2,3-Dihydroxybenzoic Acid-Containing Nanofiber Wound Dressings Inhibit Biofilm Formation by *Pseudomonas aeruginosa*

Jayesh J. Ahire, Leon M. T. Dicks

Department of Microbiology, University of Stellenbosch, Matieland (Stellenbosch), South Africa

Pseudomonas aeruginosa forms biofilms in wounds, which often leads to chronic infections that are difficult to treat with antibiotics. Free iron enhances biofilm formation, delays wound healing, and may even be responsible for persistent inflammation, increased connective tissue destruction, and lipid peroxidation. Exposure of *P. aeruginosa* Xen 5 to the iron chelator 2,3-dihydroxybenzoic acid (DHBA), electrospun into a nanofiber blend of poly(D,L-lactide) (PDLLA) and poly(ethylene oxide) (PEO), referred to as DF, for 8 h decreased biofilm formation by approximately 75%. This was shown by a drastic decline in cell numbers, from $7.1 \log_{10}$ CFU/ml to $4.8 \log_{10}$ CFU/ml when biofilms were exposed to DF in the presence of 2.0 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$. A similar decline in cell numbers was recorded in the presence of 3.0 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and DF. The cells were more mobile in the presence of DHBA, supporting the observation of less biofilm formation at lower iron concentrations. DHBA at MIC levels (1.5 mg/ml) inhibited the growth of strain Xen 5 for at least 24 h. Our findings indicate that DHBA electrospun into nanofibers inhibits cell growth for at least 4 h, which is equivalent to the time required for all DHBA to diffuse from DF. This is the first indication that DF can be developed into a wound dressing to treat topical infections caused by *P. aeruginosa*.

Pseudomonas aeruginosa is a versatile opportunistic pathogen commonly associated with a number of nosocomial infections of the respiratory tract, urinary tract, and wounds (1, 2, 3). The antibiotic-induced killing of *P. aeruginosa* is extremely poor in chronic wounds such as leg ulcers or burn wounds (4). The species protects itself from antibiotics by forming biofilms (5–7) and by doing so develops resistance to antibiotics (6, 8, 9).

Cystic fibrosis is one of the best-studied examples of chronic infections caused by biofilms of *P. aeruginosa* (10). Zhao et al. (11) have shown that biofilm formation by *P. aeruginosa* is stimulated by Psl exopolysaccharide on the cell surface. However, Singh et al. (12) and Banin et al. (13) attributed biofilm formation by *Pseudomonas* spp. to higher levels of free iron in the environment. Free iron may retard wound healing and may even be responsible for persistent inflammation, increased connective tissue destruction, and lipid peroxidation (14). These pose unique challenges in the development of antimicrobial drugs effective against *P. aeruginosa*.

Lactoferrin, an iron chelator in mammals, inhibited biofilm formation (12, 13, 15). Similar results were obtained with synthetic iron chelators such as 2,2'-dipyridyl (2DP), diethylenetriaminepentaacetic acid (DTPA), EDTA, deferoxamine mesylate (DM), and ethylenediamine-*N,N'*-diacetic acid (EDDA) (16). As far as we could determine, the effect of the iron chelator 2,3-dihydroxybenzoic acid (DHBA) on biofilm formation by *Pseudomonas* spp. has not been reported.

DHBA has been used orally as a noncytotoxic iron-chelating agent in patients suffering from β -thalassemia (17, 18). DHBA is produced during aspirin metabolism in humans and is present in many plants (19, 20). DHBA has higher binding constants for iron than transferrin and lactoferrin (21). Incorporation of DHBA in nanofiber wound dressings could thus control bacterial growth and biofilm formation in chronic wounds, especially in diabetic patients. In this study, we report on the effect that DHBA, electrospun into nanofibers, has on biofilms formed by *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *P. aeruginosa* Xen 5, a bioluminescent derivative of a clinical strain (ATCC 19660) isolated from human septicemia, was obtained from Caliper Life Sciences (Hopkinton, MA). Luria-Bertani (LB) broth, supplemented with 60 $\mu\text{g}/\text{ml}$ tetracycline, was used to maintain the plasmid containing the *lux* operon.

