauze applied to create a simulated biofilm-colonized wound bed (A); dressing placed over the simulated biofilm-colonized wound bed and hydrated with simulated wound fluid (B); application of a secondary cover dressing (C); after 4–5 days, re-challenge with fresh inoculum (D, E).

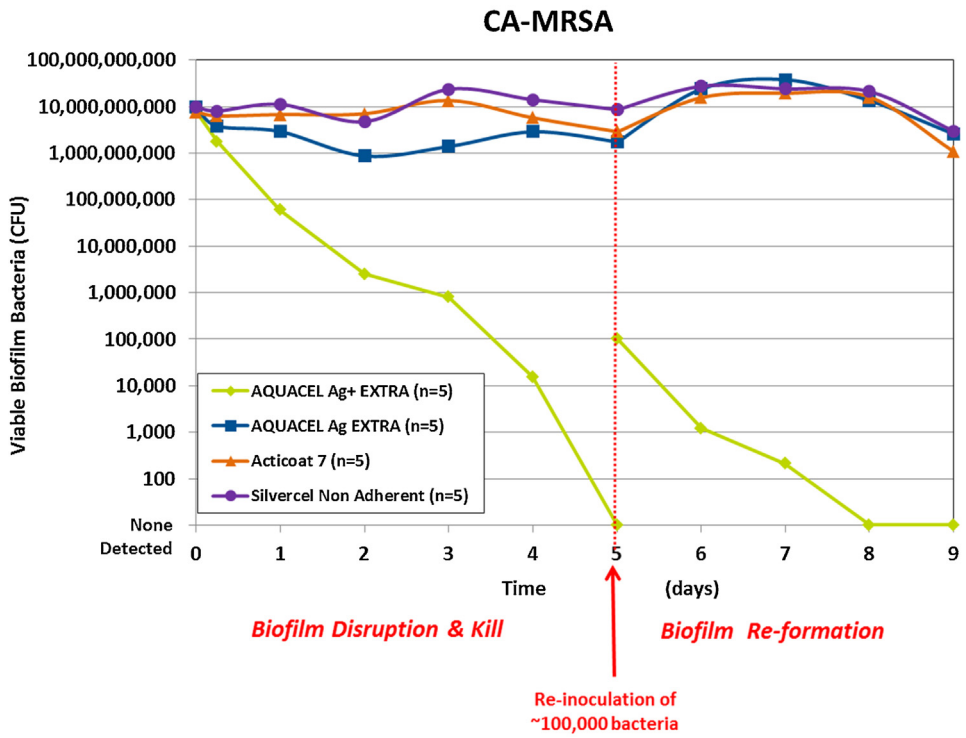

Fig. 3. Antimicrobial/anti-biofilm efficacy of an anti-biofilm silver-containing Hydrofiber® dressing (ABSH) in comparison to traditional silver dressings against CA-MRSA.

impeded chronic wounds, regular debridement has been considered to be critical in disrupting biofilm and increasing microbial susceptibility to antibiotics and antiseptics [14]. Consequently, a multi-faceted approach and use of combination therapies are clearly needed for effective clinical management of wound biofilm.

Biofilm involvement in wound healing is now an area of active research, and is considered to be one of the major local factors responsible for impaired wound healing and infection [4,5]. Non-healing wounds often fail to respond to antibiotic treatment and/or antimicrobial dressings, and it is now believed that the presence of biofilm encourages bacterial tolerance within the wound environment [3,15]. This prompts the requirement for new therapies that are able to disrupt wound biofilm and expose associated micro-organisms in their planktonic form to enable more effective action