Electrospinning of nanofibers. Nanofibers were prepared by spinning DHBA (50 mg/ml) into a blend of poly(D,L-lactide) (PDLLA) and poly(ethylene oxide) (PEO) (24%; 50:50), as described by Heunis et al. (22). *N,N*-dimethylformamide (DMF) was used as the solvent for PEO:PDLLA. The relative humidity was controlled at 50 to 55% by keeping the temperature between 28 and 30°C. Control nanofibers were produced using the same method, but without DHBA. Nanofibers containing DHBA (DF) and control nanofibers (CF) were cut into sections of 0.5 cm². Nanofibers were coated with gold to increase conductivity and images were recorded by using a Leo 1430VP scanning electron microscope (Zeiss). The diameters of the nanofibers were determined using ImageJ Software (version 1.46; Scion Corporation).

Release of DHBA from nanofibers. Nanofiber samples of 0.5 cm² (DF) were placed in 0.5 ml sterile Milli-Q water and incubated at 37°C. At specific time intervals, the nanofibers were transferred to 0.5 ml sterile Milli-Q water. Release of DHBA in the water was determined by using the universal chrome azurol S (CAS) assay (23). Five-hundred microliters of the DHBA suspension was mixed with 500 μl CAS reagent. Absorbance readings were recorded at 630 nm (A_s), with Milli-Q water as the blank. Milli-Q water with CAS reagent served as the reference (A_r). The percentage of chelator units was defined as $[(A_r - A_s)/A_r] \times 100$. Chelator units recorded for nanofibers were compared against a standard curve compiled with known concentrations of DHBA.

Received 1 November 2013 Returned for modification 18 November 2013

Accepted 17 January 2014

Published ahead of print 21 January 2014

Address correspondence to Leon M. T. Dicks, lmt@sun.ac.za.

Copyright © 2014, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AAC.02397-13

FIG 1 Schematic representation of the electrospinning of DHBA into a blend of PDLLA and PEO to produce nanofibers of 400 to 500 nm in diameter.

Biofilm formation and changes in cell numbers of *P. aeruginosa* Xen 5 when exposed to nanofibers containing DHBA. *P. aeruginosa* Xen 5 was grown overnight at 37°C in Luria-Bertani (LB) broth supplemented with 60 µg/ml tetracycline. The culture was diluted to $8.5 \pm 0.02 \log_{10}$ CFU/ml in M63 biofilm assay medium [3 g KH_2PO_4 , 7 g K_2HPO_4 and 2 g $(\text{NH}_4)_2\text{SO}_4$ in 1 liter of water], as described by O'Toole (24). The medium was supplemented with tetracycline (60 µg/ml), magnesium sulfate (1 mM), and arginine (0.4%, wt/vol) to stimulate biofilm formation. Wells in a 48-well polystyrene flat-bottom multidish (BioLite MultiDishes, Thermo Scientific, NY) were each filled with 200 µl of the cell suspension, thus $\sim 7.8 \log_{10}$ CFU per well. Nine wells each received 0.5 cm² of DF, nine wells 0.5 cm² CF, and the rest no nanofibers. The multidish was incubated at 37°C without shaking.

The total numbers of *P. aeruginosa* Xen 5 cells, dead and viable, in a biofilm were determined by staining the wells with crystal violet, as described by O'Toole (24). In brief, 250 µl of 0.1% (vol/vol) crystal violet was added to the biofilms in the wells and incubated at 25°C for 10 to 15 min. After incubation, the wells were carefully rinsed three to four times with sterile distilled water, blotted dry by placing the multidish upside down on paper towels, and then left for 4 to 5 h to air dry. Acetic acid (250 µl; 30%, vol/vol) was added to each well and left at 25°C for 10 to 15 min to solubilize the crystal violet. The density of the crystal violet in solution was determined by recording optical density at 595 nm (OD_{595}) readings in a microplate reader (model 680; Bio-Rad). Acetic acid (30%, vol/vol) served as the blank.

The number of viable cells of *P. aeruginosa* Xen 5 in the biofilm was determined by removing planktonic cells and nanofibers from the wells, followed by gentle rinsing of the wells with three volumes of sterile distilled water. Special care was taken not to disturb the biofilms. Two-hundred microliters of phosphate-buffered saline (PBS) (pH 7.3) was added to each well and gently agitated with a glass rod. The cell suspension was then transferred to a sterile tube, vortexed for 1 min, and serially diluted with sterile PBS. One-hundred microliters of each dilution was spread plated onto LB agar and the plates incubated at 37°C for 24 h.