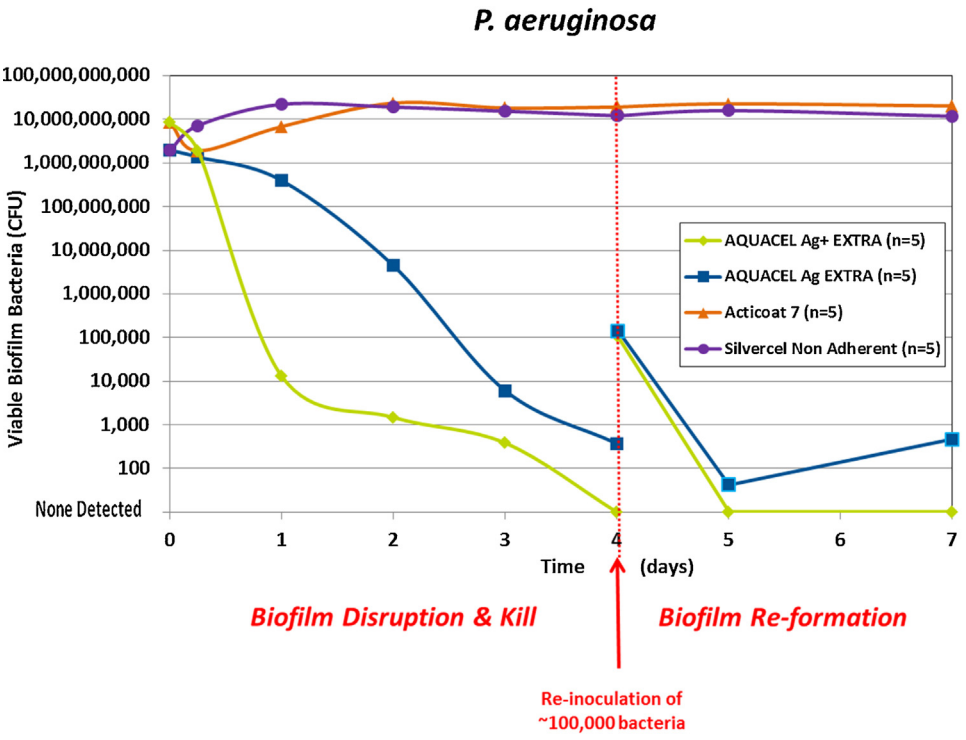

Fig. 4. Antimicrobial/anti-biofilm efficacy of an anti-biofilm silver-containing Hydrofiber® dressing (ABSH) in comparison to traditional silver dressings against *P. aeruginosa*.

of antibiotics and topical antimicrobial agents. The ABSH dressing has been designed with this approach in mind.

It has been previously reported that there is no correlation between dressing silver content and antimicrobial activity against planktonic bacteria [16] and the same observation can be made for anti-biofilm activity from this study. SH demonstrated some anti-biofilm activity (Fig. 4) and this has been previously reported elsewhere [8,9]. As the other, higher silver-content dressings showed no activity, the activity of SH must be due to an intrinsic property of the base dressing (Hydrofiber®, a swellable but insoluble carboxymethylcellulose [CMC]) rather than the silver component. Indeed a soluble CMC tested in the MBEC model demonstrated some synergistic anti-biofilm effects.

Consideration of pH may be important for several reasons: (1) an acidic pH increases the antimicrobial activity of ionic silver [8,17] and, as shown in the current study, reduces biofilm integrity thereby increasing microbial susceptibility; (2) chronic wounds are typically alkaline [18], acute wound healing is associated with decreasing pH [19], and proteolytic enzymes found in wounds have peak activities at alkaline or neutral pH [19,20], therefore low pH is generally preferable; (3) the base fibre of the ABSH dressing is mildly acidic and can reduce the pH of serum [18]. The inclusion of EDTA in the ABSH dressing will further enhance the dressing's pH buffering ability.

The scale of the synergistic effect seen between silver, benzethonium chloride and EDTA in the MBEC experiments was surprising and these observations have been confirmed in an independent model [21]. This synergy enabled the ABSH dressing to be formulated with a relatively low concentration of each of these compounds such that there was no change in tonicity, toxicity or physical performance from SH. This enhanced, synergistic effect was maintained in ABSH, in a stringent, simulated *in vitro* biofilm wound model, where the ABSH dressing demonstrated superior anti-biofilm/antimicrobial performance compared with a variety of traditional silver dressings.

Subsequent to the research, development and *in vitro* investigation of the ABSH dressing reported here, *in vivo* and clinical studies with this dressing have demonstrated favourable outcomes. In a well-characterised rabbit ear wound biofilm model, the ABSH dressing was shown to significantly reduce polymicrobial biofilm and promote wound healing *versus* active and inactive controls [22]. Clinical safety and effectiveness of the dressing has also been demonstrated in a study involving 42 subjects with recalcitrant venous leg ulcers [23], and in a separate clinical evaluation across Europe and Canada involving 113 recalcitrant wounds of varied aetiology (49% of which had a duration of greater than six months, and a majority was suspected as being biofilm-positive), the ABSH dressing was associated with considerable improvement in a high proportion of wounds over an average treatment period of 4.1 weeks [24]. A subsequent clinical evaluation in 29 recalcitrant acute and chronic wounds with suspected biofilm reported that 34% (10) of the wounds healed completely, and 90% (26) became smaller in size following application of the ABSH dressing for a median treatment period of 4.5 weeks [25].

The ABSH dressing is an innovative, next-generation antimicrobial wound dressing that has been developed to meet a previously unmet clinical need. It combines anti-biofilm and antimicrobial components that work in synergy to disrupt biofilm and expose associated microorganisms to the broad-spectrum antimicrobial action of ionic silver. It is anticipated that this dressing will contribute significantly to managing biofilm-impeded wounds and infections, and promoting wound healing in patients debilitated by recalcitrant wounds. Early experimental and clinical evidence is encouraging.

Conflict of interest

Philip Bowler and David Parsons are employed by ConvaTec Limited, UK.

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