Effect of iron on biofilm development. An overnight-grown culture of *P. aeruginosa* Xen 5 was diluted to $8.5 \pm 0.02 \log_{10}$ CFU/ml in M63 biofilm assay medium supplemented with tetracycline, magnesium sulfate, and arginine, as described elsewhere. The cell suspension was divided into three equal parts, to which 1.0 mM, 2.0 mM, and 3.0 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ were added, respectively. Wells of a 48-well polystyrene flat-bottom multidish were each filled with 200 µl of the cell suspension. Nine wells each received 0.5 cm² of DF, nine wells 0.5 cm² CF, and the rest no nanofibers. The multidish was incubated at 37°C without shaking. At specific time intervals, the cell suspensions and nanofibers were removed from the wells and biofilm formation was recorded as described above. Viable cell numbers were determined by plating onto LB agar, as described above.

In another experiment, the effects of DHBA, iron, and a combination of DHBA plus iron on the twitching mobility of strain Xen 5 were studied as described by Singh et al. (12), but with minor modifications. Overnight-grown cells of *P. aeruginosa* Xen 5 were stab inoculated into M63 agar (1%, wt/vol, agar), supplemented with DHBA (2.0 mM in 50%, vol/

FIG 2 Release of DHBA from DF. Data points show average values from three independent experiments (mean $\pm$ standard deviation).

FIG 3 Biofilm formation of *P. aeruginosa* Xen 5, expressed as optical density (OD_{595}) of crystal violet diffused from stained cells (a, c, e, and g) and viable cell numbers, $\log_{10}$ CFU/ml (b, d, f, and h) when cells were grown in the presence of nanofibers containing DHBA (DF) or nanofibers without DHBA (CF) and in the absence of nanofibers (control). The effects of different iron concentrations on biofilm formation are shown in panels c to h. Data points are the averages of three independent experiments (mean $\pm$ standard deviation). *, $P < 0.001$.

vol, DMF), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (1.0 mM in Milli-Q water), and a combination of 2.0 mM DHBA plus 1.0 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, respectively. Tetracycline (60 $\mu\text{g}/\text{ml}$) was added to the growth medium. Plates were incubated at 37°C for 3 to 4 days and examined for the presence of growth between the interface of the plate and the surface of the agar. The distance the cells migrated from the stab was measured in mm. The agar was carefully removed, the cells that adhered to the surface of the plates were stained with crystal violet (0.1%, vol/vol), and the migration patterns of the cells were studied under a light microscope.

Antimicrobial activity of DHBA. The antimicrobial activity of DF compared to CF was evaluated by using a slide culture model. In brief, 100 μl M63 molten agar (1%, wt/vol, agar), containing 60 $\mu\text{g}/\text{ml}$ tetracycline, was dispensed in a circle (15-mm diameter) on a sterile microscope glass slide. The agar was allowed to solidify and 10 μl of a *P. aeruginosa* Xen 5 cell suspension ($8.9 \pm 0.06 \log_{10}$ CFU/ml) was deposited in the center of the solidified medium. Samples of DF and CF (0.5 cm^2) were placed on the surface of the agar containing the cells. The glass slides were placed in petri dishes and incubated at 37°C . Drying out of the slides was prevented by sealing the plates with Parafilm. At specific time intervals, the slides were observed for bioluminescence using the *in vivo* imaging system (IVIS 100) of Caliper Life Sciences. The number of photons per second per cm^2 per steradian ($\text{p s}^{-1} \text{cm}^{-2} \text{sr}^{-1}$) emitted from each reactive oxygen intermediate (ROI) was calculated using the Living Image software (version 3.0) of Caliper Life Sciences. The agar was scraped from the glass slides, suspended in 1 ml sterile PBS, vortexed for 1 min, and serially diluted in sterile PBS. One-hundred microliters of each dilution was spread plated onto LB agar and the plates were incubated at 37°C . Colonies were counted after 24 h of incubation.

In another experiment, the effect of DHBA on the growth of *P. aeruginosa* Xen 5 was tested in the presence of iron. An overnight-grown culture of *P. aeruginosa* Xen 5 was inoculated into 100 ml iron-deficient LB broth to obtain $6.4 \pm 0.02 \log_{10}$ CFU/ml. The LB both was deferrated by the addition of 0.5% (wt/vol) 8-hydroxyquinolin (8HQ). After 30 min of stirring at 25°C , the Fe-8HQ complex was extracted by adding 20% (vol/vol) chloroform to the broth, in three consecutive steps, and then autoclaved. The cell suspension was divided into three equal volumes, to which DHBA (116 $\mu\text{g}/\text{ml}$ in 50%, vol/vol, DMF), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (1.0 mM in Milli-Q water), and a combination of DHBA (116 $\mu\text{g}/\text{ml}$) and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (1.0 mM) were added, respectively. The control culture contained no $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, but received 0.1 ml 50% (vol/vol) DMF. Incubation was at 37°C for 8 h. Samples were taken after every 2 h, serially diluted, and plated onto LB agar, and then colonies were counted after 24 h of incubation at 37°C .

Determination of MIC. DHBA was dissolved in 50% (vol/vol) DMF and added to LB broth to final concentrations ranging from 0.05 mg/ml to 5.0 mg/ml. Each of the test tubes was inoculated with *P. aeruginosa* Xen 5 to a final OD_{595} of 0.01 and incubated at 37°C for 24 h. The MIC was defined as the lowest concentration of DHBA producing complete inhibition of visible growth.

In another experiment, *P. aeruginosa* Xen 5 was inoculated into LB broth, incubated at 37°C to an OD_{595} of 0.01, and then treated by adding DHBA at the MIC level. Changes in optical density was recorded at 595 nm over 24 h. Cells grown in the presence of 50% (vol/vol) DMF served as controls.

Statistical analysis. Statistical analysis was performed by one-way analysis of variance (ANOVA) and with the *t* test by using GraphPad Prism (version 6.03 [Trial] for Windows; GraphPad Software, Inc., CA). Differences were considered statistically significant at *P* values of <0.05 .

RESULTS

Electrospun nanofibers. Electrospinning of DHBA into a blend of PDLLA and PEO (24%; 50:50) produced nanofibers of 400 to 450 nm in diameter. Nanofibers spun without DHBA had a diameter of 450 to 500 nm (Fig. 1).

Release of DHBA from nanofibers. Almost all DHBA was re-

FIG 4 (A) Mobility of *P. aeruginosa* Xen 5 recorded in the interface between the plate and the agar surface after 3 to 4 days of incubation at 37°C . The top three images (Aa through Ac) were taken after the agar was removed from the plates, and cells that adhered to the plates were stained with crystal violet. (Aa) Growth in the presence of 2.0 mM DHBA; (Ab) growth in the presence of 2.0 mM DHBA plus 1.0 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$; (Ac) growth in the presence of 1.0 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$. Data points presented are the average values of three independent experiments (mean $\pm$ standard deviation). (B) Microscopic images of *P. aeruginosa* Xen 5 in the presence of DHBA (a) and iron (b), as observed at $\times 10$ magnification. *, *P* < 0.01. Arrows indicate direction of cell mobility.

leased from DF within 2 h (18.8% CAS decolorization units), followed by a slow, linear release over the following 2 h to no detectable levels of DHBA (Fig. 2). Deduced from a standard curve with known concentrations of DHBA (not shown), 18.8% CAS corresponded to $116 \pm 0.03 \mu\text{g}$ DHBA per 0.5 cm^2 nanofiber.

Biofilm formation. Optical density (OD_{595}) readings recorded for biofilms that formed after 8 h in the presence of DF were 1.2 and significantly (*P* < 0.001) lower than OD readings of 1.7 recorded for biofilms that formed in the presence of CF and 1.8 in the absence of nanofibers (Fig. 3a). The number of viable cells in an 8-h-old biofilm and in the presence of DF were $6.4 \log_{10}$ CFU/ml and significantly lower (*P* < 0.001) than the cell numbers of $7.4 \log_{10}$ CFU/ml in biofilms exposed to CF and $7.5 \log_{10}$ CFU/ml in biofilms not exposed to nanofibers (Fig. 3b).

Effect of iron on biofilm formation. After 8 h of incubation, the OD_{595} readings recorded for crystal violet-stained cells from biofilms that were not exposed to nanofibers (control) and biofilms that were exposed to nanofibers, but without DHBA incorporated (CF), were similar at each of the iron concentrations (Fig. 3a, c, e, and g). Similar results were obtained when viable cell numbers were recorded (Fig. 3b, d, f, and h). However, OD_{595} readings recorded for crystal violet-stained cells from biofilms that were exposed to nanofibers containing DHBA (DF) decreased from 1.17 in the absence of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (Fig. 3a) to 0.89 (Fig. 3c), 0.48 (Fig. 3e), and 0.46 (Fig. 3g) in the presence of 1.0,

FIG 5 (a) Effects of nanofibers containing DHBA (DF) and nanofibers without DHBA (CF) on the growth of *P. aeruginosa* Xen 5. Cell numbers (CFU/ml) are shown in bar graphs and bioluminescence in line graphs. (b) Growth in the presence of sub-MICs of DHBA (116 $\mu\text{g/ml}$) and DHBA (116 $\mu\text{g/ml}$) in the presence of 1 mM iron, and in the absence of DHBA, with and without 1 mM iron. (c) Growth of strain Xen 5 in the presence of 1.5 mg/ml DHBA (MIC levels) and in the absence of DHBA. Data points are the average values of three independent experiments (mean $\pm$ standard deviation). *, $P < 0.001$.

2.0, and 3.0 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, respectively. A slight decrease in cell numbers (from 7.5 log_{10} CFU/ml to 6.4 log_{10} CFU/ml) was recorded when biofilms that formed in the presence of 1.0 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ were exposed to DF (Fig. 3d). A drastic decline in cell numbers, from 7.1 log_{10} CFU/ml to 4.8 log_{10} CFU/ml (Fig. 3f), was

recorded when biofilms that formed in the presence of 2.0 mM were exposed to DF. A similar decline in cell numbers, from 6.8 log_{10} CFU/ml to 4.7 log_{10} CFU/ml (Fig. 3h), was recorded when biofilms that formed in the presence of 3.0 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ were exposed to DF.

Twitching mobility studies. Cells migrated up to 10 mm from the point of inoculation when incubated in the presence of 2.0 mM DHBA and 2.0 mM DHBA plus 1.0 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, but not when incubated in the presence of 1.0 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (Fig. 4A). Microscopic examination of stained cells clearly showed that cells in the presence of DHBA were more motile than cells in the presence of iron (Fig. 4B).

Antimicrobial activity of DHBA. After 8 h of incubation at 37°C, cell numbers in the presence of CF increased from 8.7 to 9.2 $\log_{10}$ CFU/ml and bioluminescence from $1.50\text{E} + 07$ to $2.97\text{E} + 07$ $\text{p s}^{-1} \text{cm}^{-2} \text{sr}^{-1}$ (Fig. 5a). However, cell numbers in the presence of DF remained between 6.5 $\log_{10}$ CFU/ml and 6.7 $\log_{10}$ CFU/ml and bioluminescent readings at approximately $3.0\text{E} + 07$ $\text{p s}^{-1} \text{cm}^{-2} \text{sr}^{-1}$ throughout the 8-h experiment (Fig. 5a).

The lowest cell numbers (6.3 $\log_{10}$ CFU/ml) were recorded in the absence of iron and in deferrated LB broth supplemented with 116 $\mu\text{g/ml}$ DHBA after 8 h of incubation (Fig. 5b). In the presence of 1.0 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, cell numbers increased to 7.0 $\log_{10}$ CFU/ml after 2 h but declined to 6.5 CFU/ml during the following 6 h (Fig. 5b). Cells exposed to the same iron concentration (1.0 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), but also to 116 $\mu\text{g/ml}$ DHBA, remained at ~ 6.5 $\log_{10}$ CFU/ml for the first 4 h and then increased to 7.3 $\log_{10}$ CFU/ml over the next 4 h (Fig. 5b).

The MIC of DHBA was 1.5 mg/ml (not shown). At this concentration, growth of *P. aeruginosa* Xen 5 was suppressed for 24 h, whereas the optical density of cells in the control (without DHBA) increased to 1.3 after 24 h (Fig. 5c).

DISCUSSION

The decrease in diameter of nanofibers electrospun with DHBA compared to nanofibers without DHBA may be ascribed to the change in viscosity and conductivity of the solution. Similar findings were reported by Heunis et al. (22) when nisin was incorporated into PDLLA:PEO nanofibers. Nanofibers with a smaller diameter have a larger surface-to-volume ratio and release incorporated compounds much more quickly (25, 26). Nanofibers with these properties are thus ideal to incorporate into dressings used for treatment of topical infections.

Almost all DHBA diffused from the nanofibers within 2 h (Fig. 2), rendering 24% PDLLA:PEO (50:50) the ideal carrier for the chelating agent. The release of DHBA from nanofibers chelated free iron, and by doing so repressed the growth of cells in the biofilm, as shown with decreases in optical density readings and a decline in viable cell numbers (Fig. 3). The drastic decline of cell numbers in iron-enriched (2.0 mM and 3.0 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) biofilms (Fig. 3f and h, respectively) is not surprising. Higher concentrations of iron are toxic to the cells (27). Based on these results, the 116 $\mu\text{g/ml}$ DHBA incorporated into the nanofibers did not chelate all the iron.

The increase in mobility of strain Xen 5 in the presence of DHBA (Fig. 4) further supports our finding that the cells are less likely to form biofilms in the presence of low concentrations (1.0 mM) of free iron. These results confirm the findings of Singh and coworkers (12), i.e., a decrease in mobility of *Pseudomonas* cells (and an increase in biofilm formation) in the presence of freely available Fe^{2+} .

Results obtained with the *in vitro* slide model (Fig. 5a) corresponded to the release of DHBA from nanofibers, i.e., DHBA released from nanofibers depleted the freely available iron levels from the environment and slowed down the growth of strain Xen

5. The decline in bioluminescence recorded when cells were exposed to DF suggested that the metabolic activity of the cells decreased, which also led to a decrease in viable cell numbers. Lowering of iron levels to $<10^{-18}$ M in wounds assists the chelating function of lactoferrin and transferrin (28). These proteins bind iron as a natural defense mechanism against bacterial infections and aid phagocytic cells and tissue fluids in resisting bacterial invasion. This is extremely important, as freely available iron can severely damage or destroy the mechanism of natural resistance and lead to rapid bacterial or fungal growth in tissue fluids.

The decrease in cell numbers recorded in deferrated LB broth supplemented with DHBA to cell numbers equivalent to those obtained in the absence of iron (Fig. 5b) indicated that the cells needed iron to sustain growth. This was confirmed by recording slightly better growth in the presence of 1.0 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, without DHBA (Fig. 5b). The increase in cell numbers (from 6.5 $\log_{10}$ CFU/ml to 7.3 $\log_{10}$ CFU/ml) after the first 4 h of incubation (Fig. 5b) is difficult to explain. It is possible that at this point in growth the remaining free iron left in the medium after chelation is at a concentration supporting cell division and hence a rapid increase in cell numbers.

DHBA at MIC levels (1.5 mg/ml) inhibited the growth of *P. aeruginosa* for at least 24 h (Fig. 5c). At this concentration, DHBA chelated iron to levels lower than required to support growth. It is also important to note that the DHBA concentrations used in the experiments with results shown in Fig. 3, 4, and 5a and b were performed with 116 $\mu\text{g/ml}$ DHBA, far below MIC levels. Reduction of biofilm formation thus was not due to a direct antimicrobial effect of DHBA but rather was due to a decrease in free iron in the medium. Nanofibers containing DHBA (DF) exhibit two modes of action, i.e., (i) a decrease in biofilm formation due to the chelation of iron and (ii) an antiseptic effect that increases in the presence of iron, a mechanism that merits further investigation.

DHBA is less cytotoxic than other chelators, such as 1,2,3,4,6-penta-O-galloyl- β -D-glucopyranose (29) and 2DP (30). The chelators DTPA, EDTA, DM, and EDDA are also regarded as non-toxic (16). Dressings prepared from nanofibers containing DHBA may thus be an alternative treatment of topical infections caused by *P. aeruginosa*.

ACKNOWLEDGMENT

J. J. Ahire is grateful for financial support from the Claude Leon Foundation (Cape Town, South Africa) for a postdoctoral fellowship (2013–2014).

REFERENCES

- Harjai K, Bala A, Gupta RK, Sharma R. 2013. Leaf extract of *Azadirachta indica* (neem): a potential antibiofilm agent for *Pseudomonas aeruginosa*. Pathog. Dis. 69:62–65. <http://dx.doi.org/10.1111/2049-632X.12050>.
- Ehrlich GD, Veeh R, Wang X, Costerton JW, Hayes JD, Hu FZ, Daigle BJ, Ehrlich MD, Post JC. 2002. Mucosal biofilm formation on middle-ear mucosa in the chinchilla model of otitis media. JAMA 287:1710–1715. <http://dx.doi.org/10.1001/jama.287.13.1710>.
- Cross A, Allen JR, Burke J, Duce G, Harris A, John J, Johnson D, Lew M, MacMillan B, Meers P, Skalova R, Wenzel R, Tenney J. 1983. Nosocomial infections due to *Pseudomonas aeruginosa*: review of recent trends. Rev. Infec. Dis. 5(Suppl 5):S837–S845. http://dx.doi.org/10.1093/clinids/5.Supplement_5.S837.
- Mulcahy LR, Isabella VM, Lewis K. 6 October 2013. *Pseudomonas aeruginosa* biofilms in disease. Microb. Ecol. <http://dx.doi.org/10.1007/s00248-013-0297-x>.
- Trøstrup H, Thomsen K, Christophersen LJ, Hougen HP, Bjørnsholt T, Jensen PØ, Kirkby N, Calum H, Høiby N, Moser C. 2013. *Pseudomonas*

- aeruginosa* biofilm aggravates skin inflammatory response in BALB/c mice in a novel chronic wound model. Wound Repair Regen. 21:292–299. <http://dx.doi.org/10.1111/wrr.12016>.
6. Zhao T, Liu Y. 2010. *N*-acetylcysteine inhibits biofilms produced by *Pseudomonas aeruginosa*. BMC Microbiol. 10:140. <http://dx.doi.org/10.1186/1471-2180-10-140>.
 7. Harrison-Balestra C, Cazzaniga AL, Davis SC, Mertz PM. 2003. A wound-isolated *Pseudomonas aeruginosa* grows a biofilm *in vitro* within 10 hours and is visualized by light microscopy. Dermatol. Surg. 29:631–635. <http://dx.doi.org/10.1046/j.1524-4725.2003.29146.x>.
 8. Poole K. 2001. Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. J. Mol. Microbiol. Biotechnol. 3:255–264.
 9. Folsom JP, Richards L, Pitts B, Roe F, Ehrlich GD, Parker A, Mazurie A, Stewart PS. 2010. Physiology of *Pseudomonas aeruginosa* in biofilms as revealed by transcriptome analysis. BMC Microbiol. 10:294. <http://dx.doi.org/10.1186/1471-2180-10-294>.
 10. Gellatly SL, Hancock RE. 2013. *Pseudomonas aeruginosa*: new insights into pathogenesis and host defenses. Pathog. Dis. 67:159–173. <http://dx.doi.org/10.1111/2049-632X.12033>.
 11. Zhao K, Tseng BS, Beckerman B, Jin F, Gibiansky ML, Harrison JJ, Luijten E, Parsek MR, Wong GC. 2013. Psl trails guide exploration and microcolony formation in *Pseudomonas aeruginosa* biofilms. Nature 497:388–391. <http://dx.doi.org/10.1038/nature12155>.
 12. Singh PK, Parsek MR, Greenberg EP, Welsh MJ. 2002. A component of innate immunity prevents bacterial biofilm development. Nature 417:552–555. <http://dx.doi.org/10.1038/417552a>.
 13. Banin E, Vasil ML, Greenberg EP. 2005. Iron and *Pseudomonas aeruginosa* biofilm formation. Proc. Natl. Acad. Sci. U. S. A. 102:11076–11081. <http://dx.doi.org/10.1073/pnas.0504266102>.
 14. Burns JL, Mancoll JS, Phillips LG. 2003. Impairments to wound healing. Clin. Plast. Surg. 30:47–56. [http://dx.doi.org/10.1016/S0094-1298\(02\)00074-3](http://dx.doi.org/10.1016/S0094-1298(02)00074-3).
 15. Kamiya H, Ehara T, Matsumoto T. 2012. Inhibitory effects of lactoferrin on biofilm formation in clinical isolates of *Pseudomonas aeruginosa*. J. Infect. Chemother. 18:47–52. <http://dx.doi.org/10.1007/s10156-011-0287-1>.
 16. O'May CY, Sanderson K, Roddam LF, Kirov SM, Reid DW. 2009. Iron-binding compounds impair *Pseudomonas aeruginosa* biofilm formation, especially under anaerobic conditions. J. Med. Microbiol. 58(Pt 6):765–773. <http://dx.doi.org/10.1099/jmm.0.004416-0>.
 17. Graziano JH, Miller DR, Grady RW, Cerami A. 1976. Inhibition of membrane peroxidation in thalassaemic erythrocytes by 2,3-dihydroxybenzoic acid. Br. J. Haematol. 32:351–356. <http://dx.doi.org/10.1111/j.1365-2141.1976.tb00938.x>.
 18. Blatt J, Taylor SR, Kontoghiorghes GJ. 1989. Comparison of activity of deferoxamine with that of oral iron chelators against human neuroblastoma cell lines. Cancer Res. 49:2925–2927.
 19. Grootveld M, Halliwell B. 1988. 2,3-Dihydroxybenzoic acid is a product of human aspirin metabolism. Biochem. Pharmacol. 37:271–280. [http://dx.doi.org/10.1016/0006-2952\(88\)90729-0](http://dx.doi.org/10.1016/0006-2952(88)90729-0).
 20. Sousa M, Ousingsawat J, Seitz R, Puntheeranurak S, Regalado A, Schmidt A, Grego T, Jansakul C, Amaral MD, Schreiber R, Kunzelmann K. 2007. An extract from the medicinal plant *Phyllanthus acidus* and its isolated compounds induce airway chloride secretion: a potential treatment for cystic fibrosis. Mol. Pharmacol. 71:366–376. <http://dx.doi.org/10.1124/mol.106.025262>.
 21. Neilands JB. 1995. Siderophores: structure and function of microbial iron transport compounds. J. Biol. Chem. 270:26723–26726.
 22. Heunis TD, Smith C, Dicks LMT. 2013. Evaluation of a nisin-eluting nanofiber scaffold to treat *Staphylococcus aureus*-induced skin infections in mice. Antimicrob. Agents Chemother. 57:3928–3935. <http://dx.doi.org/10.1128/AAC.00622-13>.
 23. Payne SM. 1994. Detection, isolation, and characterization of siderophores. Methods Enzymol. 235:329–344.
 24. O'Toole GA. 2011. Microtiter dish biofilm formation assay. J. Vis. Exp. (47):pii=2437. <http://dx.doi.org/10.3791/2437>.
 25. Liu H, Ding X, Zhou G, Li P, Wei X, Fan Y. 2013. Electrospinning of nanofibers for tissue engineering applications. J. Nanomaterials 2013:1–11. <http://dx.doi.org/10.1155/2013/495708>.
 26. Tan S, Huang X, Wu B. 2007. Some fascinating phenomena in electrospinning processes and applications of electrospun nanofibers. Polym. Int. 56:1330–1339. <http://dx.doi.org/10.1002/pi.2354>.
 27. Balasubramanian D, Schnepfer L, Kumari H, Mathee K. 2013. A dynamic and intricate regulatory network determines *Pseudomonas aeruginosa* virulence. Nucleic Acids Res. 41:1–20. <http://dx.doi.org/10.1093/nar/gks1039>.
 28. Bullen JJ, Rogers HJ, Griffiths E. 1978. Role of iron in bacterial infection. Curr. Top. Microbiol. Immunol. 80:1–35.
 29. Lin MH, Chang FR, Hua MY, Wu YC, Liu ST. 2011. Inhibitory effects of 1,2,3,4,6-penta-*O*-galloyl-beta-D-glucopyranose on biofilm formation by *Staphylococcus aureus*. Antimicrob. Agents Chemother. 55:1021–1027. <http://dx.doi.org/10.1128/AAC.00843-10>.
 30. Li S, Crooks PA, Wei X, de Leon J. 2004. Toxicity of dipyrpyridyl compounds and related compounds. Crit. Rev. Toxicol. 34:447–460. <http://dx.doi.org/10.1080/10408440490503143>.